CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

Fresh samples of *Alpinia scabra* (leaves, rhizomes, roots and pseudo stems) were collected from Genting Highlands, Pahang in April 2011 and February 2012. The samples were identified by Professor Halijah Ibrahim of Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur and deposited in the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur with voucher number of HI 1419.

3.2 Extraction and fractionation of plant samples

The different parts of *A. scabra* (leaves, rhizomes, roots and pseudo stems) were washed and ground to fine powder. The dried and ground samples were then extracted with 80 % methanol for three days at room temperature to obtain the crude methanol extract. The extractions of all the samples were further repeated two times with methanol. The crude methanol extracts of the leaves and pseudo stems were dark green in colour while the crude methanol extracts of rhizomes and roots were dark brown in colour. Part of the crude methanol extract was reserved for cytotoxicity assay while the remaining portions were fractionated with hexane, chloroform and water.

The crude methanol extract was fractionated using hexane to give the hexane-soluble extracts and hexane-insoluble residues. The hexane-insoluble residues were further partitioned with chloroform and water (1:1, 100 ml: 100 ml) to give the chloroform and water extracts. The weights of the crude methanol and fractionated

extracts (hexane, chloroform and water) were measured after solvent evaporation under reduced pressure in a rotary evaporator. All the extracts were dissolved in DMSO, except for the water extracts which were dissolved in distilled water to form stock solutions of 20 mg/ml and stored at -20 ºC before cytotoxicity testing.

3.3 *In vitro* cytotoxicity assay

3.3.1 Cell lines and culture medium

The human cell lines used for this study were hormone-dependent breast carcinoma cell line (MCF7), ovarian cancer cell line (SKOV-3) and non-cancer lung fibroblast cell line (MRC-5). The cell lines were purchased from the American Tissue Culture Collection (ATCC, USA). MCF7 cells were maintained in RPMI 1640 medium (Sigma), SKOV-3 cells in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) and MRC-5 cells were maintained in Minimum Essential Medium (MEM) (Sigma), supplemented with 10 % fetal bovine serum (FBS), 100 µg/ml penicillin and 50 µg/ml of amphotericin B. The cells were cultured in a 5 % CO_2 incubator kept at 37 ºC in a humidified atmosphere (Shel Lab, USA). The viability of the cells was checked before and after treatment by the trypan blue excursion dye methods. Frozen cell stocks were stored in liquid nitrogen (-196 ºC) prior to use. The culture was sub-cultured every two to three days and routinely check under an inverted microscope (Leica Microsystems, Germany) for any contamination.

3.3.2 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

Briefly, MCF7 and SKOV-3 cells were seeded at a density of 3 x 10^4 cells/ml in a 96-well micro titer plate (Nunc) in a volume of 100 µl, whereas MRC-5 cells were seeded at 5 x 10^4 cells/ml. The plate was incubated in a CO_2 incubator at 37 ºC for 24

hours to allow the cells to adhere and achieve 60-70 % confluence at the time of the addition of the test agents. After 24 hours, the cells were treated with extracts at six different concentrations, i.e. 1, 10, 25, 50, 75 and 100 µg/ml and incubated for 24, 48 and 72 hours in a 5 % CO_2 incubator. Cells that were treated with fractions and sub-fractions were incubated for 72 hours only. DMSO was used to dilute the extracts and final concentration of DMSO in each well was not in excess of 0.5 % (v/v). No adverse effect due to presence of DMSO was observed. Wells containing untreated cells (without addition of any extract or fraction) were regarded as negative control.

At the end of the incubation period, 20 µl of MTT stock solution (5 mg/ml in PBS pH 7.4) was added into each well and the plates were incubated for another three hours at 37 ºC. After the incubation period, the residual MTT and medium were aspirated from each well. This was done as not to disturb the formazan crystals. Then, 200 µl of 100 % DMSO were added to dissolve the MTT-formazan product. After agitation on a micro plate shaker (LT Biomax 500) for 30 minutes, the absorbance of the dissolved MTT formazan was measured with a micro plate reader (Emax, Moleculer Devices, USA) at 540 nm with 650 nm as reference wavelength. Three replicate plates were used to determine the cytotoxicity activity of each test agent.

The cytotoxic effect of each test agent was evaluated based on the percentage inhibition values which were calculated using the following formula adapted from Aisha *et al*. (2009):

$$\text{Inhibition percentage (\%)} = \frac{(\text{OD of negative control} - \text{OD sample})}{\text{OD of negative control}} \times 100\%$$

Note: OD = Optical Density

In this study, the cytotoxic activity of each extract, fraction and sub-fraction was expressed as IC_{50} value, which is the concentration of tested extract, fraction or sub-fraction that causes 50 % inhibition or cell death. IC_{50} value for each extracts and fractions was extrapolated from the graph of % inhibition plotted against the concentration of extracts. Extracts or fractions with IC_{50} values equal to or lesser than 20 µg/ml was considered active (Geran *et al*., 1972; Swanson and Pezzuto, 1990) while it was 4 µg/ml or less for pure compounds (Boik, 2001).

Selectivity Index (SI) of active extract was determined in order to investigate whether the cytotoxic activity was specific to cancer cells. To determine whether the cytotoxicity was specific to the cancer cells, the cytotoxicity of the extracts was tested and the selectivity index (SI) of active extract was determined. The selectivity index (SI) of the extracts is defined as the ratio of cytotoxicity (IC_{50} values) on normal lung fibroblast (MRC-5) cells to cancer cells (MCF7 and SKOV-3): SI = IC_{50} on MRC-5 cells/ IC_{50} on cancer cells. Test agents with SI higher than three were considered to have high selectivity towards cancer cells (Mahavorasirikul *et al*., 2010).

3.3.3 Statistical analysis

The IC_{50} values for cytotoxic activity were obtained by non-linear regression using GraphPad Prism statistical software. Data are shown as mean ± SD from three independent experiments.

3.4 Extraction, isolation and identification of chemical constituent from *A. scabra*

The leaf (hexane and chloroform) and rhizome (chloroform) extracts were selected for bioassay-guided fractionation as the extracts showed the strongest cytotoxic effect against the selected cancer cells as shown in Table 4.3, 4.4, 4.5 and 4.6.

3.4.1 Column chromatography

Column chromatography was performed using Merck Kieselgel 60 PF253 Art No 7734.1000 and 9385.1000 with particle size 0.063 - 0.200 mm and 0.040 – 0.0063 mm, respectively. The gel was made into slurry with solvent before it was packed onto the column and then allowed to equilibrate for at least an hour before use. After the extract or fraction was introduced to the column, solvent with increasing polarity gradient was used to elute the chemical compounds from the column. Fractions collected were monitored by the thin layer chromatography (TLC) and appropriate fractions were combined and where necessary subjected to further separation.

3.4.2 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was used to detect and separate various compounds present in the fraction and also to determine a suitable solvent system for the isolation of chemical compounds from the fractions. Pre-coated silica gel 60 F_{254} TLC plates from Merck were used for TLC analysis. A small spot of solution containing the sample was applied to the TLC plate about 1.0 cm from the bottom using a fine capillary tube. The solvent was allowed to completely evaporate off, otherwise a very poor or no separation will be achieved. Then, the plate was placed in a TLC developing tank with various solvent systems. The TLC plate was removed from the developing tank when it was fully developed to solvent front and dried. The plate was viewed under

UV light (254 nm and/or 343 nm) and then it was placed in an iodine vapour chamber to visualize the spots of separated chemical components.

3.4.3 Bioassay-guided fractionation of leaf chloroform extract

Initially, the methanol-containing extract obtained was initially treated with charcoal, then filtered and the filtrate was evaporated under reduced pressure to give a crude methanolic extract. Treatment with charcoal was necessary to remove the high content of chlorophyll present in the extract because chlorophyll can interfere with the chromatographic separation.

The chloroform extract of leaves (9.0 g) was subjected to vacuum liquid chromatography (VLC) which is a rapid crude fractionation system. The column was packed with 540.0 g of silica gel 60 (Merck, 0.063 – 0.200 mm) as stationary phase. The elution of components present in the extract were started with chloroform and then the polarity of the eluent was gradually increased with addition of methanol and finally with methanol (Table 3.1). Elution was monitored by TLC and the eluent vials were pooled together based on similar pattern of TLC spots into a total of 10 fractions labeled as LC1 to LC10 (Table 3.2).

Table 3.1: Vacuum liquid chromatography solvent system of chloroform extract of leaf

Solvent system	Volume (Litre)	Eluent vial
Chloroform	1.0	1 - 2
5 % Methanol in chloroform	1.5	3 - 5
10 % Methanol in chloroform	1.5	6 - 8
20 % Methanol in chloroform	2.0	9 - 12
30 % Methanol in chloroform	2.0	13 - 16
40 % Methanol in chloroform	2.0	17 - 20
100 % Methanol	1.5	21 - 23

Table 3.2: Fractions obtained from VLC of leaf chloroform extract

Sub-fractions	Vials combination	Weight (g)
LC1	1 - 2	0.067
LC2	3 - 4	0.067
LC3	5 - 7	0.052
LC4	8 - 10	4.063
LC5	11 - 13	0.977
LC6	14 - 16	0.661
LC7	17 - 18	0.356
LC8	19 - 20	0.217
LC9	21- 22	0.309
LC10	23	0.143

All the 10 fractions were then tested for cytotoxicity against MCF7, SKOV-3 and MRC-5 cell lines using MTT assay at 72 hours. Among the 10 fractions, only fraction LC4 has found to be active in the cytotoxicity screening against MCF7 and SKOV-3 cell lines. Thus, LC4 was selected for further isolation and purification work.

The active fraction LC4 which was a dark brown paste was then subjected to gravity column chromatography and VLC in order to isolate components in the fraction. The purpose of using two different types of separation techniques is to determine which technique gives more yield in order to carry out further work in this study. For gravity column chromatography, the ratio of fraction LC4 to silica gel 60 (Merck, 0.063 – 0.200 mm) was prepared at ratio 1:100 and packed in a glass column. First, the column was plugged at the bottom with a cotton plug to prevent the loss of absorbent material. Silica gel was made into slurry with chloroform and poured into the column and allowed to settle (approximately 15 cm in height). The components present in LC4 were eluted initially with chloroform and the polarity of the eluent was gradually increased by addition of higher percentage of methanol and the elution was finally performed with 100 % methanol. Table 3.3 represent the eluting solvent and volumes used in the gravity column chromatography. Eluents were collected in 25 ml vials and analysed on TLC. Fractions with similar TLC profile were pooled together to give 18 sub-fractions as shown in Table 3.4.

Table 3.3: Different composition and volume of solvent systems applied in the gravity column chromatography for separating LC4 fraction

Solvent system	Volume (Litre)	Eluent vial
Chloroform	0.8	1 - 8
2 % Methanol in chloroform	1.6	9 - 16
4 % Methanol in chloroform	1.8	17 - 25
6 % Methanol in chloroform	2.0	26 - 47
8 % Methanol in chloroform	1.6	48 - 55
10 % Methanol in chloroform	1.4	56 - 62
15 % Methanol in chloroform	1.2	63 - 68
20 % Methanol in chloroform	1.2	69 - 74
22 % Methanol in chloroform	0.8	75 - 78
25 % Methanol in chloroform	1.2	79 - 84
30 % Methanol in chloroform	1.2	85 - 90
40 % Methanol in chloroform	1.4	91 - 97
45 % Methanol in chloroform	0.8	98 - 101
50 % Methanol in chloroform	1.2	102 - 107
55 % Methanol in chloroform	1.0	108 - 112
100 % Methanol	1.2	112 - 118
100 % Methanol + trifluoroacetic acid (TFA)	1.6	119 - 126

Table 3.4: Sub-fractions obtained from gravity column chromatography of LC4

Sub-fractions	Vials combination	Weight (mg)
GC1	1 - 8	23.0
GC2	9 - 16	33.0
GC3	17 - 21	27.0
GC4	22 - 24	6.0
GC5	25 - 27	3.0
GC6	28 - 32	14.0
GC7	33 - 37	5.0
GC8	38 - 40	3.0
GC9	41 - 46	8.0
GC10	47 - 55	10.0
GC11	56 - 66	7.0
GC12	67 - 74	6.0
GC13	75 - 85	5.0
GC14	86 - 95	15.0
GC15	96 - 105	20.0
GC16	106 - 117	5.0
GC17	118 - 121	4.0
GC18	122 - 126	5.0

Besides that, the LC4 fraction was subjected to a rapid fractionation using VLC. The column was packed with 60.0 g of silica gel 60 (Merck, 0.063 – 0.200 mm) as the stationary phase and the ratio of the fraction to silica gel was 1:60. In brief, elution of components in the fraction started with chloroform and then its polarity was gradually increased with addition of methanol and finally with methanol as shown in Table 3.5. Elution of components from the column was monitored by TLC and eluent vials with similar pattern of TLC profile were combined to give 17 sub-fractions as shown in Table 3.6.

Table 3.5: VLC solvent system for LC4 fraction

Solvent system	Volume (Litre)	Eluent vial
Chloroform	0.8	1 - 4
2 % Methanol in chloroform	0.6	5 - 7
4 % Methanol in chloroform	0.4	8 - 9
6 % Methanol in chloroform	0.6	10 - 12
7 % Methanol in chloroform	0.4	13 - 14
8 % Methanol in chloroform	0.6	15- 17
9 % Methanol in chloroform	0.4	18 - 19
10 % Methanol in chloroform	0.4	20 - 21
15 % Methanol in chloroform	0.4	22 - 23
30 % Methanol in chloroform	0.4	24 - 25
40 % Methanol in chloroform	0.4	26 - 27
50 % Methanol in chloroform	0.4	28 - 29
100 % Methanol	0.4	30 - 31
100 % Methanol + trifluoroacetic acid (TFA)	0.6	32 - 34

Table 3.6: Sub-fractions obtained from VLC of LC4

Sub-fractions	Vials combination	Weight (mg)
VLC1	1	21.0
VLC2	2	44.0
VLC3	3	16.0
VLC4	4	19.0
VLC5	5	14.0
VLC6	6	14.0
VLC7	7	116.0
VLC8	8	98.0
VLC9	9	297.0
VLC10	10 - 11	78.0
VLC11	12 - 15	27.0
VLC12	16 - 17	6.0
VLC13	18 - 20	4.0
VLC14	21 - 23	5.0
VLC15	24 - 25	8.0
VLC16	26 - 29	7.0
VLC17	30 - 34	10.0

Some of the sub-fractions (weight more than 20 mg) were screened for cytotoxic activity against MCF7, SKOV-3 and MRC-5 cell lines using MTT assay at 72 hours. Sub-fractions collected from the separation of LC4 via VLC were chosen to be utilized in the present study because the yield obtained from VLC was higher than those from the gravity column chromatography. Besides that, separation via gravity column chromatography is time consuming and with the limited amount of sub-fractions obtained (Table 3.4), it was not possible to attempt any further study. A flow chart of bioassay-guided fractionation of cytotoxic leaf chloroform extract is shown in Figure 3.1.

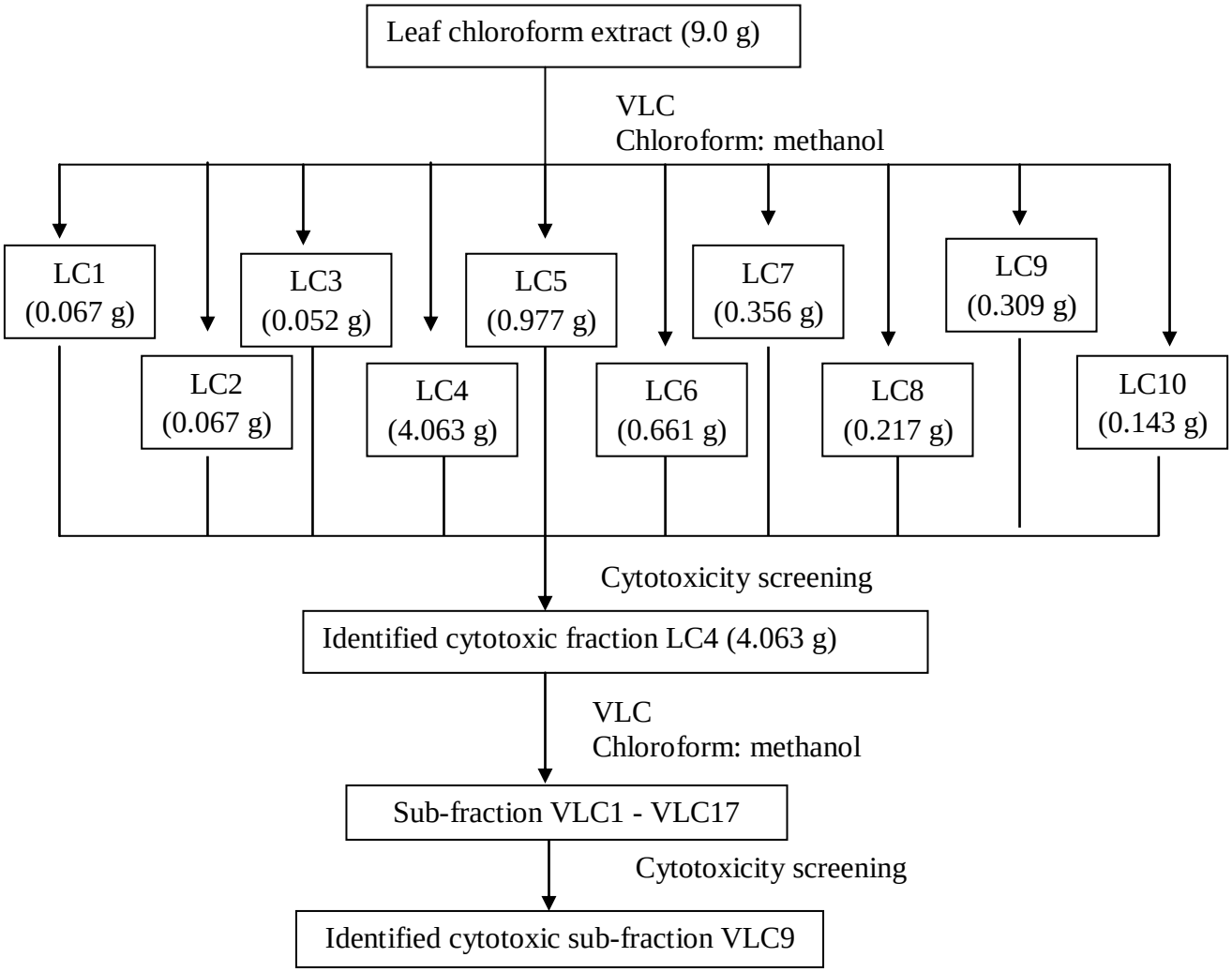

Figure 3.1: Flow chart of bioassay-guided fractionation of cytotoxic leaf chloroform extract

3.4.4 Bioassay-guided fractionation of rhizome chloroform extract

The chloroform extract of rhizomes (2.0 g) was subjected to a rapid crude fractionation using vacuum liquid chromatography (VLC). This column was packed with 120.0 g of silica gel (Merck, 0.063 - 0.200 mm) as the stationary phase and the ratio of the extract to silica gel was 1:60.

In brief, elution of components in the fraction started with the chloroform and then its polarity was gradually increased with addition of acetone and finally methanol as described in Table 3.7. Elution of components from the column was monitored by TLC. Eluent vials with similar pattern of TLC profile were combined to give 18 fractions as showed in Table 3.8.

Table 3.7: VLC solvent system of rhizome chloroform extract

Solvent system	Volume (ml)	Eluent vial
Chloroform	400	1 - 2
2 % Acetone in chloroform	200	3
4 % Acetone in chloroform	200	4
6 % Acetone in chloroform	200	5
8 % Acetone in chloroform	800	6 - 10
9 % Acetone in chloroform	400	11- 12
10 % Acetone in chloroform	600	13 - 23
11 % Acetone in chloroform	600	17 - 19
12 % Acetone in chloroform	600	20 - 22
13 % Acetone in chloroform	400	23 - 24
14 % Acetone in chloroform	200	25
16 % Acetone in chloroform	200	26
20 % Acetone in chloroform	600	27 - 29
40 % Acetone in chloroform	600	30 - 32
60 % Acetone in chloroform	400	33 - 34
80 % Acetone in chloroform	200	35
100 % Acetone	400	36
100 % Methanol	400	37

Table 3.8: Sub-fractions obtained from VLC of chloroform extract of rhizome

Sub-fractions	Vials combination	Weight (mg)
RC1	1 - 2	18.0
RC2	3	4.0
RC3	4	14.0
RC4	5	24.0
RC5	6 - 7	30.0
RC6	8 - 10	18.0
RC7	11 - 12	20.0
RC8	13 - 17	36.0
RC9	18 - 23	12.0
RC10	24 - 26	2.0
RC11	27 - 29	7.0
RC12	30	5.0
RC13	31	60.0
RC14	32	9.0
RC15	33 - 34	36.0
RC16	35	21.0
RC17	36	29.0
RC18	37	461.0

All the 18 fractions were then tested for cytotoxicity against SKOV-3 and MRC-5 cell lines. Figure 3.2 shows the flow chart of bioassay-guided fractionation of rhizome chloroform extract.

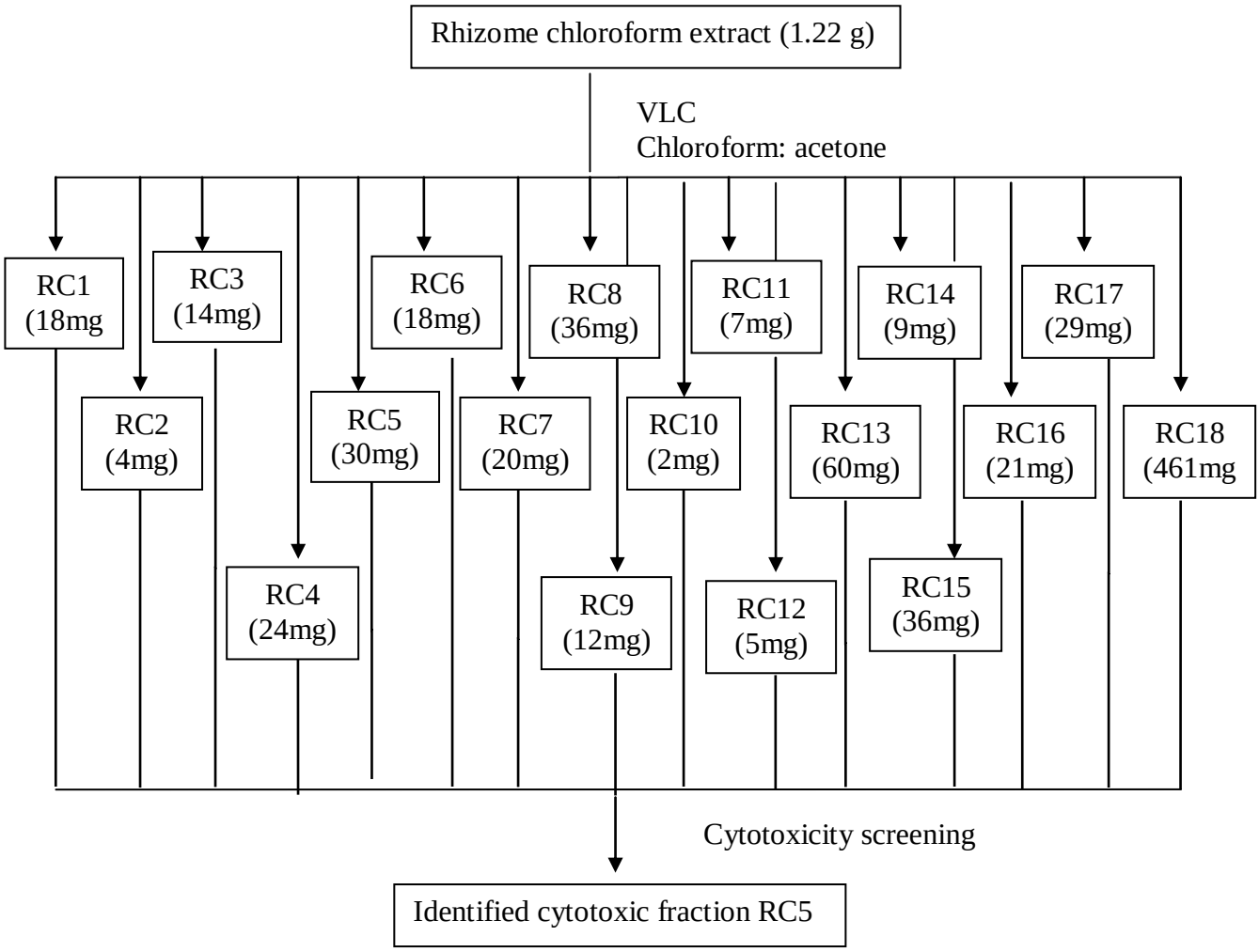

Figure 3.2: Flow chart of bioassay-guided fractionation of cytotoxic rhizome chloroform extract

3.4.5 Identification of compounds in cytotoxic extracts, fractions and sub-fraction using GC-MS analysis

Gas chromatography and mass spectral (GC-MS) analysis was performed using an Agilent Technologies 6980N gas chromatograph equipped with a 5975 Mass Selective Detector (70 eV direct inlet) using an HP-5ms capillary column (5 % phenylmethylsiloxane) with column dimensions 30.0 m x 25 mm x 25 um was initially set at 100 ºC, the temperature of the oven was then increased at 5 ºC per minute to 300 ºC and held for 10 min using helium as carrier gas at flow rate 1 ml/min. Leaf hexane and chloroform extract, rhizome chloroform extract, fraction LC4, RC5 and sub-fraction VLC9 were analysed using GC-MS. The total ion chromatogram obtained was auto integrated by ChemStation and the components were identified by comparison with an accompanying mass spectral database (W9N11 Mass Spectral Library, USA). Only mass spectral fragmentation pattern that gave greater than 90 % match were accepted.

3.5 Detection of apoptosis

3.5.1 Morphological assessment of apoptotic cells by phase-contrast inverted microscope

Analysis of cell morphological changes was carried out according to the method described by Moongkarndi *et al*. (2004). Cells (3 x 10^4 cells/ml) in the absence or presence of the active extract and fractions at IC_{50} concentrations, were incubated for 24, 48 and (or) 72 hours (based on the time point in which the extract and fraction were active) as shown in Table 4.3, 4.4, 4.5 and 4.6 in 24-well tissue culture plates. At the end of the incubation period, the culture medium was removed and cells were washed with phosphate buffer saline (PBS pH 7.4) and observed under Leica DMI 3000B phase-contrast inverted microscope (Leica Microsystems, Germany) at 200x magnification and photographed.

3.5.2 DNA fragmentation analysis by agarose electrophoresis

Detection of apoptotic fragmented DNA was performed using the Suicide-Track DNA isolation kit (Calbiochem, USA) according to manufacturer's protocol. Cells (7 x 10^5) were plated onto Petri dish and incubated in a CO_2 incubator at 37 °C for 24 hours to allow the cells to adhere before addition of the test agents. After 24 hours, the cells were treated with cytotoxic extracts, fractions, sub-fraction and doxorubicin (positive control) for 24 hours at concentration corresponding to the IC_{50} values. Negative control comprised of cells not treated with any extract.

At the end of the incubation period, the floating and trypsinized-adherant of treated and untreated cells were collected by centrifuging at 1000 x g for 5 min (Kubota 2420, Tokyo). Cell pellets were then resuspended in 55 µl of lysis buffer, followed by the addition of 20 µl of RNase solution and incubated at 37 °C for 1 hour. DNA isolation buffer was added and further incubated at 50 °C for 1 hour, followed by addition of 500 µl of resuspension buffer and mixed after the incubation period. During the DNA precipitation step, 2 µl of Pellet Paint® Co-precipitant was added to the suspension together with 60 µl of 3M sodium acetate and 662 µl of 2-propanol. Samples were mixed by inversion then centrifuged at 15 000 x g for 5 min (Thermo Scientific, Heraeus PICO 17). The resulting pellets were washed with 70 % and 100 % ethanol and the DNA samples were then air-dried and resuspended in 50 µl of resuspension buffer.

Then, 50 ml of 1.5 % agarose was melted in a microwave (National NN-7806) and poured into the gel-casting tray. The 17-wells comb was placed until its base is 1 mm from the base of the gel. After the gel has hardened (20 - 30 min), the gel comb was removed and the gel was placed in an electrophoresis tank (Gel XL Plus, Labnet) containing sufficient 1X TAE buffer to cover the gel approximately 1 mm. Individual DNA samples were gently added into each well. The power supply was turned off when the bromophenol blue dye has migrated two thirds of the way down the gel. The gel was

transferred to a container contain 0.5 µg/ml ethidium bromide solution, placed on a mini rocker (Biosan MR-1) and stain for 30 min. The stained gel was observed and photographed using gel documentation system (Gene Flash, Syngene Bioimaging).

3.5.3 Morphological detection of apoptosis using DAPI nuclear stain

The occurrence of apoptosis in MCF7 and SKOV-3 cells was evaluated using 4'6-diamidino-2-phenylindole (DAPI, Sigma) staining. Cells (1×10^6) were plated onto 6-well tissue culture plate and incubated in a CO_2 incubator at 37 °C for 24 hours. After 24 hours, the cells were treated with cytotoxic extracts, fractions, sub-fraction and doxorubicin (positive control) for 24 hours at concentration corresponding to the IC_{50} values. Negative control comprised of cells not treated with any extract. After the incubated period, the cells were then harvested and washed with PBS. The resulting cell pellet was fixed with acetone at -20 °C for 30 min. The cells were then stained with DAPI solution (1 µg/ml) at 4 °C for 30 minutes. Stained cells were spotted onto a slide and cover slips were then mounted onto glass microscope slides and observed under fluorescence microscopy (Olympus BX51) using a 358 nm excitation and 460 nm emission fluorescent filter.