CHAPTER 2

RESEARCH METHODOLOGY

2.1 Instruments

IR spectra were recorded on a Fourier Transform Infrared Spectrometer Model Equinox 55, Bruker. ^{1}H and ^{13}C-Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a 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 unit. EI-MS data were recorded by MAT 95 XL Mass Spectrometer which runs low resolution technique with direct insert probe (DIP probe). Analysis of oil was carried out by Gas chromatography/Mass spectrometry with a Hewlett-Packard HP 5890 Series II Plus GC-HP 5972 Mass Selective Detector. The operating conditions were as follows: inlet temperature 200 °C, oven initial temperature 80 °C (hold for 1 min). It was used with column HP-5 length 10 m, film thickness 0.17 μm and ID 0.25 mm. Ultraviolet spectra (UV) were measured (scanning mode) in the wave length 200-600 nm with Hewlett Packard 8452A Diode Array Spectrometer. The absorbance for free radical scavenging activity was measured at 520 nm and 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.). 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).

2.2 Plant Materials

The parts of plants, which were reported to be used against cancer by folk doctors in Thailand, were collected from several regions of Thailand from January to March, 2003. Places of collection and plant parts exhibited in Table 1 and Figures 2-1 to 2-12. Authentication of plant materials was 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. Others of these plants have been kept as specimens in the herbarium of Southern Center of Thai Medicinal Plant at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

2.3 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 practiced by Thai traditional doctors, e.g. water extraction and ethanolic extraction.

2.3.1 Water extracts

To produce the water extract of each plant, dried ground plant materials (250g) were boiled for 30 minutes in distilled water and filtered by filter cloth. Then the filtrate was concentrated and dried by lyoplillizer.

2.3.2 Ethanol extracts

For the ethanolic extracts, dried ground plant material (250g) 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.

Table 2-1 Plants and part of plants used in cancer preparation by folk doctor

Plant Species	Source (Amphor, Province)	Part Used
Bridelia ovata Decne (SKP 071 02 15)	Hat-yai, Songkhla	leaf
Curcuma zedoaria (Berg)Roscoe (SKP 206 03 26)	Meung, Yala	rhizome
Derris scandens (Roxb.) Benth (SKP 141 04 19)	Jana, Songkhla	stem
Dioscorea membranacea Pierre (SKP 062 04 13)	Natawee, Songkhla	rhizome
Drynaria quercifolia Linn (SKP 152 04 17)	Jana, Songkhla	rhizome
Erythrophleum teysmannii Craib (SKP 034 05 20)	Meung, Nakornrajchasrima	stem
Moringa oleifera Lamk. (SKP 118 13 15)	Hat-yai, Songkhla	bark
Nardostachys jatamansi DC. (SKP 201 14 10)	specimens from folk medicine shop, Songkhla	flower
Rhinacanthus nasutus (L.) Kurz. (SKP 001 18 14)	Hat-yai, Songkhla	root
Sapindus rarak DC. (SKP 170 19 18)	Mueng, Krabi	fruit
Smilax corbularia Kunth (SKP 179 19 03)	Vichienburi, Pechaboon	rhizome
Strychnos nux-vomica L. (SKP 185 19 14)	specimens from folk medicine shop, Songkhla	seed

Figure 2-1 Leaves of *Bridelia ovata* Decne

Figure 2-2 Rhizomes of *Curcuma zedoaria* (Berg)Roscoe

Figure 2-3 Stems of *Derris scandens* (Roxb.) Benth

Figure 2-4 Rhizomes of *Dioscorea membranacea Pierre*

Figure 2-5 Rhizomes of *Drynaria quercifolia* Linn.

Figure 2-6 Stems of *Erythrophleum teysmannii* Craib

Figure 2-7 Barks of *Moringa oleifera* Lamk

Figure 2-8 Flowers of *Nardostachys jatamansi* DC

Figure 2-9 Roots of *Rhinacanthus nasutus* (L.) Kurz.

Figure 2-10 Fruit of *Sapindus rarak* DC.

Figure 2-11 Rhizomes of *Smilax corbularia* Kunth

Figure 2-12 Seeds of *Strychnos nux-vomica* L.

2.4 *In vitro* Assay for Cytotoxic Activity

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).

2.4.1 Human cell lines

Two different kinds of human cancerous cell lines and one normal cell line were used in this study. The large cell lung carcinoma (COR-L23) was established and kindly provided by Dr. P. Twentyman and Dr. P. Rabbitts of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK., the human androgen-insensitive prostate cancer cell line (PC3) was obtained from Dr. Chavaboon Dechsukum, Faculty of Medicine, Prince of Songkla University, Thailand and one type of normal cell line, human fibroblast cell line (10FS) which was a non-cancerous cell line was kindly provided by Associate Professor Dr. Aureeporn Kejaroon of Faculty of Dentistry, Prince of Songkla University, Thailand. The cancer cell lines were cultured in RPMI 1640 medium (GIBCO^{TM}) supplement with 10% heated foetal bovine serum (GIBCO^{TM}), 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (GIBCO^{TM}) (Keawpradub *et al.,* 1997). The human fibroblast cell was grown in DMEM culture medium (GIBCO^{TM}) containing 10% foetal bovine serum and 1% of 10,000 U penicillin and 10 mg/ml streptomycin. The cells were

maintained at 37°C in an incubator with 10% CO_2 and 95% humidity.

2.4.2 Testing procedure

According to their growth profiles, the optimal plating densities of the cell line COR-L23, PC3 and 10FS were determined to be $1x10^3$, $1x10^3$ and $4x10^3$ 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 25 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 $1x10^4$, $1x10^4$ and $4x10^4$ cells/ml for COR-L23, PC3 and 10FS respectively. 100 µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24h the cells were treated with the extracts and pure compounds. Each extract was initially dissolved in a quantity of DMSO (Sigma) for ethanolic extracts, or sterile distilled water for water extracts and vinblastine sulphate (Sigma) used as positive control. 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. According to National Cancer Institute guidelines (Boyd, 1997) extracts with IC_{50} values < 20 µg /ml were considered active. The active extracts were further diluted in medium to produce the required concentrations. 100 µl/well of each concentration was added to the plates to obtain final concentrations of 0.5, 1, 2.5, 5, 10, 50, 100 µg/ml for the active extract, 0.1, 1, 10, 50 µM for pure compound and 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 nM for vinblastine sulphate, 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. 50µl 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.

2.5 Assay for antioxidant activity

The antioxidant activity of these plant extractions was evaluated by DPPH radical scavenging assay which was originally described by Blois (1958).

2.5.1 DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered a stable free radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as a deep violet color and shows a strong absorption band at 520 nm. The DPPH radical can accept an electron or hydrogen radical to become a stable

diamagnetic molecule, at which the absorption vanishes and the resulting decolorization is stomachiometric with the number of electrons taken up; the solution has pale violet color (Blois, 1958). A DPPH solution having a concentration of $6x10^{-5}$ M was used in the present study since at this low concentration the color is not too dense and the Lambert-Beer law is obeyed. If the tested substance is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has an antioxidant effect by the mechanism of free radical scavenging activity. The following assay procedure was modified from those described by Yamasaki *et al.*, (1994).

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

2.5.2 Preparation, testing procedure and data processing

Samples for testing were dissolved in either alcohol (e.g. absolute ethanol and methanol) or distilled water to obtain the highest concentration of 200 µg/ml. Each sample was further diluted for at least 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of the sample solution (500 µl) was mixed with an equal volume of 6×10^{-5}M DPPH (Fluka, in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxytoluene, Fluka), a well known synthetic antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the reduction in the intensity of DPPH. The result was expressed as percentage inhibition:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}]\times 100$$

EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations.

2.6 Bioassay-guided fractionation

Water and ethanolic extracts from the twelve medicinal plants were studied preliminarily for free radical scavenging activity (section 2.4) and cytotoxic activity (section 2.3).

Results from the preliminary assays for free radical scavenging activity (section 3.1.1) and cytotoxic activity (section 3.1.2) of twelve plants found that the ethanolic extracts of *Curcuma zedoaria* and *Dioscorea membranacea* gave the strongest evidence of active ingredients with both activity. So separation of the two active extracts was undertaken.

An aliquot of the ethanolic extract of the rhizome of *Curcuma zedoaria* (2.5 g) was separated by vacuum liquid chromatography (VLC), using hexane (10x100 ml), $CHCl_3$ (10x100 ml) and MeOH (10x300 ml). Drying and evaporation of each fraction yielded residues of 0.0526 g, 0.9164 g and 1.1753 g, respectively these fractions being denoted as FA1, FA2 and FA3.

An aliquot of the ethanolic extract of rhizome of *Dioscorea membranacea* (7g) was separated by vacuum liquid chromatography (VLC), using $CHCl_3$ (10x100 ml), $CHCl_3$:MeOH (9:1) (10x100 ml) and MeOH (10x100 ml). These fractions were evaporated to dryness and denoted as FB1, FB2 and FB3. The percentages of the yields were 0.704 g, 0.047 g and 1.69 g, respectively. These six fractions were tested for cytotoxic activity (section 3.1.3).

2.7 Isolation of chemical constituents from *Curcuma zedoaria*

An aliquot (7g) of the ethanolic extract of the rhizome *C. zedoaria* (CZ) was chromatographed over a silica gel column using a gradient of solvents, hexane:$CHCl_3$ (1:9) (2000 ml); $CHCl_3$ (2000 ml); $CHCl_3$:MeOH (9:1) (2000 ml); $CHCl_3$:MeOH (8:2) (2000 ml); $CHCl_3$:MeOH (7:3) (2000 ml); $CHCl_3$:MeOH (6:4) (2000 ml) finally being washed with MeOH (2000 ml). Each fraction consisted of 100 ml. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure as follows:

Fractions 1-5 were combined and obtained CZV, as a yellow oil (2061.4 mg). Further chemical analysis by using GC/MS showed in the mixture compounds.

Fractions 6-8 were obtained as dark orange liquid (496.9 mg) with orange crystals. The total dark orange liquid was further separated by crystallization from methanol to afford CZS1 (a pure compound) as orange crystals (60.0 mg).

Fraction 89 was obtained as dark brown amorphous solid. The mixture was washed with methanol to obtain CZS2 (a pure compound) as a white amorphous solid (2.3 mg).

2.8 Isolation of chemical constituents from *Dioscorea membranacea*

An aliquot (10g) of ethanolic extract of rhizome of *D. membranacea* (DM) was chromatographed over a silica gel column using a gradient of solvents, hexane:$CHCl_3$ (6:4) (2000 ml); hexane:$CHCl_3$ (2:8) (2000 ml); $CHCl_3$:MeOH (95:5) (2000 ml); $CHCl_3$:MeOH (9:1) (2000 ml); $CHCl_3$:MeOH (7:3) (2000 ml); $CHCl_3$:MeOH (6:4) (2000 ml) finally being washed with MeOH (2000 ml). Each fraction consisted of 20-30 ml. Fractions with similar TLC chromatogram characteristics were combined and evaporated to dryness under reduced pressure. Compound **DMS 1** as white amorphous solid (22.6 mg) was isolated from fractions 360-390. Compound **DMS 2** as pale yellow crystal (17.7 mg) was isolated from FB1 by crystallization from methanol. Compound **DMS 3** as red crystal (6.5 mg) was isolated from FB2 by crystallization from acetone.

2.9 Compounds from *Dioscorea membranacea*

Dioscorealide B, stigmasterol, β-sitosterol, diosgenin 3-*O*-α-*L*-rhamnopyranosyl (1→2)-β-*D*-glucopyranoside and diosgenin 3-*O*-β-*D*-glucopyranosyl (1→3)-β-*D*-glucopyranoside were taken from Arunporn Itharat,

Department of Pharmacognosy and Pharmaceutical botany, Faculty of Pharmaceutical Sciences, Prince of Songkhla University and their chemical constituents were described in Itharat (2002). These compounds had not been previous report about cytotoxicity against PC3 and apoptosis. Therefore, their cytotoxic activity and apoptosis were undertaken.

2.10 Assay for apoptosis activity

The DeadEnd™ Colorimetric TUNEL System is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells in situ at the single cell level. The system can be used to assay apoptotic cell death in both tissue section and cultured cells by measuring nuclear DNA fragmentation, an important biochemical indicator of apoptosis in many cell types (DeadEnd™ Colorimetric TUNEL System, 2005).

2.10.1 Assay principle

The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme activity. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown (DeadEnd™ Colorimetric TUNEL System, 2005).

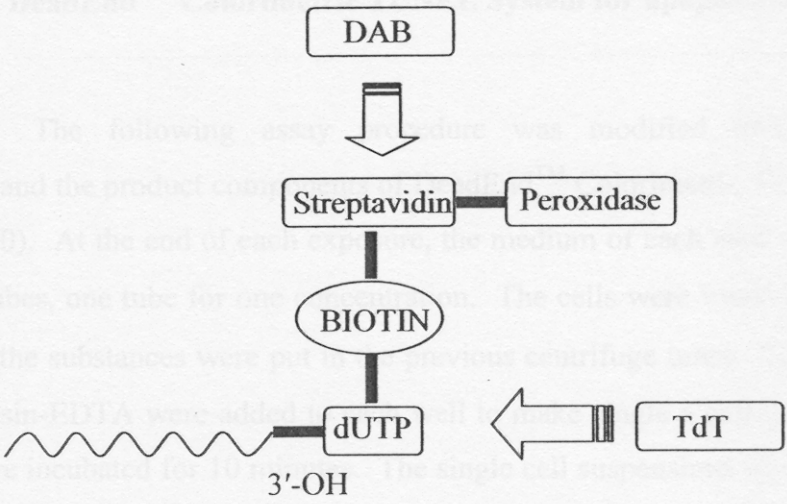

Figure 2-13 Diagram of the DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA

2.10.2 Preparation of cells for the apoptosis assay

Cells growing as monolayer in 25 cm^3 flask were washed with phosphate buffer saline (PBS) pH 7.4 free of magnesium and calcium. The PBS was decanted and cells were detached with 0.025% trypsin-EDTA to make single cell suspension. The viable cells were counted by trypan blue exclusion in haemocytometer (Freshney, 1994) and diluted with medium to give a final concentration of $3x10^4$cells/well for COR-L23 and PC3. The 300 µl/well of these cell suspensions were seeded in 24-well microliter plates and incubated for cell attachment. After 24 hours, the cells were treated with the active pure compounds. Each compound was initially dissolved in DMSO and the 300 µl/well of each concentration was added to the plates in to obtain final concentrations of 1, 5, 10 µM or 5, 10 µg/ml (depending on the IC_{50} of compounds). The final mixture used for treating the cells did not contain more than 0.1% of the solvent, the same as in solvent control wells. The plates were incubated for the selected exposure duration of 48 hours.

2.10.3 DeadEnd™ Colorimetric TUNEL System for apoptosis detection

The following assay procedure was modified from Promega's information and the product components of DeadEnd™ Colorimetric TUNEL System (Cat.# G7360). At the end of each exposure, the medium of each well was moved to centrifuge tubes, one tube for one concentration. The cells were washed with 100 µl of PBS and the substances were put in the previous centrifuge tubes. Then 200 µl of 0.025% trypsin-EDTA were added to each well to make single a cell suspension and the cells were incubated for 10 minutes. The single cell suspensions were removed to the previous centrifuge tubes and then the cells were spun at 1,000 rpm for 10 minutes. Then, the supernatant solutions were removed and the cells were resuspended with PBS.

50 µl of single cell suspensions were topped on the Poly-L-Lysine-coated slides, 25×75×10 mm size (MENZEL-GLASER®, Menzel GmbH&Co KG) and then spun with the Cytospin machine (Cytospin3, SHANDON) after that the slides were allowed to air-dry for 15-30 minutes. Cells were fixed by immersing slides in 10% buffered formalin, 4% paraformaldehyde solution or 10% buffered formalin in PBS in Coplin jar for 25 minutes at room temperature. Then, the slides were washed by immersion in fresh PBS for 5 minutes at room temperature and this step repeated (after this, slides may be stored in PBS at 4°C or in 70% ethanol at -20°C). Cells were permeabilized by immersing the slides in 0.2% Triton® X-100 solution in PBS for 10-15 minutes at room temperature. Slides were rinsed by immersion in fresh PBS for 5 minutes at room temperature and then repeated. Excess liquid was removed by tapping the slides then cover the cells with 30 µl of Equilibration Buffer and equilibrated at room temperature for 5-10 minutes. While the cells are equilibrating, the Biotinylated Nucleotide mix was thawed on ice and sufficient rTdT reaction mix prepared for all experimental and control reaction (reaction mix: 98 µl of Equilibration Buffer, 1 µl of Biotinylated Nucleotide and 1 µl of rTdT Enzyme) and kept it on ice. The equilibrated areas were blotted around with tissue paper and 30µl of rTdT reaction mix added to the cells on slide (the cells were not allow to dry). The cells were covered with Plastic Coverslips to ensure even

distribution of the reagent and incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur. Then, the Plastic Coverslips were removed and the reactions terminated by immersion the slides in 2X SSC (1:10 with deionized water) in a Coplin jar for 15 minutes at room temperature. The slides were washed by immersion in fresh PBS for 5 minutes at room temperature (this wash was repeated twice to remove unincorporated biotinylated nucleotides). Then, the endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 5 minutes at room temperature and the slides washed by immersion in PBS for 5 minutes at room temperature (repeated twice). Add 30 µl of the Streptavidin HRP solution 1:500 in PBS to each slide and incubated for 30 minutes at room temperature. The slides were washed by immersion in PBS for 5 minutes at room temperature (repeated twice). Add 30 µl of DAB solution, the mixture of 50 µl of DAB Substrate 20X buffer, 50 µl of the DAB 20X Chromogen and 50 µl of Hydrogen Peroxide 20X in 950 µl of deionized water, to each slide and developed until there was a light brown background (10-15 minutes, the background was not allowed to become too dark). The slides were rinsed several times in deionized water and the cells dyed by immersing the slides in methyl green solution for 5 minutes then rinsing the slides several times in deionized water. The slides were dehydrated by immersing in 70%, 95%, 100% alcohol and xylene, respectively (each immersion twice times for 5 minutes). The slides were mounted in a permanent mounting medium.