APPENDICES

APPENDIX A

List of chemicals and materials used in the study

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

Chemicals	Source
1,9-Dimethylene blue	Serva, Feinbiochemica, Heidelberg, German
96-well ELISA-plate (Nunc®, Maxisorb)	Nunc, Denmark
Absolute ethanol	Merck, Darmstadt, Germany
Acetone	Merck, Darmstadt, Germany
Acrylamide-bis acrylamide	Bio-Rad Laboratories, USA
Agarose gel	Vivantis, UK
Alcian blue	Sigma-Aldrich, St. Louis, MO, US
Alkaline Phosphatase chromogen (BCIP/NBT)	Abcam, UK
Anti-mouse mAnti-type II collagen antibody	Calbiochem, USA
Aurum total RNA purification kit	Amersham Science
BC-3 antibody	Caterson's laboratory, UK
Boric acid	Sigma-Aldrich, St. Louis, MO, USA
Bovine serum albumin	Sigma-Aldrich, St. Louis, MO, USA

Bovine testicular	Sigma-Aldrich, St. Louis, MO, US
Bradford protein assay	Bio-Rad Laboratories, USA
Bromophenol blue (Sodium salt)	Sigma-Aldrich, St. Louis, MO, USA
Carbazole reagent	Sigma-Aldrich, St. Louis, MO, USA
Chondroitin-6- sulfate	Sigma-Aldrich, USA
Chloramine T	Sigma-Aldrich, St. Louis, MO, USA
Chondroitinase ABC	Sigma-Aldrich, USA
CM5 sensor	Amersham Science
Collagenase	Calbiochem, USA
DAB substrate	Sigma-Aldrich, St. Louis, MO, USA
Dexamethasone	Sigma, Poole, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Dulbecco's Modified Eagle's medium (DMEM)	Gibco, UK
ECL detection system	KPL, USA
Ethidium bromide	Bio-Rad Laboratories, USA
Ethylacetate	Merck, Darmstadt, Germany
ethylenediamine tetra-acetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA
Fetal bovine serum	Cambrex, Wokingham, UK
Ficoll	Sigma-Aldrich, St. Louis, MO, USA
Gelatin	Sigma-Aldrich, St. Louis, MO, USA
Gentamycin	T.P. drug laboratories (1996) co.ltd, Thailand
Glacial acetic acid	Merck, Darmstadt, Germany

Glutaraldehyde, 30% (w/v)	Merck, Darmstadt, Germany
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
HEPES	Promega, Corp., Madison, WI, USA
HRP-conjugated goats anti-mouse IgM	Sigma-Aldrich, St. Louis, MO, USA
Hexane	Merck, Darmstadt, Germany
Horse-radish peroxidase-conjugated anti-mouse IgG Ab	Sigma-Aldrich, St. Louis, MO, US
Horse-radish peroxidase-conjugated anti-rabbit IgG Ab	Sigma-Aldrich, St. Louis, MO, USA
Hydrochloric acid	Merck, Darmstadt, Germany
Hydrogen peroxide	Merck, Darmstadt, Germany
ICE	Sigma-Aldrich, St. Louis, MO, USA
IL-1β	R&D system, USA
keratinase I and II	Sigma-Aldrich, St. Louis, MO, USA
Lipopolysaccharide	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Merck, Darmstadt, Germany
Millipore filter membrane (0.22 um)	Pall corporation, MI, USA
Millipore filter membrane (0.45 um)	Pall corporation, MI, USA
Mouse anti-IκBα Ab	Cell Signaling Technology, USA
Mouse anti-phosphorylated IκBα Ab	Cell Signaling Technology, USA
Mouse mAnti-MMP-1 Ab	Calbiochem, USA
Mouse mAnti-MMP-13 Ab	Calbiochem, USA
Mouse mAnti-MMP-3 Ab	Calbiochem, USA
MTT	Sigma-Aldrich, St. Louis, MO, USA

N,N,N',N',-tetramethylethelenediamide (TEMED)	Bio-Rad Laboratories, USA
Nitrocellulose membrane	Amersham Bioscience
Non fat dried milk	Bio-Rad Laboratories, USA
OPD substrate	Sigma-Aldrich, St. Louis, MO, USA
Papain	Sigma-Aldrich, St. Louis, MO, USA
Penicillin-streptomycin	Cambrex, Wokingham, UK
Phosphate buffer saline	Sigma-Aldrich, St. Louis, MO, USA
Potassium chloride	Sigma-Aldrich, St. Louis, MO, USA
Potassium hydrogen phosphate	Sigma-Aldrich, St. Louis, MO, USA
Propan-2-ol	Merck, Darmstadt, Germany
Rabbit anti- NFκB p65 Ab	Cell Signaling Technology, USA
Rabbit anti-IKKβ Ab	Cell Signaling Technology, USA
Rabbit anti-phosphorylated NFκB p65 Ab	Cell Signaling Technology, USA
Rabbit Anti-phosphorylated- SAPK/JNK Ab	Cell Signaling Technology, USA
Rabbit anti-phosphorylated-IKKβ Ab	Cell Signaling Technology, USA
Rabbit mAnti - SAPK/JNK Ab	Cell Signaling Technology, USA
Rabbit mAnti-β-actin Ab	Cell Signaling Technology, USA
Rabbit pAnti –phosphorylated-p44/42 MAPK Ab	Cell Signaling Technology, USA
Rabbit pAnti p44/42 MAPK Ab	Cell Signaling Technology, USA
Rabbit pAnti-phosphorylated-p38 MAPK Ab	Cell Signaling Technology, USA
Rabbit pAntip38 MAPK Ab	Cell Signaling Technology, USA

RevertAidTM First Stand cDNA synthesis kit	Fermentas, Canada, USA
RPMI	Gibco, UK
Silica gel column	Merck, Darmstadt, Germany
Sodium azide	Sigma-Aldrich, St. Louis, MO, USA
Sodium bicarbonate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories, USA
Sodium formate	Merck, Darmstadt, Germany
Sodium hydrogen phosphate (NaH_2PO_4)	Merck, Darmstadt, Germany
Sodium lauryl sulfate	Bio-Rad Laboratories, USA
Sodium tetraborate (borax)	Merck, Darmstadt, Germany
Sulfuric acid	Merck, Darmstadt, Germany
Tissue culture flask	Greiner bio-one, Germany
Tris(hyroxymethyl) amino-methane	Sigma-Aldrich, St. Louis, MO, USA
Triton-X100	Sigma-Aldrich, St. Louis, MO, USA
Trypsin	Gibco, UK
Tween-20	Amersham Bioscience
type IV-S hyaluronidase (Cat. No. H3631)	Sigma-Aldrich, St. Louis, MO, USA
Whatman filter paper	Whatman
β-mercaptoethanol	Bio-Rad Laboratories, USA

APPENDIX B

List of instrument used in the study

Instrument	Source
Analytical balance (HK160)	Mettler Instrument , Switzerland
Autoclave S4-240	Tomy Seiko Co.Ltd., Tokyo, Japan
BIAcore^{TM} 2000 system	BIACORE AB, Uppsala, Sweden
Block incubator	Astex, USA
CO_2 incubator	Nu-Air
Electrophoresis unit	Bio-Rad Laboratory, USA
ELISA plate reader (Titertek multiscan Mcc/340)	ICN, Flow, USA
Freeze dryer	Martin Christ, Germany
Hemocyanometer	
High Performance Liquid Chromatography	Thermo Corp., USA
High speed refrigerated micro centrifuge	ALC 4239R centrifuge, ALC srl, Milano,Italy
Hot air oven	Heraeus, Germany
Laminar flow	Fasted
Light microscope (CHK-H)	Olympus, Japan
Microcentrifuge (Microcen13)	Herolab, Germany

Microplate shaker (MTSA)	Janke & Kunkel Gmblt& Co. KG, Germany
pH meter (SevenEasy)	Mettler Toledo, USA
Phase contrast microscope	Zeiss, USA
Sonicator	Bandelin, Germany
Ultracentrifuge	Beckman Couter
Vortex mixer (Vortex-Genie)	Scientific industry
Water bath (Imperial III)	Labline, USA

APPENDIX C

Reagent and buffers preparation

1. Reagent for cell culture

1.1 DMEM medium

DMEM power	13.5 g
HEPES	3.57 g
$NaHCO_3$	3,7 g
Antibiotic (Penicillin-streptomycin)	10 ml

Add distilled water to 1 liter and adjust pH to 7.4 and sterile by Millipore filter membrane (0.22 μm).

1.2 RPMI medium

RPMI 1640 power	13.5 g
Antibiotic (Penicillin-streptomycin)	10 ml

Add distilled water to 1 liter and sterile by Millipore filter membrane (0.22 μm).

1.3 Complete DMEM or RPMI medium

DMDM or RPMI	90 ml
Fetal bovine serum	10 ml

2. Reagent for colorimetric analysis

2.1 BCA reagent

Reagent A

BCA	1.00 g
Na_2CO_3-H_2O	1.72 g
Sodium tartate	0.16 g
NaOH	0.40 g
$NaHCO_3$	0.95 g

All regents were dissolved in 80 ml of distilled water, pH was adjusted to 11.25 with 1N NaOH. Made up volume to 100 ml and store at 4^{o}C

Reagent B

Four grams of $CuSO_4$:5H2O were dissolved in 100 ml of distilled water and store at 4^{o}C until used.

Working solution

Solution A and B at ratio 50:1 was prepared immediately prior to be used.

2.2 Farndale reagent; DMMB assay

1,9-dimethylene blue	4 mg
Glycine	0.76 g
Sodium chloride	0.59 g
0.1 M hydrochloric acid	23.75 ml

All reagent except 1,9-dimethylene blue were dissolved in distilled water and made up volume to 100 ml, adjust pH to 3.0 by conc. hydrochloric acid. Then

dissolved 1,9-dimethylene blue in this solution. Stored in dark bottle at room temperature.

3. Reagent for ELISA

3.1 Phosphate buffer saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
Na_2PO_4	0.24 g

All reagents were dissolved in distilled water and made up volume to 1 L.

3.2 Tris Incubation buffer

BSA	1.0 g
Tween-20	1.0 ml
NaCl	8.77 g
Tris-HCl	1.21 g

All reagents were dissolved in 900 ml of distilled water, adjust pH to 7.4 and made up volume to 1 L. Stored at 4^oC.

3.3 Citrate phosphate buffer

Citric acid monohydrate	10.30 g
$Na_2HPO_4:3H_2O$	18.16 g

All reagents were dissolved in 900 ml of distilled water, adjusted pH to 5.0 and made up volume to 1 L. Stored reagent at 4^oC.

3.4 Substrate solution

OPD	8 mg

Citrate phosphate buffer 12 ml

30% H_2O_2 5 µL

Prepare reagent fresh for 1 plate; keep in dark before use.

4. Reagent for enzyme digestion

4.1 Chondroitinase ABC buffer

0.5 M Tris-HCl, pH 8.0 and 0.06 M CH_3COONa

4.2 Keratanase I buffer

0.1 M Tris acetate buffer, pH 7.5

4.3 Keratanase II buffer

0.1 M Tris acetate buffer, pH 6.0

5. Carbazole Assay

Stock solution of glucuronic acid (40 µg/ml)

Reagent A: 0.025 M $Na_2B_4O_7$ in Conc. H_2SO_4

Reagent B: Carbazole 50 mg in 40 ml Absolute ethanol

Working solution for standard curve

Glucuronic acid (μg)	DDI water (μL)	Glucuronic acid (μL)
0	60	0
0.48	48	12
1.0	35	25
1.5	22.5	37.5
2.0	10	50
2.4	0	60

Procedure

Cool sample and standard in ice-water

↓

Add 300 μl of Reagent A, mix well

↓ 100 ^{o}C, 10 min

Cooled in ice water

↓

Add 12 μl of Reagent B, mix well

↓ 100 ^{o}C, 15 min

Cool in ice water to room temperature and reading absorbance at 530 nm.

6. HPLC Buffer

Buffer A: 1 M NaH_2PO_4 (156.01 g dissolved in distilled water 1 L)

Buffer B: 16 mM NaH_2PO_4 (2.5 g dissolved in distilled water 1 L)

Buffer was filtered through 0.45 μm filter paper and degassed before use.

PUBLICATIONS FOR THIS THESIS

Pothacharoen P, Choochip K, **Phitak T**,Pompimon W , Premanode B, Hardingham T, Kongtawelert P. Effect of *Alpinia galanga* extract on cartilage degradation and gene expression in human chondrocyte and synovial fibroblast. Central European Journal of Biology. 2006 1(3): 1-21.

Phitak T, Choocheep K, Pothacharoen P, Pompimon W, Premanode B, Kongtawelert P. The effects of *p*-hydroxycinnamaldehyde from Alpinia galanga extracts on human chondrocytes. Phytochemistry. 2009 Jan;70(2):237-43. Epub 2008 Dec 30.

Thanyaluck Phitak, Peraphan Pothacharoen and Prachya Kongtawelert. Comparison of glucose derivatives effects on cartilage degradation. BMC musculoskeletal disorders. 2010, 11(62) [In press].

CURRICULUM VITAE

Name Miss Thanyaluck Phitak

Date of birth 13th, August, 1983

Education

1995-2000 Primary, Secondary and High school at Sansai Wittayakom School, Chiang Mai Thailand

2001-2004 B.Sc. (Medical Technology, First honor), Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai Thailand

2005-Now Ph.D. (Biochemsitry), Faculty of Medicine, Chiang Mai University,Chiang Mai, Thailand.

Thesis Title: Molecular Investigations of Phytochemicals on Human Chondrocyte Metabolism

Field of specialization

Tissue Engineering Study of cartilage tissue engineering and chondroprotective effects of the Thai medicinal plants

Research experience

- June 2001- Now : Ph.D. student in Thailand Excellence Center for Tissue Engineering at Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

- May 2009- August 2009 : Special research student in Connective Tissue Biology Center, Cardiff School of Biosciences, Cardiff University, UK.

Presentations

- **Thanyaluck Phitak,** Peraphan Pothacharoen, Wilart Pompimon and Prachya Kongtawelert. Chondroprotective effect of *p*-hydroxycinnamaldehyde isolated from *Alpinia galaga.* RGJ Seminar Series LII (Biomedical Research and Application: Bringing Health to Life, 4th September 2007, Chiang Mai Unversity, Chiang Mai, Thailand. (Oral presentation)
- **Thanyaluck Phitak,** Wilart Pompimon and Prachya Kongtawelert. Chondroprotective effect of *p*-hydroxycinnamaldehyde isolated from *Alpinia galaga.* The 7th Annual Biochemical Research Meeting, 18th -19th October 2007, Chiang Mai, Thailand. (Poster presentation)
- **Thanyaluck Phitak,** Peraphan Pothacharoen, Prachya Kongtawelert. Chondroprotective and anti-inflammatory effects of sesamin: *In vitro* study.The 9th Annual Biochemical Research Meeting, 8^{th}-9^{th} October 2009, Chiang Mai, Thailand, (Poster presentation)
- **Thanyaluck Phitak,** Peraphan Pothacharoen, Bruce Caterson and Prachya Kongtawelert. Chondroprotective and anti-inflammatory effects of sesamin. The RGJ-Ph.D. Congress XI, 1^{st}-3^{rd} April 2010, Pattaya, Thailand, (Poster presentation)
- **Thanyaluck Phitak,** Peraphan Pothacharoen, Bruce Caterson and Prachya Kongtawelert. Chondroprotective and anti-inflammatory effects of sesamin. The RGJ Seminar series LXXII, 2^{nd} July 2010, Chiang Mai, Thailand, (Oral presentation)

Honor

2005-2010	RGJ scholarship student from Thailand Research Fund.
2010	Outstanding poster presentation award in the RGJ-Ph.D. Congress- XI
2010	Excellent Oral Presentation in RGJ Seminar Series LXXII

Publication

Original articles

- Pothacharoen P, Choochip K, **Pitak T**, Pompimon W , Premanode B, Hardingham T, Kongtawelert P. Effect of Alpinia galanga extract on cartilage degradation and gene expression in human chondrocyte and synovial fibroblast. Central European Journal of Biology. 2006, 1(3): 1-21.
- **Phitak T**, Choocheep K, Pothacharoen P, Pompimon W, Premanode B, Kongtawelert P. The effects of p-hydroxycinnamaldehyde from Alpinia galanga extracts on human chondrocytes. Phytochemistry. 2009 Jan;70(2):237-43.
- **Thanyaluck Phitak**, Peraphan Pothacharoen and Prachya Kongtawelert. Comparison of glucose derivatives effects on cartilage degradation. BMC musculoskeletal disorders. 2010, 11(62) [In press].

Articles' in process to publish

- Pothacharoen P, **Phitak T**, Vanajivin O, Panthong A, Kongtawelert P. Alpha-mangostin from Garcinia mangostana Linn. inhibits chondrocyte MMP-3 and

MMP-13 gene expression by p38 and JNK of MAP Kinase signaling cascade. In process to publish in Ethnopharmacology.

- **Thanyaluck Phitak**, Peraphan Pothacharoen, Jongkolnee Settakorn, Bruce Caterson and Prachya Kongtawelert. Chondroprotective and anti-inflammtory effects of sesamin. In process to publish in Osteoarthritis and Cartilage

Patents

- Thailand patent, (0701001212) The extract from Thai galanga (*Alipinia galanga*) and its effect on the decreasing of degrdation, March 8, 2007.

- Internation patent, (PCT/SG2009/000340) Phytochemical CompositionsIncluding Sesamin For Anti-Inflammatory, Anti-Cytokine Storm, And Other Uses, September 14, 2009.