CHAPTER 3

RESULTS

3.1 Content of total phenolics and total flavonoid in crude acetone extract and ethyl acetate-soluble fraction of dried longan pulp and seed.

The total phenolic content in dried longan extracts was measured by Folin-Ciocalteu method, expressed as gallic acid equivalent (GAE). Content of total phenolics in the crude acetone extract of pulp and seed were 7.25 ± 0.25 and 387.60 ± 6.50 mg GAE/g extract, respectively. The total phenolic content in the ethyl acetate-soluble fraction of pulp (EFLP) and seed (EFLS) were 145.75 ± 11.86 and 768.10 ± 9.04 mg GAE/g extract, respectively.

The total flavonoid content in the dried longan extracts was measured by the aluminum chloride colorimetric assay, expressed as catechin equivalent (CE). Content of total flavonoids in the crude acetone extract of pulp and seed were < 1.00 and 47.71 ± 4.91 mg CE/g extract, respectively. The total flavonoid content in EFLP and EFLS were 20.30 ± 2.83 and 131.40 ± 6.63 mg CE/g extract , respectively. Content of total phenolics and total flavonoids in dried longan extract are shown in Table 3.1

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Table 3.1 Content of total phenolics and total flavonoids in the dried longan extract

Extract	Part of fruit	Total phenolics* mg GAE/g extract	Total flavonoids* mg CE/g extract
Crude acetone extract	pulp	7.25 ± 0.25	<1.00
	seed	387.60 ± 6.50	47.71 ± 4.91
Ethyl acetate-soluble fraction	pulp	145.75 ± 11.86	20.30 ± 2.83
	seed	768.10 ± 9.04	131.40 ± 6.63

* Results were expressed as mean ± SD, n = 3

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3.2 Free radical scavenging activity of dried longan extracts

Dried longan extracts were evaluated for their abilities to neutralize the stable free radicals such as DPPH radicals. The results expressed as SC_{50} values (the concentration that scavenges 50% of the DPPH radical). The scavenging activity of seed extract is more higher than pulp extract and the scavenging effect of the ethyl acetate-soluble fraction (both pulp and seed) on DPPH radical is superior to crude acetone extract. The highest scavenging activity was found in the ethyl acetate-soluble fraction of dried seed (SC_{50} = 30.83 ± 0.9 µg/ml). Table 3.2 show free radical scavenging activities of dried longan extracts.

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Table 3.2 Free radical scavenging activities of dried longan extracts and ascorbic acid

Extracts	DPPH assay SC_{50} (μg/ml)
Crude acetone extract of pulp	>1000
Crude acetone extract of seed	74.22 ± 2.2
EFLP	517.88 ± 3.4
EFLS	30.83 ± 0.9
Ascorbic acid	30.35 ± 2.8

SC_{50} = The concentration of extract that scavenges 50% of DPPH radical.

EFLP = The ethyl acetate-soluble fraction of dried longan pulp.

EFLS = The ethyl acetate-soluble fraction of dried longan seed.

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3.3 Effect of dried longan extract on the growth of colon cancer cells

The effect of dried longan extract on the proliferation of colon cancer cells, HCT-15 and RKO cells were evaluated. Both cell lines were treated with different concentrations of dried longan extract such as crude acetone extract of dried pulp or seed, EFLP and EFLS. EFLS induced a concentration-dependent reduction in the proliferation rate of both HCT-15 and RKO cells (Figure 3.1). The dosages required for 50% inhibition (IC_{50}) of HCT-15 and RKO at 48 h were 282 and 227 µg/ml, respectively. In addition, EFLS were also investigated for its cytotoxic property to NIH3T3 cells (a normal mouse fibroblast). IC_{50} of EFLS for NIH3T3 at 48 h was > 500 µg/ml. EFLP and the crude acetone extract of seed had less cytotoxic to HCT-15 and RKO cells (Figures 3.2 and 3.3) whereas the crude acetone extract of pulp did not have cytotoxic effect to both kind of cells (Figure 3.4).

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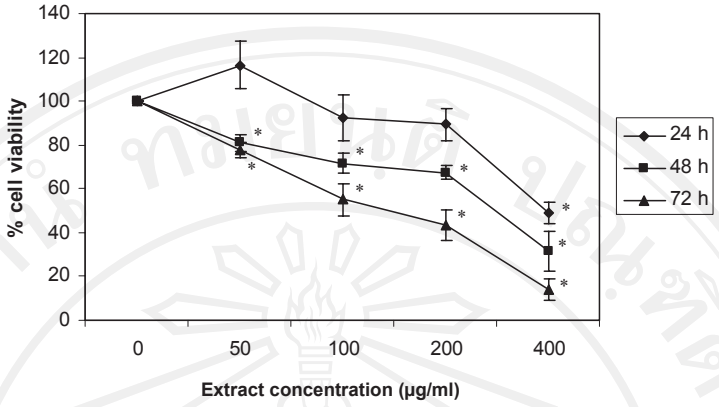

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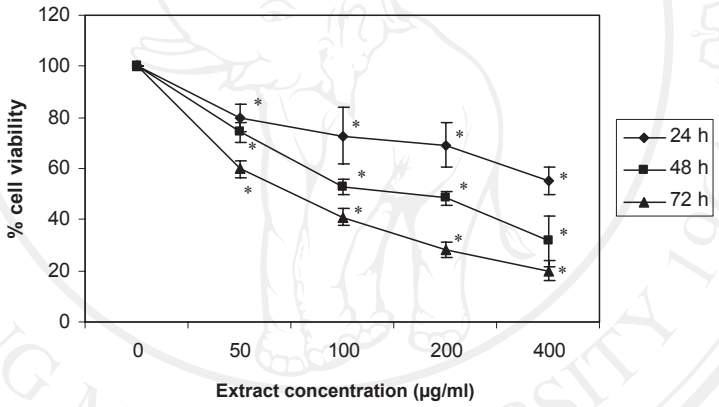

Figure 3.1 Effect of EFLS on the proliferation of colon cancer cell lines, HCT-15 (A) and RKO (B). Colon cancer cell lines (1 x 10^4 cells/well) were treated with various concentrations of EFLS (50-400 µg/ml) for 24, 48 and 72 h, respectively. Proliferation was evaluated by MTT assay. Without any extract, the percentage of cell viability value is 100. The results were expressed as the percentages of proliferation compared to the control wells (mean ± SD of three independent experiment, *, $p < 0.05$, significantly different from the control).

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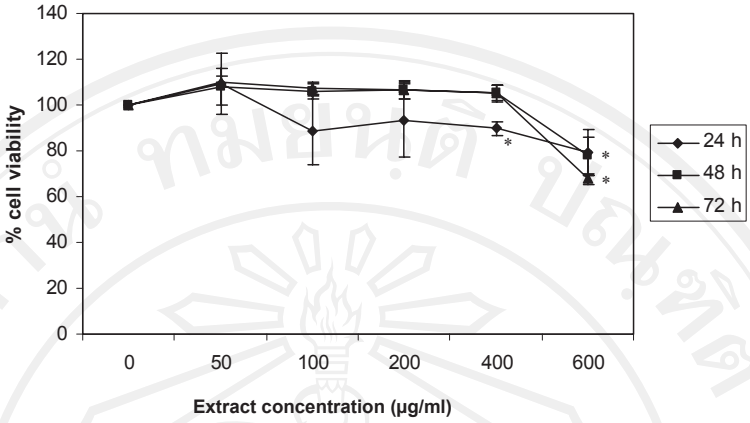

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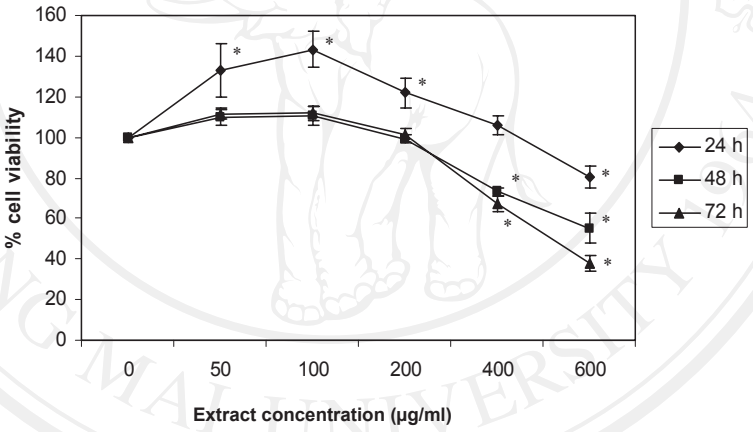

Figure 3.2 Effect of EFLP on the proliferation of colon cancer cell lines, HCT-15 (A) and RKO (B). Colon cancer cell lines (1 x 10^4 cells/well) were treated with various concentrations of EFLP (50-600 µg/ml) for 24, 48 and 72 h, respectively. Proliferation was evaluated by MTT assay. Without any extract, the percentage of cell viability value is 100. The results were expressed as the percentages of proliferation compared to the control wells (mean ± SD of three independent experiment, *, $p < 0.05$, significantly different from the control).

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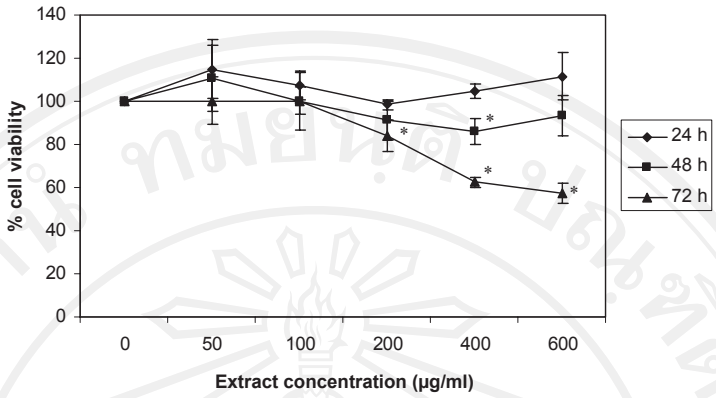

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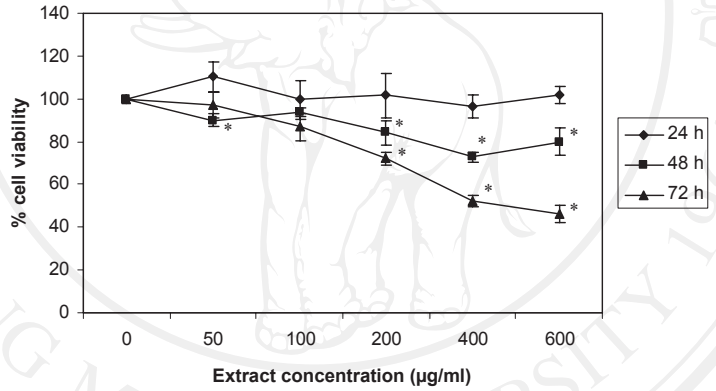

Figure 3.3 Effect of crude acetone extract of dried longan seed on the proliferation of colon cancer cell lines, HCT-15 (A) and RKO (B). Colon cancer cell lines (1 x 10^4 cells/well) were treated with various concentrations of crude acetone extract of dried seed (50-600 µg/ml) for 24, 48 and 72 h, respectively. Proliferation was evaluated by MTT assay. Without any extract, the percentage of cell viability value is 100. The results were expressed as the percentages of proliferation compared to the control wells (mean ± SD of three independent experiment, *, $p < 0.05$, significantly different from the control).

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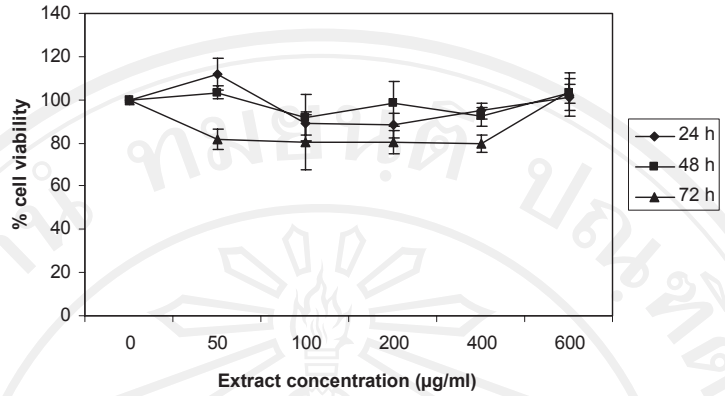

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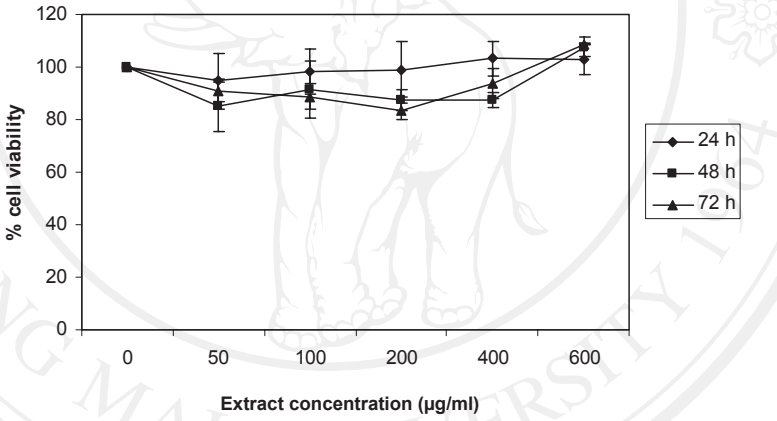

Figure 3.4 Effect of crude acetone extract of dried longan pulp on the proliferation of colon cancer cell lines, HCT-15 (A) and RKO (B). Colon cancer cell lines (1 x 10^4 cells/well) were treated with various concentrations of crude acetone extract of dried pulp (50-600 µg/ml) for 24, 48 and 72 h, respectively. Proliferation was evaluated by MTT assay. Without any extract, the percentage of cell viability value is 100. The results were expressed as the percentages of proliferation compared to the control wells (mean ± SD of three independent experiment, *, $p < 0.05$, significantly different from the control).

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3.4 DNA fragmentation

DNA ladder formation was observed in HCT-15 and RKO cells treated with EFLS or EFLP for 48 h. DNA ladders were observed in both kind of cells after treatment with EFLS for 48 h (Figure 3.5). At a concentration of 750 µg/ml of EFLP, DNA fragmentation was observed in RKO cells but not appeared in HCT-15 cells (Figure 3.6). Effect of EFLS on growth inhibition and DNA ladder formation observed in this study indicated the induction of apoptosis in both HCT-15 and RKO cells.

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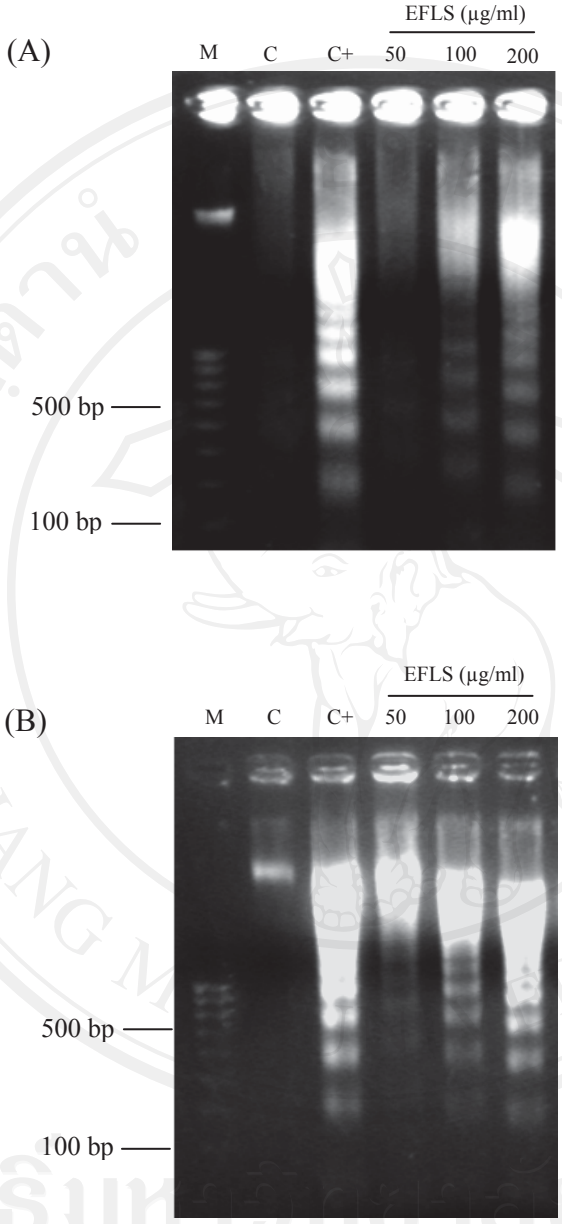

Figure 3.5 DNA fragmentation in HCT-15 (A) and RKO (B) cells treated with EFLS. Cells (1 x 10^6) were cultured in the absence or presence of 50-200 µg/ml of extract for 48 h. Total DNA was then extracted from the cells and analyzed by 2% agarose gel electrophoresis. M, DNA size markers (100 bp ladders). C+, positive control (camptothecin).

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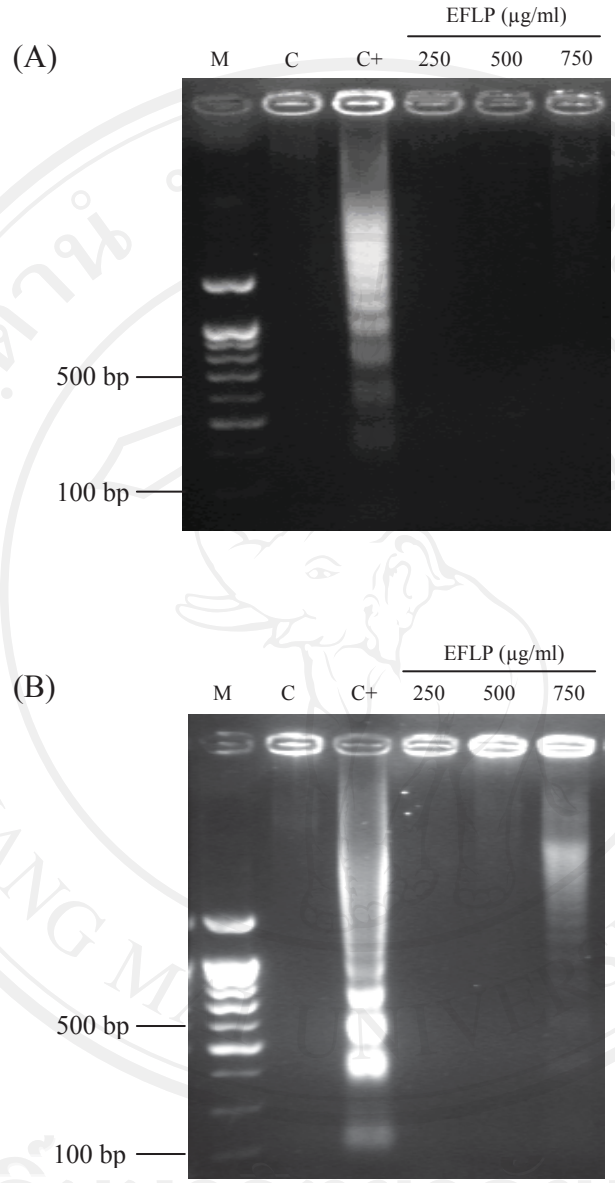

Figure 3.6 DNA fragmentation in HCT-15 (A) and RKO (B) cells treated with EFLP. Cells (1 x 10^6) were cultured in the absence or presence of 250-750 µg/ml of extract for 48 h. Total DNA was then extracted from the cells and analyzed by 2% agarose gel electrophoresis. M, DNA size markers (100 bp ladders). C+, positive control (camptothecin).

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3.5 Caspase-3 activity in HCT-15 and RKO cells

Caspase-3 activity of HCT-15 and RKO cells was increased in a dose-dependent manner after treatment with EFLS for 48 h (Figure 3.7).

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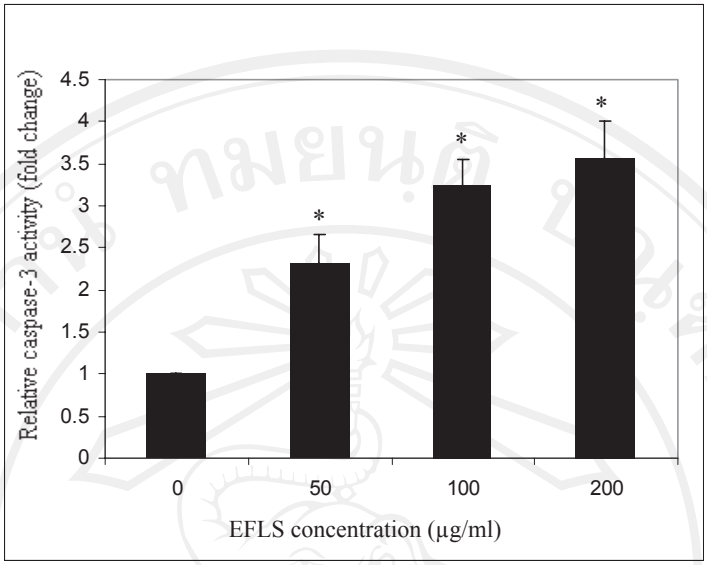

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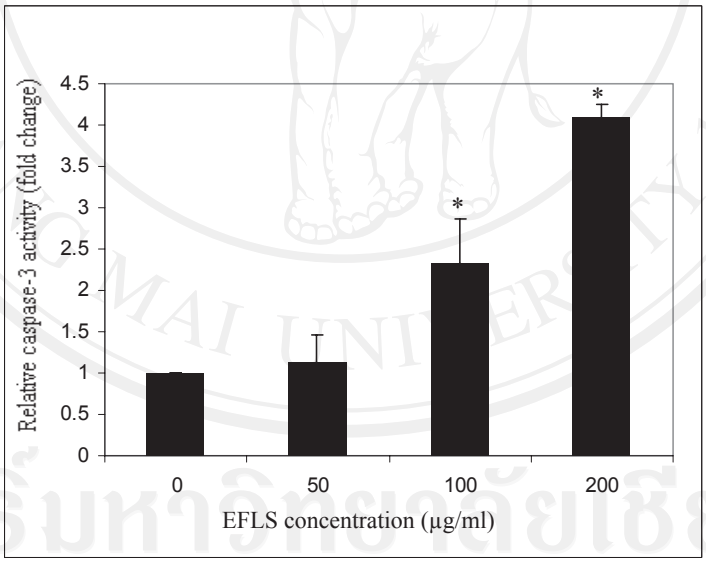

Figure 3.7 Caspase-3 activity in HCT-15 (A) and RKO (B). Cells were incubated with EFLS for 48 h. Data represented as the mean ± SD of three independent experiments, * = $p < 0.05$, significantly different from control.

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3.6 The expression of Akt, p-Akt, Bcl-2 and Bax in HCT-15 and RKO cells

Levels of Akt, p-Akt, Bcl-2 and Bax proteins in HCT-15 and RKO cells were detected by Western blot analysis (Figure 3.8). When HCT-15 and RKO cells were exposed to 50, 100 μg/ml of EFLS for 48 h, the level of Bax protein increased, but expression of p-Akt protein was decreased. The expression of Bcl-2 was decreased in HCT-15 cells but was not changed in RKO cells. However, the level of Akt protein was not changed in both HCT-15 and RKO cells.

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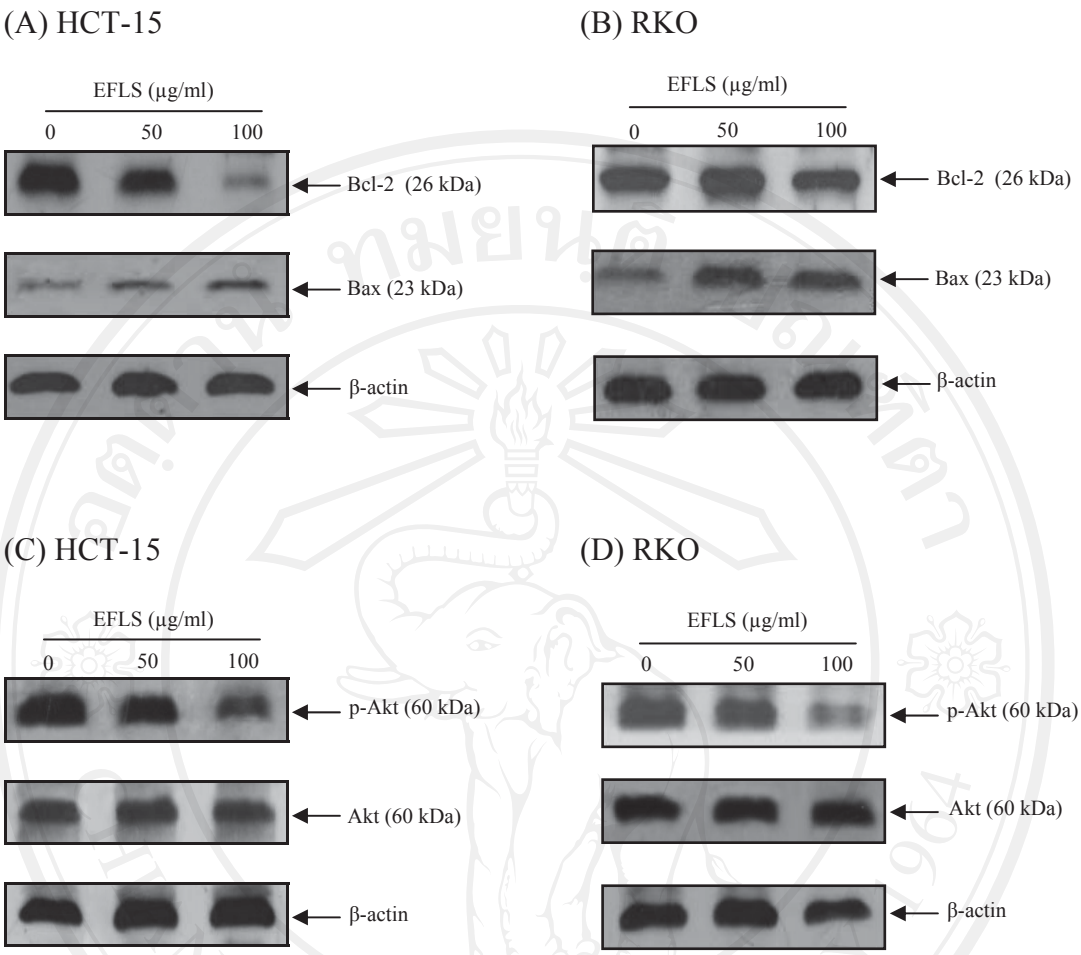

Figure 3.8 The expression levels of Bcl-2 and Bax proteins in (A) HCT-15 and (B) RKO cells after treatment with 50 and 100 μg/ml of EFLS were determined using Western blot analysis. Forty μg of protein were loaded in each lane in 12% SDS-polyacrylamide gel electrophoresis, and β-actin was detected as an internal control. The protein recognized by each antibody is indicated on the side. The level of Akt and p-Akt proteins in HCT-15 and RKO cells after treatment with 50 and 100 μg/ml of EFLS are shown in (C) and (D).

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3.7 Cell cycle analysis by flow cytometry

EFLS induced S cell cycle arrest in HCT-15 cells and G_1/S transition arrest (or S phase arrest) in RKO cells. The effect of EFLS treatment on cell proliferation was evaluated by measuring the distribution of cells in different phases of the cell cycle by flow cytometry. The cells treated with 50 and 100 µg/ml of EFLS for 24 h and 48 h were subjected to flow cytometric analysis after staining their DNA. Histograms of the flow cytometric data are shown in Figure 3.9. Exposure of HCT-15 cells to the EFLS extract (50 and 100 µg/ml) for 24 h let to S phase cycle arrest. Forty eight hour exposure of HCT-15 cells to the extract further induced evidently S phase cycle arrest (Figure 3.10) and resulted in a significant increase in accumulation of cells in sub-G_1 peak, indicating appearance of apoptotic cells, 6% (50 µg/ml), 11% (100 µg/ml) and 14% (200 µg/ml) as compared to those of control (3%) (Figure 3.12). Within 24 h of exposure of RKO cells to the EFLS extract (50, 100 µg/ml) let to transient G_1 phase cycle arrest. Following exposure of RKO cells to the EFLS extract (100 µg/ml) for 48 h, marked S phase cycle arrest was noted as revealed by increasing the percentage of cells to about 36% as compare to those of control (25%)(Figure 3.11). Sub-G_1 peak was observed at concentrations of 50, 100, 200 µg/ml EFLS after 48 h exposed in RKO cells (3%, 6% and 13%, respectively) (Figure 3.12). These results indicated that EFLS induced cell cycle arrest and led to apoptosis induction in consequence.

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A. HCT-15

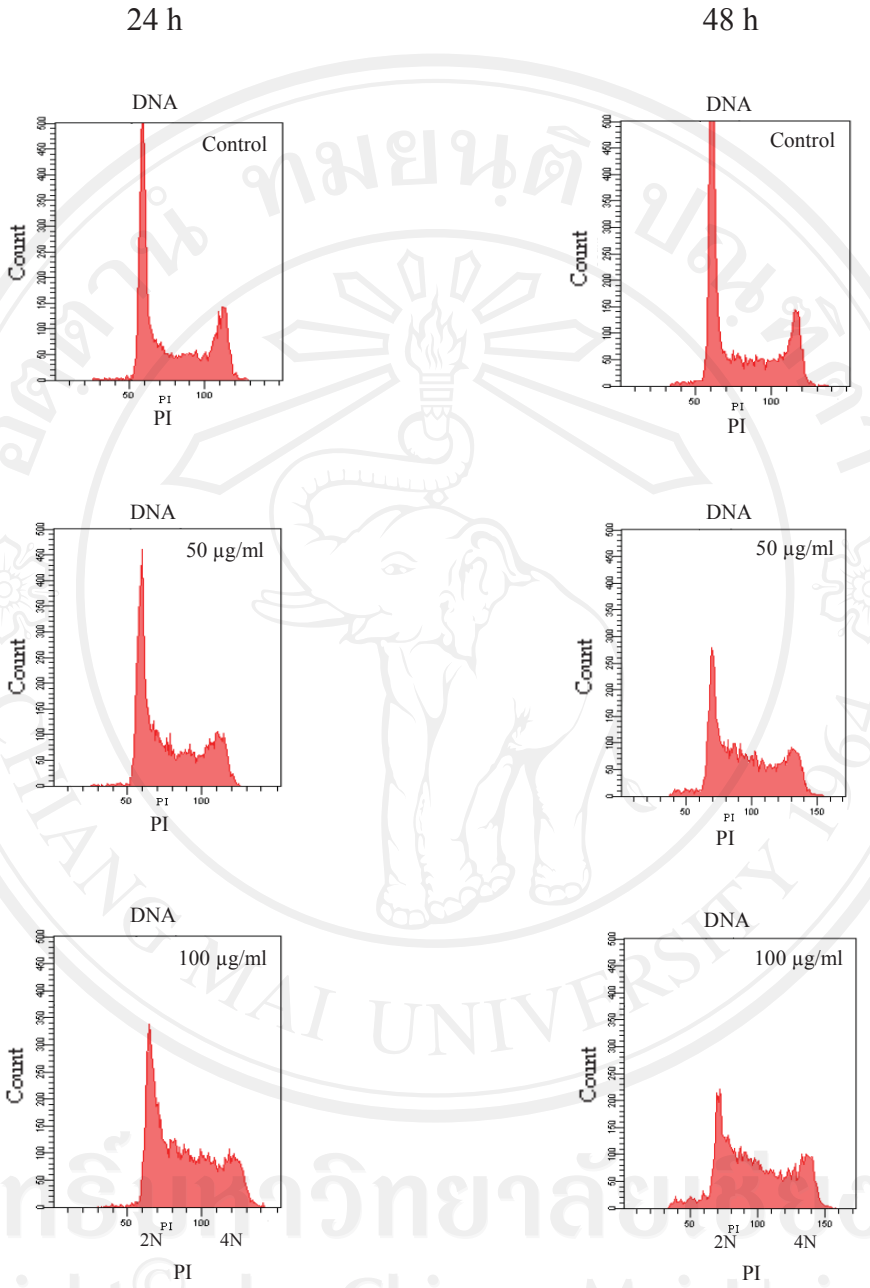

Figure 3.9 Flow cytometric analysis of the cell cycle. HCT-15 (A) and RKO (B) cells were treated with EFLS at concentrations of 50 and 100 µg/ml for 24 and 48 h. Cell number was plotted against DNA content measured in terms of PI fluorescence. 2N and 4N indicate the location of diploid (G_0-G_1) and tetraploid (G_2-M).

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B. RKO

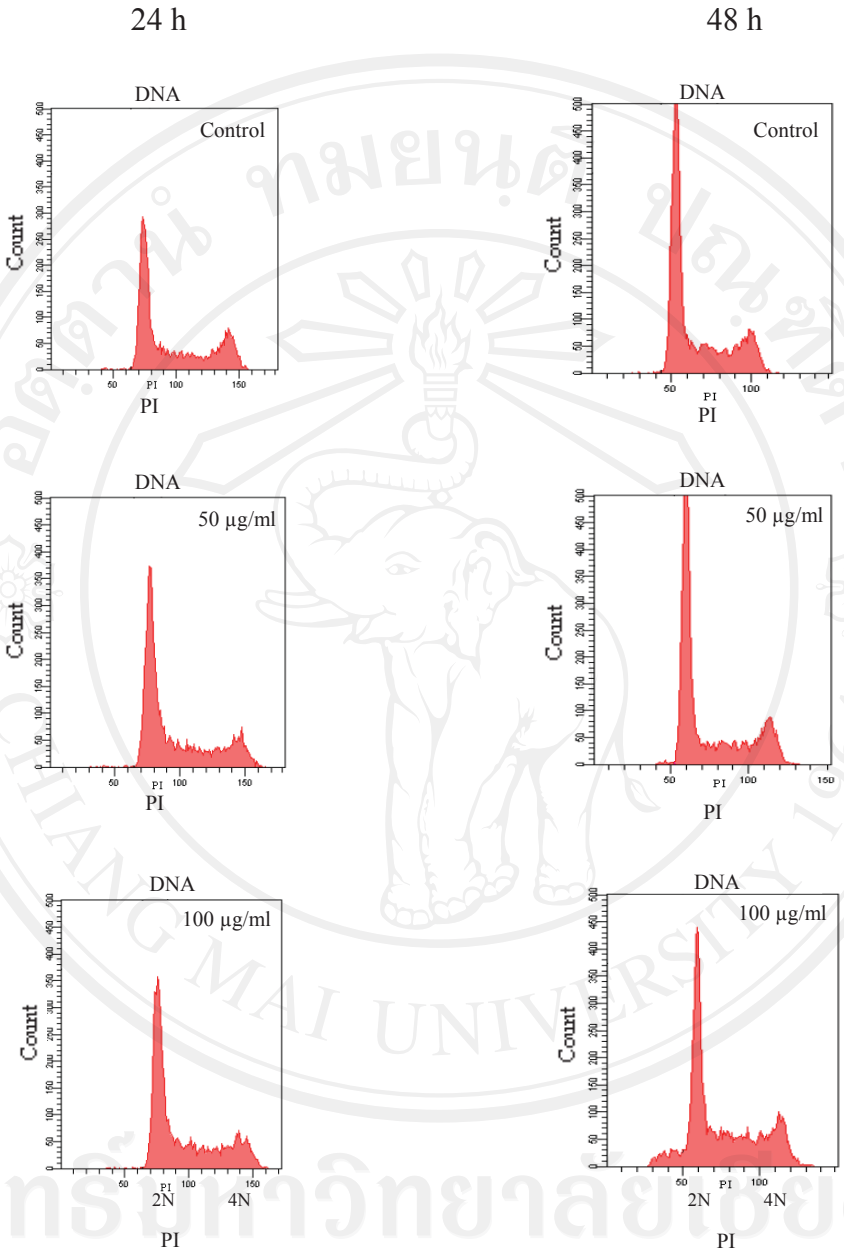

Figure 3.9 Flow cytometric analysis of the cell cycle. HCT-15 (A) and RKO (B) cells were treated with EFLS at concentrations of 50 and 100 µg/ml for 24 and 48 h. Cell number was plotted against DNA content measured in terms of PI fluorescence. 2N and 4N indicate the location of diploid (G_0-G_1) and tetraploid (G_2-M).

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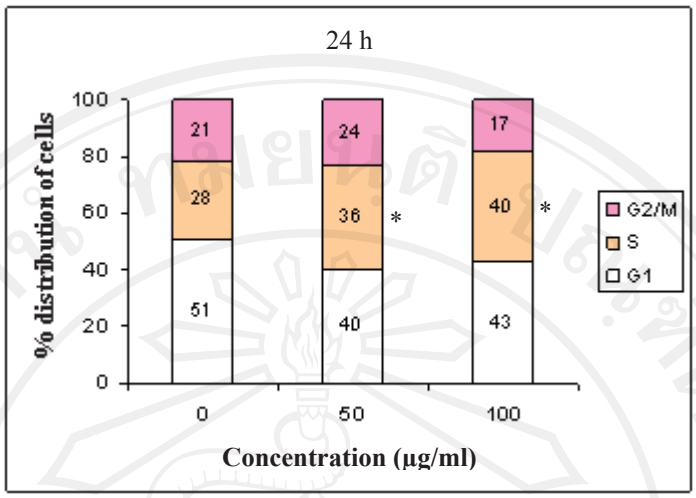

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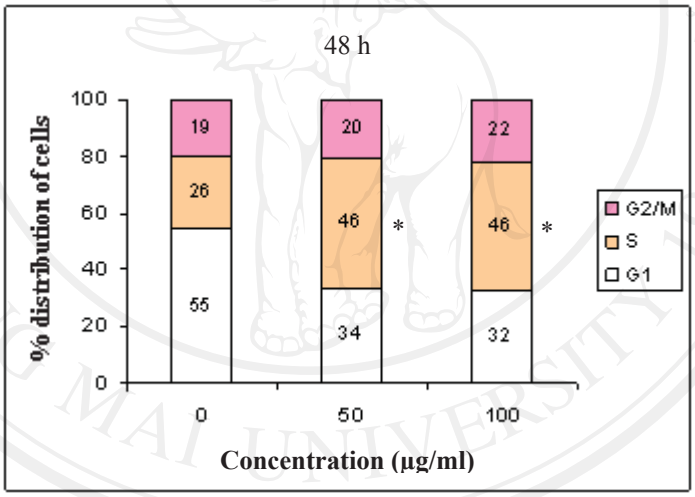

Figure 3.10 Cell cycle distribution of HCT-15 colon cancer cell treated with 50 and 100 µg/ml of EFLS for 24 h (A) and 48 h (B). The data presented here are average of three independent experiments. Each of the stacked bars is the relative distribution of cells in G_1, S and G_2/M phases of cell cycle. * = $p < 0.05$, significantly different from untreated cells.

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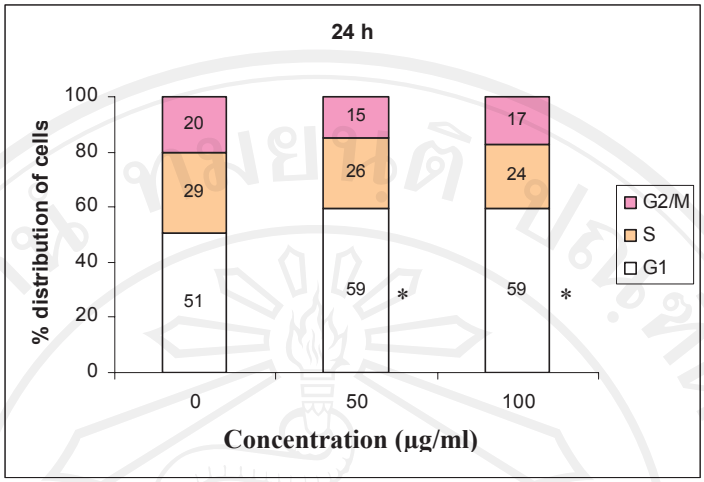

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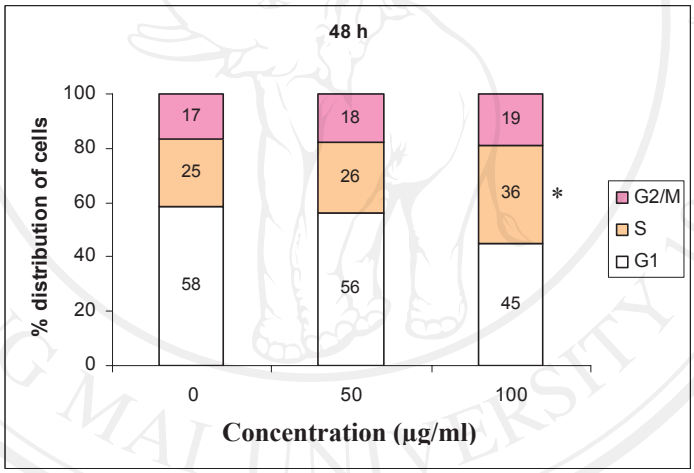

Figure 3.11 Cell cycle distribution of RKO colon cancer cell treated with 50 and 100 µg/ml of EFLS for 24 h (A) and 48 h (B). The data presented here are average of three independent experiments. Each of the stacked bars is the relative distribution of cells in G_1, S and G_2/M phases of cell cycle. * = $p < 0.05$, significantly different from untreated cells.

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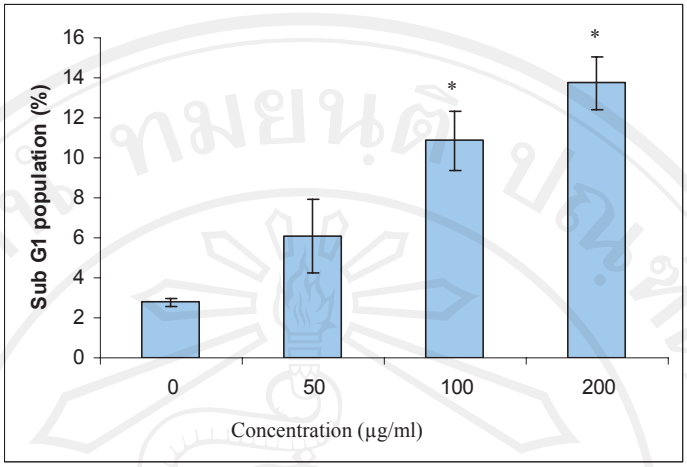

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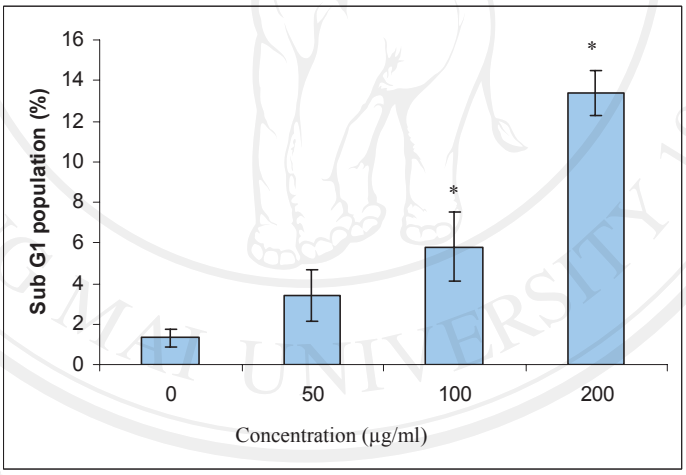

Figure 3.12 The bar charts represent changes in sub G_1 population (apoptotic cell populations) in (A) HCT-15 and (B) RKO cells after treatment with 50, 100 and 200 µg/ml of EFLS for 48 h. The data are the mean ± SD of three independent experiments, * = $p < 0.05$, significantly different from control.

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