APPENDICES

APPENDIX A

List of chemicals and materials used in this study

Chemicals/Materials	Source
Absolute ethanol	Merck, Darmstadt, Germany
Absolute methanol (analytical grade or HPLC grade)	Merck, Darmstadt, Germany
Acrylamide	Wako Pure Chemical Industries, Japan
Agarose	Wako Pure Chemical Industries, Japan
Ammonium persulfate (APS)	Pharmacia Biotech, Sweden
BCA™ Protein Assay Kit	Pierce, Rockford, USA
Boric acid	Fisher Scientific, UK
Bromophenol blue	USB Corp., Ohio, USA
CaspACE™ Assay System, Fluorometric	Promega Corp., WI, USA
Dimethylsulfoxide (analytical grade)	Sigma Chemical Co., Dosert, UK
Disodium hydrogenphosphate	Fluka A.G., Buchs, Switzerlan
Dithiotheritol (DTT)	Wako Pure Chemical industries, Japan
DNA marker	Bio-Rad Laboratories, USA
Dulbecco's Modified Eagle's Medium (DMEM)	GIBCO-BRL, Grand Island, Japan
Ethidium bromide	Sigma Chemical Co., Dosert, UK
Ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA)	Fisher Scientific, UK

Fetal bovine serum (FBS)	GIBCO-BRL, Grand Island, Japan
Filter paper No.1	Whatman International Ltd., England
Glycine	USB Corp., Ohio, USA
Glycerol	Chemical Industries, Japan
Goat anti rabbit IgG-HRP	Santa Cruz Biotech, USA
HEPES (2-[4-(2-Hydroxyethyl)-1 --piperazinyl]-ethanesulfonic acid)	Sigma-Aldrich, St. Louis, MO, USA
Hydrochloric acid	Sigma Chemical Co., Dosert, UK
Magnesium chloride	Wako Pure Chemical Industries, Japan
Millipore membrane	Whatman International Ltd., England
Minimal essential medium (MEM)	GIBCO-BRL, Grand Island, Japan
MTT dye	USB Corporation, USA
2-mercaptoethanol	Pharmacia Biotech, Sweden
N'N'-bis-methylene acrylamide	Pharmacia Biotech, Sweden
Ninety six-well flat bottom tissue culture plate	Nunc Inc., Hereford, UK
Nitrocellulose membrane	Amersham, Japan
Penicillin	General Drug House Co., Ltd
Potassium chloride	Wako Pure Chemical Industries, Japan
Propidium iodide	Wako Pure Chemical Industries, Japan
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich, St. Louis, MO, USA
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich, St. Louis, MO, USA
Proteinase K	Wako Pure Chemical Industries, Japan
Protease Inhibitor Cocktail	Sigma-Aldrich, St. Louis, MO, USA

Rabbit polyclonal anti-Akt antibody	Santa Cruz Biotech, USA
Rabbit polyclonal anti-p-Akt antibody	Santa Cruz Biotech, USA
Rabbit polyclonal anti-Bax antibody	Santa Cruz Biotech, USA
Rabbit polyclonal anti-Bcl-2 antibody	Santa Cruz Biotech, USA
Rabbit anti-β-actin antibody	Santa Cruz Biotech, USA
RNase A (DNase free)	Amresco, OH, USA
Skim milk powder	Merck, Darmstadt, Germany
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, SA
Sodium chloride	Merck, Darmstadt, Germany
Sodium dodecyl sulfate	Wako Pure Chemical Industries, Japan
Sodium hydroxide	Wako Pure Chemical Industries, Japan
Sodium pyruvate	Merck, Darmstadt, Germany
Streptomycin sulfate	M&H Manufacturing Co., Ltd
Sucrose	Fisher Scientific, UK
TEMED	USB Corp., Ohio, USA
Tissue culture flask	Nunc Inc., Hereford, UK
Tris-base	Wako Pure Chemical Industries, Japan
Triton X-100	Wako Pure Chemical Industries, Japan
Trypan blue	Sigma Chemical Co., Dosert, UK
Trypsin	Biochrom AG., Ge
Tween-20	Sigma Chemical Co., Dosert, UK
X-ray film	Kodak, USA

APPENDIX B

List of instrument used in this study

Instrument	Source
Analytical balance	Sartorius, Germany
Autoclave S4-245	Tomy Seiko Co.Ltd., Tokyo, Japan
Block incubator	Astex, USA
Centrifuge CR3i	Jouan, France
CO_2 incubator (BL-1600)	ASTEC Co.Ltd., Fukuoka, Japan
Electrophoresis system Mupid-ex	CosmoBio, Japan
Flow cytometer	BD Biosciences, USA
Freeze dryer	Martin Christ, Germany
Hot air oven	Ikemoto, Japan
Laminar Air flow (VH-1300BHT-2BS)	Nippon Medical & Chemical Instruments Co. Ltd, Osaka, Japan
Light microscope	Nikon DIAPHOT 300, Tokyo, Japan
Liquid nitrogen tank	International Cryogenics Inc.
Microplate reader	Corona Electric, Japan
Micro ultracentrifuge (himac CS120GX)	Hitachi, Japan
PAGE apparatus/power supply	Amersham, US
pH meter	Horiba, Japan
Refrigerator (-20^{o}C)	Nihong Freezer, Japan
Refrigerator (-80^{o}C)	Nihong Freezer, Japan

Semi-Dry Electrophoretic Transfer Cell	Bio-Rad Laboratories, USA
Shaker water bath	Ikemoto, Japan
Speed vacuum concentrator	Tomy Seiko, Tokyo, Japan
UV/Vis Spectrophotometer	Shimadzu, Japan
Vacuum rotating evaporator	Tokyo, Rikakikai Co., Ltd., Japan
Vortex Mixer	Bio-Active Co., Ltd.,Korea
Water bath incubator	Yamato, Japan

APPENDIX C

Reagents and buffers preparation

1. Reagent for determination of phonolic content

1.1 7 % Na_2CO_3

Na_2CO_3	7	g
DI	100	ml

1.2 Folin-Ciocalteu's phenol reagent

Dilute before use with DI 1:1

2. Reagent for determination of flavonoid content

2.1 5% $NaNO_2$ solution

$NaNO_2$	5	g
DI	100	ml

2.2 10% $AlCl_3$ solution

$AlCl_3$	10	g
DI	100	ml

2.3 1 M NaOH

NaOH	4	g
DI	100	ml

3. Reagents for DPPH assay

3.1 Acetic acid solution

Glacial acetic acid	1.42	ml
DW to 250 ml		

3.2 0.1 M Acetate buffer (pH 5.5)

CH_3COONa	2.05	g
DW	200	ml
Adjust pH to 5.5 by acetic acid solution		
Adjust volume to 250 ml		

3.3 DPPH solution (freshly prepare)

DPPH	0.3	mg
Absolute EtOH	1	ml

4. Reagents for cell culture

4.1 DMEM medium

DMEM powder	13.5	g
HEPES	3.9	g
$NaHCO_3$	3.7	g
Penicillin G	0.0625	g
Streptomycin sulfate	0.1	g
Add DW to 1 liter and adjusted pH to 7.4 and sterile by		
Millipore filter membrane (0.22 μm)		

4.2 Complete DMEM medium

DMEM	90	ml

Fetal bovine serum	10	ml

4.3 MEM medium

MEM powder	10.1	g
$NaHCO_3$	1.5	g
Sodium pyruvate	0.11	g
Penicillin G	0.0625	g
Streptomycin sulfate	0.1	g

Add DW to 1 liter and adjust pH to 7.4 and sterilize by

Millipore filter membrane (0.22 μm)

4.4 Complete MEM

MEM	90	ml
Fetal bovine serum	10	ml

4.5 Phosphate buffered saline (PBS pH 7.4)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
DW	800	ml

adjust pH 7.4 using 1 N HCl then adjust volume to 1 liter

4.6 0.025% EDTA

EDTA.2Na	0.025	g
PBS	100	ml

Sterilize by autoclave 121 ^{o}C, 15 min

4.7 0.05% trypsin-0.02% EDTA

0.25% trypsin	20	ml
0.025% EDTA	80	ml

4.8 0.2% trypan blue

Trypan blue	0.2	g
PBS	100	ml

5. Reagent for MTT assay

5.1 MTT solution

MTT	5	mg
PBS	1	ml

Filtrate through 0.22 μm filter, keep at 4 ^{o}C

6. Reagent for DNA ladder

6.1 Lysis buffer

1 M Tris-HCl buffer, pH 7.4	0.1	ml
0.5 M EDTA, pH 8.0	0.2	ml
10% Triton X 100	0.5	ml

DW to 10 ml

Sterilize by autoclave 121 ^{o}C, 15 min

6.2 TE buffer

1 M Tris-HCl buffer, pH 7.4	1	ml
0.5 M EDTA, pH 8.0	0.2	ml
DW	100	ml

Sterilize by autoclave 121 ^{o}C, 15 min

6.3 RNaseA 10 mg/ml

RNaseA	10	mg
Sterilize DW	1	ml

Keep at -20 ^{o}C

6.4 Proteinase K 10 mg/ml

Proteinase K	10	mg
Sterilize DW	1	ml

Keep at -20 ^{o}C

6.5 5 M NaOH

NaOH	20	g
DW	100	ml

6.6 1 M Tris-HCl buffer (pH 7.4)

Tris (Hydroxymethyl) aminomethane HCl	15.8	g
DW	80	ml

Adjust pH to 7.4 by HCl then adjust volume to 100 ml

Sterilize by autoclave 121 ^{o}C, 15 min

6.7 0.5 M EDTA (pH 8.0)

EDTA.2Na	18.6	g
DW	80	ml

Adjust pH to 8.0 by 5 M NaOH then adjust volume to 100 ml

Sterilize by autoclave 121 ^{o}C, 15 min

6.8 TBE buffer 10X

Tris base	108	g

Boric acid	55	g
0.5 M EDTA	40	ml
DW to 1000 ml		

6.9 TBE buffer 0.5X

TBE buffer 10X	50	ml
DW to 1000 ml		

6.10 0.5X TBE buffer plus ethydium bromide (f.c. 0.5 µg/ml)

0.5X TBE	1000	ml
10 mg/ml ethydium bromide	0.05	ml

6.11 Loading buffer

Bromophenol blue	25	mg
Sucrose	4	g
DW to 10 ml		

6.12 Preparation of 2% agarose gel

Agarose powder	3.2	g
TBE buffer	160	ml

Heat 100 ^{o}C, 10 min

Pour warm gel solution into gel tray

Keep in TBE buffer, room temperature

7. Reagent for caspase assay

7.1 1 M DTT

DTT	0.1542	g
Sterilized DW	1	ml

Keep at 4 ^{o}C

8. Reagent for Western blot

8.1 Hypotonic cell lysis buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5mM EDTA, 5 mM DTT, 2 mM PMSF)

1 M HEPES pH 7.5	500	µl
1 M $MgCl_2$	100	µl
1 M EDTA	100	µl
1 M DTT	100	µl
1 M PMSF	40	µl
Sterilized DW	19.16	ml
Total	20	ml

Protease inhibitor cocktail (add immediately before use) containing

AEBSF	1	mM
Leupeptin	1	µM
Aprotinin	150	nM

8.2 1 M HEPES

HEPES	2.3831	g
DW	8	ml

Adjust pH to 7.5 by NaOH

Adjust volume to 10 ml

Sterilize with 0.22 µM filter and store at 2-8 ^{o}C

8.3 1 M $MgCl_2$

$MgCl_2.6H_2O$	3.113	g

DW	10	ml

Sterilize by autoclave 121 ^{o}C, 15 min

8.4 1 M EDTA

EDTA.2Na	3.72	g
Sterilized DW to	10	ml

8.5 1 M DTT

DTT	0.1542	g
Sterilized DW	1	ml

8.6 1 M PMSF

PMSF	0.1742	g
Sterilized DW	1	ml

8.7 SDS-PAGE

30% T, 2.7% C Bis-Acrylamide

Acrylamide	29.2	g
Bisacrylamide	0.8	g

DW to 100 ml

Filtrate through 0.45 μM filter and keep at 4 ^{o}C (within 1 month)

8.8 0.5 M Tris-HCl pH 6.8

Tris	6.055	g
DW	80	ml

Adjust pH to 6.8 with HCl then adjust volume to 100 ml and keep at 4 ^{o}C

8.9 1.5 M Tris-HCl pH 8.8

Tris	18.165	g
DW	80	ml

Adjust pH to 8.8 with HCl then adjust volume to 100 ml and keep at 4 ^{o}C

8.10 10% Sodium dodecyl sulfate (SDS)

SDS	10	g
DW	100	ml

Keep at room temperature

8.11 10% ammonium persulfate (APS) (preparation before use)

APS	0.1	g
DW	1	ml

8.12 Separating gel preparation (12% gel)

DW	3.30	ml
1.5 M Tris-HCl pH 8.8	2.5	ml
10% SDS	0.1	ml
Acrylamide/Bis (30%T, 2.7% C)	4	ml
10% APS	50	µl
TEMED	5	µl
Total	10.005	ml

Added APS and TEMED into the solution after completely set the gel apparatus

8.13 Stacking gel preparation

DW	1.48	ml
0.5 M Tris-HCl pH 6.8	1	ml
10% SDS	0.04	ml
Acrylamide/Bis (30% T, 2.7% C)	0.51	ml
10% APS	0.025	ml

TEMED	0.005	ml

8.14 0.5% Bromophenol blue

BPB	0.05	g
DW	10	ml

8.15 Stock sample buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.025% BPB)

0.5 M Tris-HCl pH 6.8	0.12	ml
10% SDS	0.2	ml
Glycerol	0.1	ml
0.5% BPB	0.1	ml
DW	0.48	ml

8.16 SDS reducing buffer

Stock sample buffer	0.95	ml
2-mercaptoethanol	0.05	ml
(add immediately before use)		
Total	1	ml

8.17 Electrode buffer (1X Running buffer) (0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS, pH 8.3)

Tris-base	3	g
Glycine	14	g
10% SDS	10	ml

DW to 1000 ml

Keep at 4 ^{o}C

8.18 Transfer buffer 10X pH 8.3

Tris	15.15	g
Glycine	72	g
Distilled water	500	ml

8.19 Transfer buffer 1X

Transfer buffer 10X	200	ml
Methanol	400	ml
Distilled water	1400	ml

8.20 Tris-buffered saline (TBS) pH 7.5 (100 mM Tris-HCl, 0.9% w/v NaCl)

Tris	12.1	g
NaCl	9	g
Distilled water	800	ml

Adjust pH to7.5 then adjust volume to 1000 ml keep at 4 ^{o}C

8.21 TTBS (Tween 20/TBS)

TBS	999	ml
Tween 20	1	ml

Keep at 4 ^{o}C

8.22 Blocking buffer (prepare before use)

Skimmed milk	5	g
TTBS	100	ml

APPENDIX D

Table 1.3 Characteristics of colon cancer cell lines (derived from www.atcc.org)

Cell line	**Growth properties**	**Tumor stage**	**Oncogene**	**Disease**
HCT-15	Adherent	Dukes' type C	p53 mutation (Ser-241 to Phe mutation)	Colorectal carcinoma
RKO	Adherent	Dukes' type C	Wild-type p53	Colorectal carcinoma

APPENDIX E

Standard curve

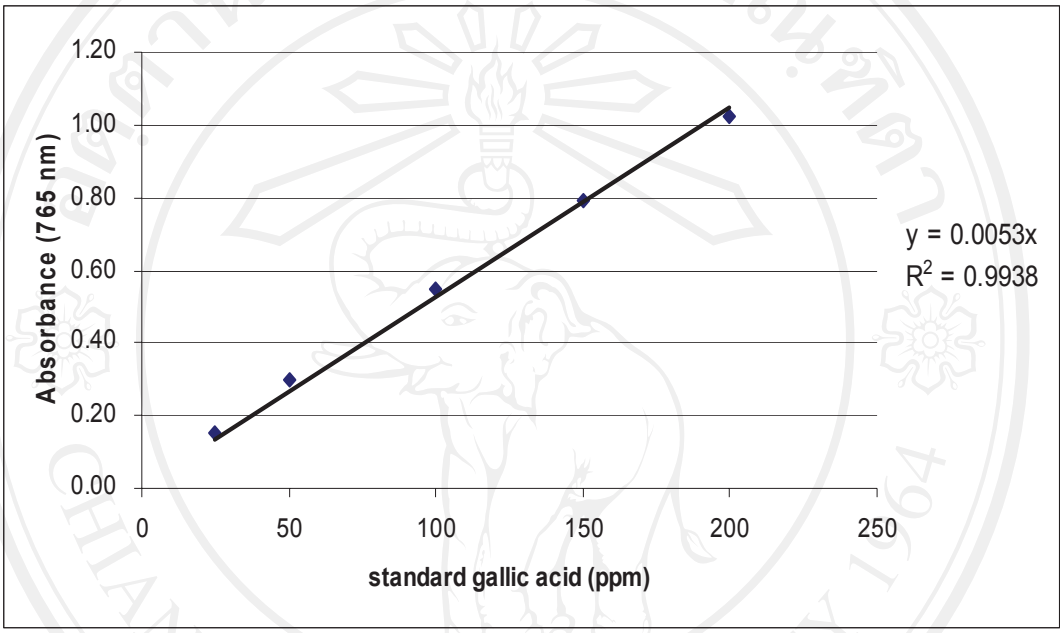

Figure 3.13 Standard curve of gallic acid

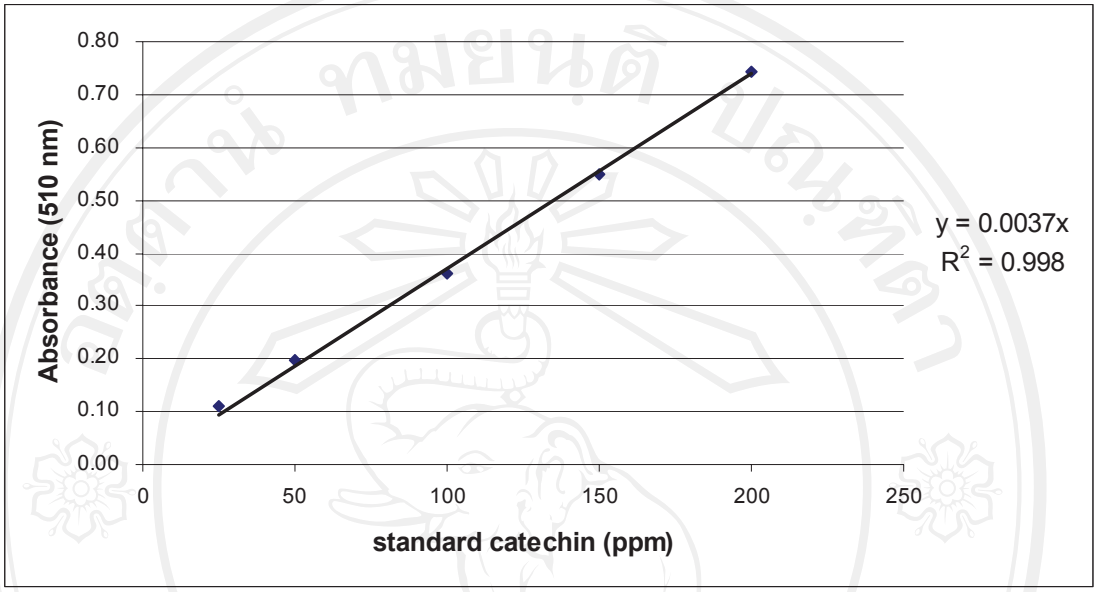

Figure 3.14 Standard curve of catechin

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2002-2005 B.Sc. (Chemistry), Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. (Second Class Honors)

Presentation

1. Phromnate N., Vinitketkumnuen U. Cytotoxic effects on colon cancer cell lines by the ethyl acetate-soluble fraction from dried longan seed crude extract. The 9th National Cancer Conference. Bangkok,Thailand, December 12-14, 2007. (Poster presentation)
2. Phromnate N., Vinitketkumnuen U. Cytotoxic effects and induction of apoptosis by the ethyl acetate-soluble fraction of dried longan in human colon cancer cells. The 2nd International Conference on Natural Products for Health and Beauty (NATPRO). Naresuan University at Phayao, Phayao, Thailand, December 17-19, 2008. (Poster presentation)
3. Phromnate N., Vinitketkumnuen U. Induction of apoptosis through cell cycle arrest in human colon cancer cells by dried longan seed extract. The 9th Annual Biochemical Reserch Meeting. Chiang Mai University, Chiang Mai, Thailand, October 8-9, 2009. (Poster presentation)