CHAPTER II

MATERIALS AND METHODS

2.1 Preparation of experimental diets

The experimental diet (0.5%, 1.0% and 1.5% w/w of ginger) was prepared as follows. Fresh ginger was purchased from the local market, peeled, washed, coarsely minced, air dried and pulverized with a blender to fine powder (Figure 12). This was added (w/w) to already pulverized feed and throughly mixed so as to obtain a diet containing 0.5%, 1.0% and 1.5% ginger.

Ingredients were mixed together and were baked in an oven at 60°C until final weight was one kilogram.

- 0.5% ginger diet: diet 99.5 g + ginger powder 0.5 g
- 1.0% ginger diet: diet 99.0 g + ginger powder 1.0 g
- 1.5% ginger diet: diet 98.5 g + ginger powder 1.5 g

2.2 Administration of Methyl Parathion

Commercially available Methyl parathion 50% was used in this study. Methyl parathion was obtained from local commercial sources. The animals were treated intraperitonealy (i.p.) with the vehicle (PG) and MP (0.5, 1.0, 1.5 mg/kg).

2.3 Animals

Male and Female Sprague-Dawley rats (180-200 g) were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. Rats were divided into 12 group, six animals in each group, and were housed individually. The temperature (20-22°C), humidity (50+10%) and lighting (12h day/night cycle) were constantly controlled. The rats were acclimatized one week before the beginning of the experiment.

Water was given *ad lib* to all the animals. Food consumption, general condition and any other symtoms were observed daily and body weight were recorded weekly by using standard worksheet of Hippocratic screening test.

2.3.1 Selection the optimal dose of methyl parathion (MP)

Male and female rats were divided into 4 groups, six animals in each group. The animals were treated with MP 0.5, 1.0 and 1.5 mg/kg/day for1, 2, 3 and 4 weeks. The rats were fed normal diet and water *ad lib*.

2.3.2 Selection the optimal dose of ginger diet.

Male and female rats were divided into 4 groups, six animals in each group. The animals were treated with 0.5%, 1.0% and 1.5% w/w ginger diet for 1, 2, 3 and 4 weeks. The rats were fed water *ad lib*.

2.3.3 Treatment of animals with the optimal dose of methyl parathion and ginger diet.

Male and female rats were divided into 4 groups, six animals in each group. The animals were treated for 1 month. The rats were fed water *ad lib*.

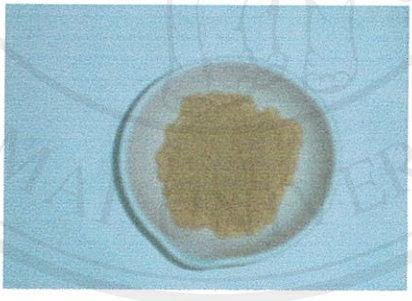


Figure 12 Ginger powder

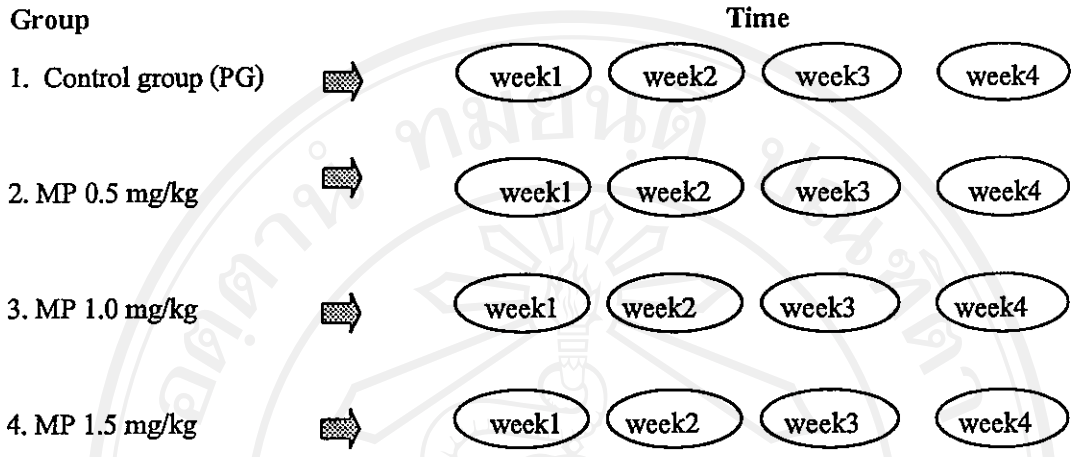


Figure 13 Selection of the optimal dose of methyl parathion.

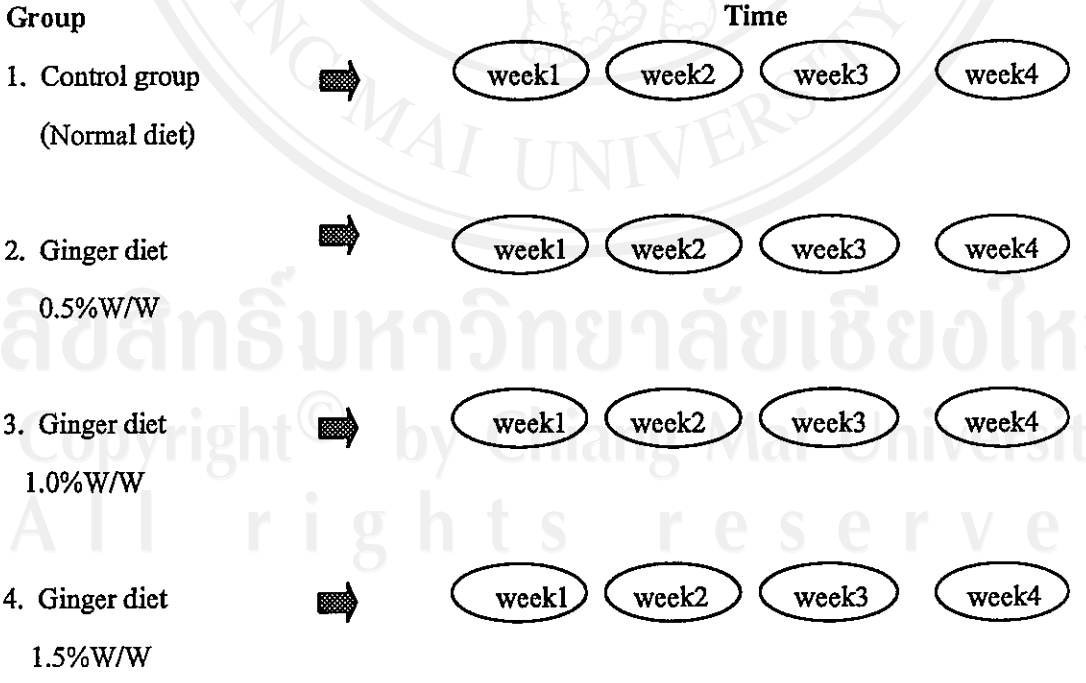


Figure 14 Selection of the optimal dose of ginger diet

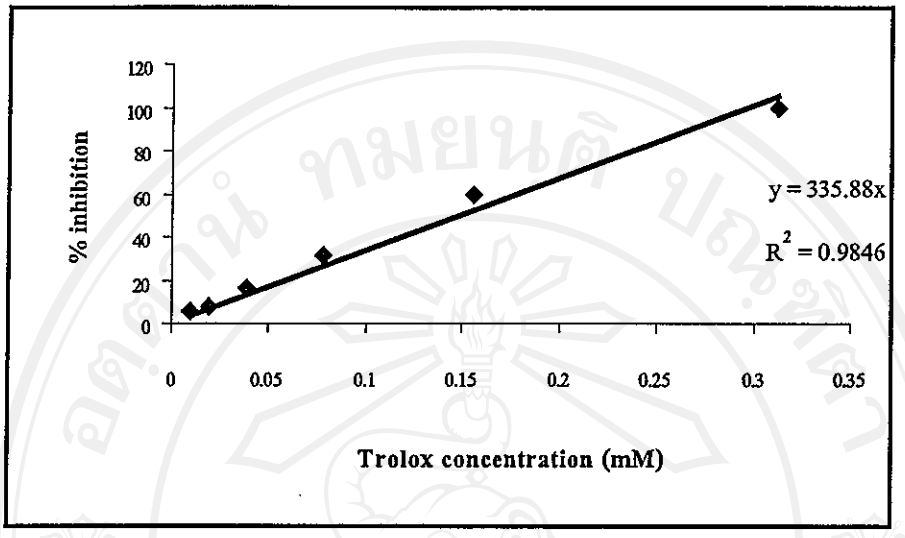
2.4 Sample collection

After overnight fasting, animals were sacrificed and EDTA blood samples were collected. Whole blood samples were also collected and sera separated for the biochemical investigations. The liver was immediately removed and kept at -70°C until used. The muscle was removed and fixed in 0.4% formalin at 4°C.

2.5 ABTS radical cation decolorization assay.

This method is modified from Re *et al* (1999)'s protocol. Briefly, ABTS is dissolved in water to a 7 mM concentration. ABTS radical cation, $ABTS^{\bullet+}$, was produced by reacting ABTS stock solution with 7 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hrs. before use. For the study the action of ginger, the $ABTS^{\bullet+}$ solution was diluted with distilled water and measured at an absorbency of 0.7-0.8 at 734 nm at room temperature. Stock powder of ginger was dissolved in distilled water (20 mg/ml) and centrifuge at 12,000 rpm for 10 minutes.

The supernatant was separated. After addition of 1,300 µl of dilution $ABTS^{\bullet+}$ solution (A 734 nm=0.7-0.8) to 300 µl of distilled water and 100 µl of sample, absorbency reading was taken until the absorbency is stable. All determination will be carried out at least three times, and in triplicate, on the standard and sample. Trolox is use as the standard reference. The data represents the percents (%) of reduction (A412 nm).



Figuer 15 Percent reduction of ABTS by Trolox

2.6 Thiobarbituric acid reactive substances (TBARS) assay.

2.6.1 Serum preparation

Blood sample was centrifuged for 15 minutes at 3,000 rpm at room temperature, Then serum was separated and stored at -20°C until analysis.

The lipid peroxidation levels in serum was measured following a modification of the methodology described by Santos *et al* (1980).

Sample (100 µl) was mixed with 450 µl of normal saline and 200 µl of thiobarbituric acid (TBA) and then added with 1,000 µl of thrichloroacetic acid (TCA). After heating for 30 minutes at 95-100°C and cooling, TBARS solution was added with 2,000 µl of distilled water and was centrifuged at 3,000 rpm for 10 minutes. The absorbance of the solution was measured at 532 nm. The amount of TBARS was calculated as malondialdehyde (MDA) equivalents using 1, 1, 3, 3-tetramethoxypropane as standard.

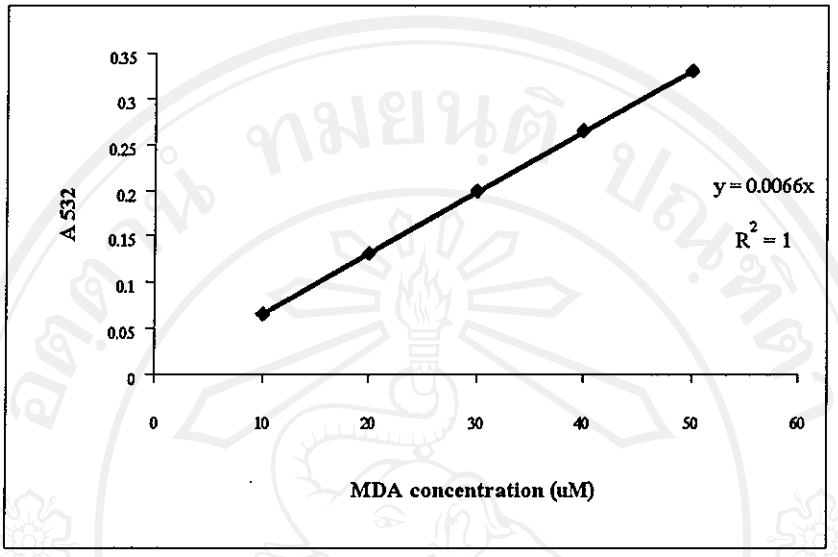


Figure 16 The TBARS assay standard curve for lipid peroxidation

2.7 Determination of Acetylcholinesterase (true cholinesterase) in red blood cells

Acetylcholinesterase in red blood cells was measured following the method described by Moffat (1986). Packed red blood cell (20 µl) was diluted in 200 µl of distilled water, then 20 µl of diluted sample was mixed in 3,000 µl of 5, 5-dithiobisnitrobenzoic acid solution for setting zero. Then 5% Acetylthiocholine iodide (50 µl) was added and mixed. The absorbance of the solution was measured at 405 nm. Acetylcholinesterase level was calculated and reported in U/L.

2.8 Glutathione determination

Glutathione (GSH) level was measured following the method described by Beutler *et al* (1963) and Ernest *et al* (1963). Whole blood (400 µl) was added with 1,600 µl of distilled water, to hemolyzed, then 3,000 µl of preciptiation solution was added and mixed. The solution was incubated for 5 minutes at room temperature and centrifuged at 2,000 g for 10 minutes and then filltered through coarse-grade filter paper Whatman No.1, Duplicate 200 µl of supernatant and

added 1,000 µl of phoshated buffer solution, 250 µl of DTNB. The absorbance was measured at 412 nm within 5 minutes. GSH level was calculated using the following equation:

GSH level

$$= \frac{\text{(Optical density of the test)} \times \text{(Standard GSH concentration)} \times 1.25 \text{ (dilution factor)}}{\text{Optical density of the standard}}$$

= mg/dl of whole blood

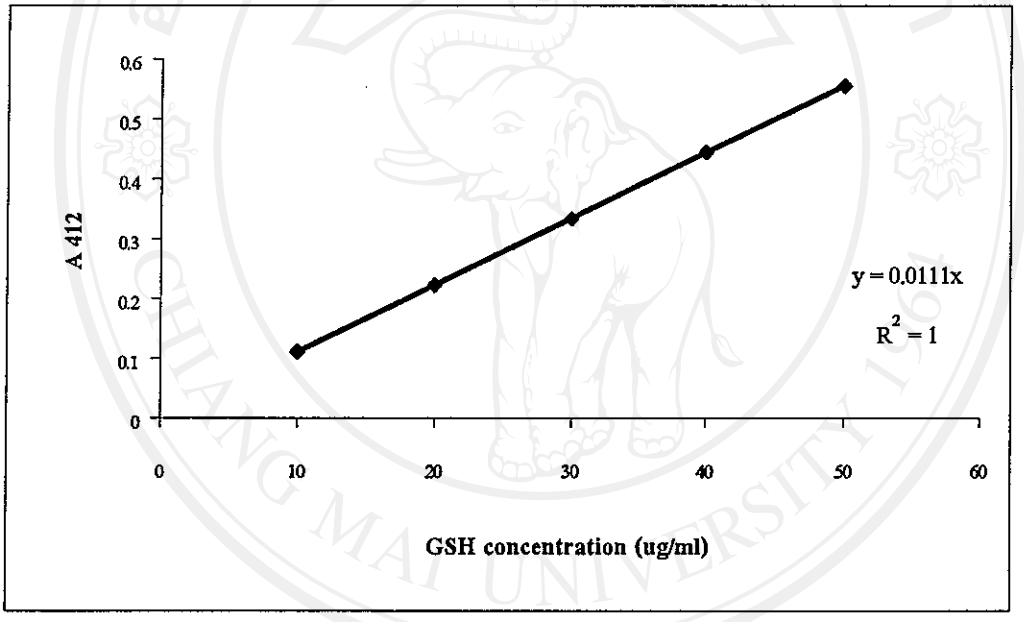


Figure 17 The GSH standard curve

2.9 Glutathion-S-transferase (GST) activity assay

2.9.1 Preparation of cytosolic fraction

The procedures were performed at a temperature ranging 0-4°C. The liver was perfused with saline to remove blood. The liver was homoginized by homoginizer and 154 mM KCl solution, pH 7.4 was used for isotonicity. The homoginates were centrifuged at 9000 g for 30 minutes. The supernatant was aliquoted and stored at -20°C until analysis for GR and GST activity. The protein concentration was determined by BCA assay kit from Sigma Co.

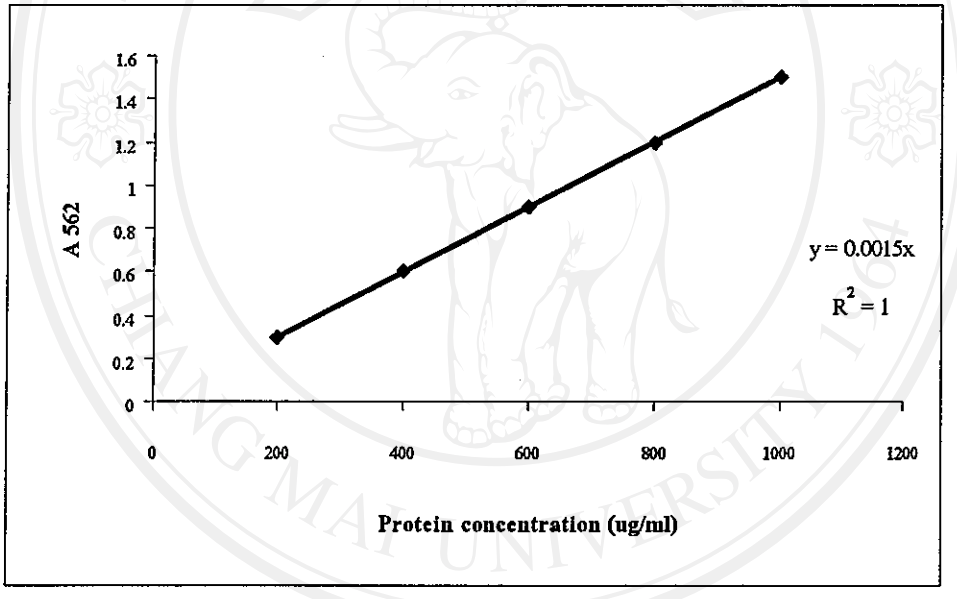


Figure 18 The BCA assay standard curve for protein

The GST activity assay was based on the GST-catalyzed reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH). The enzyme activity was determined according to the method of Habig *et al* (1974). The reaction mixture consisted of 10.5 mM CDNB and 20 mM GSH in potassium phosphate buffer (pH 6.5). The reaction was carried out at 30°C, started by addition of cytosolic fraction. The increase in absorbance (A) at 340 nm (ε=9.6 $nm^{-1}.cm^{-1}$) was measured.The GST activity was calculated using the following equation.

Enzyme activity = $\frac{\Delta \text{ A340/min sample} - \Delta \text{ A340/min blank} \times 1000 \times \text{dilution factor}}{9.6 \text{ (molar ext. coefficient)}}$

Specific enzyme activity = enzyme activity / mg of protein

(Unit = mmol/ min/ mg protein)

2.10 Glutathione reductase (GR) assay

This assay is based on the reduction of glutathione (GSSG) by NADPH in the presence of glutathione reductase. The reaction was measured by the decrease in absorbance at 340 nm using an extinction coefficient (ε^{mM}) of 6.22 for NADPH as following by Glutathione reductase assay kit from Sigma Co.

The reaction mixture consists of 500 µl of oxidized glutathione, 350 µl of assay buffer,100 µl of cytosolic fraction and 50 µl of NADPH. The reaction is stared by the addition of the NADPH solution. Mixing the solution by inversion of the cuvette, placing the cuvette in the spectrophotometer, and starting the kinetic program. Runin the reaction with assay buffer instead of enzyme sample solution as a blank. The concentration of enzyme can be calculated using the formula:

$$\text{units/ml} = \frac{(\Delta \text{ Asample} - \Delta \text{ Ablank}) \times (\text{dilution factor})}{\varepsilon^{mM} \times (\text{Voloum of sample in ml})}$$

2.11 Histochemical methods for demonstration of acetylcholinesterase activity in rats skeletal muscle

The method described by El-Badawi *et al* (1967) was modified as follows. The techniques have been applied with equally good result on rat tissue. Gastrocnemius muscle was fixed in ice-cold (-4-4°C) 4% neutral formalin. Sections (60μm) are cut in sliding microtome at –30°C, mounted on slides and rapidly dried at room temperature (18-25°C) by a current of air. Sections were incubated in incubation medium at 4°C for 2 hours. The medium is preapred no earlier than 30 minutes before use by adding to the substrate (acetylthiocholine iodide). Sections were dehydrated and cleared in 95% alcohol (30 sec), absolute alcohol (30 sec) and three times xylene (30 sec), then mount in Permount. The sections are examined by ordinary light microscopy. Sites of AChE activity are marked by a sharply defined granular reddish brown precipitate. The color of product develops as a result of reduction of ferricyanide to ferricyanide ion with subsequent precipitation of Cu^{++} as curpric ferrocyanide (Hatchett,s brown). AChE activity was depends upon the number of motor endplate and the intensity of reddish brown precipitate. Strongly positive AChE activity showed a bright sharply defined granular reddish brown precipitate. Weakly positive AChE activity showed lack appear granular.

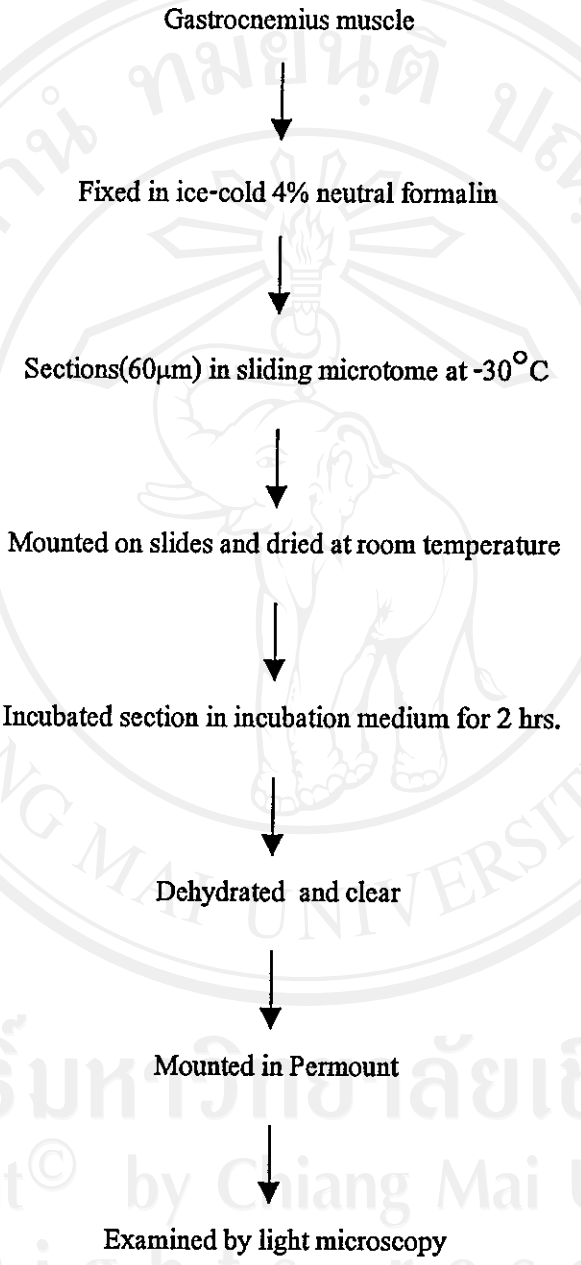


Figure 19 Scheme of histological methods

2.12 Statistical analysis

The level of AChE, GSH, GST, GR, TBARS and number of motor endplate positive AChE activity evaluated for statistic significance by ANOVA. Independent-Samples T-test was used for comparison of the difference of rat's body weight and expressed as mean±SD (Post Hoc: Tukey, $p< 0.05$). The descriptive statistics was used for evaluated of the morphology of motor endplate.