CHAPTER 3

Materials and Methods

3.1 Chemicals and materials

List of chemicals, reagents, equipments and instruments are shown in Appendix A and B, whereas the details of reagents and buffer solution used in this study are shown in Appendix C.

3.2 Preparation of plant extracts

Moringa oleifera Lam. and *Pseuderanthemum palatiferum* (Nees) Radlk. were obtained from a market in Muang District, Chiang Mai Province. The plants were identified by a botanist and the herbarium specimens were deposited at the Queen Sirikit Botanical Garden, Chiang Mai, ID: WP2614 for *M. oleifera* and ID: WP2615 for *P. palatiferum*. Fresh leaves of both plants were washed with clean tap water and ground in a ceramic mortar. One hundred grams of the ground leaves were then soaked in 1,000 ml of each solvent i.e. distilled water, 80% ethanol and hexane for 4 hours and filtered through a piece of satin cloth. The filtrates were evaporated with rotating evaporator and freeze dried. The powdered extracts were kept at -5ºC. Prior to use, the extract was dissolved in water to yield the concentrations of 12, 24, 36 and 48 mg/ml. for *M. oleifera* and 1, 2, 3 and 4 mg/ml. for *P. palatiferum*.

3.3 Phytochemical screening

Chemical tests were carried out on the water, 80% ethanol and hexane extracts of *M. oleifera* and *P. palatiferum* using standard procedures to identify the constituents as described by Jigma and Sumitra (2007); Harborne (1973); Trease and Evans (1989) and Sofowora, (1993)

3.3.1 Tannin: *Ferric chloride test*: Few drops of 5% aqueous $FeCl_3$ solution was added to 2 ml of aqueous, 80% ethanol or hexane extract of *M. oleifera* and *P. palatiferum*, A bluish black color, which disappears on addition of a few ml of dilute H_2SO_4 was followed by the formation of yellowish brown precipitate.

3.3.2 Alkaloids: *Meyar's reagent* (potassium iodide) 1.3 g of mercuric chloride was dissolved in 60 ml distilled water and 5.0 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. Few drops of this reagent was added to 1.0 ml of aqueous, 80% ethanol or hexane solution of samples. Formation of white or pale yellow precipitate showed the presence of alkaloids.

3.3.3 Glycosides: The extract was hydrolyzed with HCl solution and neutralized with HCl solution and neutralized with NaOH solution. A few drops of Fehling's solution A and B were added. Red precipitate indicates the presence of glycosides.

3.3.4 Cardiac glycoside: Glacial acetic acid (1 ml) and 1-2 drops of $FeCl_3$ were added to 2 ml alcoholic filtrate. Then, concentrated H_2SO_4 (1 ml) was added. Appearance of brown ring at the interface indicates presence of cardiac glycosides (A violet ring may also appear below the brown ring)

3.3.5 Coumarins: One milligram of the extract was dissolved in 2 ml of water. The solution was divided into 2 portions. To first portion, 0.5 ml of 10% ammonia solution was added. The second portion was used as a reference. The occurrence of an intense fluorescence under ultraviolet light indicated the presence of coumarins and its dericatives.

3.3.6 Phlobatannins: The extract (0.5 ml) was dissolved in distilled water and filttered. The filtrate was boiled with 2% HCl solution. Red precipitate showed the presence of phlobatannins.

3.3.7 Terpenoid: Chloroform (5 ml) was added to 2 ml of aqueous, 80% ethanol and hexane extract of *M. oleifera* and *P. palatiferum*, Acetic anhydride (2 ml) and 3 ml of concentrated H_2SO_4 were added carefully to form layer. Reddish brown coloration of interface indicated terpenes.

3.3.8 Flavonoid: Extract (0.2) g was dissolved in diluted NaOH and HCl was added. A yellow solution that turns colourless, indicated the presence of flavonoids.

3.3.9 Saponin: About 0.2 g of the extract was shaken with 5 ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) showed the presence of saponins.

3.3.10 Phenolic: To 2 ml of aqueous, 80% ethanol and hexane extract of *M. oleifera* and *P. palatiferum*, 1 ml of 1% ferric chloride solution was added. Blue of green color indicated phenols.

3.3.11 Steroid: Acetic anhydride (2 ml) was added to 0.5 g of each and 2 ml of H_2SO_4 was added. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

3.3.12 Reducing sugar: The extracts was shaken with distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for minutes. An orange red precipitate indicated the presence of reducing sugars.

3.3.13 Anthraquinones: The extracts (0.5 g.) were boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heat. Formation of rose-pink colour indicated the presence of authraquinones.

3.4 Safety evaluation of the extracts (Ames Test)

The safety of each extract was primarily evaluated using the mutagenicity test in *Salmonella typhimurium* (TA98), according to the method of Maron and Ames (1983). Different concentrations of the extracts were prepared and 50µl of each concentration were mixed with 500 µl of the enzyme (S9 mix) or phosphate buffer in a test tube. Overnight culture (0.1 ml) of *S. typhimurium* was then added to the mixture and shaken at 30ºC for 30 minutes. Two milliliters of molten agar was later added and mixed for 1-2 minutes. The mixtures were poured onto minimal glucose agar plate and incubated at 37ºC for 48 hours. Mutant colonies were counted under a stereomicroscope. Only the plates without the killing effect which may arise from the substances used in the experiment were taken into account. The number of mutant colonies obtained from each concentration were substracted from the background colonies of control groups (DMSO or distilled water) and mean values of three independent tests were then calculated (Appendix D). Only the non-mutagenic extracts will be selected for the investigations in 3.5 and 3.6

3.5 Investigation of the antiproliferative effect on colon cancer cell lines

The cancer cells used in the experiment were colon cancer cell line types HCT15, SW48 and SW480 (Appendix E). Primary investigation had been done on the proper amount of cells for cultivation in 96 wells culture plate. The initial number of cell was $2x10^4$ cells/well cultured in DMEM mixed with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C with 5% CO_2.

Cells were cultured overnight in a 96 well plate and treated with the extracts that were declared safe by Ames test. The concentrations of 100, 250, 500 and 1,000 µg/ml for 24, 48 and 72 hours. The culture medium with the antibiotics were then changed to a new medium with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrasolium bromide (MTT) at the concentration of 0.5 mg/ml in each well. Culturing was carried on for 4 hours and the medium with MTT was taken out and 150 µl of DMSO was added to all the wells. The colorimetric measurement was then conducted at 540 nm. The effectiveness of the

extracts in causing cell death was deduced through the calculation of percent of cell viability and median inhibitory concentration (IC_{50}). The reported value was the mean of three independent assays (Tansuwanwong *et al*., 2007).

3.6 Determination of antioxidant property

The antioxidant property was conducted in 2 sections. In the first section, the extracts were directly examined for their antioxidant activities by ABTS, DPPH, ascorbic acid and total phenolic assays. The extract from each plant that showed the high antioxidant activity was then be used to treat the laboratory rats to determine its capacity to reduce free radicals in the rats by using TBARS and SOD assays.

3.7 Experimental animal

Male wistar rats (*Rattus norvegicus*) were purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Thailand. Two rats were housed in one cage under standard conditions (12 h light/12 h dark cycle) at 25 $\pm$ 2^{o}C and with normal feeding of water and standard diet (Chalernpokkapun Co., Ltd. No. 082) *ad libitum* at the Animal Facility Unit, Department of Biology, Faculty of Science, Chiang Mai University. They were accustomed to the housing conditions for at least 1 week before the experiment. All the experimental procedures were in accordance with institutional regulations for the Animal Care and Use (no. RE 002/10), Department of Biology, Faculty of Science, Chiang Mai University.

3.7.1 Test of antioxidant activities in the plant extracts

3.7.1.1 ABTS assay

The method of Re *et al*. (1999) was modified for determining ABTS radical scavenging activity. The ABTS radical cation decolorization activity was measured by the level of blue color product of the reaction between ABTS and sample extract compared with Trolox analog. ABTS was dissolved in deionized water to a 7.0 mM concentration. ABTS radical cation (ABTS•+) was produced by reacting ABTS

stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hrs before use. The stock ABTS radical cation were diluted in deionized water to reach an absorbance of 0.70 (± 0.02) at 734 nm. 10 µl of each extract or Trolox solution at various concentrations was mixed in 1.0 ml of working ABTS radical cation. Then, the mixed solution was measured by spectrophotometer at 734 nm. The percentage inhibition in ABTS radical due to the extracts was calculated by: % inhibition = ((absorbance control - absorbance sample)/ absorbance sample) x 100 and the median inhibition concentration (IC_{50}) was determined. To evaluate the antioxidant capacity of the extracts, the trolox equivalent antioxidant capacity (TEAC) was calculated by: IC_{50}Trolox / IC_{50} sample. Data were expressed as TEAC µg/mg extract. (Appendix F)

3.7.1.2 DPPH assay

The principle of DPPH method is to spectrophotometrically measure the deep violet color of DPPH (1,1-diphenyl-2-picrylhydrazyl), a stable free radicals. When a solution of DPPH is mixed with a substance with antioxidant activity, then this gives rise to the reduced form with the loss of violet color. With respect to this principle and to the method of Brand-Williams *et al*. (1997) the extracts at various concentrations were mixed with DPPH and Tris-HCl solutions in methanol and the absorbance was recorded at 517 nm. The percentage inhibition in DPPH radical due to the extracts was calculated by: % inhibition = ((absorbance control - absorbance sample)/absorbance control) x 100 and the median inhibition concentration (IC_{50}) was determined. To evaluate the antioxidant capacity of the extracts, the gallic acid equivalent (GAE) was calculated by: IC_{50} Gallic acid/ IC_{50} sample. Data were expressed as GAE µg/mg extract. (Appendix G)

3.7.1.3 Ascorbic acid assay

According to the method of Schlessier *et al.* (2002), the vitamin C-analog antioxidant was measured by extracting vitamin C from the samples by adding trichloro-acetic acid. Dinitrophenylhydrazine reagents before heating at 60 °C. The mixture was cooled in ice tank and added with H_2SO_4. After keeping in the dark for 20 min the absorbance was recorded at 520 nm. Vitamin C concentration was calculated as compared to standard ascorbic acid. (Appendix H)

3.7.1.4 Total Phenolic assay

To determine antioxidant activity of substances in the phenolic group, phosphomolybdic acid and phosphotungtic acid, the constituents in Folin-ciocalteu reagent were used to react with the tested extracts. By following the method of Singleton and Rossi (1965), the extracts were mixed with Folin-ciocalteu reagent in $Na_2CO_3.10H_2O$ for 2 hrs. The mixture was then spectrophotometrically measured at 750 nm. Total polyphenol was calculated using gallic acid monohydrate as a standard. (Appendix I)

3.7.2 Test of antioxidant activities in laboratory rats

3.7.2.1 Animal treatments

To avoid the fluctuation of sex hormones, male rats were used in this study. Male wistar rats, 4-5 weeks of age, weighing between 150-180 g. were divided into 9 groups of 8 rats each.

Group I (control group) received distilled water only.

Groups II-V were treated with 60, 120, 180 and 240 mg/kg BW of *M. oleifera* extract respectively.

Groups VI-IX were treated with 5, 10, 15 and 20 mg/kg BW of *P. palatiferum* extract respectively.

After 6 0 days, the animals were anesthetized with ether and blood and liver tissue samples were collected.

Serum and erythrocytes preparation

Blood samples were centrifuged at 3,500 rpm for 10 min at room temperature. Serum and erythrocytes were stored separately at -40 °C until further use for MDA and SOD assays.

Tissue preparation for lipid peroxidation

Twenty grams of homogenated liver tissue was put in 1 ml of 0.1 M phosphate buffer solution (PBS), pH 7.4 at -4°C. The mixed samples were centrifuged at 3,500 rpm for 10 minutes and the supernatant was stored at-4°C for examination of MDA within 24 hours.

3.7.2.2 TBARS assay

Thiobarbituric acid (TBA), reactive MDA products in serum and liver of treated rats was determined by modification of a procedure previously described by Buege and Aust (1978). Briefly, 100 µl of the sample (serum and liver) was assayed by adding 450 µl of normal saline, 200 µl of TBA and 1,000 µl of TCA. The mixture was heated for 30 minutes at 100ºC, cooled with running tap water and then added with 2,000 µl of distilled water with shaking by vortex mixer and centrifuging at 3,000 rpm for 10 minutes. The absorbance at 532 nm was compared with MDA standard curve.

3.7.2.3 Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) is an important enzyme that acts as antioxidant against superoxide free radicals. The erythrocytes samples of rats in all groups was lysed by distilled water and diluted with phosphate buffer before measuring SOD activity. SOD standard or diluted RBC lysate sample was mixed with carbonate buffer and xanthine oxidase (XO). Absorbance was read every 20 seconds continuously for 3 min with a spectrophotometer at 500 nm. The SOD content was determined by comparing with SOD standard curve. (Xin *et al*., 1991)

3.8 Statistical analysis

All the results are presented as mean ± standard deviation (SD) of three replicated determinations and were analyzed with one-way ANOVA by the SPSS statistical software program version 16 for windows. Significant differences between the means of treatments were determined by Least Significant Difference (LSD) test.