Chapter 2

Literature Review

2.1. Neem *Azadirachta indica* A. Juss.

General classification of *A. indica* is :

Kingdom Plantae

Division Spermatophyta

Class Angiospermae

Subclass Dicotyledoneae

Order Rutales

Family Meliaceae

Genus *Azadirachta*

Species *Azadirachta indica*

Common name : Neem

Thai name : Sadao

Neem *Azadirachta indica* A. Juss. (Syn. *Melia indica* (A. Juss.) Brand and *Melia azadirachta* L.) belongs to the Meliaceae family. Neem tree is a hardy, fast-growing evergreen tree with a straight trunk, long, spreading branches, moderately thick bark and round crown. Mature trees attain heights of 7-20 metres with a spread of 5-10 metres. They start producing fruits in 4-5 years, become fully productive in 10 years and may live for more than 200 years (van der Nat et al., 1991). In Thailand, there is another variety of *A. indica*. Known as Thai neem. It is *A. indica* var *siamensis* Valeton. Some authors distinguish this variation as a species *Azadirachta siamensis* (Sombatsiri, 1997).

Figure 2-1 The neem tree, *Azadirachta indica* A. Juss.

A. indica is endemic in the Indo-Pakistan sub-continent (van der Nat et al., 1991). It grows in tropical zone, altitudes between 50 and 1000 metres, as little rainfall as 130 mm per year and long stretches of drought (Ketkar, 1982). It is found in South Asia in India, Pakistan, Bangladesh, upper Myanmar and the drier part of Sri Lanka. In Southeast Asia the species occurs in Thailand, Southern Malaysia and Indonesian islands (van der Nat et al., 1991).

Chemical constituents

Many chemical constituents were isolated from the extract of neem. They were divided into several structure classes according to van der Nat et al. (1991) as presented below.

a. Terpenes and Steroids

The diterpenes were isolated from bark while triterpenes/steroids including a major group, limonoids (nortriterpenoids) were isolated from many parts of the tree such as trunk wood, fresh leaves, dried fruits, dried seeds and seed oil. Some well-known active compounds included in the group of limonoids were azadirachtin (Figure 2-2), azadirachtol, nimbin, nimbolide, nimbolin and salannin.

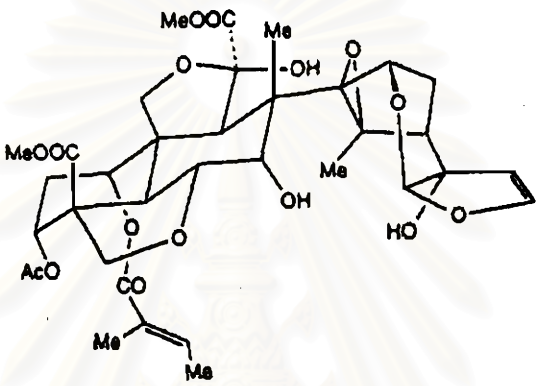

Figure 2-2 Structural formula of azadirachtin, an active constituent of neem seed extract (Schroeder and Nakanishi, 1987).

b. (Poly) phenolics

The flavonoids were found in heartwood, flowers and leaves. Flavonolglycosides were found in flowers and leaves. Dihydrochalcones were isolated from fruits. Tannins were condensed from bark, and coumarins were extracted from leaves.

c. Carbohydrates and proteins

The gum exuded from the stem of *A. indica* is a very complex combination of proteins and heteropolysaccharides. Some amino acid composited in *A. indica* were reported such as aspartic acid, glutamic acid, leucine, valine, serine, proline and glycine (Anderson, Hendrie and Munro, 1972). The stem bark has been reported to contain several biologically active carbohydrates such as arabinose, glucose, galactose and glycosamine.

d. Sulphurous compounds

Some parts of *A. indica* have strong garlic odour and its medicinal properties have been attributed to the presense of sulphur containing compounds. Mubarak and Kulatilleke (1990) indentified some sulphurous compounds from volatiles of fresh neem seeds. They are dipropyl disulphide, cis and tran-1-propenyl-1-propyl disulphide.

e. Others

In neem fruits there is aromatic ester, nimbocetin. Oxalic acid, acetic acid, pyruvic acid and tiglic acid are found in leaves and seeds. The seed oil contains some fatty acids.

Biological activities

Several studies about biological activities of various neem preparations have been documented in a wide spectrum. Several groups of the biological effects were classified by van der Nat et al. (1991) into 11 groups. They were the effects on insects anti-inflammatory antirheumatic and anti-arthritic effects, antipyretic effects, antimalarial effects, antimicrobial activity, antitumor effects, effects on the central nervous system, cardiovascular effects, antiulcer effects, antidiabetic effects and antifertility effects. Some other activities were reported by Ketkar (1982) for example antifungal activity, antiprotozoal activity, antiallergic activity and effects on dental diseases.

The effects of neem on insects resulted in using of neem products as insect repellent and insecticide. Neem insecticides have multifactorial action on insect. Their insecticidal and related properties were documented including insecticidal, antifeedant, oviposition deterrent, systemic growth regulating and synergistic property (Ketkar, 1982). Larvicidal activity of neem products were also documented. Monzon et al. (1994) reported larvicidal potential of neem extract against two mosquito species *Aedes*

aegypti Linn. and *Culex quiquefasciatus* Say. A study by Miller and Chamberlain (1989) indicated that azadirachtin is efficacious against larval horn flies, stable flies and house flies (Diptera: Muscidae). In addition, the effects of azadirachtin were also reported on insect hormones, ecdysone and 20-hydroxy ecdysone activity (Barnby and Klocke, 1990; Warbrick et al., 1993) and on ecdysone-20-monooxygenase activity (Smith and Mitchell, 1988; Mitchell et al., 1997). This information supported the growth regulating property of neem products. Moreover, current study by Su and Mulla (1998) revealed the ovicidal activity of azadirachtin against *Culex* mosquitoes (Diptera: Culicidae). From the information above, it can be said that neem products affected wide range of insect species in wide range in mode of action.

Reproductive property of neem products have been studies for long time especially the antifertility effects. Studies on antifertility of the products were conducted on several animal models range from insects, rats, rabbits and monkeys to human. In red cotton bug, *Dysdercus cingulatus* Fabr., oocyte differentiation, fecundity and hatchability were inhibited by neem product (Thomas and Hiradhar, 1993). Adverse effects of neem extracts on egg laying and hatching were reported in the tick *Boophilus microplus* (Canest) (Williams, 1993). The gonotrophic cycle of female *Anopheles stephensi* and *An. Culicifacies* (Diptera: Culicidae) was impaired, as well as oviposition was suppressed by exposure to volatiles of neem (Dhar et al., 1996). In mammals, a study of antifertility effect of neem oil in female albino rats showed 80 pecent antifertility activity by oral route (Lal et al., 1986). Another study on female albino Wistar rats suggest that the active conpounds of neem oil were absorbed through the vaginal mucosa into the circulation and exerted antifertility efffect (Riar et al., 1988). The reversible antifertility effects of neem oil were also reported in female Wistar rats (Upadhyay et al., 1990) and bonnet monkeys (Upadhyay et al., 1994). In male reproductive system, Garg et al. (1993) stated that a purified extract from the dried seeds of neem showed high spermicidal activity in rabbits and monkeys after intravaginal application. Moreover, there was a report revealed that the seed oil of neem

proved spermicidal against rhesus monkey and human spermatozoa *in vitro* (Sinha et al., 1984 cited in van der Nat et al., 1991). Because of its botanical origin, insecticide from neem was classified as a biopesticide. Neem biopesticides were increasingly used throughout the world including in Thailand. Their wide spectrum in mode of action and their low persistance made them to be used as a new safer choice for farmer in pest management nowaday.

Toxic effects

Although the biopesticide from neem were known to be safer and less persistent in environment when compared with other xenobiotic control agents, they were not without risk. Current study revealed that azadirachtin is as persistent as the carbamates and pyrethroids in water and soil (Stark, 1997). Many toxic effects of neem products were documented in many toxic levels on several species.

Nontarget effects of neem-based insecticides on aquatic invertebrates were reported by Kreutzweiser (1997). A study by Ibrahim et al. (1992) documented the toxic effects of neem leaves on growth, haematological constituents, spleen, liver and kidney of Brown Hisex chicks. In fish species, haematological alterations were also reported on Nile tilapia (Tangtong, 1997) as well as hepatotoxic effects (Janart, 1997). Pathogenesis in liver of children caused by neem oil poisoning were also stated in studies of Sinniah et al. (1989) and Sundaravalli, Bhaskar Raju and Krishnamoorthy (1982). From the study of acute toxicity of neem oil on rats and rabbits pointed out that lungs and central nervous system were target organs of toxicity on these animals (Gandhi et al., 1988). Studies of margosa (neem) oil poisoning were also reported the toxic encephalopathy by ingestion in Malaysian and Indian childrens (Sinniah et al., 1989; Lai, Lim and Cheng, 1990)

Furthermore, mutagenic activity of neem products, nimbolide and nimbic acid was documented on some bacteria strains (Rojanapo et al., 1985). Consequently,

current study by structural analyses showed that biopesticide from neem has the potential for acting as a genotoxic carcinogen (Rosenkranz and Klopman, 1995). Cytotoxic effects on neuroblastoma (mouse), osteosarcoma (human) and Sf9 cultured cell lines of insects were also reported (Rembold and Annadurai, 1993; Cohen, Quitads and Casida, 1996.)

2.2 Nile tilapia *Oreochromis niloticus* Linn.

General classification of *O. niloticus* is:

Kingdom Animalia

Phylum Chordata

Subphylum Vertebrata (Craniata)

Superclass Gnathostomata

Class Osteichthyes

Order Perciformes

Suborder Percoidei

Family Cichlidae

Genus *Oreochromis*

Species *Oreochromis niloticus*

Common name: Nile tilapia, Nile mouth-brooder

Thai name: Pla Nil

The family Cichlidae is widely distributed in Africa and Palestine, South and Central America, Southern India and Sri Lanka. The tilapias originated exclusively from the African continent and from Palestine. The original distribution of *O. niloticus* is the african continent. The species, originating from the upper Nile in Uganda evidently moved southwards, colonize all the western Rift lakes down to Lake Tanganyika. It also colonize central and western Africa, via the Chad and Niger basins. Introduction of tilapias outside Africa was begun since 1939. Now tilapias occur in natural waters

throughout the tropics, even in Australia (Philippart and Ruwet, 1982). They are fishes of economic importance in tropical and subtropical countries.

O. niloticus was first introduced to Thailand in March 1965 by His Royal Highness Akihito, the Prince of Japan. Consequently, they were given to the Department of Fisheries for further development of culturing by His Majesty the King of Thailand (Phumipat, 1981). Nowaday, Nile tilapia is an essential food fish, widely cultured in many areas throughout Thailand.

Nile tilapia is a well known fresh water fish of the big order Perciformes. The species is distinguished from other perch-like fishes in having one nostril on each side of the snout. Its body is fairly elongate, moderately deep and greatly compressed. Dorsal and ventral profiles about equally convex. 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. Caudal peduncle broadly short. Mouth slightly oblique, portractile, broad with swollen lips. The variation of color is influenced by breeding season and its wide habitats or distribution. The upper posterior margin of fins is covered by a black spot. Vertical fins, usually bordered with red, marked with many broden dark crosed bands (Wongratana, 1996).

All the tilapias, in the broad sense, have in common a mainly herbivorous diet. Structural adaptations to this diet are the long, coiled intestine, the bicuspid and tricuspid teeth of the jaws and the small, sharp pharyngeal teeth. (Trewavas, 1982).

2.3 Reproductive biology of Tilapia

Determination of sex and maturity

It is difficult to sex the Nile tilapia during juvenile stage or during nonbreeding season. Only one external feature used to determine the sex of tilapia is genital papillae. The female is distinguished from the male in having genital papillae with two openings while the male have only urogenital pore on their genital papillae (Tangtrongpairoj et al., 1993).

Advanced sexual maturity in some species of fish is accompanied by obvious external changes in pigmentation (Crim and Glebe, 1990). In breeding time, the tilapia express their sexually dimorphic pigment patterns so their sex can be detrmined easily. Higher pigmentation is presented in the male than in the female (Tangtrongpairoj et al., 1993; Phumipat, 1981).

Determination of maturity by sizing is low accurate because the size to which the fish grow, and at which they mature, varied greatly. Fish in poor condition mature at, and grow to, a smaller size than fish in good condition (Lowe-McConnell, 1982). The relative gonad weight or gonadosomatic index (GSI) is commonly used as a simple index of reproductive maturity of fish. Besides, full reproductive maturity may be externally detected by the easy expulsion of eggs or sperm when the abdomen is pressed (Crim and Glebe, 1990). At that time of reproductive development their fecundity may ranges from a few hundred eggs to several thousands per spawning (Guerrero III, 1982).

Reproductive behavior

All tilapias exhibit a high degree of parental care and in this function they are divided into substrate-spawners (guarders) and mouthbrooders (bearers). *O. niloticus* is a maternal mouthbrooder. The species have a reproductive schema that excludes the males from the care of the brood. The female takes the eggs as soon as they are fertilized to special nursery areas where she holds them in her mouth until the yolk is sufficiently reduced for them to swim freely (Trewavas, 1982). Spawning frequency of

the female is several times but not more than six or seven times in one year (Mires, 1982).

Female reproductive histology

Reproductive development and reproductive histology in female are well understood by histological study. Histology is the most accurate method to determine the reproductive state of female fish (West, 1990). The ovarian histological pattern of teleosts was described according to Crim and Glebe (1990). They proposed the division of ovarian tissues into seven or eight stages of maturity based upon the dominant gametogenic cell type present. Previtellogenic (immature) oocytes are small, spherical ovarian cells containing a central nucleus and increasing amounts of cytoplasm (stages 1-3). Vitellogenic (maturing) oocyte (stages 3-6) incorporate the yolky materials produced by the liver. Yolk granules aggregate first at the periphery and later towards the center of the egg. Mature oocytes (stage 7) are the largest and are filled with yolk. The spent ovary (stage 8), found in females that have spawned, contains empty follicles and postovulatory structures termed *corpora lutea*.

Basic study on histology of Nile tilapia, *O. niloticus* is still limited especially in reproductive system. Some basic knowledge was mentioned by Husssain, Penman and McAndrew (1996) that diploid ovaries from the fish of six to eight months of age contained oogonia and maturing previtellogenic and vitellogenic oocytes with irregular nuclei and vacuolated cytoplasm associated with endogenous and exogenous yolk formation.

2.4 Toxicology

Toxicology is both a science and an art. The science of toxicology is defined as the observational and data-gathering phase, whereas the art of toxicology is the

predictive phase of the discipline (Gallo and Doull, 1991). Toxicology is the study of the adverse effects of chemicals on living organisms. The variety of potential adverse effects and the diversity of chemicals present in our environment in combination make toxicology a very broad science. So modern toxicology are diversed and widespreaded in different areas. Toxicologist usually divide the exposure of animals to chemicals into four categories: acute, subacute, subchronic, and chronic. The principal goals of the subchronic study are to establish a no-observable effect level and to further identify and characterize the specific organ(s) affected by the test compound after repeated exposure (Klaassen and Eaton, 1991).

Aquatic toxicology is the science which concerned with the effects of toxicants on aquatic organisms. The purposes of aquatic toxicity tests is first to determine which concentrations of a substance are harmful to fish and other aquatic organisms and which have no apparent effect. The data provided by the tests may be assembled to derive water quality criteria. The second reason of the tests is to monitor the toxicity of effluents including runoffs or evaluate the quality of surface waters. The third purpose in aquatic toxicology is to provide basic in any scientific research (Sprague, 1990).

Reproductive toxicology is the occurrence of adverse effects on the male and female reproductive system that result from exposure to chemical or physical agents (Klaassen and Eaton, 1991). The three-part system of reproductive toxicology test adopted by the FDA in 1966 (Palmer, 1977) is basically consists of:

1. An investigation of effects on fertility and general reproductive performance.
2. An investigation of effects during pregnancy and in particular the potential to cause malformation of the offspring.
3. An investigation of effects during late pregnancy and lactation, in particular the potential to cause damage during late fetal and early neonatal development.

Effects on aquatic organisms

By the end of the 20th century numerous toxic effects of chemicals and other anthropogenic materials on various aquatic organisms have been reported. Begin with small biota, effects on protozoan and algal species by metal and pesticide contamination in freshwater ecosystem were documented (Fernandez-Leborans and Novillo, 1995; Wellnitz and Sheldon, 1995; Fargasova and Kizlink, 1996). Several studies revealed the toxic effects of pesticides on some aquatic invertebrates such as *Daphnia magna*, *Gammarus fasciatus* and *Dreissena polymorphia* (Fisher et al., 1991; Muñoz, Ramos and Tarazona, 1996; Barry et al., 1996; Amyot et al., 1996). Moreover, Ashley et al. (1996) demonstrated 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) toxicity in crayfish *Pacifastacus leniusculcus* which the induction of cytochrome P450 was presented.

In fish species there were many studies about the toxicity of various toxicants. For example, Pereira et al. (1994) reported the decline of the Striped bass in San Francisco Bay-Delta Estuary resulting from toxicity of organochlorine compounds. Camusso, Vigano and Balestrini (1995) reported the accumulation of trace metals in many target organs of Rainbow Trout in non severe polluted sites. Bainy et al. (1996) found that polluted waters induced oxidative stress in erythrocytes, gill, liver and kidney of Nile tilapia.

Histopathological findings of aquatic toxicology were evidenced in some studies. Histopathological changes in gills, intestine, liver and kidney of *Oreochromis mossambicus* were detected after exposure to the molluscicides Aquatin and Brestan (Cruz, de la Cruz and Suñaz, 1988). Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium *Microcystis aeruginosa*, on common carp (*Cyprinus carpio* L.) were reported by Rabergh, Bylund and Eriksson (1991). Benerjee and Bhattacharya, (1997) also documented the histopathological changes induced by

chronic nonlethal levels of elsan, mercury and ammonia in liver of *Channa punctatus* (Bloch).

Even in aquatic mammals, Beck et al. (1997) reported contamination of heavy metals in livers of Bottlenose Dolphins in South Carolina. Organochlorine levels were also detected in Mediterranean monk seal (*Monachus monechus*) from the western Mediterranean and Sahara coast in level that can cause immune depression and reproductive impairment (Borrell, Aquila and Pastor, 1997).

Reproductive schema of aquatic toxicology

Reproductive toxicology in many fish species have been documented especially in a past few decades when environmental damage and its adverse effects were concerned.

Dey and Bhattacharya (1989) pointed out the chronic toxicity of elsan, mercury and ammonia on ovary of *Channa punctatus.* A remarkable decrease in both the number and the diameter of mature oocytes occurred in all cases of treatment. They concluded that all diverse xenobiotics have equal reproductive toxicity in fish.

Sumpter and Jobling (1995) proposed vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. They found that effluent from sewage treatment works contain chemical that induces vitellogenin systhesis in male rainbow trout *Oncorhynchus mykiss.*

Ungerer and Thomas (1996) reported the binding of *o,p'*-DDT by plasma lipoproteins and its accumulation in the ovaries of Atlantic croaker (*Micropogonias undulatus*). The results suggest that the pesticide is carried in the plasma via

lipoproteins and that oocyte uptake of triglyceride-rich VLDL is a major mechanism of *o,p'*-DDT accumulation in croaker ovaries.

Mondal, Mukhopadhyay and Bhattacharya (1997) reported the induction of 3β-hydroxysteroid dyhydrogenase and the accumulation of progesterone in the oocyte of *Channa punctatus* after $HgCl_2$ intoxication. They concluded that inorganic mercury is able to initiate translatable messenger RNA synthesis in fish oocyte at a low degree of intoxication.

Lye et al. (1997) used gonad morphology, hepatosomatic index (HIS) and serum levels of the egg protein vitellogenin (VTG) as indicators to determine reproductive health of wild populations of a marine fish.

Teitge et al. (1998) reported the reproductive toxicology and disposition of 2,3,7,8-tetrachlorodibenzo-p-dioxin in adult brook trout (*Salvelinus fontinalis*) following a dietary exposure. The concentration of TCDD in ovaries and spawned eggs was estimated to be 61 and 39% of the whole body TCDD concentrations, respectively. Accordingly to the delay of spawning in the highest treatment group, they suggested that TCDD might have affected ovulation of the fish.

Usage of histopathological evidence for evaluation of reproductive toxicity in fish was found in a study by Wester and Canton (1986). They carried out histopathological study of *Oryzias latipes* (medaka) after long-term β-hexachlorocyclohexane exposure. The development of testis-ova in males and induction of vitellogenesis in either sex were detected and implied with estrogen-like activity. A study by Holm, Norrgren and Linden (1991) reported the reproductive and histopatological effects of long-term exposure to bis(tributyltin)oxide on the three-spined stickleback, *Gasterosteus aculeatus*. The effects on GSI and frequency of resorbing oocytes were noted.