CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and reagents

The details concerning chemicals and reagents used in this study are shown in Appendix A.

2.2 Cells and cell culture conditions

Leukemic cell lines used in this study were human erythroid leukemia (K562), human promyeloid leukemia (HL-60), human monocytic leukemia (U937), and human lymphoblastic leukemia (Molt4). These cell lines were cultured in RPMI 1640 medium with 110 mg/mL pyruvate, 10 mM HEPES, 100 units/mL penicillin, 100 µg/mL streptomycin and supplemented with 10% fetal bovine serum. The cell lines were grown at 37 ^{o}C under 5% CO_2 atmosphere.

2.3 Mangosteen peel fractions extract

Mangosteen fraction extracts used in this study were extracted from the pericap of the mangosteen. Ten kilograms of fresh mangosteen peels were dried at 50^{o}C, until completely dried (dry weight was approximately 1 kg). After drying the magosteen peels were ground to a powder, after which 400 g of the powdered dry magosteen peel were extractd with 95% ethanol for 24 hours and this was repeated 3 times. The ethanol extract was evaporated to yield a crude extract (fraction 1). Dried mangosteen peel powder (600 g) was extracted with hexane for 24 hours for a total of 3 times and the supernatant was evaporated to obtain a crude extract (fraction 2). The sediment product from fraction 2 was dried at 50^{o}C and further extracted with ethyl acetate for 24 hours for a total of 3 times, and then evaporated to obtain a crude extract (fraction 3). The sediment product from fraction 3 was extracted with butanol and methanol. The ethanol fraction was precipitated with bovine serum albumin (BSA) to obtain

fraction 6. The pure xanthone and mangostin were generous gifts from Assist. Prof. Dr. Chadarat Ampasavate.

2.4 Mangosteen peel fraction extracts treatments

After reaching 80% confluent with cultured cells, the leukemic cells were washed 3 times with sterile PBS and then counted for cell viability with 0.2% trypan blue. K562 cells at a concentration of 1.5 x 10^5 cells/mL and HL-60, U937, and Molt4 cells at concentrations of 1.0x10^5 cells/mL were cultured with each of the various mangosteen peel extracts. These tretments were cultured in complete RPMI 1640 medium for 48 h at 37°C under 5% CO_2 atmosphere.

2.5 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay measures the conversion of tetrazolium 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan in living cells. The reaction is catalyzed by mitochondrial succinate dehydrogenase and requires NADH, which must be supplied by the living cells, thus providing an indication of cell viability. The formazan crystals are then solubilized with dimethyl sulfoxide (DMSO). The amount of solubilized formazan product is spectrophotometrically measured using an ELISA plate reader.

The MTT assay was used to detect cytotoxicity of mangosteen peel fraction extracts on the four types of leukemic cell lines. 1.5 x 10^5 cells were placed into flat-bottom 96 well plates and cultured overnight. Various concentrations of mangosteen peel extracts dissolved in 100 µL medium were added and incubated for 48 h. Then 100 µL of medium were removed and 15 µL of MTT dye (sigma-Aldrich; USA) were added, and then incubated for 4 h. After incubation the supernatant from each well was removed. A volume of 200 µL DMSO was added to each well to dissolve the formazan crystals. The optical density was measured by an ELISA reader at 540 nm with a reference wavelength of 630 nm. Percentage of cell survival was calculated by the following formula.

$$\% \text{ Cell survival} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}} \times 100$$

2.6 Preparation of total RNA extract

2.6.1 Total RNA extraction

After the mangosteen peel extract treatment, leukemic cells were washed 3 times with ice cold PBS. The cells were counted for viability with 0.2% trypan blue. The cell pellet was resuspended in TRIzol® reagent (1 mL per 5-10 x 10^6 cells) for cell lysing and homogenized using a glass homogenizer on ice for 30 strokes. Then, the cell homogenate was centrifuged at 12,000 rpm for 10 min to precipitate insoluble material such as extracellular membrane, polysaccharide and high molecular weight DNA; the supernatant contained RNA. The supernatant was transferred to a fresh sterile microcentrifuge tube and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 mL of chloroform was added to the tube and the tube was shaken vigorously by hand for 15 sec. The sample was incubated at room temperature for 2 to 3 min and centrifuged at 12,000 rpm for 15 min. Following centrifugation, the mixture was separated into a lower red phase, called the phenol-chloroform phase or interphase, and a colorless upper aqueous phase. Total RNA remained exclusively in the aqueous phase. After transferring the aqueous phase to a new sterile microcentrifuge tube, 0.5 mL of isopropyl alcohol was added to the tube and mixed vigorously to precipitate the total RNA form the aqueous phase. The solution was incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min. The total RNA forms a gel-like pellet on the side and bottom of the tube. After centrifugation, the supernatant was removed. The RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 10,000 rpm for 5 min. Then, 75% ethanol was removed and the RNA pellet was briefly air-dried for 5 to 10 min. Finally, DEPC-treated water was added to dissolve the RNA pellet and 0.5 µL (40 U/µL) RiboLock™ ribonuclease inhibitor (Fermentas, USA) was added for RNA protection. The RNA solution was stored at –70°C until examination.

2.6.2 Measurement of purity and concentration of total RNA extract

After RNA extraction, total RNA extract was measured for purity and concentration by spectrophotometry at an absorbance of 260 nm/280 nm ratio and the RNA concentration was calculated by the following formula.

RNA concentration (µg/mL) = Absorbance 260 nm x 40 µg/mL x Dilution factor

2.7 Effect of mangostin fractions on *WT1* gene expression

In this study, Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using SuperScript™ III One-step RT-PCR System with Platinum® *Taq* DNA polymerase reagent (Invitrogen™ USA) Briefly, a master mix containing reaction buffer, *Taq* DNA polymerase and sets of primers was prepared in a single tube and then aliquoted into individual thin-walled PCR tubes. DEPC-treated water and 1µg of total RNA were then added. The reaction mixture was kept on ice until the PCR cycling had started.

The sets of primers used in this study were WT1 primers for the *WT1* gene and GAPDH primers for the *GAPDH* gene, which was used as housekeeping gene. For WT1, the sense primer (5'GGCATCTGAGACCAGTGAGAA-3') and the anti-sense primer ((5'GAGAGTCAGACTTGAA AGCAGT-3') were used. These sequences corresponded to residues 780 to 800 on exon 7 and residues 1232 to 1253 on exon 10, respectively. For GAPDH, the sense primer (5'CGAAGTCAACGGATTTGGTCGTA T–3') and the anti-sense primer (5'-AGCCTTCTCGGTGGTGAAGAC-3') were used. These sequences corresponded to residues 888 to 911 and 1174 to 1194, respectively.

The RT-PCR cycling condition was started as one cycle of cDNA synthesized at 94°C for 30 min and denatured at 94°C for 2 min. PCR amplification was performed for 35 cycles of sequential denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. After amplification, the mixture was incubated at 72°C for 3 min. The resulting PCR products were 474 and 306 bp for *WT1* and *GAPDH* gene, respectively. For a negative control, deionized distilled water was amplified using the same condition to detect any possible contamination. A total of 15 µL of each PCR product were electrophoresed on a 1% agarose gel at 100 volts for 35 min. Then the gel was visualized with ethidium bromide staining (2 mg/mL).

2.8 Preparation of nuclear protein extract for WT1 protein measurement

2.8.1 Nuclear protein extraction

In this step, NE-PER® Nuclear and Cytoplasmic Extraction Reagents (PIERCE, USA) were used to extract nuclear membrane protein from the leukemic cell lines. After mangosteen peel fraction treatments, leukemic cells were washed three times

with ice cold PBS. The cells were counted for viability with 0.02% Trypan blue. The supernatant of the cell suspension was then carefully removed and the cell pellet was made as dry as possible. After that, 200 µL of ice cold Cytoplasmic Extraction Reagent I (CER I) were added to the cell pellet to disrupt the cell membranes. The cell pellet was fully resuspended by vortexing the tube vigorously for 15 sec. The cell suspension was incubated on ice for 10 min. Then, 11 µL of ice-cold Cytoplasmic Extraction Reagent II (CER II) were added and the tube was vortexed vigorously for 5 sec and incubated on ice for 1 min. The tube was centrifuged at 13,500 rpm for 5 min to separate the cytoplasmic extract (in the supernatant). The supernatant fraction was transferred into a new tube and kept on ice until storage. Then, the insoluble fraction, which contained nuclei, was resuspended with 100 µL of ice-cold Nuclear Extraction Reagent (NER). The tube was incubated on ice and vortexed for 15 sec every 10 min, for a total of 40 min. Finally, the tube was centrifuged at 13,500 rpm for 10 min and the supernatant fraction, which contained nuclear protein extract, was then removed into a new tube and kept at -70°C until analyzed.

2.8.2 Measurement of protein concentration

The protein concentration was measured by the Folin-Lowry method. The basis of this method is the reaction of protein with copper (II) ion under alkaline conditions and the Folin-Ceocalteau phosphamolybdicphosphate acid reduction to heteropolymolybsdenum blue by the copper-catalyzed oxidation of aromatic amino acids.

The protein standard curve was constructed by preparing BSA in various concentrations from stock 1 mg/mL BSA, as shown in Table 8 and Figure 7. An aliquot (20 µL) of solubilized protein sample was diluted with 480 µL of deionized distilled water. The 2.5 mL alkaline copper solution (Reagent C) was added and mixed. After standing at room temperature for 10 min, 250 µL of Folin-phenol reagent (Reagent D) was added, mixed gently and allowed to stand for 30 min at room temperature. The concentrations of standard BSA and test samples were determined by spectrophotometry at an absorbance of 750 nm.

Table 8 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

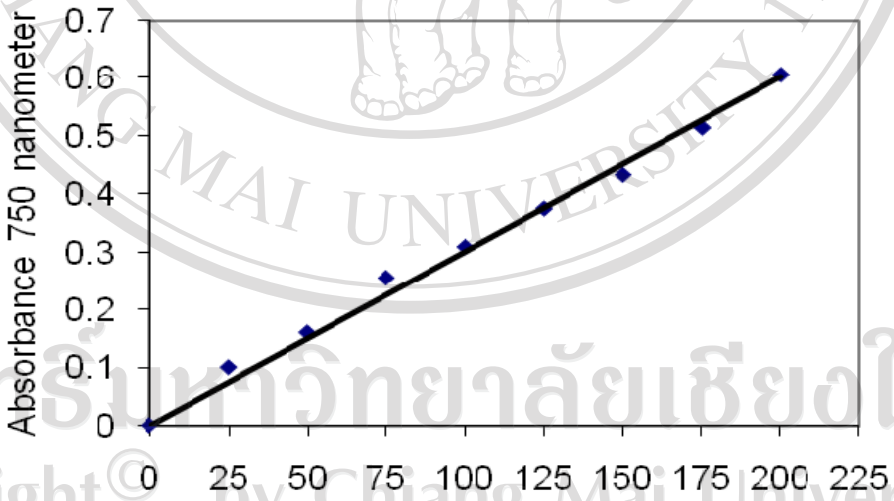

Figure 8 Standard curve of BSA

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 applied for analyzing the WT1 protein and its molecular size. The SDS-PAGE procedure separates proteins according to their apparent sizes *via* the mechanism of the anionic detergent SDS making the surface charge uniformly negative. When the nuclear membrane protein extract is applied onto a gel matrix and placed in an electrical field, the negatively charged protein molecules move toward the positively charged electrode at rates dependent upon their molecular weight. A small protein molecule can move through the gel easily and hence migrate faster than a larger molecule. The size of a protein can be estimated by comparison of its migration distance with that of a known molecular weight standard protein marker. The concentration of acrylamide used for the gel depends on the size of the proteins to be analyzed. Low acrylamide concentrations are used to separate high molecular weight proteins, while high acrylamide concentrations are used to separate low molecular weight proteins. Improved resolution of protein bands is achieved by the use of a discontinuous gel system having stacking and separating gel layers.

Electrophoresis was run as follows. The glass plates were first cleaned in detergent, washed with water and ethanol and allowed to dry. They were aligned and taken to the clamp. The separating gel monomer solution (12%) was prepared and poured onto the glass plate quickly before the acrylamide polymerized. The monomer solution was immediately overlaid with distilled water. The gel was allowed to polymerize for 20 min. The overlay solution was completely rinsed off with distilled water. Then, the staking gel monomer solution (4%) was prepared and poured onto the top of the separating gel. The comb was inserted into the gel solution and the gel was allowed to polymerize for 15 min. After polymerization was completed, the comb was replaced by pulling it straight up slowly and gently. The wells were completely washed with distilled water. After that, the clamp assembly gel sandwich was released form the casting stand. The gel was placed in the electrophoresis chamber. The electrode buffer was prepared and used to fill the reservoir. The pre-running step was performed at 100 volts for 30 min.

The nuclear protein (100 µg/lane) of tested samples, which was prepared by mixing four volumes of the protein sample with one volume of 5X reducing buffer, was loaded into wells under the electrode buffer. After sample application, electrophoresis was carried out using 100 volts for 1 h. Then, the separated proteins on the gel were transferred to the nitrocellulose membrane by using 30 volts of electricity overnight in transferring buffer. After transblotting, electrophoretically resolved proteins were analyzed by Western blot analysis.

2.9.2 Western blot analysis for WT1 protein detection

After blotting, the nitrocellulose membrane was incubated sequentially with 5% skimmed milk in PBS (blocking solution) for 2 h with shaking at room temperature to block non-specific binding. Then, the membrane was incubated with primary rabbit polyclonal anti-WT1 antibody (WT1; C-19); (SantaCruz, CA, USA) buffer (0.1% PBS-tween) six times, 5 min each to remove excess primary antibodies. In the next step, the membrane was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP); (PIERCE, USA) at a 1:15,000 dilution in blocking buffer for 1 h with shaking at room temperature. Then, the membrane was washed with washing buffer six times to remove excess antibodies. Finally, bound proteins were detected by using the SuperSignal® West Pico Chemiluminescent Substrate (PIERCE, USA). This is a two-component substrate that contains a SuperSignal® West Pico luminal/enhancer solution and a stable peroxide solution. These two components were mixed together at a one-to-one ratio to give sufficient cover to the membrane (750:750 µL). The detection reagent was added to the protein side of the membrane. The membrane was incubated in the detection reagent for 5 min at room temperature. The excess buffer was drained and the membrane was placed on a piece of Saranwrap. Then, the wrapped membrane was placed into the film cassette. The work was carried out as quickly as possible in order to minimize the delay between incubation the membrane in the detection reagent and exposing it to the film. After that, Kodak Medical X-ray Film (Kodak, USA) was placed on the top of the membrane for 5 to 20 min. The film was removed and developed by incubating in developing solution for 1 min and fixed in fixative solution for 1 min. Finally, the protein band was quantified using a scan densitometer (BIO-RAD, USA).

2.9.3 Western blot analysis for GAPDH protein detection

After the stripping procedure, the nitrocellulose membrane was incubated sequentially with blocking solution for 30 min with shaking at room temperature to block non-specific binding. Then, the membrane was incubated with primary rabbit polyclonal anti-GAPDH antibody (GAPDH; FL-335); (SantaCruz, CA, USA) at a 1:1,000 dilution in blocking solution for 1 h with shaking at room temperature. The membrane was washed with washing buffer six times, 5 min each to remove excess primary antibodies. The membrane was next incubated with goat anti-rabbit IgG conjugated with HRP; (PIERCE, USA) at a 1:15,000 dilution in blocking buffer for 1 h with shaking at room temperature. Then, the membrane was washed with washing buffer six times to removed excess antibodies. Finally, bound proteins were detected by using the SuperSignal®West Pico Chemiluminescent Substrate (PIERCE, USA). The detection reagent was added to the protein side of the membrane. The membrane was incubated in the detection reagent for 5 min at room temperature. The excess buffer was drained and the membrane was placed on a piece of Saranwrap. Then, the wrapped membrane was placed into the film cassette. The work was carried out as quickly as possible in order to minimize the delay between incubating the membrane in the detection reagent and exposing it to the film. After that, Kodak Medical X-ray Film (Kodak, USA) was placed on the top of the membrane for 5 to 20 min. The film was removed and developed by incubating in developing solution for 1 min and fixed in fixative solution for 1 min. Finally, the protein band was quantified using a scan densitometer (BIO-RAD, USA)

2.10 Cytotoxicity of mangosteen peel fractions on leukemic cell lines by MTT assay

To determine the cytotoxicity of magosteen peel fraction extracts on the four leukemic cell lines, cells (1×10^5cells/well) were plated in 100 µL of medium containing magosteen peel extract and incubated for 48 h. The cell viability in each well was determined by MTT assay as described in section 2.5.

2.11 Determination of the levels of WT1 mRNA in leukemic cell lines

The levels of WT1 mRNA in four cell lines were measured. The cell lines were cultured in complete RPMI 1640 medium at 37ºC under 5% CO_2 atmosphere. After

reaching 80% confluent, the cells were removed and washed 3 times with ice cold PBS. The total RNA was extracted using the TRIzol® reagent (Invitrogen™ USA) and its concentration measured as described in sections 2.6.1 and 2.6.2. RT-PCR was used to determine the level of WT1 mRNA in all of the leukemic cell lines. One microgram of total RNA was amplified via the SuperScript™ III One-step RT-PCR System with Platinum® *Taq* DAN polymerase reagent (Invitrogen™ USA) as described in section 2.7.

2.12 Determination of the levels of WT1 protein in leukemic cell lines

The levels of WT1 protein in four cell lines were measured. The cell lines were cultured in complete RPMI 1640 medium at 37ºC under 5% CO_2 atmosphere. After reaching 80% confluent, the cells were removed and washed 3 times with ice cold PBS. The total nuclear membrane proteins from four cell lines were extracted and their concentrations were measured as described in section 2.8.1 and 2.8.2. WT1 protein levels were determined using Western blot analysis as described in section 2.9.

2.13 Effect of magosteen peel fractions on *WT1* gene expression

To study the effect of magosteen fractions on *WT1* gene expression, each leukemic cell line was cultured in complete RPMI 1640 medium with ethanol ethylacetate hexane butanol and methanol as described in section 2.4 for 2 days at 37ºC under 5% CO_2 atmosphere. After 2 days, the total RNA was extracted and RT-PCR was carried out as described in sections 2.6 and 2.7. The level of WT1 mRNA was compared with the vehicle control of each experiment.

2.14 Effect of magosteen peel fractions on *WT1* gene expression in a dose dependent manner

After data analysis the magosteen peel fraction that had the strongest inhibitory effect on WT1 mRNA level in each type of leukemic cell line, was used to study its effect on WT1 mRNA in a dose dependent manner. Non-toxic concentrations of each fraction were used for each treatment. DMSO at 0.05% was used as a vehicle control. After 2 days of incubation, the total RNA was extracted and RT-PCR was carried out as described in sections 2.6 and 2.7. The level of WT1 mRNA was compared with the vehicle control of each experiment.

2.15 Effect of magosteen peel fractions on *WT1* gene expression in a time dependent manner

After data analysis, the magosteen extract that had the strongest inhibitory effect on WT1 mRNA level in each type of leukemic cell line was used to study its effect on WT1 mRNA in a time dependent manner. The leukemic cell lines were treated with each fraction and cultured at 37ºC under 5% CO_2 atmosphere for 1, 2, and 3 days. For the vehicle control, the cells were incubated with 0.05% DMSO for 3 days. After incubation, the total RNA was extracted and RT-PCR was carried out as described in sections 2.6 and 2.7. The level of WT1 mRNA was compared with the vehicle control of each experiment.

2.16 Effect of magosteen peel fractions on WT1 protein expression

To study the effect of magosteen fractions on WT1 protein expression, each leukemic cell line was cultured as described for the effect of magosteen fractions on *WT1* gene expression (section 2.13). The nuclear protein was extracted and Western blot analysis was carried out as described in sections 2.8 and 2.9. The levels of WT1 protein expression were compared with the vehicle control of each experiment.

2.17 Effect of mangosteen peel fractions on WT1 protein expression in a dose dependent manner

After data analysis, the magosteen fraction that had the strongest inhibitory effect on WT1 protein level in each leukemic cell line was used to study its effect on WT1 protein in a dose dependent manner. Non-toxic concentrations of mangosteen peel fractions were used for each treatment. DMSO at 0.05% was used as a vehicle control. After 2 days of incubation, the nuclear protein was extracted and Western blot analysis was carried out as described in sections 2.8 and 2.9. The level of WT1 protein was compared with the vehicle control of each experiment.

2.18 Effect of magosteen fractions on WT1 protein expression in a time dependent manner

After data analysis, the magosteen peel fraction that had the strongest inhibitory effect on WT1 protein level in each type of leukemic cell line was used to study its effect on WT1 protein in a time dependent manner. The leukemic cell lines were

treated with magosteen peel fractions and cultured at 37ºC under 5% CO_2 atmosphere for 1, 2, and 3 days. For the vehicle control, the cells were incubated with 0.05% DMSO for 3 days. After incubation, the nuclear protein was extracted and Western blot analysis was carried out as described in sections 2.8 and 2.9. The level of WT1 protein was compared with the vehicle control of each experiment