Chapter III

Materials and Methods

1 The plant materials

1.1 Plant powder preparation

The tuberous roots of *P. mirifica*, *B. superba* and the whole stem of *M. collettii* were collected from the various sites indicated in Figure 4, 5 and 6. (Cherdshewasart unpublished data). The plant materials were washed, sliced and dried in hot-air oven at 70 °C. The dried materials were grinded into powder at 100 Mesh size. The powder was bottled and kept in a cool, dry and dark place.

1.2 Crude extraction

Fifty g powder was extracted with 500 ml 95% 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.

Figure 3 Sources of plant materials; *P. mirifica* in the experiments.

No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
4	Phayoa
5	Nan
6	Lampang
7	Phrae
8	Lamphun
9	Uttharadith
10	Sukhothai
11	Tak
12	Phitsanulok
13	Phetchabun
14	kamphaeng phet
15	Nakhon Sawan
16	Uthaithani
17	Sakon Nakhon
18	Nong Bua Lam Phu
19	Chaiyaphum
20	Nakhon Ratchasima
21	Saraburi
22	Lop Buri
23	Kanchanaburi
24	Phrachin Buri
25	Ratchaburi
26	Phetchaburi
27	Prachuap Khiri Khan
28	Chumphon

Figure 4 Sources of plant materials; *B. superba* in the experiments.

No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
4	Lampang
5	Uttharadith
6	Tak
7	Phitsanulok
8	Phetchabun
9	Nakhon Sawan
10	Sakon Nakhon
11	Loei
12	Nong Bua Lam Phu
13	Khon Kaen
14	Chaiyaphum
15	Nakhonratchasima
16	Srisaket
17	Kanchanaburi
18	Lop Buri
19	Saraburi
20	Ratchaburi
21	Prachinburi
22	Chachoengsoa
23	Chonburi
24	Chantaburi

Figure 5 Sources of plant materials; *M. collettii* in the experiments.

2. 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 EMEM medium supplemented with 10% heat-inactivated Newborn Calf Serum (NCS; BioWittaker, USA), 10,000 unit/L Penicillin G and 1mg/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.

2.1 Subculture

MCF-7 cells were subcultured twice a week. The culture medium was removed from the flask and rinse with 3-4 ml of Phosphate Buffer Solution (PBS). The solution was removed and 2-3 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 cells were propagated 3 days before the experiment as follows:

2.2.1 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. The cells were trypsinized with 4 ml of 0.25% Trypsin, stand 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 suspension from 2.2.1 were transferred to micro-well plate. The cells 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, 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. (Figure 7)

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)

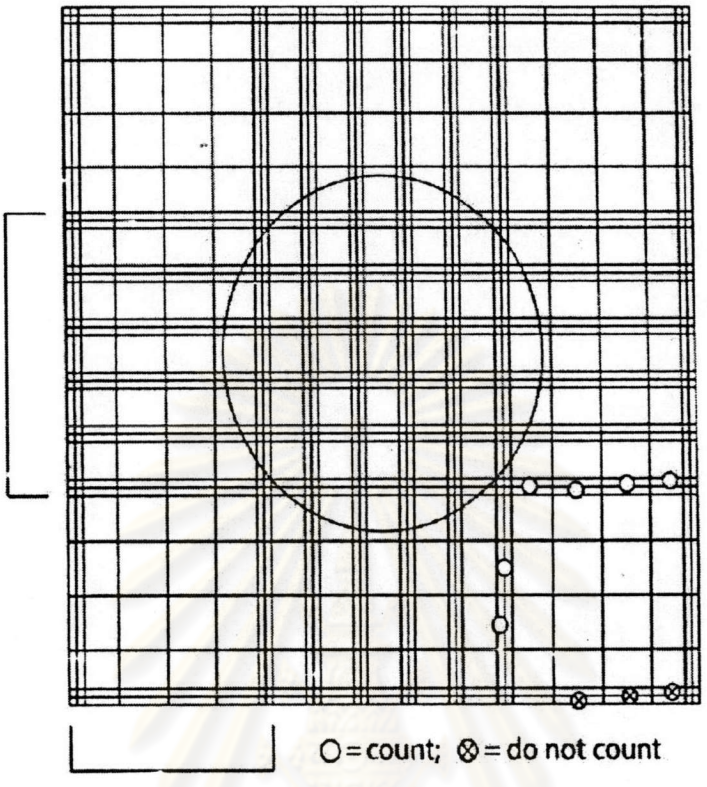

Figure 6. Corner square (enlargement). Cells were counted on top and left touching the middle line and not counted touching the middle line at the bottom and right.

3. Puerarin test

One hundred microliter of diluted MCF-7 cell suspension (from 2.2.2) were inoculated in the 24 multi-well plates (at $5x10^4$ cell/ml approximately) in DMEM medium supplemented with 10% Newborn Calf Serum (NCS) and Penicillin / Streptomycin. Stock solutions (10^{-5} M.) of puerarin and stock solution (10^{-9} M.) 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 puerarin (at concentration 10^{-7}, 10^{-8}, 10^{-9},10^{-10},10^{-11}, 10^{-12},10^{-13} M., 3 wells per concentration) and estradiol (at 10^{-10}, 10^{-11}, 10^{-12} M., 3 wells per concentration) and without the puerarin and estradiol (control D_3). The multi-well plates were incubated at 37°C in a 5% CO_2 incubator for 3 days.

4. Cytotoxicity test

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

One hundred microliter of diluted MCF-7 cell suspension (from 2.2.2) were inoculated in the 24 multi-well plates (at $5x10^4$ cell/ml approximately) in DMEM medium supplemented with 10% Newborn Calf Serum (NCS) and Penicillin / Streptomycin. Stock solutions (10000 µg/ml) 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, 3 wells per concentration) and without the extract (control D_3). The multi-well plates were incubated at 37°C in a 5% CO_2 incubator for 3 days.

4.2 Inhibition concentration (IC_{50}) at D_3 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_3) and incubated at 37°C in a 5% CO_2 incubator for 3 days.

5. MTT assay (modified from Mosmann, 1983)

The culture flasks were medium drained by inversion. The adherent cell layer was rinsed once with 1 ml DMEM and subsequently feed into the micro-well plates with 900 µl fresh medium and 100 µl MTT solution was added to all wells and incubate for 3 hrs. in a darkness at 37°C. The medium and MTT was removed from wells. The remaining MTT-formazan crystals were dissolved by addition of 900 µl

DMSO to all of the wells together with 125 µl glycine buffer per well. The absorbance of the solution was recorded at 570 nm with 900 µl DMSO and 125 µl Glycine buffer as blank for the spectrophotometry.

5.1 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.

6. Statistical analysis

The results were shown as mean ± standard error (SE) of three replicated experiments. One Way 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 3 days were calculated by Probit analysis. All statistical analysis is performed by SPSS version 10.0 (SPSS INC.). The P value of less than 0.05 was considered to be statistically significant.