CHAPTER III

MATERIALS AND METHODS

3.1. Plant material

P. mirifica seeds of 2 clones were collected from wild plants in Chiang Dao district (E98°58°/N19°23°) and Doi Tao district of Chiang Mai province (E98°42°/N17° 49°), respectively. The plants were identified by Cherdshewasart with the reference (Kahshemsanta *et al.*, 1952) in comparison with the voucher specimen No. BCU 11045 (Cherdshewasart *et al.*, 2004[a]) deposited at the Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok Thailand and were assigned as PM-III and PM-IV. Seedlings of PM-III were established under greenhouse condition. Plantlets of PM-IV were established with asexual propagation from the same plant. The 3 months-old plants, 36 plants per cultivar, were subsequently transferred to field trial in Banpong district of Ratchaburi province (E99°52°/N13°37°).

3.2. Tuber harvest

Single tuberous roots, weight at least 1 kg of the 3 year-old plants were randomly harvested from 3 plants in each clone since March 2005 to February 2006 The collected samples were cleaned, sliced and dried in hot air oven at 70°C, then ground into fine powder.

3.3. Tuberous size and weight

The collects were mesure the size in circum ference, height and width and weight in kilogramm.

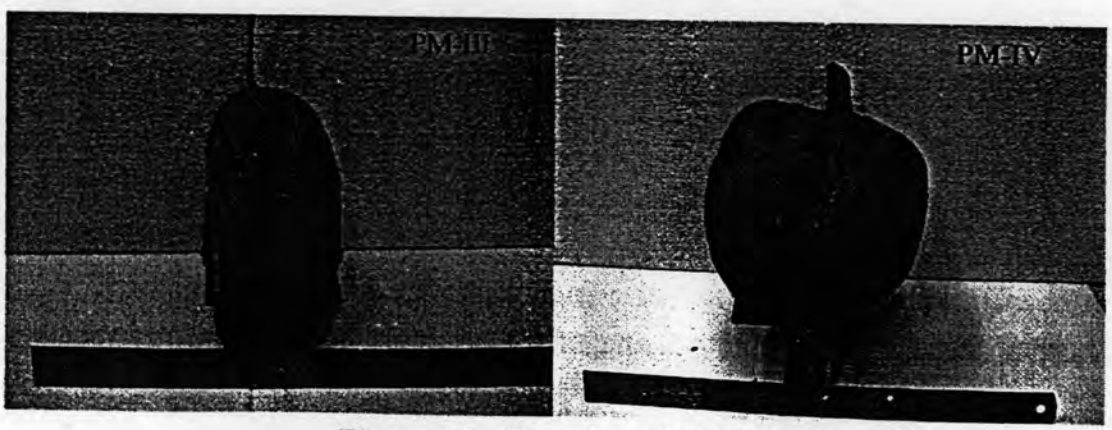

Figure 3.1 The tuber of PM-III and PM-IV

3.4. Leaf morphometry

Fifty leaves were collected and measured in paramerers of leaf including, petiole length (PL), petiole diameter (PD), rachis length (RL), petiolet length (PLL), terminal leaflet length (TLL), terminal leaflet breadth (TLB), stipule length (SPL), angle of first leaf border (A^B)^{o} and number of pairs of primary veins (NPV).

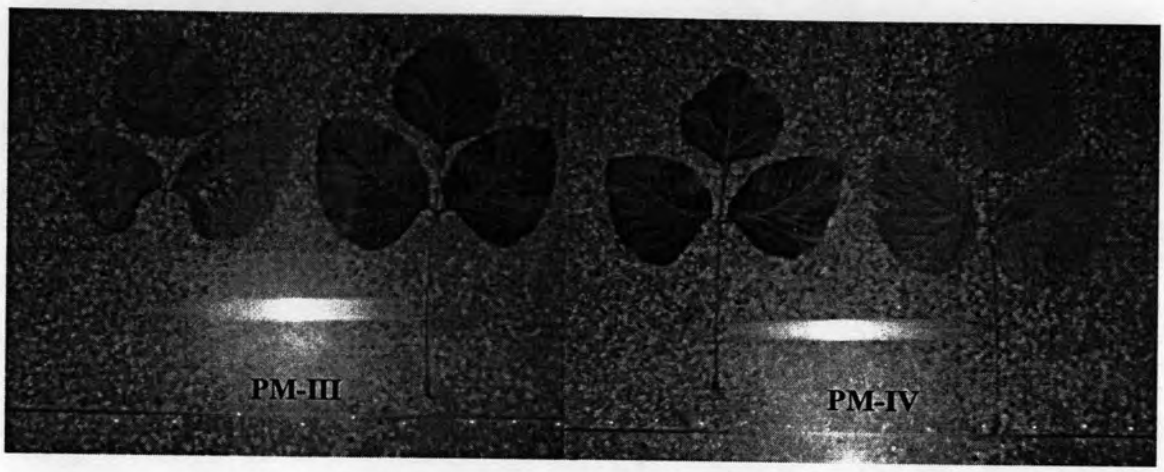

Figure 3.2 The leaf of PM-III and PM-IV.

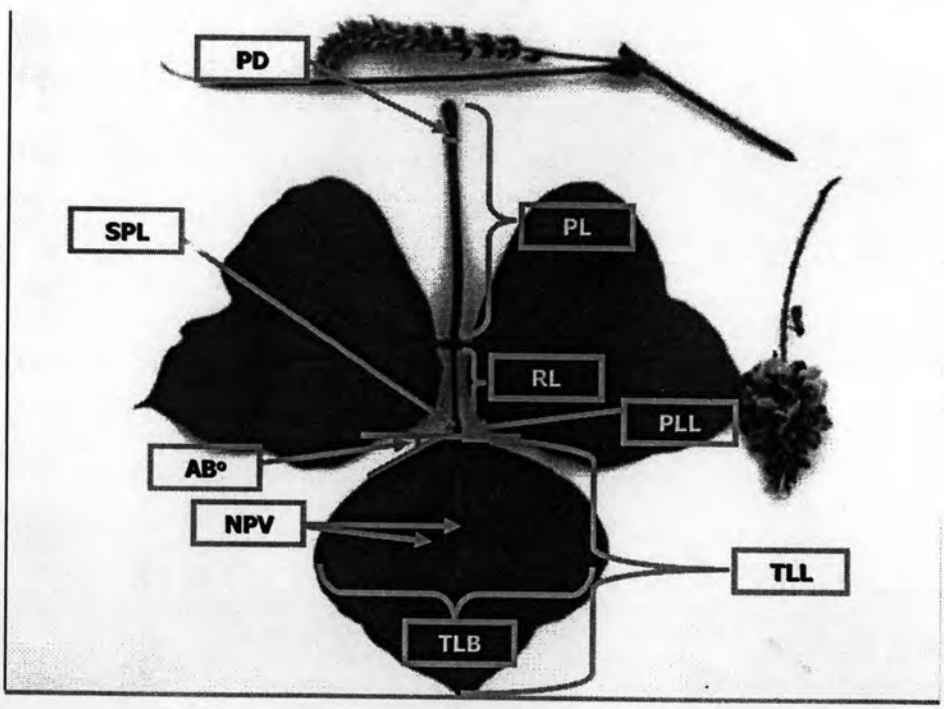

Figure 3.3 Morphometry parameters of *P. mirifica* leaf. (petiole length – PL,petiole diameter – PD, rachis length – RL, petiolet length – PLL, terminal leaflet length – TLL, terminal leaflet breadth – TLB, stipule length – SPL, angle of first leaf border – (A^B)^{o}, number of pairs of primary veins – NPV.

3.5. Pod morphometry

Fifty pods were collected and measured in parameters of pods including, width and length. The seeds were counted as seed per pod.

Figure 3.4 Pods of PM-III and PM-IV.

3.6. Plant crude extraction

Fifty g powder derived equally from the 3 collected tubers for each plant sample was extracted at room temperature with 500 ml 95% ethanol for 7 days. The supernatants were collected by filtration (Whatman filter paper No.4, Whatman, USA) and subsequently evaporated in the rotary evaporator (Buchi, Germany) at 40 °C until completely dried. The plant crude extracts were collected and bottled and kept in a cool, dry and dark place.

3.7. Analysis of isoflavonoid contents

3.7.1. Chemicals and equipments

Isoflavonoid standards, including puerarin, genistin, daidzein and genistein were purchased from Sigma, St. Louis, MO, USA., daidzin was purchased from Fluka Biochemika (Buchs, Switzerland). The organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. The water of over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA). HPLC system control and data processing were carried out by a Waters™ apparatus (Waters-717 plus Autosampler, Waters-600 Controller, Waters-2996 Photodiode Array Detector). The reversed phase C18 column (250 x 4.6 mm) was filled with 5 μm ODS2 (Waters Spherisorb®, Ireland), pre-filtered with Waters Spherisorb®

S5 ODS2 (4.6x10 mm) guard cartridge. The filter set was Millipore membrane at 0.45 μm pore size with 13 mm diameter for the sample and 47 mm diameter for the mobile phase, of HA type for aqueous solution and HV type for organic solvent. The chromatography manager software Empower™ was operated on a personal computer.

3.7.2. HPLC sample preparation

One g tuberous powder was extracted at room temperature with 10 ml methanol (Merck) with the aid of sonication for 30 min. The supernatant was collected after filtration with Whatman No.1 filter paper and filtrated with a 0.45 μm pore size, 13 mm diameter membrane.

3.7.3. Quantitative HPLC

Methods for isoflavonoid analysis were modified from those previously described (Cherdshewasart *et al.*, 2007[a]) by setting the linear gradient system for 50 min from 100: 0 to 55: 45 with 0.1 % acetic acid: acetonitrile, with a flow rate of 1 ml/min and analyzed at the wavelength of 254 nm. The standard isoflavonoids were serially diluted from 1:1 to 1:16 with methanol to establish the concentrations of 1/4, 1/8, 1/16, 1/32 and 1/64 mg/ml, to generate a five point calibration curve. Calibration curves were obtained for all isoflavonoids by plotting the standard concentration as a function of peak area from HPLC analysis of a 10 μl injection volume. The concentrations of standard were chosen to cover the range of isoflavonoid concentrations in the samples. The analyses of the samples were run in triplicate and identified by comparing the retention times and quantified for the amount using standard curves of peak area of the isoflavonoid standards.

3.8. Study of estrogenic activity of the plant extract (MCF-7 test)

3.8.1. Cells and cell culture

MCF-7 the ERα positive human mammary adenocarcinoma cells were obtained from the National Cancer Institute, Thailand. The cell lines were cultured in MEM medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS; BioWittaker, USA), 10,000 unit/L Penicillin G and 1 mg/L Streptomycin sulfate (Antibiotics; BioWittaker, USA) at 37°C in a humidified atmosphere of 5% CO_2 incubator. The cells

were routinely subcultured once every 3-4 days to maintain the optimum conditions for the exponential growth.

3.8.2. Subculture

MCF-7 cells were subcultured twice a week. The culture medium was removed from the flask and rinsed with 3-4 ml of Phosphate Buffer Solution (PBS), then the solution was removed and 2-3 ml of 0.25% Trypsin was added. The flask was stood at room temperature (or at 37°C in incubator) for 30-45 seconds or until the cells were detached, then the solution was removed. 5 ml of fresh MEM was added, aspirate, dispensed into the new culture flasks. Fresh MEM was added to the final volume at 5 ml and finally incubated in the 37°C, 5% CO_2 incubator.

3.8.3. Cell suspension preparation for assay

MCF-7 cells were propagated 3 days before the experiment as follows:

3.8.3.1. Cell digestion

The medium was removed after 3 days cultured. The cells were rinsed with 3-4 ml PBS followed by removal of the solution, then 4 ml 0.25% trypsin was added. The flask was stood at room temperature (or at 37°C in incubator) for 1-1.5 minutes before removal of the solution. The fresh DMEM was added and aspirate gently with the aid of a pipette in order to dissociate into single cells.

MCF-7 cell suspensions were transferred to micro-well plate, cells were counted and diluted.

3.8.3.2. Cell count and dilution

The 0.4% trypan Blue dye solution and hemocytometer were applied to determine the viable cell number. 0.2 ml of trypan Blue and 0.2 ml of cell suspension were transferred to a test tube and gently mixed thoroughly by Pasture pipette.

The cover slip of the hemocytometer was placed. Trypan Blue-cell mixture was transferred to the chamber by touching the tip of pasture pipette to the edge of the cover slip and allowing the chamber to be filled by the capillary action. The suspension was not allowed to overfill or underfill the chamber. The viable cells, not stained with Trypan Blue were counted in four-1 mm square on the corner and on-1 mm square in the middle of the hemocytometer (Figure3.1).

The cells in each square area of the hemocytometer were equivalent to approximately 1 mm, represent a total volume of 0.1 mm^3 and the subsequent cell density per ml was calculated using the following calculation:

Cell density (cell per ml) = (total cell count/5) x 2 $x10^4$

Then, calculate for dilution (desired cell density = 4 x 10^4)

$$C_1V_1 = C_2V_2$$

C_1: cell density

V_1: cell volume

C_2: desired cell density

V_2: final volume

Dilute cells suspension with DMEM to desirable volume

$$z = y - x$$

x: cell volume

y: desirable volume

z: media volume

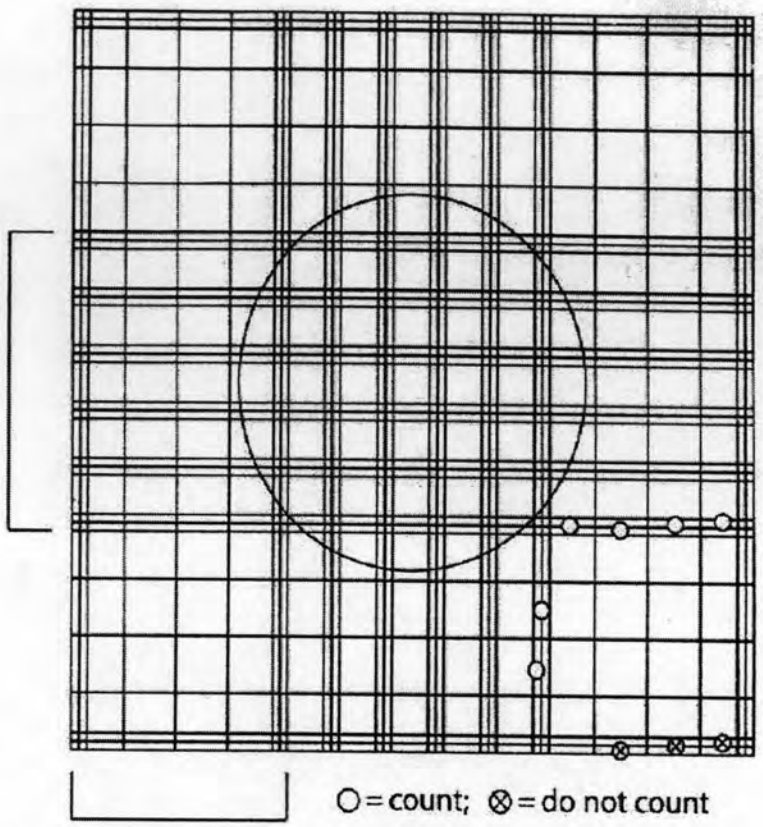

Figure 3.5 Haematocytometer. Corner squares (enlargement). Cells were counted on top and left touching the middle line and not counted touching the middle line at the bottom and right.

3.8.4. Preparation of plant solution for MCF-7 test

Plant crude extract was dissolved in 100% DMSO (2000 µg/ml). Stock solution was diluted to various concentrations by 100% DMSO. Final concentrations of DMSO did not exceed 2% of the total volume, an amount that was not toxic to the cells.

3.8.5. Preparation of S9 mix for MCF-7 test

Rat liver is the general source of activating enzyme. It is the efficient detection of a wide variety of carcinogens requiring metabolic activation.

The S9 fraction at -80 ^{o}C was thawed at room temperature. 10 ml S9 mix composed of the ingredients; 6.6 ml, 0.2 M phosphate buffer pH 7.4, 0.4 ml, 0.1 M NADP solution, 0.5 ml, 1 M G-6-P solution, 0.5 ml, 0.16 M $MgCl_2$ solutions and 2 ml S9

fraction (see Appendix B). The S9 mix was freshly prepared and kept on ice during experiment.

3.8.6. MCF-7 test

The assays were prepared by incubating MCF-7 cells with the plant extracts (at the concentrations of 0.1, 1, 10, 100 and 1000 µg/ml, 3 triplicates per concentration) and without the extract (negative control). 17 β-estradiol (at the concentrations of 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12} M, 3 per concentration) was set as a positive control. In addition, this study tested the estrogenic activity of the metabolite form by S9 reaction. After the cells were treated, the 96 multi-well plates were incubated at 37°C in a 5% CO_2 incubator for 3 days.

3.8.7. Standard test

The assays were prepared by incubating MCF-7 cells with isoflavonoid standards including puerarin, daidzin, genistin, daidzein and genistein (at the concentrations of 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12} M, 3 triplicates per concentration) and without isoflavonoid standards (control). The 96 multi-well plates were incubated at 37 °C in a 5% CO_2 incubator for 3 days.

3.8.8. Cell proliferation assay

Cell proliferation assay was performed by the MTT Z3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric method. After each incubation period, MTT (5 mg/ml) was added at the amount of 10 µl/well, the incubation was carried out for an additional 4 hours at 37 °C in the dark. The solution was discarded, 150 µl of DMSO and 25 µl of 0.1 M glycine buffer (pH 10.5) were added into each well to dissolve the insoluble formazan crystals. Plates were kept agitation for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative was analysed on a microplate reader at a wavelength of 540 nm. The percentage of cell viability was calculated according to the following formula.

% of cell viability = (OD of treated cells x 100)/OD of control cells

The IC_{50} values were obtained by plotting the percentage of cell viability versus the concentrations. Repeating experiments at selected time to confirm results were carried out and the IC_{50} were determined. All data were expressed as mean ± S.E.M. and

analyzed by one-way of variance (ANOVA) using SPSS software (version 11.5 for windows).

3.8.9. The calculation for cell growth

The absorbent value of the control D_3 was given as 100% of cell growth. The absorbent values of the others were calculated to percentage value compared to that of control D_3.

3.9. Statistical analysis

The mean ± S.E.M. of isoflavonoid contents from the samples of *P. mirifica* were analyzed for statistical significant by the un-paired T-test, Factorial analysis and Duncan analysis of variance at the significance level of $P < 0.05$.

3.10. Correlation analysis of isoflavonoid content and estrogenic activity

The correlation analyses in PM-III and PM-IV are the correlations of the growth response percentage of MCF-7 in the presence and absence of S9 mix with isoflavonoid contents, and the correlations of isoflavonoid contents with temperature and amount of rainfall, were analyzed by Spearman's rho.