CHAPTER I

GENERAL INTRODUCTION

A. PERSPECTIVE ON TRADITIONAL MEDICINE AND *MUCUNA* SPECIES

Traditional medicine has been proven to be of significance in modern communities. The WHO estimates that between 65% and 80% of the world's population relies on traditional medicine as their primary form of health care (Manyam and Sanchez-Ramos, 1999). In traditional societies, people use their natural resources to supply fuel, vegetables, fruits, building materials and medicine (Myers, 1983; Balick and Cox, 1996). Plants continue to provide new drugs for treating various diseases before the actual active constituent being chemically synthesized. Herbs, which have been the principal form of medicine in the developing countries, have recently become popular in the developed world (Chevallier, 2000). Not only the direct consumptive use of these plants, in medicines that are recently produced synthetically by chemists, many of the original structures were first discovered in a wild species used in traditional medicine (Primack, 1998). Over 40% of prescription drugs sold in the United States contain ingredients derived from nature, and 25% of them contain at least one component derived from the flowering plants (Foster and Duke, 1990; Primack, 1998). Examples of the plant derived synthetic drugs included an acetyl salicylic acid (aspirin) derived from extracts of willow tree bark, *Salix alba* (Primack, 1998), and a digoxin from the common foxglove, *Digitalis purpurea*, which has been used for treatment of heart failure (Chevallier, 2000).

Eventhough the world's tropical forests occupy less than 10% of the land area, they possess the greatest species diversity. Among the estimated 250,000 plant species believed to exist in the world, tropical rain forests inhabit more than 100,000 plant species (Myers, 1980). These natural resources are being continually screened for new plants that can be used to fight human diseases. One of the approaches used by pharmaceutical companies is to target traditional medical plants for bioactive compound screening (Primack, 1998). Great species diversity in the tropical rain forests and the basis of traditional remedy are thus the invaluable resources of new medicine.

Many species of plants in genus *Mucuna* (family Fabaceae) have been recognized as potential natural medicine. For example, a Chinese medicinal plant, *M. birdwoodiana*, used to treat stagnant blood disease because it contains phenolic compounds that can inhibit prostaglandin biosynthesis (Goda et al., 1987). *M. pruriens*, *M. nivea* and *M. utilis* (Prakash et al., 2001) *M. aterrima*, *M. deeringiana*, *M. gigantea*, *M. holtonii*, *M. mutisiana*, *M. prurita*, *M. sloanei* and *M. urens* (Dexenbichler et al., 1972) contain an active amino acid L-dopa (3-(3,4-dihydroxyphenyl)-L-alanine) generally used in Parkinson's disease treatment. Traced back to an ancient time of Indian system of medicine (about 1,000 B.C.) known as Ayurveda, *M. pruriens* was used in treatment of paralysis agitans (Manyam, 1990). It is now widely utilized by many pharmaceutical companies as an important source of L-dopa. However, the use of *M. pruriens* powder was proven to be more effective than the use of L-dopa alone in Parkinson's disease animal model (Manyam et al., 1996). Reproductive effects of some *Mucuna* species have also been reported. *M. pruriens* is used in traditional medicine of Indo-Pakistan subcontinent for treating male sexual dysfunction. The effect of *M. pruriens* in normal male rats includes improving of mating behavior, libido and mating

potency (Amin et al., 1996). In contrast, *M. urens* has been observed to be a potential male antifertility agent in guinea-pigs. Histological observations at high dose of *M. urens* crude extract showed complete degeneration of spermatozoa in testicular tubules. The spermatid, primary and secondary spermatocytes showed pyknosis (Udoh and Ekpenyong, 2001). In Ayurveda, *M. pruritans* is used in treatment of gynecological disorders (Jadhav and Bhutani, 2005). In China, there is also a report on use of *Mucuna* species in traditional remedy as fertility regulating agents (Kong et al., 1986).

B. THE BLACK KWAO KRUA, *MUCUNA MACROCARPA*

The Northern Thai traditional remedy of Luang Anusarnsoontorn of Chiang Mai had considered three types of herbs known as 'Kwao Krua' as potential treatment for reproductive dysfunction. White type of 'Kwao Krua', *Pueraria mirifica* (Fabaceae), is believed to have effects on female reproduction. Some phytoestrogens have been found in this plant extract including miroestrol (Cain, 1960). Some other isoflavonoids were also found in the extract of the white Kwao Krua, for example, puerarin and mirificin (Ingham et al., 1986). Red type of 'Kwao Krua', *Butea superba* (Fabaceae), is known to have effects on male reproduction. Black type of 'Kwao Krua', the *Mucuna macrocarpa* Wall. (Figure 1-1; synonym: *Mucuna collettii* Lace) has been used as crude drug for treating male sexual dysfunction and believed to have stronger effects on male reproduction than the red type. In Xishuang Banna at the border of Burma and Laos, *M. macrocarpa* has also been used by villagers in traditional medicine (Pei, 1985). Due to the closely related uses of *B. superba* and *M. macrocarpa* in the traditional remedy, documents of *B. superba* might yield some clues about their reproductive effects. Study on chemical constituents of *B. superba* revealed flavonoid (3, 7, 3'-trihydroxy-4'-

methoxyflavone) and flavonoid glycoside (3, 3'-dihydroxy-4'-methoxyflavone-7-O-β-D-glucopyranoside) (Roengsumran et al., 2000). Subchronic toxicity test on male Wistar rats showed the reductions of luteinizing hormone (LH) and testosterone levels in some treatment groups (Bhuntaku, 2000). However, in the treatment on female Wistar rats, there is no effect on physiological changes (Posachai, 2000).

The black Kwao Krua, *M. macrocarpa*, is a large woody climber (30-40 m long), scattered by streams in evergreen forest of Thailand. Leaves are trifoliate with 11-22 leaflets. Inflorescences hang on stem up to 30 cm long with flowers consisting of 5 sepals united into a bell-shaped tube and covered with rough, brown hair (Smitinand, 1977). This plant has been screened for its chemical constituents, of which 3 active compounds were isolated and identified as kaempferol, quercetin and hopeaphenol (Figure 1-2; Roengsumran et al., 2001). Among these 3 compounds, hopeaphenol was found as the highest yield at 1.5×10^{-4}% w/w of fresh stems, whereas quercetin was found at 5.0×10^{-5}% w/w of the fresh stems and kaempferol was found at 3.0×10^{-5}% w/w of the fresh stems (Sookkongwaree et al., unpublished manuscript). Kaempferol and quercetin are flavonoids that have been reported to have many effects on animal reproduction especially on the males (Aravindakshan et al., 1985; Eckberg, 1983; Li et al., 1997; Khanduja et al., 2001; Nass-Arden and Breitbart, 1990). Whereas, hopeaphenol is a stilbenoid tetramer of a well-known phytoestrogen, resveratrol. This suggests that *M. macrocarpa* could affect both male and female reproductions. Recently, there is a report on reproductive effects of *M. macrocarpa* in rat that it had no effect on sex hormone levels and reproductive organs of the males whereas it altered plasma sex hormone levels without histopathological alteration in the ovarian tissues of the females (Thansa, 2003). As suggested in traditional remedy, there is a possibility

that *M. macrocarpa* may be developed and used as natural medicine. Basic knowledge about mode of action of this plant product is thus crucial for safety in pharmaceutical use in human in the future. Until now, scientific reports about the biological effect of crude product from *M. macrocarpa* on reproductive system of animal are limited. It is of interest to know whether it can elicit reproductive effect *in vivo* and what effect it can produce.

C. A FISH MODEL IN REPRODUCTIVE SCREENING ASSAY

Although the effect of natural products on human may be assessed retrospectively, this approach has several limitations and the exact mechanism of injury is not well understood. Screening assay using animal model has been regarded as a superior solution since it allows closer examination on toxic responses and resolves difficulties by providing a direct measure of the effect.

Among wide varieties of animal model used in research, fish model is widely used in toxicological research especially in the field of aquatic toxicology. History of using fish as an experimental model is well summarized in the book *The Laboratory Fish* by Ostrander (2000) which mentioned the onset of using fish in toxicity test since 1940s. Standard procedures for toxicity tests in fish have been written by world renowned organizations such as American Society for Testing and Materials (ASTM), Food and Agriculture Organization of the United Nations (FAO) and U.S. Environmental Protection Agency (EPA). For reproductive screening assay, fish model is frequently used as evidenced in the EPA report on the fish screening assays for endocrine disruption. A set of *in vivo* tests is recommended to conduct on fish model for identifying and characterizing endocrine effects of pesticides, industrial chemicals

and environmental contaminants (EPA, 2002). In assessment of environmental effects of chemicals, acute mammal and fish mortality and chronic sublethal effects in fish are also assigned in "A Method for Ranking and Scoring Chemicals by Potential Human Health and Environmental Impacts" by EPA (1994).

Benefits of a fish model on reproductive toxicity screening include convenience to maintain in laboratory condition, ease of handling and exposing to the test material both via diet and water. Fish gonad produces large number of eggs and sperm in various stages synchronously making it an ideal target organ in reproductive toxicology. Fish also produces a large number of progeny from the same parent allowing a large sample size in an experiment to obtain the median lethal concentration (LC_{50}) with strong statistical significance. Furthermore, among non-mammalian vertebrates, fish is closer to mammals in the activation and detoxication pathways as well as in mode of toxic action (Hodgson and Levi, 2000) suggesting it as the excellent non-mammalian model for the reproductive toxicity study.

Fish reproductive system, though differs from other animals to certain extent, shares similar ultimate goal of producing and supporting male and female germ cells necessary for successful reproduction. Reproductive events in females include proliferating of oogonia in ovary, becoming oocytes, undergoing vitellogenesis, maturation and ovulation, internal/external fertilization and internal/external development of embryos (Connaughton and Aida, 1999). Male reproductive system produces the gametes through spermatogenesis in testis as well as seminal components in accessory ducts necessary for successful breeding (Le Gac and Loir, 1999).

The gonad is unique in the diversity of the regulatory factors involved in normal function. Sex steroid hormones synthesized concurrently in the gonad play roles to

support the aforementioned reproductive processes (Johnson and Everitt, 1995). The major events occur in gonad, both gametogenesis and steroidogenesis, are controlled along the hypothalamo-pituitary-gonadal (HPG) axis by gonadotropin (GTH) from pituitary. Two forms of GTH exist in fish, GTH-I and GTH-II, which express follicle-stimulating hormone (FSH)- and luteinizing hormone (LH)-like effects, respectively. Gonadotropin releasing hormone (GnRH) secreted by the hypothalamus directly controls gonadotropin output. Furthermore, feedback mechanisms act at each level to enhance or diminish various factors and hormone secretions (Connaughton and Aida, 1999).

The Nile tilapia, *Oreochromis niloticus* Linn., is an economic teleost which has been adapted in freshwater for a long time. It is a good model for reproductive screening assay because a homogeneous population can be obtained from one couple of parents to reduce genetic difference between individuals. It is easy to maintain and culture in laboratory condition. Moreover, it is an asynchronous spawner so that the reproductive effect will be screened in all stages of gametes contained in gonad. Basic knowledge on its gonadal morphology and cytology, sexual maturation and reproductive endocrinology has been well documented (Alves et al., 1983; Nakamura and Nagahama, 1985; Nakamura and Nagahama, 1989; Nakamura et al., 1993; Herrera, 1996; Hines et al., 1999; Srijunngam and Wattanasirmkit, 2001).

D. APPROACH USED IN THIS STUDY

The study of subchronic effect of *M. macrocarpa* on reproductive system of the Nile tilapia could be of importance for pharmaceutical research in using this natural product as medicine in the future, as well as aquaculture research in developing plant-

based xenoendocrine substances to increase fish stock production in substitution of synthetic hormones. In toxicological study, a set of appropriate toxicity tests must be assigned in order to obtain information that can be used to evaluate the toxic effects. There are two main principles underlie all descriptive animal toxicity testings, 1) the effects produced by a chemical in laboratory animals, when properly qualified, are applicable to humans and 2) the exposure of the animals to toxic agents in high doses is a necessary and valid method of discovering possible hazards in humans (Eaton and Klaassen, 2001). According to these principles, we thus designed an acute toxicity test as the first experiment to determine the median lethal concentration which is the crucial information for further experiment.

Since the effects of xenobiotics in biological system are not produced unless they and their metabolites reach appropriate sites in the body at a concentration and for a length of time sufficient to produce the effects (Eaton and Klaassen, 2001), reproductive toxicity of xenobiotics is generally assessed in a long-term, low-level experiment. A subchronic study was then assigned to determine the specific effects on the gonad after long-term exposure to the plant extract. Effects of xenobiotics on germ cell development can be assessed using morphology as markers. The endpoint may be positive healthy histological structure with a sign of normal gametogenesis or negative histological alterations or pathogenesis in gonadal tissue. Histology is a good qualitative to semi-quantitative tool widely used in several studies on reproduction in fish and has been recognized as the most accurate for staging reproductive development in fish (West, 1990). The reproductive effect can be best identified morphologically at both cellular and subcellular levels using histological methods for light and electron microscopy.

The effects on steroidogenesis can be assessed using histochemical biomarker. It was firstly reported in 1951 that the steroid producing cells in ovary and testis possess activity of an enzyme which oxidizes Δ^5-3-hydroxysteroids to α,β unsaturated ketones in the presence of diphosphopyridine nucleotide (DPN) as a hydrogen acceptor (Samuel et al, 1951). This enzyme has been known later on as 3β-hydroxysteroid dehydrogenase (3β-HSD) which is an enzyme in early steroidogenic pathway that catalyzes conversion of pregnenolone to progesterone (Johnson and Everitt, 1995). Wattenberg (1958) coupled the oxidation to the reduction of a tetrazolium salt, making histological localization of the reaction possible in tissue sections (Baillie et al., 1966). Histochemical assay for 3β-HSD activity has been used in many studies as a key biomarker of steroidogenesis in target tissues (Guraya, 1976). In addition to the histochemically detected activity, cytological ultrastructures have also been used by many researchers to confirm the steroidogenic potential of the tissue as reviewed by Guraya (1976).

In this study, the reproductive effects of the crude extract from the black Kwao Krua on gonadal structure and function of the Nile tilapia were studied by subchronic experiment. The endpoints for the effects on gonadal structure were determined by histological and ultrastructural biomarkers whereas the endpoints for gonadal steroidogenic function were determined by histochemical and ultrastructural biomarkers.

FIGURES

Figure 1-1: The black Kwao Krua, *Mucuna macrocarpa* Wall. (synonym: *Mucuna collettii* Lace). (A) Inflorescences hanging on stem. (B) Trifoliate leaves. (C) An inflorescence with blackish-purple flowers. (D) Pods with black flattened seeds.

Description from: Smitinand, 1977

Picture source: (A-C) Chuakul et al., 1996; (D) Dr. Wichai Cherdshewasart

Figure 1-1

Figure 1-2: Chemical constituents found in the crude extract from *M. macrocarpa*. (A) Quercetin. (B) Kaempferol. (C) Hopeaphenol.

Picture source: Roengsumran, 2001

Figure 1-2