CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and Reagents

The details of chemicals and reagents are shown in Appendix.

2.2 Preparation of bitter melon extracts

Fresh whole plants, leaves, fruits and tendrils of bitter melon were purchased from Lampang Medicinal Plants Conservation Assembly, Lampang, Thailand. Plant materials were dried at 30-45 °C and ground. Dried powdery plant samples were extracted exhaustively with 80 % ethanol by maceration. Treated the extracts by mixing with charcoal for decolorized. Dried ethanolic extracts were obtained after removing the solvent by evaporation under reduced pressure in evapolater, then lyophilized. Dried residue was weighed and stored at –20 °C. The scheme of extraction method was shown in Figure 7.

The extracts were used in all experiment were from the same lot of plant materials. How ever the HPLC fingerprint of each extracts were recorded for further reference (Appendix A)

The HPLC system for gradient elution

Column : Inertsil ODS-3, 253 X 4 X 6 mm

Guard column : Inertsil 4.6 X 50 mm

Mobile phase : gradient of 0.1 % TFA in water and methanol

Flow rate : 1.0 ml/min

Injection volume : 10 µl

Run time : 40 min

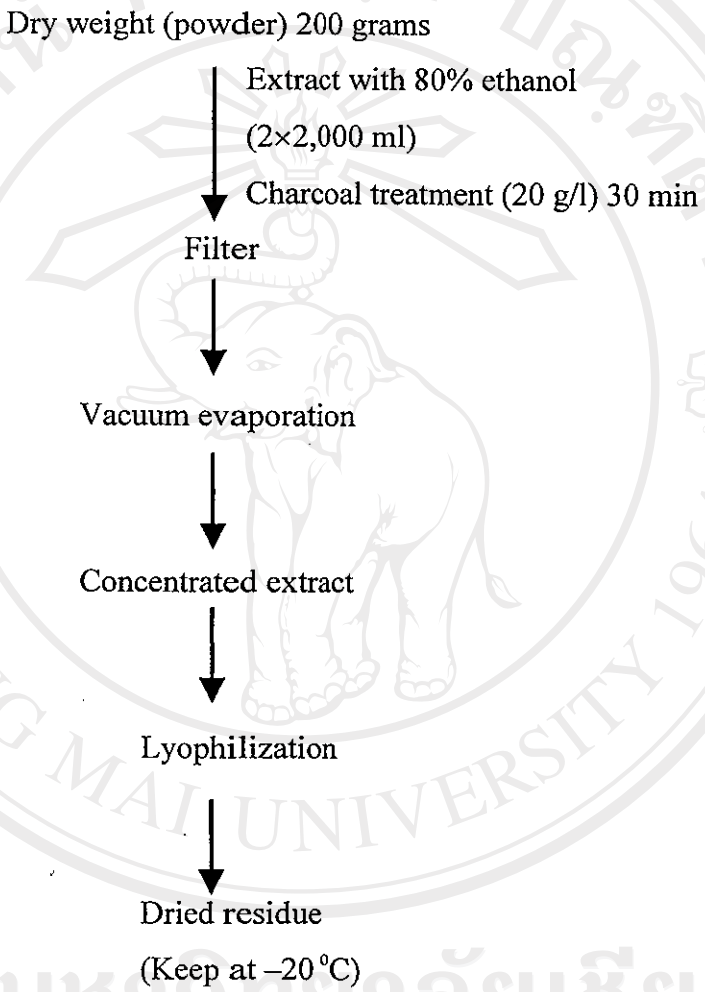

Figure 7. Protocol for preparing ethanolic extracts from bitter melon

2.3 Cell lines

Cell lines used in this research are human cervical carcinoma. All of the cell lines were provided by Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD., USA). The cell lines were divided into two types as follows:

KB-3-1 cell lines: The drug sensitive parental cell lines was derived from the primary cervix carcinoma of a 31-year-old black woman. The parent cell line for the drug resistant, KB-3-1, was derived from a single clone of human KB epidermoid carcinoma cell after two subclonings.

KB-V-1 cell lines: The multidrug resistant subline was derived from the parental line KB-3-1 by serial passage with increasing vinblastine concentrations. KB-V-1 cell line was maintained in the presence of 1 μg/ml vinblastine. These cells overexpress P-gp and possess 100-fold amplification of *MDR1* gene.

2.4 Cell culture conditions

KB-V-1 cells (multidrug resistance cervical carcinoma cell line) and KB-3-1 cells (drug sensitive cervical carcinoma cell line) were cultured in DMEM with 4.5 g of glucose/l plus 10% fetal calf serum, L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin; 1 μg/ml or 0.5 μg/ml of vinblastine was added only to the KB-V-1 culture medium. These two cell lines were maintained in humidified incubator under 95 % air and 5% CO_2 at 37°C. When the cells reached confluency, they were harvested and plated for consequent passages or for drug treatments.

2.5 Cells survival measurement

Cell survival is determined by using the colorimetric MTT [3-(4,5 dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide] assay. MTT assay was introduced by Mosman [86] in 1983. This assay measures the reduction of MTT by mitochondrial succinate dehydrogenase. The yellow tetrazolium salt MTT enters the cells and passes into mitochondria where it is reduced to insoluble purple formazan product (Figure 8). The formazan crystals are then solubilized with a variety of solvents, such as dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS) in phosphate buffer saline solution (PBS). The solubilized formazan product is spectrophotometrically measured using an ELISA plate reader. Since reduction of

MTT can only occur in metabolically active cells and the level of activity is a measure of the viability of the cells, the color products will not found in dead cells.

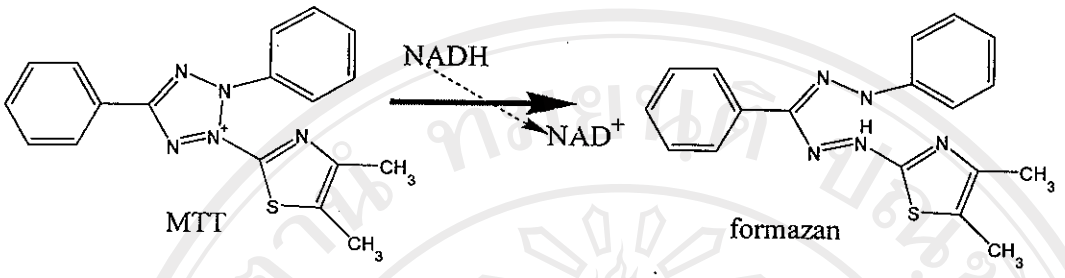

Figure 8. The reaction of MTT by succinate dehydrogenase in cell mitochondria

The procedure of MTT assay was performed by plating cells in 96 well plates (3 x10^3 cells/well), in 100 µl medium, and incubating before drug treatment at 37°C for 24 h. After 24 h, various drugs or the extracts were added in medium (100 µl) and incubated for another 48 h. The metabolic activity in each well was determined by the MTT assay [87] and compared to untreated cells. Just after removal of 100 µl medium, MTT stock dye solution (5 mg/ml in PBS) 20 µl was added to each well. The plates were incubated at 37°C under 5% CO_2 for 4 h. Most of the medium from each well was removed, leaving about 10-20 µl medium, an purple formazan crystals. In the well DMSO 200 µl was added to dissolve the crystals and the plates were shaken for 10 min. The absorbance at 540 nm with a reference wavelength of 630 nm was read on ELISA plate reader. The fractional absorbance was calculated by the following formula.

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

2.6 Radiolabeled drug accumulation and efflux

The effect of bitter melon extracts on drug accumulation was determined by intracellular radiolabeled drug (^{3}[H]-vinblastine) accumulation by the method modified from Plouzek *et al.*,1999 [88]. KB-V-1 cells (6.5 x10^5 cells/ well) were cultured in complete DMEM in 6-well plate for 24 h. Cells were exposed to 0.05 μCi ^{3}[H]-vinblastine/ml (10.8 Ci/mmol) in 5% CO_2 incubator for 60 min while vehicle control is 0.4% DMSO. The medium was removed and the plates were washed with ice-cold PBS pH 7.4. The cells were then harvested by centrifugation at 10,000 rpm at 4°C for 3 min. The cells were dissolve with 200 μl of 3 N NaOH, then neutralized with 100 μl of 6 N HCl. The 250 μl of cell lysate was pipetted into the scintillation vial containing 3 ml of scintillation fluid. The radioactivity was determined by β-counter. The protein concentration was determined by Bradford method using 10 μl of cell lysate in 96-well plate. The amount of intracellular radioactivity (counting unit) was calculated as DPM/mg protein.

For determination of drug efflux, cells were plated out as described for drug accumulation experiments. Cells were incubated at 37°C for 1 h with 0.05 μCi ^{3}[H]-vinblastine/ml and 50 μM verapamil in order to load the cells with radiolabeled drug. After the incubated cells were washed with ice-cold PBS pH 7.4, and then medium containing bitter melon extract or 0.4% DMSO. After incubation at 37°C for 30 min, the cells were washed with ice-cold PBS pH 7.4 and harvested. The amount of intracellular radioactivity was determined by scintillation counting.

2.7 Plasma membrane preparation from KB-V-1 and KB-3-1 cell lines

KB-V-1 or KB-3-1 cells were plated out at 6.5 x 10^6 cells per 15 ml medium in T-75 cm^2 culture flasks. After overnight incubation at 37 °C in 5% CO_2 incubator. Cells were then washed with ice-cold PBS (pH 7.4) and centrifuged at 3,000 g, for 5 min. After the cells were harvested by scraping and homogenized in a solution of 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM PMSF and 10 mM Tris-HCl, pH 7.4, for 30 strokes on ice [89]. The cell homogenates were centrifuged at 4,000 g at 4°C for 10 min. The supernatant was collected and then centrifuged again at 100,000 g at 4°C for 1 hour. The pellet (plasma membrane) was resuspended in Laemmli buffer

[90] and divided into 50 μL aliquots and stored at -20°C. The membrane protein concentration was measured by the Folin-Lowry method [91].

2.8 Protein determination

The membrane protein concentration was determined by Folin-Lowry method. The principle of this method is based on the reactivity of the CO-NH- inpolypeptide chain react with Cu^{2+} in an alkaline solution to give a blue colored complex. In addition, tyrosine and tryptophan residiue of protein cause the reduction of the phosphomolybdate and phosphotungstate component of the Folin-Ciocalteau reagent to give product which contribute forwards enhancing the sensitivity of the method. It is comonly used method for determination of proteins in cell free extracts because of its high sensitivity and quantiy as low as 20 μg protein can detected.

The procedure of protein determination by Folin-Lowry method is as follows:

1. Bovine serum albumin (BSA) standard solution with various concentrations (25-200 μg/tube) were prepared from stock 1 mg/ml BSA as shown in Table 9.
2. Twenty μl of protein samples were diluted with 480 μl of distilled water.
3. Alkaline copper solution, reagent C (see Appendix), 2.5 ml was added to each test tube and incubated at room temperature for 10 min.
4. Folin-phenol reagent (1M) 250 μl was added to each tube, mixed immediately and incubated at room temperature for 30 min.
5. The absorbance at 750 nm of Standard and samples were determined by spectrophotometer.
6. Standard curve was constructed by plotting absorbance and μg protein and then sample's protein concentrations were determined from this curve.

Table11. Preparation of bovine serum albumin standard solution

BSA concentration (μg/tube)	BSA stock (μl)	Distilled water (μl)
0	-	500
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

2.9 Western blot analysis and ECL detection

The cell membrane proteins (20 μg/lane) were electrophoresed on a 7.5 % SDS- polyacrylamide gel electrophoresis (SDS-PAGE) at 100 volts for 2.5 h and then electroblotted onto nitrocellulose filters (GIBCO-BRL) at 30 volts overnight in the transfer buffer (blotting buffer). The filters were incubated sequentially with 5% skim milk in PBS (blocking solution) for 2 h at room temperature (for blocking non-specific binding) and primary mouse monoclonal anti-P-gp clone F4 [92] at 1: 5,000 in blocking buffer for 1 h at room temperature. The nitrocellulose membrane was then washed with washing solution buffer (0.1% PBS-tween) for 6 times (5 min in each) to remove excess antibodies. Next, the membrane was blocked for non-specific binding of antibody by incubating with goat anti-mouse IgG linked to peroxidase (HRP-conjugated goat anti-mouse IgG) at a 1: 20,000 dilution in blocking buffer for 1 h at room temperature. The membrane was then washed again by washing buffer, and, finally, bound proteins were detected by using the SuperSignal® protein detection kit (enhanced chemiluminescence, ECL), and then exposed to Kodak X-Omat film (approximately 1-5 min) and quantitated by scanning densitometry.

A membrane containing molecular weight marker (high range, BIO-RAD) was stained by amido black.

ECL Western blotting is a light emitting nonradioactive method for detecting immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labeled antibodies. The principles of this methods are showed in Figure 9.

The principles of ECL detection are as follows: luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence the excitation is effected by chemical reaction. The chemical reaction of cyclic diacylhydrazides such as luminol has been widely used in chemical analysis. One of the most clearly understood systems is the HRP/hydrogen peroxide catalyzed oxidation of luminol under alkaline conditions. Immediately following oxidation, the luminol is in an excited state, which then decays to ground state via a light-emitting pathway. Enhanced chemiluminescence (ECL) is achieved by performing the oxidation of luminol by HRP in the presence of chemical enhancers such as phenols. This itching can increase the light output approximately 1,000 folds and extending the time of light emission. The light produced by this enhanced chemiluminescent reaction peaks after 5-20 min and decays slowly thereafter with a half-life of approximately 60 min. The maximum light emission at 425 nm can be detected by a short exposure to blue-light sensitive autoradiographic film.

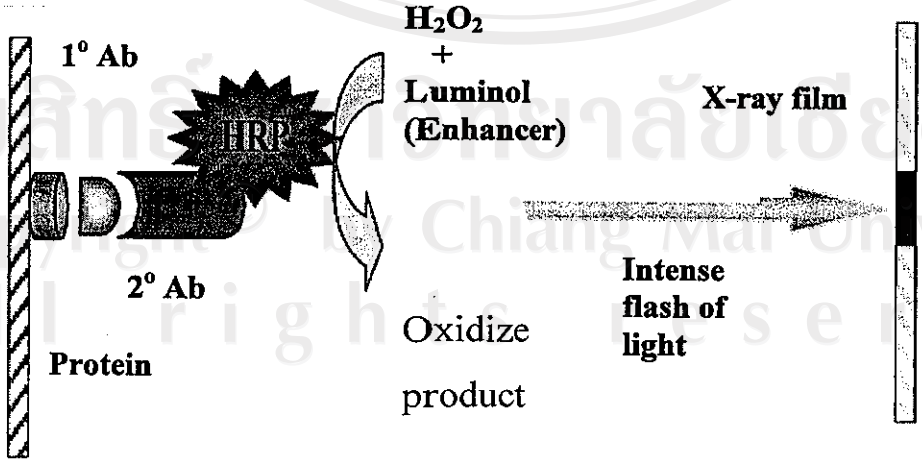

Figure 9. The principle of Enhanced Chemiluminescence (ECL) system [93]

2.10 Statistical Analysis

Data were the mean ± standard deviation of the mean from duplicate or triplicate samples of three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Statistical significance was considered when $P < 0.05$.

2.11 P-gp expression (protein level) in KB-V-1 and KB-3-1 cell lines

To determine P-gp level in KB-V-1 and KB-3-1 cell lines, membrane fractions were prepared from KB-V-1 and KB-3-1 cell lines. After that the membrane protein (20 μg) was separated by SDS-PAGE, and P-gp level was determined by Western blot and ECL detection as described in Section 2.9

2.12 Cytotoxicity of bitter melon extracts on KB-V-1 and KB-3-1 cell lines

To determine the cytotoxicity of bitter melon extracts (whole plants, leaves, fruits and tendrils) in KB-V-1 and KB-3-1 cell lines, cells were plated in 96 well plates (3.0×10^3 cells/well), in 100 μl medium, and incubating before sample treatment at 37 °C for 24 h. After 24 h, 100 μl of medium containing 0-200 μg/ml bitter melon extracts were added and incubated for another 48 h. The metabolic activity in each well were determined by the MTT assay and compared to untreated cells as described in Section 2.5

2.13 Modulating effect of bitter melon extracts on MDR phenotype in KB-V-1 and KB-3-1 cell lines

To study effect of bitter melon extracts (whole plants, eaves, fruits and tendrils) on the cytotoxicity of vinblastine, KB-V-1 or KB-3-1 cells (3.0×10^3 cells/well), in 100 μl medium were grown in the presence or absence of bitter melon extracts in combination with different concentrations of vinblastine. The number of viable cells was determined by MTT assay described in Section 2.6

2.14 Effect of bitter melon extracts on P-gp function in KB-V-1 and KB-3-1 cell lines

2.14.1 Effect of bitter melon extracts on radiolabeled drug accumulation

To study the effect of bitter melon extracts (whole plants, leaves, fruits and tendrils) on drug accumulation, KB-V-1 or KB-3-1 cells (6.0×10^5 cell/well) were cultured in complete DMEM in a 6-well plate for 24 h. Cells were treated with 25, 50, 75 and 100 μg/ml of bitter melon extracts and 10 μM cyclosporin A (positive control) in the presence of 0.05 μCi ^{3}[H]-vinblastine/ml for 60 min while vehicle control is 0.4% DMSO. Cells were then harvested and amount of intracellular radioactivity was measured using β counter as described in Section 2.6

2.14.2 Effect of bitter melon extracts on radiolabeled drug efflux

For determination of drug efflux, cells were plated out as described for drug accumulation experiments. Cells were incubated 60 min at 37°C with 0.05 μCi ^{3}[H]-vinblastine/ml in the presence of 50 μM verapamil in order to load cells with radiolabeled drug. Cells were then washed with ice-cold PBS pH 7.4, following with medium containing 25, 50, 75 and 100 μg/ml bitter melon extracts or 0.4% DMSO (vehicle control). After incubation at 37°C for 30 min, cells were washed with ice-cold PBS pH 7.4 and harvested. The amount of intracellular radioactivity was determined by scintillation counting as described in Section 2.6

2.15 Phytochemical properties [94]

2.15.1 Phytochemical test

Testing procedures and positive results of each chemical test are presented as follows

1 Phenolic moiety test

To an alcoholic solution of the sample extract, a drop of ferric chloride solution was added. The positive test will give green/blue.

2 Flavonoids test (Shinoda's test)

A few pieces of magnesium and 1-3 drops of concentrated hydrochloric acid were added to an alcoholic solution of the sample extracts. The positive test will give pink or red.

3 Triterpenes/Steroids test (Libermann-Burchard test)

A drop of acetic acid anhydride and a drop of concentrated sulfuric acid were added to small quantity of the dried sample extract. The positive test will give Green color of steroids and violet color of triterpeneds.

4 Saponin test (Foam test)

An alcoholic solution were leavily shaken; a honey comb will appear for the positive test.

5 Alkaloids test

A drop of 1 N HCl, tannic acid, Hay's reagent, Mayer, Valser and Wanger's reagent were sequencingly added to the extracts. The precipitates are formed for the positive test.

6 Anthaquinone

The test compound was burned at 160-180 °C, then a drop of diluted KOH was added, the pink color appeared for the positive test.