CHAPTER III

MATERAIL AND METHOD

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3.1 Plant material

Seeds from 5 clones of the wild *Pueraria mirifica* with high yield of isoflavonoid contents were collected from Prachuab kirikhan province (E99°48′/N11°48′), Saraburi province (E100°45′/N14°45′), Chiang Dao district (E98°58′/N19°23′), Doi Tao district (E98°42′/N17°49′) of Chiang Mai province and Pakchong district (E101°24′/N14°42′) of Nakorn Ratchasima province, respectively. The plants were identified by Cherdshewasart with the reference (Kashemsanta *et al.*, 1952) in comparison with the voucher specimen No. BCU 11045 (Cherdshewasart *et al.*, 2004[b]) deposited at the Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok Thailand and were assigned as PM-I, PM-II PM-III, PM-IV and PM-V. The seeds were germinated in the green house condition at Chulalongkorn University for 3 months and subsequently transfer to field trial in Banpong district of Ratchaburi province (E99°52′/N13°37′). Tuberous roots were randomly harvested in mid – summer, mid-rainy season and mid-winter.

3.1.1. Tuber harvest

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

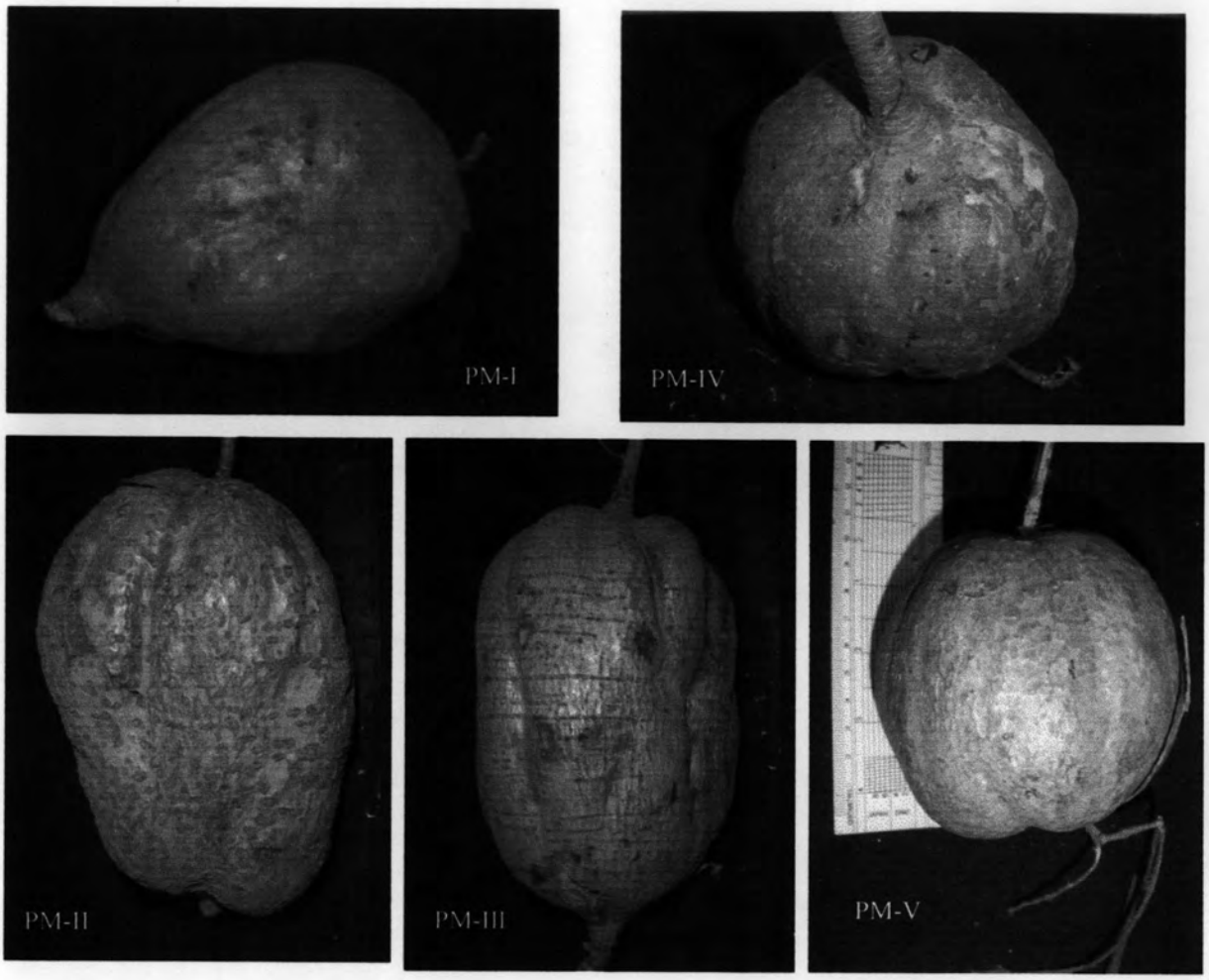


Figure 3.1 The tuber of PM-I, PM-II, PM-III, PM-IV and PM-V.

3.1.2. Tuberous weight

The collects were weight in gram.

3.1.3. Leaf morphometry

Fifty leaves were collected and measured in parameters 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)° and number of pairs of primary veins (NPV).

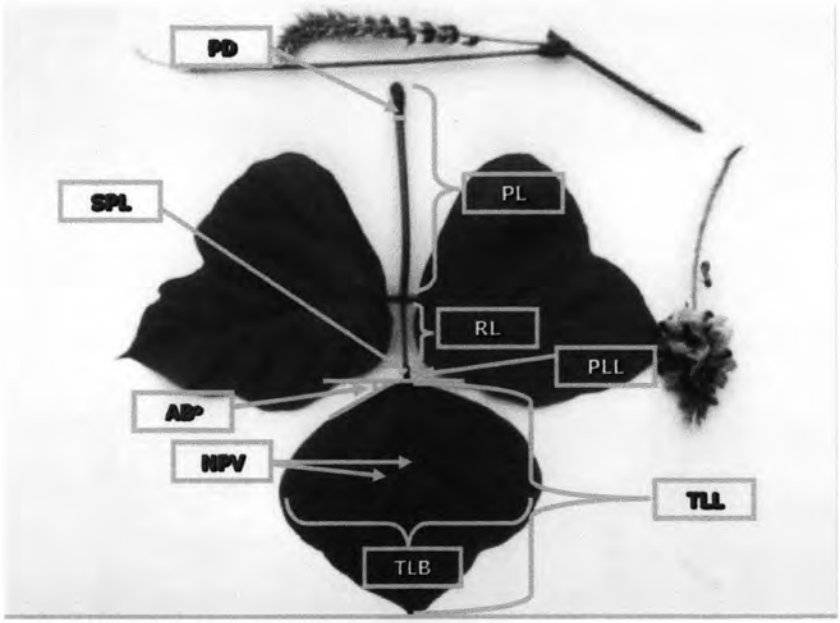


Figure 3.2 Morphometry parameters of *P. mirifica* leaf. (petiole length – PL, petiole diameter – PD, rachis length – RL, petiolet length – PLL, terminal leaflet length – TLL, terminal leaflet breadth – TLB, stipule length – SPL, angle of first leaf border – (A^B)°, number of pairs of primary veins – NPV

3.1.4. Pod morphometry

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

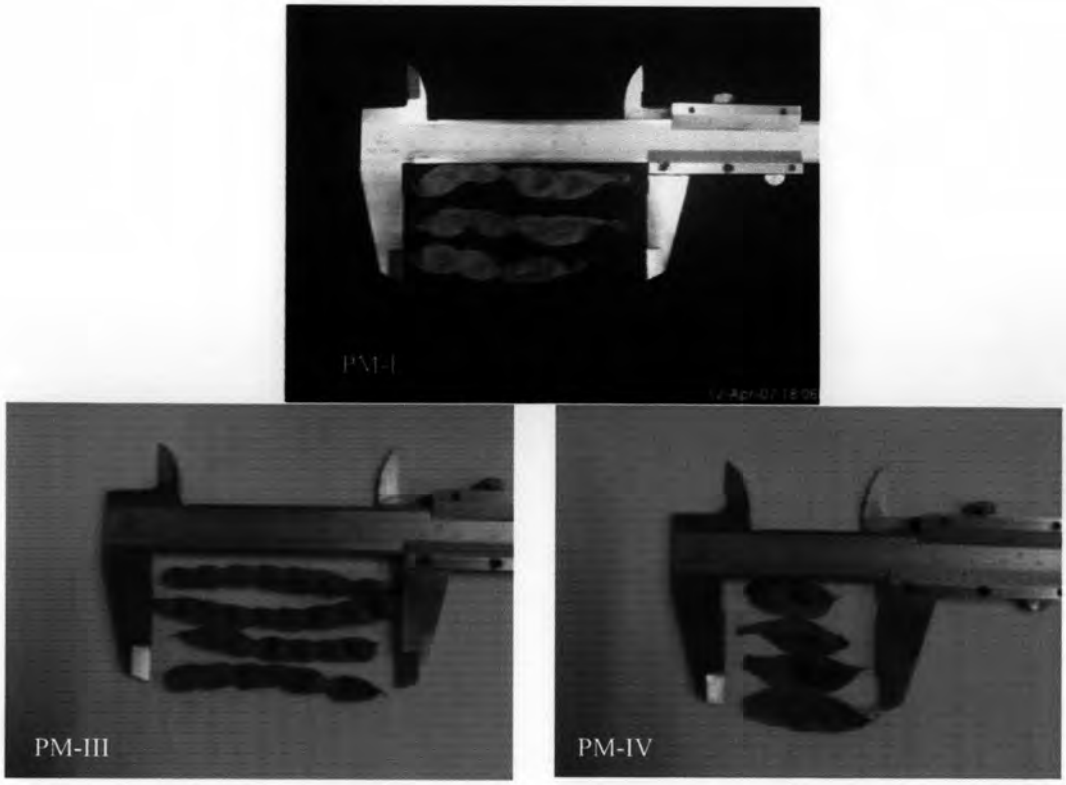


Figure 3.3 Pod of *P. mirifica*

3.2. HPLC analysis

3.2.1 Crude extraction

One g powder of tuberous roots powder was mixed with methanol 10 ml and macerated under sonication; water bath, room temperature, 30 min. The filtrates are collected by filtration (Whatman filter paper No.1, Whatman, USA) and the plant extracts are filtrated again by 0.45 μm pore size, 13 mm diameter membrane then injected directly at the volume of 10 μl analyzed by HPLC. The procedure was repeated in triplicate.

3.2.2 Chromatographic condition

The mobile phase was optimized using the linear gradient system. The solvent gradients created by pumping system with varying amount of solvent as 0.1%(v/v) acetic acid: acetonitrile (100:0 to 55:45) for 50 min, with flow rate 1.0 ml/min. The filter set was Millipore membrane, 0.45 μm pore size with 13 mm diameter for the sample and 47

mm diameter for mobile phase, of HA type for aqueous solution and HV type for organic solvent.

Authentic standards of isoflavonoids including, puerarin, genistin, daidzein and genistein, were purchased from Sigma, USA., daidzin was purchased from Fluka, 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 Waters instruments (Waters-600 Controller analytical workstation, Waters-717plus Autosampler, Waters-2996 Photodiode Array Detector). The reverse phase C18 column Spherisorb (4.6x250 mm) filled with 5 μm. ODS2 (Waters Sperisorb®, Ireland), pre-filtered with Waters Sperisorb® S5 ODS2 (4.6x10 mm) guard cartridge.

3.2.3 Standard preparation and calibration curve

Commercial standard of puerarin, daidzin, genistin, daidzein and genistein were dissolved in methanol and used generating calibration curves. The five concentrations 0.0001, 0.001, 0.01, 0.1, 1 mg/ml of standard solution are injected to generate a five points calibration curve for the standard compounds separately. Calibration curves are obtained for most isoflavonoid by plotting the standard concentration as a function of peak area from HPLC analysis of 10 μl injection. The concentrations of standard are chosen to cover isoflavonoids in the samples. The standard curvs are alternatively set by Empower program (Waters).

3.2.4 Calculation for isoflavonoid contents

The samples are run in triplicate. Empower program (Waters) was used to calculate for the concentration of isoflavonoid. The results are expressed as mg isoflavonoid per 100g powder of the sample. Statistical analysis was done by SPSS.

3.3. Bioassays

Crude extraction for antioxidant and MTT assay

Fifty g of the plant powder are dissolved in 450 ml ethanol for 1 week. The supernatants are filtered through Whatman filters No.1. The filtrates were dried in vacuo and stored at 4 °C.

3.3.1. Antioxidant assay

3.3.1.1. Preparation of plant samples

Six mg crude extracts of *P. mirifica* were dissolved in 1 ml absolute ethanol for stock solution 1000 μM /100ml in absolute ethanol. 50 μM of α-tocopherol is adopted as a positive control. Puerarin, daidzin, genistin, daidzein and genistein were prepared at 10^{-5}-10^{-9} M as positive controls for *P. mirifica*.

3.3.1.2. Experimental protocol

DPPH (radical 2,2 diphenylpicryhydrazyl, Fluka Chemical) solution was freshly prepared at the concentration of 100 μM in absolute ethanol and kept in the dark until used. The absorbance of 100 μM DPPH solution was measured at 517 nm for setting up of reagent blank. Diluted *P. mirifica* crude extracts and α-tocopherol with absolute ethanaol. DPPH solution 190 μl was mixed with the plant extracts 10 μl at the concentration of 0.325, 0.75, 1.5, 3 and 6 mg/ml and serial amount of α-tocopherol of 0.625 1.25, 2.5, 5, and 10 μg/ml. The mixture was shaken vigorously and incubated in a dark chamber at 37°C for 30 min. The solution was placed in 96-well microtiter plates. The absorbance was measured at 517 nm and performed in triplicate in three independent experiments by microtiter plate reader (SUNRISE-TECCAN ASTRIA GMBTL).

3.3.1.3. Statistical analysis

The mean ± S.E.M. of % scavenging of samples were analyzed for statistical significant by One Way Anova, LSD test and Duncan analysis of variance at the significance level of $P < 0.05$. The IC_{50} values at 95% confidence limits of the extracts after 3 days were calculated by Probit Analysis.

3.3.2. MTT assay

3.3.2.1. Cell and cell culture

MCF-7, ERα positive human mammary adenocarcinoma cells were obtained from the Section of Experimental Oncotherapy, Research Division, National Cancer Institute, Ministry of Public Health, Thailand. The cells were grown as a monolayer in EMEM medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; BioWittaker, USA), 10^4 unit/l Penicillin G and 1 mg/l Streptomycin sulfate (Antibiotics; BioWittaker, USA) and 1g/l sodium bicarbonate to basal medium and incubated in a 5% CO_2 incubator at 37°C. The cells were refreshed once every 3-4 days to maintain the optimum conditions for exponential growth.

3.3.2.2. Subcuture

MCF-7 cells were subcultured twice a week to maintain the optimum conditions for the exponential growth. The culture medium was removed from the flask and cell were detached from the surface of the 25 cm^2 T-flask by trypsinization with 1.5 ml 0.05% Trypsin solution for 30-45 seconds at room temperature, then the solution was removed. The cell were re-suspended in 5 ml of fresh DMEM medium containing with serum, dispense into the new culture flasks. Fresh DMEM was added to the final volume at 10 ml and finally incubated in 37 °C, 5% CO_2 incubator.

3.3.2.3. Cell suspension preparation for assay

MCF-7 cells were dissociated into single cells by trypsinization. Fresh EMEM was added to the final volume at 5 ml and finally the cells were incubated in the 37°C, 5% CO_2 incubator. After 3 days of culturing, the medium was removed and the cells were rinsed with 3-4 ml PBS. The cells were trypsinized with 1.5 ml of 0.05% trypsin. The fresh DMEM was added and aspirated gently. The cells were counted with the application of 0.4% Trypan Blue dye solution and a haemocytometer. One hundred microliters of diluted MCF-7 cell suspension (at approximately $5x10^4$ cell/ml) were

inoculated in 96 multi-well plates in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin / Streptomycin.

3.3.2.4. Cell count and dilution

The 0.4% Trypan blue dye solution and hemacytometer were applied to determine the viable cell number. Make 1:1 dilution of cell suspension with 0.4% Trypan blue and gently mixed thoroughly by pasture pipette. The cover slip was place on haemacytometer. Trypan blue-cell mixture was transferred to chamber by touching tip of pasture pipette to edge of the cover slip and allowing the chamber to be filled by capillary action. The hemacytometer was placed on the stage of microscope focus with the counting chamber. Trypan blue stain will only enter across the membranes of non-viable cells and the viable cell not stained. The viable cells were counted in 4 of 1 mm^2 on the corner and on 1 mm^2 in the middle of the hemacytometer.

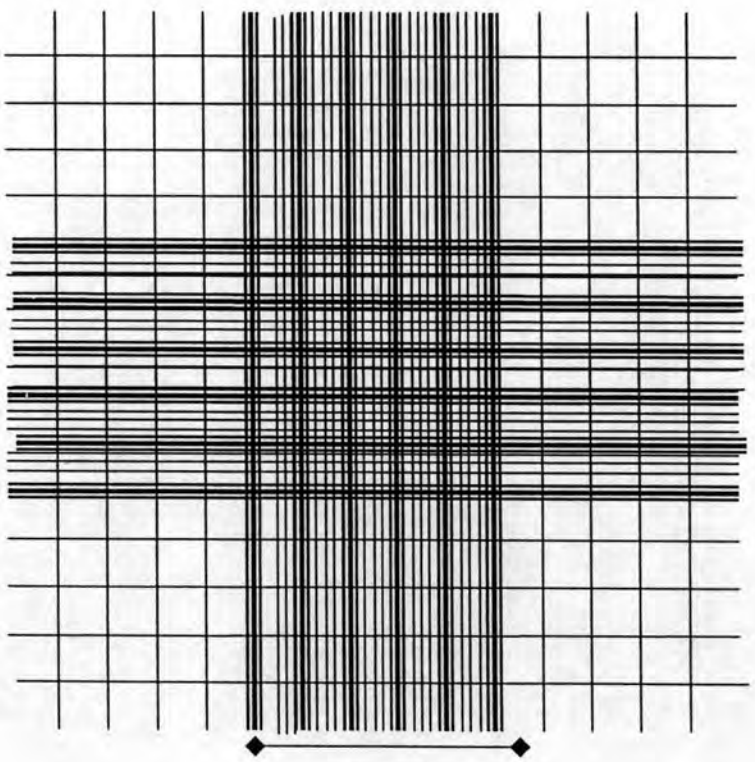


Figure 3.4 Magnified view of the cell counting chamber grid. The central 1 mm^2 area is divided into 25 smaller squares, each 1/25 mm^2. There are enclosed by triple rule lines and are further subdivided into 16 squares, each 1/400 mm^2.

The cells in each square of the hemacytometer 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 equation:

Cell density (cell per ml) = (total cell count /5) x 2 x 10^4

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

Dilution factor (x) = cell per ml / 4 x 10^4

Diluted cell suspension with DMEM to desirable volume (y)

Media x-1 ml : Cell 1 ml

Media y ml : Cell z ml

(z = cell volume for dilution)

3.3.2.5. Preparation of S9 mixture 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 fractions keep at –80 °C was thawed at room temperature. 10 ml of S9 mix composed of the ingredients; 6.6 ml, 0.2M phosphate buffer pH 7.4, 0.4ml, 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). The S9 mix was freshly prepared and kept on ice during experiment.

3.3.2.6. Standard test

One hudred microliter of dilute MCF-7 cell suspension (from 3.3.4) were inoculated in 96-well microtitre plates (at 5x10^4 cell/ml approximately) in DMEM medium supplemented. Stock solutions (10^{-5} M.) of standards (puerarin, daidzin, genistin, daidzein and genistein) and stock solution of 17-β estradiol (E_2) (10^{-8} M.) were freshly prepared in DMSO, diluted with solution control (culture medium with 2% DMSO v/v). The concentration of DMSO following dilution was 2% of the total volume, an amount that was not toxic to the cells. The assays were prepared by incubating the cells with the

standards (at concentration 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M. and 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M. of E_2 4 well per concentration)

3.3.2.7. Cytotoxicity test

The plant crude extract was dissolved in 100% DMSO and adjust the concentration to 200,000 μg/ml. Stock solution was diluted to the test concentration by 100% DMSO which did not exceed 2% of the total volume. The assays were prepared by incubating cells with the plant crude extract (at 0.1, 1, 10, 100 and 1000 μg/ml, 4 replicate per concentration) and without extract as a negative control. The 96-well microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 3 days (72 h).

3.3.2.8. Cell Proliferation assay

Cytotoxic testing can be measured by clonal growth, net change in population size, a change in cell mass or metabolic activity. The 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphynyl tetrazolium bromide (MTT) assay is one of the metabolic activity measurements. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form purple formazan crystals. The color can be measured spectrophotometrically at 540 nm. MTT assay offers a quantitative, reproducible, rapid and convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or decrease in growth. Application is used as clinical, pharmaceutical, cosmetic and chemical industries, viability and cytotoxicity analysis.

Cell proliferation assay was performed by MTT colorimetric method (Carmichael et al., 1987 and Twentyman, 1987) and with S-9 reaction : Each well of 96-well plate were filled with 125 μl of S9 reaction (S9 with buffer, NADP, G-6-P and $MgCl_2$) , 75 μl DMEM and 50 μl of cell. After 72 h incubation period, 96-well plate was carried out to add MTT 5 mg/ml 10 μl/well and incubated 3 h under darkness at 37°C. The medium and MTT was removed from wells. The remaining MTT-formazan crystals were dissolved by addition of 150 μl DMSO. Plates were kept agitation for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative was analyzed by microplate reader at the wavelength 540 nm with 250 μl DMSO as a blank. The percentage of cell vialbility was calculated according to the following formula.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells x100}}{\text{OD of control cells}}$$

The IC_{50} value was obtained by plotting the percentage of cell viability versus the concentration.

3.3.2.9. Statistical analysis

The results were shown as mean ± standard error (SE) of three replicate experiments. One Way Anova was submitted for the comparison of the difference within group and between groups (LSD test) and Duncan. The IC_{50} values at 95% confidence limits of the extracts after 3 days were calculated by Probit Analysis. All statistical analysis is performed by SPSS version 13 (SPSS, INC.). The P value of less than 0.05 was considered to be statistically significant.

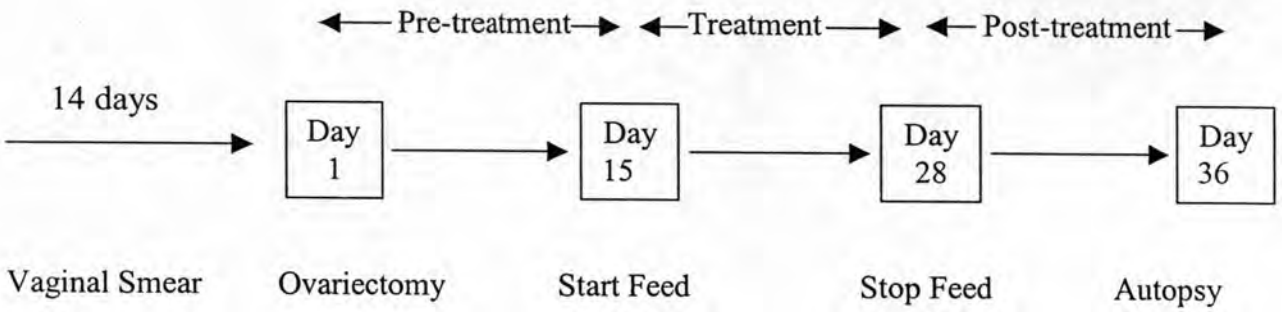
3.3 Ovariectomized rat assay

Adult female Wistar rats were supplied from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed in stainless steel cages (5 animals/cage) with a standard animal husbandry environment of controlled lighting (lights on 06.00-20.00 h) and temperature (25 ± 1 °C) at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Thailand. Rat chow and tap water were provided *ad libitum*. The animal experiments conducted in the morning between 08.00 and 10.00 h. The experiment protocol was approved by the Animal Ethical Committee in accordance with the university guideline for the care and use of laboratory animals.

The rats used were 60-days old and 200-250 gm in weight, and each had at least 3 consecutive estrous cycles (4-5 days) before the start of this study. The 5 rats in each group were ovariectomized under ether anesthesia in the diestrous phase of the 4th estrous cycle. The day of ovariectomy was designated as the 1st day of the study period. The rats were divided into the following 3 groups; *P. mirifica*, negative control, and positive control. The treatment schedule was separated into the following 3 periods: pretreatment for 14 days, treatment for 14 days, and post-treatment for 7 days. In the pretreatment and

post-treatment periods, the rats were administered 0.7 ml distilled water. In the treatment period, the rats of the *P. mirifica* group were fed 100 or 1,000 mg/kg BW of *P. mirifica* powder suspend in 0.7 ml distilled water daily, the rats of the negative control group were fed 0.7 ml distilled water daily, and the rats of the positive control group were subcutaneously injected with 0.2 mg/100g BW of 17β-estradiol (Sigma, St. Louis, MO, USA) dissolved in corn oil daily, respectively.

Experimental plot



3.3.1. Vaginal cornification assay

The vaginal epithelium was checked daily between 08.00 and 09.00 h with the aid of a small glass rod sterilized with 70% alcohol solution and soaked in 0.9% normal saline solution before use. The vaginal cells were smeared onto a slide with a drop of 0.9% normal saline solution, observed under a light microscope (100×), and identified and then their cell types were recorded. Vaginal cells were categorized into the following 3 types: leucocyte cells (L), nucleated cells (O), and cornified cells (Co). The representative cell-type was determined by selecting the type of cells that comprised the majority of cells. The results of examination of vaginal smear cells from 5 rats in each treatment group were expressed as a mode value (the most frequently occurring cell type in 5 rats). The appearance of cornified cells (or a majority of Co-type cells) was used as an indicator of estrogenic activity. Thus, 25 cultivars of *P. mirifica* were ranked according to their estrogenic activity. The criteria for ranking were as follows: 1) earlier stimulation of Co-type cell appearance (or the first day of cornified cell appearance) after 100 mg/kg BW of *P. mirifica* was judged as the higher estrogenic activity, and 2) if the first day of cornified cell appearance was the same between two (or more) cultivars of *P. mirifica* after judgement with the first criterion, earlier stimulation of Co-type cell

appearance after 1,000 mg/kg BW of *P. mirifica* was used for subsequent judgement for the next step.

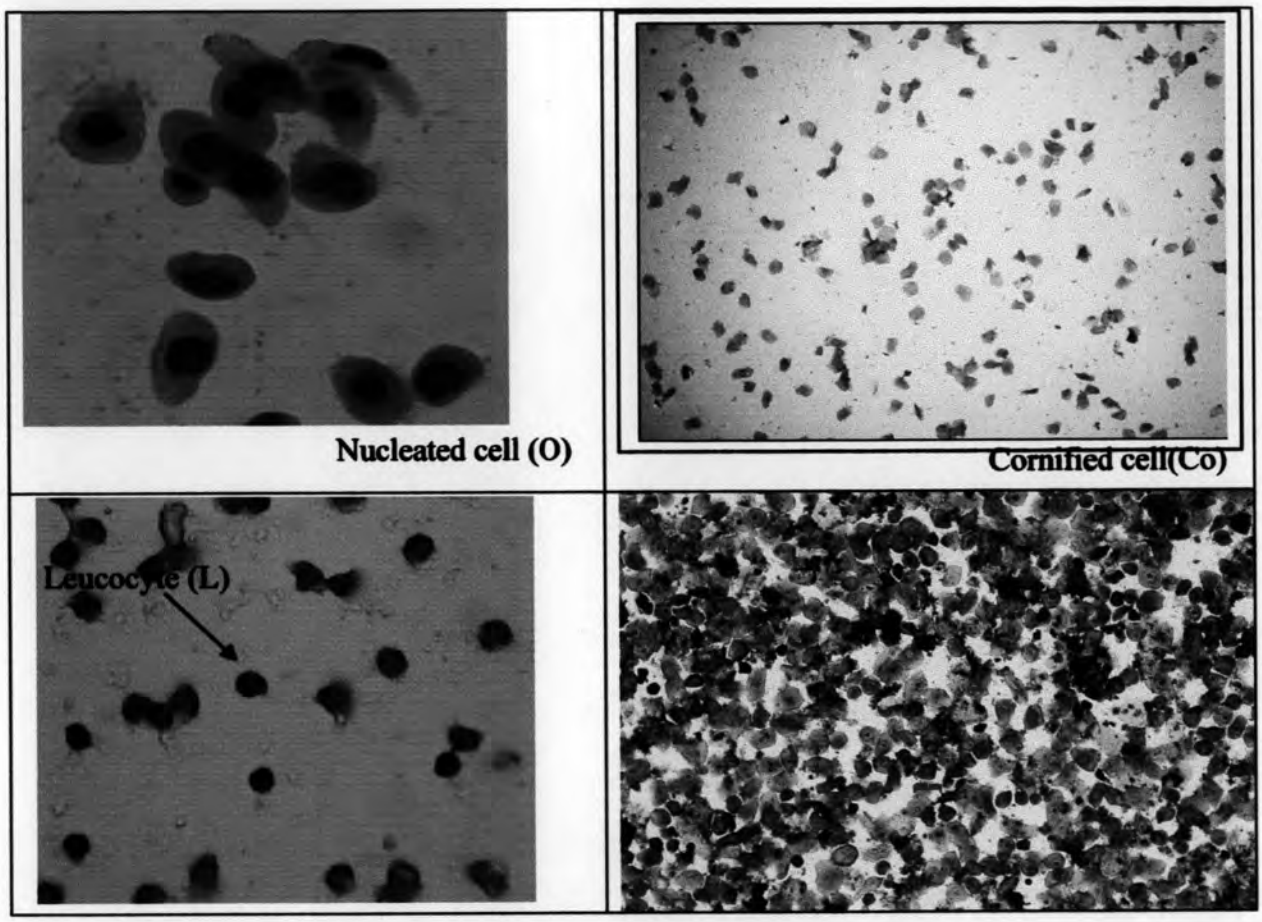


Figure 3.5 Vaginal epithelium cells found in the different phase of estrous cycle in rats. L-type cells, O-type cells, Co-type cells indicated lucocyte, nucleated and cornified cells respectively.

Additionally, the percentage of cornified cells was randomly calculated for the negative control (distilled water) group, positive (17β-estradiol) control group, and the five cultivars of *P. mirifica* in the treatment groups. In brief, after evaluation of vaginal smears the 3 types of vaginal cells were counted randomly for total of 100 cells and the percentage of cornified cells were then calculated. The percentage of cornified cells were calculated according to Ternius(1971) as follows;

$$\text{Percentage of cornified cells} = \frac{\text{Number of cornified cells}}{\text{Number of leucocyte + cornified + nucleated cells}} \times 100$$

3.3.2. Uterotrophic assay

The uterus was removed, trimmed the fat tissue and cut above junction between the cervix and the uterine horns. The uterus was weight and fixed in 10% buffer formalin at least 24 hr. and processed according to the standard histological technique (Humanson, 1967).

3.3.3. Histology

3.3.3.1 Uterine gland number

The uterine gland numbers were counted under microscopic field (4x). The three uterine tissues from five tissues in each experiment were counted for the total gland for each section.

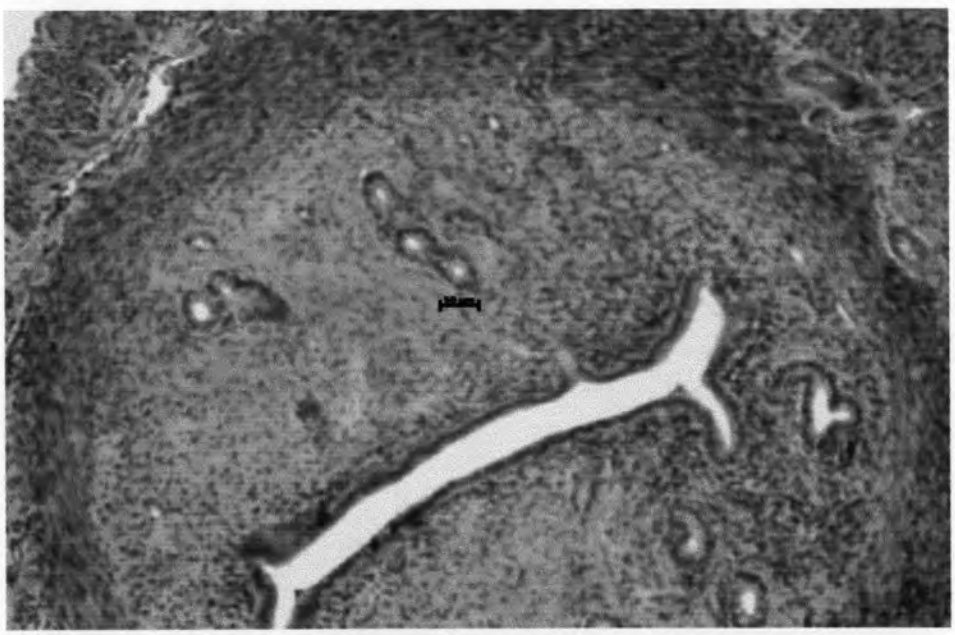


Figure 3.6 The uterine glands of uterus tissue in ovarictomized rat.

3.3.3.2. Cross-section area of uterine tissue assay

The cross-section area of uterine tissue was measured under microscopic field (10x) by Image-Pro express program version 2. The result was determined as cross-section area of endometrium, myometrium and lumen of uterine tissue.

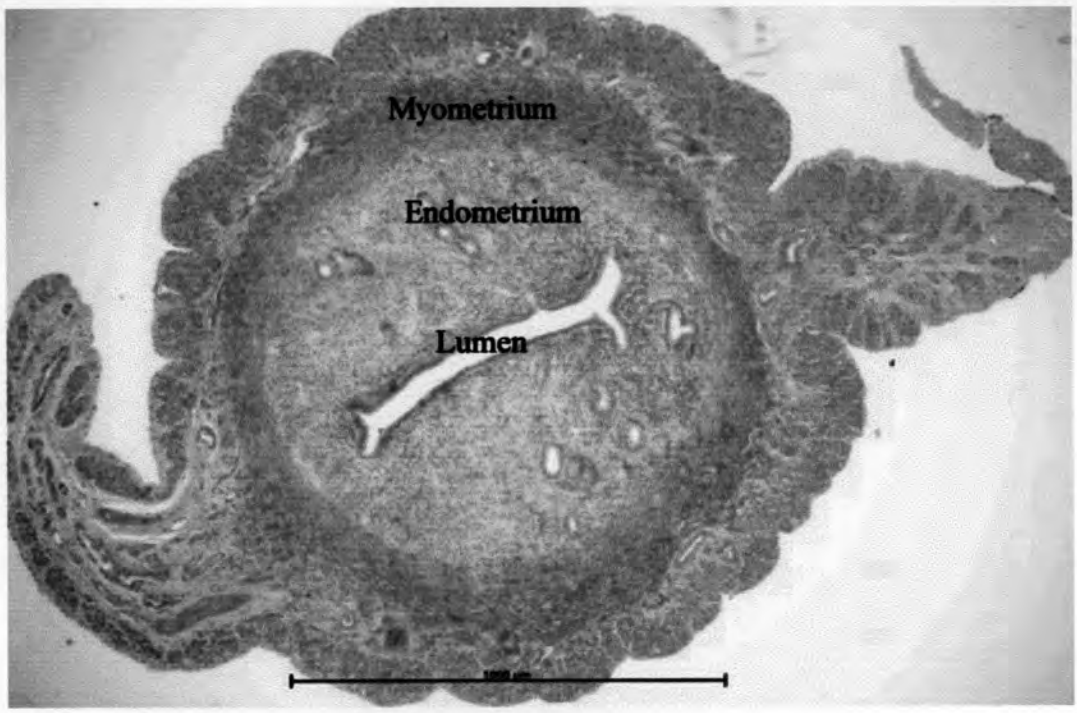


Figure 3.7 The area of three layers of uterine tissue (4x) in ovariectomized rat (myometrium, endometrium and lumen)

3.3.4. Statistical analysis

The results from vaginal cytology were compared between dosage and clone to seek for high estrogenic activities that have the earliest and longest response.

Uterine weight, body weight, uterine gland number and X-section area of uterine tissue were analyzed by one way analysis of variance using PC-vase version of the Statistical Program for the Social Science (SPSS) program version 13.

The correlation between estrogenic activity determined by vaginal cytology assay and physical factor, or between the estrogenic activity determined by vaginal cytology and the isoflavonoid content were also analyzed by SPSS version 13 (SPSS, INC.). The significant level was taken at $P < 0.05$.

3.4. Correlation analysis

3.4.1. Correlation of isoflavonoid contents between tuber and leaf

Isoflavonoid contents of tuber and leaf collected from the same plant at the same period will be compared to establish a correlation analysis pattern.

3.4.2. Correlation of tuberous isoflavonoid contents and bioassays

Isoflavonoid contents of tubers and bioactivity of the crude extract derived from the same tuber will be compared to establish a correlation analysis pattern.