CHAPTER 3

METHODOLOGY

3.1 Chemicals and Instruments

^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Fourier Transform NMR spectrometer (FTNMR), Varian UNITY INOVA 500 MHz using either operating solvent or tetramethylsilane (TMS) as an internal standard. Spectra were recorded as chemical shift parameter (δ) value in ppm scale (J in Hz). The absorbance (OD) of each well in cytotoxic activity assay was measured at 492 nm, using a Power Wave X plate reader (Bio-TEK Instruments, Inc.). The High performance liquid chromatographic (HPLC) apparatus was a Constametric® 4100 Bio equipped (TSP, USA) with ultraviolet visible (UV-vis) detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500) was made to evaluate piperine and plumbagin content. Chromatographic separation was carried out at room temperature using a Phenomenex Luna 5µ C18(2) 100A analytical column (250 x 4.60 mm 5 micron).

Silica gel 60 (Merck, 0.063-0.200 mm) was used for vacuum liquid chromatography (VLC). Silica gel 60 (Merck, 0.040-0.060 mm) was used for column chromatography (CC).

3.2 Plant Materials

The parts of plants, which used to treat cancer patients by folk doctors in Thailand, were collected from all parts of Thailand in January to March 2006. Place of collection were all region of Thailand and exhibited in Table 3.1 and Figures 3.1 to 3.5. Authentications of plant materials were carried out at the herbarium of the Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept to specify plant and species identified. Another one of these plants have been kept to specimen in the herbarium of Southern Center of Thai Medicinal plant at Faculty of Pharmaceutical Science , Prince of Songkhla University, Songkhla.

3.3 Preparation of plant extracts

Parts of these plants were washed with water to remove the remaining sand and to reduce the microbial load. The cleaned plant materials were cut into small pieces and dried at 50 °C, powdered and extracted in a similar way to that practice by Thai traditional doctors, e.g. ethanolic extraction. The formula drug "Benjakul" was also extracted in the same procedure.

3.3.1 Ethanolic extracts

For the ethanolic extract, dried ground plant material (1 kg) was macerated with 95% ethanol for 3 days, filtered and concentrated to dryness under pressure. The marc was macerated 2 times and dried by evaporator. All extracts of each plant was combined and calculated percentage of yield. For Benjakul extract, all plants in equal portion were mix and extracted as the same method.

Table 3.1 The summarized data of the investigated plant species which ingredients in Benjakul preparation were collected by folk doctors.

Plant Species	Source (Amphur, Province)	Part of Used	Voucher numbers
Piper longum Linn.	Khaosaming, Chanthaburi	Fruit	SKP 146160301
Piper sarmentosum Roxb.	Chombueng, Ratchaburi	Root	SKP 146161901
Piper interruptum Opiz.	Phupan, Sakhonnakhon	Stem	SKP 146160901
Plumbago indica Linn.	Thalingchan, Bangkok	Root	SKP148160901
Zingiber officinale Roscoe.	Khaokho, Phetchabun	Rhizome	SKP206261501

Figure 3.1 Dried fruit of *Piper longum* Linn.

Figure 3.2 Dried root of *Piper sarmentosum* Roxb.

Figure 3.3 Dried stem of *Piper interruptum* Opiz.

Figure 3.4 Dried root of *Plumbago indica* Linn.

Figure 3.5 Dried rhizome of *Zingiber officinale* Roscoe.

3.4 Study on biological fingerprint of Benjakul preparation

3.4.1 *In vitro* assay for cytotoxic activity by SRB assay

The antiproliferative assay, SRB (sulphorhodamine B) assay, was performed according to the method of Skehan *et. al*. (1990). This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. The principle of SRB, which is a bright pink aminoxanthene dye, is that it is an anionic protein stain containing two sulphonic groups, which bind electrostatically to basic amino acid residues of cellular protein under mildly acidic condition. The protein-bound dye is extracted from cells and solubilized for spectrophotometry by weak bases. This colorimetric assay can be used to estimate cell number indirectly

only for monolayer by providing a sensitive index of total cellular protein content which is linearly related to cell density (Skehan *et. al.*, 1990). This assay was found to give good results over both high and low cell densities (Freshney, 1994).

3.4.2 Human cell lines

Four different kinds of human cancerous cell lines and one normal cell line were used in this study. The human breast adenocarcinoma cell line (MCF-7; ECACC No.86012803) and the human cervical cancer cell line (Hela) were established and kindly provided by Cancer Research Institutes of Thailand, the human large cell lung carcinoma cell line (COR-L23) and the human liver cancer cell line (HepG2) were obtained from Assoc. Prof. Dr. Arunporn Itharat, Faculty of Medicine, Thammasat University, Thailand and one type of normal normal lung fibroblast cell line (MRC5) which was a non-cancerous cell line was kindly provided by Prof. Houghton King College, London, England. The MCF-7 were cultured in Minimum Essential Media (MEM) with Earle's salt (without glutamine) (GIBCO™) supplement with 10% heat-inactivated foetal bovine serum (GIBCO™), 50 IU/ml penicillin and 50 µg/ml streptomycin (GIBCO™) and 1% non-essential amino acid (GIBCO™). Hela were cultured in MEM with 10% heat-inactivated foetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. HepG2 were cultured in MEM with 10% heat-inactivated foetal bovine serum, 50 IU/ml penicillin, 50 µg/ml streptomycin and 0.3% hepes (GIBCO™). COR-L23 were cultured in RPMI 1640 medium (GIBCO™) supplement with 10% heated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (Keawpradub *et. al.*, 1997). MRC5 were cultured in Dulbecco's modified Eagle's (DMEM) culture medium (GIBCO™) containing 10% foetal bovine serum and 1% of 10,000 U penicillin and 10 mg/ml streptomycin. The cells were maintained at 37^{o}C in an incubator with 10% CO_2 and 95% humidity.

3.4.3 Testing procedure

According to their growth profiles, the optimal plating densities of MCF-7, Hela, COR-L23, HepG2 and MRC5 were determined to be $3x10^3$, $3x10^3$, $1x10^3$, $3x10^3$ and $5x10^3$ cells/well, respectively to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay (Skehan *et al.*,

1990). Cells growing as monolayer in a 75 cm^3 flask were washed with magnesium and calcium free phosphate buffer saline (PBS) pH 7.4 (AMRESCO®). PBS was decanted and cells detached with 0.025% trypsin-EDTA (GIBCO™) to make a single cell suspension. The viable cells were counted by trypan blue (GIBCO™) exclusion in haemacytometer (Freshney, 1994) and diluted with medium to give a final concentration of $3x10^3$, $3x10^3$, $1x10^3$, $3x10^3$ and $5x10^3$ cells/ml for MCF-7, Hela, COR-L23, HepG2 and MRC5 respectively. One hundred microlitres per well of these cells suspensions were seeded in 96-well microtiter plates and incubated to allow for cells attachment. After 24 hr the cells were treated with the extracts and pure compounds. Each sample was initially dissolved in a quantity of DMSO (Sigma) for ethanolic extracts and pure compounds. The first screening was 50 µg/ml of each extract, which was tested against all cancer cells, and the results of the percentage of cell survival less than 50 % at an exposure time of 72 hours were considered to be active. The active extracts were further diluted in medium to produce the required concentrations. One hundred microlitres per well of each concentration was added to the plates to obtain final concentrations of 1, 10, 50, 100 µg/ml for the active extract and 0.1, 1, 10, 50 µg/ml for pure compound, the final mixture used for treating the cell contained not more than 0.1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40% trichloroacetic acid (TCA, Aldrich Chemical) per well, incubated at 4 °C for 1 hour in the refrigerator and washed 5 times with tap water to wash non viable cells, so viable cells were fixed as monolayer in each well. Fifty microlitres of SRB solution (0.4% w/v in 1% acetic acid, Sigma) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with 1% acetic acid until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base [tris (hydroxy methyl) aminomethane, pH 10.5] (Sigma) was added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (6 replicate) was read on a Power Wave X plate reader at 492 nm as an indication of cell number.

Cell survival was measured as the percentage absorbance compared with the control (non-treated cells). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines (Boyd, 1997) extracts with IC_{50} values < 20 µg /ml were considered active.

3.5 Bioassay-guided fractionation

Ethanolic extract from the five medicinal plants and Benjakul preparation were studied preliminarily for cytotoxic activity (section 3.4).

Results from the preliminary assays for cytotoxic activity (section 4.1) of five medicinal plants and Benjakul formula found that the ethanolic extracts of all plants and Benjakul formula gave the strongest evidence against COR-L23. So separation of the ethanolic Benjakul extracts was undertaken.

An aliquot of the ethanolic extract of the Benjakul perparation (40.01 g) was separated by vacuum liquid chromatography (VLC), using hexane (8×250 ml), hexane:chloroform (1:1) (8×250 ml), chloroform (40×100 ml), chloroform:methanol (1:1) (8×250 ml) and methanol (8×250 ml). Drying and evaporation of each fraction yielded residues of 0.2160, 0.4601, 5.6134, 21.7254 and 6.2616 g, respectively these fractions being denoted as BEN1, BEN2, BEN3, BEN4 and BEN5. These five fractions were tested for cytotoxic activity against COR-L23 (section 3.4). BEN3 showed the highest cytotoxic activity against COR-L23 (IC_{50} = 7.38 µg/ml), so it was used for isolated pure compounds.

3.6 Isolation of chemical constituents from Benjakul preparation

3.6.1 Isolation of BEN3 fraction

An aliquot (2.03 g) of BEN3 was dissolved in hexane:ethyl acetate (8:2). After that, BENS1 (a pure compound) was precipitated, filtered and washed the BENS1 with hexane:ethyl acetate (8:2) until obtained light yellow crystal (158.5 mg). It was identified by using ^{1}H-NMR as piperine.

The filtrate was concentrated to dryness under pressure (1.79 g) and chromatographed over a silica gel column using hexane:ethyl acetate (8:2) as eluent finally being washed with methanol. Ten milliliters of each fraction were collected and tested each fraction. The fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

Fractions 12-31 were obtained BENS2 (a pure compound), as yellow orange crystal (74.9 mg). It was identified by using ^{1}H-NMR as plumbagin.

Fractions 66-73 were obtained BENS3 (a pure compound), as yellow brown sticky (9.6 mg). It was identified by using ^{1}H-NMR as 6-gingerol.

These three pure compounds were tested for cytotoxic activity COR-L23 (section 3.4).

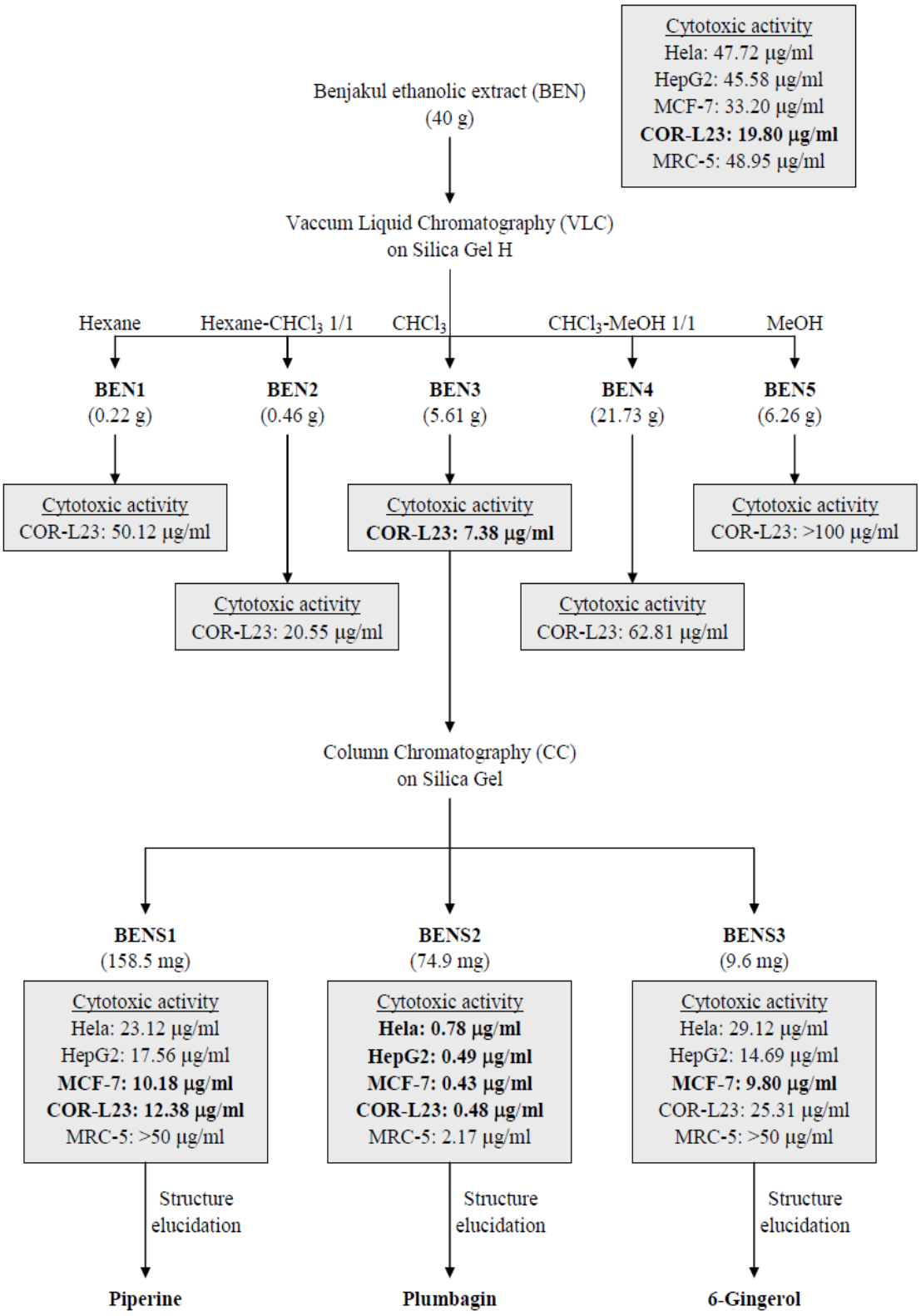

Figure 3.6 Bioassay-guided fractionation of the ethanolic extract of Benjakul preparation.

3.7 Study on chemical fingerprint of Benjakul preparation

3.7.1 Chemicals and Reagents

Standard plumbagin was purchased from Sigma–Aldrich (Seelze, Germany), with Piperine purchased from Merck (Bangkok, Thailand), acetronitrile and purified water (HPLC grade) from Labscan (Bangkok, Thailand).

3.7.2 Apparatus and chromatographic conditions

The study on chemical fingerprint will be carried out using High performance liquid chromatography (HPLC) system (Constametric® 4100 Bio), with ultraviolet visible (UV-vis) detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). Data were analyzed with TSP PC1000 software. A reversed-phase column, Phenomenex Luna 5µ C18(2) 100A analytical column (250 x 4.60 mm 5 micron), with guard column of the same material will be used.

The mobile phase was composed of water-acetronitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100. The mobile phase was filtered under vacuum through a 0.45µm membrane filter before use. The flow rate was 1 ml/min with UV absorbance detection at 256 nm. The operating temperature was maintained at room temperature.

3.7.3 Benjakul sample preparation

Benjakul ethanolic extract was dissolved with acetronitrile, sonicated for 5 minutes, and filtered through a 0.45 µm membrane filter before use.

3.7.4 Standard Preparation

One milligrams of reference standard was weighed accurately and dissolved in 1 ml of acetronitrile.

3.8 Validation of HPLC method

The study on chemical fingerprint of ethanolic extract of Benjakul preparation will be including the study on linearity, precision, accuracy, limit of detection and limit of quantitation for validate the HPLC method that describes below (Wang & Huang, 2005).

3.8.1 Specificity validation

For specificity validation, standard piperine (0.2 mg/ml) and plumbagin solution (0.05 mg/ml) and sample solution of the ethanolic extract of Benjakul preparation (10 mg/ml) were prepared with acetronitrile. The acetronitrile was used as a control. A volume of 10 µl was injected into the HPLC column individually.

3.8.2 Linearity validation

For linearity validation, standard compound (piperine and plumbagin) solutions at least 5 concentrations were prepared and 10 µl was injected into the HPLC column. Triplicate analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

3.8.3 Limit of detection and limit of quantitation

For limit of detection (LOD) and limit of quantitation (LOQ), serial dilutions of piperine and plumbagin were made with acetronitrile, and were then analyzed with HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

3.8.4 Precision validation

For precision validation, standard compound at least 3 concentrations (eg. 50, 100, and 150 µg/ml) were prepared and 10 µl was injected into the HPLC column. Concentrations of standard compound from the experiments were calculated with a linear equation of the standard curve. Triplicate analyses were conducted. The intra- and inter-day precisions were obtained by triplicate analyses in a day and per day over 3 days, respectively. Coefficient of variation (CV) was calculated as standard deviation (SD) to the mean value from the results of triplicate testing and not more than 2%.

3.8.5 Accuracy validation

For accuracy validation, standard compound at least 3 concentrations (eg. 50, 100, and 150 µg/ml) were prepared and mixed with Benjakul sample solution. The three injections for each concentration were done per day over three different days (3 injections × 3 concentrations × 3 days). Recoveries of standard compound were calculated as % Recovery.

3.9 Stability testing of Benjakul ethanolic extract

3.9.1 Stability testing under accelerated condition

The stability testing was carried out in triplicate using transparent vials. All samples were exposed during a 4-month period, under 45±2°C with 75±5% RH as accelerated testing (Koseywattana, 2002). The apparatus utilized for these tests are the desiccators and incubators. Samples were sampling at day 0, 7, 15, 30, 60, 90 and 120. The content of marker compounds (piperine and plumbagin) was evaluated by using HPLC method.