APPENDIX A

CHEMICALS AND REAGENTS

Name of chemical/ reagent	Source/ Company
Absolute ethanol	Merck, Darmstadt, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamide	Bio-Rad, Richmond, CA, USA
Ammonium persulfate (APS)	Bio-Rad, Richmond, CA, USA
Bis (N, N'-Methylene-bis-acrylamide)	Bio-Rad, Richmond, CA, USA
Bovine serum albumin	PIERCE, Rockford, IL, USA
Bromphenol blue	Sigma-Aldrich, St. Louis, MO, USA
Coomasie brilliant blue R-250	Sigma-Aldrich, St. Louis, MO, USA
Copper sulfate	Merck, Darmstadt, Germany
Developer and replenisher	Kodak, NY, USA
Diethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA LAB-SCAN, Bangkok, thailand
Disodium hydrogen phosphate	Merck, Darmstadt, Germany Fluka, Buchs, Switzerland
Dithiothreitol (DTT)	Roche Applied Science, Mannheim, Germany
DyNAmo™ Probe q PCR Kit	Finzymes, Espoo, Finland

Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Fetal bovine serum	GIBCO-BRL, Grand Island, NY, USA
Folin & Cocalteu's phenol reagent	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Amresco, Ohio, USA
HEPEPS	Sigma-Aldrich, St. Louis, MO, USA
High Pure RNA Isolation Kit	Roche Applied Science, Mannheim, Germany
HRP conjugated goat anti-rabbit IgG	Promega, Madison, WI, USA
Hydrochloric acid (HCL)	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
L-glutamine	GIBCO-BRL, Grand Island, NY, USA
Magnesiumchloride	Merck, Darmstadt, Germany
Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Methanol	LAB-SCAN, Bangkok, Thailand
MTT	Sigma-Aldrich, St. Louis, MO, USA
PageBlue™ Protein Staining Solution	Fermentas, Maryland , USA
PageRuler™ Prestained Protein Ladder	Fermentas, Maryland , USA
Penicillin-streptomycin	GIBCO-BRL, Grand Island, NY, USA
Phenylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich, St. Louis, MO, USA
Potassium chloride	Merck, Darmstadt, Germany

Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Primers	Operon, Huntsville, Alabama, USA
Probes	Operon, Huntsville, Alabama, USA
Rabbit polyclonal anti-GAPDH antibody (GAPDH; FL-335)	Santa Cruz Biotechnolygy, CA, USA
Rabbit polyclonal anti-GAPDH antibody (WT1; C-19)	Santa Cruz Biotechnolygy, CA, USA
Restore™ Western Blot Stripping Buffer	PIERCE, Rockford, IL, USA
RPMI-1640 powder	GIBCO-BRL, Grand Island, NY, USA
Skim milk	Fluka, Buchs, Switzerland
Sodium bicarbonate	Merck, Darmstadt, Germany
Sodium carbonate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, St. Louis, MO, USA
Sodium potassium Tartrate	Sigma-Aldrich, St. Louis, MO, USA
SuperSignal® West Pico Chemiluminescent substrate	PIERCE, Rockford, IL, USA
TEMED	Bio-Rad, Richmond, CA, USA
Transcriptor high fidelity cDNA synthesis kit	Roche Applied Science, Mannheim, Germany
Tris	Vivantis, Oceanside, CA, USA
Trypan blue	Sigma-Aldrich, St. Louis, MO, USA
Tween 20	Sigma-Aldrich, St. Louis, MO, USA

Water, sterile, nuclease-free	Amresco, Ohio, USA

APPENDIX B

INSTRUMENTS

Instrument	Company
Analytical balance	Ohaus corporation, USA
Autoclave	Huxley, Taiwan
Automatic pipette	Biohit, Finland; Gilson, USA; Labmate,UK; and Bio-Rad, USA
Automatic pipette tip	Bioline, UK
Carbon dioxide incubator	Jouan, UK
Centrifuge	Clay Adams (BD), USA
Chromo4 Real-time PCR detection system	Bio-Rad, USA
15 or 50 ml centrifuge tube	Greiner bio-one and Corning incorporation
Deionized distilled water machine	PK water text
Clear blue X-ray film	Thermo fisher scientific, USA
Freezer (-80)	PTW ultra cold
Freezer (-20)	Sanyo, Japan
Gel documentation	Bio-Rad, USA
10 cm glass plate	PYREX, USA; and PETRIO
Homogenizer	Pargus, Japan

Hot air oven	Thai stianless argon, Thailand
Inverted microscope	Olympus, Japan
Larminar flow biological cabinet	Clean
Light microscope	Olympus, Japan
MJ Opticon Monitor analysis system version 3.1	Bio-Rad, USA
PVDF membrane	Pall corporation, USA
Magnetic stirrer	Sybron/ Thermolyne
Microcentrifuge, bench-topped	Eppendorf, Germany
Microcentrifuge	CLP
Millipore filter paper	Pall corporation, USA
Mini protein II slab gel	Bio-Rad, USA
Pipette-aid	Drummond, USA
Pasture pipette	Pyrex, USA
PCR amplifier	Eppendorf, Germany
pH meter	Thermo Orion, USA
Power supply	E-C apparatus corporation, USA
Real time PCR amplifier	Roche, Germany
Refrigerator	Toshiba, Japan
Serological pipette	Pyrex, USA
Spectrophotometer	Shimadzu, Japan

25 or 75 cm^3 T-flask	SPL life Sciences, Korea
Thin-wall PCR strip tube	Roche, Germany
Thin-wall PCR tube	Molecular bio products
Trans-blot® electrophoretic transfer cell	Bio-Rad, Richmond, CA, USA
Vortex mixer	Gemmy industrial corporation
Water bath	Witeg, Korea

APPENDIX C

REAGENTS AND BUFFERS PREPARATION

Human leukemic cell lines culture

1. **Incomplete RPMI 1640 medium**

RPMI 1640 powder (GIBCO-BRL)	10.4	g (1 pack)
HEPES	3.57	g
$NaHCO_3$	2.0	g
0.34% 2-Mercaptoethanol	1.0	mL

All substances were dissolved in 800 mL of deionized distilled water and adjusted to pH 7.2-7.4. After that the volume was adjusted to 1,000 mL in a volumetric flask and sterilized by filtration through suction filter with 0.2 µm filter membrane. Medium was checked for sterility before use and stored at 4˚C.

2. **Complete RPMI 1640 medium**

Incomplete RPMI 1640	88.5	mL
10,000 Units/mL Penicillin/ 10,000 µg/mL Streptomycin	1	mL
200 mM L-Glutamine	0.5	mL
Fetal bovine serum	10	mL

3. **Freezing solution**

8% DMSO in fetal bovine serum

Fetal bovine serum	9.2	mL
DMSO (biological grade)	0.8	mL

Stored at 4˚C

4. Phosphate buffer saline (PBS) pH 7.4

KH_2PO_4	0.24	mL
Na_2HPO_4	1.44	mL
NaCl	8.0	mL
KCl	0.2	mL

All substances were dissolved in 800 mL of deionized distilled water and adjusted to pH 7.2. After that volume was adjusted to 1,000 mL in volumetric flask and sterilized in an autoclave.

Cell survival measurement

1. 0.2% (w/v) Trypan blue

Trypan blue	0.2	g
PBS	100	mL

2. MTT dye

MTT dye	1.0	g
PBS	200	mL

The dissolved MTT dye was filtrated through membrane filter size 0.2 µm to remove any non-soluble powder and then kept at 4˚C in dark container.

Real-time polymerase chain reaction (Real-time PCR)

1. **Total RNA preparation**

High Pure RNA Isolation Kit (From lab direction)

Reagent contents	**Components**	**Preparation**
Lysis/-Binding Buffer	[4.5 M guanidine-HCl, 50 mM Tris-HCl, 30% Triton X-100 (w/v), pH 6.6]	
DNase I, recombinant, lyophilizate	10 KU lyophilized DNase I	Resuspend in 0.55 mL Elution Buffer
DNase Incubation Buffer	[1 M NaCl, 20 mM Tris-HCl and 10 mM MnCl2, pH 7.0]	
Wash Buffer I	[5 M guanidine hydrochloride and 20 mM Tris-HCl, pH 6.6] final concentrations after addition of 20 mL ethanol	Add 20 mL ethanol p.a. before first use
Wash Buffer II	[20 mM NaCl, 2 mM Tris-HCl, pH 7.5] final concentrations after addition of 40 mL ethanol	Add 40 mL ethanol p.a. before first use
Elution Buffer	Nuclease-free, sterile, double distilled water	

2. **DEPC treated water**

Deionized distilled water	1	L
Diethylpyrocarbonate (DEPC)	100	µL

The DEPC treated water was shaken vigorously and stored at room temperature overnight and DEPC removed by autoclaving.

Real-time polymerase chain reaction (Real-time PCR)

1. cDNA preparation

Transcriptor high fidelity cDNA synthesis kit

- **Template-primer mix preparation** (1 reaction)

Component	**Volume (µL)**	**Final concentration**
Total RNA	Variable	0.5 µg
Random Hexamer primer	2	60 µM
Sterile nuclease free water	Variable	
Total	11.4	

- **Reverse transcriptase mix preparation**

Component	**Volume (µL)**	**Final concentration**
Reaction buffer	4	8 mM
Protector Rnase inhibitor	0.5	20 U
Deoxynucleotide mix	2	1 mM
DTT	1	5 mM
Reverse transcriptase	1.1	10 U
Template-primer mix	11.4	
Total	20	

2. Primer and Probe preparation

WT1 primers and probe

- **WT1 forward primer (F1):**

  5’GATAACCACACAACGCCCATC3’

  436.43 µg, OD 14.22

  MW 6316.14 µg/ µmole, 50 nmole, 691.31 µl for 100 µM

- **WT1 reverse primer (R1):**

  5’CACACGTCGCACATCCTGAAT3’

  631.5 µg, OD 19.72

  MW 6335.17 µg/ µmole, 50 nmole, 996.82 µl for 100 µM

- **WT1 probe:**

  5’FAM-ACACCGTGCGTGTGTATTCTGTATTGG-TAMRA3’

  324.5 µg, OD 9.88

  MW 9768.92 µg/ µmole, 0.2 µmole, 332.17 µl for 100 µM

β-actin primers and probe

- **β-actin forward primer:**

  5’CCCAGCACAATGAAGATCAAGATCAT3’

  721.8 µg, OD 24.6

  MW 7941.25 µg/ µmole, 50 nmole, 908.93 µl for 100 µM

- **β-actin reverse primer:**

  5’ATCTGCTGGAAGGTGGACAGCGA’3

  636.67 µg, OD 20.87

MW 7153.71 µg/ µmole, 50 nmole, 889.99 µl for 100 µM

- **β-actin probe:**

  5'FAM-TGAGCGCAAGTACTCCGTGTGGATCGGCG-TAMRA3'

  188.45 µg, OD 5.86

  MW 10431.28 µg/ µmole, 0.2 µmole, 180.66 µl for 100 µM

Primers and probes were dissolved in sterile nuclease free water, following the description to obtain primer and probe concentrations of 100 µM for stock 1. During use, primers and probes were diluted from stock 1 to a final concentration of 10 µM in sterile nuclease free water.

3. Real-time PCR

- **Reagent components**

Reagent in final concentration	**Reagent in final concentration**
cDNA (≤10 ng/mL)	cDNA (≤10 ng/mL)
1x reaction mix - hot start *Tbr* DNA polymerase - Optimized PCR buffer - $MgCl_2$ - dNTP mix including dUTP	1x reaction mix - hot start *Tbr* DNA polymerase - Optimized PCR buffer - $MgCl_2$ - dNTP mix including dUTP
0.5 µM WT1 forward primers	0.5 µM β-actin forward primer
0.5 µM WT1 reverse primers	0.5 µM β-actin reverse primer
250 nM TagMan probes (WT1)	250 nM TagMan probe (β-actin)

- **Template-primer-probe mix preparation (1 reaction)**

Components	**Volume (µL)/ final volume 20 µL**
2x reaction mix	10
WT1/ β-actin forward primer [10 µM]	0.8
WT1/ β-actin reverse primer [10 µM]	0.8
WT1/ β-actin probe [10 µM]	0.4
Template RNA	2
Sterile nuclease free water	6
Total	20

Nuclear protein extraction

1. **1 M H HEPES**

   HEPES (MW 238.30) 5.9575 g

   Deionized distilled water 25 mL

   The pH was adjusted to 7.9 by using conc. NaOH.

2. **4.2 M $MgCl_2$**

   $MgCl_2.6H_2O$ (MW 203.31) 42.663 g

   Deionized distilled water 50 mL

3. **100 mM DTT**

   Dithiothreitol (DTT) (MW 154.2) 0.0154 g

   Deionized distilled water 1 mL

4. 100 mM PMSF

Phenylmethanesulfonylfluoride (MW 174.2)	0.0871	g
Isopropanol	5	mL

5. Buffer A

1 M HEPES pH 7.9	2	mL
4.2 M $MgCl_2$	72	µL
KCl	0.1492	g
100 mM DTT	1	mL

Deionized distilled water was added until the volume reached 200 mL.

6. Buffer A + 0.1% NP40

100% NP40	100	µL

Buffer A was added until the volume reached 10 mL.

7. Modify Laemmli buffer: Laemmli buffer + PMSF +DTT

Laemmli buffer

Deionized distilled water	6.25	mL
1 M Tris-HCl, pH 6.8	0.625	mL
10% SDS	2	mL
Glycerol	1	mL

All substances were mixed together and then 90 µL of Laemmli buffer was mixed with 5 µL of PMSF and 5 µL of DTT for preparing Modify Laemmli buffer.

Protein measurement

1. **Reagent A**

   2% (w/v) Na_2CO_3 in 0.1 N NaOH

NaOH	2.0	g
Na_2CO_3	10.0	g
Deionized distilled water	500	mL

   NaOH solution was prepared before adding Na_2CO_3.

2. **Reagent B :**

   0.5% (w/v) $CuSO_4 \cdot 5\ H_2O$ in 1% (w/v) $NaKC_4H_4O_6 2H_2O$ (Na-K Tartrate)

   0.5% (w/v) $CuSO_4 \cdot 5\ H_2O$

$CuSO_4 \cdot 5\ H_2O$	0.5	g
Deionized distilled water	50	mL

   1% (w/v) $NaKC_4H_4O_6 2H_2O$ (Na-K Tartrate)

Na-K Tartrate	1.0	g
Deionized distilled water	50	mL

   Reagent B was prepared by mixing $CuSO_4$ and Na-K Tartrate ratio 1:1.

3. **Reagent C**

   Reagent C was freshly prepared by mixing Reagent A and Reagent B ration 50:1.

4. **Folin-ciocalteau phenol reagent 1N**

   Folin-ciocalteau phenol reagent 2 N was diluted to 1N by using deionized distilled water.

SDS-PAGE analysis

1. **Stock solution A**

Separating gel buffer 1.5 mM Tis-HCl, pH 8.8

Tris-base	18.15	g
Deionized distilled water	80	mL

The pH was adjusted to 8.8 and the volume was adjusted to 100 mL in a volumetric flask. Non-soluble powder was filtrated by using membrane filter pore size 0.2 µm, and stored in a dark container.

2. **Stock solution C**

Stock acrylamide solution (30% T, 2.7%)

Acrylamide	29.2	g
Bis (Estaman)	0.8	g
Deionized distilled water	70	mL

Volume was adjusted to 100 mL in volumetric flask. Non-soluble powder was filtrated by using membrane filter pore size 0.2 µm, stored in dark container.

3. **Stock solution D**

Stacking gel buffer 0.5 mM Tri-HCl, pH 6.8

Tris-base	6.8	g
Deionized distilled water	70	mL

The pH was adjusted to 6.8 and the volume was adjusted to 100 mL in a volumetric flask. Non-soluble powder was filtrated by using membrane filter pore size 0.2 µm, and stored in a dark container.

4. Electrode Buffer (Running buffer)

Tris-Base	3.0	g
Glycerol	14.4	g
SDS	1.0	g

All substances were dissolved in 1,000 mL of deionized distilled water and filtered through a suction filter with a 0.2 µm filter membrane and stored at 4˚C.

5. 5X non-reducing buffer

1.0 M Tris-HCl, pH 6.8	0.625	mL
Glycerol	1.0	mL
1% Bromphenol	0.125	mL

Volume was adjusted to 10 mL with deionized distilled water.

6. 6X reducing buffer

5X non-reducing buffer	475	µL
2-mercaptoethanol	25	µL

7. Coomasie blue stain

Coomasie blue	0.25	g
Methanol	20	mL
Acetic acid	10	mL

Deionized distilled water is topped up to 100 mL.

8. Coomasie blue destaining solution

Methanol	20	mL
Acetic acid	10	mL

Deionized distilled water was topped up to 500 mL.

9. Stock ammonium persulfate solution

10% (w/v) APS in deionized distilled water

Ammonium persulfate	0.1	g
Deionized distilled water	1.0	mL

10. Stock 10% SDS solution

SDS	0.2	mL
Deionized distilled water	1.0	mL

11. Separating gel 12% (1 gel)

Deionized distilled water	1.75	mL
1.5 mM Tris-HCl, pH 8.8 (solution A)	1.25	mL
10% SDS	50	µL
Acrylamide/Bis (solution C)	4.0	mL
10% APS	25	µL
TEMED	2.5	µL

12. Stacking gel 4% (1 gel)

Deionized distilled water	1.525	mL
1.0 mM Tris-HCl, pH 6.8 (solution D)	0.625	mL
10% SDS	25	µL
Acrylamide/Bis (solution C)	0.325	mL
10% APS	12.5	µL
TEMED	2.5	µL

Western blot analysis

1. **Transfer buffer (Blotting buffer)**

Tris-base	3.03	mL
Glycine	14.4	mL
Methanol	200	mL

Deionized distilled water was topped up to 1,000 mL and filtered through a suction filter with a 0.2 µm filter membrane and stored at 4˚C.

2. **Phosphate buffer saline (PBS), pH 7.4**

NaH_2PO_4	0.204	g
Na_2HPO_4	1.3	g
NaCl	7.28	g

All substances were dissolved in 800 mL of deionized distilled water and adjusted to pH 7.4. After that the volume was adjusted to 1,000 mL in a volumetric flask and sterilize by using an autoclave.

3. **Blocking reagent**

Skim milk	5	g
PBS, pH 7.4	100	mL

4. **Washing buffer**

PBS, pH 7.4	500	mL
Tween 20	500	µL

CURRICULUM VITAE

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Publication

1. **Chueahongthong F**, Ampasavate A, Okonogi S, Anuchapreda S. Cytotoxic effects of crude kaffir lime (Citrus hystrix, DC.) leaf fractional extracts on leukemic cell lines. *J Med Plants Res*. 2011. (accepted)

Meeting presentations

1. **Chueahongthong F**, Anuchapreda S. Inhibitory effect of kiffir lime leaf crude

extracts on leukemic cell lines. The 2nd BMB Conference: Biochemistry and Molecular Biology for Regional Sustainable Development, Department of Biochemistry, Faculty of Medicine and Department of Biochemistry, Faculty of Science, Khon Kaen Universtiy. May 7-8, 2009. Khon Kaen, Thailand (Poster).

2. **Chueahongthong F**, Ampasavate A, Okonogi S, Anuchapreda S. The study of cytotoxic effects of crude kaffir lime leaf fraction extracts on leukemic cells lines. 5th Chiang Mai Academic day, Chiang Mai University. November 26-27, 2009. Chiang Mai, Thailand (Poster).
3. **ฟ้า เชื้อหงษ์ทอง**, ชฎารัตน์ อัมพเศวต, ศิริพร โอโกโนกิ, ทรงยศ อนุชปรีดา. ผลของสารสกัดหยาบแยกส่วนจากใบมะกรูดต่อการยับยั้งการแสดงออกของยีนวิล์มทูเมอร์วันในเซลล์มะเร็งเม็ดเลือดขาวชนิด HL60, U937 และ K562. การประชุมวิชาการประจำปีสมาคมเทคนิคการแพทย์ ครั้งที่ 34. โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน พัทยา . 20-22 เมษายน 2553. (โปสเตอร์)