CHAPTER III

MATERIALS AND METHODS

A. Endometrial cells

1. Normal endometrial cell isolation

The immature pig uterus, aged 3-6 months, was selected as a model for this study to minimize the variability due to the effect of hormone cycling. The porcine endometrial cell isolation was done following the method of Deachapunya and O'Grady (1998) which proven to contain endometrial gland 90% purity. The pig uteri from slaughter house were transported in standard porcine ringer solution (mM: 130 NaCl, 6 KCl, $3CaCl_2$, 0.7 $MgCl_2$, 20 $NaHCO_3$, 0.3 NaH_2PO_4, 1.3 Na_2 HPO_4, pH 7.4) which was kept on ice. Upon received, the selection criterion were the total length between 10-15 cm, the pinkish color with no swollen and the intact ovary contained no visible follicles or corpus luteum. Then the uterus was cleaned and trimmed to remove the serosa and connective tissues, the uterine horns were clamped with artery forceps to make a closed system, cut and wash porcine ringer containing Kanamycin (50x). After washing step, the above uterine horn was minced into smaller pieces (~ 1-2 cm), and cut longitudinally to expand into a rectangle, spread and placed on plastic plate, the basal epithelial side on top. The serosa and muscle layer were peeled off and the rest was then washed in the porcine ringer containing Kanamycin. These endometrium tissues were further incubated in Ca^{2+}, Mg^{2+} free PBS containing penicillin–streptomycin (2X) and 1% fungizone for 45-60 min at 4 °C (3-5 pieces per 40 ml). While incubating, the collagenase was prepared by dissolved the collagenase in DMEM containing antibiotic at a final concentration of 2 mg/ml. The endometrium was then cut into smaller pieces and incubated in prepared collagenase solution for 24 hrs at 37 °C. The digested endometrium was further dissociated by pipetting through Pasteur pipette, and the PBS was then added to stop the collagenase reaction. The tissue solutions were then filtered through a mesh screen. The endometrial gland was isolated by centrifuge at 500 x g for 10 min at 4°C. The resulting pellets were washed with PBS containing antibiotic and allowed to settle by gravity force for 15 min. This step was then repeated until the supernatant was cleared in color. Finally, the pellet was washed with DMEM containing

antibiotic and transferred to cell culture dishes. The standard culture media was DMEM supplemented with 3.7 g/L $NaHCO_3^-$, 10% FBS, 5 μg/ml insulin, 1% non-essential amino acid, 5 μg/ml fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin. The cells were allowed to settle in incubator (37°C, 5%CO_2) overnight. The media was changed on the next day and every 2 days, thereafter. The endometrial cells were maintained in standard DMEM until confluence in 100 mm culture plate.

2. Endometrial cancer cells (RL95-2)

The endometrial carcinoma cell line, RL95-2 purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA), was cultured in DMEM supplemented with 5 μg/ml insulin (Sigma), 2.0 g/l $NaHCO_3^-$, 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2.

B. Chemicals

The ethanol extractions of *Peuraria mirifica* root are kindly provided as a blended, light beige powder by Bio-Botanica Inc., Thailand. The *P. mirifica* used in all experiments were freshly prepared in distilled water at the concentration of 0.1 g/ml. After leaving for sedimentation, the supernatant was then collected and sterile-filtered before testing with the cells. The main active ingredient in 100 g powder elution contains miroestrol, 35.7 mg; daidzin, 12.8 mg; puerarin, 24.4 mg; genistein, 1.4 mg, standardized from company using High Performance Liquid Chromatography (HPLC).

17β-Estradiol ((1, 3, 5 [10]-Estratriene-3, 17 β -diol), E-8875, FW 272.4, lot no.120H0126, Sigma, St. Louis, MO)) was dissolved in 95% ethanol, then mixed to give a stock concentration of 0.1 M. Genistein (98% purity, FW 270.24, lot no.034K0852, Sigma) was dissolved in dimethylsulfoxide (DMSO), and then mixed to give a stock concentration of 0.1 M. Daidzein (98% purity, FW 254.20, lot no. 025K4102, Sigma) was dissolved in dimethylsulfoxide (DMSO), and then mixed to give a stock concentration of 0.1 M.

All other chemicals and cell culture materials were purchased from Sigma and Gibco (Invitrogen, Bangkok, Thailand).

C. Experimental protocols

Experiment 1: To determine the effects of ethanol extracted of *Pueraria mirifica* root, daidzein, genistein and 17β-estradiol on the proliferation of normal porcine endometrial cells

Normal porcine endometrial cells were divided into 5 groups:

1. Control group; cells were treated with media or vehicle
2. *P. mirifica* group; cells were treated with ethanol extracted *P. mirifica* root.
3. Daidzein group; cells were treated with Daidzein
4. Genistein group; cells were treated with Genistein
5. 17β-estradiol group; cells were treated with 17β-estradiol

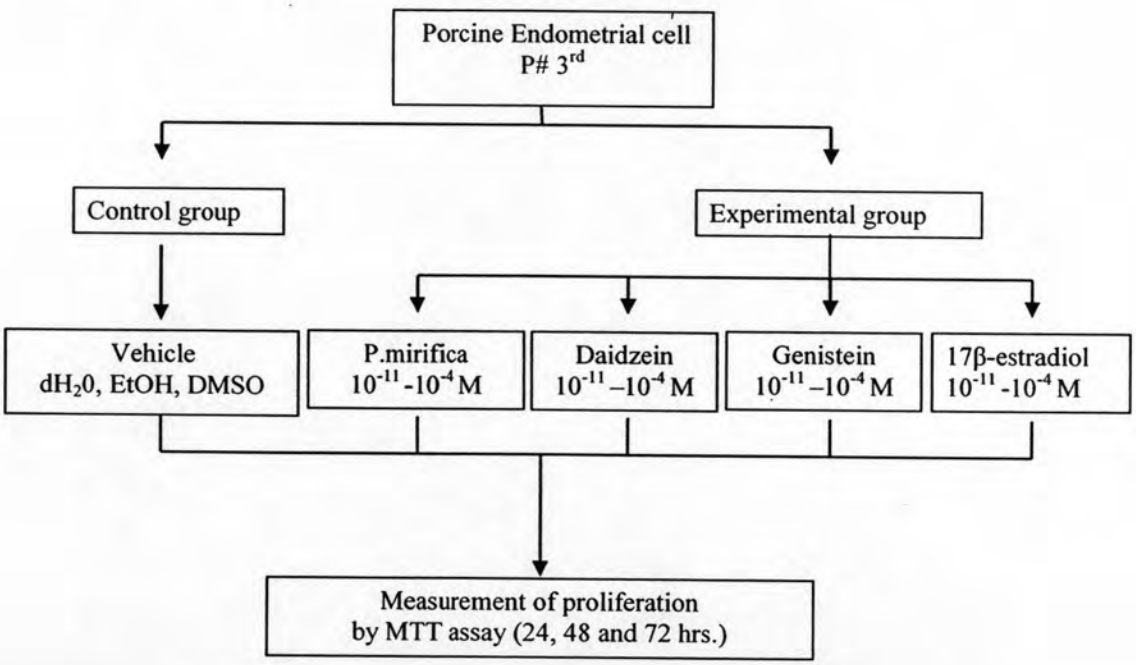

Figure 3-1 Diagram of the experimental protocol 1

Experiment 2: To determine the effects of ethanol extraction of *Pueraria mirifica* root, daidzein, genistein and 17β-estradiol on the proliferation of RL-95 endometrial cancer cells

RL-95 endometrial cancer cells were divided into 5 groups:

1. Control group; cells were treated with media or vehicle
2. *P. mirifica* group; cells were treated with ethanol extracted *P. mirifica* root.
3. Daidzein group; cells were treated with Daidzein
4. Genistein group; cells were treated with Genistein
5. 17β-estradiol group; cells were treated with 17β-estradiol

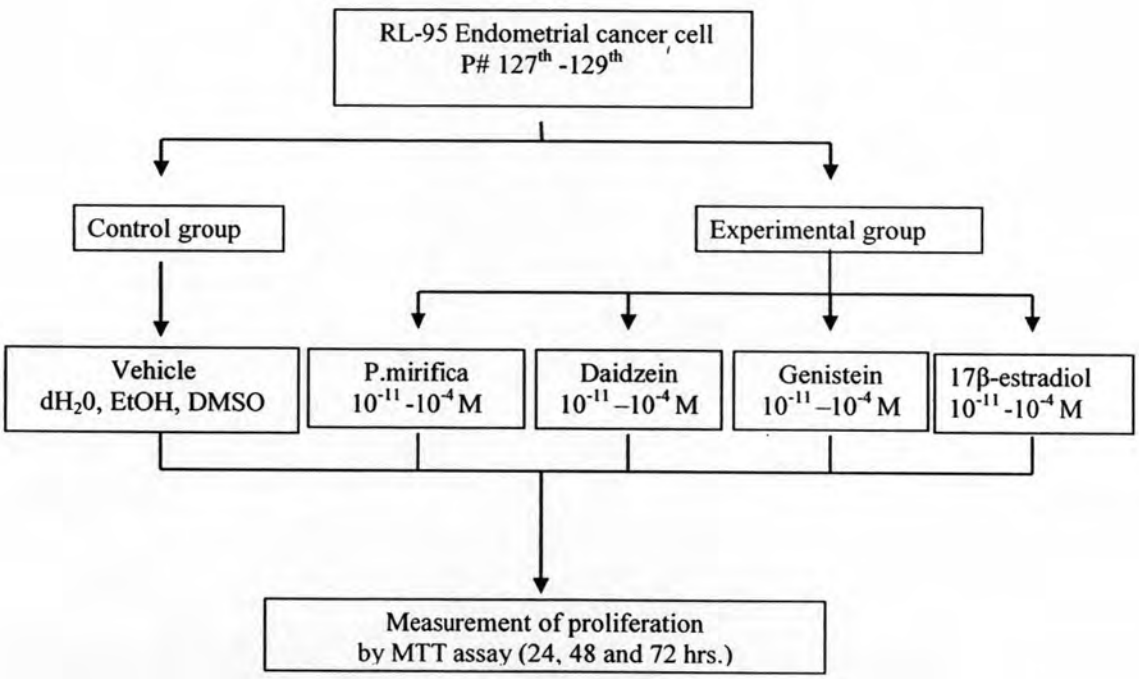

Figure 3-2 Diagram of the experimental protocol 2

Experiment 3: To quantitate estrogen receptor protein expression in normal endometrial cells and endometrial cancer cells treated with ethanol-extracted *Pueraria mirifica* root, daidzein, genistein and 17β-estradiol.

PE-I and RL-95 were divided into 5 groups:

1. Control group (with media or vehicle)
2. Cells were treated with uneffective and effective concentration (from Exp.1 and 2) of ethanol extraction of *P. mirifica* root
3. Cells were treated with uneffective and effective concentration (from Exp.1 and 2) of daidzein
4. Cells were treated with uneffective and effective concentration (from Exp.1 and 2) of genistein.
5. Cells were treated with uneffective and effective concentration (from Exp.1 and 2) of 17β-estradiol

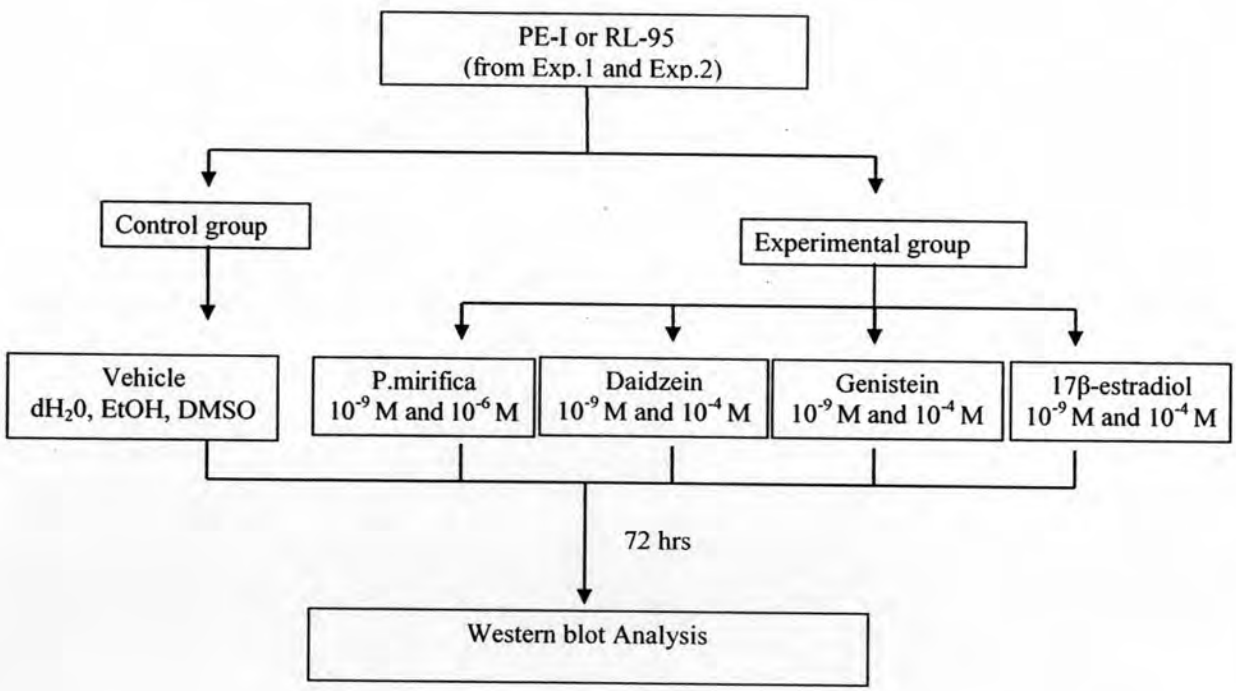

Figure 3-3 Diagram of the experimental protocol 3

D. Methods

1. Cell culture

Cultures of normal endometrial gland and RL-95, endometrial cancer cells were seeded into 100 mm plates in standard DMEM containing 10% FBS and grown until confluent. The fresh standard media was replaced every 2 days. In order to reduce the proliferative effect of serum contained in growth media, the cell culture media of plate containing confluent cells were substituted by DMEM/ 2% FBS. Two days later, cells were trypsinized and seeded into 48-well plate (5×10^4 cells/ml/well) in the above condition media. After 48 hrs, cells were treated with either PM, Ge, Di or 17β-estradiol with varying concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M. In addition, some cells were added with the equal volume (10 μl/ml/well)) of the vehicle used in this study. At 24, 48 and 72 hours after treatment, cell proliferation was assessed by MTT assay. For Western blot analysis, cells were plated and pretreated in 100 mm plate; 48 hrs later, the selected concentrations of each chemical either of uneffective or effective concentration were added and the proteins were collected 72 hrs after the drug treatment. The proliferation assay was done in duplicate.

2. Measurement of cell proliferation by MTT assay

The MTT assay is based on the mitochondrial metabolic reduction of soluble MTT to insoluble formazan crystals by mitochondria dehydrogenase of viable cells (Alley et al., 1988; Scudiero et al., 1988). At 24, 48 or 72 hrs after treatment, media was replaced with 125 μl DMEM containing MTT solution (62.5 μg/ml) and incubated for 3 hrs in CO_2 incubator. The MTT medium was then removed and the cells were dissolved with 100 μl dimethyl sulfoxide (DMSO) to dissolve formazan crystal. At this step, plates were wrapped in aluminum foil and incubated for 45 minutes in CO_2 incubator. The DMSO-solubilized cell solution was transferred to a 96-well plate. The absorbance of formazan and non-specfic background was respectively read at 570 nm and 620 nm by multiwell scanning spectrophotometer (Tecan Sunrise™, Tecan trading AG, Switzerland). Relative cell numbers are proportional to the specific absorbance of formazan products which are the subtraction of the 620 nm from 570 nm reading values. Cell numbers in the vehicle

control group are considered 100 % and those in the treatment groups were calculated as percent of controls. In this study, the interassay coefficient of variation of 10.44% was calculated from 7 duplicated RL-95 treated with vehicle control (H_2O) at 24 hrs.

2.1 MTT solution preparation

MTT [3-(4,5-dimethythiazol-2-yl)-2 5-diphenyltetrazolium bromide] was weighed and dissolved in distilled water to the final concentration of 5 mg/ml, then filtered in sterile hood and kept at 4°C.

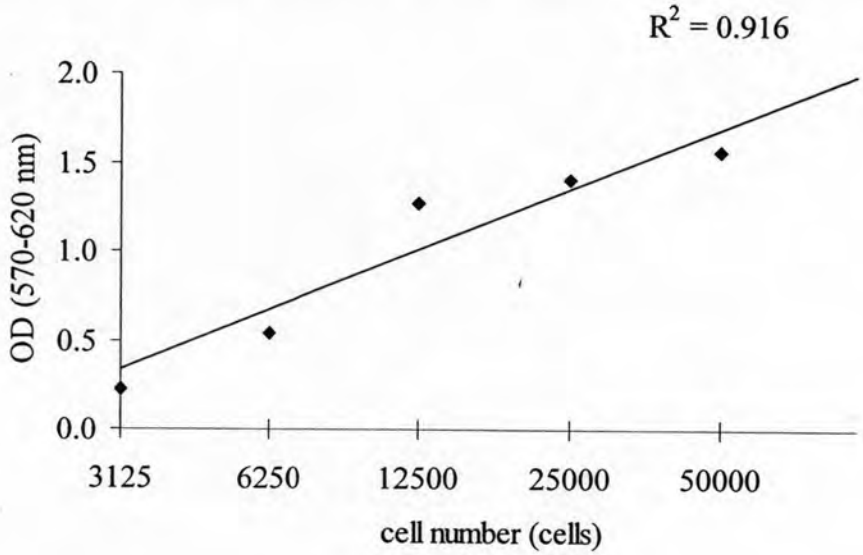

Figure 3-4 Graph relationship between absorbance and cell numbers

3. Western blot analysis

3.1 Electrophoresis

Cells were harvested and total cell lysates made with lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0). After incubating on ice for 30 min, cell lysates were centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined by BCA™ protein assay kit (Pierce Biotechnology, Inc., IL, USA). The supernatant was collected and stored at -20°C for further Western blot analysis. Samples (20 μg protein/lane) were resolved by 10% sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis using vertical minigel system (Bio-Rad laboratories Inc., Hurcules, CA, USA). Each sample was separated in triplicate.

3.2 Immunodetection for ER-α and ER-β

Subsequent to separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Arlington Heights, IL) in Tris-glycine transfer buffer. Blotted membranes were then blocked with 5% nonfat powdered milk in Tris-buffered saline (TBS) for 4 hour at room temperature. For identification of proteins, membranes were washed (2 × 5 min) and incubated overnight at 4°C with the primary antibodies diluted in 1% milk. The primary antibodies were anti-human ER-α or ER-β rabbit polyclonal antibody against the carboxyl terminal domain of human (HC-20 and HC-150, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at the dilution of 1:500 and 1:300, respectively. Following the primary antibody incubation, the membranes were washed and then incubated for 1 h in 1:10,000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Zymed Laboratories Inc., San Francisco, USA) at room temperature. This incubation step was terminated with several washes and the immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, USA) according to manufacturer's instructions. Membranes were exposed to film (Hyperfilm-ECL; Amersham Biosciences, Piscataway, NJ, US) for times adequate to visualize chemiluminescent bands. Blots were reprobed with 1:300,000 monoclonal anti-β-actin (clone AC-15; Sigma) and 1:10,000 horseradish peroxidase-conjugated anti-mouse secondary antibody (Zymed Laboratories Inc), respectively. Comparisons were made with known molecular weight standards. Differences in protein immunoreactivity between treatments were determined by scanning densitometry in proportion to β-actin immunoreactive bands (Scion Image; Scion Corporation, Frederick, MD).

The ratios of ER-α or ER-β to β-actin and the ratio of ER-β to ER-α were calculated as followed;

$$\text{Ratio of ER / beta actin} = \frac{\text{Density of ER-}\alpha\text{ or ER-}\beta}{\text{Density of }\beta\text{-actin}}$$

$$\text{Ratio of ER-}\beta\text{ / ER-}\alpha = \frac{\text{Ratio of ER-}\beta\text{ / beta actin}}{\text{Ratio of ER-}\alpha\text{ / beta actin}}$$

4. Measurement of protein concentration

Total protein concentrations used Western blot analyses were measured based on biocinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation using a unique reagent containing bichinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly with increasing protein concentrations over a broad working range (20-2,000 ug/ml).

4.1 Reagents

Reagent A, was sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M NaOH. Reagent B was 4% cupric sulfate. Bovine serum albumin 2 mg/ml (BSA) was used as a standard.

4.2 Procedure

Sample or standard 20 µl was added with 300 µl of BCA mixed (BCA A: BCA B; 50: 1 and incubated for 30 min at room temperature) for 30 min at 37°C, incubator. The absorbance was then measured at 570 and 620 nm. A standard curve of absorbance was plotted as a linear correlation for determination of the unknown protein concentration.

5. Statistical analysis

In the experiment 1 and 2, the proliferative effect was measured as percent change from control, and analyzed by one way analysis of variance (ANOVA) followed by the Newman Keuls comparison test as appropriate. In the experiment 3, the unpaired *t*-test was used to compare between vehicle control and treatments. In all cases, a value of $P < 0.05$ was considered significant.