CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and Reagents

The details of chemicals and reagents are shown in Appendix A.

2.2 Preparation of *Stemona tuberosa Lour.* extract

The roots of *Stemona tuberosa Lour*. were used in this study. Fresh roots of *Stemona tuberosa Lour*. were collected from Udornthani, Thailand. Plant materials were dried at 30-45 ^{o}C and ground. Dried powdery plant samples were extracted exhaustively with 95 % ethanol by maceration twice. Dried ethanolic extract was obtained after removing the solvent by evaporation under reduced pressure in evaporator, then lyophilized. Dried residue was weighed and stored at –20 ^{o}C. The scheme of extraction method is shown in Figure 2.1

The extract used in all experiments was from the same lot of plant material. The HPLC profile of the extract was 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

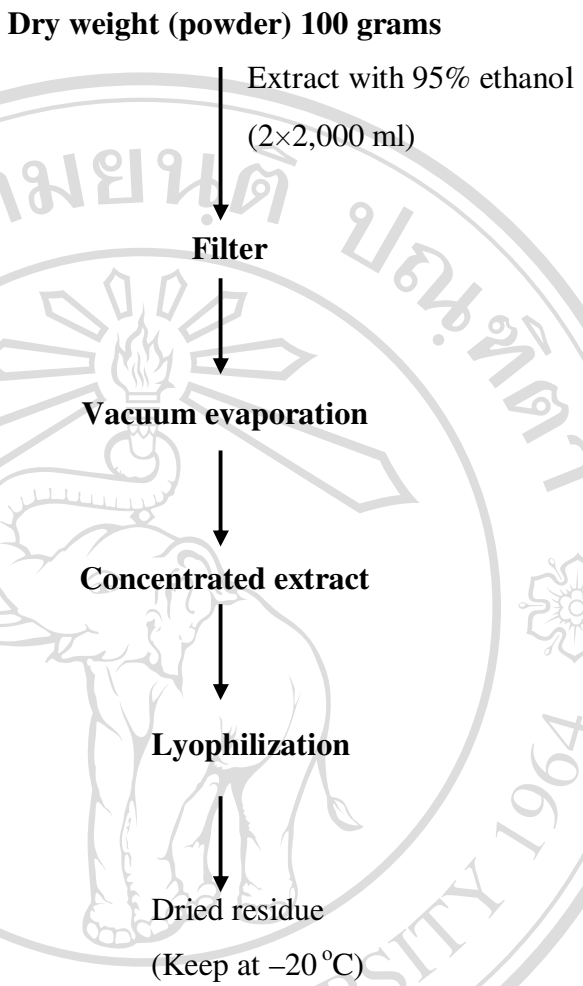

Figure. 13 Protocol for preparing crude ethanolic extract of *Stemona tuberosa Lour.*

2.3 Cell lines

Human cervical carcinoma cell lines were used to study of P-glycoprotein and tranfected human embryonic kidney for MRP-1 . All of the cell lines were provided by Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD., USA).

KB-3-1 cell lines: The parental cell line was obtained from the primary cervix carcinoma of a 31-year-old black woman. The KB-3-1 drug sensitive cell lines was derived from a single clone of human KB epidermoid carcinoma cells 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-glycoprotein and possess 100-fold amplification of *MDR1* gene.

HEK293/pcDNA 3.1: The drug sensitive human embryonic kidney tranfected with empty vector p cDNA 3.1. These cell lines do not express MRP-1.

HEK293/pcDNA 3.1 MRP1-H10: The drug resistant human embryonic kidney tranfected with vector that carry MRP-1 gene. These cell lines overexpress MRP-1.

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. After thawing, the increments of vinblastine were added only to the KB-V-1 culture medium. The cells were maintained in drug - free culture medium for 3-6 days before experimenting. The scheme of vinblastine increments was shown in Figure 14. 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 subsequent passages or for drug treatments.

HEK293/pcDNA 3.1 MRP1-H10 cells (multidrug resistance human embryonic kidney cell line) and HEK293/pcDNA 3.1 cells (drug sensitive human embryonic kidney 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. After thawing, 800 μg/ml geneticin was added to both culture media but 5 μM etoposide was only added to HEK293/pcDNA 3.1 MRP1-H10 cells. The cells were maintained in drug and selected agent - free culture medium for 3-6 days before experimenting. These two cell lines were cultured 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.

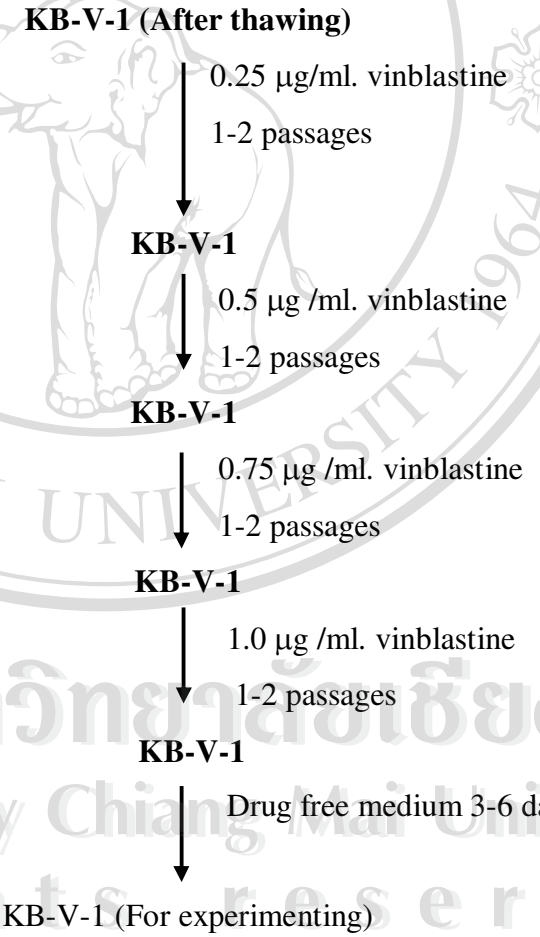

Figure. 14 The scheme of vinblastine increments for KB-V-1

2.5 Cells survival measurement

Cell survival is determined by using the colorimetric MTT [3-(4,5 dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide] assay. Mosman (1983) introduced MTT assay. 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 15). The formazan crystals are then solubilized in a different solvents, such as dimethyl sulfoxide (DMSO), or sodium dodecyl sulphate (SDS) in phosphate buffer saline solution (PBS). The solubilized formazan product is spectrophotometrically monitored 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 be found in dead cells.

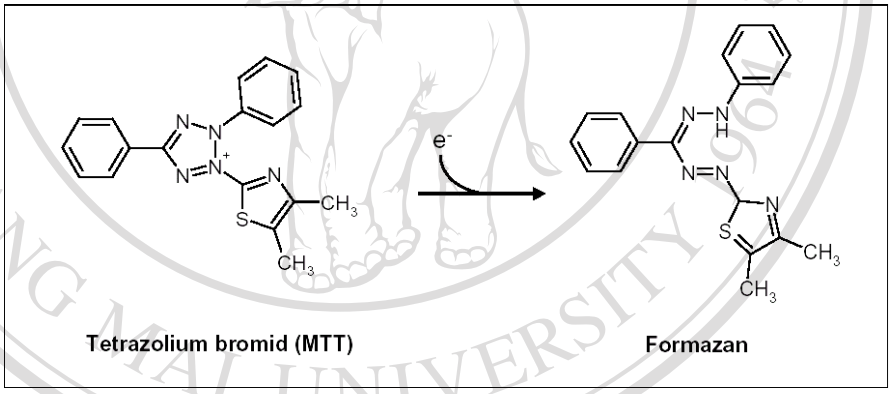

Figure. 15 The reaction of MTT by succinate dehydrogenase in cell mitochondria.

The procedure of MTT assay was performed by plating cells in 96 well plates (3×10^3 cells/well) in 100 µl medium, and incubating before drug treatment at 37°C for 24 h. After 24 h, various drugs or the extract were added to medium and incubated for another 48 h. The metabolic activity in each well was determined by the MTT assay and compared to untreated cells. Just after removal of 100 µl medium, 20 µl of MTT stock dye solution (5 mg/ml in PBS) was added to each well. The plates were incubated at 37°C under 5% CO_2 for 4 h. Most of the medium was removed from each well, leaving only about 10-20 µl medium. DMSO (200 µl) was added to dissolve the

crystals in each well and the plates were shaken for 10 min. The absorbance at 540 nm with a reference wavelength of 630 nm was read on the 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 uptake and retention assay

The effect of *S. tuberosa* extract on drug uptake was determined by intracellular radiolabeled drug (^{3}[H]-vinblastine) accumulation by the method modified from Plouzek *et al.*1999. KB-V-1 cells (6.0 x10^5 cells/ well) were cultured in complete DMEM in 6-well plate for 24 h. Cells were co-incubated with 0.05 μCi ^{3}[H]-vinblastine/ml (10.8 Ci/mmol) and *S. tuberosa* extract in 5% CO_2 incubator for 60 min while vehicle control is 0.5% 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 12,000 rpm at 4°C for 2 min. The cells were dissolved 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 - scintillation 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 retention, cells were plated out as described for drug uptake experiments. Cells were incubated at 37°C for 1 h with 0.05 μCi ^{3}[H]-vinblastine/ml and 30 μM verapamil in order to load the cells with radiolabeled drug. After incubation the cells were washed with ice-cold PBS pH 7.4, and then medium containing *S. tuberosa* extract or 0.5% DMSO was added. 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 Protein assay

The protein concentration was determined by Coomassie Plus Protein Kit (Pierce, IL, USA). The principle of this method is based on the reactivity when coomassie dye binds to protein, an intermediate shifts in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. Protein concentrations are estimated from standard curve of known protein concentration.

The procedure of protein determination by Coomassie Plus Protein Kit is as follows:

1. Bovine serum albumin (BSA) standard solution with various concentrations (25-750 µg/ml) was prepared from stock 2 mg/ml BSA as shown in Table 9.
2. 10 µl of standard protein or samples was pipetted into 96-well plate.
3. 300 µl of Coomassie Plus Protein Kit reagent was added to each well and gently mixed.
4. Measure the absorbance at 570 nm.
5. Prepare standard curve by plotting the absorbance of each BSA concentration vs. BSA concentration.
6. Protein concentration was determined by comparing the absorbance to BSA standard curve.

Table 9. The various concentrations of bovine serum albumin (BSA) for preparing the standard curve

BSA concentration (µg/ml)	**BSA stock (µl)**	**Distilled water (µl)**
0 (vial I)	0	400
25(vial H)	100 µl of vial G	400
125(vial G)	325 µl of vial F	325
250(vial F)	325 µl of vial E	325
500(vial E)	325 µl of vial C	325
750(vial D)	175 µl of vial B	175
1,000(vial C)	325 µl of stock	325
1,500(vial B)	375 µl of stock	125
2,000(vial A)	300 µl of stock	0

2.8 Statistical Analysis

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

2.9 Antiproliferation effect of *S. tuberosa* extract on KB-V-1 and KB-3-1 cell lines

To determine the cytotoxicity of *S. tuberosa* in KB-V-1 and KB-3-1 cell lines, cells were seeded in 96 well plates (2.0×10^3 cells/well of KB-V-1 and 1.0×10^3 cells/well of KB-3-1), containing 100 µl medium. After 24 h, 100 µl of fresh medium containing 0-400 µg/ml *S. tuberosa* extract was added and incubated for another 48 h. The metabolic activity in each well was determined by the MTT assay and compared to untreated cells as described in Section 2.5

2.10 Antiproliferation effect of verapamil on KB-V-1 and KB-3-1 cell lines

To determine the cytotoxicity of verapamil in KB-V-1 and KB-3-1 cell lines, cells were seeded in 96 well plates (2.0×10^3 cells/well of KB-V-1 and 1.0×10^3 cells/well of KB-3-1), containing 100 µl medium. After 24 h, 100 µl of medium containing 0-100 µM verapamil was added and incubated for another 48 h. The metabolic activity in each well was determined by the MTT assay and compared to untreated cells as described in Section 2.5

2.11 Modulating effect of *S. tuberosa* extract on drugs sensitivity in KB-V-1 and KB-3-1 cell lines

To investigate effect of *S. tuberosa* extract on the sensitivity of vinblastine, paclitaxel and colchicine. KB-V-1 and KB-3-1 cells (2.0×10^3 cells/well of KB-V-1 and 1.0×10^3 cells/well of KB-3-1) were seeded in 96 well plates. After 24 h, 100 µl of fresh medium containing DMSO or 50 µg/ml and 150µg/ml of *S.tuberosa* extract with various concentrations of vinblastine, paclitaxel and colchicine was added; 20 µM verapamil was a positive control. The cells were incubated for 48 h and the number of viable cells was determined by MTT assay described in section 2.5.

2.12 Effect of *S. tuberosa* extract on P-gp function in KB-V-1 and KB-3-1 cell lines

2.12.1 Effect of *S. tuberosa* extract on radiolabeled drug uptake

To study the effect of *S. tuberosa* extract on radiolabeled drug uptake, KB-V-1 and KB-3-1 cells (6.0 x 10^5 cells/well) were plated in a 6-well plate containing complete DMEM for 24 h. Cells were treated with 0-500 μg/ml of *S. tuberosa* extract and 10 μM verapamil (positive control) in the presence of 0.05 μCi ^{3}[H]-vinblastine/ml for 60 min; vehicle control was 0.5% DMSO. Cells were then harvested and amount of intracellular radioactivity was measured using β counter as described in Section 2.6.

2.12.2 Effect of *S. tuberosa* extract on radiolabeled drug retention

For determination of drug efflux, cells were plated out as described for drug uptake experiments. Cells were incubated 60 min at 37°C with 0.05 μCi ^{3}[H]-vinblastine/ml in the presence of 30 μM verapamil in order to load cells with radiolabeled drug. Cells were then washed with ice-cold PBS pH 7.4, followed by adding medium containing 0-500 μg/ml *S. tuberosa* extract or 0.5% DMSO (vehicle control); 10 μM verapamil was a positive 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.

2.13 Antiproliferation effect of *S. tuberosa* extract on HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells

To determine the cytotoxicity of *S. tuberosa* in HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells, cells were seeded in 96 well plates (2.5 x10^3 cells/well), with 100 μl medium. After 24 h, 100 μl of fresh medium containing 0-400 μg/ml *S. tuberosa* extract were added and incubated for another 96 h. The metabolic activity in each well was determined by the MTT assay in comparison to untreated cells as described in section 2.5.

2.14 Antiproliferation effect of indomethacin on HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells

To determine the cytotoxicity of indomethacin in HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells, cells were seeded in 96 well plates ($2.5x10^3$ cells/well), with 100 µl medium. After 24 h, 100 µl of medium containing 0-100 µM indomethacin was added and incubated for another 96 h. The metabolic activity in each well was determined by the MTT assay and compared to untreated cells as described in section 2.5.

2.15 Modulating effect of *S. tuberosa* extract on drugs sensitivity in HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells

To study effect of *S. tuberosa* extract on the sensitivity of etoposide. HEK293/pcDNA 3.1 MRP1-H10 cells and HEK293/pcDNA 3.1 cells ($2.5 x10^3$ cells/well) were seeded in 96 well plates. After 24 h, 100 µl of fresh medium containing DMSO or 25 µg/ml. and 50 µg/ml.of S.tuberosa extract in the pressence various concentrations of etoposide was added; 40 and 70 µM indometahcin were used as positive control. The cells were incubated for 96 h and the number of viable cells was determined by MTT assay described in section 2.6

2.16 Phytochemical properties (Farnsworth, 1996)

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

(1) Phenolic moiety test

A drop of ferric chloride was added to an alcoholic solution of the extracted sample. 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 extract. 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 extracted sample. The positive test will give Green color for steroids and violet color for triterpenes.

(4) Saponin test (Foam test)

An alcoholic solution was vigorously shaken; a honeycomb appears 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 sequentially added to the extract. The precipitates are formed for the positive test.

(6) Anthaquinone glycosides test

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