CHAPTER II

ACUTE TOXICITY OF THE CRUDE EXTRACT FROM

MUCUNA MACROCARPA ON THE NILE TILAPIA

INTRODUCTION

Many species of plants of the genus *Mucuna* have been recognized as potential natural medicines. The black Kwao Krua, *Mucuna macrocarpa* has been used in traditional Thai remedy for a long time for treatment of male sexual dysfunction. The study on chemical constituents of this plant extract revealed three interesting compounds including two flavonoids, kaempferol and quercetin, and a stilbenoid, hopeaphenol (Roengsumran et al., 2001). The biological effects of these compounds are well documented. Whereas the reports on biological effects of *M. macrocarpa* in form of crude extract as being used in traditional medicine are still limited especially in toxicological perspective.

Because of the possibility that the products from the black Kwao Krua, *M. macrocarpa* may be developed and become commercially available, basic toxicity assessment is required. Among basic toxicity testing procedures in use today, acute toxicity test is the first procedure that performed on a new chemical (Gallo, 2001). Acute toxicity is toxicity elicited as a result of short-term exposure to a toxicant (LeBlanc, 2000). It refers to adverse effects on, or mortality of, organisms after a single or multiple exposures to a chemical agent in a short time period and it is the effect that occurred within the first few days after exposure (Hodgson and Levi, 2000). Acute exposure thus provides the most rapid assessment of toxic effects of a

chemical and suggests an appropriate approach for further studies on its biological effects.

As, until now, there is no report specifying active substances in this plant that elicit reproductive effects, crude extract of *M. macrocarpa* was used in this study. The acute toxicity of *M. macrocarpa* crude extract was assessed using the Nile tilapia, *Oreochromis niloticus* as a model. Toxicological endpoints of fish acute toxicity assessment are the median lethal concentration (LC_{50}) and the non-observed effect concentration (NOEC) (EPA, 1994). We used the standard method for acute toxicity testing in fish by ASTM (1980) and FAO (1982) to determine the LC_{50} at 96 hours and the NOEC of the plant extract. In addition, assessment of acute toxicity may involve postmortem examination of target organ in order to obtain meaningful scientific information of the toxic effect (Gallo, 2001). Liver is a target organ of acute toxicity study because it is the organ where metabolism of chemicals, especially the detoxification of toxic substances, occurred after entering the organism. In this study, we thus determined the acute hepatic effects of the plant extract on the tilapia using histological methods.

MATERIALS AND METHODS

Fish procurement and maintenance

O. niloticus brood stock (3 weeks post-hatching) was obtained from the Aquatic Animal Breeding Research Station at Pathumtani, Department of Fisheries, the Ministry of Agriculture and Cooperatives of Thailand. The fish were acclimatized for 7 days in 325-L glass aquarium with aerated water. They were maintained on a 14 h light/10 h dark photoperiod at 27-29 °C and fed with commercial fish food (CP Company) twice daily. Fish were fasted for 24 hours prior to the experiment.

Preparation of M. macrocarpa crude extract

Whole stems and tubers of *M. macrocarpa* were ground and dried. The plant powder was extracted with absolute ethanol at room temperature for 2 weeks. Solvent extraction ratio was 1:10, powder (g): solvent (ml). The solution was filtered and the solvent was evaporated out by a rotary evaporator at 40°C at the Natural Products Research Unit, Department of Chemistry, Chulalongkorn University. The crude extract was dried in an oven at 40°C. The extraction process gave approximately 1.51% yield. All crude extracts yielded from each extraction process were pooled together before use in order to minimize variation in chemical composition between each batch.

Acute toxicity bioassay

Standard method for acute static toxicity bioassay (ASTM, 1980; FAO, 1982) was carried out on the tilapia at the age of 1 month. This standard method includes the

Range-finding test and the Definitive test which were conducted in 14-L glass jars containing 10 L of the test solution. The crude extract was dissolved in 1 ml dimethyl sulfoxide (DMSO) prior to further dilution with filtered water. In the Range-finding test, a concentration series of 1, 10, 100 and 300 ppm of crude extract were used, while the Definitive test consisted of a series of 20, 40, 60 and 80 ppm of crude extract. In each test, the control group exposed to filtered water and the solvent control group exposed to 100 ppm DMSO in filtered water were included. There were 3 replicates in each treatment consisting of 10 fish per replicate (jar). Mortality responses of the fish were observed at 24, 48, 72 and 96 hours after exposure. The LC_{50} value of *M. macrocarpa* crude extract after 96 hours at 95% confidence intervals was calculated by Probit Analysis (Finney, 1971) using Probit Analysis program in SPSS for Windows version 9.05 (Chicago, IL). The non-observed effect concentration (NOEC) and the lowest-observed effect concentration (LOEC) were based on the Definitive test results, and the maximum acceptable toxicant concentration (MATC) was calculated from NOEC and LOEC (Rand and Petrocelli, 1985; Sprague, 1990). Finally, the application factor (AF) was calculated as follows:

$$AF = \frac{MATC}{LC_{50}}$$

Subchronic concentration of the extract was determined at the level lower than the MATC by multiplying the MATC with the AF.

Histology

The livers of the tilapia from the control, the solvent control and the treated groups at 100 ppm and 300 ppm were removed and fixed in 10% neutral bufferred formalin. For treated groups, the livers were fixed immediately after death. The control fish were sacrified after the end of the experiment. Twelve liver tissues (3 livers per group) were processed using standard method for paraffin section (Humason, 1979), sectioned at 5 μm and stained with hematoxylin and eosin in order to study histopathological effects by light microscopy.

RESULTS

Mortality of the tilapia upon the acute exposure to the crude extract is shown as number of dead fish after 24, 48, 72 and 96 hours of exposure (Table 2-1). There was no mortality observed in both control and solvent control groups. The concentration that induced 100% mortality of the fish after 24 hours was observed at 80 ppm. The responses were concentration-related with NOEC at 20 ppm and LOEC at 40 ppm. The MATC was estimated as a median value between NOEC and LOEC at 30 ppm.

The LC_{50} at 96 hours calculated by Probit analysis program (SPSS 9.05) was at 65.72 ppm (Table 2-2). The Probit transformed responses are shown in Figure 2-1. From the MATC and the LC_{50}, AF was calculated to be 0.46. Therefore, the subchronic concentration for further long-term experiment was determined at 14 ppm.

Normal histological structures of hepatic tissues of the control and the solvent control tilapia are shown in Figure 2-2A and Figure 2-3A. The basic architecture of hepatic tissue consisted of polyhedral hepatocytes organized as a plate along sinusoids (sinusoidal capillaries) arrayed from central vein. The hepatocyte possesses concentric nucleus with prominent nucleolus and cytoplasm stained light purple with some fat droplets. Histopathological alterations in the livers of the fish exposed to the plant extract at 100 ppm (Figure 2-2B-D) included large fat droplets accumulation and accumulation of some eosinophilic droplets in hepatocytes, foci necrosis with both pyknotic nuclei and karyolysis and infiltration of white blood cells. The lesions were found severely around hepatic and central vein. The normal hepatic tissue architecture was lost. In the livers of the fish exposed to 300-ppm extract (Figure 2-3B-D), the more severe lesions were observed. More abundant

eosinophilic droplets and large fat droplets were found in the treated hepatocytes. Necrosis with pyknotic nuclei and extensive foci and diffuse necrosis with karyolysis were observed. A number of macrophages were found infiltrated into the necrotic areas of the hepatic tissues.

DISCUSSION

Acute toxicity test in the tilapia revealed the LC_{50} at 96 hours of *M. macrocarpa* crude extract at 65.72 ppm. This value indicates high toxicity of this plant extract according to classification by Matsumura (1985). Based on classification criteria for dangerous substances by the Commission of the European Communities of the European Union, as the LC_{50} (96 hr) in fish acute toxicity test falls in the range of 10-100 ppm, the plant extract can be classified in R52 Phrase as, "harmful to aquatic organisms", and in R53 Phrase as, "may cause long-term adverse effects in the aquatic environment", in Annex VI of Directive 2001/59/EC (EC, 2001). In comparison with other plant products, *Apodytes dimidiate* crude extract (pesticide), extract of yellow oleander *Thevetia nerifolia* (piscicide) and tannic acid (phenolic compound from plant barks), which showed LC_{50} at 96 hours on the tilapia *O. mossambicus* at 790 ppm, 129.02 ppm and 107.2 ppm, respectively (Brackenbury and Appleton, 1997; Sambasivam et al., 2003; Saha and Kaviraj, 1996), *M. macrocarpa* crude extract may be considered as a more potent toxicant. Whereas, in acute toxicity study of lapachol (medicinal plant product) on *O. niloticus*, the 24-hr LC_{50} at 6.3 ppm and 2.05 ppm were reported for lapachol and isolapachol salts, respectively (Lima et al., 2002). However, differences due to variation of experimental conditions, age and species of the fish tested are on accounted. In the similar *O. niloticus* test system performed earlier at this laboratory, the following LC_{50} at 96 hours of these plant extracts were found: 4.41 ppm for *Derrris trifoliata* extract (piscicide, pesticide), 36.25 ppm for *Azadirachta indica* extract (pesticide) and 11.54 ppm for *Chromolaena odorata* extract (pesticide) (Wattanasirmkit et al., 1996; Srijunngam, 1998; Wuthipanyarattanakun and Wattanasirmkit, 2003).

The responses of the tilapia to *M. macrocarpa* extract included the acute death since the 100% mortality was observed in the first 24 hours of exposure at 80 ppm. The non-observed effect concentration (NOEC) was estimated from mortality data at 20 ppm since there was no mortality of the fish at this concentration. The data of effective concentrations from Probit analysis (Table 2-2) also indicated that safe concentration is below 34.87 ppm (probit 0.01). Thus the maximum acceptable toxicant concentration (MATC) estimated at 30 ppm may be considered to be a safety guideline for the use of this plant extract in aquatic environment.

The histological study revealed histopathological alterations in the livers of the fish exposed to the plant extract in concentration-related manner. The major lesions are large fat droplets accumulation and necrosis, both pyknosis and karyolysis, which indicated high hepatic toxicity of the plant extract on the fish. In the acute cadmium exposure (10 ppm CdCl$_2$) in white seabass (*Lates calcarifer*), the liver tissues of the treated fish show vacuoles and dark granules accumulations and pyknotic nuclei in some cells (Thophon et al., 2003). In comparison, the acute hepatic effects of the plant extract occurred in tilapia seem to be more severe. In the acute exposure to *Derris trifoliata* extract and *Chromolaena odorata* extract, the liver of the tilapia *O. niloticus* also showed the similar histopathological lesions including hydropic swelling, necrosis (both of pyknosis and karyolysis) and degeneration of hepatic plate (Wattanasirmkit et al., 1996; Wuthipanyarattanakun and Wattanasirmkit, 2003).

The reports on toxicity tests of *M. macrocarpa* extract are still limited. Cytotoxicity tests on anti-proliferative effect of the plant extract were done on human tumor cell cultures and revealed high anti-proliferative activity in MCF-7 and HeLa

cell lines with ED_{50} at 85.36 µg/ml and 393.85 µg/ml respectively, suggesting cytotoxicity of the plant extract (Cheewasopit, 2001; Cherdshewasart et al., 2004a; Cherdshewasart et al., 2004b). The observed toxic effects of this plant extract are correlated with the properties of its chemical constituents. Quercetin is a flavonoid which has been reported to induce apoptosis in human cancer HPB-ALL cells (Russo et al., 1999), promyelocytic leukemia HL-60 and murine leukemia L1210 cells (Čipák et al., 2003) and in rat liver tissue (Iwao and Tsukamoto, 1999). Hopeaphenol is also reported to be cytotoxic with a very low ED_{50} at 1.2 µg/ml for human nasopharynx cancer KB cells and 4.2 µg/ml for breast cancer MCF-7 cells (Ohyama et al., 1999). Quercetin and resveratrol, a monomer of hopeaphenol, also synergistically induced apoptosis by caspase-3 activation with EC_{35} at 6.4 µmol/l (Mertens-Talcott and Percival, 2005).

This study is the first report of *in vivo* acute toxicity of *M. macrocarpa* extract in fish model. The mortality responses, the LC_{50} at 96 hours, the NOEC and the histopathological alterations of liver tissues of the treated fish clearly indicate the acute toxicity of *M. macrocarpa* crude extract. The results will provide essential information for further studies in long-term assessment including study on its specific effects on fish reproductive system.

TABLES AND FIGURES

Table 2-1: Number of mortality of the tilapia responded to acute exposure of the

crude extract from *M. macrocarpa.*

Concentration (ppm)	Number of Subjects	Observed Number of Mortality at			
		24 hours	48 hours	72 hours	96 hours
0	30	0	0	0	0
20	30	0	0	0	0
40	30	2	2	2	2
60	30	2	5	5	5
80	30	30	30	30	30
100	30	29	29	29	29

Table 2-2: Effective concentrations and 95% confidence limits of *M. macrocarpa* crude extract from Probit analysis program (SPSS 9.05).

| Probit	Concentration (ppm)	95% Confidence Limits	
		Lower	Upper
.01	34.86748	-74.99744	51.27163
.05	43.90572	-35.32690	57.58220
.10	48.72398	-14.63877	61.40643
.15	51.97483	-1.02926	64.33529
.20	54.55850	9.46350	66.98670
.25	56.77507	18.12744	69.59930
.30	58.76561	25.53559	72.31781
.35	60.61014	31.98124	75.25603
.40	62.36043	37.62697	78.51468
.45	64.05384	42.57291	82.18384
.50	**65.72041**	**46.89562**	**86.33962**
.55	67.38698	50.67098	91.04276
.60	69.08040	53.98366	96.34516
.65	70.83068	56.92678	102.30642
.70	72.67522	59.59745	109.01954
.75	74.66576	62.09553	116.64822
.80	76.88232	64.52858	125.49170
.85	79.46600	67.03352	136.13093
.90	82.71685	69.84046	149.86237
.95	87.53511	73.55690	170.65828
.99	96.57335	79.77111	210.42518
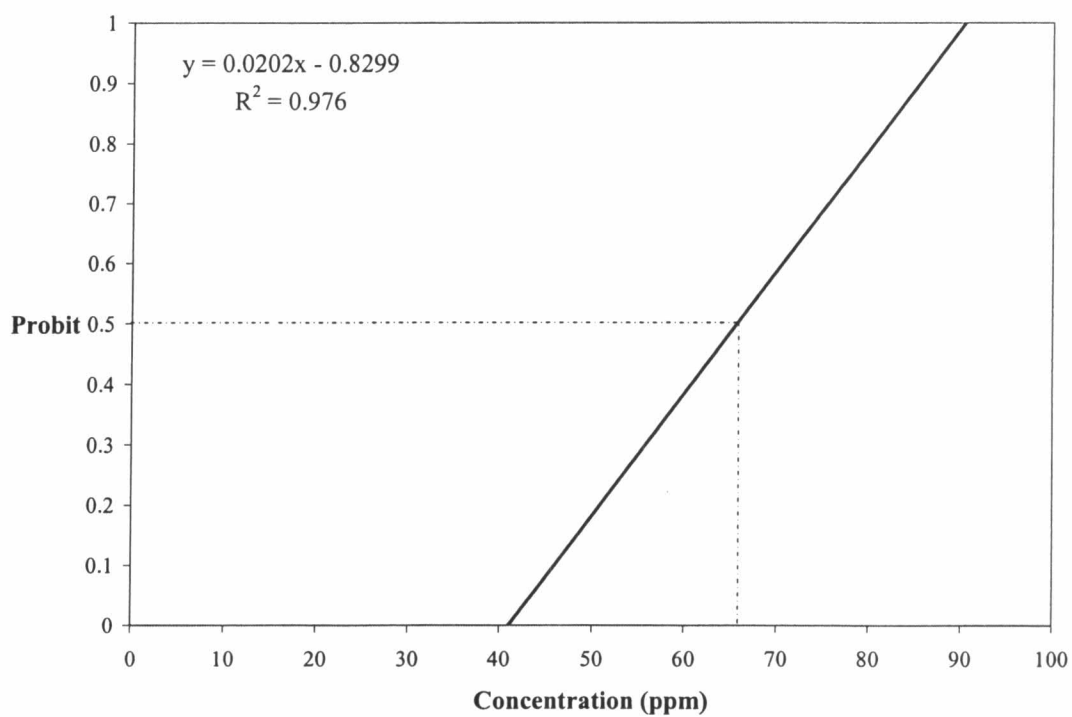
Figure 2-1: Probit transformed responses from Probit analysis program (SPSS 9.05) of the tilapia exposed to *M. macrocarpa* crude extract after 96 hours.

Figure 2-2: Photomicrograph of liver tissues of *O. niloticus*. (A) Control liver showing normal tissue architecture. (B) Treated liver at 100 ppm showing necrosis (*) and a loss of normal tissue architecture. (C) Treated liver at 100 ppm showing fat (*) and eosinophilic droplets (arrow) accumulations. (D) Treated liver at 100 ppm showing infiltration of macrophages (M) and lymphocytes (L) near hepatic portal vein. *Bar*: 30 μm.

CV central vein, *H* hepatocyte, *S* sinusoid, *HV* hepatic vein.
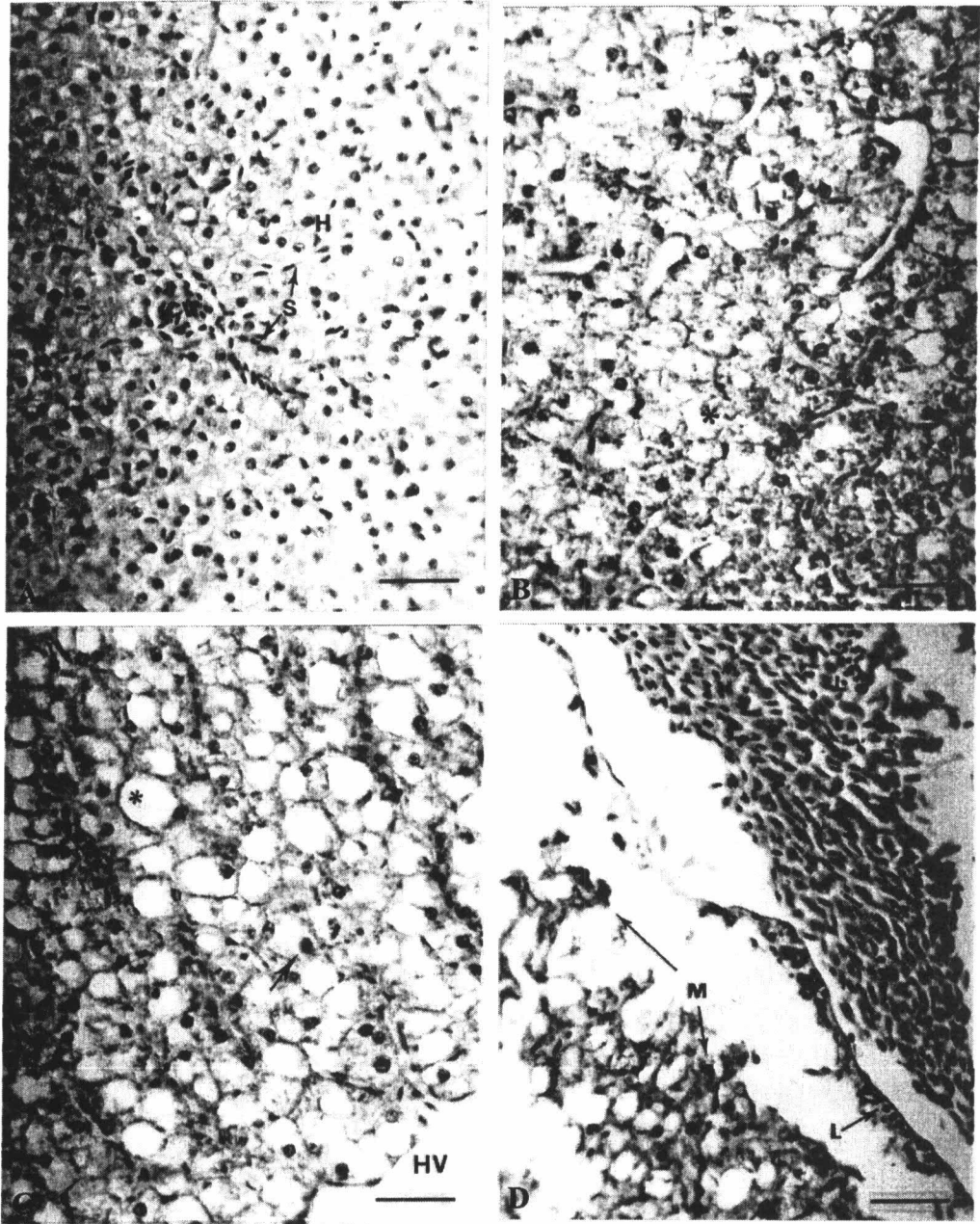
Figure 2-2

Figure 2-3: Photomicrograph of liver tissues of *O. niloticus*. (A) Solvent control liver showing normal tissue architecture. (B) Treated liver at 300 ppm showing diffuse necrosis with karyolysis (*) and infiltration of macrophages (M) and lymphocytes (L). (C) Treated liver at 300 ppm showing pyknotic nuclei (arrow) and severe foci necrosis with karyolysis (*). (D) Treated liver at 300 ppm showing eosinophilic droplets (arrows) and large fat droplets (*) accumulations. *Bar*: 30 μm.

CV central vein, *H* hepatocyte, *S* sinusoid, *P* pancreas.

Figure 2-3