# CHAPTER II

# LITERATURE REVIEW

## 2.1 Aquatic toxicology

Aquatic toxicology has been the qualitative and quantitative study of the adverse or toxic effects of chemicals and other anthropogenic materials or xenobiotics on aquatic organisms. (Rand and Petrocelli, 1985).

Toxic effects may include lethality and sublethal effects, for expample, changes in growth, development, reproduction, pharmacokinetic responses, pathology, biochemistry, physiology and behavior. The quantifiable criteria may be used for monitoring the effects such as the number of organisms killed, percent egg hatchability, changes in length and weight, percent enzyme inhibition, number of skeletal abnormalities and tumor incidence (Rand and Petrocelli, 1985).

Adverse effects may be produced by acute or chronic exposure to chemicals or other potentially toxic agents. In general, an acute exposure concerns a short period of time compared to the life cycle of an organism, the test lasts about a week or less for fish. The effect usually occurs within 4 days. Mortality is the end point. On the other hand, a chronic exposure may involve the entire reproductive life cycle. Exposure that are intermediate in duration, a month to several months, are less than a complete reproductive life cycle and include exposure during sensitive early stages of development. In mammalian toxicology, it usually signifies exposures lasting one-tenth of a lifetime or longer. Therefore, it is sometimes used to mean a full life cycle test in aquatic toxicology (Schreck and Moyle, 1990).

### 2.1.1 Acute toxicity test

Generally, the purposes of any acute toxicity test are to discover and report any adverse health effect which could be attributed to the chemical under study. Effects of acute toxicity test are relatively severe.

The most common one measured in aquatic organisms is lethality or mortality. Acute toxicity studies are often designed to express the potency of the toxicant in terms of the median lethal concentration ($LC_{50}$), the estimated concentration in the environment to which the animals are exposed that will result in 50% mortality of the population of animals under the conditions defined for the study. The period of time is relatively short, such as 96 h to 14 days (Rand and Petrocelli, 1985).

### 2.1.2 Chronic toxicity test

The aims of chronic study are to make certain the biological effects of repeated administration of the test agent on potential target organs of the body at concentration that do not elicit acute toxicity, to establish a concentration-effect relationship between biochemical, physiological and morphological effects over the concentration range and the time intervals of exposure of the agent, to make certain the maximum concentration level that produces no discernible ill effects following repeated exposure, and to explore the possible mechanism(s) by which the toxicant elicits its effect(s) (Ecobichon, 1992).

Chronic toxic tests permit evaluation of the possible adverse effects of the chemical under conditions of long-term exposure at a sublethal concentration. The most common chronic effects may be behavior changes, physiological changes, biochemical changes and histological changes. Consistent with these, aquatic toxicology has evolved as a multidisciplinary field of study, which borrows freely from several other basic sciences (Rand and Petrocelli, 1985).

## 2.2 Routine haematological methods

Knowledge of the composition of fish blood and of the function of blood components is fundamental to a comprehensive understanding of the normal and pathobiology and biochemistry of fish. Moreover, such information is particularly important when interpreting the changes induced by water pollutants or other abnormal environmental factors. As aquatic poikilotherms, fish respond readily to alterations in their environments, and these are able to occur hourly and daily as well as seasonally (Schreck and Moyle, 1990). To establish reliable diagnostic blood tests for identifying systemic and metabolic dysfunctions of fish, more fundamental information is needed on normal fish blood, fish

metabolism, and the variability of metabolic component (Christensen et al., 1978).

Descriptions of some routine haematological methods for examining fish blood including haemoglobin estimation, haematocrit, erythrocyte counts, erythrocyte sedimentation rate, total and differential leukocyte counts, and cytochemical staining were suggested as a possible means of assessing fish health but there was a need for establishing values in health, disease and various stress conditions before their value in diagnosis could be evaluated (Blaxhall and Daisley, 1973). An enormous literature pertinent to these studies has existed. Such McCarthy et al.(1973) observed that some blood parameters of the rainbow trout *Salmo gairdneri* Richardson of the American Kamloops strain including erythrocyte sedimentation rate, haemoglobin content, packed cell volume, erythrocyte count, erythrocyte diameters, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), differential leukocyte count and plasma total protein fell within narrow ranges and the total leukocyte counts and glucose levels were more widely spread than other strains. Ezzat et al. (1974) reported the results of a study on haematology of *Tilapia zillii* (Gervais) in Egyptian waters. They found five types of leukocytes including lymphocytes, monocytes, neutrophils, eosinophils and basophils and mentioned that the differential count of leukocytes in *T. zillii* showed seasonal variation. Also, Courtois (1976) reported that juvenile striped bass *Morone saxatilis* changed in haemoglobin, haematocrit, total protein and serum eletrolytes that correlated to different enviromental factors such as salinity and temperature. In the same time, Ferguson (1976) studied about the response of plaice *Pleuronectes platessa* leukocytes to the injection of carbon particles, and also divided white blood cells into four main types which included lymphocyte, thrombocyte, monocyte and neutrophil. However, Williams and Warner (1976) observed that there were eight cellular blood elements of channel catfish *Ictalurus punctatus* including thrombocytes, lymphocytes, neutrophils, monocytes, eosinophils, basophils, granular anucleate bodies and erythrocytes. The predominant leukocytes were thrombocyte, lymphocyte and neutrophil.

In 1976, Fourie and van Vuren noted that yellowfish *Barbus holubi* contained different haemoglobin during the season (summer, autumn and midwinter) caused by different erythrocyte populations were present. Siddiqui and Naseem (1979) noted that there were variations in haematological parameters of *Labeo rohita* such as haematocrit values, haemoglobin concentration, erythrocyte counts, leukocyte counts, erythrocyte sedimentation rate and clotting time in relation to size and weight of the fish. Males had higher haematological values than the females, seasonal and variations due to maturation of gonads were also apparent. Morrow and Pulsford (1980) classified the peripheral blood leukocytes of the dogfish *Scyliorhinus canicula* as lymphocytes, plasma cells , monocytes, thrombocytes and granulocytes. The granulocytes were further classified into four types according to the structure of their granule. At the same period, Yamamoto et al. (1980) mentioned that haematocrit and haemoglobin concentrations of yellowtail *Seriola quinqueradiata* blood elevated more than 40% just after a severe exercise by 5 min chasing which caused by erythrocyte supply from spleen. While Doggett et al. (1987) described and identified the morphological characteristics of the peripheral leucocytes of the cichlid *Oreochromis mossambicus* into four main types including lymphocytes, thrombocytes, monocytes and granulocytes. Two distinct types of granulocyte were found. Furthermore, Watson and colleagues (1963) studied in this fish species and reported that the principle cellular components of the peripheral blood of *Carassius auratus* consisted of erythrocyte, thrombocyte, lymphocyte, neutrophil, eosinophil and basophil. However, Fujimaki and Isoda (1990) noted that there were eight types of leukocytes in the goldfish *C. auratus*. They consisted of neutrophil, eosinophil, large granular leukocyte, medium-sized granular leukocyte, small granular leukocyte, fine granular leukocyte, lymphocyte and monocyte.

Different types of leukocytes were observed in different species of fish. Data about the percentage of various types of leukocytes in some species of fish were collected and shown in Table 2.1.

**Table 2.1 A comparison between differential leukocyte count in some fish species**

| Species | Differential leukocyte count | | | | | | | References |
|---|---|---|---|---|---|---|---|---|
| | Lymphocytes (%) | Monocytes (%) | Granulocytes (%) | Neutrophils (%) | Eosinophils (%) | Basophils (%) | Other (%) | |
| *Anguilla japonica* | 77.50 | 2.50 | - | 20.00 | - | - | - | Sano (1957) |
| *Carassius auratus* | 92.50 | - | - | 5.10 | 2.20 | 0.20 | - | Watson et al.(1963) |
| *Cyprinus carpio* | 34.90 | - | - | 65.10 | - | - | - | Onada (1934) |
| *Cyprinus cyprinus* | 22.00 | - | 78.00 | - | - | - | - | Onada (1934) |
| *Misgurnus fossilis* | 40.00 | - | 60.00 | - | - | - | - | Onada (1934) |
| *Perca flavescens* | 67.70 | - | - | 32.40 | - | - | - | Yokayama (1947) |
| *Prosopium illiamsoni* | 33.90 | - | - | 66.10 | - | - | - | McKnight (1966) |
| *Salmo gairdneri* | 93.50 | - | 4.80 | - | - | - | 1.80 | McCarthy et al.(1973) |
| *Salmo namaycush* | 91.20 | 8.20 | 0.60 | - | - | - | - | Lieb et al.(1953) |
| *Salmo trutta (L.)* | 90.00 | - | - | 6.60 | - | - | 3.33 | Blaxhall and Daisley(1973) |
| *Salvelinus fontinalis* | 36.20 | - | - | 5.90 | - | - | 57.40 | Christensen et al.(1978) |
| *Tilapia zillii* | 61.00 | 7.75 | - | 24.50 | 6.25 | 0.50 | - | Ezzat et al.(1974) |

Todate, there have also been several researches about effects of various environmental toxicants monitoring by fish blood. Such Buckley (1977) reported that coho salmon *Oncorhyncus kisutch* exposed to chlorinated wastewater exhibited symtoms of hemolytic anaemia including an increase in numbers of circulating immature erythrocytes, pathological changes in erythrocytes and a reduction in packed cell volume and haemoglobin levels. Heinz bodies were also observed. Chliamovitch and Kuhn (1977) has noted that there were the increasing of the packed cell volume, the haemoglobin concentration and erythrocyte count in *Salmo gairdneri* Richardson exposed to 0.023 to 1.17 mg/l bis (tri-*n*-butyltin) oxide (TBOT) for 24 hr. In 1979 Srivastava and Agrawal investigated haematological anomalies in a fresh water teleost *Colisa fasciatus* on acute exposure to cobalt. The decline in blood clotting time with a concomitant increase in the abundance of circulating thrombocytes was noted in treatment. However, there was no differences in total erythrocyte count, haematocrit, erythrocyte sedimentation rate and haemoglobin values between the control and treated fish. As van Vuren (1986) studied about the effects of toxicants on the haematology of *Labeo umbratus*. These toxicants include detergent, ammonium sulphate, fertilizer and Metasystox. The results showed statistically significant changes between the values of parameters of experimental and control fish.

Gill and Pant (1985) reported that morphological aberrations in mature erythrocytes including cytoplasmic vacuolation, hypochromia, deterioration of cellular membrane, basophilic stipping of cytoplasm, clumping of chromatin material and extrusion of nuclei, and schistocytosis occurred in the cyprinid fish *Puntius conchonius* exposed to 0.63 and 0.84 mg/l cadmium chloride (1/20 and 1/50 of 96-hr $LC_{50}$). Anomalous basophils and monocytes were also encountered though less frequently. Decreased erythrocyte counts, haemoglobin concentrations and haematocrit values were also associated with chronic cadmium poisoning. After 30 days exposure to cadmium, the mean corpuscular haemoglobin and mean corpuscular volume were higher but mean corpuscular haemoglobin concentration exhibited unclear change. While Srivastava and Narain (1985) studied about catfish *Heteropneustes fossilis* blood chemistry under environmental stress. The results showed an alteration in the level of blood glucose, serum cholesterol, blood urea, acid and alkaline phosphatase activity in serum . In 1987 Bielinska reported that there was a reduction in erythrocyte count, haematocrit,

haemoglobin concentration, mean corpuscular haemoglobin concentration and mean corpuscular haemoglobin in fish *Cyprinus carpio* exposed to a sublethal concentration of sodium alkylbenzene sulphonate (8.0 mg/l).

Murad and Houston (1988) have observed that gold fish *Carassius auratus* exposed to sublethal concentrations of cadmium (90, 270 and 445 µg/l) for 3 weeks, has exhibited significant reductions in total leukocyte counts because of the results of decreases in lymphocyte and thrombocyte numbers. Observations of neutrophil, eosinophil and basophil numbers have been found higher. At the same time, Williams and Eddy (1988) reported that there was an increase in methaemoglobin levels from around 3% to over 60% in rainbow trout *Salmo gairdneri* exposed to 0.5 mmol/l nitrite for periods varying from 2 to 24 hr. A decrease in the concentrations of plasma potassium, sodium and chloride exhibited after 2 hr nitrite exposure, followed 2 hr later by an increase in intra-erythrocyte potassium and sodium concentration with increased red cell volume. Exposure to nitrite for 12 hr led to an increase in the red cell population, the new cells being smaller and containing less haemoglobin. After 24 hr exposure fell into two groups including nitrite-intolerant fish with high levels of plasma nitrite and methaemoglobin and nitrite-tolerant fish with low plasma levels. All fish surviving 24 hr nitrite exposure had lower plasma potassium levels than unexposed fish. Cyriac et al.(1989) noted that the haemoglobin content and haematocrit values in the fish *Oreochromis mossambicus* (Peters) after short-term exposure to 100 µg/l and 200 µg/l copper were significantly lower than the control at 24 hr and in fish exposed to 100 µg/l and 150 µg/l mercury did not exhibit any significant differences in the haemoglobin content. Haematocrit values did not alter significantly in both metal-treated fish at 24 h the copper-treated fish and fish exposed to 150 µg/l mercury exhibited a statistical increase in haematocrit values.

Recently, Rao et al.(1990) have described that five species of fish grey mullet *Mugil cephalus*, Borneo mullet *Liza macrotepis*, goldspot mullet *Liza parsia*, long whiskered catfish *Mystus gulio* and the Indian mackerel *Rastrelliger kanagurta* from complex polluted waters of Visakhapatnam harbour (in India) had shown significantly lower erythrocyte numbers, haematocrit, haemoglobin content and thrombocyte percentage and significantly higher mean cell volume (MCV), leukocyte numbers and lymphocyte percentage, which compared with the controls.

Corresponding, Ruparelia et al.(1990) observed that there was a significant decrease in haemoglobin content, haematocrit and red blood cell counts in blood of tilapia *Oreochromis mossambicus* (Peters) during prolonged exposure to cadmium. In case of red blood cell indices such as MCV, MCH and MCHC showed certain changes in test fish. MCV and MCH increased significantly but MCHC was reduced significantly from control.

Also, Dutta et al.(1992) studied about malathion induced changes in haematological parameters of an Indian catfish *Heteropneustes fossilis* (Bloch). Noticeable differences were observed in the total RBC and WBC count in addition to haemoglobin content. There was a decrease in the number of red blood cell and haemoglobin content in test fish. Conversely, the total WBC count showed a slight decrease in treated fish. Moreover, Sampath et al.(1993) have found the decreasing in the erythrocyte count, haemoglobin concentration and mean corpuscular volume (MCV), leading to microcytic anaemia in *Oreochromis mossambicus* exposed to sublethal levels of Ekaluk, an organophosphate pesticide. The increasing in the total leukocyte counts have been also found. In the same period, Allen (1994) investigated the alterations in the haematological profile of the cichid *Oreochromis aureus* (Steindachner) during acute inorganic mercury intoxication. The fish exposed to 0.5 ppm mercury casued elevated leukocyte and erythrocyte counts within 24 hr. Furthermore, a decrease of mean corpuscular volume (MCV) and an increase of mean cell haemoglobin concentration (MCHC) were observed in fish exposed to 0.1 ppm mercury for 1 week. Consistent with this, Alkahem (1994) has reported that there has been a decrease in total leukocyte counts and an increase in erythrocyte count, haemoglobin concentration and haematocrit in fish *Oreochromis niloticus* exposed to sublethal concentrations of nickle (1.5, 3.0 and 5.0 mg/l). Differential leukocyte count has also showed a significant fall in the numbers of thrombocytes and lymphocytes and an increase in neutrophil, eosinophil and monocyte numbers.

Nussey et al.(1995) studied the effect of copper on the haematology of the Mozambique tilapia *Oreochromis mossambicus*. The result showed the effects of leukocytosis and erythrocytopenia in fish exposed to 0.16 mg/l and 0.40 mg/l copper for 96 hr and 4 weeks ,respectively. Heaton et al.(1995) studied about the effects of consumption of environmental contaminants contained in carp *Cyprinus*

*carpio* from Saginaw Bay, Michigan on various haematological parameters of adult female mink *Mustela vison*. The results showed a decrease in erythrocyte count in mink treated with carp from Saginaw Bay, while the number of white blood cells was higher. Significant differences in the numbers of neutrophils, lymphocytes, monocytes and eosinophils were also reported between the control and carp-fed groups. In the same year, Srivastava et al.(1995) reported that fish *Heteropneustes fossilis* exposed to malachite green at concentration of 0.20 mg/l (1/5 of 96 $LC_{50}$) for 96 hr exhibited a significant decrease in the serum calcium and protein levels and a significant increase in the total cholesterol levels of blood. Recently, Jeney et al.(1996) noted that there was a significant decrease of leukocrit and total plasma protein and a significant increase of blood glucose in the roach *Rutilus rutilus* L. from waters that contaminated with bleached kraft pulp and paper mill effluent. Balint et al. (1995) also reported that the blood glucose level was 30% higher in Carp *Cyprinus carpio* L. treated with deltramethrin, a type-II pyrethroid. Corresponding, a significant increase in blood glucose level that was found in rainbow trout *Oncorhynchus mykiss* exposed to mercurial compounds for 168 hr (Bleau et al., 1996). As Schwaiger et al.(1996) evaluated the toxic effects of sublethal concentrations of the fungicide triphenyltinacetate (TPTAc) on rainbow trout *Oncorhynchus mykiss*. An increase of the total number of erythrocytes, haemoglobin content and the packed cell volume were presented. The number of leukocytes tended to decrease at higher concentrations. The percentage of lymphocytes within the differential blood cell count decreased. Also, Soldatov (1996) has observed that the flounder *Pleuronectes flesus luscus* was maintained in water with low oxygen content (2.6-2.7 mg/l, saturation), has showed an increase in erythrocyte number in blood.

## 2.3 Blood chemistry of liver diseases

The number of metabolic functions performed by the liver has been enormous. Metabolic functions of the liver consisted of synthesis and degradation of carbohydrates and protein as well as regulation of lipid metabolism. Regulation of blood levels of glucose and amino acids have depended on a normally levels of several other metabolites. They include haemoglobin breakdown products, coagulation proteins, albumin, cholesterol, hormones, and ammonia. Alterations in the levels of these compounds may be ocurred when the liver is injured and

because of a number of extrahepatic organs are affected secondarily. Therefore, measurement of blood levels of several such components help determine the nature and extend of the liver injury (Kumar et al., 1992).

### 2.3.1 Marker of hepatocellular necrosis

Glutamic oxaloacetic transaminase (GOT) or aspartate aminotransferase (AST) and glutamic pyruvic transaminase (GPT) or alanine aminotransferase (ALT) activities in the serum have been the most often measured as indicators of liver disease. These enzymes catalyze the transfer of the α-keto group of aspartate and alanine to the α-keto group of ketoglutalic acid, resulting in the formation of oxaloacetic acid and pyruvic acid, respectively (Fig.2.1). The products of the GOT and GPT reactions, oxaloacetate and pyruvate, reduce enzymatically to malate and lactate, respectively, with concomitant oxidation of NADH to NAD. This event is able to be followed spectrophotometrically since NADH, but not NAD, absorbs light at wavelength 340 nm.

GOT has been in several types of tissues. These include heart, skeletal muscle, kidney, and brain in addition to liver. GPT appears to be localized primarily in the liver. Serum GOT and GPT have been elevated to some extent in almost all liver diseases. The highest elevations occur with vital hepatitis or toxin induced hepatic necrosis, and with circulatory shock (ischemia). Decrease in GOT and GPT in serum could be a sign of recovery, but they can also monitor a poor necrosis, reflecting a paucity of remaining hepatocytes (Zakim and Boyer, 1982).

### 2.3.2 Marker of cholestasis

The elevation of the serum alkaline phosphatase (ALP) value has been a very important feature of cholestasis. The elevating of enzymes levels result from increased synthesis rather than decreased excretion. In the human body, ALP has been identified in liver, bone, intestine, placenta, kidney, and leukocytes. Thus ALP in serum has been presumed to represent enzyme liberated from tissues and elevated serum levels may be encountered in nonhepatic disorder. An increase in the level of this enzyme has been a very sentitive marker of impaired biliary excretion. Studies in humun subjects demonstrate that ingestion a fatty meal

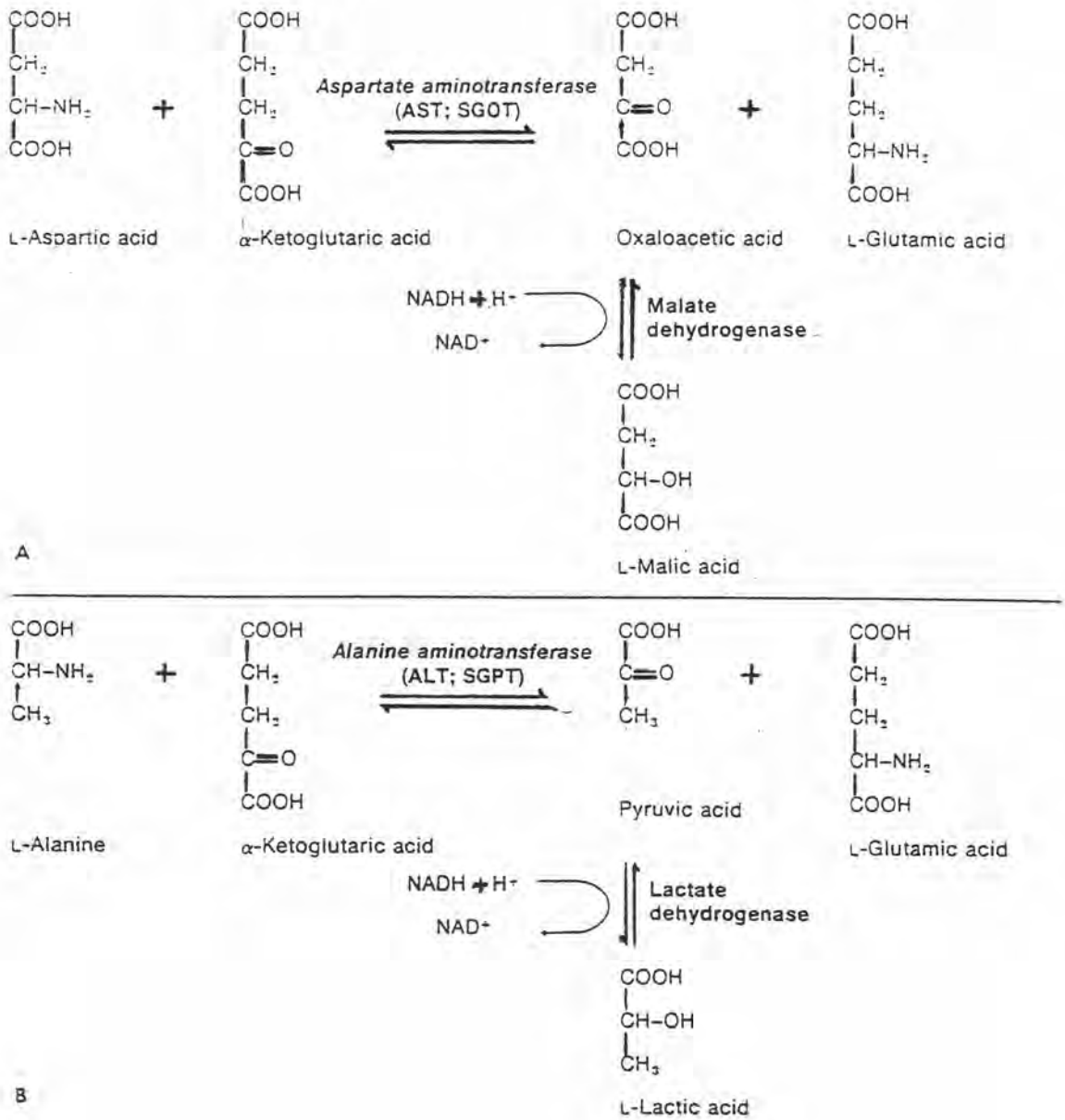


**Fig.2.1** Biochemical determination of serum transaminases. For asparte aminotransferase (AST : SGOT), (A) aspartic acid and α-ketoglutamic acid serve as co-substrates, which are converted oxaloacetic acid and glutamic acid. The addition of malate dehydrogenase and NADH catalyzes the conversion of oxaloacetic acid to malic acid. This reaction is followed by the loss of NADH in this coupled reaction. For alanine aminotransferase (ALT ; SGPT) (B) alanine and α-ketoglutaric acid serve as co-substrates, which are converted to pyruvic acid and glutamic acid. The addition of lactate dehydrogenase and NADH catalyzes thr conversion of pyruvic acid to lactic. Thus, measurement of the second reaction (loss of NADH) optimizes the sensitivity of the reaction of each transaminase (Zakim and Boyer, 1982).

increases ALP in the blood. This suggests that a possible role for the enzyme in lipid absorption and transport (Zakim and Boyer, 1982).

Most of researchers stress on these three enzymes as an index for investigation of hepatotoxic effects and some diseases related with alteration of these enzymes. For example, Buttar et al.(1976) observed the activities of GOT and GPT in acute and subacute acetaminophen-treated rats to induce the hepatotoxicity. At the same period, Sammoms et al.(1976) studied about changes in blood serum constituents and haematologic values in *Macaca mulatta* with Rockey Mountain spotted fever. The results showed a significant increase of GOT and ALP concentrations in macaques inoculated intravenously with yolksac-grown *Rickettsia rickettsii.* Also, Sharma (1977) measured the quantity of ALP, GOT and GPT in rabbits induced arthritis by mycobacterial adjuvant, the significant increase of these three enzyme exhibited an abnormal pattern in adjuvant animals. In 1978 Dikshith et al. used the activities of GOT, GPT and ALP in liver and serum to evaluate the toxic effects of benzene hexachloride on liver. As Knight (1978) studied about the effects of experimental infections with *Fasciola hepatica* of ovine and bovine origin in homogous and heterologous hosts by estimating GOT, GPT and ALP. At the same time, Strubelt et al.(1978) studied about the increased carbon tetrachloride hepatotoxicity after low-level ethanol consumption by determination of serum activities of GOT and GPT. Furthermore, Suarez and Bhonsle (1978) also observed the enhanced hepatotoxicity of carbon tetrachloride following sodium nitrite pretreatment by measuring the rise in serum GOT and GPT.

In 1979 Cook and co-workers used the plasma GOT and GPT activity for study about resistance of essential fatty acid-deficient rats to endotoxic shock. Strubelt et al.(1979) also studied about the mechanism of paracetamol which induced protection against paracetamol hepatotoxicity by determination of serum GOT and GPT. Balis et al. (1979) studied about glucocorticoid and antibiotic effects on hepatic microcirculation and associated host responses in lethal gram-negative bacteremia by estimating changes in level of GOT and GPT. While Ahmad et al.(1980) measured the quantity of serum GOT and GPT to investigate the effect of zinc on myocardial damage in albino rats. Singh et al.(1980) studied about the effect of phenelzine, a monoamine oxidase inhibitor , on isoproterenol-induced myocardial by estimating the increases of serum GOT and GPT.

Recently, Wang et al.(1994) examined the alteration of ALP, GOT and GPT in 84 male and 36 female ferrochromium-producing workers for urinary biochemical indicator of renal injury. They mentioned that GPT and ALP were early sensitive indicators of the most valuable for evaluating the renal injury. In 1997 Hirazawa and colleague measured serum concentration of ALP, GOT and GPT before and after experiment to investigate the reversible obstruction jaundice in rat. There has been many reports about using the data of blood biochemistry, GOT and GPT to evaluate the toxic effects of contaminants on aquatic organisms. McCorkle et al. (1979) evaluated the effects of seasonal change on tissue enzymes in the channel catfish *Ictalurus punctatus* by determining the activities of GOT and GPT. While Jeney et al. (1996) reported that fish *Rutilus rutilus* L. that was caught from the polluted lake exhibited lower values of GOT and GPT. Recently Rawat et al. (1997) used ALP, GOT and GPT level for study the hepatoprotective activity of Punarnava *Boerhaavia diffusa* L. roots on albino rat *Rattus norvegicus*.

## 2.4 Neem *Azadirachta indica* A. Juss.

### 2.4.1 Botanical characteristics and distribution

Neem is a medicinal plant botanically known as *Azadirachta indica* A. Juss (synonymy : *Melia indica* and *Melia azadirachta*) and belongs to the family Meliaceae (Fig.2.2). In English it is named Indian Liac, neem tree or margosa tree. Vernacular names are neem or nim (Hindi), neeb (Arabic), azad dirakht (Persian), nimba (Sanskrit) and Sadao India (Thai) (van der Nat et al., 1991).

It is a hardy, fast - growing evergreen tree with a straight trunk, long, spreading braches, moderately thick bark and round crown. The neem tree is undemanding and grows well on moist, dry, stony, clayey or shallow soils. The roots seem to have an unusually great ability to extract nutrients and moisture though from highly leached, sandy soils. It propagates easily by seed, without pre-treatment. Transplanting is good for 9 to 12 months-old seedling. Mature trees attain heights of 7-10 m. with a spread of 5-10 m. Fruiting begins after five years, become fully productive in 10 years and may live for more than 200 years. The fruit is a fleshy drupe, yellow when ripe (Atal and Kapur, 1982; van der Nat et al., 1991).

Neem is endemic in the Indo-Pakistan subcontinent. At present, its appearance is in South Asia in India, Pakistan,Bangla Desh, upper Burma and the drier parts of Sri Lanka. In Southeast Asia, the species is found in Thailand, Southern Malaysia and in the drier Indonesian islands east of Java. Moreover, it has been introduced to the Phillipines , Fiji and Mauritius and has spread to other islands in the South Pacific. In the Middle East it has been introduced in Yemen and Saudi Arabia. In Africa, it is wide distributed in Ghana, Nigeria, Sudan, East Africa and West Africa. In the New World it is planted in Haiti and Surinam and has been introduced recently to Cuba and Central American as a plantation tree (van der Nat et al., 1991).

**Fig.2.2** The photograph of neem *Azadirachta indica* A. Juss. tree.

### 2.4.2 Chemical constituents

Neem has been the subject of extensive phytochemical studies because of the strong biological effects of its preparations used for agricultural and medicinal purposes. Serveral chemical constituents have been isolated and characterized from various part of neem tree. The chemical investigation of neem leaves was reported that it consisted of flavonoid, nimbosterol, glutamic acid, tyrosine, aspartic acid, alanine, proline, glutamine and cystine like amino acids (Atal and Kapur, 1982). Garg and Bhakuni (1985) isolated a new meliacin that related to salannin, 2,3-dehydrosalannol from the leaves of neem. Siddiqui et al. (1986) isolated a triterpenoid named nimbocinone from fresh, undried winter leaves of neem. The sterol compound including sitosterol and stigmasterol were also identified. While the part of trunk bark contain nimbin, nimbinin, nimbosterol, essential oil, tannins, a bitter principle margosine and desacetylnimbin (Atal and Kapur, 1982). On the other hand, the components of neem seed were noted that there were two active principles, meliantriol and azadirachtin ($C_{35}H_{44}O_{16}$) (Fig.2.3) (Jotwani and Srivastava, 1981).

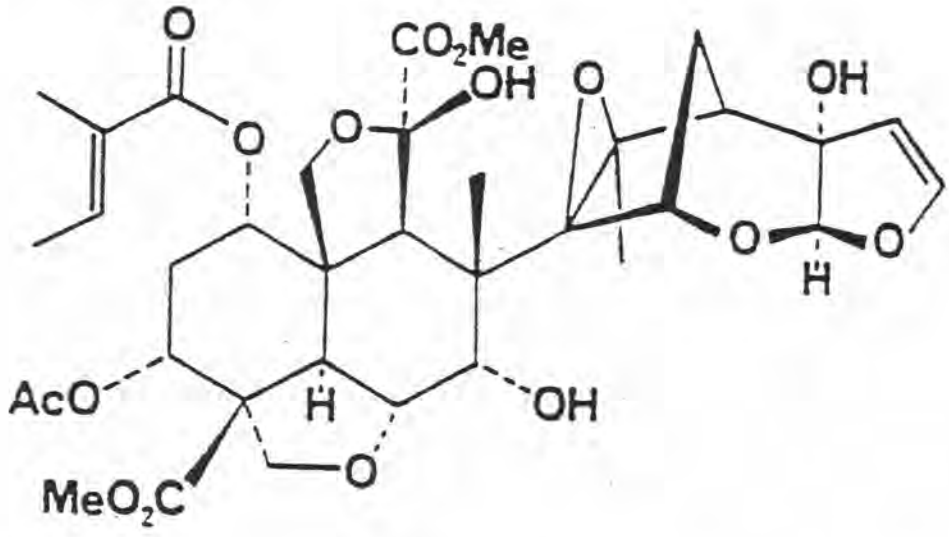


**Fig.2.3** The chemical structure of azadirachtin (Mulla et al., 1997)

As Atal and Kapur (1982) reviewed that this part of neem tree also composed of two sulphur contents, laevorotatory and desacetylnimbin, in addition to azadirachtin and salannin. A triterpenoid named azadirachtol has also been isolated from the fruits of neem (Siddiqui et al., 1985).

The further contents of neem seed were investigated by Wealth of India (Upadhyay et al., 1992). These included proto-meliacins, meliacins, pentanortriterpenoids, hexanortriterpenoid and a variety of non-terpenoidal components such as fatty acids, sterols, phenol, flavonoids and carbohydrates. Van der Nat et al. (1991) have reviewed on several chemical constituents of neem and classified into six groups including terpenes and steroids (e.g., diterpenes, triterpenes / steroids and limonoids or nortriterpenoids), (poly) phenolics (e.g., flavonoids, flavonoglycosides, dihydrochalcones, tannins and coumarins), carbohydrates and proteins, sulphurous compounds, alkaloids and others (e.g., nimbocetin and acids). Recently, a new tetranortriterpenoid, 13,14-desepoxyazadirachtin-A has been isolated from neem kernel extract by preparative HPLC (Govindachari and Gopalakrishnan, 1997). Also, Krause et al.(1981) found six new tetranortriterpnoids from the extract of neem seed. These consisted of 1α-methoxy-1,2-dihydroepoxyazadiradione(1), 1,β,2β-diepoxyazadiradione(2), 7-acetylneotrichilenone (3), 7-desacetyl-7-benzoyl-azadiradione(4), 7-desacetyl-7-benzoylepoxyazadiradione(5) and 7-desacetyl-7-benzoylgedunin(6). However, azadirachtin, a limonoid of tetranortriterpenoid type is the major potential component of neem in which has been interested most because of its properties for pest control and neem seed is the part that is found this component in a very high quantity. The terpenes and steroids of neem seed are listed in Table 2.2.

### 2.4.3 Ethnopharmacology

The information on use of traditional medicine from *A. indica* preparations has been widely display in application. The Wealth of India reported that it was a remedy in some chronic skin diseases, ulcer, rheumatism, diabetes, leprosy, dental troubles, asthma and was also used as a spermicidal and early abortificient agent (Upadhyay et al., 1992). Consistent with this, Bakhiet and Adam (1995) reviewed on the neem bark product decoction that was used in the treatment of pyrexia and influenza to relieve muscular pains. Oliver (1959) noted that this plant was used primarily for treat malaria, fever, abdominal disorders, hemorrhoids and as anthelmintics in Nigeria (Isman et al., 1990). In Sudan and other African countries, the leaf extract has been widely used as an antimalarial agent (Ibrahim et al., 1992).

**Table 2.2** Terpenes and steroids of neem seed *A. indica*

| Compound | Reference |
|---|---|
| 1. Meliantriol | Lavie et al.(1967) |
| 2. 6-Desacetylnimbin | Narayanan and Lyer (1967) |
| 3. Meldenin | Connolly et al.(1968) |
| 4. Nimbin | Harris et al.(1968) |
| 5. Salannin | Henderson et al.(1968) |
| 6. Nimbinin | Narayanan et al.(1969) |
| 7. Nimbidinin | Mitra et al.(1970) |
| 8. Nimbidic acid | Mitra et al.(1970 |
| 9. 17-Epi-azadiradione | Kraus and Cramer (1978) |
| 10. 17-β-Hydroxyazadiradione | Kraus and Cramer (1978) |
| 11. 7-Desacetyl-7-benzoylazadiradion | Kraus et al.(1981) |
| 12. 1α-Methoxy-1,2-dihydroepoxyazadiradione | Kraus et al.(1981) |
| 13. 1β,2β-Diepoxyazadiradione | Kraus et al.(1981) |
| 14. 7-Desacetyl-7-benzoylepoxyazadiradione | Kraus et al.(1981) |

**Table 2.2** Terpenes and steroids of neem seed *A. indica* (continue)

| Compound | Reference |
|---|---|
| 15. 7-Desacetyl-7-benzoylepoxyazadiradione | Kraus et al.(1981) |
| 16. 7-Actylneotrichilenone | Kraus et al.(1981) |
| 17. 7-Desacetyl-7-benzoylgedunin | Kraus et al.(1981) |
| 18. 1,3-Diacetylvilasinin | Kraus and Cramer (1981a) |
| 19. 3-Desacetylsalannin | Kraus and Cramer (1981a) |
| 20. Salannol | Kraus and Cramer (1981a) |
| 21. Salannolide | Garg and Bhakuni (1984) |
| 22. Nimbandiol | Kraus and Cramer (1981b) |
| 23. 6-*O*-Acetylnimbandiol | Kraus and Cramer (1981b) |
| 24. Nimbinen | Kraus and Cramer (1981b) |
| 25. 6-Desacetylnimbinen | Kraus and Cramer (1981b) |
| 26. 22,23-Dihydro-23-β-methoxyazadirachtin | Kraus et al.(1985) |
| 27. 4-Epinimbin | Devakumar and Mukerjee (1985) |
| 28. Azadirachtin | Kraus et al.(1985) |
| 29. 3-Tigloylazadirachtol | Klenk et al.(1986) |
| 30. 13,14-Desepoxyazadirachtin-A | Govindachari and Gopalakrishnan (1997) |

However, there were many researches that investigated about those properties of neem *Azadirachta indica* extract. Such Okpanyi and Ezeukwu (1981) studied about the anti-inflammatory and antipyretic activities of neem *A. indica* extract on rat and concluded that it was a potent anti-inflammatory agent and also has a fairly good antipyretic effect. Similar activity was reported in rabbit by Khattak et al. (1985).

The effect of antihyperglycemia was also reported. For example, Dixit et al. (1986) investigated the effect of neem *Azadirachta indica* seed oil on the blood glucose concentration of normal and alloxan-diabetic rats. The results showed that the seed oil possessed active constituents capable of lowering blood glucose in both normal and alloxan-induced diabetic rats. Consistent with this, Peter and Peter (1997) reported that exposure of perches to various doses of nimbecidine, a neem-based pesticide, produced significant changes in the levels and activity of various parameters of growth functions, particularly a decrease in the glucose concentration in serum, suggesting hypoglycemic effect.

Furthermore, the immunomodulatory activity was observed by van der Nat et al. (1987). Effect of an aqueous extract from stem bark of *Azadirachta indica* on human immune system was investigated. The results showed strong anticomplementary effects of this extract which were dose and time-dependent and most pronouced in the classical complement. Moreover, a dose-dependent decrease in the chemiluminescence of polymorphonuclear leukocytes was observed as well as a dose-dependent increase in the production of migration inhibition factor by lymphocytes. Besides, effect of an alternative pathway activation of human complement was also reported by van der Nat et al. (1989). In addition to the effect on the cardiovascular system was reported by Chattopadhyay (1997). The result of this study was demonstrated that the neem leaf extract was able to produce a dose-dependent fall in blood pressure without altering the amplitude or rate of respiration in cat.

The effect of antifertility of neem *Azadirachta indica* extract was evaluated. Such Prakash et al. (1988) reported that at subcutaneous doses up to 0.3 ml/kg body weight, neem *A. indica* oil did not possess any estrogenic, anti-estrogenic or progesteronal activity and appeared not to disturbed the action of progesterone in rat. These observations were

confirmed using the histo-architecture of the uterus of treated rats because the post-coital contraception effect of neem *A. indica* oil seemed to be non-hormonal. So it would be expected to elicit less side effects than the steroidal. The activity of antifertility was also reported by Talwar et al.(1997). In addition to spemicidal, anti-microbial, anti-fungal and anti-viral properties were also observed (Talwar et al., 1997).

### 2.4.4 Pharmacology

Bioactivities of *A. indica* has been reported.

**Effects on insects**

There are a great bioactivities of *A. indica* against insects. It included insecticidal, antifeedant, oviposition deterrent, systemic, growth regulating and synergistic properties (Atal and Kapur, 1982). Consistent with this, Barnby and Klocke (1990) reported that the final-instar larvae of the tobacco budworm *Heliothis virescens* (Fabr.) exhibited a reduction whole body and haemolymph titres of the moulting hormones, ecdysone and 20-hydroxyecdysone after oral injections of 1 μg of the plant chemical azadirachtin. Also, Isman et al.(1990) observed that there was a larval growth inhibition and antifeedant activity against the variegated cutworm (*Peridroma saucia*) treated with neem seed oil. The moulting disrupting activity against the mink weed bug (*Oncopeltus fasciatus*) was occurred.

The cytotoxicity effects of neem constituents including nimbolide, epoxyazadiradione, salannin, nimbin, deacetylnimbin and azadirachtin were also evaluated in Sf9, cell line of insect *Spodoptera frugiperda*, compared with the two mammalian cell lines, N1E-115 neuroblastoma (mouse) and 143B.TK$^-$ osteosarcoma (human). The most potent of these limonoids was nimbolide. Nimbolide at 10 μM acted rapidly in the neuroblastoma cells to induce blebbing associated with disruption of plasma membranes instantaneously and 50% loss of cell viability within 30 min. At 5 μM nimbolide, the cells became elongated and assumed a neuronal shape accompanied by spikes and lamellipodia within 1-2 hr followed shortly thereafter by extensive cytological changes and vacuolization associated with irreversible processes leading to cell death (Cohen et al., 1996). However, Mulla et al.(1997) studied about the activity and efficacy of neem products against mosquito larvae *Culex*

*quinquefasciatus* and reported that neem products were good potential for the control mosquito larvae in aquatic habitats. Corresponding, Singh and Srivastava (1997) investigated the effect of two neem seed extracts, an oil-based and a cake-based against mango leaf hopper *Idioscopus nitidulus* (Walker). The result showed a significant reduction in the number of insect population treated with both neem seed extracts.

**Effects on aquatic organisms**

Osuala and Okwuosa (1993) reported that the stem bark extract of *A. indica* caused a hundred percent mortality when tested against three common snail intermediate host species, *Biomphalaria pfeifferi*, *Bulinus truncatus*, and *Lymnaes natalensis* at a concentration of 100 mg/l for 24 hr. A similar work was carried out on fish, *Aphyosemon giardneri* a 96 hr $LC_{50}$ of 15.1 mg/l was recorded (Osuala and Okwuosa, 1993).

Recently, Singh et al.(1996) studied about molluscicidal properties of *A. indica* against the snails *Lymnaea acuminata* and *Indoplanorbis exustus*.The molluscicidal activity of the leaf, bark, cake, neem oil and the neem-based pesticides, achook and nimbecidine were observed both time-and dose-dependent. Whereas the toxic effect of pure azadirachtin against both the snails was greater than the synthetic molluscicides. While Wan et al.(1996) evaluated the acute toxicity of azadirachtin, neem extract, and neem-based products on juvenile Pacific Northwest salmon. The result of the 96-hr $LC_{50}$ value of azadirachtin (49% purity) for this fish was recorded greater than 4 mg/l. The most toxic neem material to young salmon was neem extract. However, the toxicity of neem-based products to the fish depended on the solvents and emulsifiers used in formulating the materials, with 96-hr $LC_{50}$ values ranging from 4 mg/l to 72 mg/l (Wan et al., 1996).

In 1997 Tangtong and Wattanasirmkit also determined the 96-hr $LC_{50}$ of neem seed extract and reported that the $LC_{50}$ value of neem seed extract for 96 hr was 80.27 ppm for tilapia *Oreochromis niloticus* at the age of 4 weeks. Moreover, the investigation of the acute toxicity of neem extract on blood of tilapia *Oreochromis niloticus* was studied. Noticeable differences were observed in various types of leukocyte number including lymphocyte, monocyte, neutrophil, basophil and eosinophil number. There was a significant decrease in lymphocyte number and a significant increase in neutrophil, basophil and eosinophil number in fish

exposed to 80 ppm neem extract for 96 hr. In case of monocyte number, there was no obviously change. Moreover, morphological anomalies of erythrocyte were altered in the nuclear chromatin, location, size and the shape of the nucleus in addition to the occurrence of spiny plasma membrane (Tangtong and Wattanasirmkit, 1997).

**Effects on other vertebrates**

Cardiovascular effects of *A. indica* leaf extract on guinea pigs and rabbits were evaluated by Thompson and Anderson (1978). The effects including profound hypotension and a minimal negative chronotropic effect increased at higher doses. The heart rate was obviously decreased in rabbits but remained nearly constant in the guinea pig, this difference was species related. As Sinniah et al.(1989) described that Margosa oil, an extract of the seeds of neem was exhibited to cause a Reye-like syndrome with death from hepatoencephalopathy in children of Malasia and India. The experiment performed with Margosa oil administered mice presented the development of hepatic lesions with many features of this disease. Besides, Lai et al.(1990) also noted that Margosa oil caused toxic encephalopathy particularly in infants and young children. The results of leukocytosis and metabolic acidosis were significant laboratory findings. However, Tewari et al.(1989) noted that subcutaneous administration of neem oil to cyclic rats caused significant damage to the luminal epithelium of the uterus and uterine glands. It also decreased in glycogen and total protein contents in the ovary and uterus, while the activity of acid phosphatase in these organs was significantly increased. Recently, Upadhyay et al.(1992) studied about the immunomodulatory effects of neem oil in mice and demonstrated that neem oil had immunomodulatory properties.

The data on toxic effects of neem on vertebrates have been also examined by using the haematological and biochemical parameters as a biomonitoring. Such Gangopadhyay et al.(1979) noted that Murrah buffaloes given neem seed cake with food did not show any haematological changes. However, they reported that there was a decrease in serum protein when the content of neem cake in the concentrate mixture was higher. Also, Vijjan et al.(1982) reported that there was no significant difference in the values of blood glucose, haemoglobin content and urea nitrogen in control and experimental lambs fed neem cake. Pillai and Santhakumari (1984) mentioned that

nimbidin, a component of neem seed affected the increasing of haemoglobin level and decreasing in eosinophil number in albino rats received nimbidin at 25, 50 and 100 mg/kg. Furthermore, a dose-related effects including a significant increase in liver glycogen and reduction in serum protein were shown in 100 mg/kg group. For the result of alkaline phosphatase activity in serum, there was no change in all groups. While Gandhi et al.(1988) reported that the significant increase in serum values of total bilirubin and GOT were observed in rats and rabbits treated with neem oil and reported that an early damage of liver function ocurred.

Akah et al.(1992) investigated the hepatotoxic effect of *A. indica* leaf extract in rabbits, using enzymes indices of hepatic dysfunction (GOT GPT and ALP).The result of the significant rise of the enzymes was not found in rabbits given 1746 mg/kg but increasing the dose of the extract to 2328 mg/kg resulted in a significant rise of the enzymes. These results suggested that in high dose, the leaf extract of neem might have some hepatotoxic effects. While Ibrahim et al.(1992) reported that Brown Hisex chicks fed diets containing 2% and 5% neem leaf exhibited the clinicopathological changes including the increasing of lactic dehydrogenase, glutamic oxaloacetic transaminase, alkaline phophatase activities, uric acid and bilirubin concentrations and the decreasing of the total protein levels in serum. Changes in values of erythrocyte count, haemoglobin concentration, packed cell volume, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were remarkable. These reults indicated severe liver damage.

## 2.5 Nile tilapia *Oreochromis niloticus* Linn.

### Taxonomy

Phylum Vertebrata
Class Osteichthyes
Order Perciformes
Family Cichlidae
Genus Oreochromis
Species *Oreochromis niloticus*

Common name : Nile mouth-Brooder and Nile tilapia
Thai common name : Pla Nil

**Charateristics and biology**

*Oreochromis niloticus* is endemic to Africa and Palestine. The distribution of species has been widened by artificial introductions, mainly since the 1950s, to include much of the tropics and subtropics. It was introduced to Thailand via Japan in March 1965. Nowaday, it is one of the most economically freshwater fish for various kind of aquaculture throughout the country.

The species is distinguished from other perch-like fish in having one nostril on each side of the snout. Its body is fairly elongate, moderately deep and greatly compressed. It has a dorsal fin with long base, spinous dorsal fin with 16-17 spinous finrays, followed by 11-15 soft finrays. Its anal fin is pretty short, consisting of 3 spinous finrays and 8-11 soft finrays. Scale is fairly large, cycloid, 2-3 series on cheek. The variation of color is influenced by breeding season and its wide habitats or distribution. The upper posterior margin of gill is covered by a black spot. Vertical fins, usually bordered with red, marked with many broken dark crossed bands (Wongratana, 1996). Nile tilapia exhibit a high degree of parental care. The female takes the eggs as soon as they are fertilized to special nursery areas where she holds in her mouth until the yolk is sufficiently reduced for them to swim freely (Lowe-McConnell, 1959).

In many developing countries, it is a major protein source. Furthermore, it has many attributes that recommend for culture. Since Nile tilapia shows rapid growth rates on low protein diets, tolerate wide ranges of environmental conditions, exhibits little susceptibility to disease and is amenable to handling and captivity. Also, it has a short generation time and breeds in captivity. Most important of all, it enjoys wide acceptance as food fish because of its good taste. With all these advantages, there is a promotion of this fish culture in many ways. Such Little et al.(1996) reported that Nile tilapia *Oreochromis niloticus* was the major species stocked in rice fields. Besides, it is also used as an experimental animal for many purposes of study. For example, Yi et al. (1996) evaluated influence of Nile tilapia *O.niloticus* stocking density in cages on their growth and yield in cages and in ponds containing the cages. At the same time, Gunasekera et al. (1996) studied about influence of protein content of broodstock diets on larval quality and performance in Nile tilapia *O.niloticus*. In the same period, Tacon et al. (1996)

observed the relationships between the expression of maternal behavior and ovarian development in the mouthbrooding cichlid fish *O.niloticus*. The study of toxic effects of various substances also used *O.niloticus* as a model species. Such as Alkahem (1994) studied about the toxicity of nickel and the effects of sublethal levels on haematological parameters and behavior of the fish *O.niloticus*. Bainy et al. (1996) evaluated oxidative stress in gill, erytrocytes, liver and kidney of Nile tilapia *O. niloticus* from a polluted site.

Therefore, Nile tilapia *O.niloticus* is widely studied in many fields. Also, in this study it was selected to study in the topic of haematological alteration after long-term low level exposure to neem *Azadirachta indica* seed extract on Nile tilapia *O.niloticus*. Because of haematological evaluation of fish blood is one of the most reliable tools to be an aid in the diagnosis of diseases and pathological state caused by chemicals and other anthropogenic materials or xenobiotics. Moreover, it is used as biomarkers to assess the health status of aquatic organisms and to obtain early-warning responses of environmental risks.

## 2.6 Fish blood

Blood is a specialized form of connective tissue and consists of formed elements or blood cells, and fluid intercellular substance, the blood plasma. The functions of the two components are occasionally separate and share by both component. The remaining components of plasma include a limited number of inorganic ions and a wide variety of organic compound relating to most metabolic functions. In case of cellular composition, it consists of discrete cells having distinctive anatomy and discrete functions (Smith, 1991).

### 2.6.1 Type of cells

There are several types of blood cell that are identified in varoius species of fish. Species difference were related to type of cells which ranging from 4-8 types. For example, the classification of peripheral blood cells of brown trout *Salmo trutta* (L.) was divided into five types including erythrocyte, small lymphocyte, large lymphocyte neutrophil and blast cell (Blaxhall and Daisley, 1973). McCarthy et al. (1973) oberved that there were three kinds of white blood cell in the rainbow trout *Salmo gairdneri* Richardson which included lymphocyte,

granulocyte and thrombocyte. As blood cells of *Tilapia zillii* consisted of lymphocyte, monocyte, neutrophil, eosinophil and basophil (Ezzat et al., 1974). Moreover neutrophil was identified into metamyelocyte and myelocyte. There were six types of blood cells in the channel catfish *Ictalurus punctatus* that consisted of thrombocyte, lymphocyte, neutrophil, monocyte, eosinophil, basophil, granular anucleate bodies, and erythrocyte (Williams and Warner, 1976). In the plaice *Pleuronectes platessa*, leukocyte was identified into four main types such lymphocyte, thrombocyte, monocyte and neutrophil (Ferguson, 1976). For the blood cells of the dogfish *Scyliorhinus canicula* were also reported by Morrow and Pulsford (1980) that they included lymphocyte, plasma cell, monocyte, thrombocyte and granulocyte. The peripheral blood leukocytes of *Oreochromis mossambicus* were lymphocyte, thrombocyte, monocyte and granulocyte (Doggett et al., 1987). While Watson and colleague (1963) reported that the blood of goldfish *Carassius auratus* consisted of erythrocyte, thrombocyte, lymphocyte, neutrophil, eosinophil and basophil. However, Fujimaki and Isoda (1990) observed that the blood of this fish included eight types of blood cell including neutrophil, eosinophil, large granular leukocyte, medium-sized granular leukocyte, small granular leukocyte and fine granular leukocyte in addition to lymphocyte and monocyte.

### 2.6.2 Morphology of blood cells

Various types of fish blood cell were identified according to their general form and affinity to dyes. The main types of blood cells which were often found in many fish, were erythrocyte, thrombocyte, leukocytes including lymphocyte, monocyte, neutrophil, basophil and eosinophil.

**Erythrocyte**

In the channel catfish *Ictalurus punctatus*, this cell was an oval shape with an eccentric nucleus staining a deep bluish color and the chromatin material is densely clumped. The cytoplasm stained a pale reddish color with Wright's stain (Williams and Warner, 1976).This description was consistent with given in the brown trout *Salmo trutta* (Blaxhall and Daisley, 1973). Watson et al.(1963) also reported that mature erythrocyte in the blood of goldfish *Carassius auratus* was nucleated elements, ellipsoidal in shape, containing a centrally located,

biconvex, elongated nucleus. The cytoplasm stained pink to orange with Wright's stain, similar to hemoglobin containing cells of other animals and the nucleus was a dense, dark blue to purple staining body with a thick purple chromatin network (Watson et al., 1963).

**Thrombocyte**

There was numerous haematological researches on fish blood that reported this cell type. In channel catfish *Ictalurus punctatus*, this cell has a thin rim of cytoplasm around the nucleus. The cytoplasm stained a light blue color with Wright's stain. However, thrombocyte shapes were variable being either round, elongated or spindle forms (Williams and Warner, 1976). Similar characteristic was observed in the brown trout *Salmo trutta* (L.) (Blaxhall and Daisley, 1973) the paice *Pleuronectes platessa* (Ferguson, 1976) and the dogfish *Scyliorhinus canicula* L. (Morrow and Pulsford, 1980). In the goldfish *Carassius auratus*, thrombocytes varied from 3.0 to 4.5 μm in diameter. This cell was found both round and spindle-shaped with stain dark blue to violet (Watson et al., 1963).

**Lymphocyte**

Lymphocyte was frequently found in fish. It was spherical in shape having a round nucleus. The nucleus stained purple blue with Giemsa stain, with a dense finely-clumped chromatin in *Tilapia zillii* (Ezzat et al., 1974). Like characteristic was investigated in *Salmo trutta* (L.) (Blaxhall and Daisley, 1973). In channel catfish *Ictalurus punctus*, this cell was the same as the description in *Tilapia zillii* (Williams and Warner, 1976). Fujimaki and Isoda (1990) mentioned that these cell were abundant in the circulating blood of the goldfish *Carassius auratus*. Ferguson (1976) reported that lymphocyte in the paice, *Pleuronectes platessa* had numerous fine pseudopodia extending from a cytoplasm.

**Monocyte**

Monocyte was found in the plaice *Pleuronectes platessa* (Ferguson, 1976) , the dogfish *Scyliorhinus canicula* L. (Morrow and Pulsford, 1980) , the goldfosh *Carassius auratus* (Fujimaki and Isoda, 1990) , the channel catfish *Ictalurus punctatus* (Williams and Warner, 1976) and *Tilapia Zillii* (Ezzat et al, 1974). However, this cell was not

found in some species of fish such *Salmo trutta* (Blaxhall and Daisley, 1973). Monocyte had an oval eccentric nucleus in *Tilapia zillii* (Ezzat et al., 1974) and the dogfish *Scyliorhinus canicula* (Morrow and Pulsford, 1980). However, nuclei were sometimes showed pleomorphism such as intended, kidney-shaped or bilobed form (Blaxhall and Daisley, 1973; Ferguson, 1976; Williams and Warner, 1976; Fujimaki and Isoda, 1990). The cytoplasm stained a blue gray color with Wright's stain. Vacuoles were present ranging from few and small to many and large size (Williams and Warner, 1976).

**Neutrophil**

Neutrophil was infrequently observed in the peripheral blood of fish. Some researchers reported that they found this leukocyte in their experimental fish such as Watson et al.(1963), Ezzat et al.(1974), Williams and Warner (1976) , Fujimaki and Isoda (1990) and Ferguson (1976). Especially Ferguson (1976) reported that neutrophil was the most numerous type in the circulating blood of the plaice *Pleuronectes platessa*. The characteristic of neutrophil in *Tilapia zillii* was spheroid and somtimes oval. It was ranged between 10.8-12.6 μm in diameter. An oval nucleus located close to the edge of the cell. The cytoplasm stained pale blue There were numerous fine granules stained dark violet with Giemsa stain (Ezzat et al., 1974). The appearence of a small nuclear appendage or "drumstick" was observed on a few neutrophilic nuclei of male channel catfish (Williams and Warner, 1976). In the goldfish *Carassius auratus*, most nuclei were band forms but sometimes kidney-shaped and bilobed. Trilobed nuclei were also occasionally observed (Fujimaki and Isoda, 1990).

**Basophil**

Most haematological research on fish blood indicated that basophil was absent in case of the peripheral blood of *Cyprinus cyprinus*, *Cyprinus 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 *Salmo trutta* (Blaxhall and Daisley, 1973) , the plaice *Pleuronectes platessa* (Ferguson, 1976) and the goldfish *Carassius auratus* (Fujimaki and Isoda, 1990). However, this cell was described in the channel catfish *Ictalurus punctatus* that the nucleus was eccentrically

located and was oval in shape. The cytoplasm stained light blue, almost colorless with Wright's stain. Also the presence of small darkly staining granules was in the cytoplasm (Williams and Warner, 1976). In *Tilapia zillii*, basophil was the smallest granulocyte which ranged between 9.7 and 11.0 μm (Ezzat et al, 1974).

**Eosinophil**

Normally, eosinophil is difficult to find in a whole blood smear of fish. There was a few information on this cell type of fish, for example, in *Tilapia zillii* (Ezzat et al., 1974), the channel catfish *Ictalurus punctatus* ( Williams and Warner, 1976) and the gold fish *Carassius auratus* (Fujimaki and Isoda, 1990). There was no report of this granulocyte in the peripheral blood of *Cyprinus cyprinus*, *Cyprinus carpio*, *Misgurnus fossilis* (Onada, 1934), *Perca flavescens* (Yokayama, 1947), *Salmo namaycush* (Lieb et al., 1953), *Anguilla japonica* (Sano, 1957), *Prosopium williamsoni* (McKnight, 1966), and the brown trout *Salmo trutta* (L.) (Blaxhall and Daisley, 1973) , and the plaice *Pleuronectes platessa* (Ferguson, 1976). This cell was an eosinophilic coarse granulocyte and was sometimes round or oval in shape. The nucleus was noted to be elliptical in shape (Williams and Warner, 1976). Similar description was observed in the goldfish *Carassius auratus* (Fujimaki and Isoda, 1990).

But granulocyte was sometimes unable to classify into neutrophil, basophil and eosinophil since these white blood cells, were shown the morphological structure of neutrophil, basophil and eosinophil no obviously. Onada (1934) reported that there were two types of leukocyte in fish species *Cyrinus cyprinus* and *Misgurnus fossilis* including lymphocyte and granulocyte.

### 2.6.3 Anomalies of blood cell

Buckley (1977) reported that the abnormality of fish blood cell was observed in fingerling coho salmon *Oncorhynchus kisutch* exposed for 2 weeks to various concentrations of treated waste water containing total residual chlorine ($TRCl_2$) with riverwater diluent under continuous flow conditions. The result of exposure to levels of $TRCl_2$ averaging 0.003-0.05 mg/l exhibited pathological changes in erythrocyte. These alteration consisted of poikilocytosis and damaged cells with an increase

nuclear interchromatin spaces. A "ragged" appearance of cytoplasmic membranes and microcytosis were also observed. Gray or basophilic cytoplasm was observed. The further anomalies such dividng cells and cytoplasmic fragments from both mature and immature erythrocyte and hypochromic erythrocyte were seen. Besides, the spindle-shaped erythrocyte and spherocytosis appeared. The anomalous erythrocyte was also found in the freshwater fish *Puntius conchonius* Ham. exposure to 0.63 and 0.84 mg/l cadmium chloride for 30 and 90 day. These morphological aberrations included cytoplasmic vacuolation, hypochromia, deterioration of cellular membrane, basophilic stippling of cytoplasm, clumping of chromatin material and extrusion of nuclei and schistocytosis. Moreover, anomalous basophils and monocytes were noted (Gill and Pant, 1985). Consistent with this, Katz, (1950) reported that some anomalous erythrocytes were seen in the blood smear of the diseased silver salmon *Oncorhynchus kisutch*. These included round shape, basophilic cytoplasm and the lack of condensed basic chromatin material in the nuclei. In addition to the vacuolated nucleus and ragged nuclear membranes were also observed.