Chapter III

Materials and Methods

1 The Plant extraction

1.1 Powder preparation

The tuberous roots of *P. mirifica*, *B. superba*, *P. Lobata* and the whole stem of *M. collettii* were collected from the selected cultivars indicated in table 4.1. All collected materials were washed, sliced and dried in hot-air oven at 70 °C. The dried material were grinded into powder. The powder was bottled and kept in cool, dry and dark place.

1.2 Extraction

The powder was extracted with absolute ethanol. The filtrates were collected by filtration (Whatman filter paper No.4, Whatman, USA) and subsequently evaporated in the rotary evaporator (Buchi, Germany) until completely dried. The extracts were bottled and kept in a cool, dry and dark place.

Table 5 Sources of plant materials in the experiments.

Species	Source
P. mirifica	Chiengmai Province in March 1999
P. lobata	Guangzhou, China in April 1999
B. superba	Lampang Province in March 1999
M. collettii	Chiengmai Province in March 1998

2. Cell and cell culture

MCF-7, the ER positive human mammary adenocarcinoma cell, and HeLa, the ER negative human cervical carcinoma cells were obtained from the National Cancer Institute, Thailand. The cell lines were cultured in EMEM medium supplemented with 10% heat-inactivated Newborn Calf Serum (NCS; BioWittaker, USA), 100000 unit/L Penicillin G and 1mg/L Streptomycin sulfate 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.

2.1 Subculture

MCF-7 and HeLa cells were subcultured twice a week. The culture medium was removed from the flask and rinse with 3-4 ml of 1 x-Hank's Balance Salt Solution (1x-HBSS). The solution was removed and 3-4 ml of 0.25% Trypsin was added. The flask was allowed to stand 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 EMEM was added, aspirate, dispense into the new culture flasks. Fresh EMEM was added to the final volume at 5 ml and finally incubated in the 37°C, 5% CO_2 incubator.

2.2 Cell Suspension preparation for assay

MCF-7 and Hela cells were propagated 3 days before the experiment and were prepared for the assay as described below:

2.2.1 Cell digestion

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

MCF-7 cell suspension from 2.2.1 were transferred to 15 ml conical tube and centrifuge at 1500 rpm for 10 minutes. The supernatant was removed. The cell was gently separated by the aid of 23Gx1 ½ needle and 5 ml syringe in order to make single-cell suspension. The cells were counted and diluted as described in 3.2.2.2

The digested HeLa cell suspension from 2.2.1 were counted and diluted as described in 3.2.2.2

2.2.2 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 over fill or under fill the chamber. The viable cells, which were not stained with Trypan Blue, were counted in four-1mm square on the corner and on-1mm square in the middle of the hemocytometer. (**Figure 3**)

The cells in each square of the hemocytometer was 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)

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

Diluted cell suspension with EMEM to desirable volume (y).

Media x-1 ml : Cell 1 ml

Media y ml : Cell z ml

(z = cell volume for dilution)

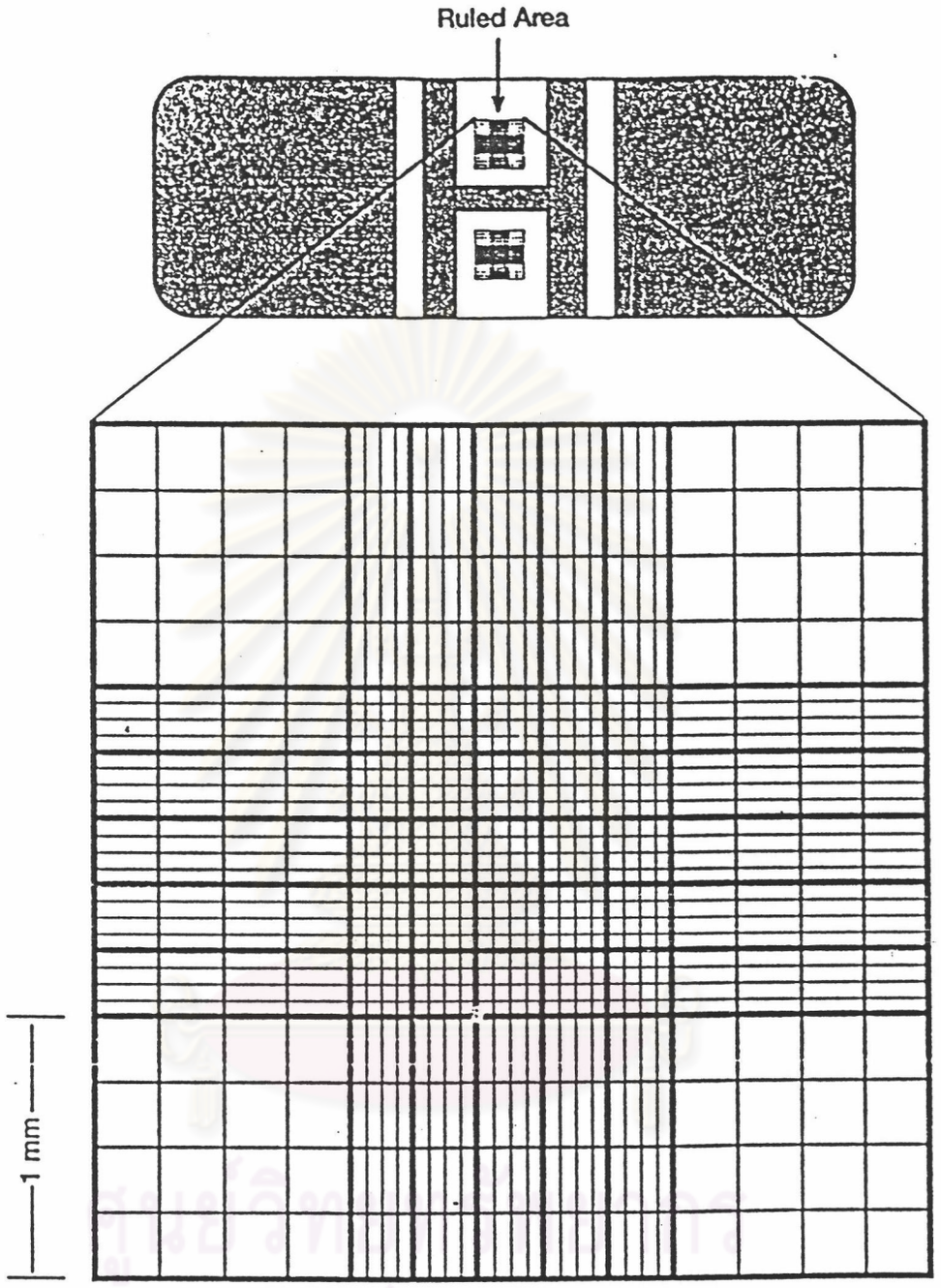

Figure 3 Corner square (enlargement). Cells were counted on top and left touching the middle line and not counted touching the middle line at the bottom and right.

3 Cytotoxicity test

3.1 Effect of the plant extracts on MCF-7 and HeLa cell line (Range finding test)

Three milliliter of diluted MCF-7 and HeLa cell suspension (from 2.2.2) were inoculated in the 25 cm^2 culture flask (at $4x10^4$ cell/ml approximately) in EMEM medium supplemented with 10% Newborn Calf Serum (NCS) and Penicillin / Streptomycin. Stock solutions of the extracts were freshly prepared in DMSO, diluted with the 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 plant extracts (at 0.1, 1, 10, 100, 1000 μg/ml, 2 flasks per concentration) and without the extract (control D_4). The flasks were incubated at 37°C in a 5% CO_2 incubator for 4 days. Control D_0 was prepared by aliquot 3 ml of cell suspension into 15 ml conical tube and centrifuge at 1500 rpm for 10 minutes. The supernatant was removed and 5 ml PBS was added. The cells were pelleted by centrifugation at 1500 rpm for 10 minutes. The supernatant was removed and the cells were stored at 4°C until protein determination. The first day of experiment was noted as day 0 (D_0) whereas the cells were collected at day 4 (D_4)

The culture media was removed and gently washed with 5ml Phosphate Buffer Solution (PBS) twice at D_4. Stored at 4°C until protein determination.

3.2 Median effective dose (ED_{50}) at D_4 analysis of the plant extracts

From the result 2.1 and 2.2, the concentration interval of the plant extracts, which caused 50% of cell death, were chosen and divided into 5 intervals. The assays were prepared by incubating the cells with the plant extracts (at chosen concentrations) and with DMSO (control D_4) and incubated at 37°C in a 5% CO_2 incubator for 4 days. The control D_0 was prepared by aliquoted 3 ml of the cell suspension into 15 ml conical tube and centrifuge at 1500 rpm for 10 minutes. The supernatant was removed, 5ml of PBS were added. The cells were recentrifuged at

1500 rpm for 10 minutes. The supernatant was removed and the cells were stored at 4°C until protein determination. The first day of experiment was noted as day 0 (D_0). The cells were collected at day4 (D_4)

The culture medium was removed at day 4 and gently washed twice with PBS. They were stored at 4 °C until protein determination.

3.3 Effect of the plant extracts in the presence of estradiol

The selected low concentration (1 μg/ml for *P. mirifica* and *P. lobata,* 10 μg/ml for *B. superba* and *M. collettii*) and high concentration (1000 μg/ml for all plants) were chosen. The experiment was divided into 6 groups. The cells were incubated with the plant extracts. 1) with proliferative dose of the plant extract only. 2) with proliferative dose and Estradiol (E_2). 3) with anti-proliferative dose 4) with anti-proliferative dose and E_2 5) with E_2 (positive control) and 6) with medium (negative control). The cells were incubated in 37°C, 5% CO_2 incubator for 4 days. Control D_0 was prepared by aliquoted 3 ml of cell suspension into 15 ml conical tube and centrifuge at 1500 rpm for 10 minutes. The supernatant was removed and 5 ml of PBS were added. The cells were recentrifuged at 1500 rpm for 10 minutes. The supernatant was removed and the cells were stored at 4°C until protein determination. The first day of experiment was noted as day 0 (D_0) .The cells were collected at day 4 (D_4)

At D_4, the culture medium was removed and gently washed twice with 5ml PBS. They were stored at 4 °C until protein determination.

4. Protein determination (modified from Oyama and Eagle, 1956)

4.1 Washing of the cell culture

The culture flasks were medium drained by inversion. The adherent cell layer was rinsed twice with 5 ml PBS. After the second rinse, the flasks were left inverted over clean tissue paper to drain for approximately 15 minutes. At this stage, the rinsed

and drained cells may be kept in the original flasks at 4°C for several days with no effect to protein determination.

4.2 Solution of cell in alkaline copper tartate

Five milliliters of Alkaline copper solution (See appendix) or Solution C was added to the flasks. The solution was allowed to contact with the cell layer for 10 minutes. The flasks were subsequently shaken to complete the dissolution of the cells. The flasks were possible to be stored for several days with no loss in protein.

4.3 Color development

One milliliter of the cell solution was pipetted into a test tube and solution C was added to a total of 5 ml. One ml of distilled water was added. 0.5 ml of the properly diluted Folin-Ciocalteau (F-C) reagent was finally added and mixed rapidly by vortex. The blank tube consisted of 1 ml distilled water, 5 ml of solution C and 0.5 ml of diluted F-C. The absorption was read after 30 minutes at 660 nm.

4.4 The calculation for cell growth

The absorbent value of the control D_4 was given as 100% of cell growth. The absorbent values of the others were calculated to percentage value compared to that of control D_4.

6 Statistical analysis

The results were shown as mean ± standard error (SE) of three experiments. Oneway anova was submitted for the comparison of the difference within group and between groups (LSD test). The ED_{50} values at 95% confidence limits of the extracts after 4 days were calculated by Probit. All statistical analysis were performed by SPSS version 10.0 (SPSS INC.). The P value of less than 0.05 were considered to be statistically significant.