CHAPTER II

Materials and Methods

2.1 Chemicals and reagents

The details concerning chemicals and reagents used in this study have been shown in Appendix C.

2.2 Leukemia cell line cultures

Molt4 (acute lymphoblastic leukemia cell line), K562 (chronic myelogenous leukemia cell line), and EoL-1 (acute myeloblastic cell line) were used as human leukemic cell models in this study, and were cultured in RPMI 1640 medium supplemented with heat-treated fetal bovine serum (10%), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified incubator with 5% CO_2.

2.3 Plant material

M. siamensis flowers were collected between February-April, 2014 in Chiang Mai Province, Thailand. A voucher specimen (J.F. Maxwell, No. 92-70) was deposited by the CMU herbarium, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The flowers were dried in hot-air oven before extraction by ethanol (EtOH).

2.4 Preparation of the *M. siamensis* flowers crude extract and fractional extracts

In order to extract the crude EtOH extract and fractional extracts from *M. siamensis* flowers, the air-dried powders of *M. siamensis* flowers were extracted with EtOH. After removal of solvent, the obtained residue was called “crude ethanolic (EtOH) extract”. After that crude EtOH extract was fractionated using a quick column chromatography packed with silica gel and was partitioned with hexane, ethyl acetate, and methanol successively to get the Hex, EtOAc, and MeOH fractions, respectively based on their polarity. The concentrated solution was completely dried in evaporator.

The four fractions were kept in the refrigerator at -20°C until used and suspended in DMSO to prepare the stock solution (25,000 µg/ml).

2.5 HPLC for screening of chemical compounds

HPLC analyses of crude and fractional extracts of *M. siamensis* flowers were carried out on a 250×4.6 mm, 5 µm (GL Sciences, Inc. Torrance, CA, USA), Inertsil ODS-3 column at 25°C with a flow rate of 0.5 ml/min. The sample isocratically run using water with acetic acid (1%) (phase A) and acetonitrile (20/80 v/v) (phase B) and the injection volume was 10 µl. The HPLC chromatograms were detected with UV detection at 280 nm. Quantitation of the compounds in the extract and in each fraction was performed by using mammea E/BB [26] as the standard compound.

2.6 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 times. In this study, Molt4, K562, and EoL-1 cells were seeded in 24-well plate with the concentration of 1.0×10^4 cells/well. Cells were counted with trypan blue dye exclusion method every day.

2.7 MTT assay

The MTT assay is based on the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into formazan crystals by succinate dehydrogenase within the mitochondria of living cells. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometry, which determines cytotoxicity of substances to cell growth.

Three leukemia cell lines were used as model to determine cytotoxic effect of *M. siamensis* flower fractional extracts (crude EtOH extract and fractional extracts of Hex, EtOAc, and MeOH) by MTT assay. Each 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 4 fractional extracts (0-100 µg/ml) and medium with and without DMSO, used as vehicle control (VC) and cell control. The period of incubation continued for another 48 h. After that 15 µl of 0.2% MTT dye solution was added for

each well, incubating for 4 h. Formed formazan crystals were dissolved with 200 µl of DMSO, and the absorbance values 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.

$$\% \text{ Cell viability} = \frac{\text{OD of test} \times 100}{\text{OD of VC}}$$

The average of cell viability that obtained from triplicate experiments was plotted as a graph. The inhibitory concentration at 50% growth (IC_{50}) value was presented as the lowest concentration that inhibits cell growth 50%, and IC_{20} value was determined as non-cytotoxic dose used for determining protein expression.

2.8 Effect of *M. siamensis* flowers crude extract and fractional extracts on Bcr/Abl, WT1, and FLT3 protein expressions in leukemic cell lines

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 WT1 expression, K562 cells were also used for Bcr/Abl protein expression and EoL-1 cells were adjusted to 5×10^5 cells/ml for WT1 and FLT3 expressions. Three cell lines were cultured with the most effective fraction (crude EtOH extract, Hex, and EtOAc fractions) in complete RPMI 1640 medium at 37°C with 5% of CO_2.

2.9 Protein preparation

2.9.1 Whole protein extraction

After *M. siamensis* crude EtOH extract and fractional extract treatment, leukemic cells were harvested and washed 3 times with ice-cold PBS, pH 7.2. Cells were counted for viable cells by using 0.4% typan blue dye solution. Thereafter, cells were centrifuged and removed supernatant 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 protease. Following this, the cell suspension was vortexed every 10 minutes in hour. Then, cell suspension

was centrifuged at 15,000×g for 15 minutes 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°C until analysis.

2.9.2 Measurement of protein concentration

The protein concentration was evaluated by the Folin-Lowry method [59]. The principle of this method is the combination between the reactions of copper II (Cu^{2+}) with the peptide bonds under alkaline conditions and the reduction of Folin-Ciocalteu reagent, a mixure of phosphotungstatic acid and phosphomolybdic acid, to heteropoly-molybdenum blue by the copper-catalytzed oxidation of aromatic protein residues (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) protein 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 2.1 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.

Table 2.1 Preparation of bovine serum albumin 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

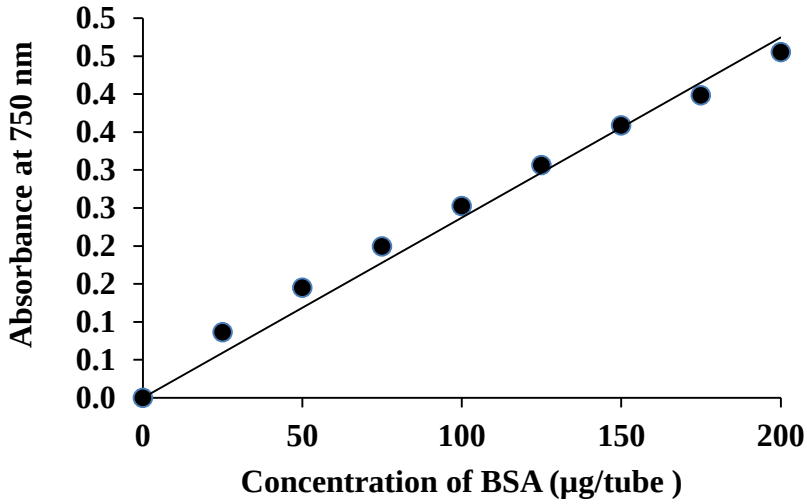

Figure 2.1 Standard curve of BSA.

2.10 Protein determination by SDS-PAGE and Western blot analysis

2.10.1 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 size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker). The concentration of acrylamide used for gel preparation depended on the size of target proteins. A low acrylamide concentration is used to separate high molecular weight of proteins, whereas a high acrylamide concentration is used to separate low molecular weight of proteins. Besides, the resolution of protein band could be improved by using a discontinuous gel system consisted of stacking and separating gel layers.

In this study, SDS-PAGE was used to determine protein levels of WT1, Bcr/Abl, FLT3 (target proteins), and GAPDH (housekeeping protein) and their molecular size. The glass plates were first cleaned with ethanol and allowed to dry. After that they were aligned and taken to the clamp. The 12% or 7.5% separating gel monomer solution was prepared and gel was allowed to polymerize for 20 min. After separating gel setting, the 4% stacking gel monomer solution was prepared and poured onto the top of separating gel. A comb was inserted into the stacking gel solution and the gel was allowed to polymerize for 15 min. After polymerization was completed, the comb was removed and the wells were washed 3 times with deionized water. The gel was placed in the electrophoresis chamber. The electrode buffer was prepared and filled into the reservoir. The pre-running step was performed at 100 volts for 30 min. After pre-running, protein samples were prepared by adding water and reducing buffer and then loaded into wells. Later, electrophoresis was carried out using 100 volts for 1 h. Polyvinylidene fluoride (PVDF) was activated by absolute methanol for 5 min. The separated proteins on gel were transferred to PVDF membrane by using 30 volts in transferred buffer overnight. After that target proteins on membrane were determined by Western blot analysis.

2.10.2 Western blot analysis and enhanced chemiluminescence (ECL) detection for WT1, Bcr/Abl, and FLT3 protein detection

After blotting, the PVDF membrane was incubated sequentially with 5% skim milk in PBS (blocking solution) with shaking at room temperature for 2 h to block non-specific binding sites. Then each membrane was incubated with primary rabbit polyclonal anti-WT1 antibody or primary rabbit polyclonal anti-Bcr/Abl antibody or primary rabbit polyclonal anti-FLT3 antibody at 1:1,000 dilution in blocking buffer and primary rabbit polyclonal anti-GAPDH antibody at 1:1,000 dilution in blocking buffer with shaking overnight. Membranes were washed 6 times for 5 min each with washing buffer (0.1% Tween20-PBS) to remove excess primary antibodies. Next, the membranes were incubated with goat anti-rabbit IgG conjugated with HRP at 1:20,000 dilution in a blocking buffer for 1 h at room temperature. After that the membranes were washed 6 times for 5 min each with washing buffer (0.1% Tween20-PBS) to remove excess antibodies. Finally, bound proteins were detected by using Luminata™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, USA). The membranes were incubated in substrate working solution for 5 min at room temperature. After the excess solution was drained, the membrane was packed with clear thin plastic wrap. Each wrapped membrane was placed onto a film cassette and AGFA film (HEALTHCARE, China) was placed on the top of membrane for 1-2 min. The film was removed from the cassette and the protein band signal was developed in developing solution for 1 min. Following this, film was fixed in fixing solution for 1 min. Finally, the protein band signals were quantified by using Quantity One, Version 4.6.3 (Bio-rad laboratories, Hercules, CA, USA).

2.11 Anti-proliferative effect of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on Molt4, K562, and EoL-1 cell lines by MTT assay

Anti-proliferative effect of crude EtOH extract and fractional extracts of Hex, EtOAc, and MeOH from *M. siamensis* flowers were determined in Molt4, K562, and EoL-1 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 2.7.

2.12 Effect of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on Bcr/Abl protein expression in K562 cell line

To determine the level of Bcr/Abl protein in K562 cell line after crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers treatments, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of crude EtOH extract and fractional extracts of Hex and EtOAc of *M. siamensis* flowers, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of Bcr/Abl protein were normalized by the intensity of GAPDH and the level of Bcr/Abl expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.13 Effect of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on WT1 protein expression in Molt4, K562, and EoL-1 cell lines

To determine the levels of WT1 protein in three leukemic cell lines after crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers treatments, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of crude EtOH extract and fractional extracts of Hex and EtOAc of *M. siamensis* flowers, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of WT1 protein were normalized by the intensity of GAPDH and the levels of WT1 expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.14 Effect of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on FLT3 protein expression in EoL-1 cell line

To determine the level of FLT3 protein in EoL-1 cell line after crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers treatments, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of crude EtOH extract and fractional extracts of Hex and EtOAc of *M. siamensis* flowers, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of FLT3 protein were normalized by the intensity of GAPDH and the level of FLT3 expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.15 Effect of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on total cell number in Molt4, K562, and EoL-1 cell lines

To determine the total cell number of three leukemic cell lines after crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers treatments, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of crude EtOH extract and fractional extracts of Hex and EtOAc of *M. siamensis* flowers, obtained from MTT assay, for 48 h as described in section 2.7. After that total cell number was counted by trypan blue dye exclusion method. The statistical analysis was performed as described in section 2.28.

2.16 Effect of time period of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on Bcr/Abl protein expression in K562 cell line

To evaluate the activity of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on Bcr/Abl protein expression in a time dependent manner in K562 cell line. The non-cytotoxic concentrations at IC_{20} values of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers were used for treatment for 12, 24, 48, and 72 h. DMSO was used as a vehicle control. After the whole protein was extracted, Bcr/Abl protein of each cell line was determined by

Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of Bcr/Abl protein were normalized by the intensity of GAPDH and the level of Bcr/Abl expression of treatments were compared to vehicle control of each time experiment. The statistical analysis was performed as described in section 2.28.

2.17 Effect of time period crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on WT1 protein expression in Molt4, K562, and EoL-1 cell lines

To evaluate the activity of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on WT1 protein expression in a time dependent manner in Molt4, K562, and EoL-1 cell lines. The non-cytotoxic concentrations at IC_{20} values of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers were used for treatment for 12, 24, 48, and 72 h. DMSO was used as a vehicle control. After the whole protein was extracted, WT1 protein of each cell line was determined by Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of WT1 protein were normalized by the intensity of GAPDH and the levels of WT1 expression of treatments were compared to vehicle control of each time experiment. The statistical analysis was performed as described in section 2.28.

2.18 Effect of time period of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on FLT3 protein expression in EoL-1 cell line

To evaluate the activity of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on FLT3 protein expression in a time dependent manner in EoL-1 cell line. The non-cytotoxic concentrations at IC_{20} values of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers were used for treatment for 12, 24, 48, and 72 h. DMSO was used as a vehicle control. After the whole protein was extracted, FLT3 protein of each cell line was determined by Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of FLT3 protein were normalized by the intensity of GAPDH and the level of

FLT3 expression of treatments were compared to vehicle control of each time experiment.

2.19 Effect of time period of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on total cell number in Molt4, K562, and EoL-1 cell lines

To evaluate the activity of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers on total cell number in a time dependent manner in Molt4, K562, and EoL-1 cell lines. The non-cytotoxic concentrations at IC_{20} values of crude EtOH extract and fractional extracts of Hex and EtOAc from *M. siamensis* flowers as described in section 2.13 were used for treatment for 12, 24, 48, and 72 h. DMSO was used as a vehicle control. After that total cell number was counted by trypan blue dye exclusion method. The statistical analysis was performed as described in section 2.28.

2.20 Effect of concentration of Hex fraction from *M. siamensis* flowers on Bcr/Abl protein expression in K562 cell line

To evaluate the activity of Hex fraction from *M. siamensis* flowers on Bcr/Abl protein expression in a dose dependent manner in K562 cell line. The non-cytotoxic concentrations at IC_{20} value (25, 30, 35, and 40 µg/ml) of Hex fraction from *M. siamensis* flowers were used for treatment for 48 h. DMSO was used as a vehicle control. After the whole protein was extracted, Bcr/Abl protein was determined by Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of Bcr/Abl protein were normalized by the intensities of GAPDH and the level of Bcr/Abl expression of treatments were compared to vehicle control of each time point. The statistical analysis was performed as described in section 2.28.

2.21 Effect of concentration of Hex fraction from *M. siamensis* flowers on WT1 protein expression in Molt4, K562, and EoL-1 cell lines

To evaluate the activity of Hex fraction from *M. siamensis* flowers on WT1 protein expression in a dose dependent manner in Molt4, K562, and EoL-1 cell lines. The non-cytotoxic concentrations at IC_{20} values of Hex fraction (0.5, 1.0, 1.5, and 2.0

µg/ml for Molt4, 25, 30, 35, and 40 µg/ml for K562 and 0.25, 0.50, 0.75, and 1.00 µg/ml for EoL-1 cells) were used for treatments for 48 h. DMSO was used as a vehicle control. After the whole protein was extracted, WT1 protein was determined by Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of WT1 protein were normalized by the intensity of GAPDH and the level of WT1 expression of treatments were compared to vehicle control of each time experiment. The statistical analysis was performed as described in section 2.28.

2.22 Effect of concentration of Hex fraction from *M. siamensis* flowers on FLT3 protein expression in EoL-1 cell line

To evaluate the activity of Hex fraction on FLT3 protein expression in a dose dependent manner in EoL-1 cell line. The non-cytotoxic concentrations at IC_{20} value of *M. siamensis* flower Hex fraction were used for treatments for 48 h. DMSO was used as a vehicle control. After the whole protein was extracted, FLT3 protein was determined by Western blot analysis as described in section 2.9 and 2.10, respectively. The band intensities of FLT3 protein were normalized by the intensity of GAPDH and the level of FLT3 expression of treatments were compared to vehicle control of each time point. The statistical analysis was performed as described in section 2.28.

2.23 Effect of concentration of Hex fraction from *M. siamensis* flowers on total cell number in Molt4, K562, and EoL-1 cell lines

To evaluate the activity of Hex fraction from *M. siamensis* flowers on total cell number in a dose dependent manner in Molt4, K562, and EoL-1 cell lines. The non-cytotoxic concentrations at IC_{20} values Hex fraction (0.5, 1.0, 1.5, and 2.0 µg/ml for Molt4, 25, 30, 35, and 40 µg/ml for K562 and 0.25, 0.50, 0.75, and 1.00 µg/ml for EoL-1 cells) were used for treatments for 48 h. DMSO was used as a vehicle control. After that total cell numbers were counted by trypan blue dye exclusion method. The statistical analysis was performed as described in section 2.28.

2.24 Effect of Hex fraction from *M. siamensis* flowers and mammea E/BB on Bcr/Abl in K562 cell line

To determine the levels of Bcr/Abl protein in K562 after Hex fraction from *M. siamensis* flowers and mammea E/BB treatments, K562 cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of Hex fraction and mammea E/BB, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of Bcr/Abl protein were normalized by the intensity of GAPDH and the levels of Bcr/Abl expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.25 Effect of Hex fraction from *M. siamensis* flowers and mammea E/BB on WT1 in Molt4 cell line

To determine the levels of WT1 protein in Molt4 cells after Hex fraction from *M. siamensis* flowers and mammea E/BB treatments, Molt4 cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of Hex fraction from *M. siamensis* flowers and mammea E/BB, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of WT1 protein were normalized by the intensity of GAPDH and the levels of WT1 expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.26 Effect of Hex fraction from *M. siamensis* flowers and mammea E/BB on FLT3 in EoL-1 cell line

To determine the levels of FLT3 protein in EoL-1 cells after Hex fraction from *M. siamensis* flowers and mammea E/BB treatments, EoL-1 cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of Hex fraction from *M. siamensis* flowers and mammea E/BB, obtained from MTT assay, for 48 h as described in section 2.7. After that the whole protein was extracted and Western blot analysis was carried out as described in section 2.9 and 2.10, respectively. The band intensities of

FLT3 protein were normalized by the intensity of GAPDH and the levels of FLT3 expression of treatments were compared to vehicle control of each experiment. The statistical analysis was performed as described in section 2.28.

2.27 Effect of Hex fraction from *M. siamensis* flowers and mammea E/BB on total cell number in Molt4, K562, and EoL-1 cell lines

To determine the total cell number of three leukemic cell lines after Hex fraction from *M. siamensis* flowers and mammea E/BB treatments, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic doses of Hex fraction from *M. siamensis* flowers and mammea E/BB, obtained from MTT assay, for 48 h as described in section 2.7. After that total cell number was counted by trypan blue dye exclusion method. The statistical analysis was performed as described in section 2.28.

2.28 Statistical analysis

The average of triplicate experiments and standard errors of mean (SEM) were used for quantification. The level of target protein expressions were compared to the vehicle control in each experiment. The results were shown as mean±SEM. Differences between the means of each experiment were analyzed by One-way analysis of variance (One-way ANOVA). Statistic significances were considered at $p<0.05$.