CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction yield of *Alpinia scabra*

Samples of *A. scabra* were dried in an oven as a method of preservation after which the samples were ground to powder. The latter was carried out to allow maximum contact of the dried sample to the extraction solvent. The solvents used in the present study were selected based on their different polarity ranges. In term of chemistry, polar substances would dissolve in polar solvents while non-polar substances will dissolve in non-polar solvents (Zuo *et al*., 2002). Methanol, chloroform and hexane (in order of decreasing polarity, respectively) were selected to enable the extraction and separation of a wide range of components that are present in the samples. Application of different extraction solvents used in this study was an attempt to produce best separation of compounds in each extract. This was achieved by gradual isolation of compound groups following different solvent polarities. Powdered samples were first soaked in methanol in to extract out the polar compounds which constitutes the bulk compounds present in the samples. Methanol is classified as high polarity solvent that can be used to extract sugar, amino acids and glycosides from the samples (Houghton and Raman, 1998). Usually, 80 % methanol and 70 % ethanol are most preferred solvents for phenolic extraction from plants (Apak *et al*., 2007).

The percentage of crude methanol extract yield as shown in Table 4.1 was based on the weight of dried and ground plant materials. The yield of crude methanol extract of pseudo stems (7.29 %) is the highest among the four samples whereas yield of crude methanol extract of rhizomes (4.47 %) is the lowest compared to the other samples.

Table 4.1: The weight and percentage yield of crude extracts from the samples of *A. scabra*

Samples	Extract	Weight (g)
Leaves	Fresh samples	1000.00
	Dried and ground plant material	600.00 (60 %)
	Methanol extract	41.22 (6.87 %)
Rhizomes	Fresh samples	7000.00
	Dried and ground plant material	400.00 (5.71 %)
	Methanol extract	17.86 (4.47 %)
Roots	Fresh samples	900.00
	Dried and ground plant material	100.00 (10 %)
	Methanol extract	7.11 (7.11 %)
Pseudo stems	Fresh samples	8000.00
	Dried and ground plant material	650.00 (8.13 %)
	Methanol extract	47.38 (7.29 %)

The crude methanol extracts were further fractionated into hexane, chloroform and water extracts. The concentrated methanol extracts were then subjected to repeated hexane extraction to acquire a mixture of volatile oils, fats and waxes (Houghton and Raman, 1998). The remaining hexane insoluble methanol extract was then partitioned with chloroform and water (ratio 1:1). This crude fractionation procedure as described by Houghton and Raman (1998) involved separation of compounds based on their relative solubility. Alkaloids, aglycones and volatile oils will be forced into the chloroform layer while the water layer embodies water-soluble carbohydrate polymers, glycosides and amino acid (Oufnac *et al*., 2007).

The percentage yield of fractionated extracts was based on the weight of crude methanol extract as shown in Table 4.2. For all the samples, the water extract was the most abundant extract (52.16 % for leaves, 67.12 % for rhizomes, 72.29 % for roots and 69.76 % for pseudo stems) among the fractionated extracts in each samples while the hexane extract gave the lowest yield which 1.21 % for leaves, 3.62 % for rhizomes, 4.36 % for roots and 1.01 % for pseudo stems.

Table 4.2: The weight and percentage yield of fractionated extracts from the crude methanol extracts of *A. scabra*

Sample	Extract	Weight (g)
Leaves (extracted from 41.22 g of methanol extract)	Hexane	0.50 (1.21 %)
	Chloroform	9.64 (23.39 %)
	Water	21.50 (52.16 %)
Rhizomes (extracted from 17.86 g of methanol extract)	Hexane	0.65 (3.62 %)
	Chloroform	1.22 (6.83 %)
	Water	11.99 (67.12 %)
Roots (extracted from 7.11 g of methanol extract)	Hexane	0.31 (4.36 %)
	Chloroform	0.63 (8.86 %)
	Water	5.14 (72.29 %)
Pseudo stems (extracted from 47.38 g of methanol extract)	Hexane	0.48 (1.01 %)
	Chloroform	3.23 (6.82 %)
	Water	33.05 (69.76 %)

4.2 Cytotoxic activities of *A. scabra* extracts

In the present study, the cytotoxic effect (IC_{50}) of the crude methanol and fractionated extracts (hexane, chloroform and water) from different parts of *A. scabra* were investigated on two human cancer cells (MCF7 and SKOV-3) and one normal non-cancer cells (MRC-5) using MTT assay in dose and time-dependent manner. MCF7 and SKOV-3 cell lines were chosen for this study is to focus on diseases related women. MTT assay is used in cell biology for the study of growth factor, cytokines and for screening of cytotoxic or chemotherapeutic agents. MTT offers a quantitative and simple method for evaluating a cell population's response to external factors. This assay is based on the reduction of yellow tetrazolium salts to form purple formazan crystals that are insoluble in aqueous solutions. This reduction process requires functional mitochondria where mitochondrial dehyrogenases of viable cells cleaves the tetrazolium ring. The trapped formazon crystals are then solubilised by addition of a detergent so that the color can be quantified by spectrophotometric means (Mosmann, 1983). The absorption of dissolved formazan in the visible region correlates with the number of intact alive cells. Cytotoxic compounds are able to damage and destroy cells, and thus decrease the reduction of MTT to formazan. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation (Carmicheal *et al.*, 1987).

According to the United States National Cancer Institute plant screening program, a plant extract is generally considered to have active cytotoxic effect if the IC_{50} value, following incubation between 48 to 72 hours, is 20 µg/ml or less (Lee and Houghton, 2005). Cytotoxicity activity (IC_{50}) and Selectivity Index (SI) of the extracts of leaves, rhizomes, roots and pseudo stems of *A. scabra* are summarized in tables 4.3, 4.4, 4.5 and 4.6, respectively. Data are shown as mean ± SD from three independent experiments.

Absorbance values and percentage of inhibition of leaves, rhizomes, roots and pseudo stems of *A. scabra* against the MCF7, SKOV-3 and MRC-5 cells are shown in Appendix B and C. Besides that, the IC_{50} value was determined by extrapolation of the graph of concentration of extract against percentage of inhibition. The IC_{50} value of the extract/fraction was calculated as the average of three replicates and an example of the calculation of the IC_{50} value is shown in Appendix D.

For MCF7 cells, hexane and chloroform extracts of leaves sample have active cytotoxic effect on MCF7 cells. The hexane extract of leaves showed high inhibition towards MCF7 cells with IC_{50} value of 15.30 µg/ml at 48 hours, in comparison to IC_{50} value at 24 hours and 72 hours, which are 19.30 µg/ml and 16.33 µg/ml, respectively. The chloroform extract of the leaves showed good cytotoxic effect with an IC_{50} value of 18.80 µg/ml at 24 hours and the rhizomes, roots and pseudo stems showed weaker cytotoxicity profile against the MCF7 cells.

Meanwhile for SKOV-3 cells, the hexane and chloroform extracts of the leaf sample and chloroform extract of the rhizome sample have active cytotoxic effect on the cells. Hexane extract of the leaf showed a remarkably high inhibition towards SKOV-3 cells with IC_{50} value of 4.93 µg/ml at 72 hours, in comparison to IC_{50} value at 24 hours and 48 hours, which are 18.00 µg/ml and 6.00 µg/ml, respectively. The chloroform extract from the leaf samples possessed the strongest cytotoxicity at 48 hours with IC_{50} value of 14.33 µg/ml in comparison to the IC_{50} values at 24 hours and 72 hours. Meanwhile, the chloroform extract from the rhizomes sample showed a remarkably high inhibition towards SKOV-3 cells with IC_{50} value of 17.30 µg/ml at 72 hours, in comparison to the IC_{50} values at 24 hours and 48 hours, which are 21.67 µg/ml and 19.33 µg/ml, respectively. Roots and pseudo stems showed weaker cytotoxicity profile against the SKOV-3 cells. MRC-5 cells have been used as control in many similar studies (Reddy *et al.*, 2012, Ramasamy *et al.*, 2012). All the 4 samples were also screened on the

MRC-5 cells and only the leaf hexane extract showed cytotoxicity at 48 hours and 72 hours with IC_{50} values of 14.64 µg/ml and 15.90 µg/ml, respectively. Selectivity of the active extracts were determined but none of the active extracts showed selectivity to the cancer cells since all the selectivity indexes are lower than 3 except for leaf hexane which showed selectivity towards SKOV-3 cells at 72 hours with the SI value of 3.2 (Table 4.3).

The leaf (hexane and chloroform) and rhizome (chloroform) extracts were selected for the bioassay-guided fractionation as it showed the strongest cytotoxic effect against the selected cancer cells.

Table 4.3: Cytotoxic activity (IC_{50} µg/ml) of leaf extracts of *A. scabra*

Extracts	Treatment duration (hour)	IC_{50}[a] (µg/ml) (SI[b])		
		MCF7	**SKOV-3**	**MRC-5**
Methanol	24	90.67 ± 6.11	47.00 ± 11.53	>100
	48	56.27 ± 9.18	37.67 ± 2.52	57.58 ± 1.25
	72	53.33 ± 6.43	34.33 ± 0.58	65.35 ± 1.68
Hexane	24	**19.30 ± 5.7 (1.6)**	**18.00 ± 2.65 (1.7)**	31.38 ± 2.31
	48	**15.30 ± 4.04 (1.0)**	**6.00 ± 1.00 (2.4)**	**14.63 ± 2.08**
	72	**16.33 ± 0.58 (1.0)**	**4.93 ± 0.12 (3.2)**	**15.90 ± 0.94**
Chloroform	24	**18.80 ± 1.06 (2.4)**	**20.00 ± 1.00 (2.3)**	45.88 ± 3.81
	48	23.67 ± 7.64	**14.33 ± 1.53 (2.3)**	32.26 ± 2.11
	72	25.00 ± 0	**14.67 ± 0.58 (2.2)**	32.90 ± 0.76
Water	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100

[a] Data are presented as mean ± SD of three replicates. Values in bold characters are considered to have cytotoxic activity (IC_{50} 20 µg/ml or less)
[b] Selectivity index (SI)

Table 4.4: Cytotoxic activity (IC_{50} µg/ml) of rhizome extracts of *A. scabra*

Extracts	Treatment duration (hour)	IC_{50}[a] (µg/ml) (SI[b])		
		MCF7	**SKOV-3**	**MRC-5**
Methanol	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100
Hexane	24	79.67 ± 10.6	40.00 ± 2.00	73.69 ± 2.23
	48	60.00 ± 5.66	25.67 ± 0.58	41.31 ± 2.26
	72	57.33 ± 1.15	24.00 ± 3.46	53.49 ± 2.71
Chloroform	24	70.67 ± 23.12	21.67 ± 4.73	78.08 ± 7.76
	48	39.00 ± 1.41	**19.33 ± 0.58 (2.3)**	43.56 ± 0.54
	72	37.67 ± 0.58	**17.33 ± 0.58 (2.6)**	44.65 ± 2.57
Water	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100

[a] Data are presented as mean ± SD of three replicates. Values in bold characters are considered to have cytotoxic activity (IC_{50} 20 µg/ml or less)
[b] Selectivity index (SI)

Table 4.5: Cytotoxic activity (IC_{50} µg/ml) of root extracts of *A. scabra*

Extracts	Treatment duration (hour)	IC_{50}[a] (µg/ml) (SI[b])		
		MCF7	SKOV-3	MRC-5
Methanol	24	70.67 ± 5.86	56.67 ± 1.15	44.98 ± 10.59
	48	47.67 ± 4.93	34.67 ± 0.58	47.70 ± 8.68
	72	64.00 ± 0.00	34.00 ± 4.36	38.94 ± 5.02
Hexane	24	56.00 ± 2.00	33.33 ± 1.15	30.59 ± 0.36
	48	33.67 ± 5.03	28.33 ± 3.51	30.71 ± 3.15
	72	40.67 ± 1.15	28.00 ± 1.73	29.59 ± 5.98
Chloroform	24	67. 33 ± 5.03	44.00 ± 5.29	30.27 ± 0.33
	48	37.33 ± 4.16	37.00 ± 0.58	51.09 ± 10.80
	72	42.67 ± 2.31	33.67 ± 3.21	38.39 ± 5.57
Water	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100

[a]Data are presented as mean ± SD of three replicates.
[b] Selectivity index (SI)

Table 4.6: Cytotoxic activity (IC_{50} µg/ml) of pseudo stem extracts of *A. scabra*

Extracts	Treatment duration (hour)	IC_{50}[a] (µg/ml) (SI[b])		
		MCF7	SKOV-3	MRC-5
Methanol	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100
Hexane	24	>100	40.67 ± 3.51	>100
	48	84.00 ± 1.00	34.00 ± 1.73	55.86 ± 16.71
	72	67.30 ± 1.15	34.67 ± 0.58	49.74 ± 1.33
Chloroform	24	80.00 ± 15.10	56.00 ± 5.29	>100
	48	66.67 ± 1.16	30.67 ± 5.13	52.86 ± 1.75
	72	59.30 ± 1.15	33.00 ± 1.73	48.18 ± 2.34
Water	24	>100	>100	>100
	48	>100	>100	>100
	72	>100	>100	>100

[a]Data are presented as mean ± SD of three replicates.
[b] Selectivity index (SI)

4.3 Extraction, isolation and identification of chemical constituents from *A. scabra*

Bioassay-guided method was used to isolate the most effective constituent of leaf and rhizome chloroform extract in inhibiting the growth of human cancer cells. Bioassay-guided fractionation of medicinal plants is a feature of routine in the attempt to isolate bioactive components from natural sources. Most of the plant compounds that have been found to be medicinally useful and interesting tend to be secondary metabolites (Fakim, 2006).

4.3.1 Bioassay-guided fractionation of the leaf chloroform extract

The cytotoxic effect of the fractions (LC1 - LC10) derived from the chloroform extract of leaf by vacuum liquid chromatography (VLC) was evaluated in order to determine the fraction that give the highest activity. The cytotoxic activities of ten fractions were tested on MCF7, SKOV-3 and MRC-5 cell lines using MTT assay at 72 hours. The fraction LC4 was the only fraction found to be active in the cytotoxicity screening against MCF7 and SKOV-3 cell lines with IC_{50} values of 18.53 and 11.12 µg/ml, respectively (Table 4.7). Hence, fraction LC4 warranted further purification by VLC and yielded 17 sub-fractions (VLC1 - VLC17). As shown in Table 4.7, sub-fraction VLC9 showed good cytotoxicity against MCF7 and SKOV-3 cell lines (IC_{50} values of 15.53 and 10.89 µg/ml, respectively) but weak cytotoxicity profile against the MRC-5 cell line. In Table 4.7, the cytotoxicity in ascending order was leaf chloroform extract < LC4 < VLC9. This may be due to the cytotoxic compounds present in VLC9 after the purification of leaf chloroform extract and LC4 *via* VLC. Thus, the active ingredients in VLC9 may lead to valuable compounds that have the ability to kill cancer cells but not toxic against normal MRC-5 cells. The incubation time of 72 hours was selected to allow sufficient time for bioactive compounds to produce effect without

having to re-feed the cells. Besides, insufficient incubation time may give a false negative indication (Riddell *et al.*, 1986; Carmichael *et al*., 1987).

Table 4.7: Cytotoxic activity (IC_{50} µg/ml) of fractions and sub-fraction obtained from leaf chloroform extract

Fraction (LC) / Sub-fraction (VLC)	IC_{50}[a](µg/ml)		
	MCF7	**SKOV- 3**	**MRC-5**
LC1	50.00 ± 5.00	92.30 ± 4.78	>100
LC2	42.20 ± 4.48	56.00 ± 6.60	>100
LC3	33.40 ± 4.05	40.50 ± 2.56	>100
LC4	**18.53 ± 1.02**	**11.12 ± 0.24**	>100
LC5	51.18 ± 4.56	39.18 ± 1.34	>100
LC6	>100	>100	>100
LC7	>100	>100	>100
LC8	>100	>100	>100
LC9	>100	>100	>100
LC10	>100	>100	>100
VLC9	**15.53 ± 0.50**	**10.89 ± 0.64**	>100
Chloroform	25.00 ± 0.00	14.67 ± 0.58	32.90 ± 0.76

[a]Data are presented as mean ± SD from three independent experiments triplicate for each. Values in bold characters are considered to have cytotoxic activity (IC_{50} 20 µg/ml or less)

4.3.2 Bioassay-guided fractionation of the rhizome chloroform extract

The cytotoxic effect of the fractions (RC1 - RC18) derived from the chloroform extract of rhizome by VLC was evaluated in order to determine the fraction that give the highest activity. The cytotoxic activities of 18 fractions were tested on SKOV-3 and MRC-5 cell lines using MTT assay at 72 hours. Table 4.8 shows the IC_{50} values of the 18 fractions from the chloroform extract of rhizome. Fraction RC5 was the only fraction which exhibited remarkable cytotoxicity (IC_{50} value of 2.84 µg/ml) and showed high selectivity (SI value of 14.15) against the SKOV-3 cells, compared to the rhizome chloroform extract. This showed the improvement of cytotoxicity and selectivity after the purification procedure. Besides that, variations in the cytotoxic activities among the chloroform extract and RC5 against SKOV-3 could be attributed to the distribution of the several identified cytotoxic compounds in different fractions/sub-fractions (Cheng *et al*., 2006).

Table 4.8: Cytotoxic activity (IC_{50} µg/ml) of fractions obtained from the rhizome chloroform extract

Fraction	IC_{50}(µg/ml) (SI[b])	
	SKOV-3	**MRC-5**
RC1	94.56 ± 4.32	95.70 ± 3.00
RC2	88.90 ± 4.78	66.00 ± 5. 66
RC3	25.49 ± 2.56	50.80 ± 1.24
RC4	23.35 ± 2.24	71.00 ± 3.40
RC5	**2.84 ± 0.60 (14.15)**	40.20 ± 1.50
RC6	39.90 ± 1.18	>100
RC7	32.63 ± 6.78	>100
RC8	50.24 ± 7.78	>100
RC9	64.50 ± 1.40	>100
RC10	66.82 ± 2.26	>100
RC11	51.30 ± 3.12	>100
RC12	45.70 ± 4.00	>100
RC13	21.60 ± 10.80	>100
RC14	29.70 ± 2.50	>100
RC15	25.33 ± 3.56	>100
RC16	24.75 ± 2.85	>100
RC17	45.08 ± 3.45	>100
RC18	43.89 ± 4.76	>100
Chloroform	17.33 ± 0.58 (2.6)	44.65 ± 2.57

[a]Data are presented as mean ± SD from three independent experiments triplicate for each. Values in bold characters are considered to have cytotoxic activity (IC_{50} 20 µg/ml or less); [b]Selectivity Index (SI)

4.3.3 GC-MS analysis of cytotoxic extracts, fractions and sub-fraction

The cytotoxic hexane leaf extracts were analysed using GC-MS (Appendix E) in the present study. The identified compounds are methyl palmitate 61.02 % and methyl stearate 24.91 % which comprise of 85.93 % of the total detected compounds in the hexane extract. Methyl palmitate EI-MS m/z (%): 270 $[M]^+$ (2), 239 (2), 227 (5), 213 (2), 199 (4), 185 (5), 171 (8), 157 (2), 143 (18), 129 (8), 115 (4), 107 (1), 97 (8), 87 (70), 74 (100), 65 (1), 55 (30). Methyl stearate EI-MS m/z (%): 298 $[M]^+$ (6), 255 (8), 241 (2), 213 (4), 199 (8), 185 (6), 143 (26), 129 (20), 111 (4), 97 (10), 87 (70), 74 (100), 55(56). All the compounds were identified by GC-MS analysis as well as comparison of its mass spectral data with reported data (Sri Nurestri *et al*., 2009). Structures of the compounds are shown in Figure 4.1. Meanwhile, many unidentified compounds were present in the leaf chloroform extract, rhizome chloroform extract, fraction LC4, RC5 and sub-fraction VLC9 (Appendix F - J). Due to an insufficient amount of the above extracts, further studies on the isolation and chemical characterization of the pure bioactive compounds were not carried out.

Previous report by Sri Nurestri *et al*. (2009) suggested that methyl esters might exert cytotoxic effect against normal MRC-5 cells but not on KB, MCF7 and HCT116 cells. This finding supports the data from the present study on the cytotoxicity of *A. scabra* extracts against MRC-5 cells. As shown in Table 4.3, the hexane leaf extract showed cytotoxic activity on MRC-5 cells at 48 and 72 hours with IC_{50} values of 14.63 and 15.90 µg/ml, respectively. This can be due to the presence of methyl palmitate and methyl stearate in the extract. Furthermore, this finding on cytotoxicity of methyl esters is supported by Takeara *et al*. (2008) which reported that methyl palmitate showed cytotoxic effect on T-cell leukemia cell line (Molt-4) with IC_{50} value of 2.28 µg/ml while methyl stearate was cytotoxic to acute promyeloblastic leukemia cell line (HL-60) and Molt-4 cell line with IC_{50} values of 3.08 and 4.65 µg/ml, respectively.

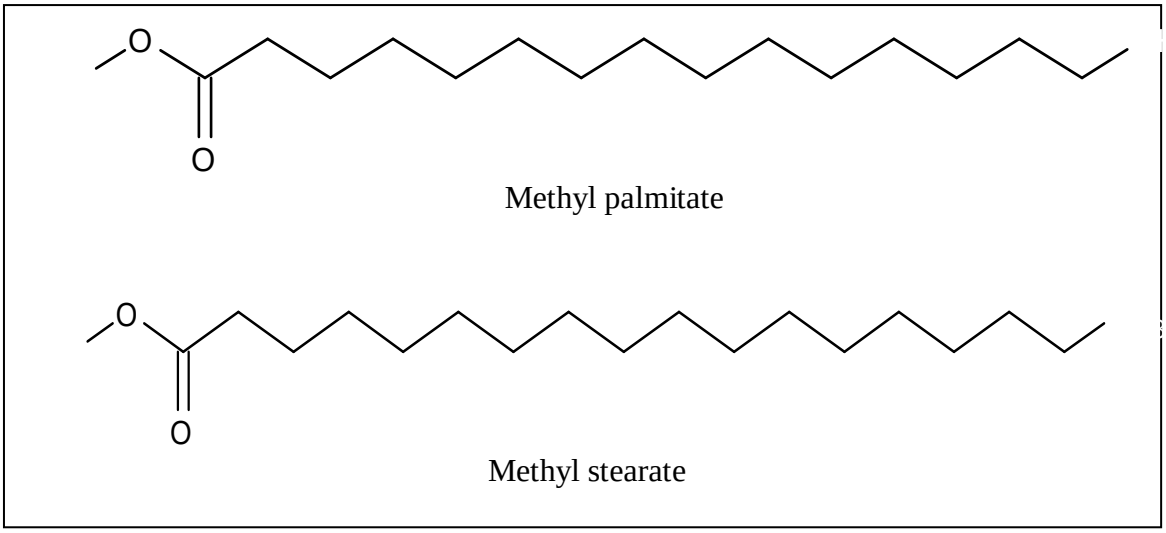


Figure 4.1: Structures of methyl palmitate and methyl stearate

4.4 Detection of apoptosis

In the present study, active extracts and fractions of *A. scabra* studied with IC_{50} value equal to or less than 20 µg/ml are considered cytotoxically active and were selected for further assessment of their apoptotic activities by various morphological and biochemical methods. Cytotoxically active extracts from leaves, rhizomes, fractions LC4, RC5 and sub-fraction VLC9 were analysed for their potential apoptotic activity on MCF7 and SKOV-3 cell lines using phase-contrast and fluorescence microscopy method and detection of DNA fragmentation by agarose electrophoresis.

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

Phase-contrast microscopy revealed the early stages of apoptosis which are characterized by the shrinkage of cells, blistering, and membrane blebbing (Xu *et al.*, 2004). The results from the present study (Figure 4.2, 4.3 and 4.4) showed that there were obvious morphological changes in MCF7 and SKOV-3 cells after treatment with the cytotoxic extracts, fractions and sub-fraction which were indicative of cell apoptosis. The untreated control MCF7 and SKOV-3 cells maintained their original morphology which are cuboids and in polygonal shapes, and were adherent to the plates. The MCF7 cells were treated with hexane and chloroform extracts of leaves (Figure 4.2) while SKOV-3 cells were treated with leaf hexane, leaf chloroform and rhizome chloroform extracts (Figure 4.3) for 24, 48 and (or) 72 hours according to the IC_{50} values (Table 4.3 and 4.4). Figure 4.4 shows MCF7 and SKOV-3 cells treated with fraction LC4, sub-fraction VLC9 and fraction RC5 at 72 hours according to the IC_{50} values in Tables 4.7 and 4.8. The most recognizable morphological features of apoptotic cells observed in this study were shrinkage of cells due to cytoplasmic condensation, rounding up of cells, bleb formation, chromatin condensation and apoptotic bodies formation. Other morphological change observed in apoptotic cells was the rounded up

cells losing contact with neighbouring cells and caused some sensitive cells to detach from the surface of the well plates. These morphological observations of apoptotic cells were in agreement with previous report (Ramasamy *et al.*, 2012). Furthermore, cell shrinkage happens only in apoptotic cell death. Cells intracellular concentration of monovalen ions (K^+ and Na^+) was able to inhibit activation of cell death cascade. As a result, the ions will be expelled out during apoptotic event and this will cause cell shrinkage. Compare to necrosis, ionic homeostatis occurs due to the drastically decrease of energy level. This event will cause the increase of cell volume and subsequently, swelling and total cell lysis will be observed (Bortner and Cidlowski, 1998; Hong *et al.*, 2002).

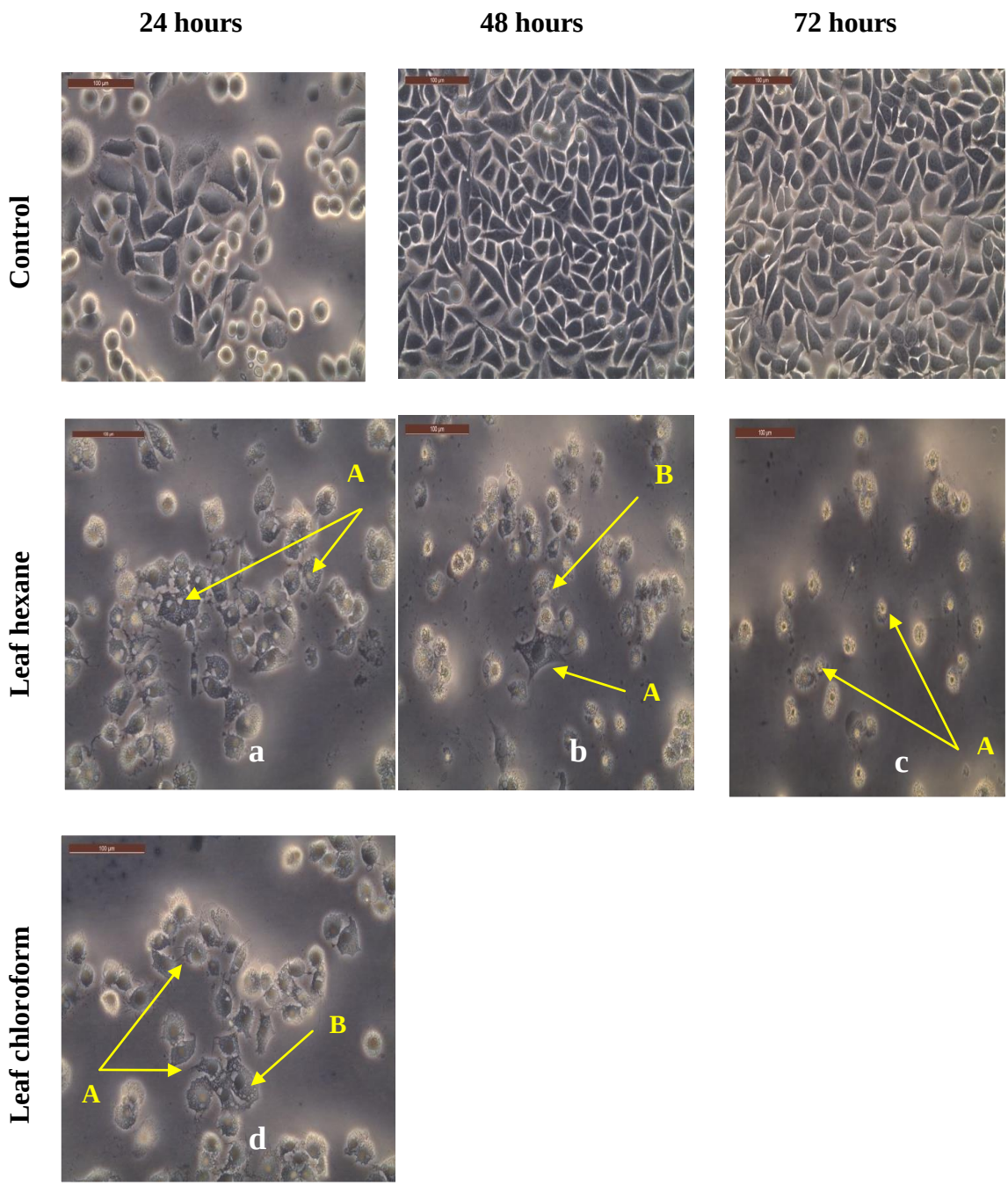


Figure 4.2: Morphological observation of MCF7 cells treated with the cytotoxic leaf hexane and chloroform extracts under phase-contrast inverted microscope (magnification 200x). Arrows indicate (A) cell shrinkage and (B) membrane blebbing as evidence of apoptosis. Note that the cells were treated with the following concentrations of extracts: a = 19.3 µg/ml, b= 15.30 µg/ml, c = 16.33 µg/ml and d = 18.80 µg/ml. Figures shown were obtained from at least three independent experiments with similar parameter.

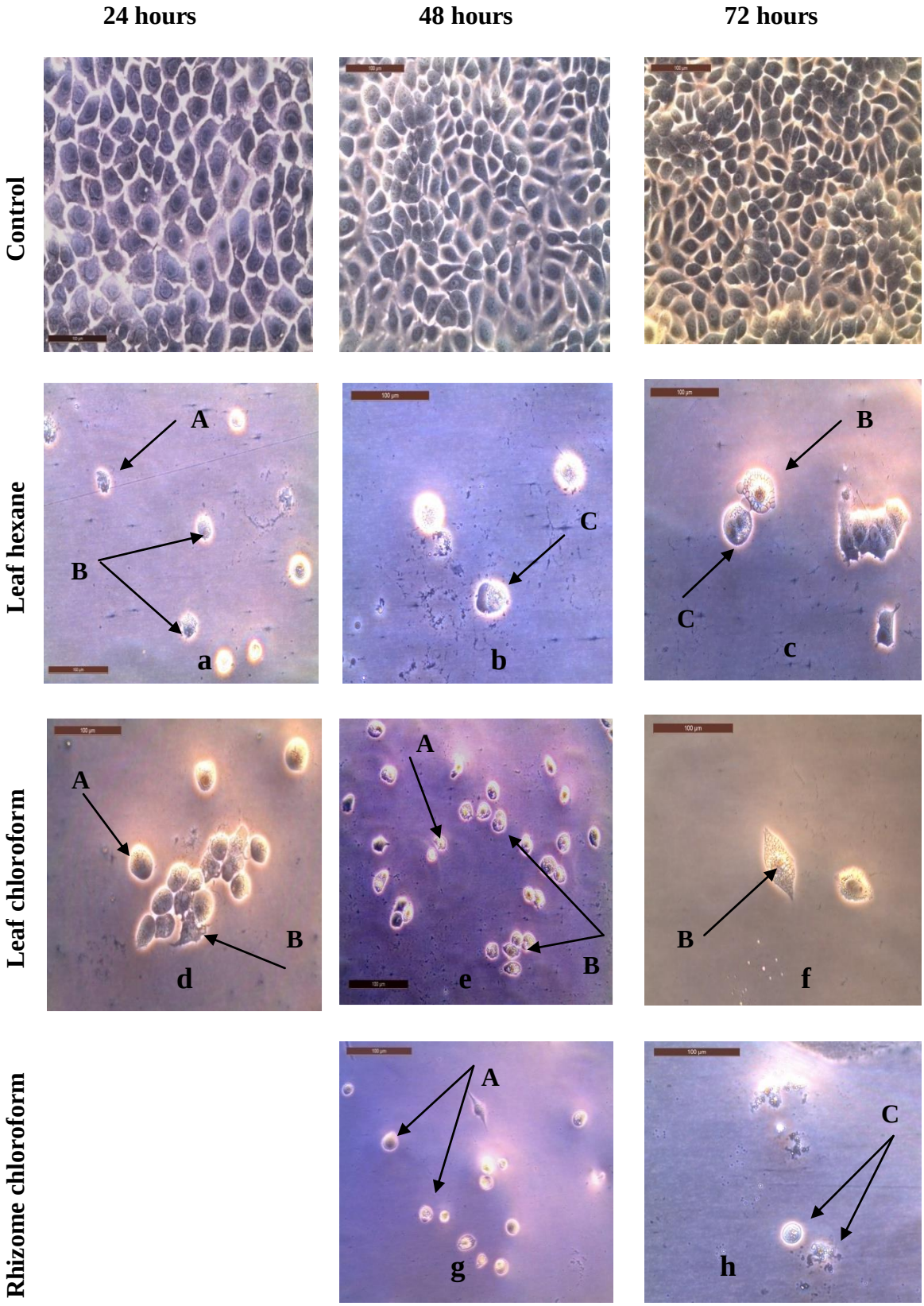

24 hours
48 hours
72 hours
Control
Leaf hexane
Leaf chloroform
Rhizome chloroform
A
B
a
C
b
B
C
c
A
B
d
A
B
e
B
f
A
g
C
h

Figure 4.3: Morphological observation of SKOV-3 cells treated with the cytotoxic leaf hexane, leaf chloroform and rhizome chloroform extracts under phase-contrast inverted microscope (magnification 200x). Arrows indicate (A) cell shrinkage; (B) membrane blebbing and (C) apoptotic bodies as evidence of apoptosis. Note that the cells were treated with the following concentrations: a = 18.00 µg/ml, b = 6.00 µg/ml, c = 4.93 µg/ml, d = 20.00 µg/ml, e = 14.33 µg/ml, f = 14.67 µg/ml, g = 19.33 µg/ml and h = 17.33 µg/ml. Figures shown were obtained from at least three independent experiments with similar parameter.

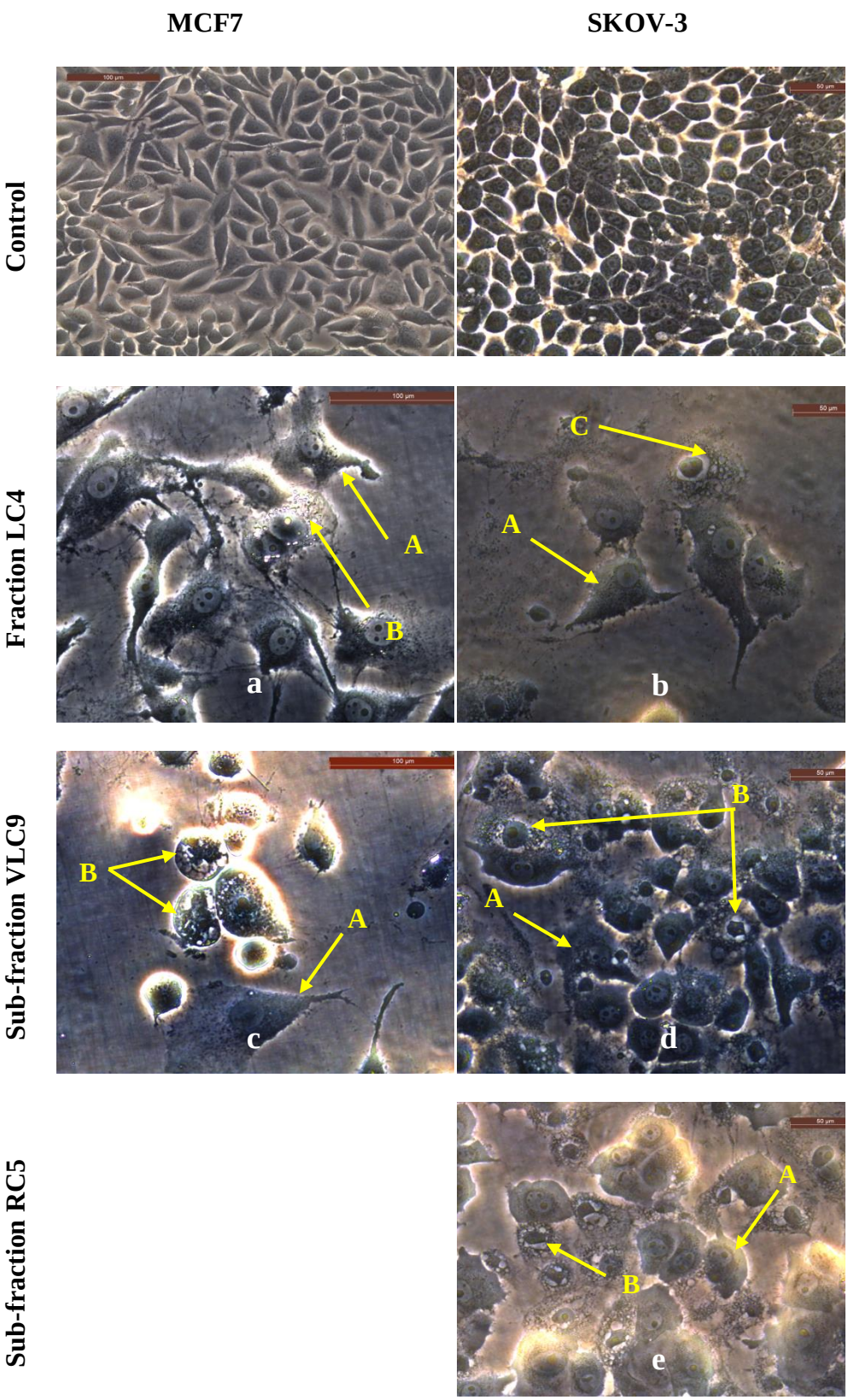

MCF7
SKOV-3
Control
Fraction LC4
Sub-fraction VLC9
Sub-fraction RC5
A
B
a
C
A
b
B
A
c
B
A
d
A
B
e

Figure 4.4: Morphological observation of MCF7 and SKOV-3 cells treated with the cytotoxic fractions LC4, VLC9 and RC5 under phase-contrast inverted microscope (magnification 200x). Arrows indicate (A) cell shrinkage; (B) membrane blebbing and (C) apoptotic bodies as evidence of apoptosis. Note that the cells were treated with the following concentrations: a = 18.53 µg/ml, b = 11.12 µg/ml, c = 15.53 µg/ml, d = 10.89 µg/ml and e = 2.84 µg/ml. Figures shown were obtained from at least three independent experiments with similar parameter.

4.4.2 DNA fragmentation analysis by agarose electrophoresis

DNA fragmentation is a biochemical hallmark of apoptotic cell death. To elucidate whether the active extracts and fractions decrease cell survival by the induction of DNA fragmentation, genomic DNA isolated from MCF7 and SKOV-3 cells were exposed according to the IC_{50} value concentration, electrophoresed and photographed as shown in Figures 4.5 and 4.6. Typical DNA ladder formation can be seen clearly in the MCF7 and SKOV-3 cells treated with doxorubicin whereas DNA from untreated MCF7 and SKOV-3 cells did not show any fragmentation or smearing. In MCF7 cells-treated with cytotoxic active extracts and sub-fractions (Figure 4.5), the formation of DNA ladder was observed less clearly as there were interspersing smear in the lanes. This pattern can be clearly observed in MCF7 cells-treated with hexane and chloroform extract of leaf, fraction LC4 and sub-fraction VLC9 at concentrations of 19.30, 18.80, 18.53 and 15.53 µg/ml, respectively. The smearing could be due to some post-apoptotic necrosis cells (Ramasamy *et al*., 2012).

For the SKOV-3 cells (Figure 4.6), the ladder-like appearance of DNA observed mildly in the cells treated with hexane and chloroform extract of leaf at concentrations of 18.0 and 20.0 µg/ml, respectively. SKOV-3 cells which are treated with chloroform extract of rhizome, fraction LC4, sub-fraction VLC9 and fraction RC5 at concentrations of 21.67, 11.12, 10.89 and 2.84 µg/ml, respectively did not show any DNA laddering or even smearing effect. This can be due to the concentration of the particular extract and sub-fractions which were used to treat the cells were low since at lower doses of treatment, only high molecular weight intact DNA was observed (Yew *et al*., 2009). Besides that, in some cases, DNA fragmentation appears to be delayed, partial, or absent in cells which otherwise meet the morphological criteria for apoptosis and maybe show more limited DNA degradation with the formation of 300- or 50-kb fragments (Zakeri *et al.*, 1993).

The large band present at the top of the gel as observed in SKOV-3 cells treated with fraction LC4 and RC5 may represent large semi-fragmented pieces of DNA and indicates incomplete apoptotic fragmentation in the sample material (Matassov *et al*., 2004).

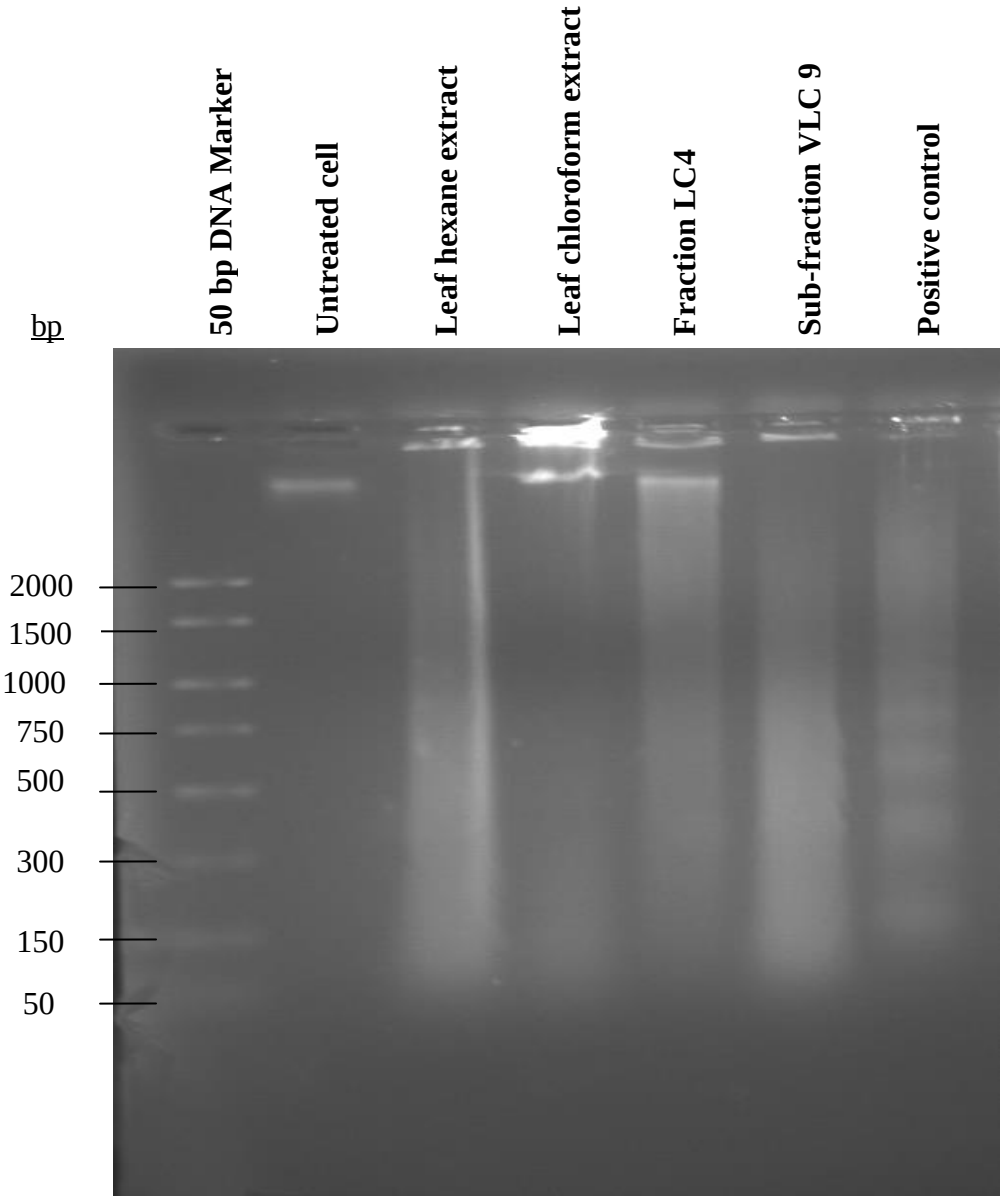


Figure 4.5: DNA fragmentation of MCF7 cells after treated with cytotoxic extracts and sub-fractions for 24 hours

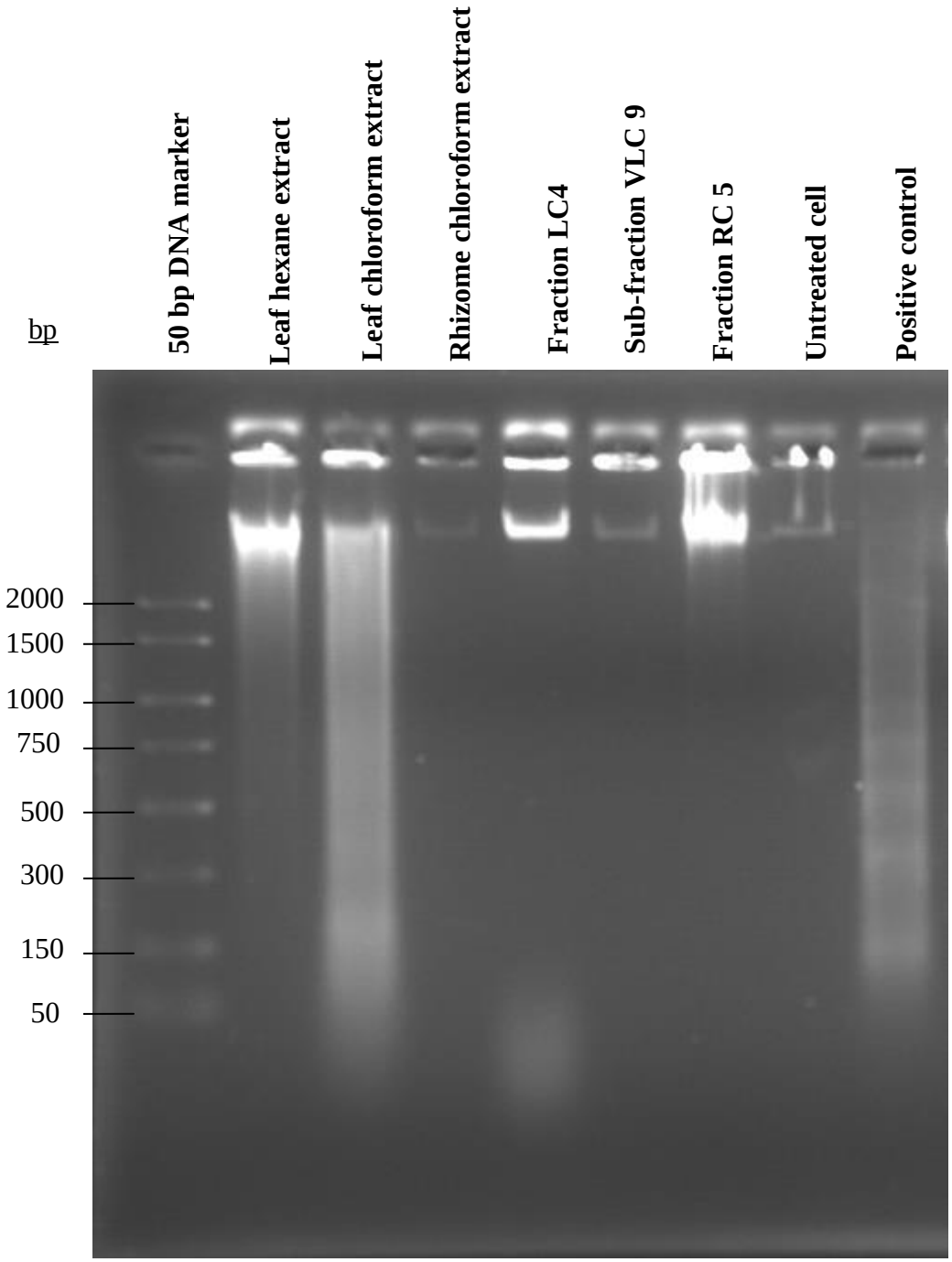
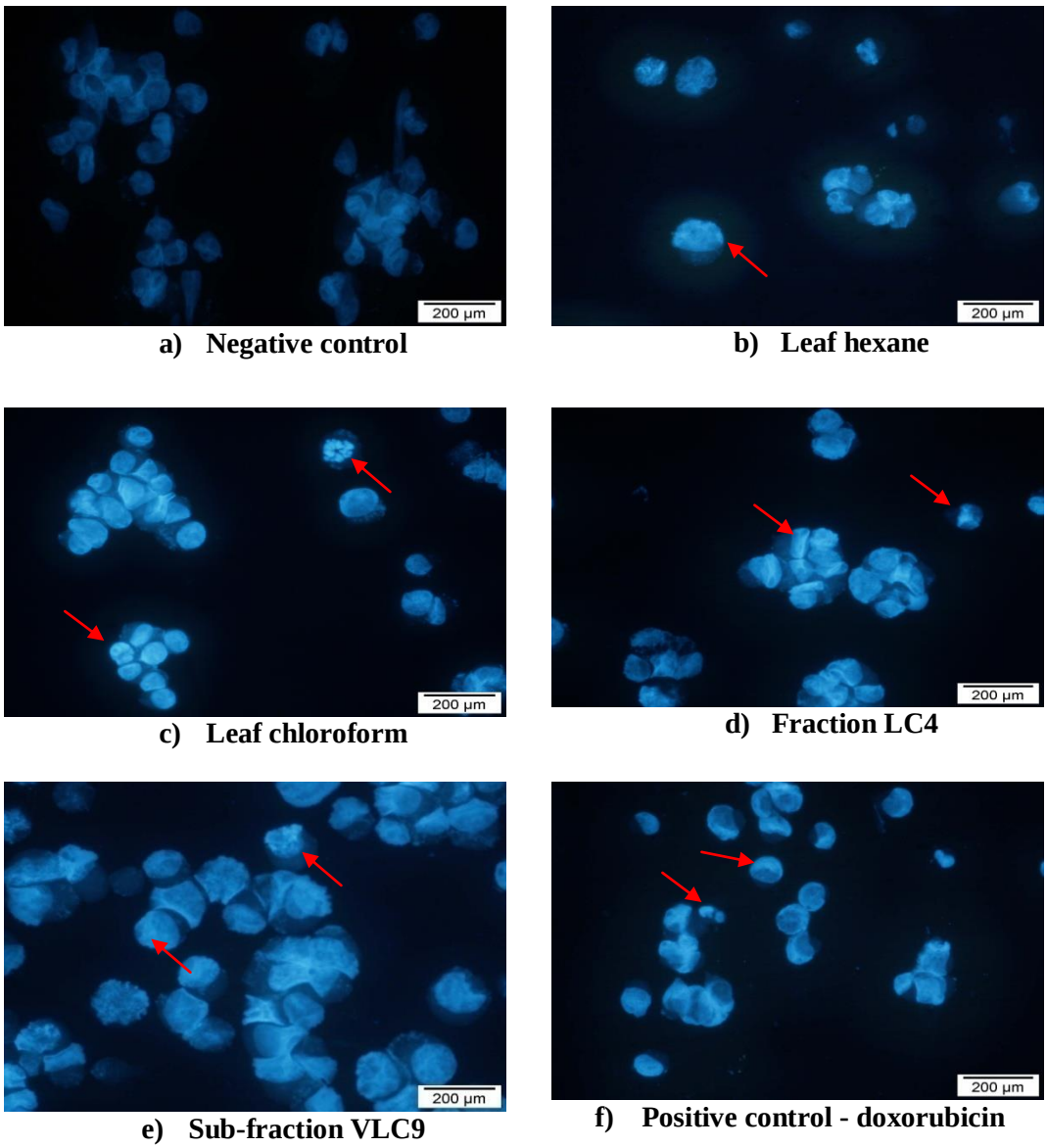


Figure 4.6: DNA fragmentation of SKOV-3 cells after treated with cytotoxic active extracts and sub-fractions for 24 hours.

4.4.3 Morphological detection of apoptosis using DAPI nuclear stain

DAPI is a fluorescent stain that allows examination of nuclei in a fluorescence microscope for morphologic assessment of changes during apoptosis (Kapuscinski, 1995). Apoptosis is initially characterized by morphological features, such as chromatin condensation, nuclear fragmentation, and membrane blebbing (Kerr *et al.*, 1972). To gain an insight on the effect of cytotoxic extracts, fractions, sub-fraction and doxorubicin on nuclear alterations, cells were stained with DAPI. Figure 4.7 and 4.8 show the apoptotic morphological characteristics, as visualized by DAPI staining, of MCF7 and SKOV-3 cells treated (for 24 hours) with cytotoxic extracts, fractions, sub-fraction and doxorubicin (positive control) according to the IC_{50} values. In the control-untreated group (Figure 4.7 (a) and 4.8(a), the cells were rounded in shape and the large nuclei were homogenously stained with a less bright blue color. This is because when healthy cells are exposed to DAPI, staining is restricted to chromatin. Treated MCF7 and SKOV-3 cells displayed bright blue fluorescence with higher intensity than untreated cells due to the highly condensed chromatin.

Besides that, signs of nuclear shrinkage and chromatin condensation which are hallmark of apoptosis were also observed as shown in Figure 4.7 and 4.8. Apoptosis is also characterized by the condensation of nuclear chromatin followed by the eventual breakup of the chromatin leading to nuclear fragmentation (Willingham, 1999). This indicated that MCF7 and SKOV-3 cells underwent apoptosis when treated with the cytotoxic extracts, fractions and sub-fraction. This nuclear morphology changes were in agreement with previous report (Hsiung and Kadir, 2011).



a) Negative control

b) Leaf hexane

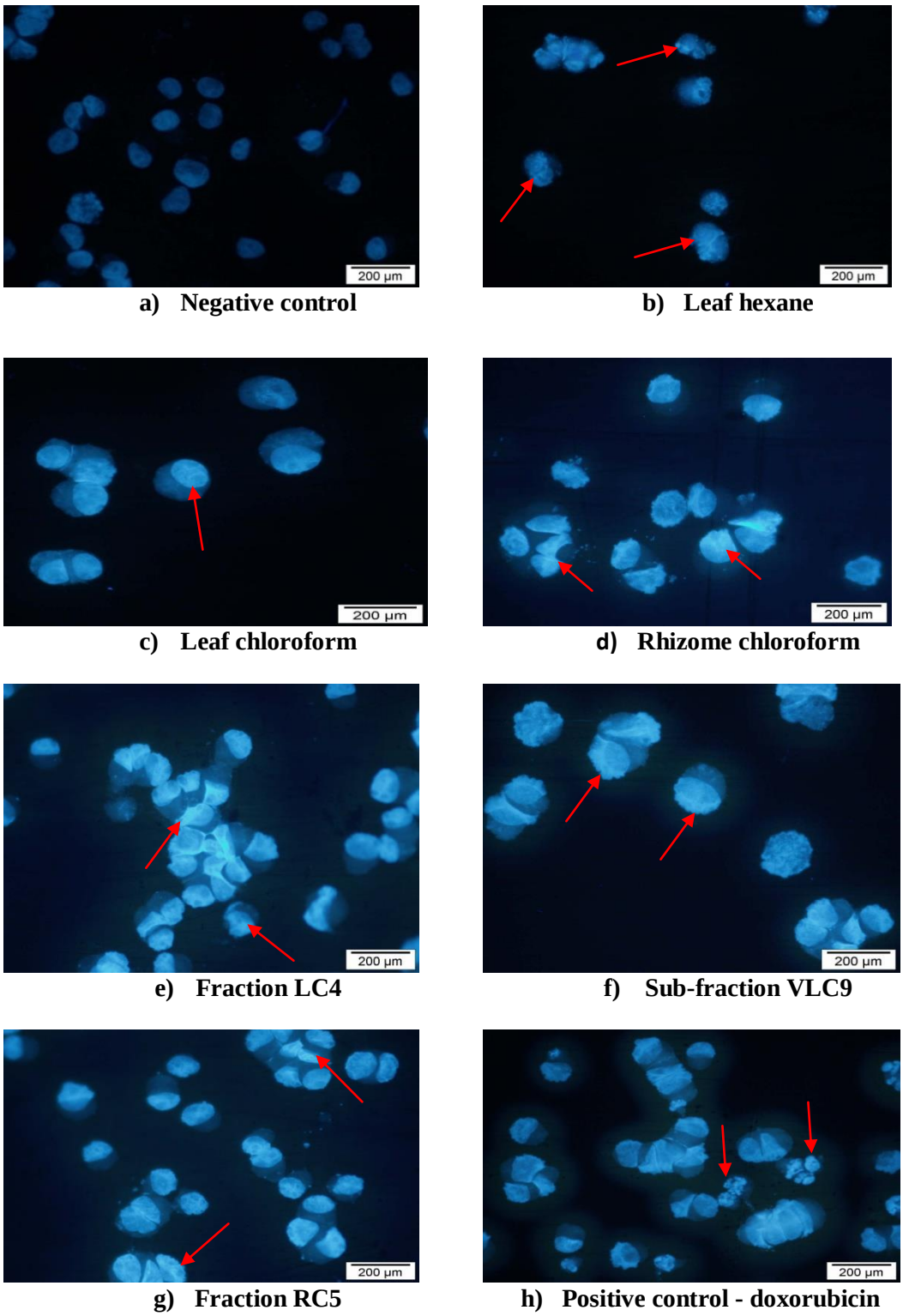
c) Leaf chloroform

d) Fraction LC4

e) Sub-fraction VLC9

f) Positive control - doxorubicin

Figure 4.7: Morphological observation with DAPI staining by fluorescence microscope for MCF7 cells at 24 hours (magnification 400x). Arrows indicate signs of nuclear shrinkage and chromatin condensation. DNA samples in the untreated cells were homogenously stained and less intense compared to those in treated cells.



a) Negative control

b) Leaf hexane

c) Leaf chloroform

d) Rhizome chloroform

e) Fraction LC4

f) Sub-fraction VLC9

g) Fraction RC5

h) Positive control - doxorubicin

Figure 4.8: Morphological observation with DAPI staining by fluorescence microscope for SKOV-3 cells at 24 hours (magnification 400x). Arrows indicate signs of nuclear shrinkage and chromatin condensation. The untreated cells were rounded in shape and the large nuclei were homogenously stained with less bright blue color.