CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Plants

1) *Hypericum hookerianum* Wight &Arn
2) *Garcinia speciosa* Wall
3) *Garcinia xanthochymus* Hook. F. ex T. Anderson
4) *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel
5) *Calophyllum polyanthum* Wall ex Choisy
6) *Schisandra verruculosa* Gagnap

2.1.2 Chemicals / cell lines

- α-Tocopherol (Sigma Co, St. Louis, MO, USA)
- Acetic acid (Merck, Germany)
- Ascorbic acid (Sigma Co, St. Louis, MO, USA)
- Chloroform (Labscan Asia, Bangkok, Thailand)
- Cyclosporin A (Sigma Co, St. Louis, MO, USA)
- Dimethylsulfoxide (DMSO, Sigma Co, St. Louis, MO, USA)
- 1,1-Diphenyl-2-picryhydrazyl (DPPH, Sigma Co, St. Louis, MO, USA)
- (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Co, St. Louis, MO, USA)
- DMEM medium (Sigma Co, St. Louis, MO, USA)

- Doxorubicin hydrochloride (Dabur Pharma, UK)
- Ethanol (Merck, Germany)
- Fetal bovine serum (FBS, Gibco BRL, UK)
- Fetal calf serum (FCS, Gibco BRL, Canada)
- Gentamicin (Sigma Co, St. Louis, MO, USA)
- Histopaque-1077 (Sigma Co, St. Louis, MO, USA)
- L-glutamine (Sigma Co, St. Louis, MO, USA)
- Methanol (Merck, Germany)
- Phosphate buffered saline tablet (Sigma Co, St. Louis, MO, USA)
- Phytohemagglutinin (PHA, Sigma Co, St. Louis, MO, USA)
- RPMI-1640 (Gibco BRL, UK)
- Sulforhodamine B (SRB, Sigma Co, St. Louis, MO, USA)
- Silica gel60 (0.063-0.200 mm) for column chromatography (Merck, Germany)
- Silica gel 60 GF_{254} for analytical and preparative TLC (Merck, Germany)
- Trichloroacetic acid (Merck, Germany)
- Tripsin (Gibco BRL, Canada)
- Tris (Merck, Germany)
- Trypan blue (Sigma Co, St. Louis, MO, USA)
- MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), UACC-62 (melanoma) cell lines (National Cancer Institute (NCI), Bethesda, MD, USA)
- HeLa (cervical), KB (epidermoid) and B16F10 (melanoma) cell lines (National Cancer Institute, Bangkok, Thailand)

2.1.3 Equipment

- Bruker AMC instrument operating at 300.13 and 75.47 MHz for ^{1}H and ^{13}C NMR
- Centrifuge tube 15 ml, 50 ml (TPP, Switzerland)
- CO_2 incubator (Shel Lab, USA)
- Hitachi Perkin-Elmer RMV-6M instrument for EI mass spectra
- Kratos Concept II 2 sector/mass spectrometer for HRMS mass spectra
- Larminar air flow cabinet (Cytair 125, Equipments Scientifiques & Industries S.A., France)
- Muti-channel autopipette (Labsystems, Finland)
- Rotation (Polax-2 L instrument, USA)
- 12 Wells flat buttom plates tissue culture grade (TPP®, Switzerland)
- 96 Well plates (Nunc®, USA)
- Well reader (Seikagaku SK601, Japan)
- Ultracentrifuge (Avanti™ 30 Centrifuge, Beckman, USA)
- UV-visible spectrophotometer (Cary 1 E)

2.2 Methods

The scope of this study was divided into 4 parts as the scheme in Figure 2.1 The details are as follows.

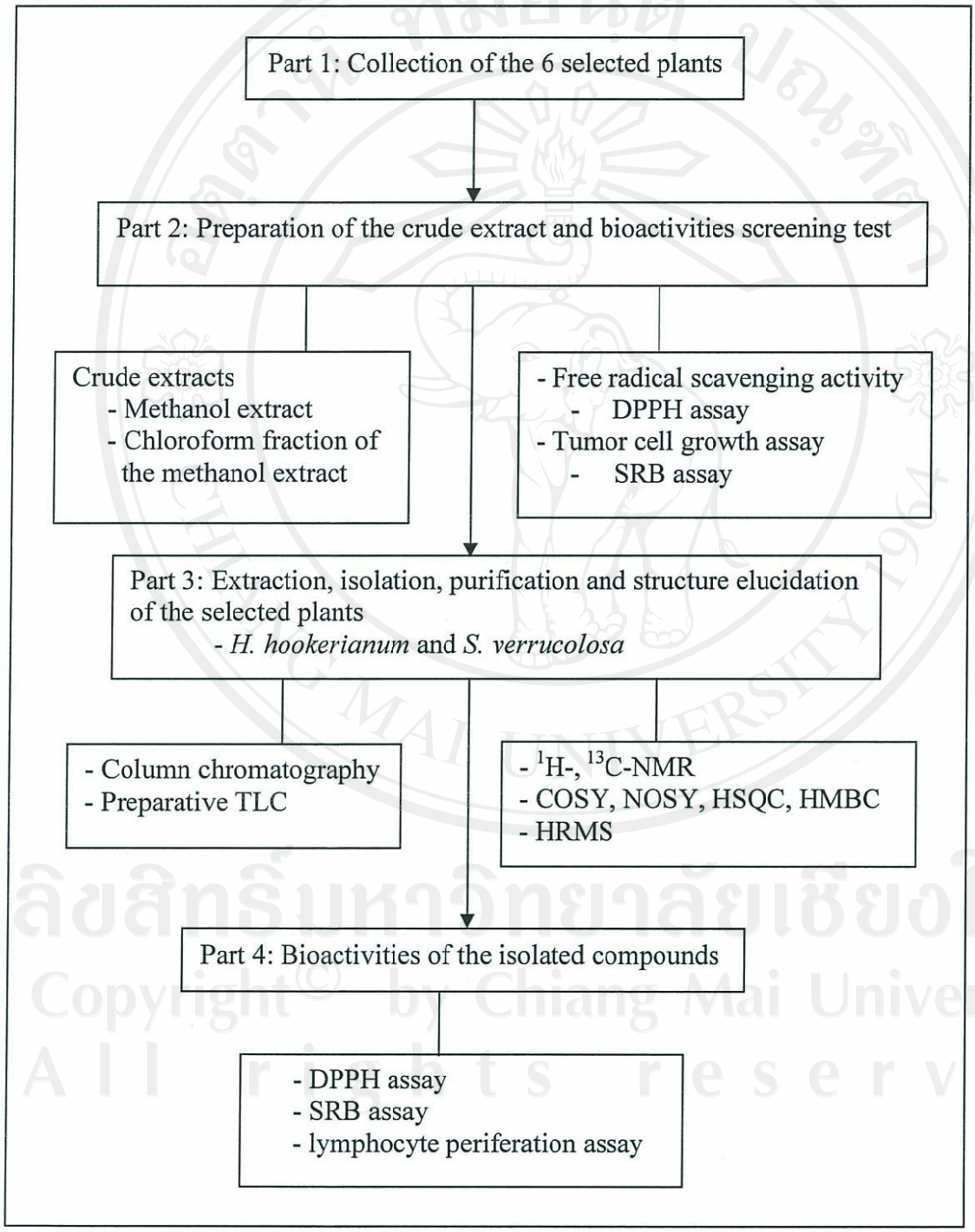

Figure 2.1 Scope of the Part 1 to Part 4 study

Part 1: Collection of the plants

The five plants from family Guttiferae and one from family Schisandraceae were selected focusing on the evidence of cytotoxicity and antioxidant activity. The plants were collected from Chiang Mai Province, Thailand during November- December 2002. The details are as follows.

1) *Hypericum hookerianum* Wight et Arn

Aerial parts of *H. hookerianum*, a woody shrub, were collected at 2500 m altitude at Doi Inthanon National Park, Chiang Mai, Thailand in November 2002. A voucher specimen (Number 91-776) (Fig. 2.2) was deposited in the CMU Herbarium, Faculty of Science, Chiang Mai University. Leaves and green branches were removed and the remaining woody stems were air dried in the shade prior to extraction.

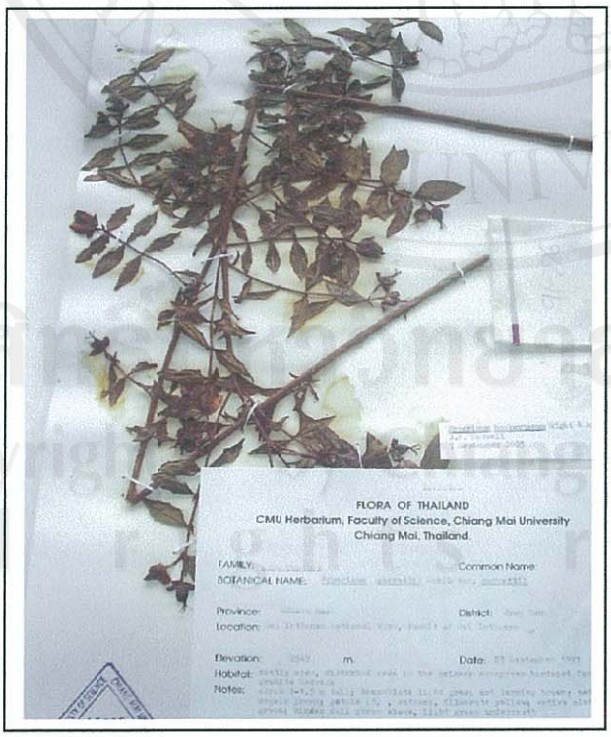

Figure 2.2 The herbarium specimen of *Hypericum hookerianum*

2) *Garcinia speciosa* Wall

Leaves and stem wood of *G. speciosa* were collected at Doi Sutep, Chiang Mai, Thailand in December 2002. A voucher specimen (Number 89-743) (Fig. 2.3) was deposited in the herbarium, Faculty of Pharmacy, Chiang Mai University. Leaves and woody stems were air dried in the shade prior to extraction.

Figure 2.3 The herbarium specimen of *Garcinia speciosa*

3) *Garcinia xanthochymus* Hook. f. ex. T. Anderson

Leaves, fruits and stem wood of *G. xanthochymus* were collected at Doi Sutep, Chiang Mai, Thailand in December 2002. A voucher specimen (Number 88-1) (Fig. 2.4) was deposited in the herbarium, Faculty of Pharmacy, Chiang Mai University. Leaves, fruits and woody stems were air dried in the shade prior to extraction.

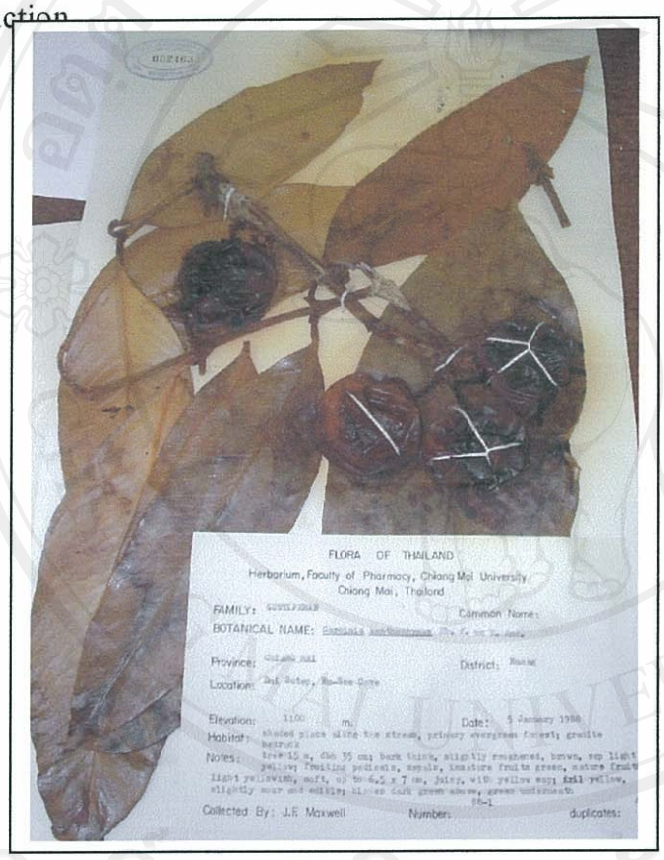

Figure 2.4 The herbarium specimen of *Garcinia xanthochymus*

4) *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel

Leaves and stem wood of *C. formosum* ssp. *pruniflorum* were collected at Doi Sutep, Chiang Mai, Thailand in December 2002. A voucher specimen (Number 87-604) (Fig. 2.5) was deposited in the herbarium, Faculty of Pharmacy, Chiang Mai University. Leaves and woody stems were air dried in the shade prior to extraction.

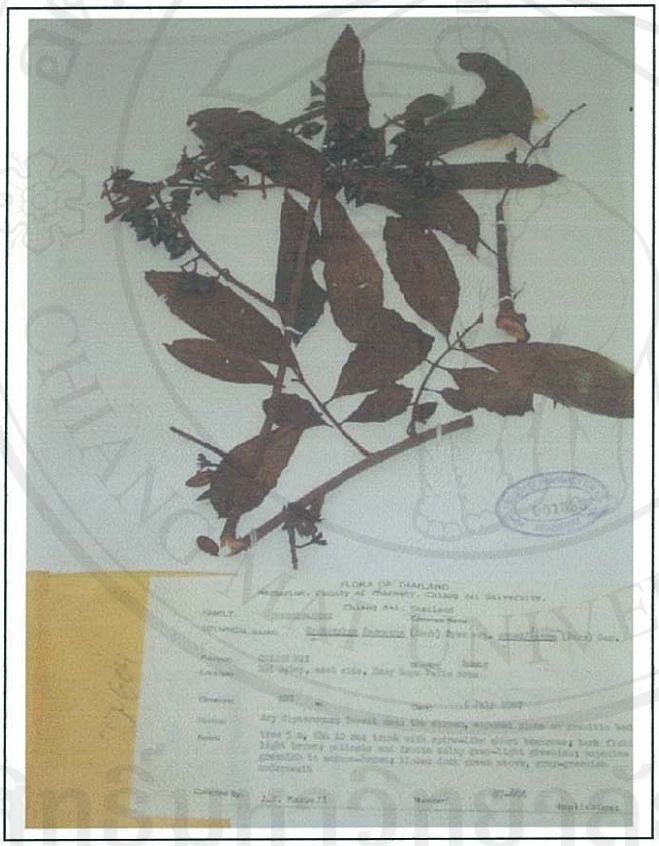

Figure 2.5 The herbarium specimen of *Cratoxylum formosum* ssp. *pruniflorum*

5) *Calophyllum polyanthum* Wall ex Choisy

Leaves and stem wood of *C. polyanthum* were collected at Doi Sutep, Chiang Mai, Thailand in December 2002. A voucher specimen (Number 1446) (Fig. 2.6) was deposited in the CMU Herbarium, Faculty of Science, Chiang Mai University. Leaves and woody stems were air dried in the shade prior to extraction.

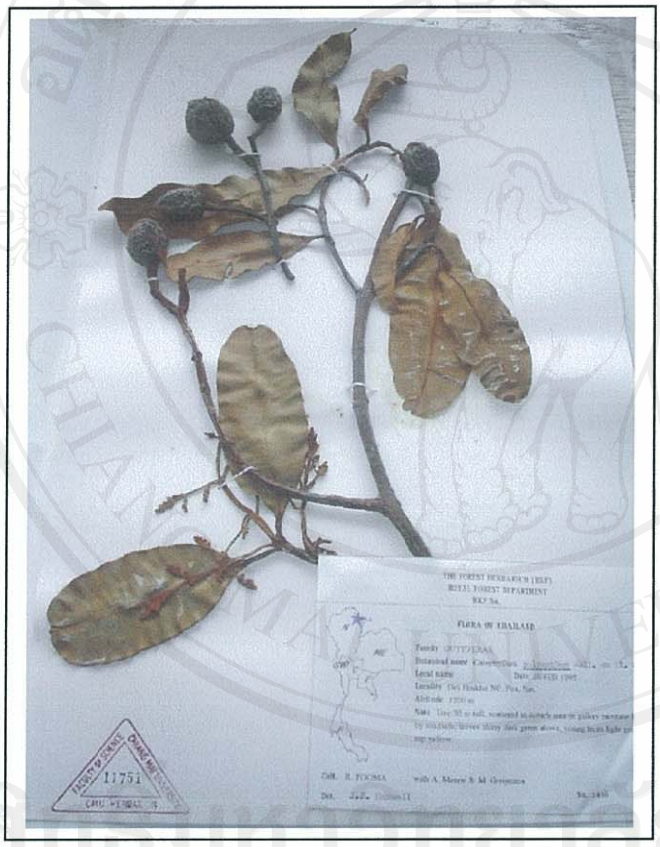

Figure 2.6 The herbarium specimen of *Calophyllum polyanthum*

6) *Schisandra verruculosa* Gagnap

Aerial parts of *S. verruculosa* were collected at Doi Mawn Ngaw, Mae Tang, Chiang Mai, Thailand in December 2002. A voucher specimen (Number 01-103) (Fig. 2.7) was deposited in the CMU Herbarium, Faculty of Science, Chiang Mai University. Leaves and woody stems were air dried in the shade prior to extraction.

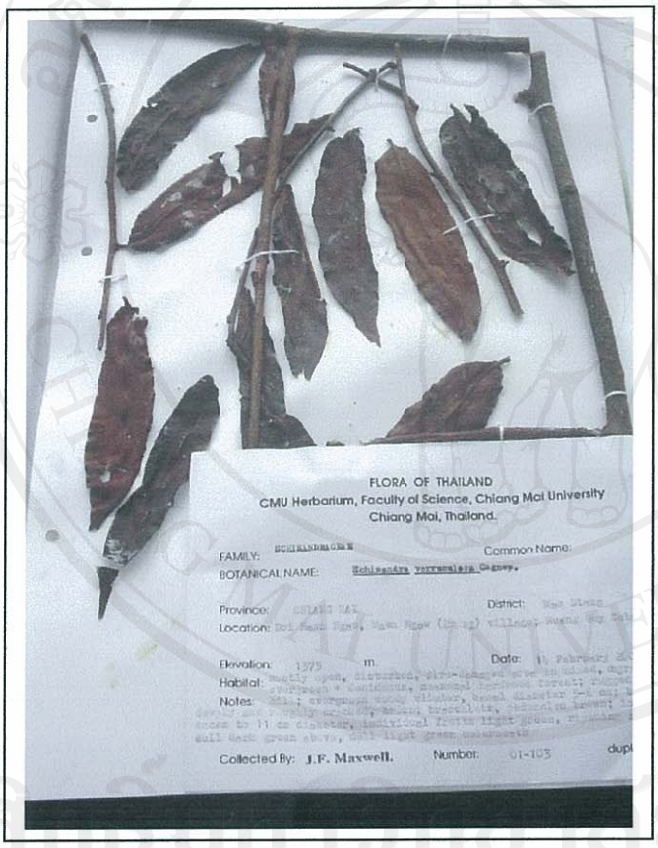

Figure 2.7 The herbarium specimen of *Schisandra verruculosa*

Part 2: Preparation of the crude extracts and bioactivities screening test

2.2.1 Preparation of the crude extracts

Wood, leaves from the six selected plants and the fruit of *G. xanthochymus* were separately reduced to small pieces, dried at 40°C in a hot air oven and comminuted to powder. The dried powder samples (100-300 g) were macerated in 95% methanol for 48 h. The solvent was evaporated under reduced pressure by a rotary evaporator. The residues were re-extracted with chloroform and concentrated by partial evaporation under reduced pressure. Twenty-four methanol and chloroform fraction of the methanol crude extracts were obtained, and the percentage yields were calculated. The diagram of extraction was shown in Figure 2.8

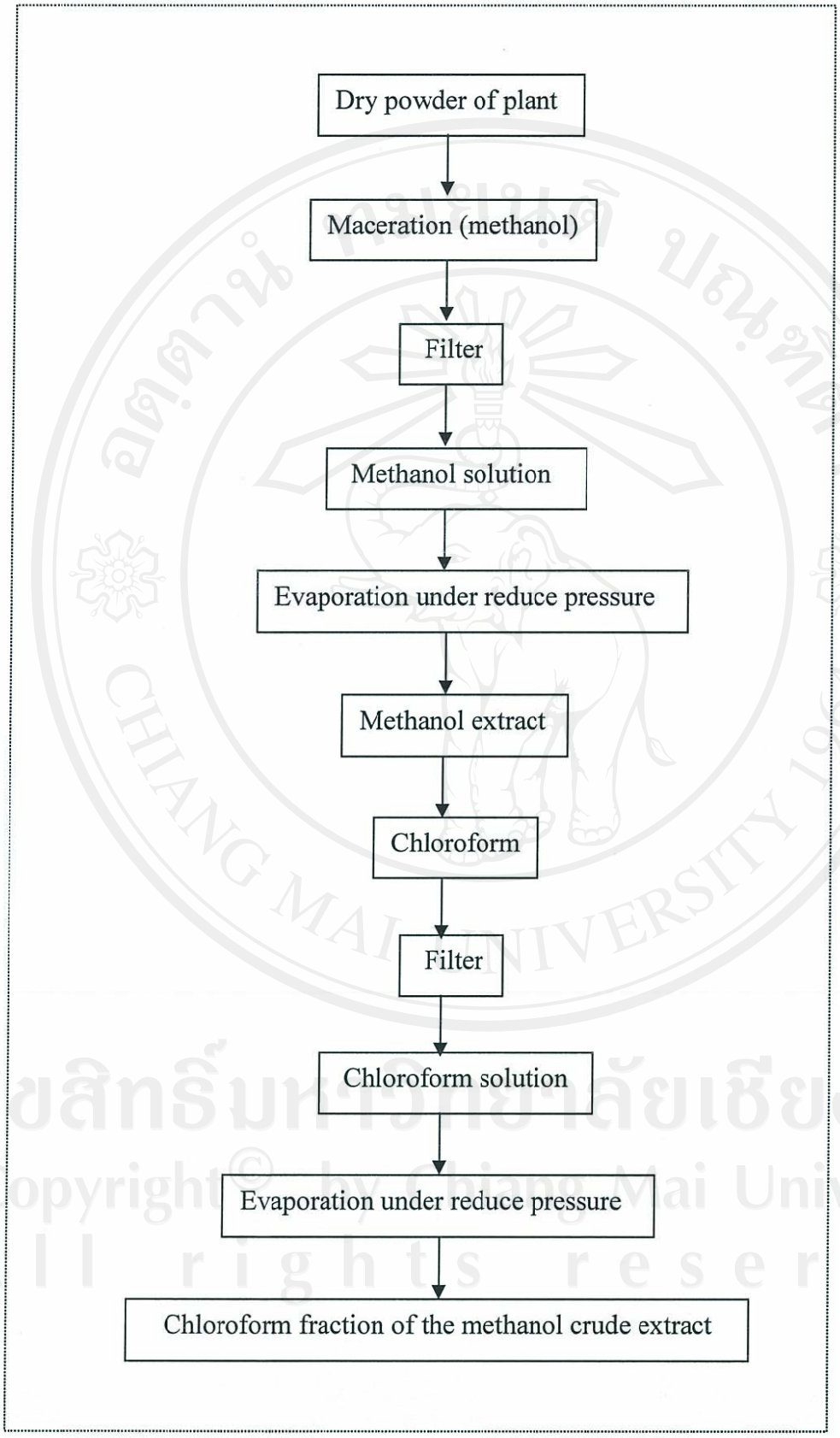

Figure 2.8 Diagram of the preparation of crude extract

2.2.1 Free radical scavenging activity study of crude extracts (DPPH assay)

The free radical scavenging activities of all extracts and the standards (ascorbic acid and α-tocopherol) were determined by a modified DPPH assay of Tachibana *et al.* (2001). DPPH was used as a stable free radical. Briefly, 75 µl of the methanol extracts (1 mg/ml-6.25 µg/ml) and 75 µl of 200 µM ethanol solution of DPPH were put into each well of a 96-well microplate. The reaction mixtures were allowed to stand for 30 min at room temperature, and the absorbance was measured at 570 nm by a Well Reader against a blank (ethanol without DPPH). The experiments were done in triplicates. The DPPH free radical scavenging activity was calculated according to the following equation:

DPPH free radical scavenging activity (%)

$$= \left(\frac{(\text{absorbance of the control} - \text{absorbance of the sample})}{\text{absorbance of the control}} \right) \times 100$$

The scavenging activity was plotted against concentrations. The concentration which showed 50% DPPH scavenging activity (IC_{50}) was determined.

2.2.3 An antitumor activity study of crude extracts (SRB assay)

Stock solutions of extracts were prepared in DMSO and stored at –20 °C. The frozen samples were diluted with cell culture medium prior to the assay. The concentration ranges of the extracts were 3 µg to 250 µg.

The effect of extracts on the growth of human cancer cell lines were evaluated according to the procedure of the NCI for the *in vitro* anticancer drug screening using the protein-binding dye, SRB to assess cell growth (Skehan *et al.*, 1990). Three human cancer cell lines, HeLa, KB and B16F10, were used. Cells were maintained as

adherent cell cultures in DMEM medium supplemented with 10% heat-inactivated FCS and 50 μg/ml of gentamicin at 37°C in a humidified air incubator containing 5% CO_2. Each cell line was plated at a density of 2.0×10^5 cells/ml for HeLa and 1.0×10^5 cells/ml for KB and B16F10 in 96-well plates (optimal cell density of three cell lines are in appendix B) and allowed to attach overnight. Cells were then exposed to five serial concentrations of extracts for 48 hours. After incubation, the adherant cells were fixed *in situ*, washed and dyed with SRB. The bound dye was solubilized by Tris and the absorbance was measured at 492 nm in a microplate reader. The dose-response curves were generated and the GI_{50}, corresponding to the concentration of compounds that inhibit 50% of the cell growth was determined as described (Monks *et al.*, 1991). Doxorubicin hydrochloride was used as positive control.

Part 3: Extraction, isolation, purification and structure elucidation of the selected plants

Stem wood of *Hypericum hookerianum* and *Schisandra verruculosa* which have high and moderate biological activity, respectively and also have never been reported for chemical constituents previously were selected for chemical and biological study.

3.1 Extraction, isolation purification and structure elucidation of *Hypericum hookerianum*

The chloroform fraction of the methanol extract from woody stem of *H. hookerianum* was prepared according to the process of the preparation of the crude extracts to keep the adequate quantity for the isolation. The crude extract was loaded on the silica gel G60 (0.2-0.5 mm), in a column, and eluted with the different polarity

of solvent mixture (Petrol-$CHCl_3$, $CHCl_3$, $CHCl_3$-acetone) with the flow rate 1 ml/min. Fractions of 100-300 ml of were collected for each fraction. All fractions were mornitored by analytical TLC and combined, according to their composition. Fractions which showed complicated spots under UV light were refractionated in the small column and subfractions were also collected. The interesting subfractions were purified by PTLC to give the isolated compounds.

In order to establish the structures of the isolated compounds from *H. hookerianum*, ^{1}H-NMR, ^{13}C-NMR , COSY, NOESY,HSQC, HMBC and also HRMS were performed.

3.2 Extraction, isolation, purification and structure elucidation of *Schisandra verruculosa*

Dried and powdered stem wood of *S. verruculosa* was also prepared for the adequate quantity of crude chloroform extract.

One part of the crude chloroform fraction of the methanol extract was dechlorophyllated following the method described by Herz and Gregor (1962) before fractionation. Another part of the crude extract was applied to Silica gel 60 column and eluted with the different polarity of solvent mixture (Petrol- $CHCl_3$, $CHCl_3$, $CHCl_3$-acetone) with the flow rate 1 ml/min. Fractions of 100-500 ml were collected for each fraction. All fractions were combined according to their composition as revealed by analytical TLC. Fractions which showed complicated spots under UV light were refractionated in the small column. Subfractions were collected and purified by PTLC to give the isolated compounds. The structures of the isolated compounds were elucidated by ^{1}H-, ^{13}C-NMR, COSY, HMBC, HSQC and HRMS.

Part 4: Bioactivities of the isolated compounds

4.1 Tumor cell growth assay

Stock solutions of compounds prepared in DMSO were freshly diluted with the different culture medium prior the assays. Final concentration of DMSO (≤ 0.25%) did not interfere with the biological activities tested. Four human cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), SF-268 (CNS cancer), UACC-62 (melanoma), were used. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine and 50 μg/ml of gentamicin at 37°C in a humidified air incubator containing 5% CO_2. Each cell line was plated at a density that ensured exponential growth throughout the experimental period according to their growth profiles (7.5×10^4 cells/ml for NCI-H460, 1.0×10^5 cells/ml for UACC-62, and 1.5×10^5 cells/ml for MCF-7 and SF-268) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 hours to five serial concentrations of compounds. Following this incubation period, the adherant cells were fixed *in situ* washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a microplate reader. For each test compound and for each cell line a dose-response curve is generated and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined as described (Monks *et al.*, 1991). Doxorubicin was used as positive control.

4.2 Human lymphocytes proliferation assay

The effect of compounds on the mitogenic response of human lymphocytes to PHA were evaluated using a modified colorimetric MTT assay (Mosman, 1983)

previously described by Gonzalez *et al* (1999). Human mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation and were adjusted to 2-3 x 10^6 cells/ml in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml of gentamicin. Mononuclear cells in 96-well plates were exposed for 4 days to seven serial concentrations of compounds. Following this period MTT solution (1 mg/ml) was added and plates were incubated for more 4 h. The water insoluble formazan dye was solubilized overnight at 37 °C. The absorbance of the colored solution was then measured in a microplate reader at 550 nm. The concentration giving 50% inhibition in the test system (IC_{50}) was calculated. Cyclosporin A was used as positive control.

4.3 Cell viability determination by trypan blue exclusion assay

Lymphocytotoxicity, determined in terms of the percentage of viable cells, was present when the viability of the exposed cells was less than 70% of the non-exposed control cells. For trypan blue exclusion assay exponential lymphocytes, growing in 12-well plates, were exposed to the maximum concentration of compound in cell proliferation assay (50 μg/ml), for 24 h. After treatment, cells were aspirated and stained with 0.2% trypan blue and counted on a hemacytometer. The percentage viability was calculated comparing treated cell to the untreated control cells (corresponding to 100% viable cells).

4.4 Free radical scavenging activity

The isolated compounds were evaluated for the free radical scavenging activity using DPPH assay. The concentration which showed 50% DPPH scavenging activity (IC_{50}) was determined.