CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and reagents

The details as to chemicals and reagents used in this study are given in Appendix A.

2.2 Cells and cell culture conditions

K562 (chronic myelocytic leukemia), Molt4 (lymphicytic leukemia), U937 (monocytic leukemia) and HL60 (promyelocytic leukemia) were used in this study, and cultured in RPMI 1640 medium (GIBCO-BRL) containing 1 mM L-glutamine, 100 units/ml penicillin, 100 µg/mL streptomycin, and supplemented with 10% fetal bovine serum (FBS) at 37˚C under atmosphere of 95% air and 5% CO_2.

2.3 Kaffir lime leaf fractional extracts preparation

Two kilograms of fresh kaffir lime leaves were dried at 50˚C. After being completely dried, the leaves were ground into powder (~980 g). A half portion of dried kaffir lime leaf powder (476 g) was macerated in 95% ethanol for 24 h and the liquid portion was collected. This step was repeated 3 times. After that, the liquid portions of extractions were pooled together and filtered. The filtrate was collected and evaporated

using a rotary evaporator and subsequently freeze-dried to obtain a crude ethanol fraction extract (fraction 1). Another half portion was subjected to sequential maceration. First, it was macerated in hexane for 24 h and the liquid portion was collected. This step was repeated 3 times. After that, the whole liquid portion was filtered. The filtrate was collected and evaporated using a rotary evaporator to obtain a crude hexane fractional extract (fraction2). The residue of kaffir lime leaf powder from fraction2 was placed under the hood to remove the hexane. The dried residue was further extracted with an ethyl acetate using the same procedure to obtain an ethyl acetate fractional extract (fraction3). The residues from fraction3 were further extracted with n-butanol and methanol to obtain crude n-butanol (fraction4) and methanol (fraction5) fractional extracts, respectively. Crude kaffir lime leaf fractional extracts were kept at -20˚C. The extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 25 mg/mL and kept at -20˚C before. (All of the fractional extracts were generous gifts from Assist. Prof. Dr. Chadarat Ampasavate.)

2.4 MTT assay

The MTT assay is a colorimetric assay, used to determine cytotoxicity of medical agents or toxic substances to cell viability and growth. The principle is to measure the activity of succinate dehydrogenase, a mitrochondrial enzyme, which requires NADH for reducing 3-4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye to insoluble purple formazan in living cells. The formazan crystals can be solubilized in DMSO and the resulting purple solution is measured by spectrophotometry. An increase

in cell number would result in the amount of formazan crystal and an increase in absorbance, indicating cell viability.

The MTT assay was used for detecting cytotoxicity of crude kaffir lime leaf fractional extracts on four leukemic cell lines. Each strain of leukemic cells was adjusted to 1.0 x 10^4 cells in 100 µL of complete RPMI1640 medium and added into each well of a flat-bottom 96-well plate. Cells were incubated at 37˚C under 5% CO_2 atmosphere overnight. Various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) of crude kaffir lime leaf fractional extracts dissolved in 100 µL of medium and medium with and without DMSO, used as vehicle and cell control, were added into each well and incubated for 48 h. Then 100 µL of medium were removed and 15 µL of MTT dye (Sigma-Aldrich; USA) were added, and cells were further incubated for 4 h. After the supernatant was removed, 200 µL of DMSO were added to each well, and mixed thoroughly to dissolve the formazan crystals. The optical density was measured using an ELISA plate reader at 540 nm with reference wavelength at 630 nm. Percentage of cell survival was calculated from the absorbance of test and control wells by the following equation.

$$\% \text{ cell viability} = \frac{\text{Absorbance of test well}}{\text{Absorbance of vehicle control}} \times 100$$

The average of percentage of cell survival at each concentration obtained from triplicate experiments were plotted as a dose response curve. The inhibitory concentration at 50% growth (IC_{50}) of each crude kaffir lime leaf fractional extract was determined as

the lowest concentration which could inhibit cell growth by 50% compared to the untreated culture, and IC_{20} of each crude extract was determined as the non-toxic concentration to be used for the study of *WT1* gene and WT1 protein expression.

2.5 Kaffir lime leaf fractional extracts treatments for the studies of effects of fractional extracts on *WT1* gene and WT1 protein expression

Cultured leukemic cells with 80% confluent were harvested and washed 3 times with sterile PBS, pH 7.2. After that cells were counted for cell viability and amount of total cells using 0.2% trypan blue dye. Then each leukemic cell line was adjusted to a concentration of 2.0 x 10^5 cells/mL and cultured with each of crude kaffir lime leaf fractional extracts in complete RPMI 1640 medium for 48 h at 37˚C under 5% CO_2 atmosphere.

2.6 Preparation of total RNA for gene expression study

2.6.1 Total RNA extraction

After being treated with crude kaffir lime leaf fractional extracts, leukemic cells were harvested and washed 3 times with ice-cold sterile PBS, pH 7.2 and then cells were counted for cell viability using 0.2% trypan blue dye. Afterward, the cell pellet was collected and resuspended in 200 µL of sterile PBS, pH 7.2. Then total RNA was extracted by High Pure RNA Isolation Kit (Roche, Germany). A volume of 400 µL of Lysis/-Binging buffer [4.5 M guanidine-HCl, 50 mM Tris-HCl, 30% Triton X-100 (w/v), pH 6.6] was added and vortexed for 15 s. After that, the sample was transferred to a High

Pure filter tube, consisting of a High filter tube inserted into collection tube, by pipetting. Sample was centrifuged at 8,000 xg for 15 s. The filter tube was removed from the collection tube. The flowthrough liquid was discarded. After re-inserting the filter tube into the used collection tube, for 1 sample, 90 µL of DNase incubation buffer [1 M NaCl, 20 mM Tris-HCl, and 10 mM $MnCl_2$, pH 7.0] and 10 µL of DNase I were added into a sterile microcentrifuge tube, mixed carefully, and pipetted into the filter tube. The sample was incubated at 15˚ to 25˚C for 15 min, and 500 µL of Wash buffer I [5 M guanidine hydrochloride, 20 mM Tris-HCl pH 6.6, and ethanol] was added and then centrifuged at 8,000 xg for 15 sec. After the flowthrough was discarded, 500 µL of Wash buffer II [20 mM NaCl, 2 mM Tris-HCl pH 7.5, and ethanol] was added into the filter tube and centrifuged at 8,000 xg for 15 s. Later flowthrough was discarded and 200 µL of Wash buffer II was added into the filter tube and centrifuged at maximum speed (13,000 xg) for 2 min to remove any residual washing buffer. After the collection was discarded, the filter tube was inserted into a clean sterile 1.5 mL microcentrifuge tube and 100 µL of Elution buffer [nuclease-free, sterile, double distilled water] were added into the filter tube. Total RNA was eluted by centrifugation at 8,000 xg for 1 min. Finally, 1 µL of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, USA) was added into microcentrifuge tube with the eluted total RNA for RNA protection. Total RNA was used directly in Real-time PCR or stored at -70˚C for later analysis.

2.6.2 Measurement of purity and concentration of total RNA

Total RNA was determined for purity by measuring optical density via spectrophotometry at 260 nm/280 nm ratio. The RNA concentration was calculated from the following formula.

Total RNA (µg/mL) = Absorbance at 260 nm x 40 µg/mL* x Dilution factor

* 1 OD = 40 µg/mL

2.7 cDNA synthesis

After determination of total RNA concentration, cDNA was synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany). Briefly, a template-primer mixture was prepared by adding 0.5 µg of total RNA, random hexamer primer and sterile nuclease free water in a thin-wall PCR tube, and then the template-primer mixture was denatured by heating the tube for 10 min at 65˚C to ensure denaturation of RNA secondary structures. After that, the tube was immediately cooled on ice. The remaining components, 5x reaction buffer, protector RNase inhibitor, deoxynucleotides mix (dNTPs), dithiothreitol (DTT) and Reverse Transcriptase, were prepared as a master mix and then added into the template-primer mixture. All reagents were mixed carefully and kept on ice until the PCR cycling had started. The tube was placed in a block cycler with a heated lid to minimize evaporation, and then the PCR cycling for cDNA synthesis was started at 55°C for 30 min and inactivation of Reverse Transcriptase by heating to 85˚C

for 5 min. Finally, the reaction was stopped by placing the tube on ice. The cDNA was kept at -20˚C until analysis.

2.8 Effect of crude kaffir lime leaf fractional extracts on *WT1* gene expression

In this study, real-time PCR analysis was performed using the DyNAmo™ qPCR Kit (Finnzymes, Finland), reagent for quantitative real-time analysis of DNA samples using probe base detection, Tagman® probe, based on the hot start Thermos brockianus (Tbr) DNA polymerase. Briefly, a PCR premix was prepared by mixing 2x reaction buffer 2x master mix (containing hot-start Tbr DNApolymerase, optimized PCR buffer, $MgCl_2$, dNTP mix including dUTP), primers, probe and sterile nuclease free water. All components were mixed thoroughly to assure homogeneity and then dispensed into Thin-walled PCR strip tubes. After that, template cDNA was added into the PCR tube containing PCR premix (for two-strep qRT-PCR, the volume of cDNA added as template should not exceed 10% of final PCR volume) and centrifuged before starting the cycling program. As for the hot start, Tbr DNA polymerase was inactive during the PCR setup step, it was not necessary to do the setup on ice.

The probes-based detection reagent used in this study was Taqman™, hydrolysis probe (Operon, USA). The sets of primers and probes included WT1 primers and a probe for the WT1 gene and β-actin primers and a probe for the β-actin gene; these were used as the housekeeping genes.

For WT1 primers, the forward primer (5’GATAACCACACAACGCCCATC3’) and the reverse primer (5’CACACGTCGCACATCCTGAAT3’) and WT1 probe

(5'FAM-ACACCGTGCGTGTGTATTCTGTATTGG-TAMRA3') were used. For β-actin primer, the forward primer (5'CCCAGCACAATGAAGATCAAGATCAT3') and the reverse primer (5'ATCTGCTGGAAGGTGGACAGCGA'3) and β-actin probe (5'FAM-TGAGCGCAAGTACTCCGTGTGGATCGGCG-TAMRA3') were used. Due to the large difference in the copy numbers of *β-actin* and *WT1* in the sample, to prevent *β-actin*, which had a larger copy number, from using all the PCR components (DNA polymerase, $MgCl_2$, dNTP, and dUTP) in the PCR amplification process, the detection of *WT1* and *β-actin* gene expression had to be done in separate tubes.

The Real-time cycling condition was started at an initial denaturation temperature of 95˚C for 10 min. This step was needed to activate the hot start Tbr DNA polymerase and to denature the template cDNA. After that, PCR amplification was performed for 50 cycles of denaturation at 95˚C for 30 s and annealing extension at 63˚C for 60 s.

The standard curves of WT1 and β-actin were obtained by making serial dilutions of the K562 cell line cDNA for validation of the $2^{-\Delta\Delta CT}$ method. A plot of the log cDNA dilution versus ΔCT was made. If the absolute value of the slope is close to zero, the efficiencies of the target and reference genes are similar, and the ΔΔCT calculation for the relative quantification of the target may be used [194].

Results of Real-time PCR were analyzed by using MJ Opticon Monitor analysis system version 3.1 (Bio-Rad, USA). The amount of cDNA of each sample was shown in Ct (Cycle threshold) value, the number of cycles required for the fluorescent signal to cross the threshold. For each sample, to normalize the amount of *WT1*, the difference

between Ct value of *WT1* and *β-actin* was calculated, resulting in the ΔCt value of *WT1*. The ΔCt of crude extract treated cells was then subtracted from ΔCt of vehicle control, giving the final difference in cycle numbers of *WT1* between the vehicle control and treated samples (ΔΔCt value). The amount of target WT1 cDNA was obtained using relative quantification [194] as shown in the following formula, and reported in % WT1 mRNA level. The β-actin gene expression was used for normalization.

$$\text{Relative quantification} = 2^{-\Delta\Delta CT}$$

2.9 Preparation of protein extraction

2.9.1 Nuclear protein extraction

Nuclear protein was extracted by using Buffer A and 0.1% NP40 and Modified laemmli buffer [195]. After 48 h of treatment, 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 0.2% trypan blue dye. Following this, the cell suspension was removed to a microcentrifuge tube and then centrifuged. The supernatant was discarded. The cell pellet was resuspended with 1 mL of Buffer A, mixed, and centrifuged at 5,000 rpm for 5 min. The supernatant was discarded. The cell pellet was suspended in 200 µL of Buffer A with 0.1% NP40 and ground with 50 strokes using a homogenizer for better cell lysis. Then the cell suspension was incubated on ice for 30 min and centrifuged at 10,000 rpm for 15 min to separate cytoplasmic protein extract. After centrifugation, the supernatant containing cytoplasmic protein was discarded and the protein pellet with nuclear protein was

resuspended in 100 µL of modified laemmli buffer and ground with 10 strokes using a homogenizer to break intact nuclei. The tube was incubated on ice for 15 min and vortexed every 5 min. Finally, the protein suspension was centrifuged at 10,000 rpm for 15 min. The supernatant fraction containing nuclear protein was removed to a new sterile microcentrifuge tube. It was used for protein measurement. Nuclear proteins were kept at -20°C until analysis.

2.9.2 Measurement of protein concentration

The protein concentration was determined by the Folin-Lowry method. 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 mixture of phosphotungstic acid and phosphomolybdic acid , to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic protein residues (tyrosine and tryptophan amino acid), obtained from the first reaction. The strong color of reduced Folin reagent results from the increase of protein concentration. The concentration of the reduced Folin reagent is measured from the absorbance at 750 nm.

The protein sample concentration was compared to the BSA protein standard curve. The BSA protein standard curve could be constructed by preparation of various concentrations of BSA from stock, 1 mg/mL, as shown in Table 8 and Figure 3. Each protein sample was diluted with deionized distilled water in the ratio of 20:480 µL. Then 2.5 mL of alkaline copper solution (solution C) were added and mixed by using vortexing. After incubating at room temperature for 10 min, 250 µL of Folin-phenol reagent were added, mixed by vortex, and further incubated at room temperature for 30 min. Finally,

the concentrations of standard and sample protein were determined by spectrophotometry at a wavelength of 750 nm [197].

Table 8 Preparation of Bovine serum albumin (BSA) standard solution

BSA concentration (µg/mL)	Stock BSA 1mg/mL (µL)	Deionized distilled water (µL)
0	0	500
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

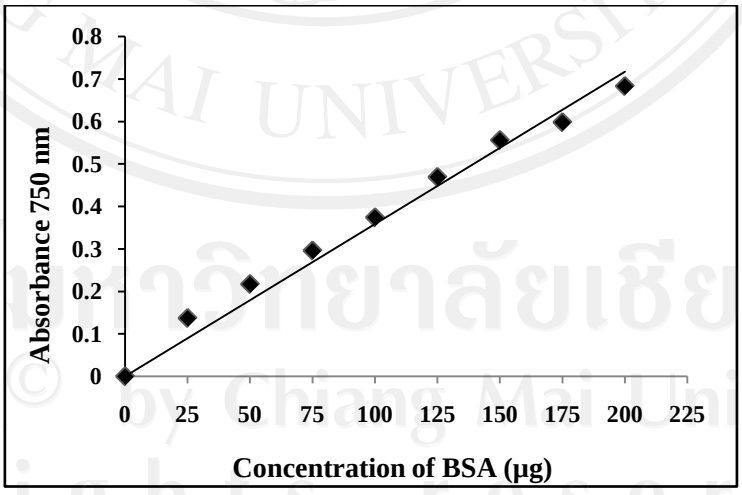

Figure 3 Standard curve of BSA

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 proteins according to their electrophoretic mobility, a function of length of polypeptide chain or molecular weight. The secondary and non–disulfide–linked tertiary structures of sample proteins were denatured by SDS, an anionic detergent which applied a negative charge to each protein in proportion to its mass, giving a near uniform negative charge along the length of the polypeptide. Thus, when the protein was applied onto the SDS gel, the negatively charged protein could be moved toward the positively charged electrode at rate depending on its molecular weight. The distance of migration through the gel could be assumed to be directly related to only the size of the protein, so that a small protein molecule could migrate more easily and faster than a larger molecule. The size of the protein of interest could be estimated by comparing the protein migration distance with a standard protein marker of known molecular weight. The concentration of acrylamide used for gel preparation depended on the size of the target protein. 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 proteins. To improve the resolution of protein bands, a discontinuous gel system consisted of staking and separating gel layers was used.

In this study, SDS-PAGE was used to analyze WT1 (target protein) and GAPDH (housekeeping protein) and their molecular sizes. First, two glass plates were cleaned with ethanol and allowed to dry. After that they were aligned and taken to the clamp. The 12% separating gel monomer solution was prepared and quickly po ured into the gap between

the glass plates before acrylamide polymerized, and then deionized distilled water was overlaid to adjust the gel surface. The gel was allowed to polymerize for 20 min. After separating gel setting, deionized 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 15 min. After polymerization was complete, the comb was removed by pulling it straight up slowly and gently, and the wells were washed 3 times with deionized 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 nuclear proteins were prepared by mixing four volumes of nuclear 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 in transfer buffer by using 30 volts of electricity overnight. After blotting, target protein on the membrane was determined by Western blot analysis.

2.10.2 Western blot analysis for WT1 and GAPDH protein detection

After blotting, the PVDF membrane with the transferred proteins was cut into 2 parts, which were used for the WT1 protein (MW 48-54 kDa) and GAPDH protein (MW 37 kDa) detection. The membranes were shaken in PBS for 5 min to remove any residues

of transfer buffer, 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) 2 times for 15 min and 5 min, respectively. Each membrane was incubated with primary rabbit polyclonal anti-WT1 antibody (WT1; clone C19; Santa Cruz, CA, USA) at a 1:1,000 dilution in blocking buffer and primary rabbit polyclonal anti-GAPDH antibody (GAPDH; FL-335; Santa Cruz, CA, USA) at a 1:500 dilution in blocking solution with shaking 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-rabbit 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 Chemiluminescent Substrate (PIERCE, USA), which included two substrate components, SuperSignal® 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 2-5 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).

2.11 Cytotoxicity of kaffir lime leaf fractional extracts on leukemic cell lines by MTT assay

Cytotoxic effects of each crude kaffir lime leaf fractional extract were determined in K562, U937, HL60, and Molt4 cell lines. Non-cytotoxic doses (IC_{20}) were used for studying *WT1* gene and WT1 protein expression. The four leukemic cell lines were prepared at a concentration of $1x10^4$ cells/100 µL at 37˚C under 5% CO_2 atmosphere, overnight. Crude kaffir lime leaf fractional extracts with various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) in 100 µL were added to the leukemic cells. The cells were incubated at 37˚C under 5% CO_2 atmosphere for 48 h. The percentage of cell viability was determined using the MTT assay as described in section 2.4.

2.12 Effects of crude kaffir lime leaf fractional extracts on *WT1* gene expression in leukemic cell lines

To determine the level of WT1 mRNA in four leukemic cell lines after crude kaffir lime leaf fractional extract treatment, the cells were cultured in complete RPMI 1640 medium with a non-cytotoxic concentration of crude kaffir lime leaf fractional extract (ethanol, hexane, ethyl acetate, n-butanol, and methanol) at 37˚C under 5% CO_2 atmosphere for 48 h as described in section 2.5. After that the total RNA was extracted and concentration the measured as described in section 2.6.2 and 2.6.2. The cDNA was

synthesized and led through the real-time PCR process as described in section 2.7 and 2.8. The levels of WT1 mRNA were compared to vehicle control (DMSO) of each experiment.

2.13 Dose dependent effect of crude kaffir lime leaf fractional extracts on *WT1* gene expression

After data analysis, the most effective crude kaffir lime leaf fractional extract that could inhibit WT1 mRNA synthesis in four leukemic cell lines was used to study the dosage pattern of its inhibitory effect on WT1 mRNA synthesis in the K562 cell line, which was a representative of all leukemic cancers. The cells were treated with the various non-toxic concentrations of the most effective crude kaffir lime leaf fractional extract at 37˚C under 5% CO_2 atmosphere for 48 h as described in section 2.5. Then the treated cells were harvested and total RNA was extracted and its concentration measured as described in section 2.6. The cDNA was synthesized and led through the real-time PCR process as described in sections 2.7 and 2.8. The levels of WT1 mRNA were compared with the vehicle control of each experiment.

2.14 Temporal pattern of the effect of crude kaffir lime leaf fractional extracts on *WT1* gene expression

After data analysis, the most effective crude kaffir lime leaf fractional extract that could inhibit WT1 mRNA synthesis in four leukemic cell lines was used to study the temporal patterns of its inhibitory effect on WT1 mRNA synthesis in the K562 cell line.

The cells were treated with the most effective crude kaffir lime leaf fractional extract at the same non-toxic concentration and incubated at 37˚C under 5% CO_2 atmosphere for 1, 2 and 3 days. Then the cells were harvested and total RNA was extracted and its concentration measured as described in section 2.6. The cDNA was synthesized and the real-time PCR process was carried out as described in section 2.7 and 2.8. The levels of WT1 mRNA of tests were compared with vehicle control of each experiment.

2.15 Effects of crude kaffir lime leaf fractional extracts on WT1 protein expression in leukemic cell lines

To determine the levels of WT1 protein in K562 and Molt4 leukemic cell lines (Anuchapreeda, 2008) after being treated with crude kaffir lime leaf fractional extracts, the cells were cultured in complete RPMI 1640 medium with crude kaffir lime leaf fractional extracts (ethanol, hexane, ethyl acetate, butanol, and methanol) as described in the study of the effect of crude extracts on *WT1* gene expression. Then the nuclear protein was extracted and WT1 protein levels determined by Western blot analysis as described in sections 2.9 and 2.10. The levels of WT1 protein of tests were compared to vehicle control of each experiment.

2.16 Dose dependence effect of crude kaffir lime leaf fractional extracts on WT1 protein expression

The most effective crude kaffir lime leaf fractional extract on WT1 protein levels in K562 and Molt4 leukemic cells was used to study the inhibitory effect on WT1 protein

synthesis in the K562 cell line, which was used as a representative of all leukemic cancers. The cells were treated with the various non-cytotoxic concentrations of the most effective crude kaffir lime leaf fractional extract as described in the study of the effect of concentrations of crude extracts on *WT1* gene expression. After that the nuclear protein was extracted and WT1 protein levels determined by Western blot analysis as described in sections 2.9 and 2.10. The levels of WT1 protein were compared to the vehicle control of each experiment.

2.17 Temporal pattern of the effect of crude kaffir lime leaf fractional extracts on WT1 protein expression

The most effective crude kaffir lime leaf fractional extract on WT1 protein levels in K562 and Molt4 leukemic cells was used to study the temporal pattern of the inhibitory the effect on WT1 protein synthesis in time dependent manner in the K562 cell line, which was used as a representative of all leukemic cancers. The cells were treated with non-cytotoxic concentrations at the IC_{20} of most effective crude kaffir lime leaf fractional extract as described in the study of effect of the duration of crude extracts on *WT1* gene expression. After that the nuclear protein was extracted and WT1 protein levels determined by Western blot analysis as described in sections 2.9 and 2.10. The levels of WT1 protein of tests were compared to the vehicle control of each experiment.