APPENDIX

Appendix A

List of chemicals and reagents used in this study

All chemicals and reagents used in this study are analytical grade and are listed as follows:

Name of chemical/ reagent	Source/Company
Absolute ethanol	Merck, Darmstadt, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamide	Bio-Rad, Richmond, CA, USA
Agarose	Sigma-Aldrich, St. Louis, MO, 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
Comercial grade curcuminoids	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 pyrocarbonate (DEPC)	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA LAB-SCAN, Bangkok, Thailand
Disodium hydrogen phosphate	Merck, Darmstadt, Germany Fluka, Buchs, Switzerland
DNA ladder - 1 kb DNA Ladder - O'GeneRuler™ 1 kb DNA Ladder - O'GeneRuler™ 100 bp DNA Ladder	 GIBCO-BRL, Grand Island, NY, USA MBI, Fermentas, Vilnius, Lituania MBI, Fermentas, Vilnius, Lituania
Ethidium bromide	Sigma-Aldrich, St. Louis, MO, USA
EDTA (Disodium)	Merck, Darmstadt, Germany
Fetal bovine serum	GIBCO-BRL, Grand Island, NY, USA
Folin & Clocalteu's phenol reagent	Merck, Darmstadt, Germany

Glycerol	Merck, Darmstadt, Germany
Glycine	Amresco, Ohio, USA
HEPES	Sigma-Aldrich, St. Louis, MO, USA
HRP conjugated goat anti-rabbit IgG	PIERCE, Rockford, IL, USA
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
L-glutamine	GIBCO-BRL, Grand Island, NY, USA
Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Methanol	LAB-SCAN, Bangkok, Thailand
MTT	Sigma-Aldrich, St. Louis, MO, USA
NE-PER® Nuclear and Cytoplasmic Extraction reagents	PIERCE, Rockford, IL, USA
PageBlue™ Protein Staining Solution	MBI, Fermentas, Vilnius, Lituania
PageRuler™ Prestained Protein Ladder	MBI, Fermentas, Vilnius, Lituania
Penicillin-streptomycin	GIBCO-BRL, Grand Island, NY, USA
Potassium chloride	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Primers	Invitrogen, California, USA
Rabbit polyclonal anti-GAPDH antibody (GAPDH; FL-335)	PIERCE, Rockford, IL, USA
Rabbit polyclonal anti-WT1 antibody (WT1; C-19)	PIERCE, Rockford, IL, USA
Restore™ Westren 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 hydroxide	Merck, Darmstadt, Germany
Sodium potassium tartrate	Sigma-Aldrich, St. Louis, MO, USA

Sucrose	Sigma-Aldrich, St. Louis, MO, USA
SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase	Invitrogen, California, USA
SuperSignal® West Pico Chemiluminescent Substrate	PIERCE, Rockford, IL, USA
TEMED	Bio-Rad, Richmond, CA, USA
Tris hydrochloride (Tris-HCl)	Sigma-Aldrich, St. Louis, MO, USA
TRIzol™ reagent	Invitrogen, California, USA
Trypan blue	Sigma-Aldrich, St. Louis, MO, USA
Tween 20	Sigma-Aldrich, St. Louis, MO, USA

Appendix B

List of instruments and equipments used in this study

Instrument	Company
Analytical balance	OHAUS CORPORATION
Autoclave	HUXLER
Automatic pipette	BIOHIT, GILSON, LABMATE, and BIO-RAD
Automatic pipette tip	BIOLINE
Carbon dioxide incubator	JOUAN
Centrifuge	CLAY ADAMS
15 or 50 ml centrifuge tube	GREINER BIO-ONE and CORNING INCORPORATION
Deionized distilled water machine	PK WATER TEXT
ECL-hyper film	PIERCE
ELISA microplate reader	SUNRISE
Freezer (-80 ºC)	PTW ULTRA COLD
Freezer (-20 ºC)	SANYO
Gel documentation	BIO-RAD
Gel electrophoresis apparatus	HANGZHOU BIOER TECHNOLOGY
10 cm glass plate	PYREX and PETRIO
Homogenizer	PARGUS (JAPAN)
Hot air oven	ไทยแสตนเลสอาร์กอน
Inverted microscope	OLYMPUS
Kodak medical X-ray Film	KODAK
Larminar flow biological cabinet	CLEAN
Light microscope	OLYMPUS
Nitrocellulose membrane	PALL CORPORATION

Magnetic stirrer	SYBRON/ THERMOLYNE
Microcentrifuge, bench-topped	EPPENDORF
Microcentrifuge tube	CLP
Milipore filter paper	PALL CORPORATION
Mini protein II slab gel	BIO-RAD
Pipet-aid	DRUMMOND
Pasture pipette	PYREX
RCR amplifier	EPPENDORF
pH meter	THERMO ORION
Power supply	E-C APPARATUS CORPORATION
Refrigerator	TOSHIBA
Serological pipette	PYREX
Spectrophotometer	SHIMADZU, BARA SCIENTIFIC
25 or 75 cm^3 T-flask	NUNC
Thin-wall PCR tube	MOLECULAR BIO PRODUCTS
Trans-blot® electrophoretic transfer cell	BIO-RAD
Vortex mixer	GEMMY INDUSTRIAL CORPORATION
Water bath	GFL

Appendix C

Preparation of some reagents and buffers used in this study

Human leukemic cell lines culture

1. **Incomplete RPMI 1640 medium**

RPMI powder (GIBCO BRL)	10.4	g (1 package)
HEPES	3.57	g
$NaHCO_3$	2.0	g
0.34% 2-mercaptoethanol	1.0	mL
Deionized distilled water	800	mL

Adjust pH to 7.2-7.4 then adjust volume to 1,000 mL and steriled by suction filter (membrane pore size 0.2 µm), stored at 4°C and checked sterility before used.

2. **Complete RPMI 1640 medium**

Incomplete RPMI 1640 medium	88.5	mL
Fetal bovine serum	10.0	mL
L-glutamine	1.0	mL
Pen/strep	0.5	mL

Stored at 4°C

3. **Freezing solution**

8% DMSO in fetal bovine serum

Fetal bovine serum	9.2	mL
DMSO	0.8	mL

Stored at 4°C

4. **Phosphate buffer saline (PBS) pH 7.4**

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

Dissolve in 800 mL deionized distilled water, adjusted pH to 7.4 then top up to 1,000 mL and steriled by autoclave.

Cell survival measurement

1. MTT stock dye solution

MTT	1.0	g
PBS pH 7.4	200	mL

After dissolve MTT dye, filtrate any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collected in dark container.

2. 0.2% (w/v) trypan blue

Trypan blue	0.2	g
PBS	100	mL

Protein determination

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

2. Reagent B

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

Two reagents,$CuSO_4$ and Na-K Tartrate, were prepared as follow:

Part A : 0.5% (w/v) $CuSO_4$ .5 H_2O

$CuSO_4$ .5 H_2O	0.5	g
Deionized distilled water	50	mL

Part B : 1% (w/v) Na-K tartrate

Na-K tartrate	1.0	g
Deionized distilled water	50	mL

Before using 0.5 mL of part A and part B were mixed.

3. Reagent C

Working solution was freshly prepared by mixing reagent A 50 mL and reagent B ratio 50:1.

4. Folin-ciocalteau phenol reagent 1N

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

SDS-PAGE analysis

1. Stock solution A: separating gel buffer 1.5 mM Tris-HCl, pH 8.8

Tris-base	18.15	g
Deionized distilled water	80	mL

Adjust pH to 8.8 then adjust volume to 100 mL and filtrate any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collect in 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

Adjust volume to 100 mL and filtrate any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collect in dark container.

3. Stock solution D: stacking gel buffer 0.5 mM Tris HCl, pH 6.8

Tris base	6.05	g
Deionized distilled water	70	mL

Adjust pH to 6.8 then adjust volume to 100 mL and filtrate any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collect in dark container.

4. Electrode buffer (Running buffer)

Tris-base	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolve in deionized distilled water 1,000 mL then filtrate by suction filter and store at 4ºC.

5. 5X non-reducing buffer

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

Adjust volume to 10 mL with distilled water.

6. 5X 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 water was top up to 100 mL.

8. Coomasie blue destaining solution

Methanol	100	mL
Acetic acid	50	mL

Deionized water was top 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%

Deionized distilled water	3.5	mL
Tris HCl, pH 8.8 (solution A)	2.5	mL
10% SDS	100	µL
Acrylamide/Bis (solution C)	4.0	mL
10% APS	50	µL
TEMED	5	µL

12. Stacking gel 4%

Deionized distilled water	3.05	mL
Tris-HCl, pH 6.8 (solution D)	1.25	mL
10% SDS	50	µL
Acrylamide/Bis (solution C)	0.65	mL
10% APS	25	µL
TEMED	5	µL

Western blot analysis

1. Transfering buffer (Blotting buffer)

Tris-base	3.03	g
Glycine	14.4	g
Methanol	200	mL

Dissolve in deionized distilled water 1,000 mL then filtrate by suction filter and store at 4ºC.

2. Phosphate buffer saline (PBS) pH 7.4

NaH_2PO_4	0.204	g
Na_2HPO_4	1.3	g
NaCl	7.28	g

Dissolve in 800 mL deionized distilled water, adjusted pH to 7.4 then adjusted volume to 1,000 mL and steriled by autoclave or filtration.

3. Blocking reagent

Skim milk	5	g
Anti-foam	20	µL

Dissolved in 100 mL PBS, pH 7.4

4. Washing buffer

PBS, pH 7.4	500	mL
Tween 20	500	µL

Reverse transcriptase polymerase chain reaction (RT-PCR)

1. Stock 0.5 M EDTA, pH 8.0

EDTA	186.1	g
DEPC treated water	1	L

Steriled by autoclave

2. DEPC treated water

Deionized distilled water	4	L
Diethylpyrocarbonate (DEPC)	400	µL

Shake it vigorously and store at room temperature for overnight and removed DEPC by autoclave.

3. 1 mg/mL Ethidium bromide

Ethidium bromide	0.001	g
DEPC treated water	1.0	mL

Stored at 4ºC in dark.

4. 6X loading dye

Bromphenol blue	0.0025	g
Sucrose	0.4	g
DEPC treated water	1.0	mL

Sucrose should be dissolved before adding phenol blue and then stored at 4ºC in dark.

5. 50X TAE (Tris acetate buffer)

Tris-base	24.2	g
Gracial acetic acid	5.71	mL
0.5 M EDTA, pH 8.0	10	mL
DEPC treated water	100	mL

Stored at room temperature

6. 1% Agarose

Agarose	1.0	g
1X TAE	100	mL
Ethidium bromide	10	µL

7. Primer preparation (WT1 primers and GAPDH primers)

- WT1(1) sense primer : 5’- GGCATCTGAGACCAGTGAGAA-3’
  352.63 µg (26 µg/OD), OD 13.40
  MW : 6505.2 µg/µmole, 54.3 nmole
- WT1(2) anti-sense primer : 5’-GAGAGTCAGACTTGAAAGCAGT-3’
  268.79 µg (26 µg/OD), OD 10.40
  MW : 6833.4 µg/µmole, 39.3 nmole
- GAPDH sense primer : 5’-CGAAGTCAACGGATTTGGTCGTAT-3’
  313.86 µg (28 µg/OD), OD 11.40
  MW : 7408.8 µg/µmole, 42.4 nmole

- GAPDH anti-sense primer : 5'-AGCCTTCTCGGTGGTGAAGAC-3'
  321.34 µg (28 µg/OD), OD 11.30
  MW : 6463.2 µg/µmole, 49.7 nmole

Disslove primers in 1 mL of sterile deionized distilled water, the concentration of primer solution of WT1(1), WT1(2), GAPDH sense primer and GAPDH anti-sense primer in stock 1 will be 54.3, 39.9, 42.4 and 49.7 µM, respectively. Kept at -20ºC.

Stock solution 2 should be diluted from the stock solution 1 in the final concentration of 10 µM in steriled deionized distilled water.

8. Component of RT-PCR

For K562, HL60 and Molt4 cell line

Components	Volume/20 µL	Final concentration
2X reaction mix	10 µL	1X
Template RNA	X µL*	1 µg
WT1 (1) sense primer	0.4 µL	0.2 µM
WT1 (2) anti-sense primer	0.4 µL	0.2 µM
GAPDH sense primer	1.7 µL	0.085 µM
GAPDH anti-sense primer	1.7 µL	0.085 µM
RT-Taq Mix	0.4 µL	-
DEPC treated water	up to 20 µL	-

For U937 cell line

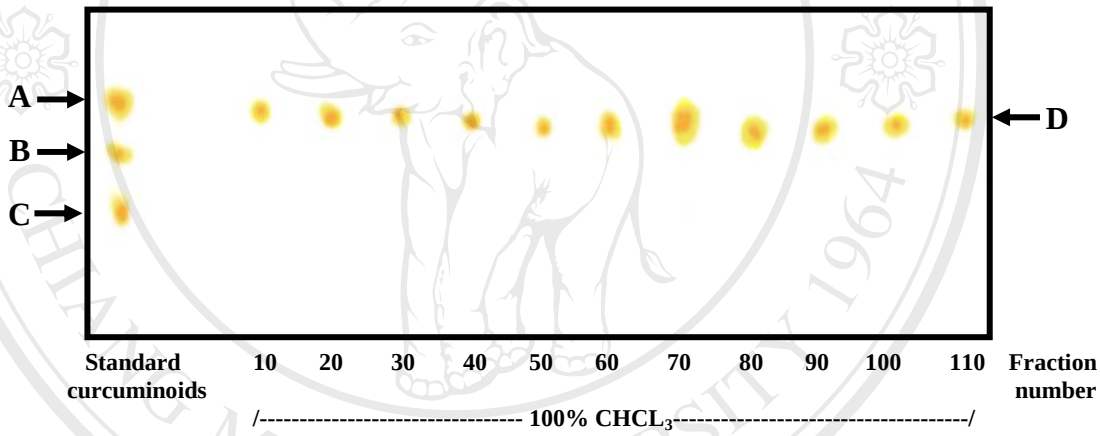
Components	Volume/20 µL	Final concentration
2X reaction mix	10 µL	1X
Template RNA	X µL*	1 µg
WT1 (1) sense primer	0.6 µL	0.3 µM
WT1 (2) anti-sense primer	0.6 µL	0.3 µM
GAPDH sense primer	1.7 µL	0.085 µM
GAPDH anti-sense primer	1.7 µL	0.085 µM
RT-Taq Mix	0.8 µL	-
DEPC treated water	up to 20 µL	-

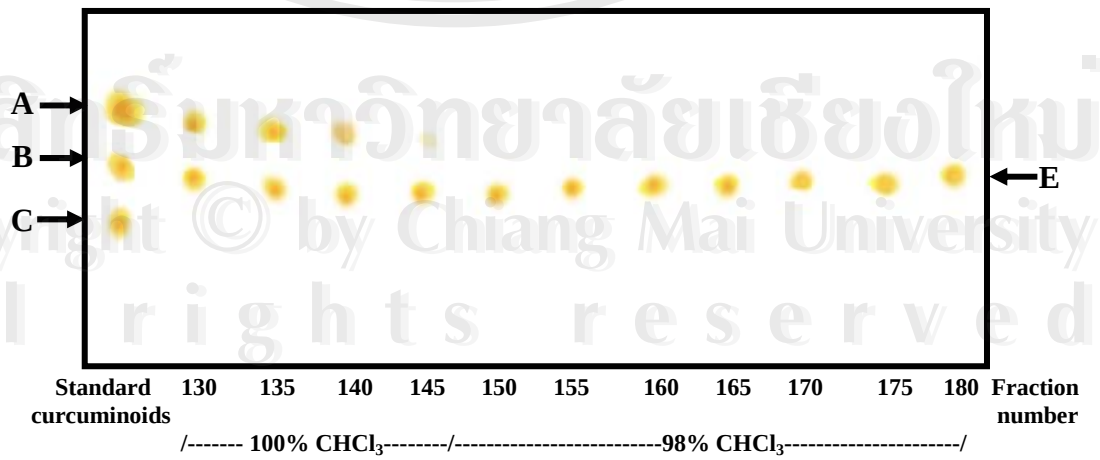
***** The volume of template RNA varies to the amount of RNA in each of experiment.

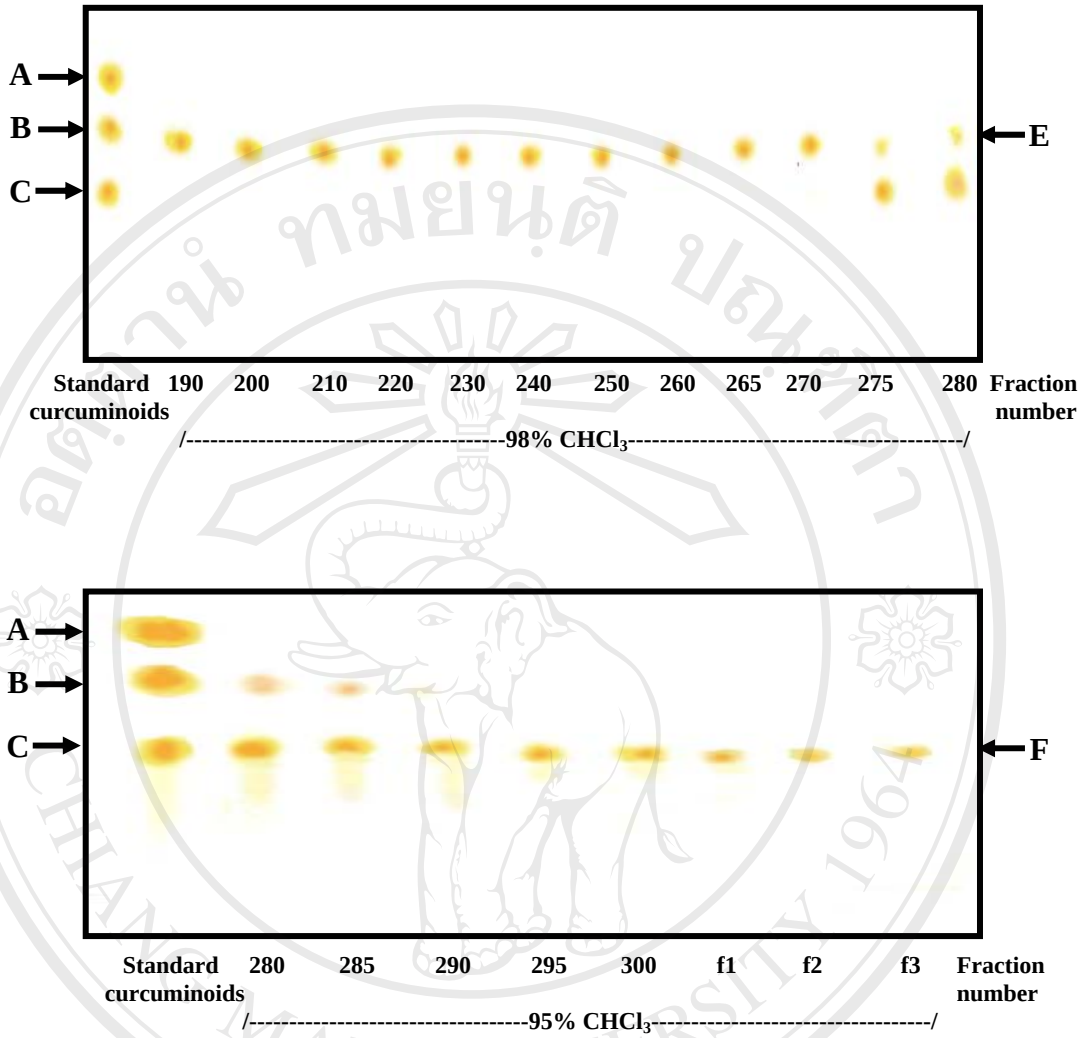
Appendix D

Curcuminoids separation by silica gel 60 column chromatography

The curcuminoid mixture after isopropanol washing were then separated into the three components by silica gel 60 column chromatography, and curcumin, demethoxycurcumin and bisdemethoxycurcumin were eluted with 100% $CHCl_3$, $CHCl_3$:MeOH (98:2) and $CHCl_3$:MeOH (95:5), respectively. The column fractions were analyzed by TLC. The fractions numbered 1-110 (curcumin), 150-265 (demethoxycurcumin) and 285 to the end of fraction (bisdemethoxycurcumin) were pooled, dried and further analyzed by HPLC.







A = Standard pure curcumin

B = Standard demethoxycurcumin

C = Standard bisdemethoxycurcumin

D = Pure curcumin

E = Demethoxycurcumin

F = Bisdemethoxycurcumin

f1 = flask no.1

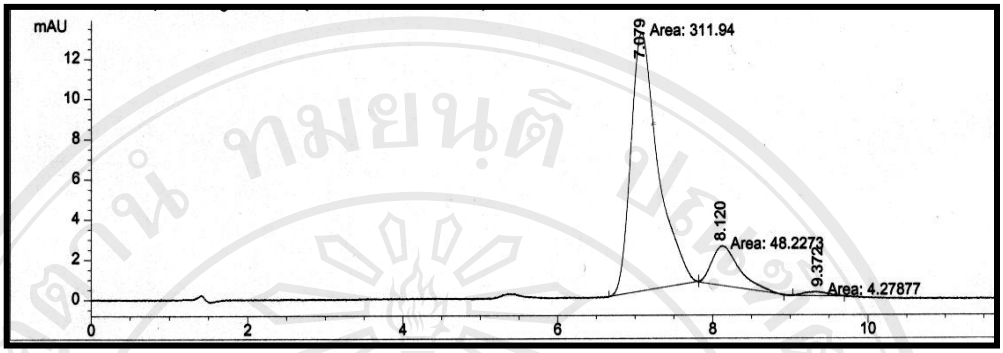
f2 = flask no.2

f3 = flask no.3

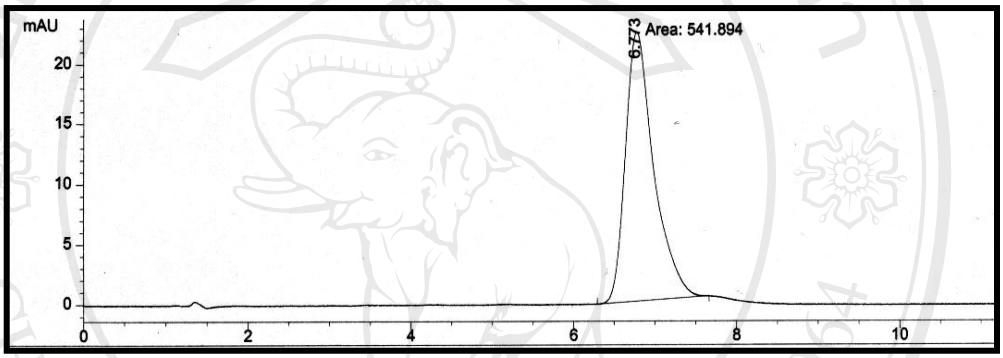
Appendix E

The HPLC fingerprints of curcuminoids

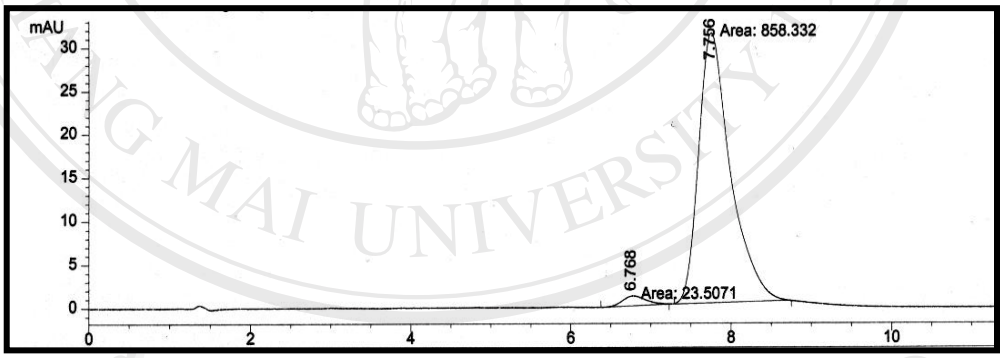
A



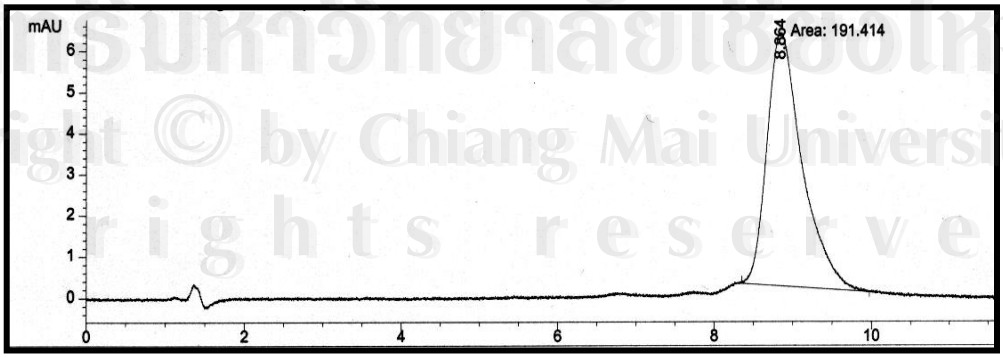
B



C



D



A = Curcuminoid mixture B = Pure curcumin

C = Demethoxycurcumin D = Bisdemethoxycurcumin

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Publications :

1. Anuchapreeda S, Wikan N, **Tima S**, Thanarattanakorn P, Sittipreechacharn S and Limtrakul P. Effect of curcumin on *WT1* gene expression in human leukemic cells. *J. Med. Tech. Assoc.* 2005, 33 (3): 1252-63.

2. Anuchapreeda S, Thanarattanakorn P, Sittipreechacharn S, **Tima S**, Chanarat and Limtrakul P. Inhibitory effect of curcumin on *MDR1* gene expression in patient leukemic cells. *Arch. Pharm. Res.* 2006. (accepted)

Meeting presentation:

1. **Tima S**, Chanarat P, Limtrakul P and Anuchapreeda S. Inhibitory effect of turmeric curcuminoids on *Wilms' tumor1* gene expression in K562 cell line. Annual Academic Meeting, Faculty of Associated Medical Sciences, Chiang Mai University. December 7-9, 2005. Chiang Mai, Thailand (Poster).

2. **Tima S**, Chanarat P, Limtrakul P and Anuchapreeda S. Inhibitory effect of turmeric curcuminoids on *Wilms' tumor1* gene expression in K562 cell line. 1st Chiang Mai Academic day, Chiang Mai University. December 8-10, 2005. Chiang Mai, Thailand (Poster).