CHAPTER III

Results

3.1 Growth curve of K562 and Molt4 cell lines

To investigate exponential growth, the total cell number was counted everyday by trypan blue exclusion method for 8 days. The patterns of leukemic cell growth were showed in Table 3.1 and Figure 3.1. The results showed that exponential growth of K562 and Molt4 cells were presented in 4-6 and 5-6 days of culture, respectively.

Table 3.1 Cell numbers of Molt4 and K562 cell lines.

Day	Total cell number (cells/mL)	
	K562	Molt4
0	10,000 ± 0	10,000 ± 0
1	20,000 ± 4,082	27,500 ± 2,886
2	55,000 ± 7,071	40,000 ± 4,082
3	95,000 ± 9,128	81,250 ± 4,787
4	282,500 ± 15,000	141,250 ± 7,500
5	587,500 ± 47,871	308,750 ± 4,787
6	787,500 ± 62,915	612,500 ± 25,000
7	337,500 ± 25,000	1,375,000 ± 144,337
8	218,750 ± 4,787	1,062,500 ± 25,000

Data are the mean values±SD of three independent experiments.

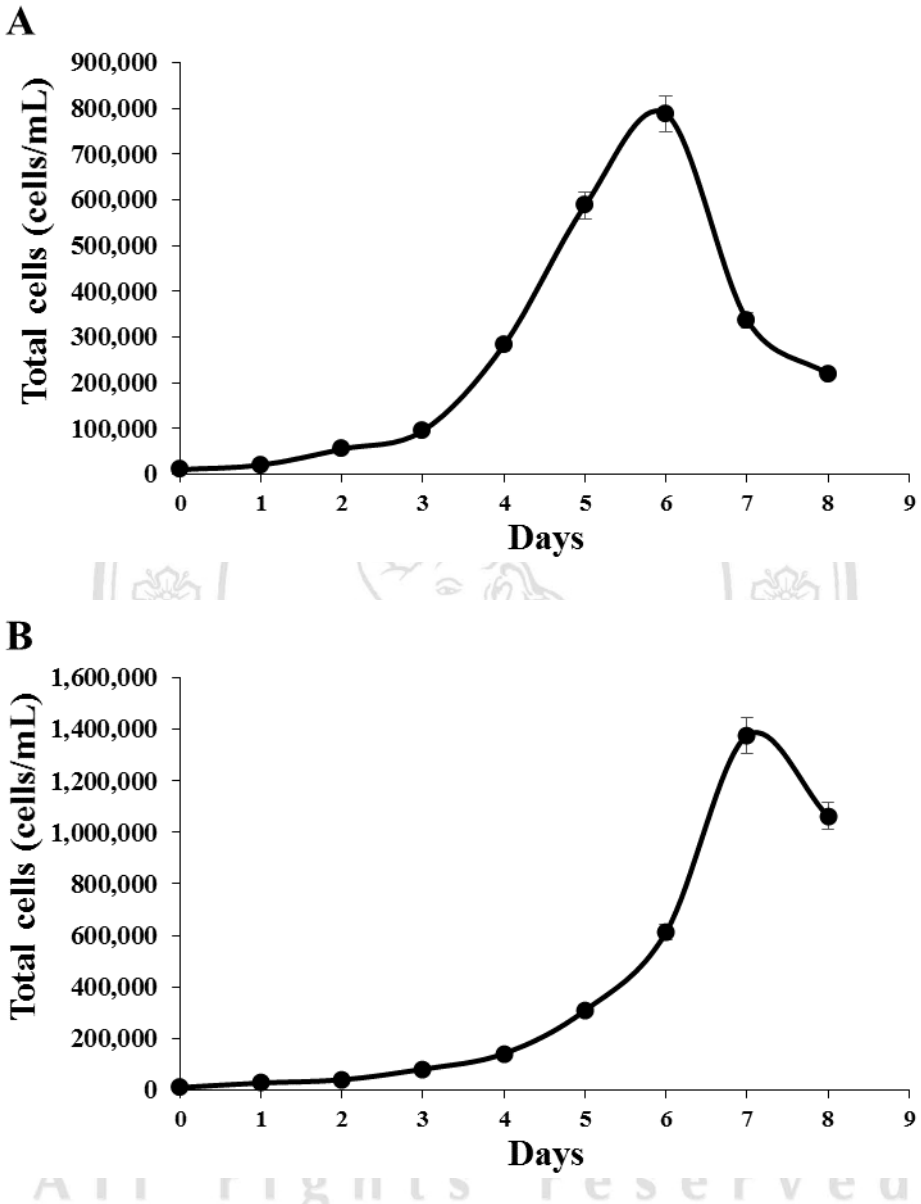

Figure 3.1 Growth curves of leukemic cell lines. The total cell numbers of (A) K562 and (B) Molt4 cells were counted by trypan blue exclusion method for 8 days.

3.2 Cytotoxicity of purified fraction No. 9, 10, and 11 of hexane fractional extract from kaffir lime leaf on K562 and Molt4 cell lines

After leukemic cell lines were treated with different concentrations of purified fraction (No.9, 10, and 11) at various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) for 48 h, the cytotoxic effects of purified fractions on K562 and Molt4 cells were investigated by using MTT assay. Cytotoxicity of each purified fractions was deter-mined by an inhibitory concentration at 50% of growth (IC_{50}). The results showed that fraction 9, 10, and 11 had cytotoxic effects on K562 and Molt4 cells. The IC_{50} values of purified fraction No. 9, 10, and 11 on K562 cells were 32.3±4.2, 48.0±0.4, and 80.0±0.3 µg/mL, respectively (Table 3.2 and Figure 3.2). The IC_{50} values of purified fractions on Molt4 cells were 24.4±3.0, 24.6±0.5, and 20.9±1.9 µg/mL for fraction 9, 10, and 11, respectively (Table 3.3 and Figure 3.3). The non-cytotoxic concentration (IC_{20}) values of all purified fractions on both leukemic cell lines were used for further studies (cell cycle arrest).

Table 3.2 The IC_{50} and IC_{20} values of purified fractions No. 9, 10, and 11 of hexane fractional extract from kaffir lime leaf in K562 cell line

Purified fraction number	Inhibitory concentration value							
	IC_{50} (µg/mL)				IC_{20} (µg/mL)			
	1	2	3	Mean±SD	1	2	3	Mean±SD
No. 9	30.3	36.8	29.0	32.0±4.2*	13.0	18.5	9.1	13.5±4.7*
No. 10	45.1	45.4	53.6	48.0±4.8	22.7	14.8	11.1	16.2±5.9
No. 11	74.0	70.3	73.0	72.4±1.9	14.8	20.4	22.3	19.2±3.9

* The most effective purified fraction as compared to other purified fractions. The data are shown as the mean values±SD of three independent experiments.

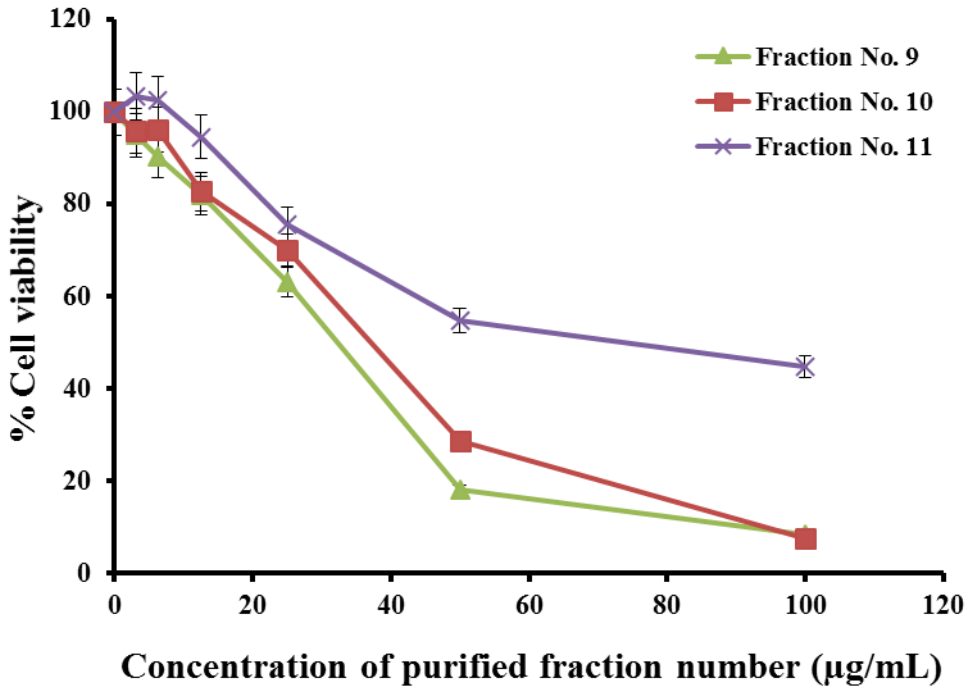

Figure 3.2 Cytotoxicity of purified fraction No. 9, 10, and 11 of hexane fractional extract from kaffir lime leaf on K562 cell line. K562 cells were seeded at a density of 1.0×10^5 cells/mL and treated with the various concentrations of kaffir lime leaf purified fractions (Fraction No. 9, 10, and 11) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value±SD of three independent experiments that were performed in triplicate.

Table 3.3 The IC_{50} and IC_{20} values of purified fraction No. 9, 10, and 11 of hexane fractional extract from kaffir lime leaf in Molt4 cell line.

Purified fraction number	Inhibitory concentration value							
	IC_{50} (µg/mL)				IC_{20} (µg/mL)			
	1	2	3	Mean±SD	1	2	3	Mean±SD
No. 9	26.7	21.0	25.6	24.4±3.0	3.1	2.9	3.2	3.1±0.1*
No. 10	24.4	25.2	24.1	24.6±0.5	6.2	5.0	5.9	5.7±0.6
No. 11	22.8	20.9	18.9	20.9±1.9*	5.9	5.0	4.3	5.1±0.8

* The most effective fraction as compared to other fractions. The data represents the mean values±SD of three independent experiments.

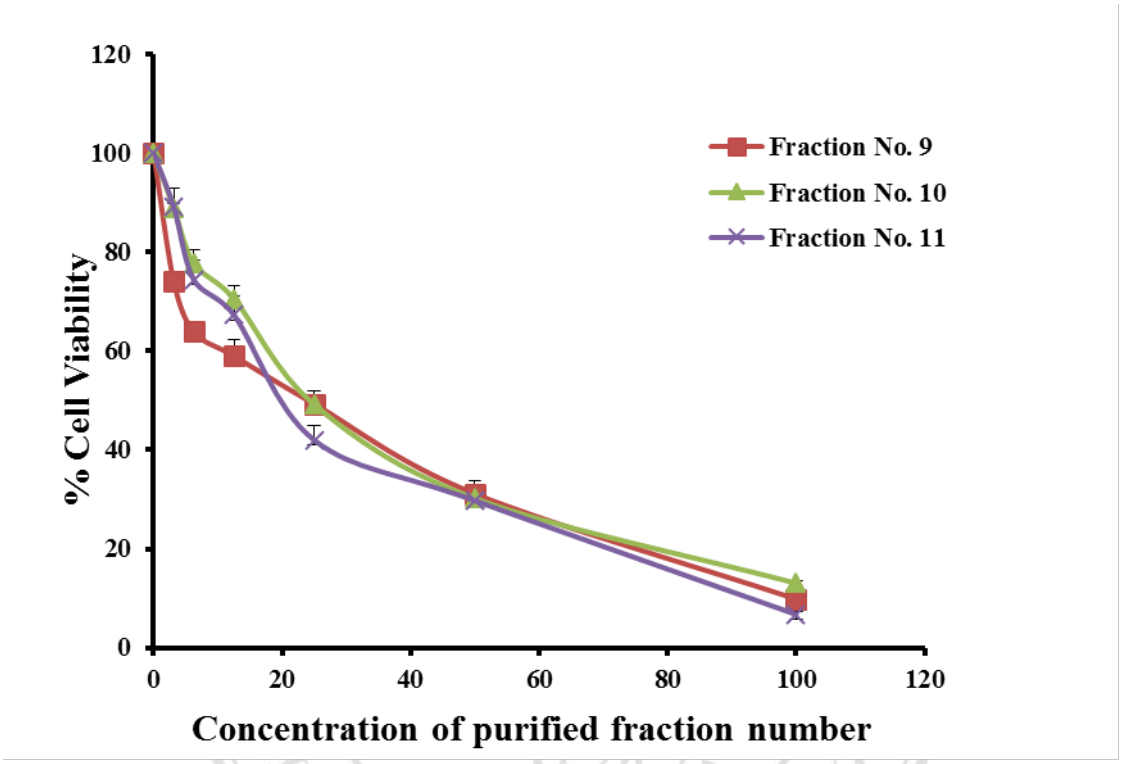

Figure 3.3 Cytotoxicity of purified fraction No. 9, 10, and 11 of hexane fractional extract from kaffir lime leaf on Molt4 cell line. Molt4 cells were seeded at a density of 1.0×10^5 cells/mL and treated with the various concentrations of kaffir lime leaf purified fractional extracts (Fraction No. 9, 10, and 11) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value±SD of three independent experiments that are performed in triplicate.

3.3 Effect of different time points of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 and Molt4 cell lines.

The purified fraction No. 9 of hexane fractional extract from kaffir lime leaf had the strongest inhibitory effect on cell cytotoxicity in both K562 and Molt4 cells. To study the effect in time increasing after purified fraction No. 9 treated on cell cycle progression was evaluated by measuring the cell distribution of K562 and Molt4 cells in different phases of cell cycle by flow cytometery.

3.3.1 Effect of different time points of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 cell line.

To determine the effect of time period treatments of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 cells, cells were cultured in complete RPMI-1640 medium containing purified fraction No. 9 at the concentrations of 10 µg/mL (IC_{20} value) and DMSO (vehicle control) for 12, 24, 36, and 48 h and determined by flow cytometric analysis after staining their DNA with PI. The flow cytometric data at 12, 24, 36, and 48 h are shown in Table 3.4 and Figure 3.4, 3.5, and 3.6, respectively. After cells were treated with purified fraction No. 9, cells significantly arrested at the G2/M phase with the increasing of percent cell population of 41.4% as compared to the vehicle control (16.8 %) at 24 h (Table 3.4 and Figure 3.5). Sub-G1 (peak of apoptotic cells) was observed after purified fraction No. 9 treatment for 36 h. However, the percent of cell dead was less than 20% by trypan blue exclusion method (Table 3.4 and Figure 3.6).

Table 3.4 Cell cycle distribution after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment for 12, 24, 36, and 48 h in K562 cells.

Treatment	Time (h)	Phase of cell cycle	% Cell population			Mean±SD
			1	2	3	
Vehicle control	12	Sub-G1	5.7	0.4	2.3	2.8±2.7
		G0/G1	31.2	37.3	44.3	37.6±6.6
		S	52.2	38.6	39.4	43.4±7.6
		G2/M	15.1	21.0	24.6	20.3±4.8
	24	Sub-G1	2.9	2.7	0.8	2.1±1.2
		G0/G1	21.3	38.9	38.2	32.8±5.8
		S	45.6	40.3	44.2	43.4±2.7
		G2/M	14.1	19.9	16.3	16.8±2.9
	36	Sub-G1	1.8	1.6	1.0	1.5±0.4
		G0/G1	33.3	38.9	27.2	33.2±5.9
		S	47.3	45.3	54.4	49.0±4.8
		G2/M	24.5	17.0	17.4	19.6±4.2
	48	Sub-G1	6.3	1.2	1.6	3.0±2.8
		G0/G1	27.3	28.6	38.8	31.5±6.3
		S	48.5	58.8	44.7	50.7±7.3
		G2/M	20.7	11.1	25.6	19.1±7.9
Purified fraction No.9	12	Sub-G1	2.9	5.8	4.3	4.4±1.5
		G0/G1	31.2	33.4	43.6	36.0±6.8
		S	44.6	40.7	43.2	42.8±2.0
		G2/M	18.4	13.2	20.8	17.5±3.9
	24	Sub-G1	4.7	5.4	4.2	4.7±0.6
		G0/G1	24.9	26.2	26.5	25.8±0.9
		S	38.4	48.1	44.8	43.8±4.9
		G2/M	37.5	41.3	45.3	41.4±3.9*
	36	Sub-G1	6.3	1.2	1.6	10.3±2.8*
		G0/G1	32.9	30.1	19.5	27.5±7.1
		S	36.7	32.8	41.9	37.2±4.6*
		G2/M	19.3	18.6	11.4	16.4±4.4
	48	Sub-G1	13.8	20.1	5.6	13.2±7.3*
		G0/G1	35.9	22.4	27.2	28.5±6.8
		S	39.8	33.5	39.2	37.5±3.5*
		G2/M	16.1	8.9	28.4	17.8±9.9

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

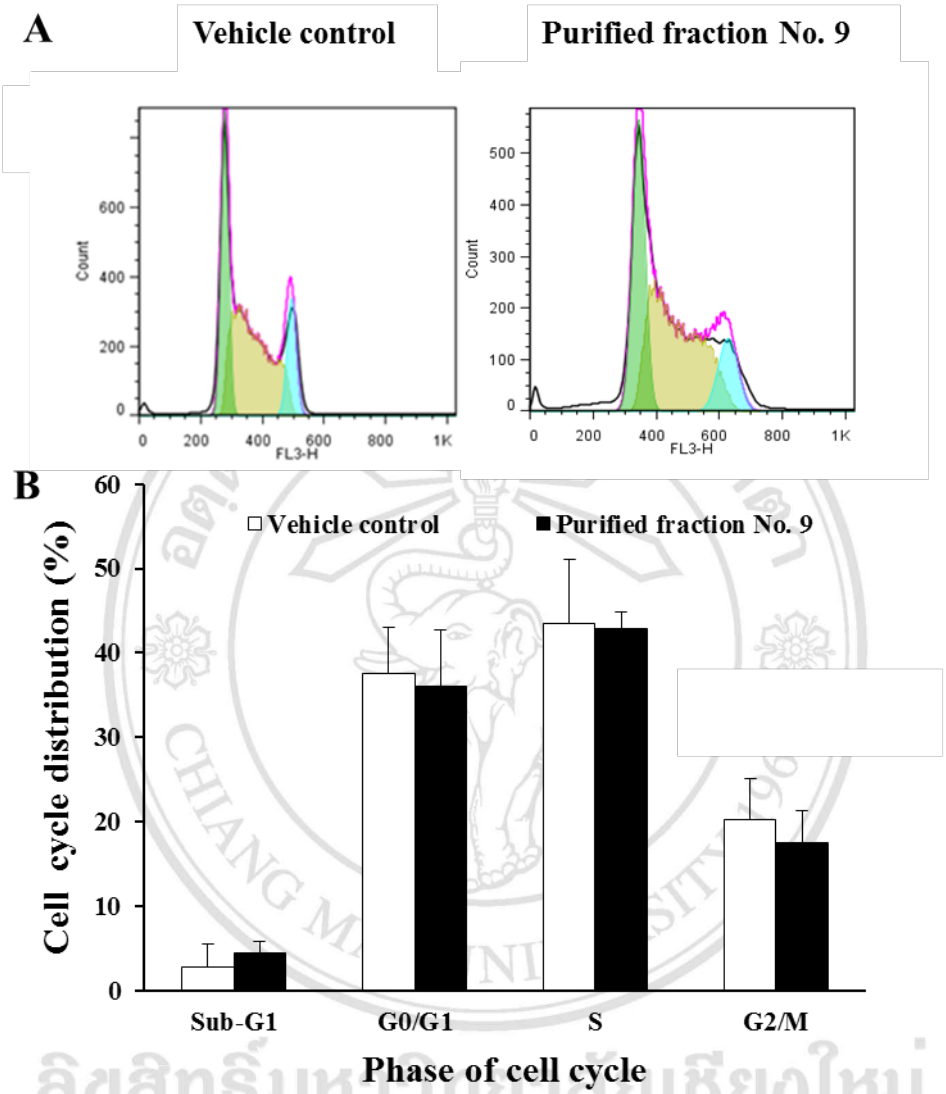

Figure 3.4 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 12 h in K562 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 10 µg/mL for 12 h. (B) Percentage of cell distribution in cell cycle phase (sub-G1, G0/G1, S, and G2/M) for 12 h. Data are the mean values±SD of three independent experiments.

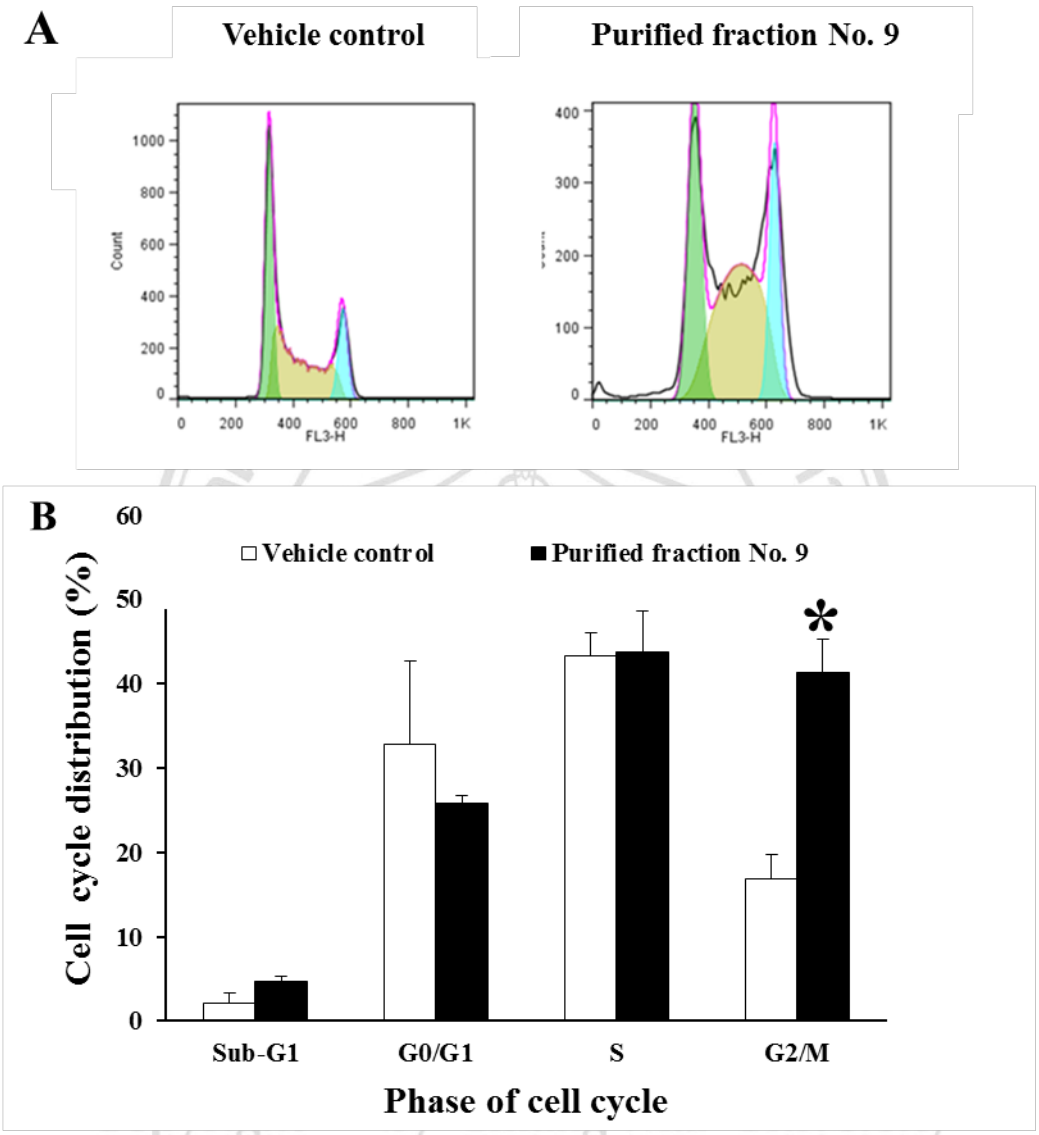

Figure 3.5 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 24 h in K562 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 10 µg/mL for 24 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) at 24 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

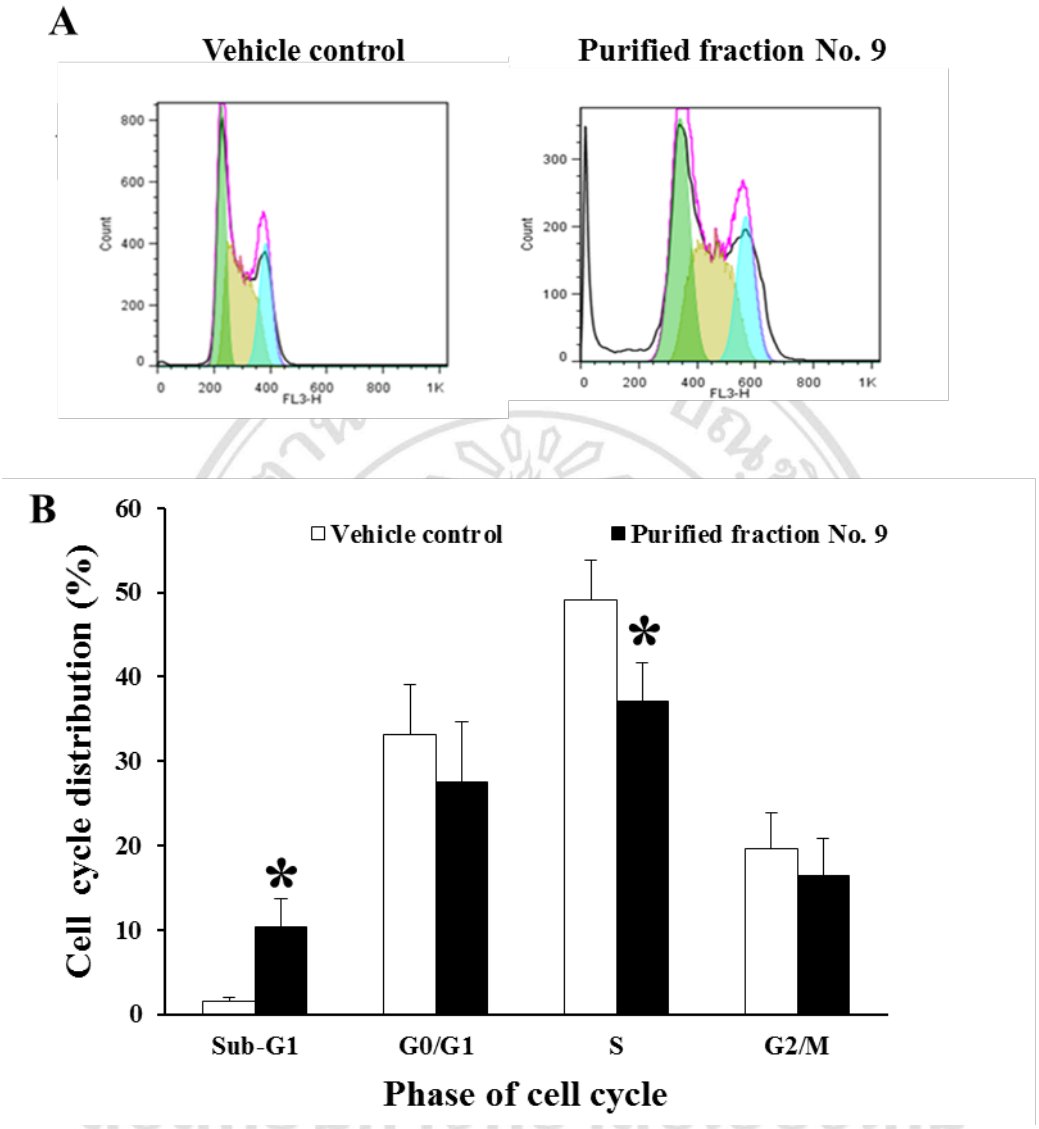

Figure 3.6 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 36 h in K562 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 10 µg/mL for 36 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 36 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

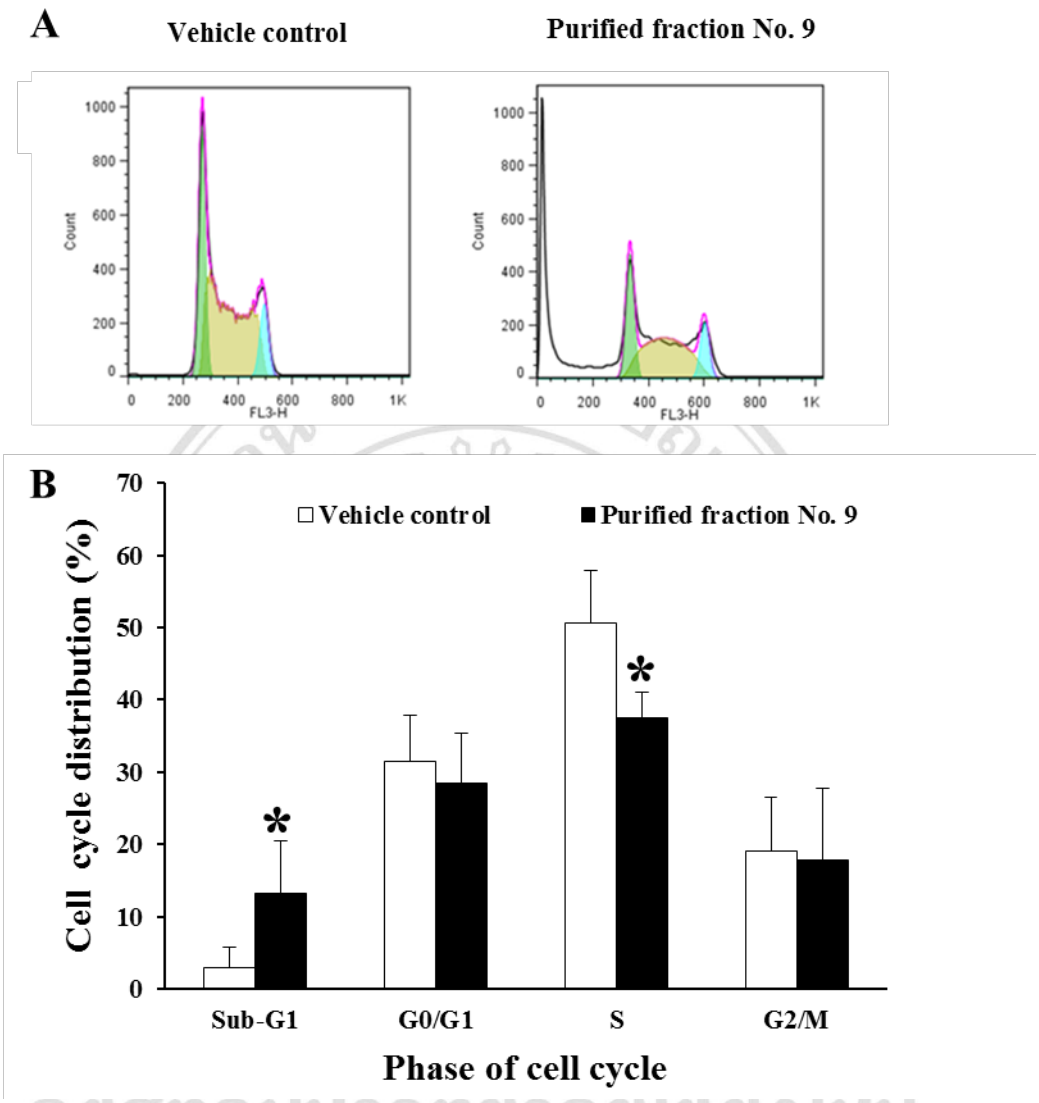

Figure 3.7 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 48 h in K562 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 10 µg/mL for 48 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 48 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

3.3.2 Effect of different time points of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle on Molt4 cell line.

To determine the effect of time period treatments of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in Molt4 cells, cells were cultured in medium containing purified fraction No. 9 and DMSO (vehicle control) at the concentration of 2.5 μg/mL (IC_{20} value) for 12, 24, 36, and 48 h and analyzed by flow cytometry after staining their DNA with PI. The flow cytometric data are shown in Table 3.5, Figure 3.8, 3.9, and 3.10, respectively. The vehicle control cells progressed with the normal distribution. After purified fraction No. 9 treatments, Molt4 cells were arrested at the G2/M phase with the increasing of percent cell population in G2/M phase by 10.3% as compared to the vehicle control (7.8%) at 24 h (Table 3.5 and Figure 3.9). The result found a significant increase in accumulation of cells in sub-G1 population, indicating appearance of apoptotic cells at 12, 24, 36, and 48 h (Table 3.5, Figure 3.9, and 3.10).

Table 3.5 Cell cycle distribution after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment for 12, 24, 36, and 48 h in Molt4 cells.

Treatment	Time (h)	Phase of cell cycles	% Cell population			Mean±SD
			1	2	3	
Vehicle control	12	Sub-G1	3.8	5.7	4.3	4.6±1.0
		G0/G1	36.0	38.2	40.2	38.1±2.1
		S	39.4	38.0	28.8	35.4±5.8
		G2/M	13.3	7.4	11.5	10.8±2.9
	24	Sub-G1	1.3	6.2	2.3	3.3±2.6
		G0/G1	55.3	42.6	49.3	49.0±6.3
		S	33.2	37.6	35.7	35.5±2.2
		G2/M	11.4	5.7	6.2	7.8±3.2
	36	Sub-G1	11.0	6.7	6.6	8.1±3.2
		G0/G1	48.1	41.3	43.2	44.2±2.5
		S	26.7	36.3	39.9	34.3±3.5
		G2/M	14.0	13.6	9.6	12.4±2.4
	48	Sub-G1	7.7	7.8	6.2	7.2±0.9
		G0/G1	56.5	44.6	62.0	54.4±5.0
		S	25.4	33.0	32.3	30.2±4.2
		G2/M	9.4	12.7	4.7	8.9±4.0
Purified fraction No. 9	12	Sub-G1	16.9	21.9	18.0	18.9±2.6*
		G0/G1	26.4	35.3	40.2	34.0±7.0
		S	35.3	27.9	35.4	32.9±4.3
		G2/M	3.7	11.1	9.3	8.0±3.9*
	24	Sub-G1	12.7	26.8	24.5	21.4±7.5*
		G0/G1	30.2	24.3	34.3	29.6±5.0*
		S	25.6	27.0	32.9	28.5±3.8
		G2/M	8.5	11.6	10.8	10.3±1.6
	36	Sub-G1	38.6	37.8	42.1	39.5±2.3*
		G0/G1	32.4	31.7	26.9	30.3±3.0*
		S	26.8	23.3	25.2	25.1±1.8
		G2/M	6.4	5.6	3.8	5.2±1.4
	48	Sub-G1	50.1	37.2	37.9	41.7±7.3*
		G0/G1	19.5	26.5	29.3	25.1±5.0*
		S	13.4	25.0	23.9	20.7±6.4*
		G2/M	5.5	7.4	7.9	7.0±1.3

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

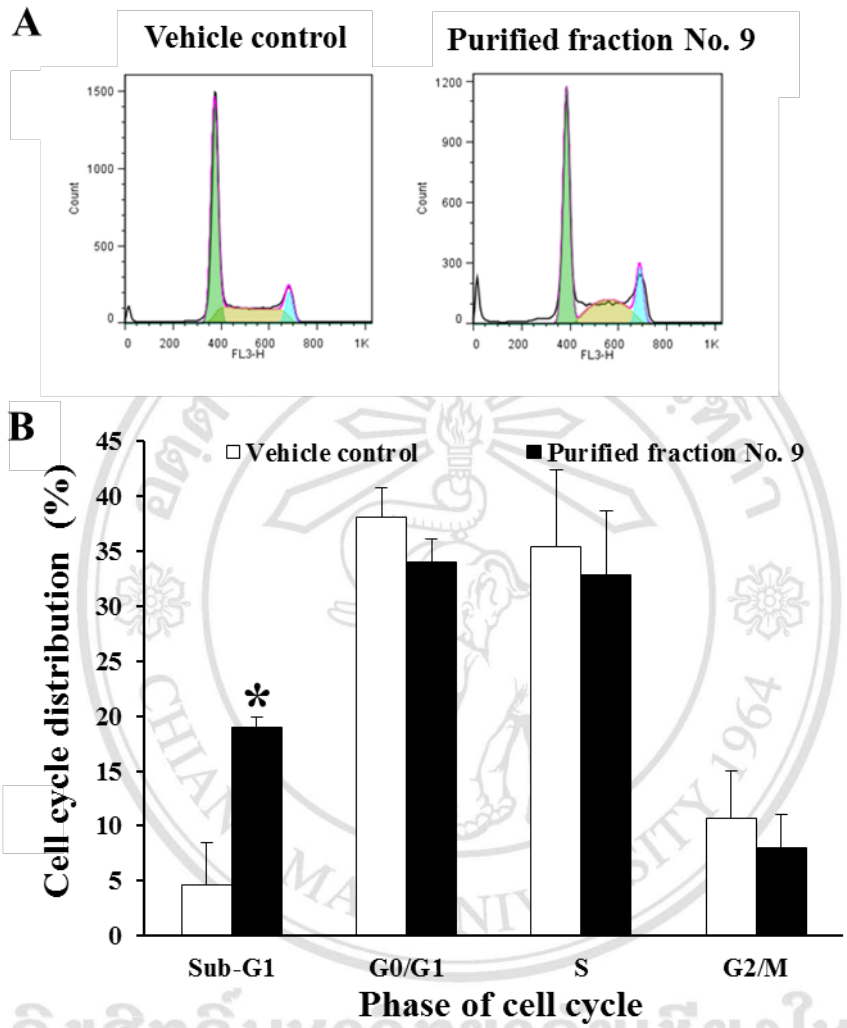

Figure 3.8 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 12 h in Molt4 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 2.5 µg/mL for 12 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 12 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

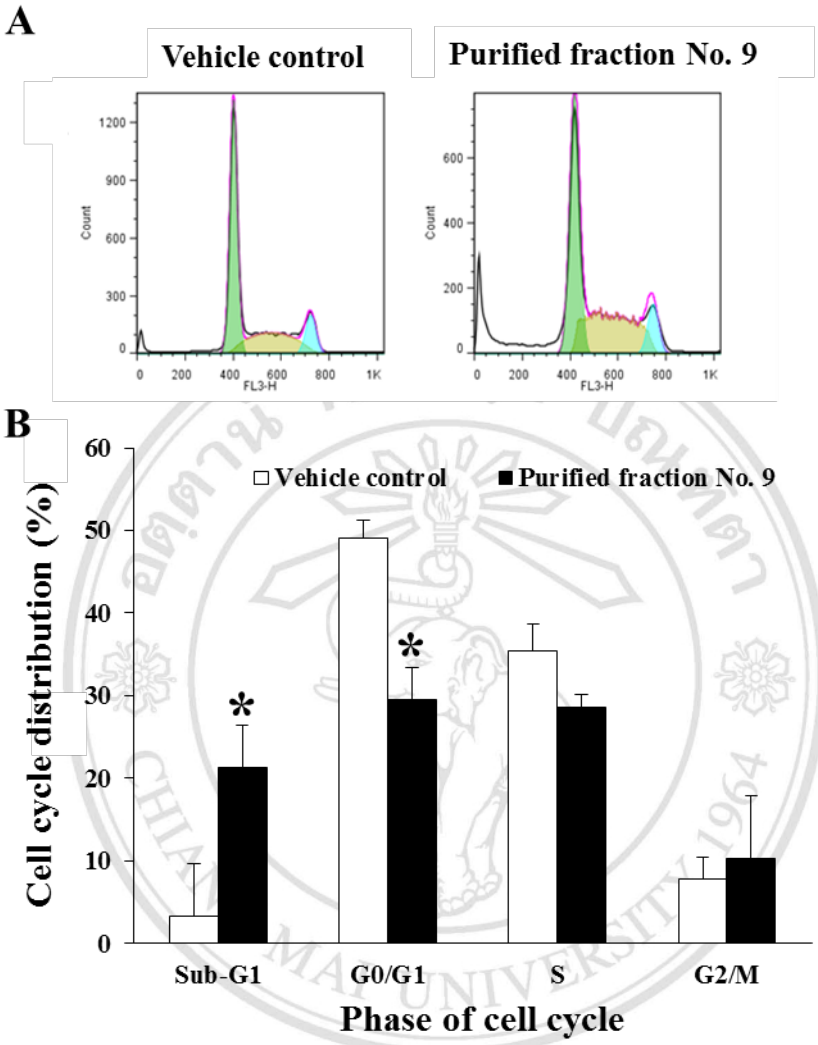

Figure 3.9 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 24 h in Molt4 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 2.5 µg/mL for 24 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 24 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

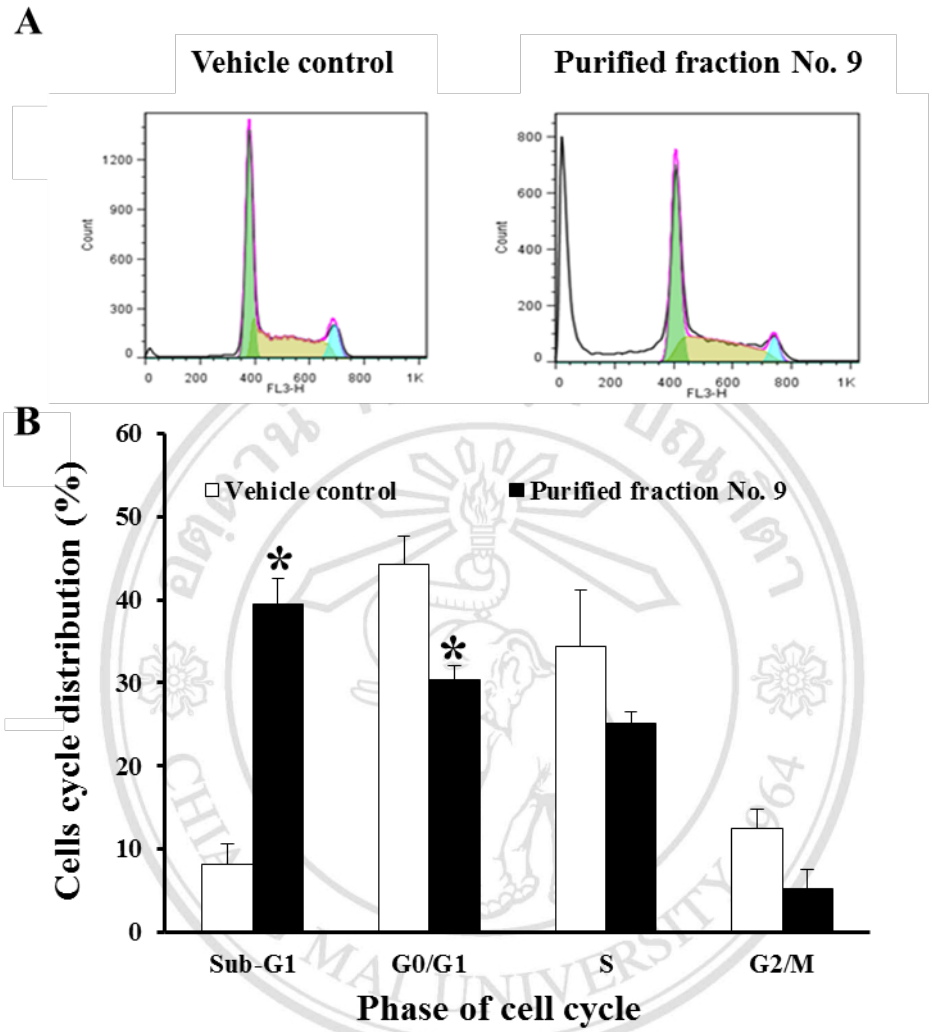

Figure 3.10 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 36 h in Molt4 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 2.5 µg/mL for 36 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 36 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

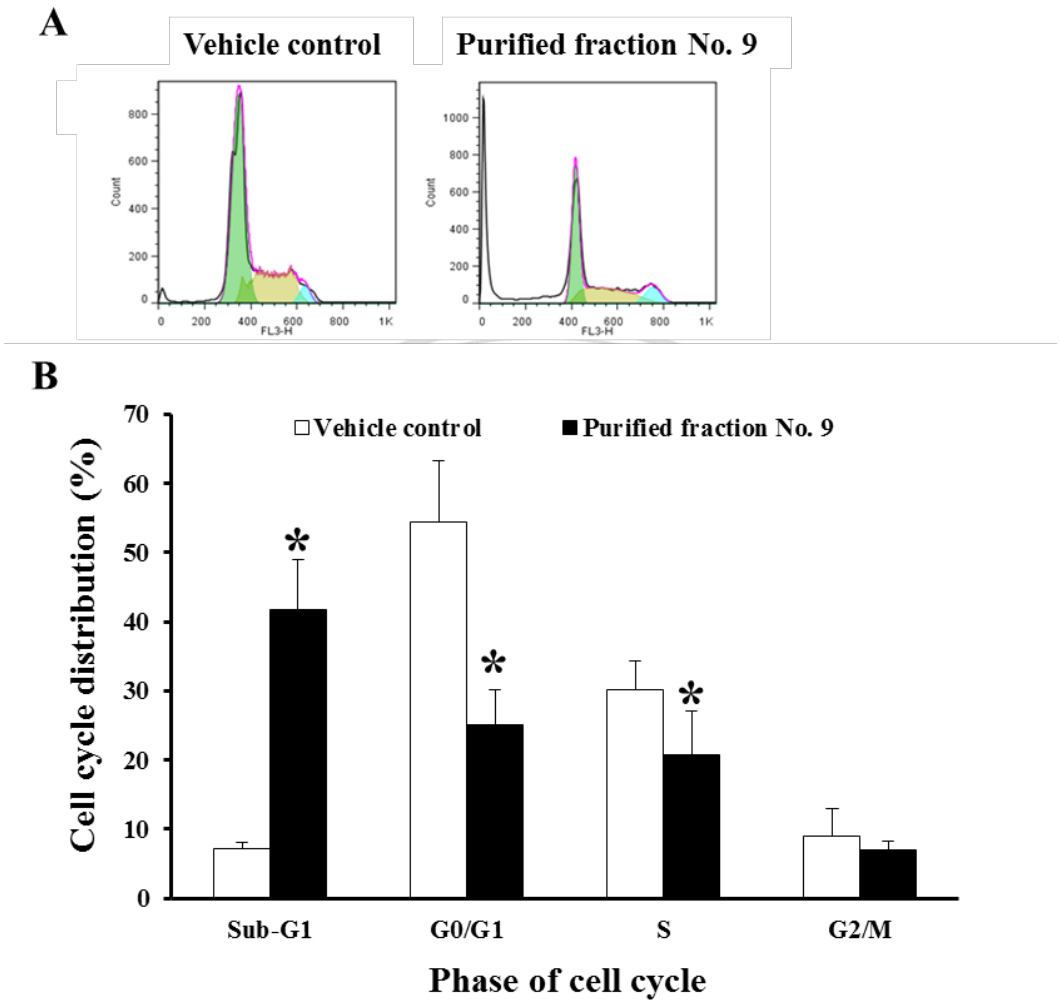

Figure 3.11 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at 48 h in Molt4 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf at the concentration of 2.5 µg/mL for 48 h. (B) Percentage of cell distribution in cell cycle phases (sub-G1, G0/G1, S, and G2/M) for 48 h. Data are the mean values±SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05)

3.4 Effect of concentrations of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 and Molt4 cell lines.

According to the results from section 3.3, the purified fraction No. 9 of hexane fractional extract from kaffir lime leaf significantly increased cell accumulation in G_2/M phase and sub-G1 at 24 h in K562 and Molt4 cells. In addition, the three different concentrations of purified fraction No. 9 were studied on cell cycle progression. To study its effect in a dose-dependent response of purified fraction No. 9 on two leukemic cell lines, K562 cells were treated with 5, 10, and 15 µg/mL and 0.04% DMSO was used as a vehicle control, while Molt4 cells were treated with 1, 2.5, and 5 µg/mL and 0.08% DMSO was used as a vehicle control at 24 h. The cell cycle distribution was evaluated by flow cytometery.

3.4.1 Effect of concentrations of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 cell line.

To determine the effects of concentrations of purified fraction No. 9 on cell cycle distribution in K562 cells, cells were cultured in complete RPMI-1640 medium containing purified fraction No. 9 and 0.04% DMSO (vehicle control) at concentrations of IC_{20} values (5, 10, and 15 µg/mL) for 24 h. After purified fraction No. 9 treatments, cells significantly arrested at G2/M phase in a dose-dependent manner when compared to vehicle control. The flow cytometric data are shown in Table 3.6 and Figure 3.12. The cell populations after purified fraction No. 9 treatments were 29.5, 32.2, and 34.8%, respectively at G2/M phases in a dose-dependent manner as compared to that of vehicle control (17.6%) at 24 h (Table 3.6 and Figure 3.12).

Table 3.6 Percentage of cell cycle distribution after purified fraction No. 9 of hexane fractional extraction kaffir lime leaf treatment at 5, 10, and 15 µg/mL for 24 h in K562 cells.

Concentration (µg/mL)	Phase of cell cycle	% Cell population			
		1	2	3	Mean±SD
0	Sub-G1	2.0	2.9	1.8	2.2±0.6
	G0/G1	38.9	39.0	29.2	35.7±5.8
	S	37.4	45.6	44.6	42.6±4.5
	G2/M	20.5	12.3	20.1	17.6±4.6
5	Sub-G1	4.1	4.3	2.0	3.5±1.3
	G0/G1	27.8	22.9	29.0	26.6±3.2*
	S	37.0	43.7	30.4	37.0±6.7
	G2/M	29.2	26.6	32.9	29.5±3.2*
10	Sub-G1	6.5	4.7	3.5	4.9±1.5
	G0/G1	25.6	22.9	23.4	23.9±1.5*
	S	31.2	41.0	27.8	33.3±6.9*
	G2/M	33.1	27.5	36.0	32.2±4.3*
15	Sub-G1	9.8	6.9	6.4	7.7±1.8
	G0/G1	25.3	18.5	25.9	23.3±4.1*
	S	29.0	39.0	27.0	31.6±6.4*
	G2/M	36.0	30.3	38.0	34.8±4.0*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

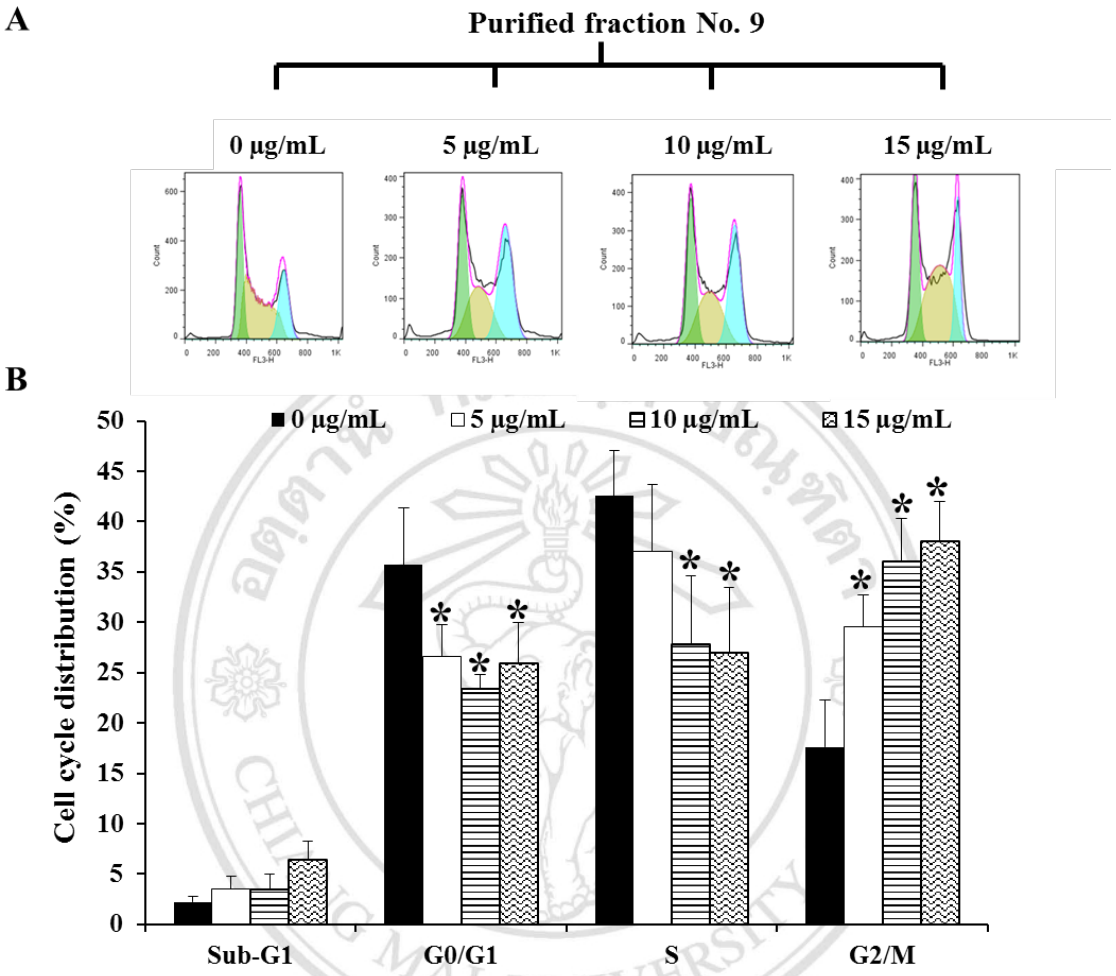

Figure 3.12 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at various concentrations in K562 cells. (A) Cell cycle distribution analysis in the fluorescence histogram, cells were treated with purified fraction No. 9 at the concentrations of 5, 10, and 15 µg/mL for 24 h. (B) Percentage of cell cycle distribution in sub-G1, G0/G1, S, and G2/M phases for 24 h. Data are the mean values±SD of three independent. Asterisk (*) denotes a significant difference from the vehicle control group (0 µg/mL) (p<0.05).

3.4.2 Effect of concentrations of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf in cell cycle distribution in Molt4 cell line.

To determine the effects of concentrations of purified fraction No. 9 on cell cycle distribution in Molt4 cells, cells were cultured in complete RPMI-1640 medium containing purified fraction No. 9 and 0.08% DMSO (vehicle control) at concentrations of IC_{20} values (1, 2.5, and 5 µg/mL) for 24 h . After purified fraction No. 9 treatments, the results did not show cell cycle arrest but it was found a significant increase of cell population in sub-G1 peak for 13.1, 20.5, and 44.2% in response to that concentration of 1, 2.5, and 5 µg/mL, respectively, indicating appearance of apoptotic cells in a dose dependent manner as compared to those of vehicle control (5.0%) at 24 h. The flow cytometric data are shown in Table 3.7 and Figure 3.13.

Table 3.7 Percentage of cell cycle distribution after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments at 1, 2.5, and 5 µg/mL for 24 h in Molt4 cells.

Concentration (µg/mL)	Phase of cell cycle	% Cell cycle distribution			
		1	2	3	Mean±SD
0	Sub-G1	2.5	4.3	8.4	5.0±3.0
	G0/G1	39.3	39.0	38.3	38.9±0.5
	S	52.6	43.8	33.9	43.4±4.7
	G2/M	19.4	16.8	17.4	17.8±1.4
1.0	Sub-G1	10.2	21.7	7.4	13.1±3.9*
	G0/G1	36.8	37.0	37.9	37.3±0.6
	S	29.5	27.6	40.0	32.4±6.7
	G2/M	19.6	12.9	17.2	16.5±3.4*
2.5	Sub-G1	23.3	20.4	17.7	20.5±2.8*
	G0/G1	30.9	32.4	37.3	33.5±3.3
	S	33.5	23.8	30.3	29.2±4.9
	G2/M	10.6	9.2	13.6	11.2±2.2*
5.0	Sub-G1	40.2	45.2	47.2	44.2±3.6*
	G0/G1	26.7	19.4	19.3	21.8±4.2
	S	23.9	15.9	23.6	21.2±4.5*
	G2/M	7.8	6.0	8.5	7.4±1.3*

Data are the mean values±SEM of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

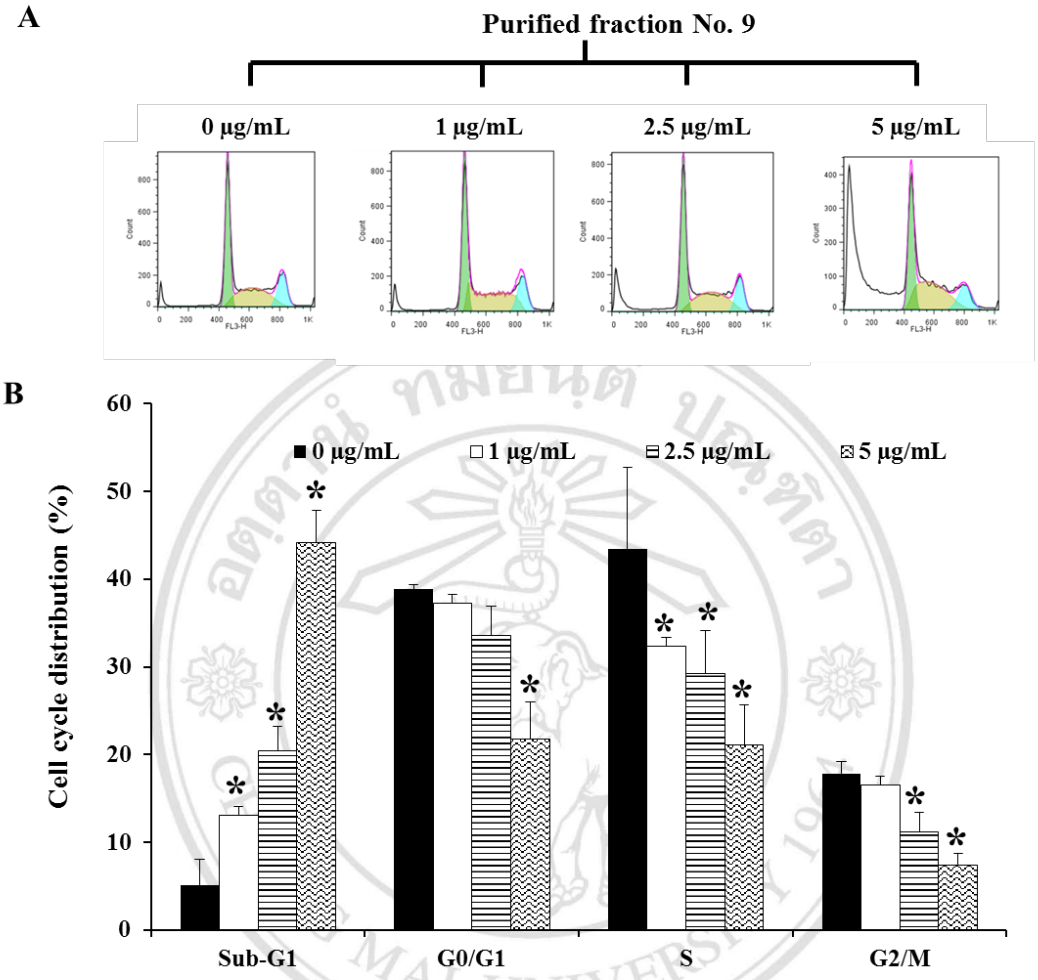

Figure 3.13 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution at various concentrations in Molt4 cell line. (A) Cell cycle distribution analysis in the fluorescence histogram,Molt4 cells were treated with purified fraction No. 9 at the concentrations of 1.0, 2.5, and 5.0 µg/mL for 24 h. (B) Percentage of cell cycle distribution in sub-G1, G0/G1, S, and G2/M phases for 24 h. Data are the mean values±SD of three independent. Asterisk (*) denotes a significant difference from the vehicle control group (0 µg/mL) (p<0.05).

3.5 Effect of incubation time periods of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on total cell numbers in K562 and Molt4 cell lines

To determine the effect of time periods of purified fraction No. 9 treatments on total cell number of K562 and Molt4 cells. Cells were treated with the non-cytotoxic doses (IC_{20} value) for 12, 24, 36, and 48 h. Then treated cells were harvested as described in section 2.4.

3.5.1 Effect of incubation time periods of purified fraction No. 9 of hexane fractional extract form kaffir lime leaf on total cell numbers in K562 cell line

To determine the effects of time periods of purified fraction No. 9 at 10 µg/mL on total numbers in K562 cells. Cells were cultured in complete RPMI-1640 medium containing 0.04% DMSO (vehicle control) and purified fraction No. 9 at the concentrations of 10 µg/mL (IC_{20} value) for 12, 24, 36, and 48 h. The total cell numbers after purified fraction No. 9 treatment were significantly decreased by 36, 29, and 35% at 24, 36, and 48 h, respectively when compared to the vehicle control. The percent of dead cells were in the range of 0-1% (Table 3.8 and Figure 3.14).

Table 3.8 Total cell number after treatment with 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf for 12, 24, 36, and 48 h in K562 cells.

Treatment	Time (h)	Survival cells ($\times 10^5$)				Dead cells ($\times 10^5$)			
		1	2	3	Mean±SD	1	2	3	Mean±SD
Vehicle control	0	8.0	8.0	8.0	8.0±0	0	0	0	0±0
	12	8.2	8.6	8.9	8.5±0.4	0	0	0	0±0
	24	12.0	15.0	18.0	15.0±3.0	0.3	0.1	0	0.2±0
	36	20.0	25.0	19.5	21.5±3.0	0	0	0	0±0
	48	25.0	24.5	29.0	26.2±2.5	0.2	0	0	0.1±0
Purified fraction No. 9	0	8.0	8.0	8.0	8.0±0	0	0	0	0±0
	12	7.0	8.4	8.5	7.9±0.8	0	0.1	0	0±0
	24	9.7	8.1	11.2	9.6±1.6	0.2	0	0.1	0.1±0
	36	15.4	14.5	16.0	15.3±0.8*	0	0.2	0	0±0
	48	15.0	16.0	20.0	17.0±2.6*	0.3	0	0.1	0.2±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).

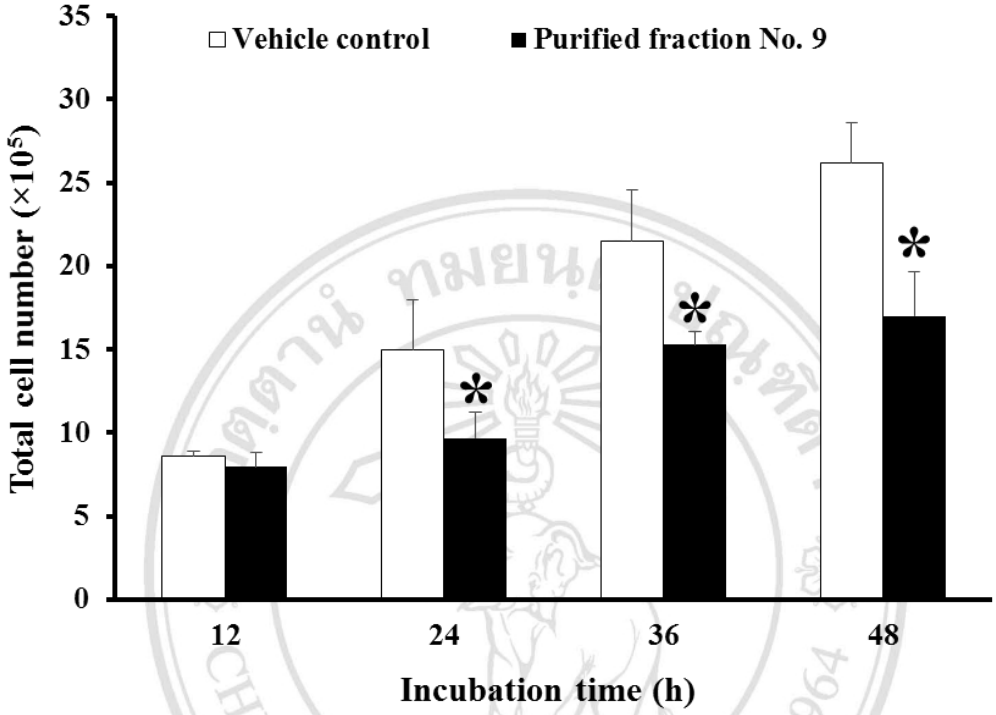

Figure 3.14 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on total cell numbers at 12, 24, 36, and 48 h in K562 cell line. Cells were counted after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.5.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on total cell numbers in Molt4 cell line

To determine the effects of time periods of purified fraction No. 9 on total numbers in Molt4 cells. Cells were cultured in complete RPMI-1640 medium containing 0.08% DMSO (vehicle control) and purified fraction No. 9 at the concentrations of 2.5 µg/mL (IC_{20} value) for 12, 24, 36, and 48 h. The total cell numbers of purified fraction No. 9 treatments were significantly decreased by 16, 39, and 38% at 24, 36, and 48 h, respectively when compared to the vehicle control. The percentages of dead cells were in the range of 0-1% (Table 3.9 and Figure 3.15).

Table 3.9 Total cell number after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Treatment	Time (h)	Survival cells ($\times 10^5$)				Dead cells ($\times 10^5$)			
		1	2	3	Mean±SD	1	2	3	Mean±SD
Vehicle control	0	8.0	8.0	8.0	8.0±0	0	0	0	0±0
	12	8.5	8.0	8.5	8.3±0.3	0	0	0	0±0
	24	9.8	10.5	15.0	11.7±2.8	0.1	0.1	0	0.1±0
	36	17.0	18.5	18.5	18.0±0.9	0	0	0	0±0
	48	21.0	27.5	23.5	24.0±3.3	0.2	0	0	0.1±0
Purified fraction No. 9	0	8.0	8.0	8.0	8.0±0	0	0	0	0±0
	12	6.7	8.0	8.5	7.3±0.9	0	0.1	0	0±0
	24	8.5	11.0	10.5	10.0±1.3	0	0	0.1	0.1±0
	36	12.0	10.5	11.0	11.0±0.8*	0.2	0	0.6	0.4±0
	48	10.5	16.0	19.0	15.0±4.3*	0.3	0	0.7	0.5±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).

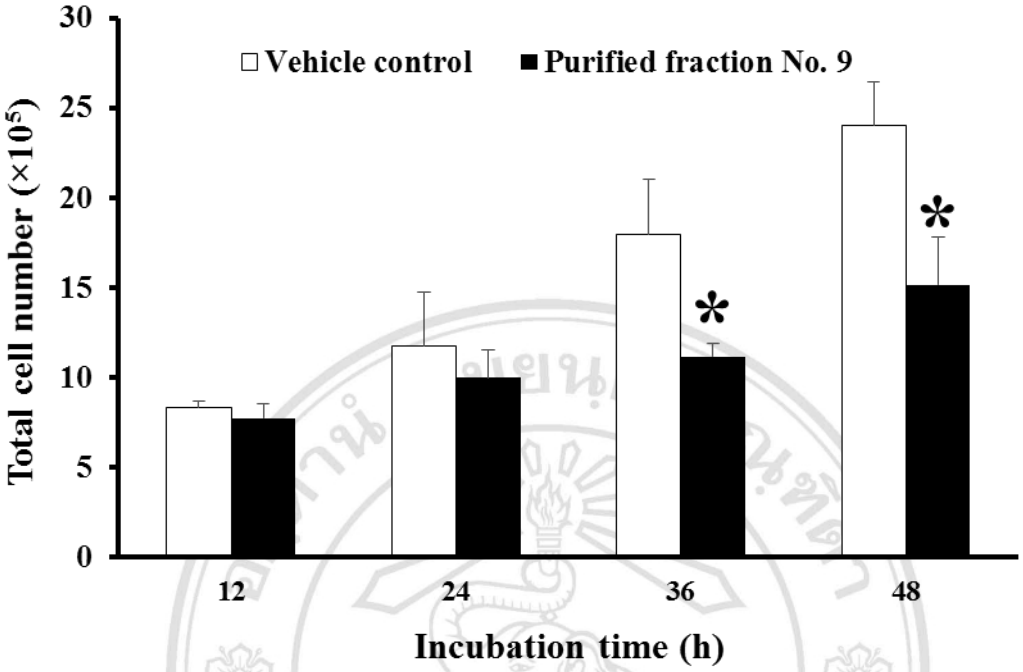

Figure 3.15 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell numbers at 12, 24, 36, and 48 h in Molt4 cell line. Cells were counted after treatment with 2.5 µg/mL of purified fraction No. 9 for 12, 24, 36, and 48 h by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.6 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on p53 protein expression in K562 and Molt4 cell lines

To determine the effect of time period of purified fraction No.9 on targeted protein involved in G2/M phase of cell cycle regulation. In this study, p53 protein was examined. Cells were treated with purified fraction No. 9 at the concentrations of IC_{20} values (non-cytotoxic doses) and DMSO for 12, 24, 36, and 48 h. Then treated cells were extracted total proteins and determined by Western blot analysis as described in section 2.9.

3.6.1 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment on p53 protein expression in K562 cell line

To study the effects of incubation times of purified fraction No. 9 treatments on p53 protein levels in K562 cells, cells were treated with 10 μg/mL (IC_{20} value) and 0.04% DMSO for 12, 24, 36, and 48 h. Cells were harvested and determined p53 protein levels by Western blotting. The levels of p53 protein were normalized by using GAPDH protein and then calculated percentage of p53 protein levels (% p53 protein level). The percentages of p53 protein levels of K562 cells were 116.3±17.4, 126.0±2.6, 108.6±9.1, and 109.7±2.2 % in response to 12, 24, 36, and 48 h, respectively. The purified fraction No. 9 extract increased the p53 protein levels in a time dependent manner at 12 and 24 h by 16 and 26%, respectively when compared to vehicle control (Table 3.10 and Figure 3.16). The purified fraction No. 9 significantly increased p53 protein levels at 24 h.

Table 3.10 Percentage of p53 protein levels after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in K562 cells.

Incubation time (h)	**% p53 protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	136.0	103.0	110.0	116.3±17.4
24	127.0	128.0	123.0	126.0±2.6*
36	117.0	110.0	99.0	108.6±9.1
48	108.0	109.0	112.0	109.7±2.2

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

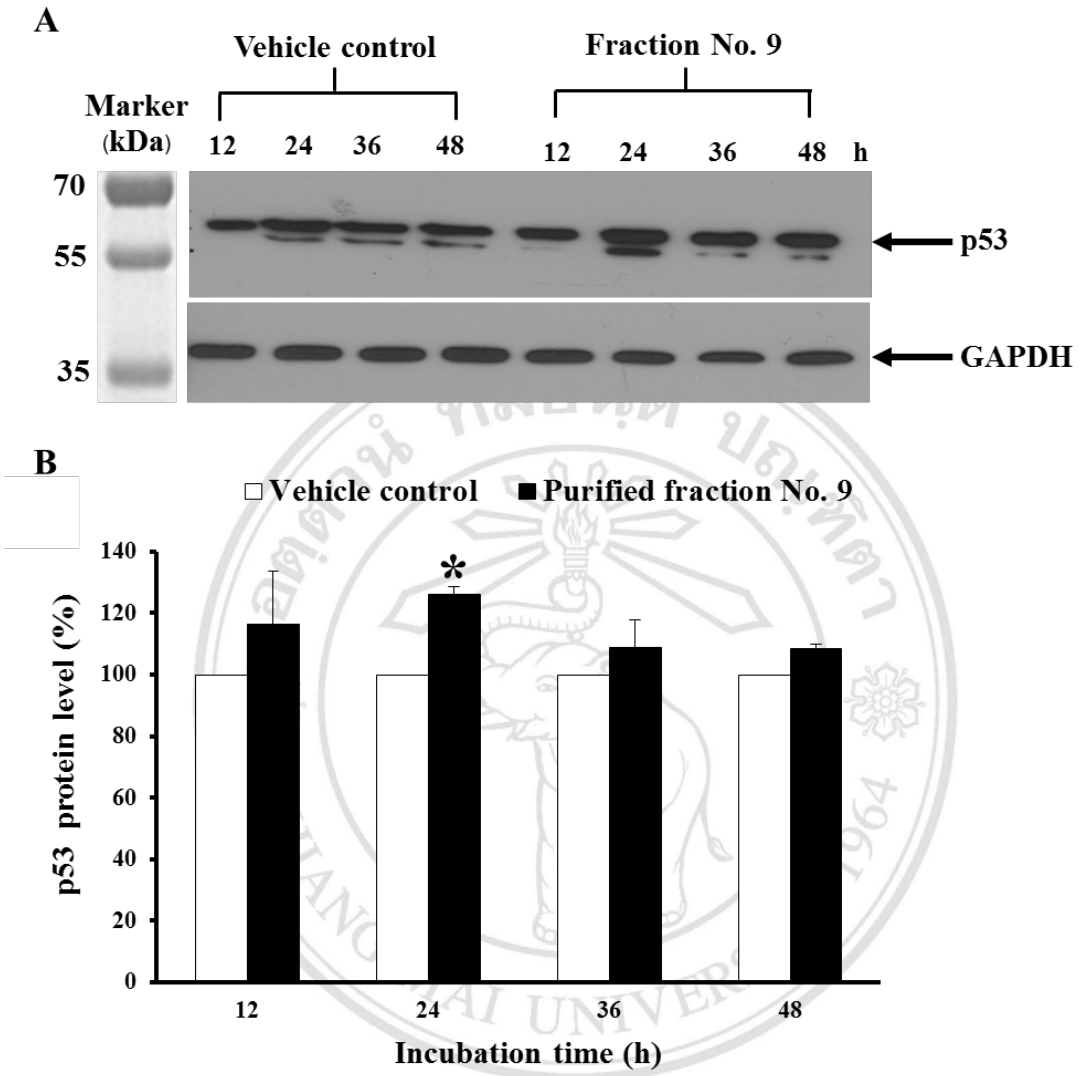

Figure 3.16 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in K562 cell line. (A) The levels of p53 protein after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.6.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in Molt4 cell line

To study the effects of incubation time of purified fraction No. 9 treatment, cells were treated with 2.5 µg/mL (IC_{20} value) and 0.08% DMSO for 12, 24, 36 and 48 h. The level of p53 protein was normalized by using GAPDH protein level and then calculated percentage of p53 protein levels (% p53 protein level). The percentages of p53 protein level of Molt4 cells were 114.3±11.7, 129.7±7.6, 137.7±13.3, and 153.0±21.8% in response to 12, 24, 36, and 48 h, respectively. The purified fraction No. 9 significantly increased the p53 protein levels in a time dependent manner at 12, 24, 36, and 48 h. by 14, 30, 38, and 53%, respectively as compared to vehicle control (Table 3.11 and Figure 3.17).

Table 3.11 Percentage of p53 protein levels after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Incubation time (h)	**% p53 protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	128.0	106.0	110.0	114.3±11.7
24	128.0	138.0	123.0	129.7±7.6*
36	123.0	149.0	141.0	137.7±13.3*
48	128.0	163.0	168.0	153.0±21.8*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

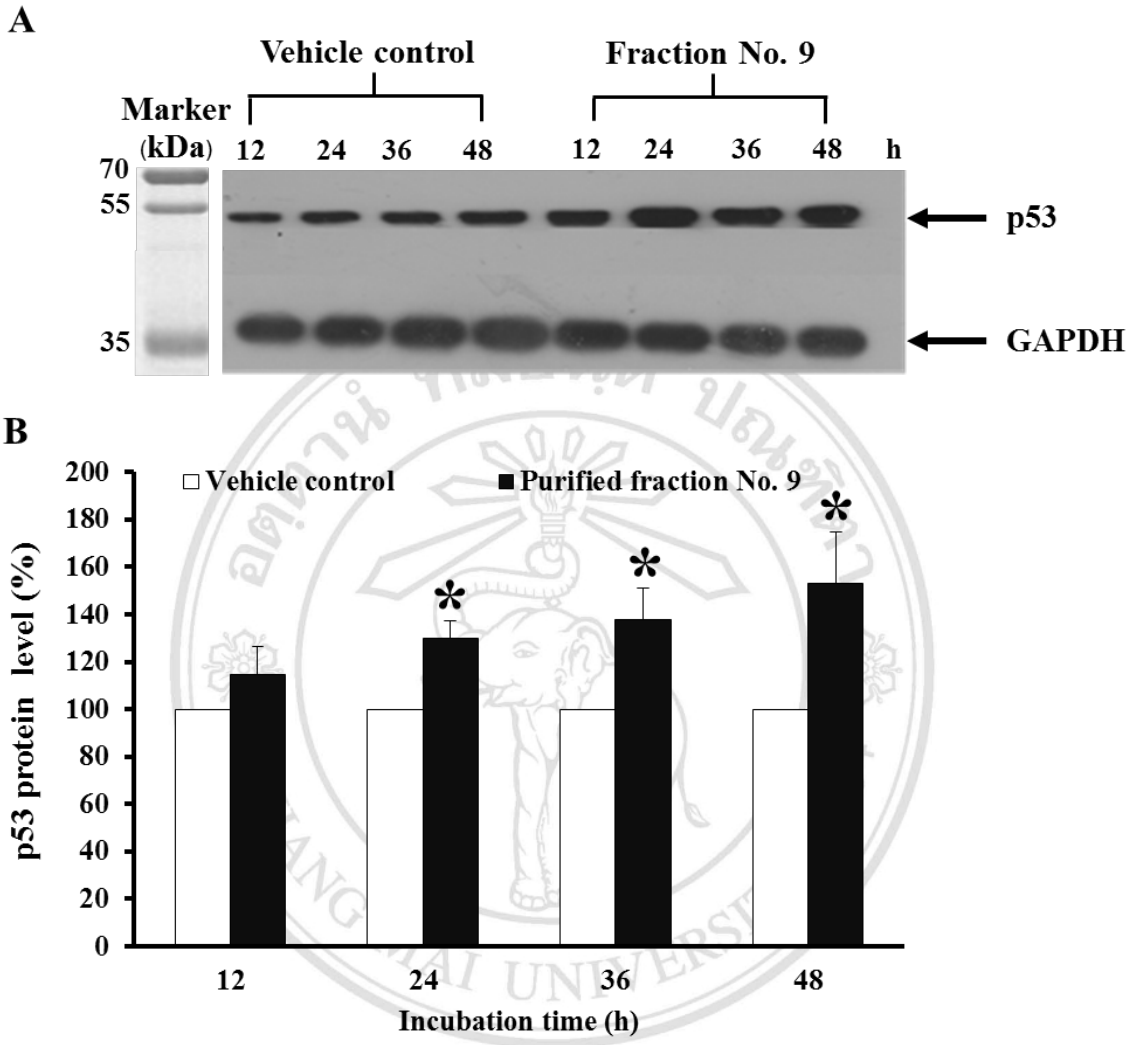

Figure 3.17 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in Molt4 cell line. (A) The levels of p53 protein after 2.5 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.7 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein expression in K562 and Molt4 cell lines

To determine the effect of incubation time periods of purified fraction No.9 treatment on targeted protein involved in G2/M phase of cell cycle regulation. This study cyclin B protein was examined. Cells were treated with purified fraction No. 9 and DMSO at the concentrations of IC_{20} values for 12, 24, 36, and 48 h. Then treated cells were extracted total protein and determined by Western blot analysis as described in section 2.9 and 2.10, respectively.

3.7.1 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf extracts on cyclin B protein levels in K562 cell line

To study the effects of incubation time of purified fraction No. 9 treatments, cells were treated with 0.04% DMSO and 10 μg/mL (IC_{20} value) for 12, 24, 36, and 48 h. The levels of cyclin B protein were normalized by using GAPDH protein and then calculated percentage of cyclin B protein level (% cyclin B protein level). The percentages of cyclin B protein levels of K562 cells were 116±14.6, 78.3±12.3, 72.6±9.3, and 52.0±9.2% in response to 12, 24, 36, and 48 h, respectively. The purified fraction No. 9 could significantly decrease the cyclin B protein levels in a time dependent manner at 24, 36, and 48 h by 22, 27, and 48%, respectively, when compared to vehicle control (Table 3.12 and Figure 3.18).

Table 3.12 Percentage of cyclin B protein levels after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in K562 cells.

Incubation time (h)	**% Cyclin B protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	121.0	128.0	100.0	116.3±14.6
24	79.0	66.0	90.0	78.3±12.0*
36	78.0	67.0	51.0	72.6±9.3*
48	61.0	44.0	51.0	52.0±9.2*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

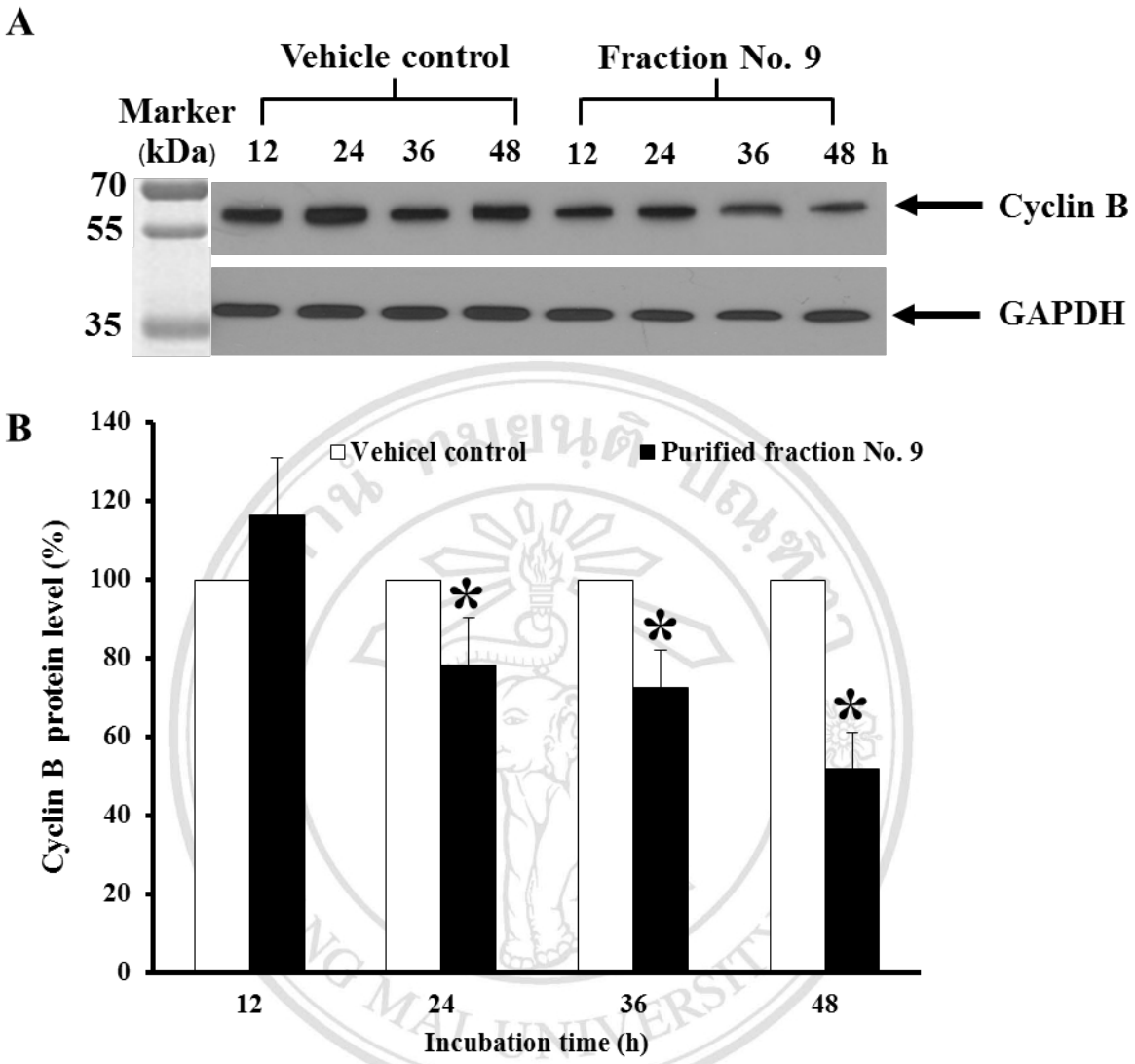

Figure 3.18 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein levels in K562 cell line. (A) The levels of cyclin B protein after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.7.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein levels in Molt4 cell line

To study the effects in a time of purified fraction No. 9 treatments, cells were treated with 2.5 µg/mL (IC_{20} value) and 0.08% DMSO for 12, 24, 36, and 48 h. The levels of cyclin B protein were normalized by using GAPDH protein and then calculated percentage of cyclin B protein level (% cyclin B protein level). The percentages of cyclin B protein levels of Molt4 cells were 74.2±21.1, 59.6±4.7, and 34.7±17.5, and 24.8±12.1 % in response to 12, 24, 36, and 48 h, respectively. The purified fraction No.9 significantly decreased the cyclin B protein levels in a time dependent manner at 12, 24, 36, and 48 h by 26, 41, 66, and 76%, respectively as compared to vehicle control (Table 3.13 and Figure 3.19).

Table 3.13 Percentage of cyclin B protein level after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Incubation time (h)	**% Cyclin B protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	98.0	67.0	57.0	74.2±21.1*
24	58.0	65.0	55.6	59.6±4.7*
36	54.2	29.8	19.9	34.7±17.5*
48	47.1	5.2	14.5	24.8±12.1*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

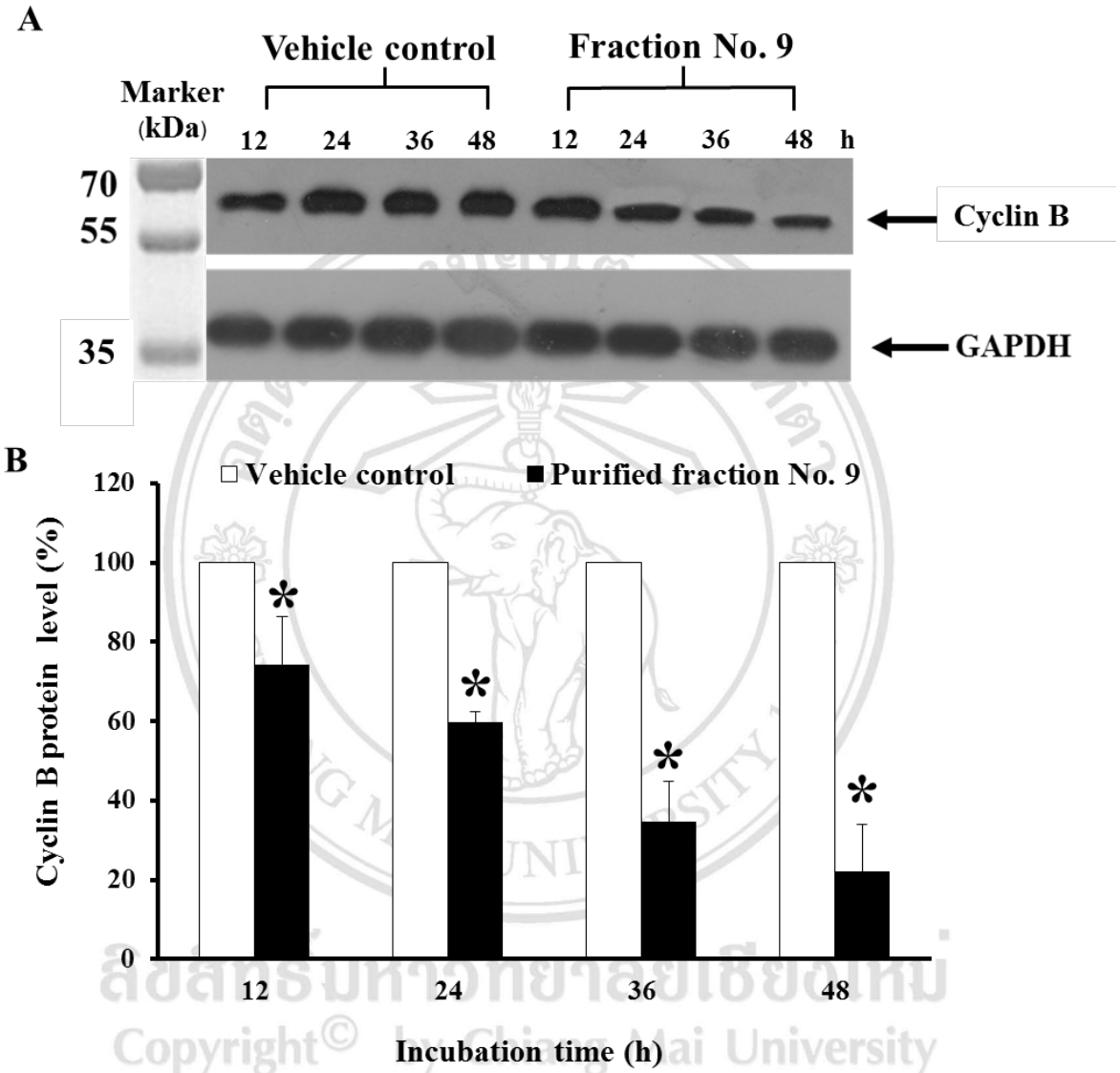

Figure 3.19 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein levels in Molt4 cells. (A) The levels of cyclin B protein after 2.5 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.8 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin A protein expressions in K562 and Molt4 cell lines

To determine the effect of incubation time periods of purified fraction No. 9 on targeted protein involved in cell cycle regulation. This study cyclin A protein was examined. Cells were treated with purified fraction No. 9 and DMSO at the concentrations of IC_{20} values for 12, 24, 36, and 48 h. Then treated cells were extracted total protein and determined by Western blot analysis as described in section 2.9.

3.8.1 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment on cyclin A protein expression in K562 cells line

To study the effects of incubation time of purified fraction No. 9 treatments, cells were treated with 0.04% DMSO and 10 µg/mL (IC_{20} value) for 12, 24, 36, and 48 h. The levels of cyclin A protein were normalized by using GAPDH protein and then calculated percentage of cyclin A protein level (% cyclin A protein level). The percentages of cyclin A protein level of K562 cells were 102.2±3.4, 84.7±7.4, 80.0±8.2, and 67.6±10.4% in the response to 12, 24, 36, and 48 h, respectively. The purified fractional No.9 significantly decreased the cyclin A protein level in a time dependent manner at 24, 36, and 48 h. by 16, 20, and 33%, respectively, compared to vehicle control (Table 3.14 and Figure 3.20).

Table 3.14 Percentage of cyclin A protein level after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in K562 cells.

Incubation time (h)	**% Cyclin A protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	109.0	99.7	98.0	102.2±3.4
24	90.0	94.0	70.0	84.7±7.4*
36	85.0	91.0	64.0	80.0±8.2*
48	84.0	68.0	48.0	66.7±10.4*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

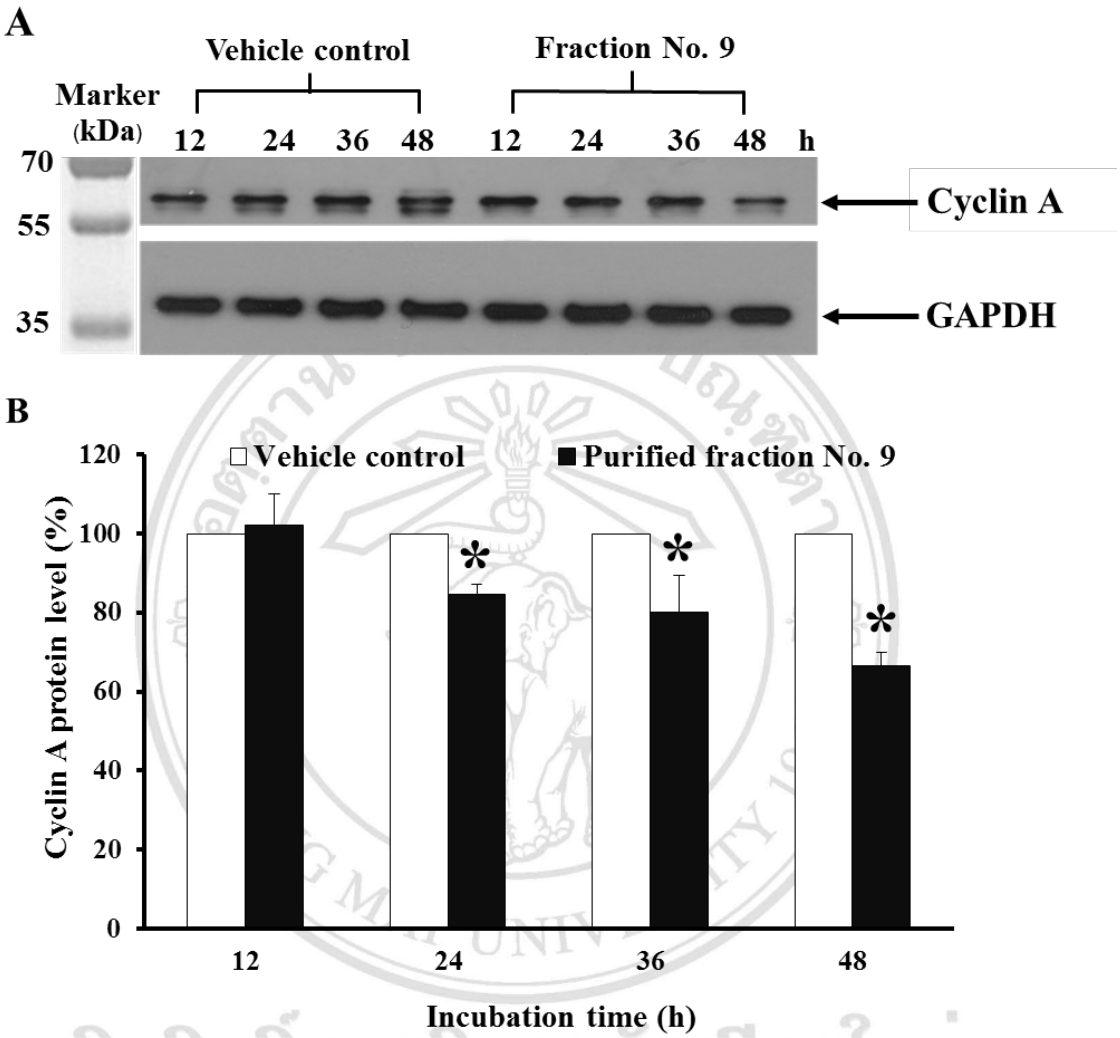

Figure 3.20 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin A protein levels in K562 cell line. (A) The levels of cyclin A protein after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.8.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment on cyclin A protein expression in Molt4 cell line

To study the effects in a time of purified fraction No. 9 treatments, cells were treated with 2.5 µg/mL (IC_{20} value) and 0.08% DMSO for 12, 24, 36, and 48 h. The level of cyclin A protein was normalized by using GAPDH protein and then calculated percentage of cyclin A protein level (% cyclin A protein level). The percentages of cyclin A protein level of Molt4 cells were 122.6±8.1, 108.0±4.4, 75.6±16.3, and 80.3±5.5% in the response to 12, 24, 36, and 48 h, respectively. The purified fraction No.9 significantly decreased the cyclin A protein levels by 24% and 20% at 36 and 48h, respectively. When compared to vehicle control (Table 3.15 and Figure 3.21).

Table 3.15 Percentage of cyclin A protein level after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Incubation time (h)	**% Cyclin A protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	124.0	130.0	114.0	122.6±8.1
24	103.0	111.0	110.0	108.0±4.4
36	83.0	57.0	87.0	75.6±16.3*
48	86.0	75.0	80.0	80.3±5.5*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

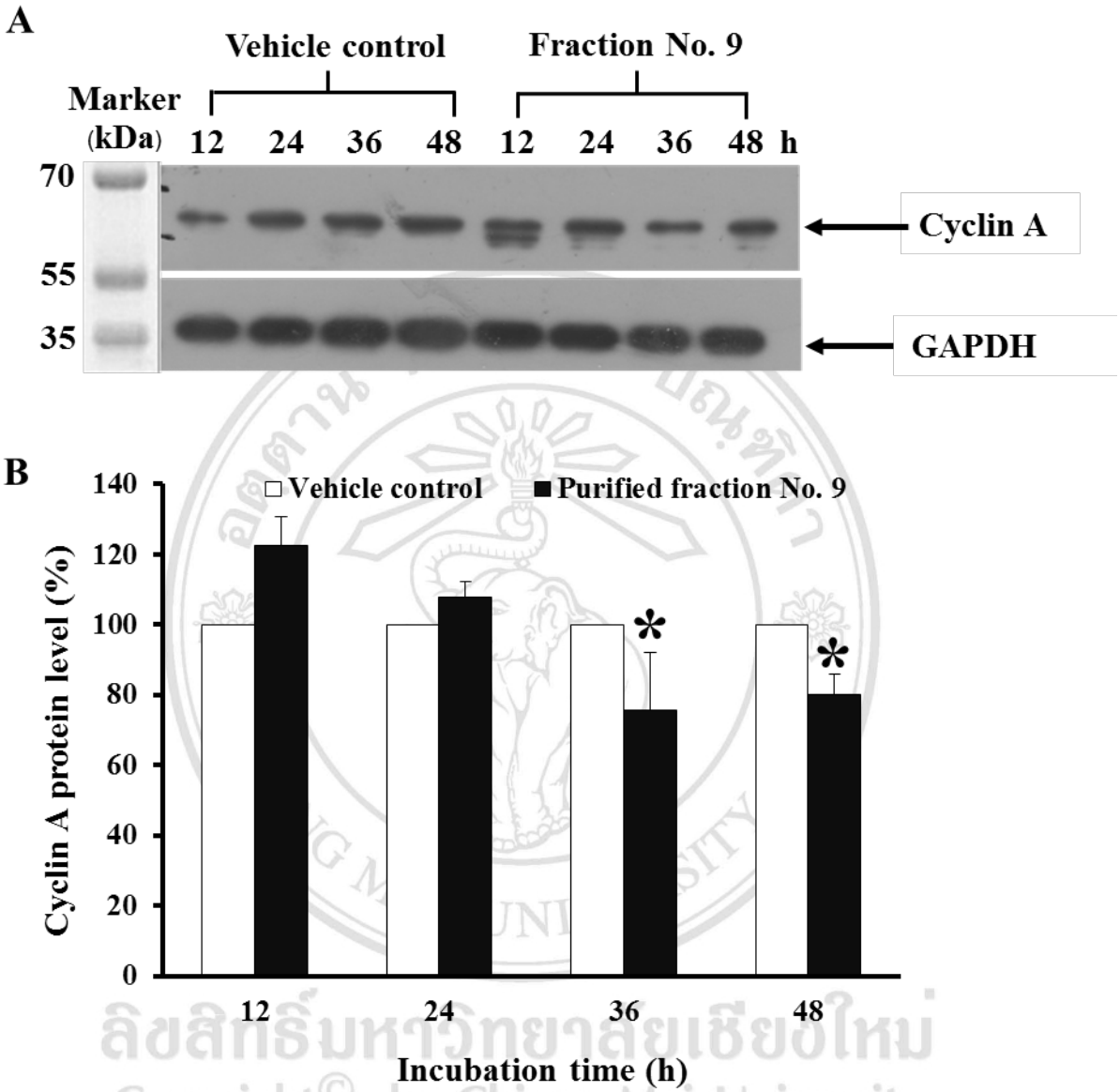

Figure 3.21 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin A protein levels in Molt4 cell line. (A) The levels of cyclin A protein after 2.5 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.9 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in K562 and Molt4 cell lines

To determine the effect of incubation time periods of purified fraction No. 9 treatment on targeted protein involved in cell cycle regulation. In this study, cyclin E protein was examined. Cells were treated with purified fraction No. 9 at the concentrations of IC_{20} values and DMSO for 12, 24, 36, and 48 h. Then treated cells were extracted total protein and determined by Western blot analysis as described in section 2.9.

3.9.1 Effect of various time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in K562 cell line

To study the effect of incubation time of purified fraction No. 9 treatments, cells were treated with 0.04% DMSO and 10 μg/mL (IC_{20}value) for 12, 24, 36, and 48 h. The levels of cyclin E protein were normalized by using GAPDH protein and then calculated percentage of cyclin E protein level (% cyclin E protein level). The percentages of cyclin E protein levels of K562 cells were 105.6±20.8, 89.8±20.0, 80.2±10.0, and 51.5±8.1% in the response to 12, 24, 36 and 48 h, respectively. The purified fraction No.9 significantly decreased the cyclin E protein levels in a time dependent manner at 24, 36, and 48 h. by 10.2, 19.8, and 48.5%, respectively, compared to vehicle control. At 36 and 48 h treatments showed statistically significant difference when compared to that of vehicle control (Table 3.16 and Figure 3.22).

Table 3.16 Percentage of cyclin E protein level after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in K562 cells.

Incubation time (h)	**% Cyclin E protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	130.0	97.0	90.0	106.3±20.8
24	112.0	84.6	72.8	89.8±20.0
36	87.6	69.3	83.6	80.2±10.0*
48	56.6	42.4	55.4	51.5±8.1*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

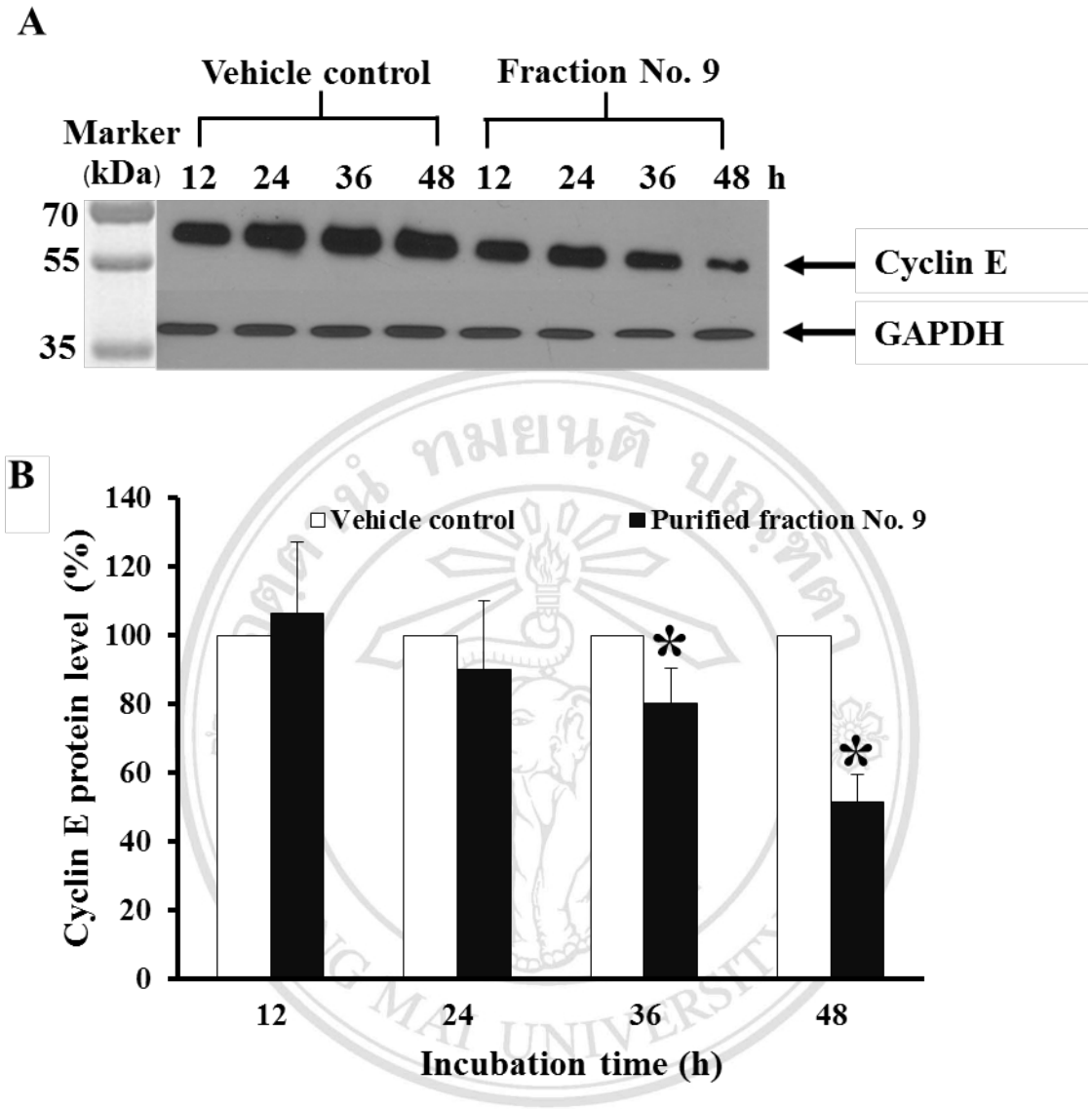

Figure 3.22 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in K562 cell line. (A) The levels of cyclin E protein after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.9.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in Molt4 cell line

To study the effect of incubation time of purified fraction No. 9 treatment, cells were treated with 2.5 µg/mL (IC_{20} value) and 0.08% DMSO for 12, 24, 36 and 48 h. The level of cyclin E protein was normalized by using GAPDH protein level and then calculated percentage of cyclin E protein level (% cyclin E protein level). The percentages of cyclin E protein levels of Molt4 cells were 94.7±12.8, 95.7±14.3, 90.7±5.5, and 81.3±7.1% in response to 12, 24, 36, and 48 h, respectively. The purified fraction No. 9 decreased the cyclin E protein level by 6.3, 5.3, 9.3, and 18.7%, respectively, compared to vehicle control (Table 3.17 and Figure 3.23).

Table 3.17 Percentage of cyclin E protein level after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Incubation time (h)	**% Cyclin E protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	108.0	84.0	89.6	94.7±12.8
24	112.0	84.0	89.6	95.7±14.3
36	87.7	96.9	86.6	90.7±5.5
48	89.0	74.5	79.8	81.3±7.1*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

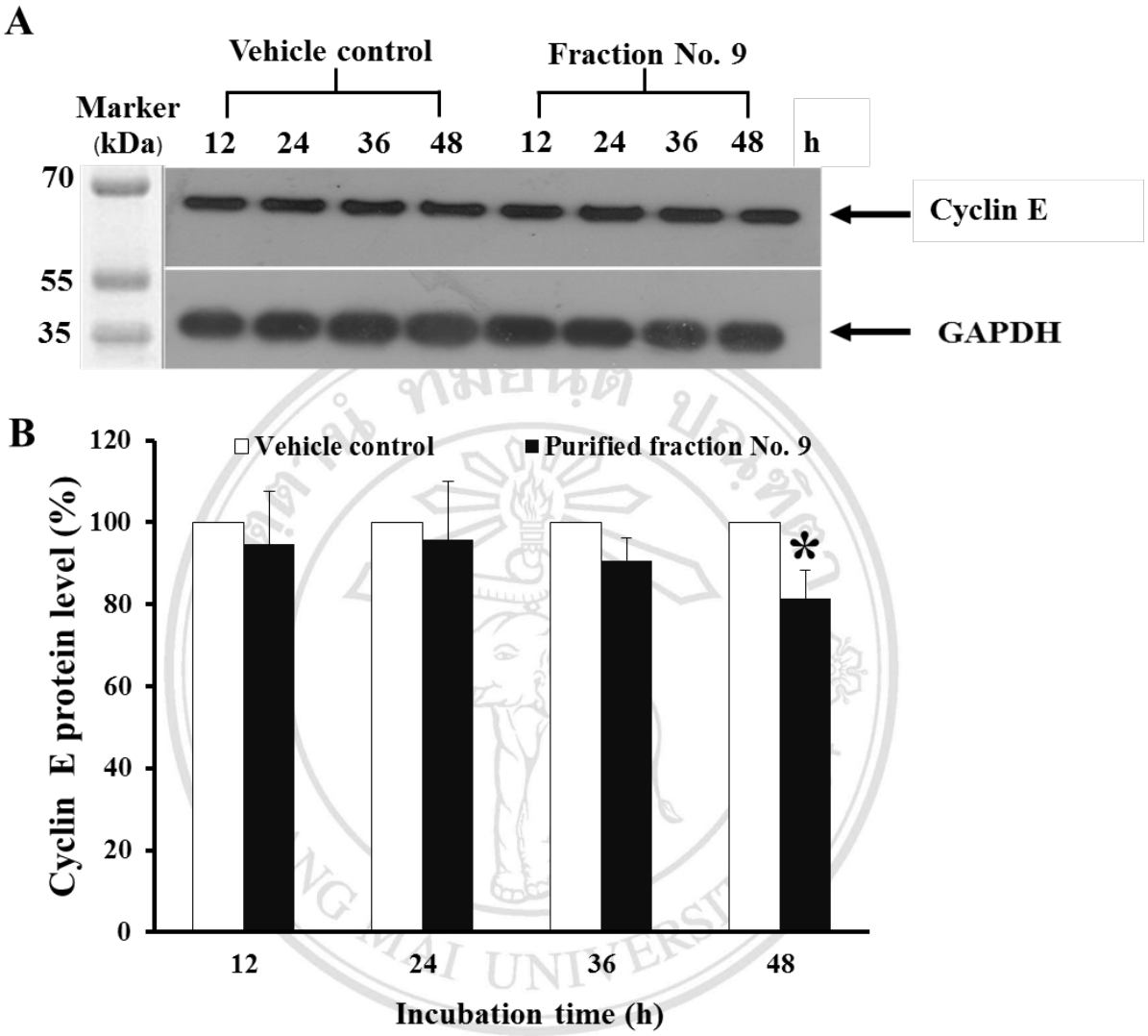

Figure 3.23 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in Molt4 cell line. (A) The levels of cyclin E protein after 2.5 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.10 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in K562 and Molt4 cell lines

To determine the effect of incubation time period of purified fraction No. 9 treatment on targeted protein involved in G2/M phase of cell cycle regulation. In this study, cdc2 protein level was examined. Cells were treated with purified fraction No. 9 at the concentrations of IC_{20} values and DMSO for 12, 24, 36, and 48 h. Then treated cells were extracted total protein and determined by Western blot analysis as described in section 2.9.

3.10.1 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in K562 cell line

To study the effects of incubation time of purified fraction No. 9 treatment, cells were treated with 0.04% DMSO and 10 µg/mL (IC_{20} value) for 12, 24, 36, and 48 h. The levels of cdc2 protein were normalized by using GAPDH protein and then calculated percentage of cdc2 protein level. The percentages of cdc2 protein levels in K562 cells were 106.3±12.9, 78.7±21.9, 48.3±17.2, and 45.0±19.1% in the response to 12, 24, 36, and 48 h, respectively. The purified fraction No. 9 significantly decreased the cdc2 protein levels in a time dependent manner at 24, 36, and 48 h by 21.3, 51.7, and 55.0%, respectively, compared to vehicle control (Table 3.18 and Figure 3.24).

Table 3.18 Percentage of cdc2 protein level after 10 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in K562 cells.

Incubation time (h)	**% Cdc2 protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	97.0	101.0	121.0	106.3±12.9
24	96.0	54.0	86.0	78.7±21.9*
36	41.0	36.0	68.0	48.3±17.2*
48	63.0	25.0	47.0	45.0±19.1*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

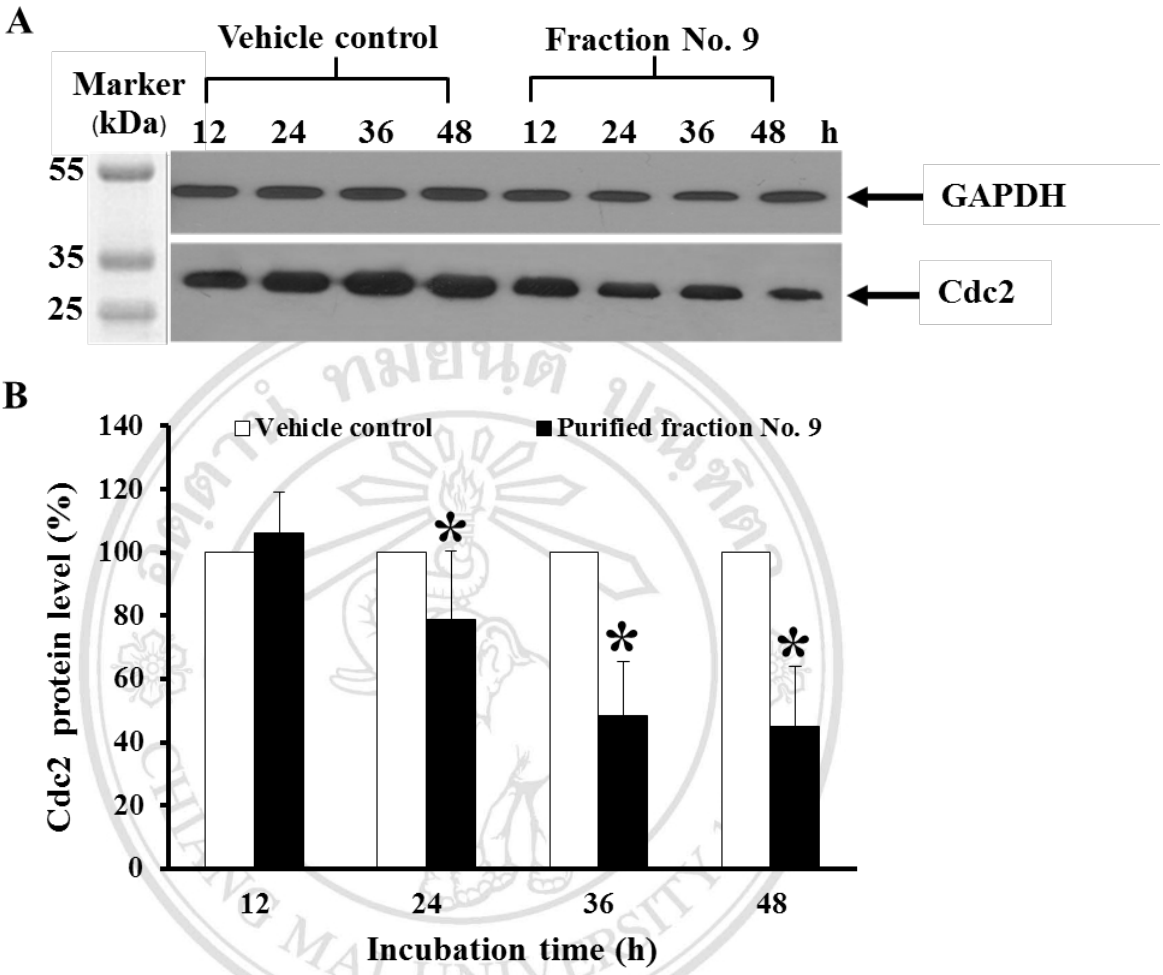

Figure 3.24 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in K562 cell line. (A) The levels of cdc2 protein levels after 10 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.10.2 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in Molt4 cell line

To study the effects in a time of purified fraction No. 9 treatments, cells were treated with 2.5 µg/mL (IC_{20} value) and 0.08% DMSO for 12, 24, 36, and 48 h. The levels of cdc2 protein were normalized by using GAPDH protein and then calculated percentage of cdc2 protein level. The percentages of cdc2 protein levels of Molt4 cells were 103.6±22.8, 94.8±15.0, and 56.3±15.0, and 52.3±17.5% in the response to 12, 24, 36, and 48 h, respectively. The purified fraction No.9 significantly decreased the cdc2 protein level in a time dependent manner at 36 and 48 h. by 43.7 and 47.7%, respectively, compared to vehicle control (Table 3.19 and Figure 3.25).

Table 3.19 Percentage of cdc2 protein level after 2.5 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 12, 24, 36, and 48 h in Molt4 cells.

Incubation time (h)	**% Cdc2 protein level**			
	1	**2**	**3**	**Mean±SD**
Vehicle control (12, 24, 36, and 48 h.)	100.0	100.0	100.0	100.0±0
12	128.0	83.0	99.0	103.6±22.8
24	110.0	80.2	94.1	94.8±15.0
36	35.0	99.1	52.0	56.3±15.0*
48	45.0	72.0	58.0	52.3±17.5*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

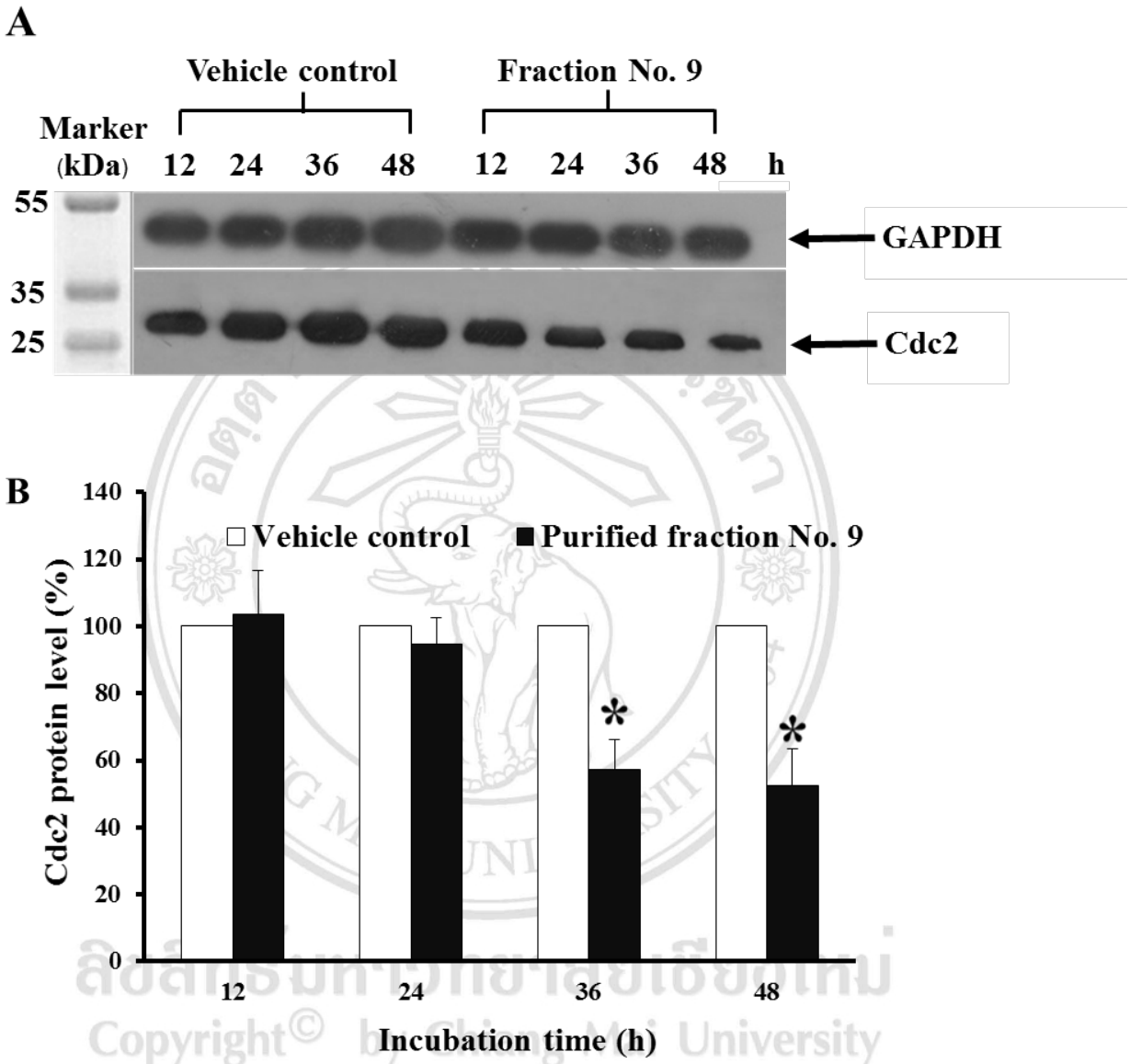

Figure 3.25 Effect of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in Molt4 cell line. (A) The levels of cdc2 protein after 2.5 µg/mL of purified fraction No. 9 treatments for 12, 24, 36, and 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.11 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in K562 and Molt4 cell lines

According to the results from section 3.6, the purified fraction No. 9 had the strongest inhibitory effect on targeted protein expressions in K562 and Molt4 cell lines. In addition, the various concentrations of purified fraction No. 9 were used to study p53 protein expression. K562 cells were treated with concentrations of 5, 10, and 15 µg/mL for 24 h and Molt4 were treated with various concentrations at 1.0, 2.5, and 5 µg/mL for 48 h. Then treated cells were extracted total protein and determined by Western blot analysis as described in section 2.9.

3.11.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in K562 cell line

To determine the effect of concentrations of purified fraction No.9 on p53 protein levels in K562 cells, cells were treated with complete RPMI-1640 medium containing 0.04% DMSO (vehicle control) and 5, 10, and 15µg/mL of purified fraction No. 9 for 24 h. Cells were harvested and determined p53 protein levels by Western blotting. The levels of p53 protein were normalized by using GAPDH protein and then calculated percentage of p53 protein levels. The percentages of p53 protein levels were 103.4±6.1, 122.1 ±7.2, and 132.3±12.5% in the response to 5, 10, and 15 µg/mL, respectively. The protein levels of p53 increased by 3.4, 22.1, and 32.3%, respectively, in response to concentrations of 5, 10, and 15 µg/mL of the purified fraction No. 9. The concentrations of 10 and 15 µg/mL significantly increased p53 protein levels as compared to the vehicle control (p<0.05) (Table 3.20 and Figure 3.26).

Table 3.20 Percentage of p53 protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments at 5, 10, and 15 µg/mL for 24 h in K562 cells.

Concentration (µg/mL)	**% p53 protein level**			
	1	**2**	**3**	**Mean±SD**
0	100.0	100.0	100.0	100.0±0
5	110.0	102.0	98.0	103.4±6.1
10	120.0	116.0	130.0	122.1±7.2*
15	132.0	145.0	120.0	132.3±12.5*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

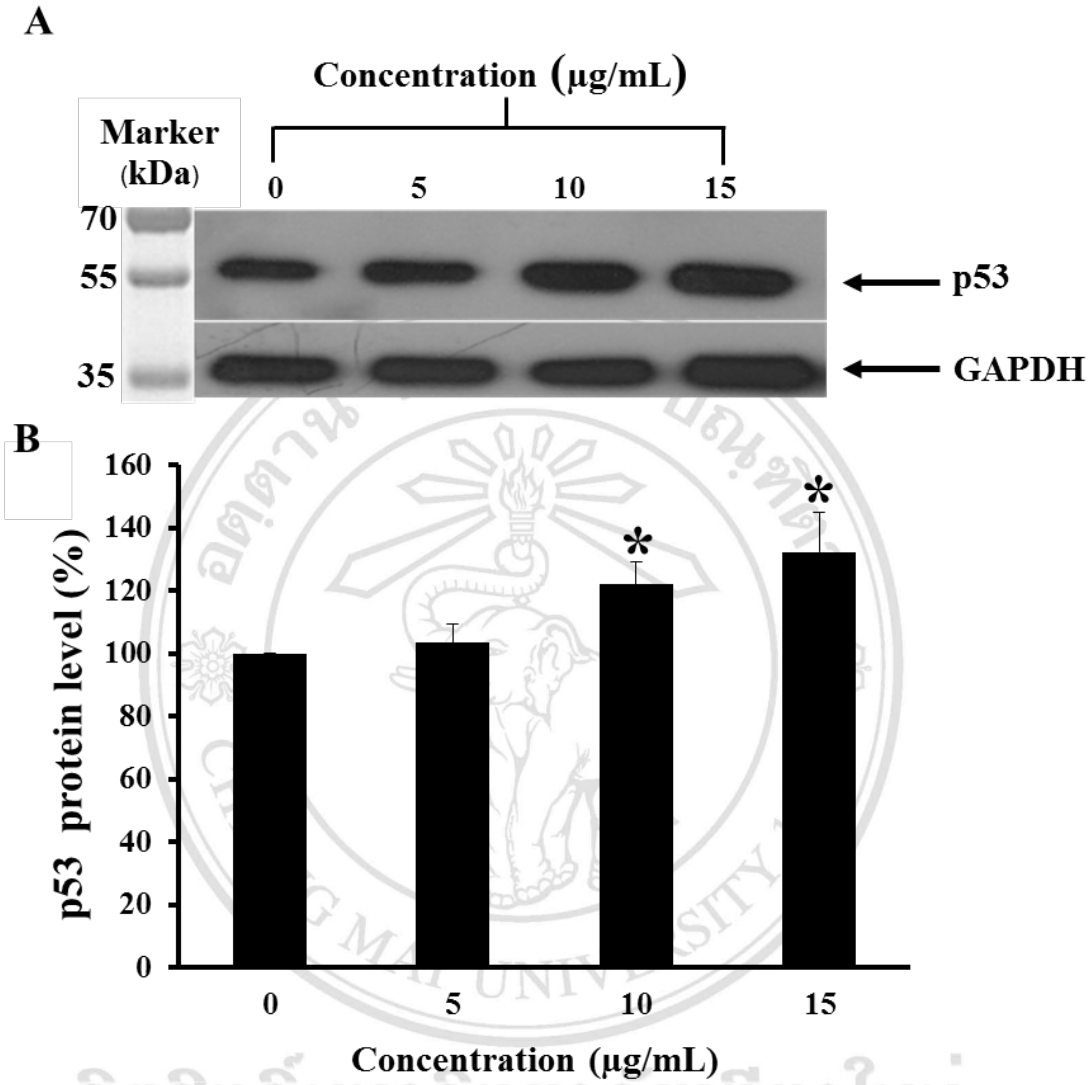

Figure 3.26 Effect of concentration of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts on p53 protein for 24 h in K562 cell. K562 cells were treated with 5, 10, and 15 µg/mL of purified fraction No. 9 for 24 h. (A) The levels of p53 protein after treatments of purified fraction No. 9 treatments by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.11.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on p53 protein levels in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 on p53 protein in Molt4 cells, cells were treated with medium containing 0.08% DMSO (vehicle control) and 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 for 48 h. The protein level of p53 was normalized by using GAPDH protein and calculated percentage of p53 protein level. The percentages of p53 protein levels were 106.3±7.6, 125.6±6.6, and 134.7±13.1% in the response to 1.0, 2.5, and 5.0 µg/mL, respectively. The p53 protein levels increased by 6.0, 25.6, and 34.7% respectively, in response to concentrations of 1.0, 2.5, and 5.0 µg/mL of the purified fraction No. 9 of hexane fractional extract from kaffir lime leaf. The concentrations of 2.5 and 5.0 µg/mL significantly increased p53 protein levels as compared to the vehicle control (p<0.05) (Table 3.21 and Figure 3.27).

Table 3.21 Percentage of p53 protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment at 1.0, 2.5, and 5.0 µg/mL for 48 h in Molt4 cells.

Concentration (µg/mL)	% p53 protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
1.0	98.0	113.0	108.0	106.0±7.6
2.5	118.0	129.0	130.0	125.6±6.6*
5.0	145.0	139.0	120.0	134.7±13.1*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

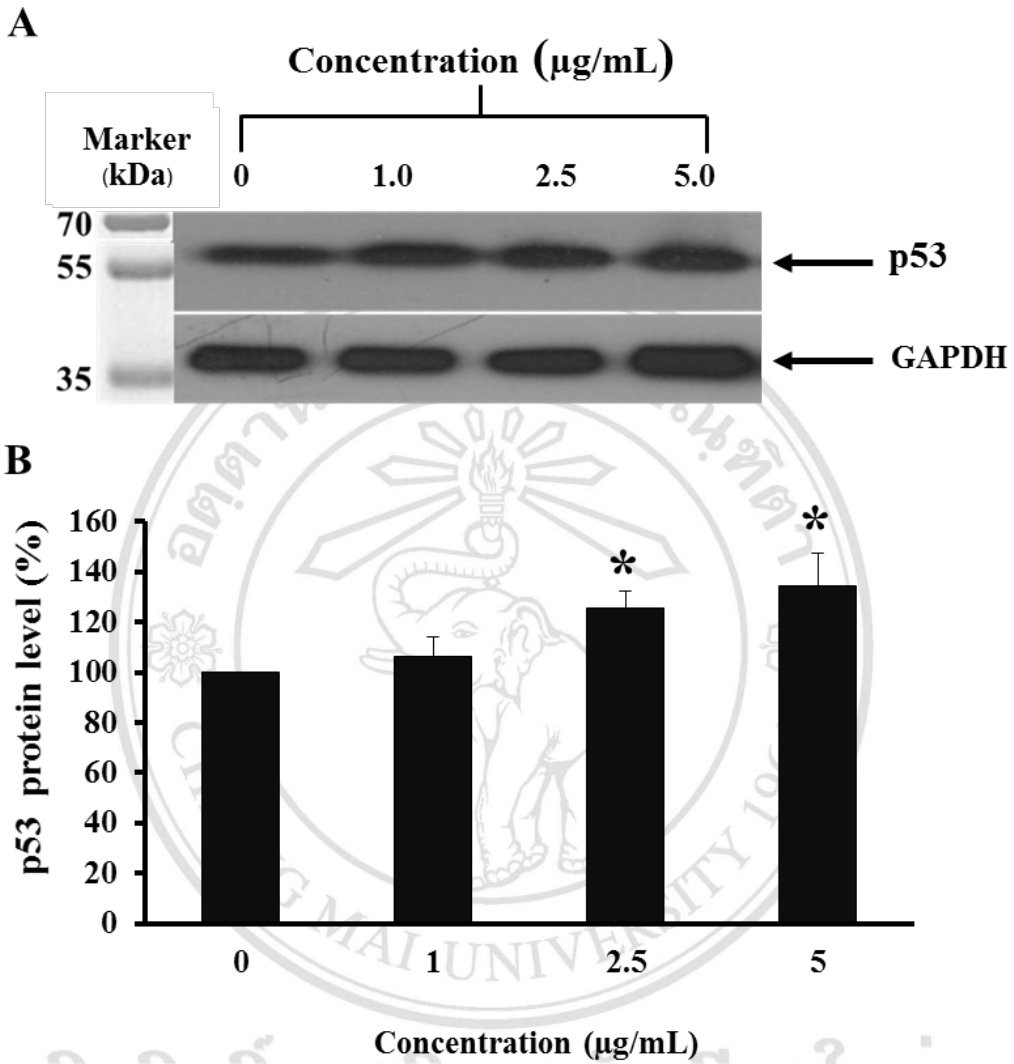

Figure 3.27 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on p53 protein for 48 h in Molt4 cells. Molt4 cells were treated with 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 for 48 h. (A) The levels of p53 protein expression after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.12 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin B protein expression in K562 and Molt4 cell lines

According to the results from section 3.7, the purified fraction No. 9 of hexane fractional extract from kaffir lime leaf had the strongest inhibitory effect on targeted protein expressions in K562 and Molt4 cell lines. In addition, the various concentrations of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf were used to study cyclin B protein expressions; cyclin B proteins. K562 cells were treated with 5, 10, and 15 µg/mL while, Molt4 cells were treated with 1.0, 2.5, and 5 µg/mL for 48 h. Then treated cells were extracted and determined by Western blot analysis as described in section 2.9.

3.12.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein levels in K562 cell line

To determine the effect of concentration of purified fraction No. 9 on cyclin B protein in K562 cells, cells were treated with complete RPMI-1640 medium containing 5, 10, and 15 µg/mL of purified fraction No. 9 and 0.08% DMSO (vehicle control) for 48 h. The level of cyclin B protein was normalized by using GAPDH protein and calculated percentage of cyclin B protein level. The percentages of cyclin B protein levels were 92.0±6.0, 64.6±12.7, and 53.3±7.7% in the response to 5, 10, and 15 µg/mL, respectively. The protein levels of cyclin B decreased by 8, 36.4, and 46.7%, respectively, in response to concentrations of 5, 10, and 15 µg/mL of the purified fraction No. 9. The concentrations of 10 and 15 µg/mL The concentrations of 10 and 15 µg/mL significantly decreased the cyclin B protein levels as compared to the vehicle control (p<0.05) (Table 3.22 and Figure 3.28).

Table 3.22 Percentage of cyclin B protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment at 5, 10, and 15 µg/mL for 48 h in K562 cells.

Concentration (µg/mL)	% Cyclin B protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
5	87.0	99.0	90.0	92.0±6.0
10	79.0	60.0	55.0	64.5 ±12.7*
15	55.0	60.0	45.0	53.4 ±7.7*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

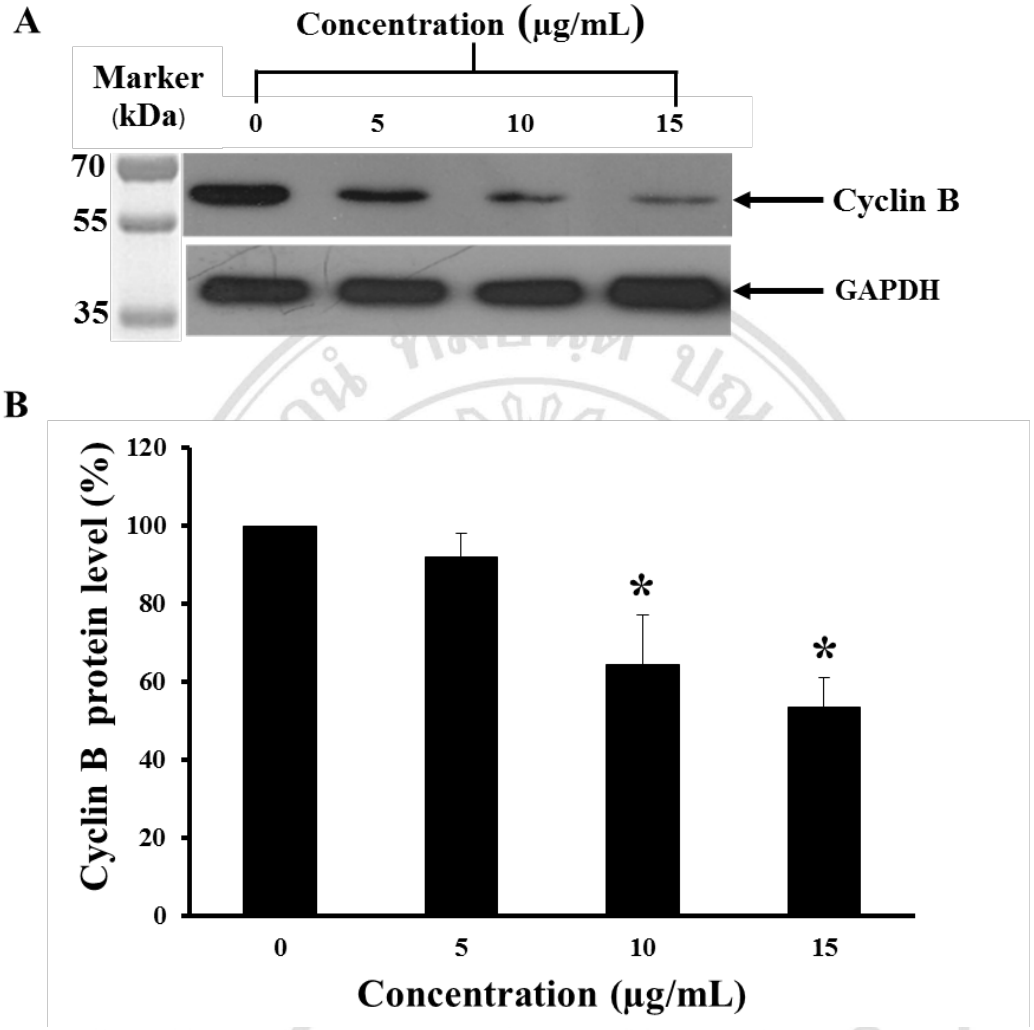

Figure 3.28 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin B protein for 48 h in K562 cell line. Cells were treated with 5, 10, and 15 µg/mL of purified fraction No. 9 for 48 h. (A) The levels of cyclin B protein expression after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.12.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin B protein expression in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 on cyclin B protein in Molt4 cells, cells were treated with complete RPMI-1640 medium containing 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 and 0.08% DMSO (vehicle control) for 48 h. The level of cyclin B protein was normalized by using GAPDH protein and calculated percentage of cyclin B protein level (% cyclin B protein level). The percentages of cyclin B protein levels were 91.6±9.3, 77.3±2.1, and 69.3±6.7% in the response to 1.0, 2.5, and 5.0 µg/mL, respectively. The cyclin B protein levels decreased by 8.4, 22.7, and 30.6%, in the response to 1.0, 2.5, and 5.0 µg/mL of the purified fraction No. 9. The concentrations of 2.5 and 5.0 µg/mL significantly decreased cyclin B protein levels as compared to the vehicle control (p<0.05) (Table 3.23 and Figure 3.29).

Table 3.23 Percentage of cyclin B protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments at 1.0, 2.5, and 5.0 µg/mL for 48 h in Molt4 cells.

Concentration (µg/mL)	% Cyclin B protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
1.0	98.0	81.0	96.0	91.6±9.3
2.5	75.0	78.0	79.0	77.3±2.1*
5.0	75.0	62.0	71.0	69.4±6.7*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

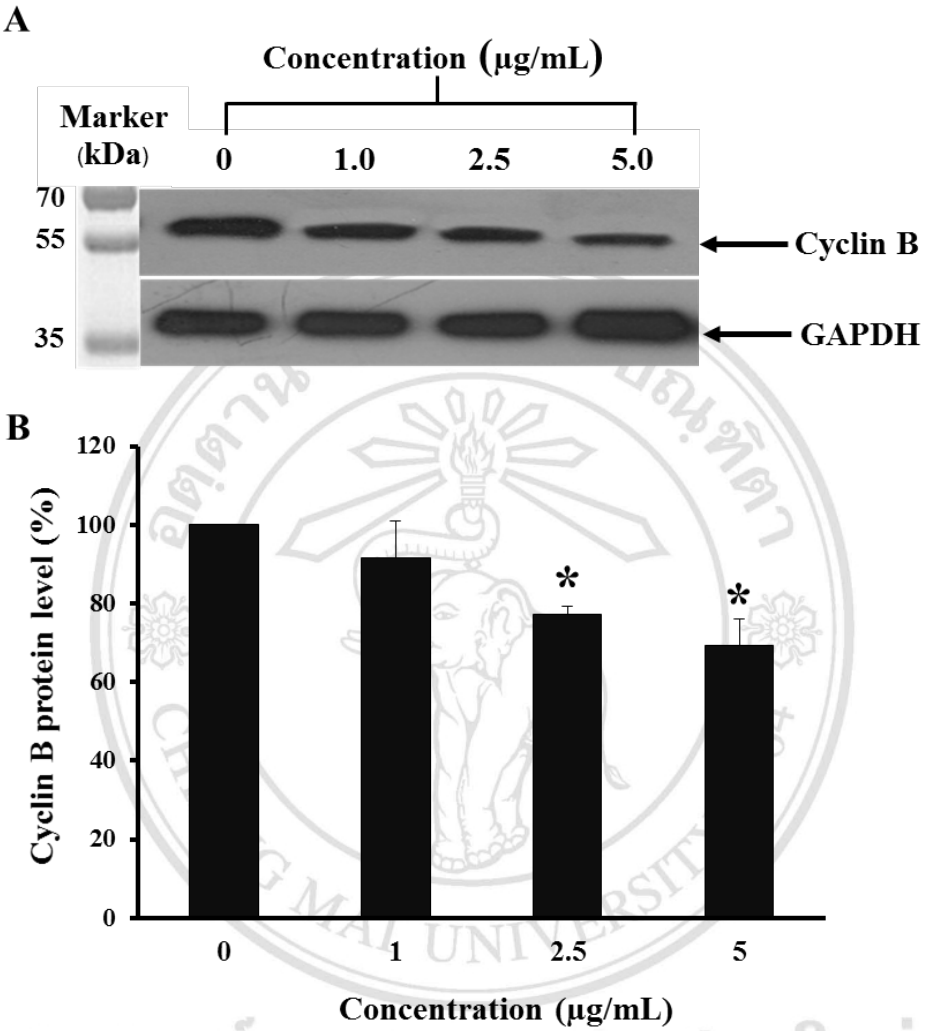

Figure 3.29 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin B protein levels for 48 h in Molt4 cell line. Cells were treated with 1.0, 2.5, and 5.0 µg/mL of purified fraction for 48 h. (A) The levels of cyclin B protein expression after purified fraction No.9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.13 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein expression in K562 and Molt4 cell lines

According to the results from section 3.8, the purified fraction No. 9 of hexane fraction kaffir lime left extracts had the strongest inhibitory effect on targeted protein expressions in Molt4 and K562 cells. In addition, the various concentrations of purified fraction No. 9 were used to study cyclin A protein expression. K562 cells were treated with 5, 10, and 15 µg/mL for 48 h While, Molt4 cells were treated with 1.0, 2.5, and 5 µg/mL of purified fraction No. 9 for 36 h. Then treated cells were extracted and determined by Western blot analysis as described in section 2.9.

3.13.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein expression in K562 cells line

To determine the effect of concentration of purified fraction No. 9 on cyclin A protein in K562 cells, cells were treated with medium containing 5, 10, and 15 µg/mL of purified fraction No. 9 and 0.04% DMSO (vehicle control) for 48 h. Cells were harvested and determined cyclin A protein levels by Western blotting. The level of cyclin A protein was normalized by using GAPDH protein and then calculated percentage of cyclin A protein level. The percentages of cyclin A protein level of K562 cells were 93.0±13.2, 82.7 ±10.1, and 70.0±5.6% in the response to 5, 10, and 15 µg/mL, respectively. The cyclin A protein level decreased in a dose dependent manner by 6.7, 17.3, and 30%, respectively, The concentrations of 10 and 15 µg/mL significantly decreased cyclin A protein levels as compared to the vehicle control (p<0.05) (Table 3.24 and Figure 3.30).

Table 3.24 Percentage of cyclin A protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatment 5, 10, and 15 µg/mL for 48 h in K562 cells.

Concentration (µg/mL)	% Cyclin A protein level			
	1	2	3	Mean±SEM
0	100.0	100.0	100.0	100.0±0
5	98.0	103.0	78.0	93.0±13.2
10	88.0	71.0	89.0	82.7±10.1*
15	65.0	76.0	69.0	70.0±5.6*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

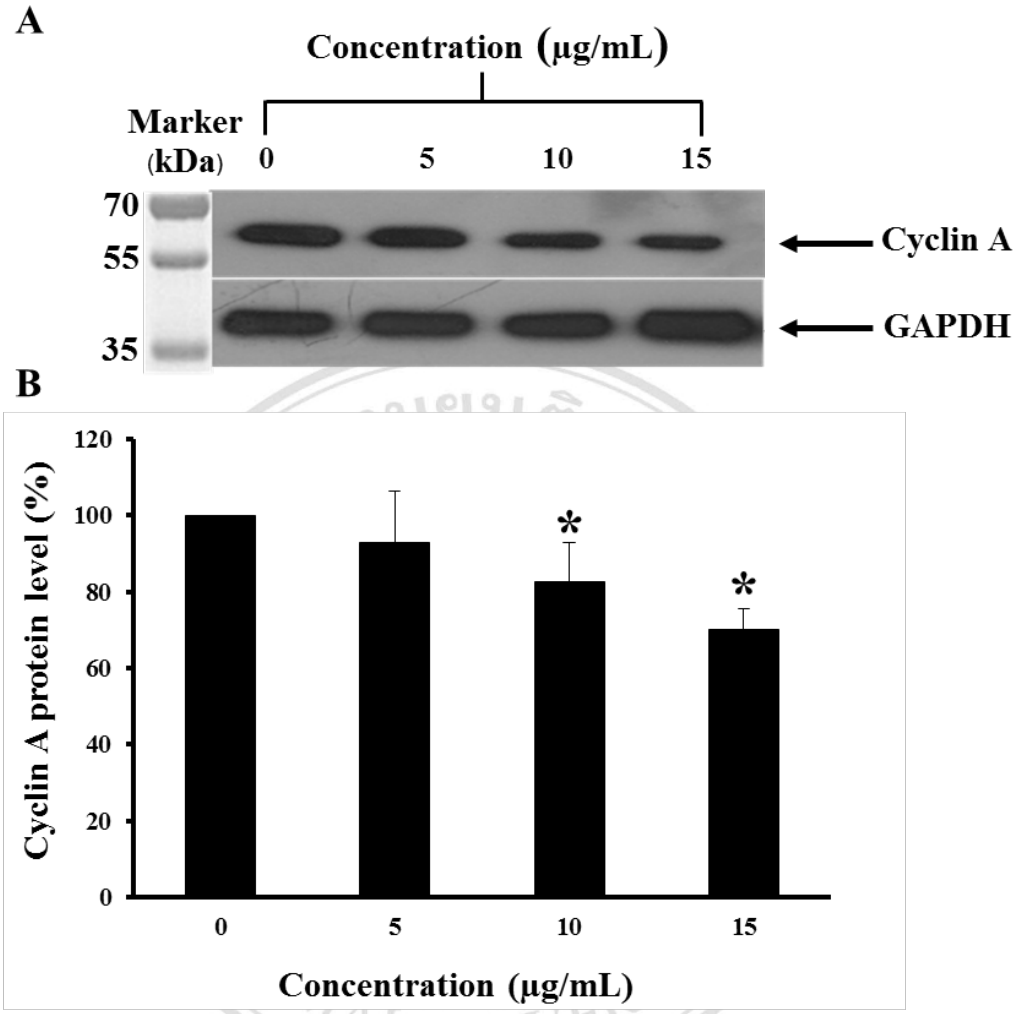

Figure 3.30 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein for 48 h in K562 cell line. Cells were treated with 5, 10, and 15 µg/mL of purified fraction No.9 treatments for 48 h. (A) The levels of cyclin A protein expression after purified fraction No.9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.13.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin A protein expression in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 on cyclin A protein in Molt4 cells, cells were treated with medium containing 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 and 0.08% DMSO (vehicle control) for 36 h. The protein level of cyclin A was normalized by using GAPDH protein and calculated percentage of cyclin A protein level. The percentages of cyclin A protein levels were 96.0±6.9, 81.3±5.6, and 72.0±10.5% in the response to 1.0, 2.5, and 5.0 µg/mL, respectively. The cyclin A protein levels decreased in a dose dependent manner by 4, 18.7, and 28.3%. The concentrations of 2.5, and 5.0 µg/mL of the purified fraction significantly decreased cyclin A protein levels as compared to the vehicle control (p<0.05) (Table 3.25 and Figure 3.31).

Table 3.25 Percentage of cyclin A protein level after purified fraction No. 9 of hexane fractional extract kaffir lime left treatment 1.0, 2.5, and 5.0 µg/mL for 36 h in Molt4 cells.

Concentration (µg/mL)	% Cyclin A protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
1.0	92.0	104.0	92.0	96.0±6.9
2.5	86.0	75.0	83.0	81.3±5.6*
5.0	83.0	71.0	62.0	72.0±10.5*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

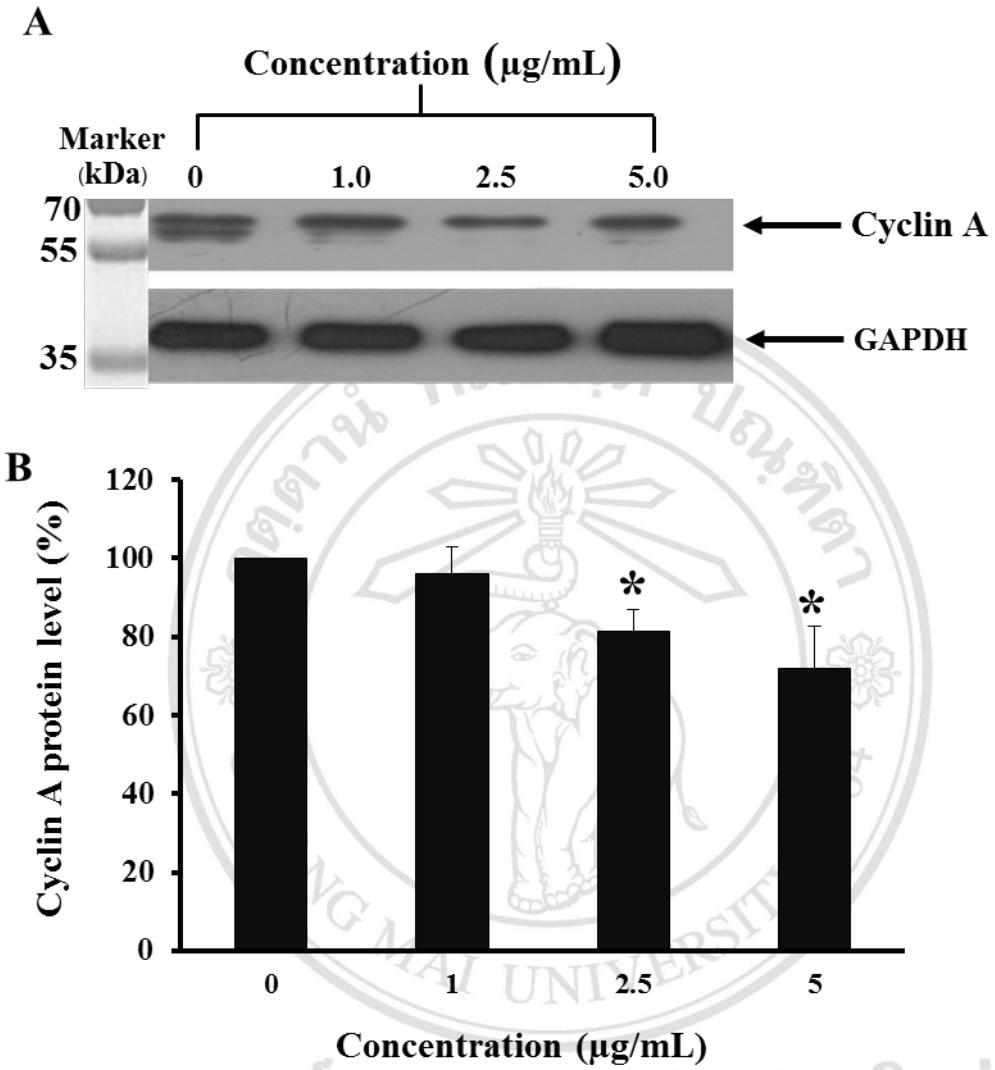

Figure 3.31 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin A protein levels for 48 h in Molt4 cell line. Molt4 cells were 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 treatments for 36 h. (A) The levels of cyclin A protein expression after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.14 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in K562 and Molt4 cell lines

According to the results from section 3.9, the of purified fraction No. 9 of hexane fraction kaffir lime left extracts had the strongest inhibitory effect on targeted protein expressions in Molt4 and K562 cells. In addition, the various concentrations of purified fraction No. 9 were used to study cyclin E protein expression. K562 cells were treated with 5, 10, and 15 µg/mL for 48 h while, Molt4 cells were treated with 1.0, 2.5, and 5 µg/mL for 36 h. Then treated cells were extracted and determined by Western blot analysis as described in section 2.9.

3.14.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in K562 cell line

To determine the effect of concentration of purified fraction No. 9 on cyclin E protein in K562 cells, cells were treated with medium containing 5, 10, and 15 µg/mL of purified fraction No. 9 and 0.04% DMSO (vehicle control) for 48 h. Cells were harvested and determined cyclin E protein levels by Western blotting. The levels of cyclin E protein were normalized by using GAPDH protein and then calculated percentage of cyclin E protein levels. The percentages of cyclin E protein level were 96.0±5.0, 82.0 ±10.1, and 68.3±10.6% in the response to 5, 10, and 15 µg/mL, respectively. The cyclin E protein levels decreased in a dose dependent manner by 4, 18, and 31.7%, respectively, The concentrations of 10 and 15 µg/mL of the fraction significantly decreased cyclin E protein levels as compared to the vehicle control (p<0.05) (Table 3.24 and Figure 3.32).

Table 3.26 Percentage of cyclin E protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments at 5, 10, and 15 µg/mL for 48 h in K562 cells.

Concentration (µg/mL)	% Cyclin E protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
5	97.0	91.0	101.0	96.0±5.0
10	84.0	71.0	91.0	82.0±10.1*
15	57.0	78.0	70.0	68.3±10.6*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

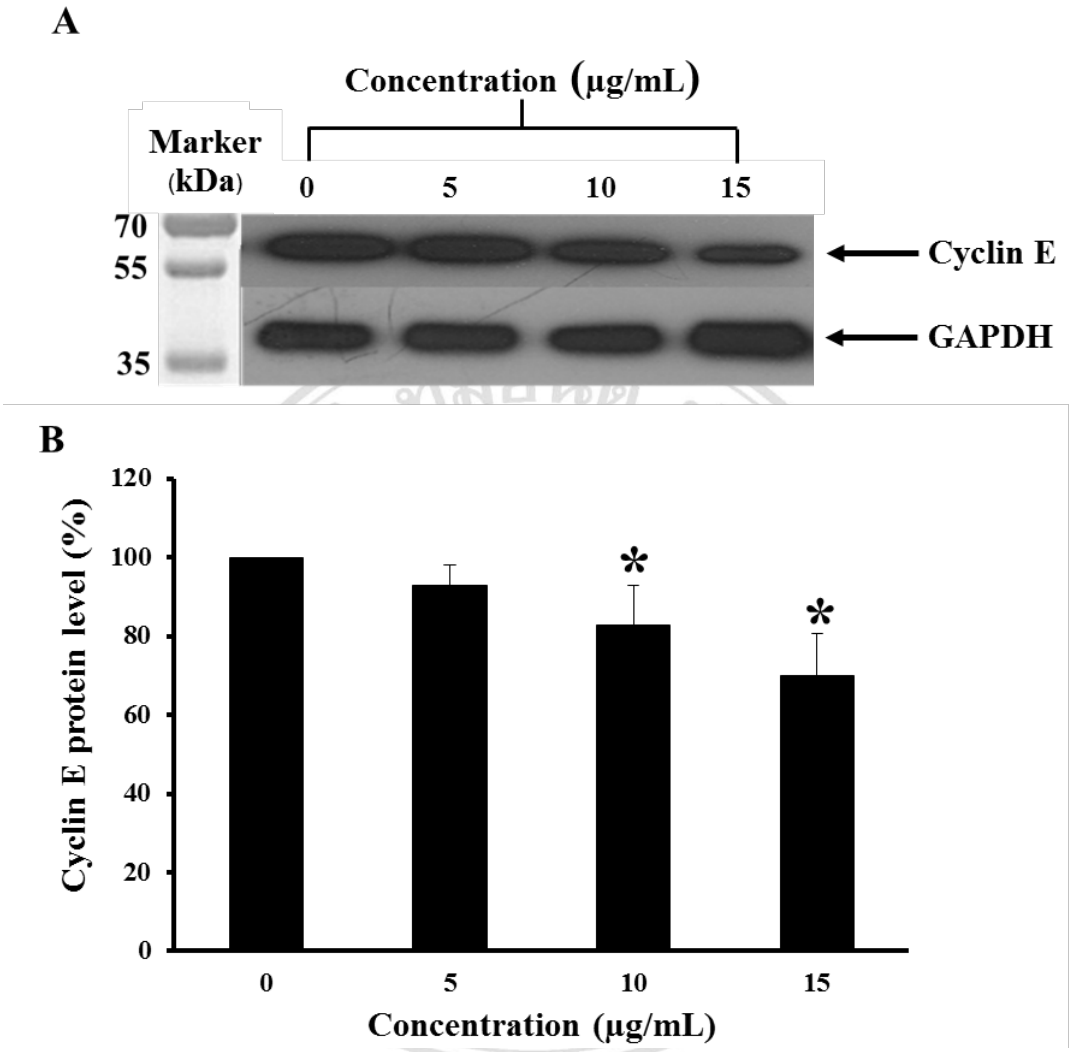

Figure 3.32 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E levels for 48 h in K562 cell line. K562 cells were treated with 5, 10, and 15 µg/mL of purified fraction No. 9 for 48 h. (A) The levels of cyclin E protein expression after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.14.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 on cyclin E protein in Molt4 cells, cells were treated with medium containing 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 and 0.08% DMSO (vehicle control) for 36 h. The protein level of cyclin E was normalized by using GAPDH protein and calculated percentage of cyclin E protein level (% cyclin E protein level). The percentages of cyclin E protein levels were 97.3±5.5, 86.6±7.5, and 84.0±4.6% in the response to 92.7, 13.4, and 16.0 µg/mL, respectively, compared to the vehicle control (Table 3.27 and Figure 3.33).

Table 3.27 Percentage of cyclin E protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime left treatment 1.0, 2.5, and 5.0 µg/mL for 36 h in Molt4 cells.

Concentration (µg/mL)	**% Cyclin E protein level**			
	1	**2**	**3**	**Mean±SD**
0	100.0	100.0	100.0	100.0±0
1.0	92.0	97.0	103.0	97.3±5.5
2.5	90.0	92.0	78.0	86.6±7.5*
5.0	89.0	83.0	80.0	84.0±4.6*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

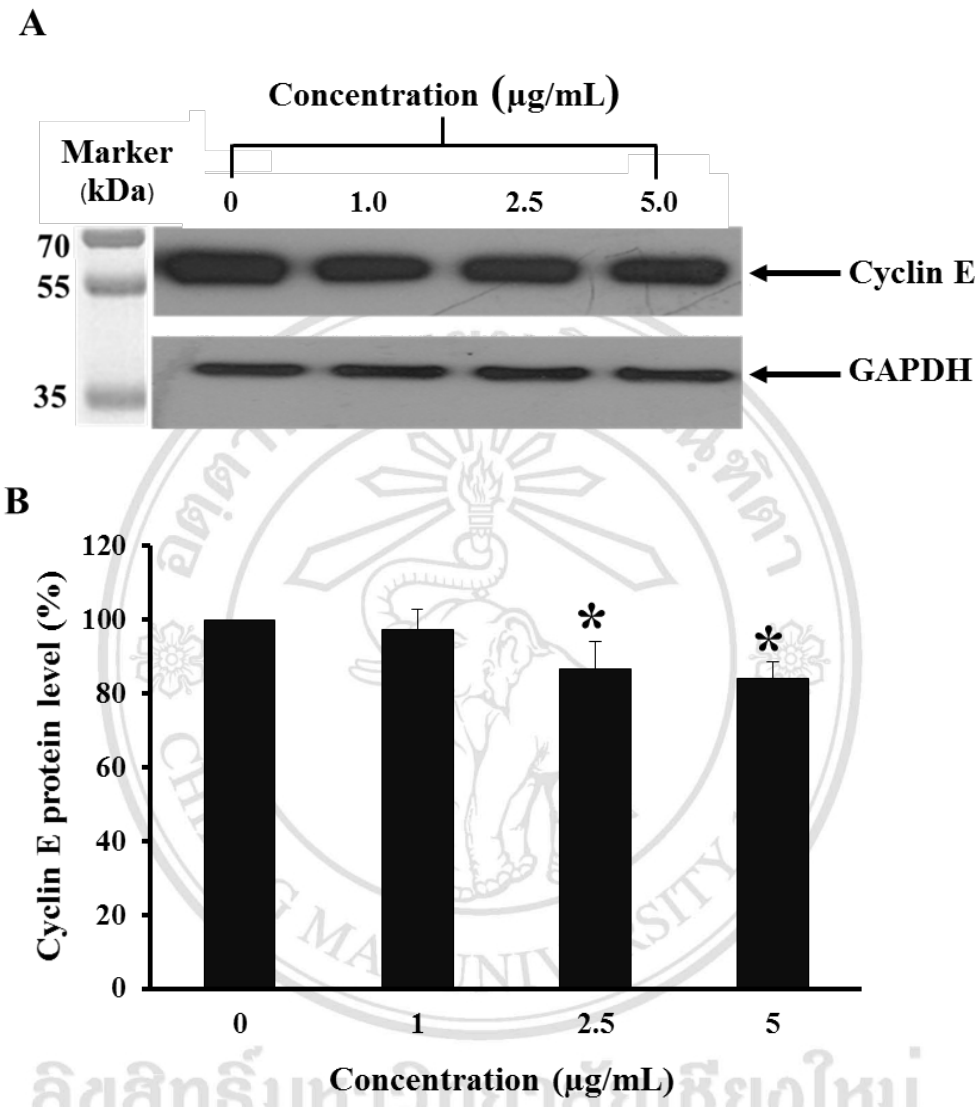

Figure 3.33 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cyclin E protein levels for 48 h in Molt4 cell line. Molt4 cells were treated with 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 for 48 h. (A) The levels of cyclin E protein expression after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments.

3.15 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in K562 and Molt4 cell lines

According to the results from section 3.9, the purified fraction No. 9 had the strongest inhibitory effect on targeted protein expressions in K562 and Molt4 cell lines. In addition, the various concentrations of purified fraction No. 9 were used to study cdc2 protein expression. K562 cells were treated with 5, 10, and 15 µg/mL for 48 h and Molt4 cells were treated with 1.0, 2.5, and 5 µg/mL for 48 h. Then treated cells were extracted and determined by Western blot analysis as described in section 2.9.

3.15.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in K562 cell line

To determine the effect of concentration of purified fraction No. 9 on cdc2 protein in K562 cells, cells were treated with medium containing 5, 10, and 15 µg/mL of purified fraction No. 9 and 0.04% DMSO (vehicle control) for 48 h. Cells were harvested and determined cdc2 protein levels by Western blotting. The levels of cdc2 protein were normalized by using GAPDH protein and then calculated percentage of cdc2 protein. The percentages of cdc2 protein levels were 91.0±3.5, 80.0±8.7, and 60.3±7.0% in the response to 5, 10, and 15 µg/mL, respectively. The cdc2 protein levels decreased in a dose dependent manner by 9, 27.7, and 39.7%, respectively, The concentrations of 10, and 15 µg/mL of the fraction significantly decreased cdc2 protein levels as compared to the vehicle control (p<0.05) (Table 3.28 and Figure 3.34).

Table 3.28 Percentage of cdc2 protein level after purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments at 5, 10, and 15 µg/mL for 48 h in K562 cells.

Concentration (µg/mL)	% Cdc2 protein level			
	1	2	3	Mean±SD
0	100.0	100.0	100.0	100.0±0
5	89.0	95.0	89.0	91.0±3.5
10	70.0	82.0	65.0	80.0±8.7*
15	65.0	64.0	67.0	60.3±7.0*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

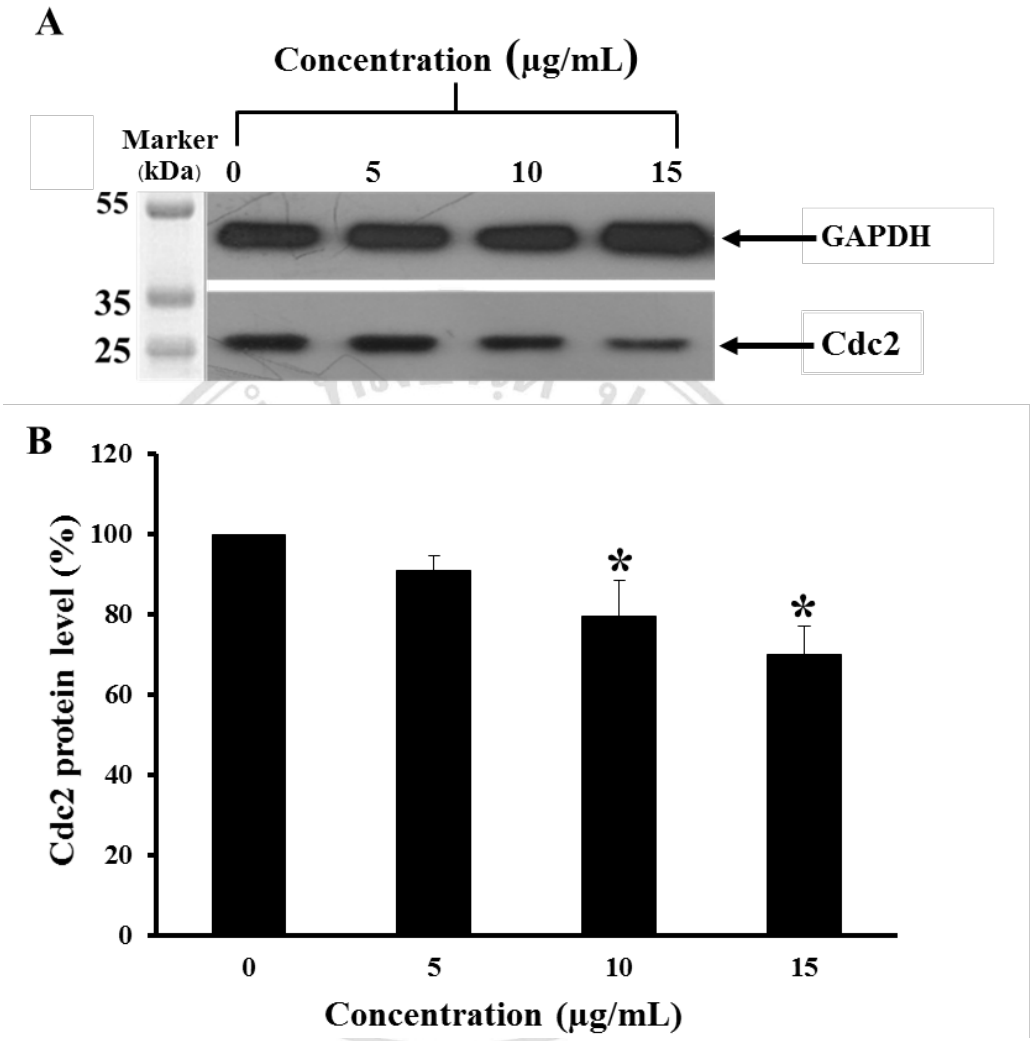

Figure 3.34 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cdc2 protein for 48 h in K562 cell line. K562 cells were 5, 10, and 15 µg/mL of purified fraction No. 9 treatments for 48 h. (A) The levels of cdc2 protein levels after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.15.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 on cdc2 protein in Molt4 cells, cells were treated with complete RPMI-1640 medium containing 1.0, 2.5, and 5.0 µg/mL of fraction and 0.08% DMSO (vehicle control) for 48 h. The protein level of cdc2 was normalized by using GAPDH protein and calculated percentage of cdc2 protein level. The percentages of cdc2 protein levels were 86.3±7.4, 75.0±9.9and 66.7±4.5 in the response to to 1.0, 2.5, and 5.0 µg/mL, respectively. The cdc2 protein level decreased in a dose dependent manner by 13.7, 24.7, and 23.3%, respectively. The concentrations of 2.5, and 5.0 µg/mL of the purified fraction significantly decreased compared to vehicle control (Table 3.29 and Figure 3.35).

Table 3.29 Percentage of cdc2 protein level after purified fraction No. 9 of hexane fractional extracts from kaffir lime left treatment at 1.0, 2.5, and 5.0 µg/mL for 48 h in Molt4 cells.

Concentration (µg/mL)	**% Cdc2 protein level**			
	1	**2**	**3**	**Mean±SD**
0	100.0	100.0	100.0	100±0
1.0	92.0	89.0	78.0	86.3±7.4
2.5	83.0	64.0	78.0	75.0±9.9*
5.0	67.0	71.0	62.0	66.7±4.5*

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

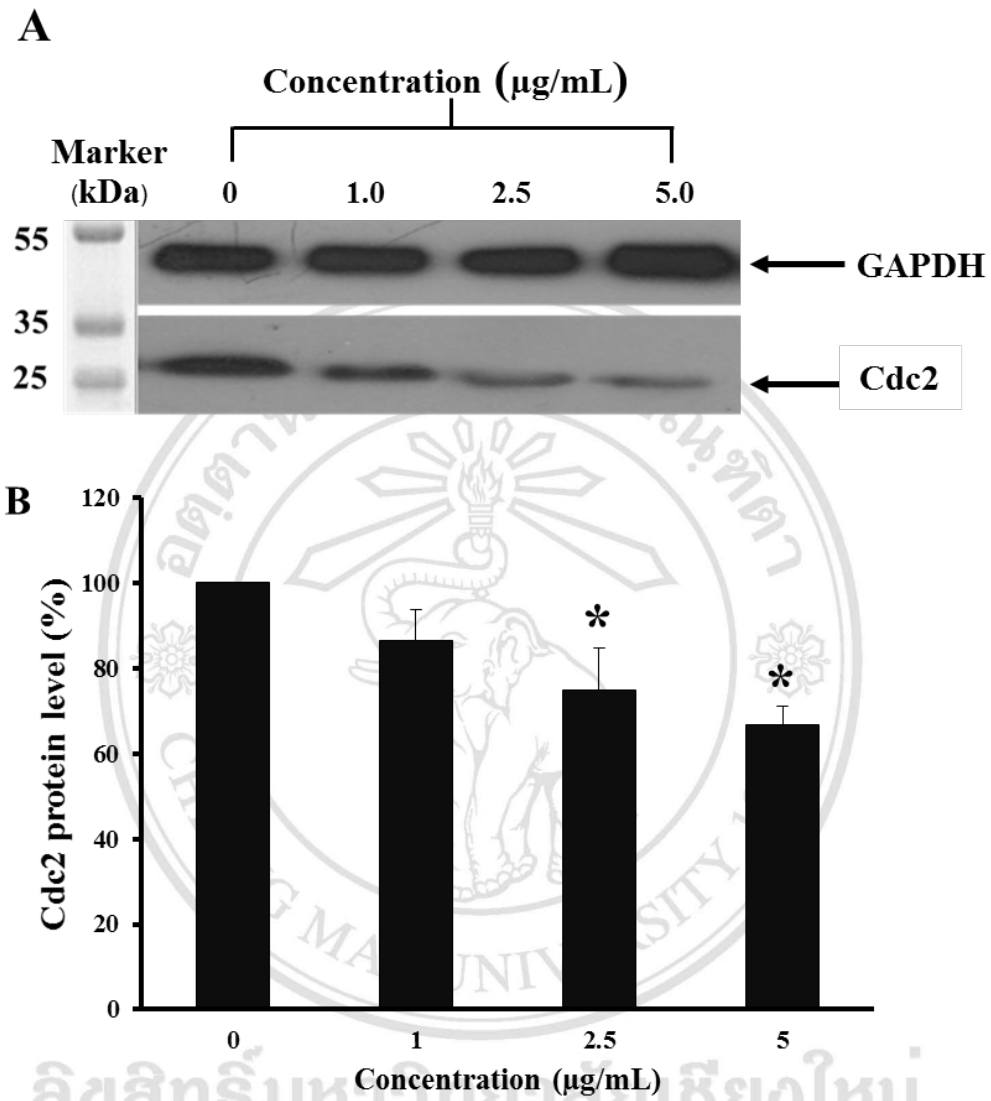

Figure 3.35 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on cdc2 protein levels for 48 h in Molt4 cell line. Molt4 cells were treated with 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 for 48 h. (A) The levels of cdc2 protein levels after purified fraction No. 9 treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer and compared to vehicle control by the percentages of vehicle control protein level was 100%. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control group (p<0.05).

3.16 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell number in K562 and Molt4 cell lines

To determine effects of purified fraction No.9 on targeted proteins of cell cycle regulation in K562 and Molt4 cell lines. K562 cells were treated with the various concentrations of purified fraction No. 9 for 24 and 48 h. Molt4 cells were treated with the various concentrations of fraction No. 9 for 36 and 48 h. Then treated cells were harvested as described in section 2.4.

3.16.1 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell number in K562 cell line

To determine the effect of concentration of purified fraction No. 9 in K562 cells, cells were cultured in medium complete RPMI-1640 containing 5, 10, and, 15 µg/mL of purified fraction No. 9 and 0.04% DMSO (vehicle control) for 24 h. The total cell numbers of treatment were significantly decreased by 7.3±0.1, 29.3±0.4, and 46.4±1.2% at concentrations of 5, 10, and 15 µg/mL of purified fraction No. 9, respectively. The percentages of dead cells were in the range of 0-0.1% (Table 3.30 and Figure 3.36).

Table 3.30 Total cell number after 5, 10, and 15 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments in K562 cells for 24 h.

Concentration (µg/mL)	Survival cells ($\times 10^5$)				Dead cells ($\times 10^5$)			
	1	2	3	Mean±SD	1	2	3	Mean±SD
0	8.0	8.2	8.5	8.2±0.3	0	0	0	0±0
5	8.5	7.0	7.2	7.6±0.8	0	0	0	0±0
10	6.0	5.5	6.0	5.8±0.3*	0	0	0	0±0
15	4.5	4.0	4.8	4.4±0.4*	0	0.1	0	0±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).

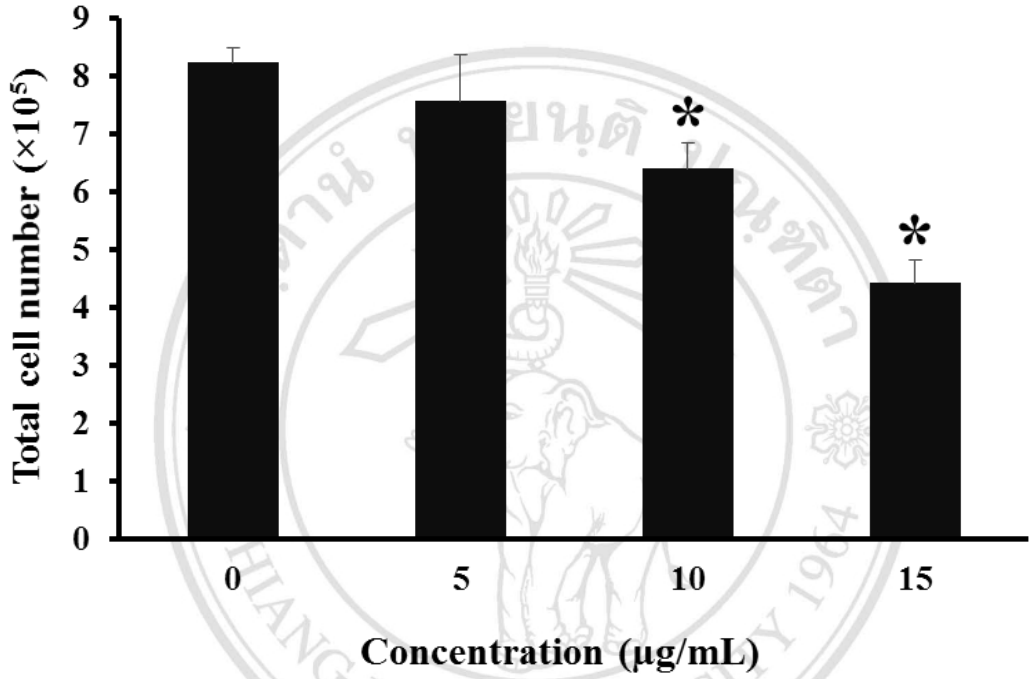

Figure 3.36 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf extracts on total cell number for 24 h in K562 cell line. Cells were counted after 5, 10, and 15 µg/mL of purified fraction No. 9 treatments by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the control group (p<0.05).

3.16.2 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell number in K562 cell line

To determine the effect of concentrations of purified fraction No. 9 treatments in K562 cells, cells were cultured in complete RPMI-1640 medium containing 5, 10, and 15 µg/mL of purified fraction No. 9 and 0.08% DMSO (vehicle control) for 48 h. The total cell number of treatment were significantly decreased by 23.75±0.3, 31.3±0.4, and 56.3±1.2% at concentrations of 5, 10, and 15 µg/mL of purified fraction No. 9, respectively. The percentages of dead cells were in the range of 0-0.2% (Table 3.31 and Figure 3.37).

Table 3.31 Total cell number after 5, 10, and 15 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments in K562 cells.

Concentration (µg/ml)	**Survival cells ($\times 10^5$)**				**Dead cells ($\times 10^5$)**			
	1	**2**	**3**	**Mean±SD**	**1**	**2**	**3**	**Mean±SD**
0	8.0	8.9	7.0	8.0±1.0	0	0	0	0±0
5	6.0	6.3	6.0	6.1±0.2*	0.2	0	0.2	0.1±0
10	5.0	5.5	6.0	5.5±0.5*	0	0	0	0±0
15	4.0	3.0	3.5	3.5±0.5*	0	0.1	0	0±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).

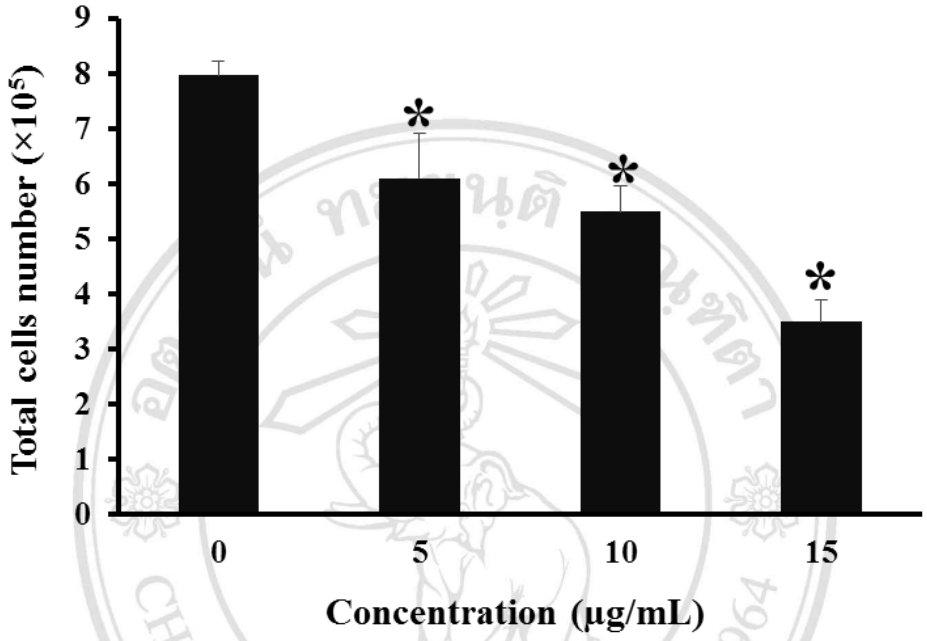

Figure 3.37 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 48 h on total cell number in K562 cell line. Cells were counted after 5, 10, and 15 µg/mL of purified fraction No. 9 treatments by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the control group (p<0.05).

3.16.3 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell number in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 treatments in Molt4 cells, cells were cultured in medium containing 1.0, 2.5, and, 5.0 µg/mL of purified fraction No. 9 and DMSO (vehicle control) for 36 h. The total cell numbers after treatments were significantly decreased by 23.7±1.2, 43.8±0.7, and 51.4±0.3% at concentrations of 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9, respectively. The percentages of dead cells were in the range of 0-0.1% (Table 3.32 and Figure 3.38).

Table 3.32 Total cell number after 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments in Molt4 cells.

Concentration (µg/mL)	Survival cells ($\times 10^5$)				Dead cells ($\times 10^5$)			
	1	2	3	Mean±SD	1	2	3	Mean±SD
0	8.0	8.0	8.0	8.0±0	0.1	0	0	0±0
1.0	6.0	6.0	6.3	6.1±0.5*	0	0	0.2	0±0
2.5	4.0	4.5	5.0	4.5±0.4*	0	0	0	0±0
5.0	3.5	4.0	4.2	3.9±0.2*	0	0.1	0	0±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).

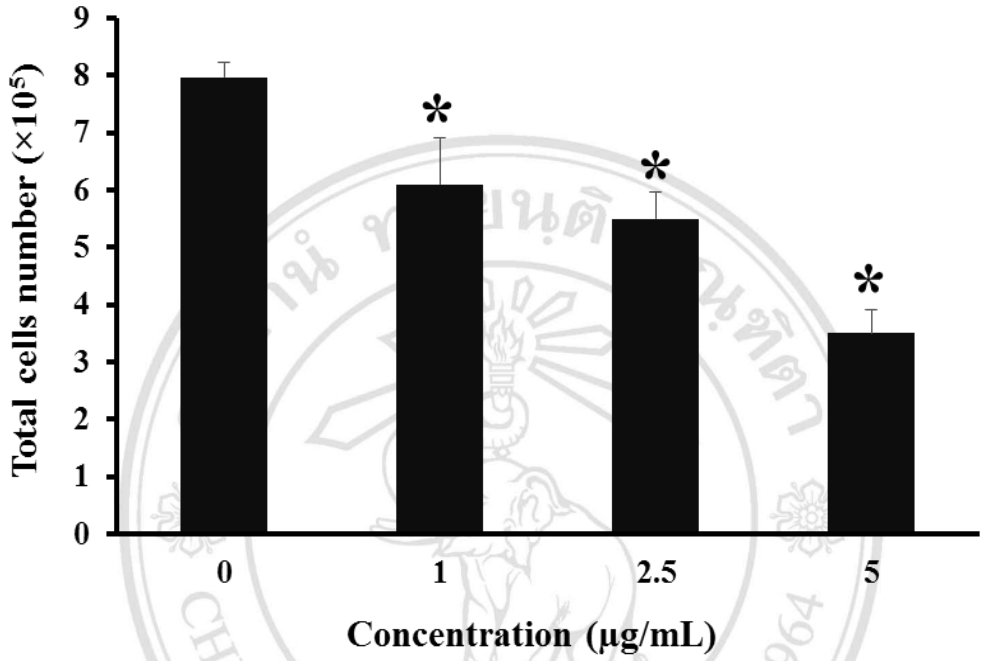

Figure 3.38 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 36 h on total cell number in Molt4 cell line. Cells were counted after 1.0, 2.5, and 5.0 µg/mL treatments by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the control group (p<0.05).

3.16.4 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments on total cell number in Molt4 cell line

To determine the effect of concentration of purified fraction No. 9 treatments in Molt4 cells, cells were cultured in medium containing 1.0, 2.5, and, 5.0 µg/mL of purified fraction No. 9 and DMSO (vehicle control) for 48 h. The total cell numbers after treatments were significantly decreased by 33.8±1.5, 67.5±0.8, and 56.2±1.6% at concentrations of 1.0, 2.5, and 5.0 µg/mL of purified fraction No.9, respectively. The percentages of dead cells were in the range of 0-1% (Table 3.33 and Figure 3.39).

Table 3.33 Total cell number after 1.0, 2.5, and 5.0 µg/mL of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments in Molt4 cells.

Concentration (µg/mL)	Survival cells ($\times 10^5$)				Dead cells ($\times 10^5$)			
	1	2	3	Mean±SD	1	2	3	Mean±SD
0	8.0	8.0	8.0	8.0±0	0	0.1	0	0±0
1.0	5.0	5.0	5.8	5.3±0.4*	0	0	0.2	0±0
2.5	4.0	4.0	4.6	4.2±0.3*	0	0	0	0±0
5.0	3.0	3.5	4.0	3.5±0.5*	0.1	0	0	0±0

Data are the mean values±SD of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).

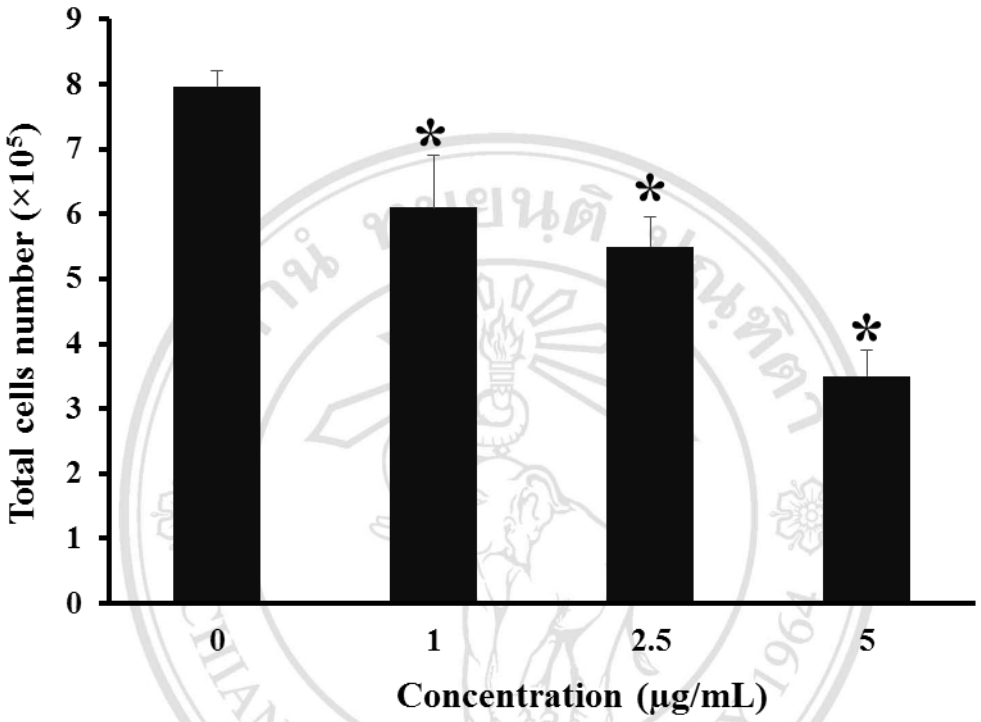

Figure 3.39 Effect of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf treatments for 48 h on total cell number in Molt4 cell line. Cells were counted after 1.0, 2.5, and 5.0 µg/mL treatments by the trypan blue exclusion method. Data are the mean values±SD of three independent experiments. Asterisk (*) denotes a significant difference from the control group (p<0.05).