CHAPTER II

Materials and Methods

2.1 Chemicals and reagents

The details of chemicals and reagents used in this study have been shown in Appendix A.

2.2 Purified kaffir lime leaf fractional extracts preparation

From the previous study of Chueahongthong *et al*, (2010) found that the hexane fraction of kaffir lime leaf extracts had the strongest cytotoxic effect against K562, Molt4, U937, and HL60 cells [9]. From this finding, the hexane crude extract (0.8 g.) was further purified by using vacuum column chromatography (VCC). Normal phase chromatography in which a mobile phase was non-polar and a stationary phase was polar siliga gel was utilized for the purification. Gradient elution with different compositions of mobile phase (hexane, dichloromethane, and methanol) as a gradient of increased polarity was generously performed by Asst. Prof. Dr. Chadarat Ampasavate, Faculty of Pharmacy, Chiang Mai University. Total of partially pure 13 fractions were collected and evaporated to dryness. From the previous study of Yuttapong Tamakaew [89] found that fraction No. 9, 10, and 11 (Table 2.1) could inhibit Wilm's tumor 1 protein expression in K562 cells by Western blot analysis. These fractions were further studied by dissolving each purified fraction in dimethyl sulfoxide (DMSO) for the final concentration of 25 mg/mL as the stock solution.

2.3 K562 and Molt4 leukemic cell cultures

The K562 leukemic cells (chronic myelocytic leukemia) and Molt4 (lympho-blastic leukemia) (from American Type Culture Collection, Rockville, USA) were cultured in RPMI-1640 (GIBCO-BRL) culture medium pH 7.2-7.4 with 100 units/mL penicillin concentration, 100 µg/mL streptomycin, 1 mM L-glutamine, and 10% fetal bovine serum (FBS) in 5% CO_2 incubator, 37°C, and 80% relative humidity.

Table 2.1 Dry weight, percent yield, and percent inhibition of WT1 protein expression in K562 cells of purified fraction No. 9, 10, and 11 from crude hexane extract after partially purified fraction by solvent of HE:DCM with the ratio of 20:80, 10:90, and 0:100, respectively.

Fraction No.	Solvent	Weight (g)	Yield (%)	WT1 inhibition* (%)
	HEX:DCM			
9	20:80	0.8	9.8	37.8±11.3
10	10:90	0.7	8.9	19.8±7.6
11	0:100	0.3	3.8	31.6±7.3

(HEX= Hexane, DCM = Dichloromethane) * WT1 protein level was normalized with GAPDH and compared to a vehicle control

2.4 Growth curve analysis of leukemic cell lines

Growth curve analysis plays an important role in cancer research. Growth curves are widely used in biology for quantities of growth. Values for the measured property are plotted on a graph between total cell number and time. In this study, K562 and Molt4 cells were cultured in 1 mL of complete RPMI-1640 medium in 24 well tissue culture plate with cell concentration of 1.0×10^4 cells/well. After that, cells were counted with trypan blue exclusion in each well every day. The cells quantity calculation required this formula;

$$\text{The total cell number (cells/ml)} = N^* \times \text{dilution fraction} \times 1000 \times 1/0.4$$

*N was counted cells. The calculated cell quantity could be written in relation graph between the total cell number and time.

2.5 MTT (3-(4,5 dimethylthiazol-2-y)-2,5 diphenyl tetrazolium bromide) assay

The MTT (3-(4,5 dimethylthiazol-2-y)-2,5 diphenyl tetrazolium bromide) is a technique for cell viability test. The principle of this technique is the enzyme succinate dehydrogenase and co-enzyme NADH in Krebs' cycle in mitochondria of living cells to changes MTT substance's color (yellow), to be a formazan crystal (purple). The purple formazan solution was directed variation to viable cell number.

The MTT assay was performed by washing K562 and Molt4 cells with PBS. Cell line was seeded at a density of 1.0×10^4 cells/well in 96-well plate, and incubated overnight at 37°C with 5% of CO_2. Then, cells were treated with 3 partially purified fraction No. 9, 10, and 11 extracts at 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL of final concentration and medium with DMSO was used as vehicle control (VC). The period of incubation continued for another 48 h. After that 15 µL of 0.2 mg/mL MTT dye solution was added for each well, incubating for 4 h. The formazan crystals were dissolved with 200 µL of DMSO, and the absorbance values of the solutions were measured at 578 nm on a microplate reader (Metertech, Taipei, Taiwan) and using the wavelength of 630 nm as reference. Percentage of cell viability was calculated by following equation.

% Cells viability = (OD average of tested well × 100) /OD average of Vehicle control

MTT assay was done in three time independent experiment. The results of three experiments were drawn to the average relation graphs between concentrations and percent cells viability. Then, the inhibitory concentration at 50% growth (IC_{50}) value was determined to compare the inhibitory of partially purified fraction of hexane fractional extract of kaffir lime leaf in each cultured leukemic cells. The calculation on the inhibitory concentration at 20% growth (IC_{20}) value was also determined for the cells cycle arrest study and cell cycle protein control that inhibited cell proliferation in K562 and Molt4 leukemic cells.

2.6 Effect of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle arrest in K562 and Molt4 leukemic cells by flow cytometry

The purified fraction No. 9 of hexane fractional extract from kaffir lime leaf was tested for their activity on cell cycle distribution by flow cytometry. If the purified

fraction could stop cell cycle progression, cells were accumulated and increased cell population in that of cycle phase. Thus, the cell distribution will be changed cell cycle pattern when compared to normal distribution of the cell control. A propidium iodine (PI) is required to stain the DNA content in cells for fluorescent detection by flow cytometry.

2.6.1 Cell preparation

After reaching 80% confluent of cultured cells, cells were washed 3 times by PBS, pH 7.2. K562 and Molt4 cells were adjusted to 1.0×10^5 cells/mL. In RPMI-1640 cells were centrifuged at 1,500×g and removed supernatant. Two cell lines were cultured with the most effective fraction (purified fraction No. 9 of hexane fraction kaffir lime leaf) at 3 concentrations or below IC_{20} for 12, 24, 36, and 48 h at 37°C with 5% of CO_2.

2.6.2 Cell preparation before flow cytometic analysis

After purified fraction No.9 treatment, cells were collected in 15 mL tube, then spin at 1,500×g for 5 min. Cells were washed 3 times in 10 mL ice-cold PBS. The last centrifugation, the cell pellet was resuspended in 300 µL of PBS then fixed cells with 700 µL ice-cold absolute ethanol for 30 min. Cells were centrifuged at 1,500×g for 5 min. The supernatant was discarded. After that cells were added with PI solution (300-500 µL) on ice and examined by flow cytometry (Cytomics FC 500, Beckman Coulter). The result was analyzed by FlowJo V10 program.

2.7 Effect of purified fraction No. 9 hexane fractional extract from kaffir lime leaf on p53, cyclin A, cyclin B, cyclin E, and cdc2 protein levels in leukemic cell lines by Western bolt analysis

After reaching 80% confluent of cultured cells, cells were washed 3 times by PBS, pH 7.2 and then cells were counted using 0.4% trypan blue dye solution. K562 and Molt4 cells were adjusted to 1.0×10^5 cells/mL for p53, cyclin A, cyclin B, and cdc2, cyclin E protein expression. K562 and Molt4 cell lines were cultured with the most effective fraction (purified fraction No. 9 of hexane fraction kaffir lime leaf) in complete RPMI-1640 medium at 37°C with 5% of CO_2.

2.8 Preparation of protein extraction

2.8.1 The whole protein lysate by RIPA buffer

After treatment with purified fraction No. 9 of hexane fractional extract from kaffir lime leaf, leukemic cells were harvested and washed 3 times with ice-cold sterile PBS, pH 7.2 and then cells were counted for cell viability by using 0.4% trypan blue dye solution. Thereafter, cells were centrifuged and supernatant was removed at 15,000×g. The cell pellet was lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) that contain protease inhibitors to inhibit activity of proteases. Following this, the cell suspension was vortexed every 10 min in 1 h. Then, cell suspension was centrifuged at 15,000×g for 15 min to separate whole protein extract. After centrifugation, whole protein lysate was collected to new centrifuge tube. It was used for protein measurement. Whole protein lysate was kept at -20^{o}C until analysis.

2.8.2 Measurement of protein concentration

The protein concentration was determined by the Folin-Lowry method [58]. The principle of this method is the combination between the reactions of copper II (Cu^{2+}) in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (tyrosine and tryptophan amino acids), obtained from the first reaction. The color of the reagent results was measured from absorbance at 750 nm, indicating protein concentration. The protein concentration of sample was equated to the bovine serum albumin (BSA) standard curve. The BSA protein standard curve could be constructed by preparation of various concentrations from stock 1 mg/mL BSA, as shown in Table 2.2 and Figure 2.1 to determine the protein concentrations of samples. Each protein sample (20 µL) was diluted with 480 µL deionized distilled water. Then alkaline copper solution (2.5 mL) were added and mixed by using vortex mixer. After incubation at room temperature for 10 min, 250 µL of Folin-phenol reagent were added and incubated at room temperature for 30 min. Finally, the concentrations of standard and sample proteins were determined by microplate reader at a wavelength of 750 nm.

2.9 Protein determination by SDS-PAGE and Western blot analysis

2.9.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate components of a protein mixture based on their size. The biological samples need to be denatured by SDS, an anionic reagent which applied a negative charge to each protein, so that they acquire uniform charge. Distance of protein migration was related to only the size of protein, so that a small molecule could migrate more easily and faster than a large molecule. The 2-mercaptoethanol destroyed disulfide bond of protein, the high pH led amphoteric protein to be anion. When the electricity on polyacrylamide gel started, protein moved from cathode to anode. The small molecule protein could fast move than large molecule protein.

Table 2.2 Preparation of bovine serum albumin (BSA) standard solution

BSA concentration (µg/tube)	Stock BSA (µL)	Deionized distilled water (µL)
0	0	0
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

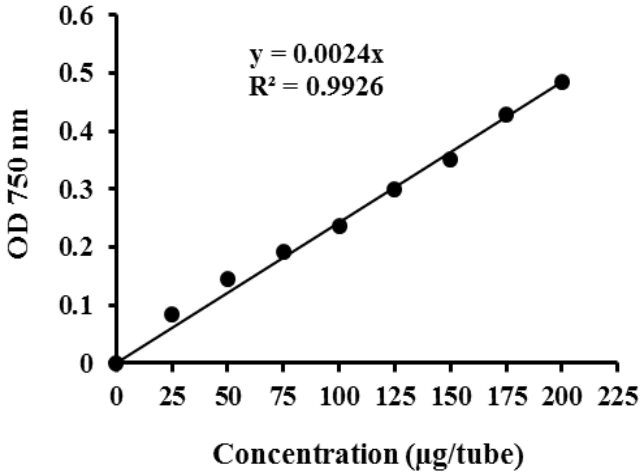

Figure 2.1 Standard curve of BSA.

In this study, SDS-PAGE was used to determine p53, cyclin A, cyclin B, cyclin E, and cdc2 proteins (target proteins) and normalize with GAPDH (housekeeping protein). First, the glass plates were cleaned with detergent, washed with water and ethanol, and allowed to dry. After that they were aligned and taken to the clamp. The separating gel monomer solution 12% was prepared and poured into the glass plates quickly before the acrylamide polymerized. After that, the overlay solution was completely rinsed off with distilled water. The gel was allowed to polymerize for 20 min. After separating gel setting, distilled water was poured out and the 4% stacking gel monomer solution was prepared and poured onto the top of the separating gel. The comb was inserted into the stacking gel solution, and the gel was allowed to polymerize for 20-30 min. After polymerization was completed the comb was removed by pulling it straight up slowly and gently, and the wells were washed 3 times with distilled water. Then the gel was removed from the casting stand and the clamp used for clasping the glass plates together was removed. The gel was placed in the electrophoresis chamber and electrode buffer was added. Later, the pre-running step was performed at 100 volts for 30 min. After pre-running, 50-100 µg of sample proteins were prepared by mixing four volumes of protein (deionized distilled water was added to adjust volume of each sample to be equal) with one volume of 5X reducing buffer. Sample proteins were loaded into well under electrode buffer. Electrophoresis was performed using 100 volts for 2 h 30 min. While proteins were running, PVDF was soaked in transfer buffer with filter paper at 4°C for 30 min. After that the separated proteins on the gel were

transferred to the PVDF membrane by using 30 volts of electricity overnight in transferring buffer. After transblotting, electrophoretically resolved proteins were analyzed by Western blot analysis and ECL detection and Luminata™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, USA).

2.9.2 Western blot analysis for p53, cyclin A, cyclin B, cyclin E, and cdc2 protein levels

After blotting, the PVDF membrane, and then incubated in 5% skim milk in PBS (blocking buffer) with shaking at room temperature for 2 h for blocking non-specific binding sites. After that the membranes were washed with 0.1% tween-PBS (washing buffer) 6 times, for 5 min, each membrane was incubated with primary antibody including mouse polyclonal anti-p53 IgG (MW 53 kDa), mouse polyclonal anti-cyclin A IgG (MW 60 kDa), mouse polyclonal anti-cyclin B IgG (MW 62 kDa), mouse polyclonal anti-cdc2 (MW 34 kDa), mouse monoclonal anti-cyclin E IgG (MW 52 kDa), and rabbit polyclonal anti-human GAPDH IgG (MW 37 kDa) at dilution 1:1,000, 1:400, 1:500,1:1,000,1:1,000, and 1:10,000, respectively, in 5% skim milk in 1X PBS with rocking on platform shaker at 4°C overnight. Membranes were washed 6 times for 5 min each with washing buffer to remove excess primary antibodies. The membranes were next incubated with goat anti-mouse IgG conjugated with HRP (Promega, USA) at a 1:10,000 dilution in blocking buffer for 1 h at room temperature. After that, the membranes were washed 6 times for 5 min each with washing buffer to remove excess antibodies. Finally, antibody-bound proteins were detected by using the SuperSignal® West Pico Chemilumi-nescent Substrate (PIERCE, USA), which included two substrate components, Super-Signal® West Pico luminal/ enhancer solution and SuperSignal® West Pico stable peroxide solution. The two substrate components were mixed at a 1:1 ratio to prepare the substrate working solution. The membranes were incubated in substrate working solution for 5 min at room temperature, after that, the excess solution was drained and the membranes were packed with clear thin plastic wrap. Each wrapped membrane was immediately placed onto a film cassette and then clear blue X-ray film (Thermo Fisher Scientific, USA) was placed on the top of the membrane for 2-20 min. The film was removed from the cassette and the protein band signal was developed in developing solution for 1 min. Film was fixed in fixing solution

for 1 min. Finally, the protein band signal was quantified by using a scan densitometer (Bio-Rad, USA).

2.10 Stripping method

The stripping method is a procedure for removing primary and secondary antibodies bound to target proteins on the membrane after Western blotting and ECL detection. Stripping methods use conditions that are effective for only low-affinity antibody-antigen interactions or are such harsh conditions that they tend to adversely alter the antigen for subsequent immunoprobing. The membrane was treated with sufficient volume of stripping buffer and then incubated for 15 min at room temperature. After that, the membrane was removed and washed with washing buffer three times, 5 min each. After this step, the membrane was used in Western blot analysis and ECL detection.

2.11 Cytotoxicity of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on leukemic cell lines by MTT assay

Cytotoxic effects of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf were determined in K562 and Molt4 cell lines. These cell lines were seeded at density of 1×10^4 cells/100 µL overnight and then 100 µL of treatments with various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) were added, incubating for 48 h. The cell viability was determined using the MTT assay as described in section 2.5.

2.12 Effect of incubation time periods of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in K562 cell line

After data analysis, the most effective purified fraction that could inhibit cell proliferation in leukemic cell lines was used. To determine the cell cycle distribution in a time dependent manner in K562 cells after purified fraction No. 9 of treatment, the cells were cultured in complete RPMI-1640 medium containing DMSO (vehicle control) and purified fraction No. 9 with a non-cytotoxic concentration at the concentration IC_{20} value which is 10 µg/mL values for 12, 24, and 48 h at 37°C under 5% CO_2 atmosphere as described in section 2.6 analyzed by flow cytometer, and analysis again with flowjo analysis program.

2.13 Effect of incubation time periods of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cell cycle distribution in Molt4 cell line

After data analysis, the most effective purified fraction No. 9 of hexane fractional extract from kaffir lime leaf which could inhibit cell proliferation in leukemic cell was further studies the on cell cycle distribution in a time dependent manner in Molt4 cells after purified fraction No. 9 treatment, the cells were cultured in complete RPMI 1640 medium containing DMSO (vehicle control) and purified fraction No. 9 with a non-cytotoxic concentration (IC_{20} value) which is 2.5 µg/mL for 12, 24, and 48 h at 37°C under 5% CO_2 atmosphere as described in section 2.6 and determined by flow cytometer, The data were analyzed by flowjo analysis program.

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

After data analysis, the most effective time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf extracts that could induce cell cycle arrest in K562 cells was used to study the dose effects in the K562 cells. The cells were treated with the various non-toxic concentrations (5, 10, and 15 µg/mL) of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts at 37°C under 5% CO_2 atmosphere for 24 h as described in section 2.6. Percentage of cell population in cell cycle phase was compared to the vehicle control of each experiment.

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

After data analysis, the most effective time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf that could induce cell cycle arrest in Molt4 cells was used to study the dose effects in the Molt4 cells. The cells were treated with the various non-toxic concentrations (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 at 37°C under 5% CO_2 atmosphere for 24 h as described in section 2.6. Percentages of cell population in cell cycle phase were compared with the vehicle control of each experiment.

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

To evaluate the activity of No. 9 of hexane fractional extract from kaffir lime leaf on total cell number in a time dependent manner in K562 and Molt4 cells. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 as described in section 2.4 were used for treatment for 12, 24, and 48 h. DMSO was used as a vehicle control. After that total cell number was counted with trypan blue dye exclusion method.

2.17 Effects 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 levels of p53 protein expression in a time-dependent manner in K562 and Molt4 leukemic cell lines. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf were used for treatments for 12, 24, 36, and 48 h. DMSO was used as a vehicle control. After being treated with purified fraction No. 9, after the whole protein was extracted, p53 protein levels determined by Western blot analysis as described in section 2.9. The band intensities of p53 protein were normalized by the intensity of GAPDH and compared to vehicle control.

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

To determine the levels of cyclin B protein expression with increased treatment time periods in K562 and Molt4 leukemic cell lines. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts were used for treatment for 12, 24, 36, and 48 h. DMSO was used as a vehicle control. After being treated with purified fraction No. 9, cells were extracted the whole protein lysate, cyclin B protein levels determined by Western blot analysis as described in section 2.9. The band intensities of cyclin B protein were normalized by the intensity of GAPDH and compared to vehicle control.

2.19 Effects of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein expression in K562 and Molt4 cell lines

To determine the levels of cyclin A protein expression with increased treatment time periods in K562 and Molt4 leukemic cell lines. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts were used for treatment for 12, 24, 36, and 48 h. DMSO was used as a vehicle control. After being treated with purified fraction No. 9, cells were extracted the whole protein lysate, cyclin A protein levels determined by Western blot analysis as described in section 2.9. The band intensities of cyclin A protein were normalized by the intensity of GAPDH and compared to vehicle control.

2.20 Effects of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf extracts on cyclin E protein expression in K562 and Molt4 cell lines

To determine the levels of cyclin E protein expression with increased treatment time periods in K562 and Molt4 leukemic cells. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts were used for treatment for 12, 24, 36, and 48 h. DMSO was used as a vehicle control. After being treated with purified fraction No. 9, cells were extracted the whole protein lysate, cyclin E protein levels determined by Western blot analysis as described in section 2.9. The band intensities of cyclin E protein were normalized by the intensity of GAPDH and compared to vehicle control.

2.21 Effects of incubation time period of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cdc2 protein expression in K562 and Molt4 cell lines

To determine the levels of cdc2 protein expression with increased treatment time periods in K562 and Molt4 leukemic cell lines. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 of hexane fraction kaffir lime leaf extracts were used for treatment for 12, 24, 36, and 48 h. DMSO was used as a vehicle control. After being treated with of purified fraction No. 9, cells were extracted the whole protein lysate, cdc2 protein levels determined by Western blot analysis as described in section 2.9. The

band intensities of cdc2 protein were normalized by the intensity of GAPDH and compared to vehicle control.

2.22 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on total cell number in K562 and Molt4 cell line

To evaluate the activity of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on total cell number in a dose dependent manner in K562 and Molt4 cells. The non-toxic concentrations at IC_{20} values of purified fraction No. 9 extracts as described in section 2.5 were used for treatment for 24 and 48 h. DMSO treatment was used as a vehicle control. After that total cell number was counted with trypan blue exclusion method.

2.23 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on p53 protein expression in K562 cell line

To determine the levels of p53 protein expression with increased treatment dose in K562 cells. The non-cytotoxic concentrations at IC_{20} values (5, 10, and 15 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined p53 protein levels by Western blotting as described in section 2.9. The band intensities of p53 protein were normalized by the intensity of GAPDH and the levels of p53 protein of tests were compared to vehicle control.

2.24 Effects of concentration of purified fraction No. 9 of hexane fraction kaffir lime leaf on p53 protein expression in Molt4 cell line

To determine the levels of p53 protein expression with increased treatment dose in Molt4 cells. The non- cytotoxic concentrations at IC_{20} values (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of p53 protein were normalized by the intensity of GAPDH and the levels of p53 protein of tests were compared to vehicle control.

2.25 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin B protein expression in K562 cell line

To determine the levels of cyclin B protein expression with increased treatment doses in K562 cells. The non-cytotoxic concentrations at IC_{20} values (5, 10, and 15 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cyclin B protein were normalized by the intensity of GAPDH and the levels of cyclin B protein of tests were compared to vehicle control.

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

To determine the levels of cyclin B protein expression with increased treatment dose in Molt4 cells. The non-cytotoxic concentrations at IC_{20} values (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cyclin B protein were normalized by the intensity of GAPDH and the levels of cyclin B protein of tests were compared to vehicle control.

2.27 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein expression in K562 cell line

To determine the levels of cyclin A protein expression with increased treatment dose in K562 cells. The non-cytotoxic concentrations at IC_{20} values (5, 10, and 15 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cyclin A protein were normalized by the intensity of GAPDH and the levels of cyclin A protein of tests were compared to vehicle control.

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

To determine the levels of cyclin A protein expression in dose dependent manner in Molt4 cells. The non-toxic concentrations at IC_{20} values (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined cyclin A protein levels by Western blotting as described in section 2.9. The band intensities of cyclin A protein were normalized by the intensity of GAPDH and the levels of cyclin A protein of tests were compared to vehicle control of each dose experiment.

2.29 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin E protein expression in K562 cell line

To determine the levels of cyclin E protein expression with increased treatment dose in K562 cells. The non-cytotoxic concentrations at IC_{20} values (5, 10, and 15 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate determined by Western blotting as described in section 2.9. The band intensities of cyclin E protein were normalized by the intensity of GAPDH and the levels of cyclin E protein of tests were compared to vehicle control.

2.30 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin E protein expression in Molt4 cell line

To determine the levels of cyclin E protein expression with increased treatment dose in Molt4 cells. The non-cytotoxic concentrations at IC_{20} values (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cyclin E protein were normalized by the intensity of GAPDH and the levels of cyclin E protein of tests were compared to vehicle control.

2.31 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cyclin A protein expression in K562 cell line

To determine the levels of cyclin A protein expression with increased treatment dose in K562 cells. The non-cytotoxic concentrations at IC_{20} values (5, 10, and 15 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cyclin A protein were normalized by the intensity of GAPDH and the levels of cyclin A protein of tests were compared to vehicle control.

2.32 Effects of concentration of purified fraction No. 9 of hexane fractional extract from kaffir lime leaf on cdc2 protein expression in Molt4 cell line

To determine the levels of cdc2 protein expression in dose dependent manner in Molt4 cells, the non-toxic concentrations at IC_{20} values (1.0, 2.5, and 5.0 µg/mL) of purified fraction No. 9 were used for treatment for 24 h. DMSO treatment was used as a vehicle control. After purified fraction No. 9 treatment, cells were extracted the whole protein lysate and determined by Western blotting as described in section 2.9. The band intensities of cdc2 protein were normalized by the intensity of GAPDH and the levels of cdc2 a protein of tests were compared to vehicle control of each dose experiment.

2.33 Statistical analysis

All the data were expressed as the mean value±standard deviation (SD) from triplicate samples of three independent experiments. Difference between the means of each experiment was analyzed by One-way ANOVA analysis. The differences were considered significant when the probability value obtained was found to be less than 0.05 (p<0.05).