CHAPTER 3

EXPERIMENTAL

Materials

Instruments and Apparatus

- UV spectrophotometer, 254, 365 nm, CHROMATO-VUE® C-70G, UVP, USA
- UV spectrophotometer (UV-2401PC), Shimadzu, Japan
- Larminar flow hood, Class II, NUAIRE™, USA
- Microplated reader EL311, BIO-TEK instruments, USA
- Inverted microscope, Nikon TMS, Japan
- Fourier transform infared spectrophotometer (FTIR 5000), Japan
- Melting point apparatus SMP_3, BIBBY, Stuart Scientific, United Kingdom
- Hematocytometer, Reichert-Jung Cambridge Instrument, USA
- Column chromatography : i.d. 7.5 cm x 60 cm length, i.d. 3 cm x 30 cm length, i.d. 2 cm x 40 cm length
- 96 – well plates, NUNCLON™, Denmark
- 25 cm^2 tissue culture flasks , NUNCLON™, Denmark
- High-performance liquid chromatography column : i.d. 4.6 x 250 mm length; 5 µm (ODS-3), Inertsil®, GL. Science, Inc., Japan
- Guard column : i.d. 4.6 x 50 mm length; 5 µm (ODS-3), Inertsil®, GL. Science, Inc., Japan
- HPLC 1100, Hewlett Packard, USA
- Nuclear magnetic resonance spectrophotometer (NMR), JEOL JNM-A500.

Chemicals and Biologicals

- Dulbeco's Modified Eagle Medium (DMEM), Gibco BRL,USA
- *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), AMRESCO®, Ohio, USA
- 5-Fluorouracil, Lot no. 31K1617, Sigma, USA
- Vinblastine sulfate, Lot no. 109HO941, Sigma, USA
- MTT (Thiazolyl blue), Lot no. 101K5300, Sigma, USA
- 95% Ethanol, commercial grade, Excise department, Chachoengsao Province, Thailand
- *n*-Hexane, 95% AR grade, Lot no. 99 12 0094, Lab-Scan, Ireland
- Chloroform AR grade, Lot no. K23545383647, Merck, Germany
- Ethyl acetate AR grade, Lot no. 9280-03, Baker Analyzer, USA
- Methanol AR grade, Lab-Scan, Ireland
- Silica gel 60 (230-400 mesh ASTM), Merck, USA
- Penicillin-Streptomycin, Lot no. 1006152, Gibco BRL,USA
- Fetal Calf Serum (FCS), Seromed, Australia

Part I Phytochemical Methodology

1. Plant Sources

Twenty Rubiaceous plants were collected from Doi Suthep-Pui National Park, Chiang Mai Province and Doi Kuhn Dtan National Park, Lampoon Province, Thailand, during April to October, 2000. Voucher specimens were collected for each plant identified by J. F. Maxwell, and deposited in the Chiang Mai University (CMU) Herbarium, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. Examples of the voucher specimens were shown in Appendix A.

2. Criteria of Cytotoxic Activity

The results were expressed as concentrations required to inhibit cell growth by 50% (IC_{50}). According to the United State National Cancer Institute (NCI), IC_{50} values are considered active when the value less than 20 µg/ml for extracts[71] and less than 4 µg/ml for pure compounds[71]. In this research, cytotoxic activity were classified as followed : IC_{50} value less than 20 µg/ml for active extracts, IC_{50} value between 20 to 100 µg/ml for moderately active extracts, IC_{50} value more than 100 µg/ml for inactive extracts, and IC_{50} value less than 4 µg/ml for active pure compounds.

3. Methods

- Crude Extraction of Rubiaceae

All samples were washed, cut, dried at a temperature under 50 °C for 24 hours, and pulverized. Powder of samples (approximately 4 gm) was macerated three times during a week period, with 120 ml of 95% ethanol. The extracts were combined and evaporated to dryness under reduced pressure. Crude extracts were used for cytotoxic activity tests and the selection of a potential plant. The

definition of a potential plant was the plants which their crude extract gave the IC_{50} less than 20 μg/ml.

- Extraction of Active Components

Apart from *Gardenia sootepensis* HutCh., *Gardenia obtusifolia* Roxb. ex Kurz was another species which exhibited high cytotoxic activity against both cell lines and selected for further studies. Dried powder from the leaves of *Gardenia obtusifolia* Roxb. ex Kurz (1.2 kg) was macerated three times in 3 liters of 95% ethanol. The extracts were combined and evaporated to under reduced pressure at 50 °C yielding 159 grams of dark and sticky crude extract. One half of the resulting crude extract (79.8 gm) was then dissolved in an aqueous phase (a mixture of ethanol and distilled water) for further separation. Based on liquid-liquid partition procedure, four solvents with different polarity, *i.e.*, *n*-hexane, chloroform and ethyl acetate were utilized to extract compounds of interest. After this process, the solvents were removed by evaporation under reduced pressure at 50 °C. The extracts from *n*-hexane (7.9 gm), chloroform (51.4 gm), ethyl acetate (6.5 gm), and the aqueous phase (5.4 gm) were tested for their cytotoxic activity test. The test revealed that the chloroform extract possessed significant cytotoxic activity against MCF-7 and KB-3-1 carcinomas ($IC_{50} \leq 20$ μg/ml), so it was chosen for further investigation (Figure 9).

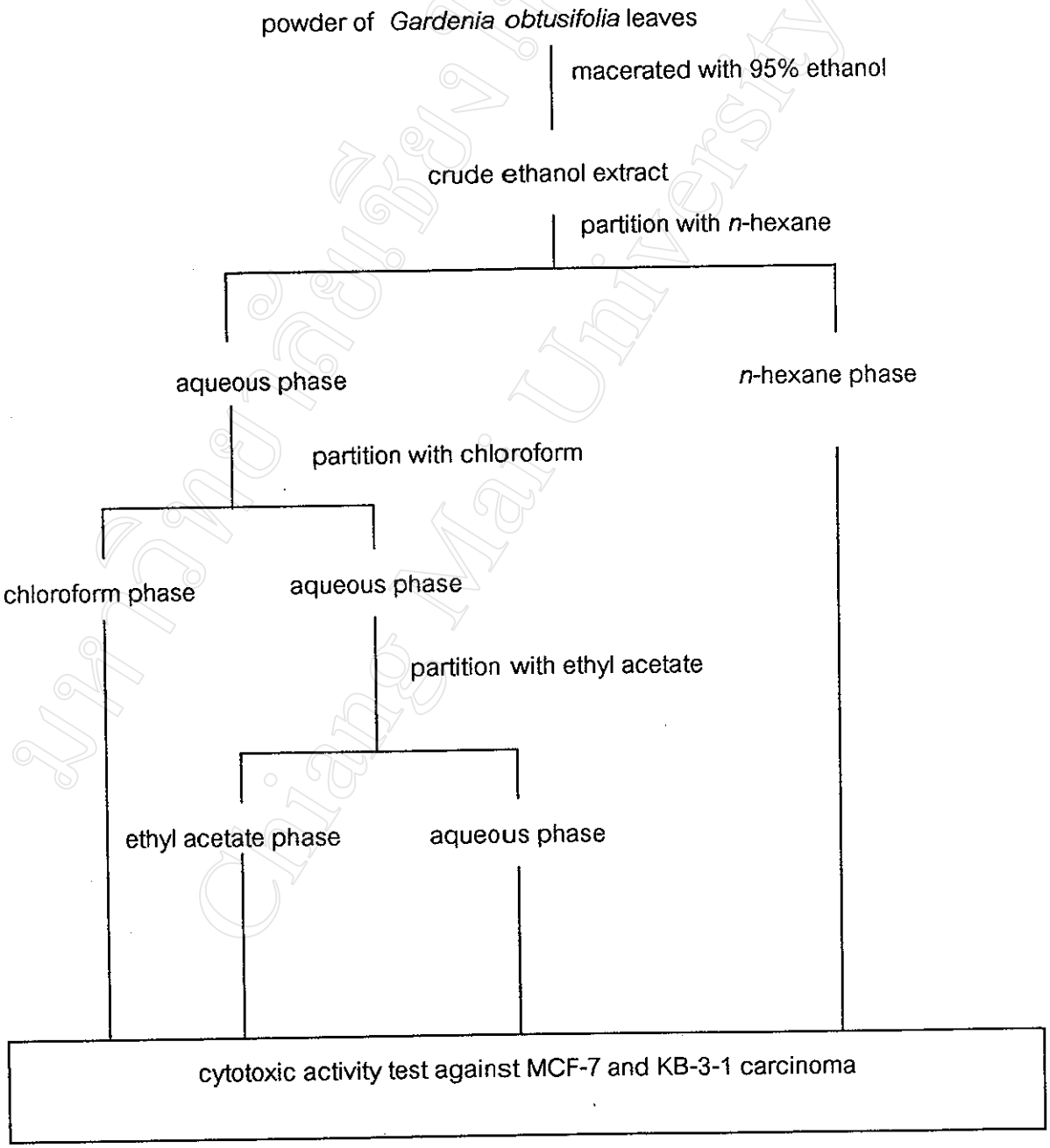

Figure 9. Extraction Scheme for *Gardenia obtusifolia*.

- Fractionation of the Chloroform Extract

The chloroform extract was isolated using conventional column chromatography. Silica gel 60 (500 gm) was suspended in chloroform and wet packed into a glass column (7.5 cm i.d. x 35 cm length). The chloroform extract (20 gm) was triturated with silica gel 60 (15 gm) and dried with a hair dryer. The powder mixture was put into the top of silica gel 60 column. Gradient elution was performed with different compositions of a mobile phase (*n*-hexane, chloroform, ethyl acetate, and methanol) as a gradient of increasing polarity. Aliquots of eluate were tested for their chromatographic similarity with TLC. The aliquots with similar TLC patterns were combined. After this process, solvents were removed with an evaporator, which gave 12 fractions. All fractions were tested for their cytotoxic activity (Figure 9). Among all fractions, chloroform:ethyl acetate (1:1) fraction was effective against KB-3-1 and MCF-7 cells ($IC_{50} \leq 20$ µg/ml). Thus, this fraction was subjected to the next step.

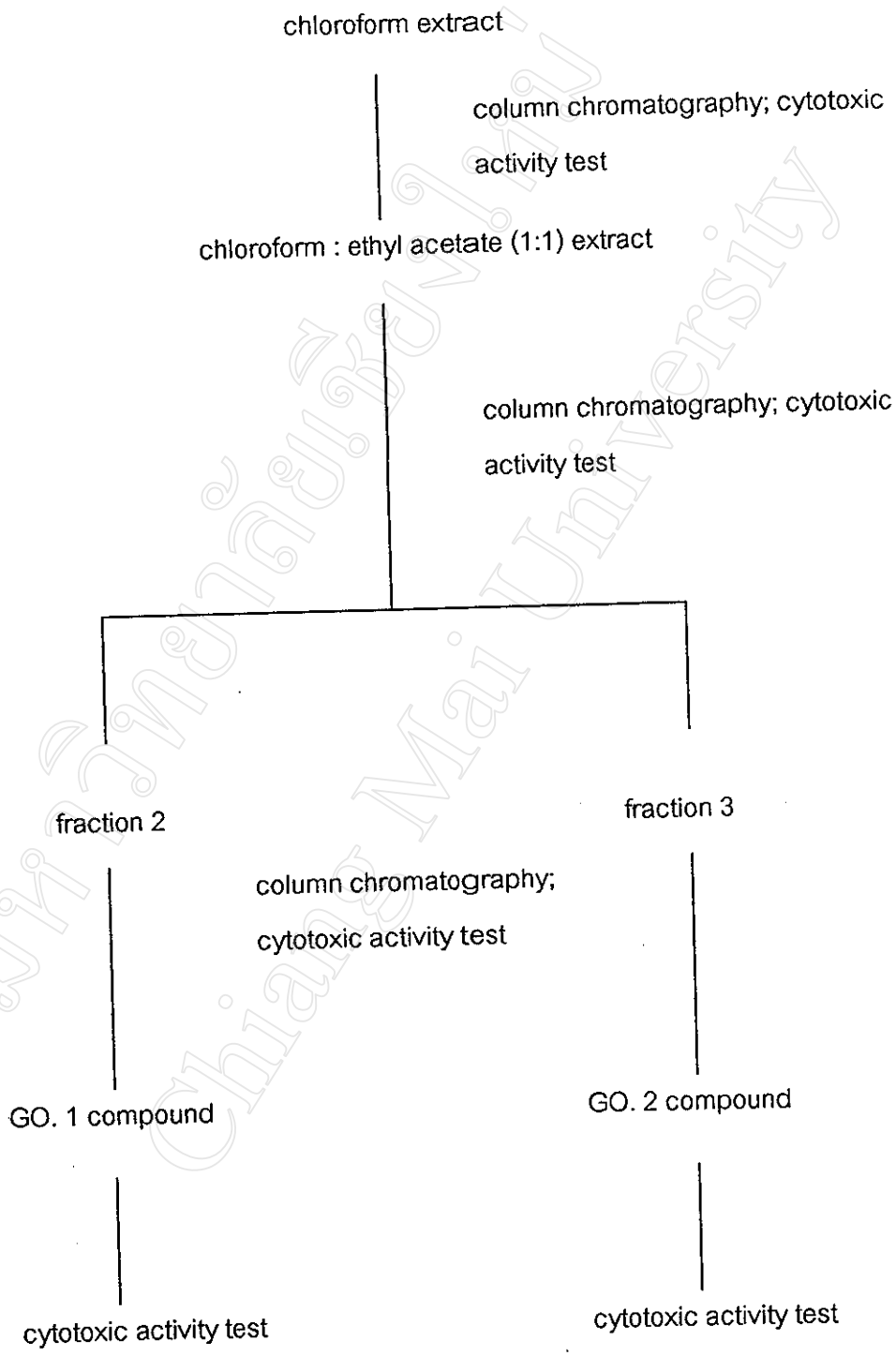

Figure 10. Isolation Scheme active components from chloroform extract.

- Isolation of Active Compounds

Isolation of active compounds from the chloroform extract was achieved by using the bioassay-guided fractionation procedure. Conventional column chromatography was utilized for purification.

- Isolation from Chloroform : Ethyl acetate (1:1) Fraction

The chloroform:ethyl acetate (1:1) fraction (3.2 gm) was rechromatographed on a silica gel 60 column (3 cm i.d. x 25 cm length) with chloroform. Twenty milliliters aliquots of the eluate were collected and examined with thin layer chromatography (TLC), which combined into eight distinctive fractions. All fractions were tested for their cytotoxic activity. Fractions 2 and 3 had the highest cytotoxic activity against MCF-7 and KB-3-1 cells (active compounds, $IC_{50} \leq 20$ µg/ml), which were subjected to next chromatographic process.

- Isolation of Fraction 2

Fraction 2 (0.8 gm) was rechromatographed on a silica gel 60 column (2 cm i.d. x 35 cm length), using a mixture of *n*-hexane:chloroform (2% v/v hexane) as a mobile phase. Twenty milliliters aliquots of the eluate were collected and combined into eight distinctive fractions (fraction i-viii). All fractions were evaporated to dryness under reduced pressure. Fractions iii, iv, v were concentrated, resulting in the crystallization of a yellow compound. This compound was washed with petroleum ether, and designated as GO.1. All dried fractions and the GO.1 were tested for their cytotoxic activity. The GO.1 compound was further studied for its physiochemical properties.

- *Isolation of Fraction 3*

Fraction 3 (0.5 gm) was rechromatographed on a silica gel 60 column (2 cm i.d. x 26 cm length). Solvents used for the elution were consisted of *n*-hexane:chloroform (10% *n*-hexane), chloroform, a gradient of chloroform in diethyl ether, diethyl ether, and methanol, respectively. Twenty milliliters aliquots of the eluate were collected and combined into six distinctive fractions (fraction a-f). All fractions were evaporated to dryness under reduced pressure. Fraction d was concentrated, then the resulting yellow compound was crystallized. This compound was washed with petroleum ether and designated as GO. 2. All dried fractions and GO.2 were tested for their cytotoxic activity. The GO.2 was studied for its physiochemical properties.

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4. Phytochemical Tests

Testing procedures and positive results of each chemical test were presented as followed :

- *Phenolic moiety test*[72]

To an alcoholic solution of the sample extract, a drop of ferric chloride solution was added. The resulting colour was green and blue.

- *Flavonoids test (Shinoda's test)*[72]

To an alcoholic solution of the sample extract, a few pieces of magnesium and 1-3 drops of concentrated hydrochloric acid were added. The resulting colour was pink or red.

- *Sugars test (Molish's test)*[72]

To an alcoholic solution of the sample extract, a drop of 5% α-naphthol in alcohol and a few drops of concentrated sulfuric acid were added. A violet ring appeared.

- *Unsaturated lactone ring (Kedde's test)*[72]

To an alcoholic solution of the sample extract, a drop of Kedde's reagent A and B was added. A violet colour appeared.

- *Deoxy sugar test (Keller-Kiliani test)*[72]

To an alcoholic solution of the sample extract, a drop of ferric chloride reagent and a drop of concentrated sulfuric acid were added. A red ring appeared.

- *Triterpenes / Steroids test (Libermann-Burchard test)*[72]

To a small quantity of the dried sample extract, a drop of acetic anhydride and a drop of concentrated sulfuric acid were added. Green colour of steroids and violet colour of triterpenes appeared.

- *Saponin test (Foam test)*[72]

An alcoholic solution (dilute with H_2O) of the sample extract was shaken. A honey comb froth appeared.

- *Alkaloids test (Dragendroff's test)*[72]

A drop of Dragendroff's reagent was added to a small quantity of the dried sample extract. An orange precipitate appeared.

- *Anthraquinones test (Borntrager test)*[72]

To an alcoholic solution of the sample extract, a drop of dilute acid was added. After this solution was boiled for 5 minutes and approximately ethyl acetate was added. After that, this solution was added to a basic solution. A pink colour appeared in a basic solution.

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5. Physicochemical Properties[73]

- *Melting points*

Melting points were determined by a melting point apparatus SMP_3, BIBBY, Stuart Scientific, United Kingdom.

- *Ultraviolet-Visible spectra*

The ultraviolet-visible spectra of GO. 1 and GO. 2 compounds in methanol were scanned with a UV 2401PC Shimadzu spectrophotometer.

- *Infrared spectra*

The IR spectra were obtained on FTIR 5000, using dry film on sodium chloride cells and pressed disc technique (dried KBr).

- *NMR spectroscopy*

The ^{1}H-NMR ($CDCl_3$, D_2O, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) spectra were obtained on a JEOL JNM-A500 .

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

Part II Cytotoxic Activity Tests

1. Cell Lines

The cell lines used in this study were breast carcinoma (MCF-7) and cervix carcinoma (KB-3-1) which were obtained from Dr. Gotteaman, National Institute of Health, National Cancer Institute, Besthesda, Maryland (USA). Cancer cell lines were maintained in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (10,000 units/ml), streptomycin (10,000 µg/ml), and incubated in 37°C humidified 5% CO_2 incubator as a subconfluent monolayer. Both cell lines were propagated by subculturing twice weekly. Cancer cells were in exponential growth phase (log phase) over the period of the assay.[74-75]

2. Preparation of Plates

- Cells in growth phase were washed twice with TD-EDTA (washing buffer; Appendix B) buffer by centrifugation in an incompleted medium (DMEM without FCS and penicillin- streptomycin).
- Viable cell numbers were determined using trypan blue dye exclusion technique. The cells were then suspended in the growth medium with a final concentration of 3,000 and 5,000-10,000 cells/well for KB-3-1 and MCF-7 plates, respectively.
- Aliquots (100 µl) of cell suspension were dispensed into each well of 96-well plates. Negative and positive control wells were included in each plate, which were blank medium and cells in a medium with known cytotoxic agents, respectively. The plates were incubated in 37 °C humidified 5% CO_2 incubator for 24 hours for KB-3-1 and 48 hours for MCF-7.

3. Preparation of Crude Extracts for Cytotoxic Activity Test

- A 200 µl aliquot of dimethyl sulfoxide (DMSO) was added to 2 mg of crude extracts, yielding a concentration of 10,000 µg/ml.
- Three concentration levels of crude extracts in a cultured medium were prepared at concentrations of 5, 50, and 100 µg/ml. DMSO concentrations were less than 0.5% in each well. Positive control cytotoxic agents, 5-FU for MCF-7 cells and vinblastine for KB-3-1 cells, were included in each experiment at appropriate concentrations.
- After a period of cell incubation, various dilutions of crude extracts (100 µl) were added to the prepared plate. The plate was further incubated for 48 hours.

4. Colorimetric End-point Determination[76]

- Five milligrams of MTT dye powder was diluted with 1.0 ml of phosphate buffer saline solution (PBS).
- After 48 hours of incubation with the extracts, an aliquot of MTT dye solution (20 µl) was added to each well. The plate was then incubated for 4 hours.
- The medium from each well was sucked off after the incubation with MTT dye. Two hundred microliters of DMSO was added to each well to dissolve the formazan crystals and the contents in each well were mixed together.
- Absorbance of each well were read at 540 nm and 630 nm (reference wavelength) on a Bio – TEK instruments® microtiter plate reader.
- A dose–response curve was constructed using the average cell viability obtained from triplicate determinations at each concentrate.
- The 50% inhibition concentration (IC_{50}) of the active substances were determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture. The IC_{50}s obtained were compared for their activities. Plants with high cytotoxic activity were selected for further study.