CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

These chemicals were purchased from Sigma: D-(+)-Glucose, D-(+)-Glucuronic acid γ-lactone, D-(+)-Glucosamine hydrochloride, chondroitin sulfate C, collagenases, gelatin. Glucosamine sulfate was obtained from Rottapharm and IL-1β and LPS was purchased from R&D. Coomassie brilliant blue R250 and Aurum total RNA purification kit were purchased from Bio-Rad Laboratories, Hercules, CA and the RevertAid^{TM} First Stand cDNA synthesis kit was purchased from MBI Fermentas, Germany.

2.2 Preparations of extracts

2.2.1 Preparations of *Alpinia galanga* extracts

We obtained 5 kg of air-dried powder of *A. galanga* rhizomes, specimen BKF no. 102287, deposited at the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Bangkok, Thailand. Then, we percolated it with 17 liters of hexane, 19 liters of ethylacetate, 17 liters of acetone and 20 liters of methanol. The whole process was conduced at room temperature with five repetitions of each percolation and took up to 7 days. Finally, we used filtration and evaporation techniques to produce extracts of hexane (72.84 g), ethylacetate (89.35 g), acetone (52.92 g) and methanol (115.38 g), the diagram

of extraction method is shown in figure. 2.1.

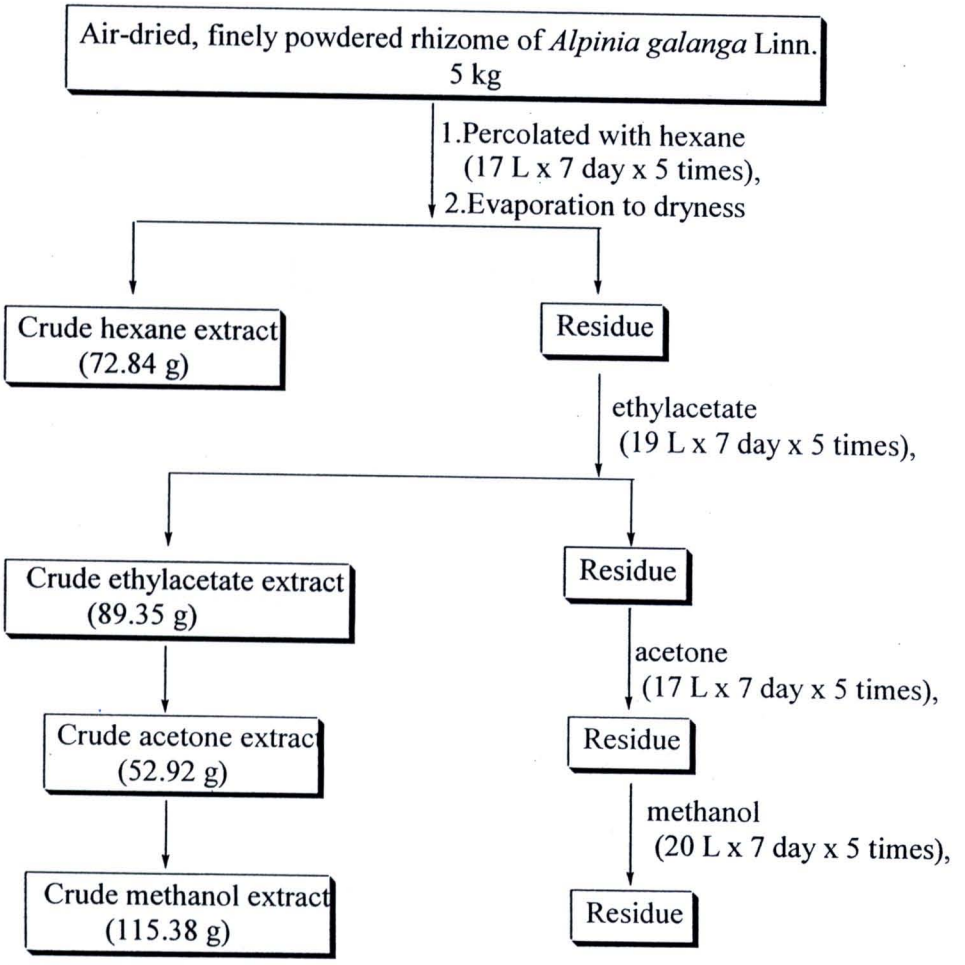

Figure 2.1 Diagram represents the method of *A. galanga* extraction and the dried weight of each crude extract.

2.2.2 Isolation of active compound of acetone fraction of *A. galanga*

The acetone extract (52.93 g) was firstly subjected to a coarse separation on a column (silica gel 1.8 kg, Merck No. 7734, Mesh 70-230 ASTM). Gradient elution was conducted initially with *n*-hexane, gradually enriched with ethylacetate, followed by increasing amount of hexane : ethylacetate in ratio 50:50. Fractions were collected (300 mL each) and combined on the basis of their TLC behavior. The solvents were evaporated to dryness to afford seven fractions ($F_1 - F_7$).

Fraction F_5 (29.48 g), eluted by 10% ethylacetate in *n*-hexane, was obtained as a semisolid. Further separation by column chromatography over silica gel (150 g) was performed by eluting with *n*-hexane, then with various proportions of ethylacetate:*n*-hexane, followed by increasing amount of hexane : ethylacetate in ratio 10:90. Fractions were collected, combined and the solvents were removed under reduced pressure to afford three fractions $A_1 - A_3$. The second subfraction (0.41 g), was separated using column chromatography over silica gel (30 g, Merck No. 7734, Mesh 70-230 ASTM) by gradient eluting with pure *n*-hexane then with various proportions of ethylacetate:*n*-hexane, followed by increasing amount of hexane : ethylacetate in ratio 90:10. The collected fractions were combined on the basis of their TLC behavior. The solvents were evaporated to dryness to afford further three subfractions.

The second subfraction (0.34 g), eluted by 10% ethylacetate-*n*-hexane, yielded a white solid which crystallized as white powder (0.34 g) after addition of methanol and further analyzed by MS/NMR. The crystal was identified as *p*-hydroxycinnamaldehyde. The diagram of isolation method was shown in figure. 2.

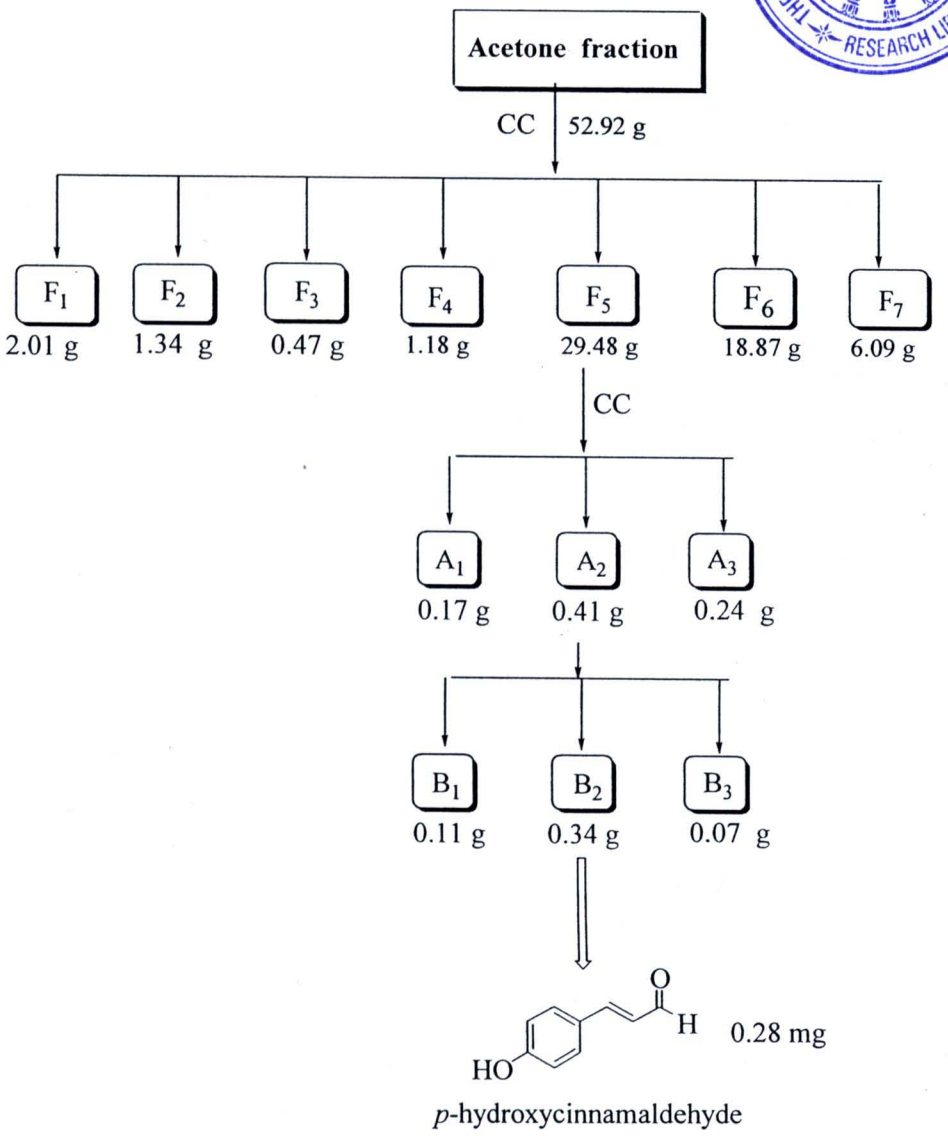

Figure 2.2 Diagram represents the method of crude acetone extract isolation for finding out of the active compound. The final isolations and investigations found that *p*-hydroycinnamaldehyde was the active compound.

2.2.3 Preparation of sesamin from *Sesamum indicum* Linn.

The seeds of *sesamin indicum* Linn. were collected from Lampang province of Thailand and voucher specimen (BKF no. 138181) has been deposited at the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environments, Bangkok, Thailand. Four hundred grams air-dried and finely powdered seed of *S. indicum* had been percolated 6 times with 4 liters hexane for 3 days at room temperature. The extracts were combined and evaporated to dryness under reduced pressure to afford a crude hexane extract (162 .30 g).

The hexane extract was separated using column chromatography over silica gel (Merck No. 7734, 500 g). Elution started with hexane, gradually enriched with ethylacetate in hexane up to 20 % ethylacetate. Fractions (500 mL each) were collected, monitoring by TLC behavior, and combined. The solvent was evaporated to dryness to afford eight fractions (F1-F8). Fractions 7 (5.94 g, eluted with 15% ethylacetate) was found to contain mainly colorless crystals.

Fraction F7 was rechromatographed on a silica gel column (Merck No. 7734, 120 g). Elution started with hexane, gradually enriched with various proportions of ethylacetate in hexane up to 50 % ethylacetate. Fractions (125 mL each) were collected and combined based on their TLC behavior. The solvents were evaporated to dryness to afford three subfractions SF1-SF3. Subfraction 3 (1.32 g, eluted with 20 % ethylacetate) was further purified by crystallization with ethanol to yield colorless needle crystal which was identified by NMR and mass spectrometry as sesamin (430 mg, 0.11 % yield).

2.3 Tissue/Cell cultures and treatments

2.3.1 Porcine cartilage explant preparation and treatment

Articular cartilage, freshly dissected from a metacarpophalangeal joint of a 20-24 week old porcine was incubated in serum-free-DMEM containing 200 units/ml penicillin and 200 μg/ml streptomycin. The explants were maintained in culture in a humidified incubator with 5% CO_2 at 37°C.

Explants were maintained in media without serum for 24 hours prior (day 0 media) to the first treatment. Conditioned media were collected on day 3 for the sort-term culture and day 4, 7, 14, 21 and 28 in long-term culture. Conditioned media were stored at -20°C until analyzed. Recombinant human interleukin-1β (10 ng/ml) was added to induce cartilage degradation. To evaluate the effects of the extracts, the various concentrations of each extract were added to the cartilage explant. Each experiment was performed three times and each time was triplicated using tissue from one animal donor.

2.3.2 Human articular chondrocytes (HACs) culture and treatment

Non-inflammatory human cartilage was obtained with fully informed patient consent from the arthroscopic diagnosis of a flat pad syndrome patient at Maharaj Nakorn Chiang Mai Hospital, (Department of Orthopedic, Faculty of Medicine, Chiang Mai University, Thailand, ethic approval code is 070CT111016). Chondrocytes were isolated by overnight trypsin (Gibco®, UK) at 4°C and 3 h digestion with collagenase (Sigma-Aldrich®, type IA) at 37°C. The cells were washed with PBS and grown in DMEM containing 10% FCS as high-density primary monolayer cultures until confluence. The HACs at passage 4 were maintained in

serum free DMEM for 24 h prior to co-treatment with various concentrations of sesamin (0.25 -1.0 μM) or *p*-hydroxycinnalmaldehyde (20-160 μM) or glucose or glucuronic acid or glucosamine-sulfate or glucosamine hydrochloride (5-20 mM) together with 10 ng/ml IL-1β (R&D systems, Inc) for 24 h to investigate the mRNA and protein expressions of MMP-1, -3 and -13 and for 10 or 15 min to study the activation of NFκB and MAPK pathway, respectively.

2.4 *In vivo* experiment

The use of animals was conformed with international and national guideline for ethical conduct on the care and use of animals and animal use was ethical proved by the committee in Faculty of Medicine, Chiang Mai University. Fifteen Wistar rats (8 weeks rats were obtained from National Laboratory Animal center, Mahidol University, Thailand) were randomly separated into 5 groups: (1) a control group of normal rats; (2) a sesamin group of normal rats with 10 μM sesamin; (3) OA group of rats in which OA was induced using papain; (4) OA rats treated with 1 μM sesamin; (5) OA rats treated with 10 μM sesamin. The OA papain model was performed as previously described (234). A solution of 4% (w/v) papain solution in saline was sterilized and then injected into the right knee of rats on day 1, 4 and 7 of the experimental period. As a control, the same volume (20 μl) of sterile saline was injected into the right knee of rats in the control group at the same time intervals. Five days after the last injection of papain, rats were intra-articular injected with 1 μM or 10 μM sesamin (20 μl) every 5 days during 5 weeks. Animals in the sesamin-treated group were injected intra-articularly with 20 μl of 10 μM sesamin. The control groups were injected intra-articularly with 20 μl of sterile saline. Animals were sacrificed at

were injected intra-articularly with 20 µl of sterile saline. Animals were sacrificed at the end of experiment, and cartilage samples were removed from the tibial plateau in the right knee. The cartilage biopsy samples were fixed in 4% paraformadehyde overnight and embedded, then cut into 5 µm thick sections perpendicular to the surface. Sections were stained with hematoxylin-eosin (H&E), Safranin *O* and immunohistochemical stained with anti type II collagen. All stained-sections were performed by pathologist.

2.5 Analytical methods

2.5.1 Cytotoxicity detections

PBMC viability was assessed by AlamarBlue assay following the vendor's instructions (235). Briftly, PBMCs were plated and co-treated with various doses of each extract. Ten percentage (v/v) AlamarBlue fluorescent dye was added for 4 h at 37°c, one hundred micro liters of media were removed, cleared by centrifugation and measured the absorbance at 540 and 630 nm.

HAC viability was tested using MTT assay. Cells ($1x10^4$) were plated in triplicate in a 96-well plates and incubated overnight. Cells were treated with different concentrations of each extract for 24 h. After incubation, culture media were discarded and new culture media which containing 10% of 5 mg/ml MTT (3,[4,4-dimethy thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were added, and incubated for 4 h. Then, the culture media were discarded and followed by adding 0.2 ml of dimethyl sulfoxide (DMSO) to each well to solubilize the formed formazane crystals. The absorbance was measured at 540 nm using microplate reader.

Percent of cell survival was calculated as follow:

2.5.2 Measurement of s-GAG levels

The level of glycosaminoglycan appearing in the conditioned medium was determined using the dimethylmethylene blue (DMMB) assay for sulfated glycosaminoglycan (236), using chondroitin sulfate C (shark cartilage extract; Sigma-Aldrich, USA) as a standard. The DMMB solution was added to the diluted sample, standards and appropriate blank solution prior to absorbance reading at 620 nm by micro-plate reader spectrophotometer.

The release of extracellular matrix (ECM) biomolecules from cartilage induced by IL-1β was calculated as follow:

% change = {(Day3 medium – Day 0 medium)/ Day 0 medium} x 100

2.5.3 Measurement of HA levels

The HA level in conditioned medium was measured using a competitive inhibition-based-ELISA as previously described, with modifications (237). Briefly, culture media samples containing unknown amounts of HA (175 µl), as well as a standard containing known concentrations of a highly purified HA preparation (Healon®) were placed in small polypropylene tubes with appropriate concentrations of biotinylated-HA binding proteins (B-HABPs) (175 µl) and incubated at room temperature (25°C) for 1 h. Aliquots (100 µl) of this reaction mixture were applied to umbilical cord HA-coated and BSA-blocked 96-well-plate and incubated at 25°C for 1 h. The wells were then washed with phosphate buffered saline solution (0.05% Tween-20), then, the appropriate dilution (1:2000 in PBS) of anti-biotin peroxidase conjugate (Zymed Lab Inc, San Francisco, CA, USA) was added to each well, incubated at 25°C for 1 hour and washed, then peroxidase substrate (OPD, o-

phenylenediamine) was added. After the color development at 25°C for 20 minutes, the reaction was stopped by the addition of 50 ml 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using a Titertek Multiskan M340 microplate reader. The levels of HA in the culture media samples were determined by their abilities to inhibit color development in the assay relative to a standard curve generated from the purified HA preparation.

2.5.4 Gelatin zymography

Pro-MMP-2 in the conditioned medium was detected by gelatin zymography as previously described (238). The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel containing 0.1 mg/ml of gelatin (Sigma-Aldrich, USA) under non-reducing conditions at 4°C. After electrophoresis, SDS in the gel was removed by rinsing with 2.5% Triton-X 100 pH 7.5. The gel was then incubated at 37°C in the incubating buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.02% NaN_3) for 18 hr. After incubation, the gel was stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA) in 50% methanol /10% acetic acid, and destained with 10% acetic acid /50% methanol. The gelatinolytic activity was analyzed by using Scion image densitometer and analysis software.

2.5.5 Quantitation of uronic acid remaining in cartilage tissue

The uronic acid remaining were measured in papain-digested cartilage discs by colorimetric assay using m-hydroxydiphenyl (239). Glucuronic acid lactone was used as standard. Concentrated sulfuric acid-birate reagent (300 μl) was added into

both sample and standard and incubated at 100°C for 10 min and cooled down to room temperature. Then 12 µl of carbazole solution (50 mg carbazole in 40 ml ethanol) was added and incubated at 100°c for 10 min. The absorbance of the pink to red color was read at 540 nm. The remaining of uronic acid content in the cartilage was calculated as follow:

% remaining of UA content = {UA(Day treated)-UA(Day3 control)}X100

2.5.6 Measurement of hydroxyproline release and remaining

Papain-digested-cartilage or conditioned media were hydrolyzed with 6N HCl for 24 h at 110°C. After hydrolyzation, samples were freeze-dried and topped up with distilled water. The hydroxyproline in sample was oxidized to a pyrrole with chloramine T at pH 6. This intermediate gave a pink color with 4-dimethylaminobenzaldehyde. Samples were added with diluent solution (67% propan-2-ol) and oxidant solution (50 mM chloramine T), and color reagent (7.5% dimethylamino benzadehyde in propan-2-ol). Reaction was performed at 70°C for 10-20 min. An absorbance of peach color was measured at 540 nm.

2.5.7 Gene expression analysis

RNA was extracted from monolayer cells using an Aurum total RNA purification kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacture's guidelines. Total RNA (500ng) of each sample was reverse-transcripted into complementary DNA (cDNA) using RevertAid^{TM} First Stand cDNA synthesis kit (MBI Fermentas, Germany). Primer and probe sets were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet

Taqman® requirements and were designed to bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. The reactions of RT-PCR were performed in PCR machine of Appendorf International and reactions of real time PCR were performed in Applied Biosystem (ABI) GeneAmp PCR system 2007.

For semi-quantitative PCR, the amplified products were electroporesed on 2%(w/v) agarose gel, stained with ethidium bromide, and then imaged using a Bio-Rad Gel-Doc fluorescent image analysis analyzer. The integrated densities were then calculated using Scion Image analysis software and normalized to the house-keeping gene GAPDH (glyceraldehydes-3- phosphate dehydrogenase) to permit semi-quantitative comparisons in mRNA levels as previously described (240, 241). The sequences of primers were shown in table 2.1.

Table 2.1 Primers used for semi-quantitative RT-PCR and quantitative real time PRC.

Gene	Annealing temperature (°c)	Product size (base pairs)	Sequences (5' to 3')	Gene Bank accession number
AGG	62	110	Forward: ACTTCCGCTGGTCAGATGGA Reverse: CAACACTGCCAACGTCCAGAT	NM_013227
COL2A1	65	106	Forward: CAACACTGCCAACGTCCAGAT Reverse: ATGATTAGTAACGATAGGCAAT	NM_000393
SOX9	68	101	Forward: ACACACAGCTCACTCGACCTTG Reverse: GGAATTCTGGTTGGTCCTCTCTT	NM_000346
MMP1	68	84	Forward: CTGTTCAGGGACAGAATGTGCT N Reverse: TCGATATGCTTCACAGTTCTAGGG	NM_002422
MMP3	65	138	Forward: TTTTGGCCATCTCTTCCTTCA Reverse: TGTGGATGCCTCTTGGGTATC	NM_002421
MMP13	65	96	Forward: TCCTCTTCTTGAGCTGGACTCATT Reverse: CGCTCTGCAAACTGGAGGTC	NM_002427
GAPDH	60	225	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTTC	NM_002046

2.5.8 Measurement of total protein level

Protein concentration was determined using the Bradford protein assay (242). Bovine serum albumin (BSA) standards (0-1000 μg/ml) and samples were added to the microtitre plates (20 μl/well) in duplicate. The 200 μl of acidic solution of Coomassie® Brilliant Blue G-250 dye was added and then measured the absorbance at 595 nm. The maximum absorbance of dye shifts from 465 nm to 595 nm when the binding to protein occur. Protein concentrations were determined from a standard curve.

2.5.9 Protein extraction and western blot analysis

After treatment, cells were harvested and washed with ice-cold PBS, added 300 μl of 1X loading buffer (0.5 M Tris-HCl containing glycerol, 10% SDS and 0.1% (w/v) Bromophenol Blue) containing 0.1% Mercaptoethanol. The cell suspensions were put on ice for 10 min, then mixed on vortex every few minutes and centrifuge at 15,000xg for 10 minutes at 4°C. Supernatants were transferred to new tubes and the debris pellets were discarded. For secreted MMP-13 protein, conditioned media were collected, and the protein was then concentrated using Amicon Ultra-4® (Ultracel-30kDa) and measured for concentration. Fifty micrograms protein of each sample was subjected to SDS-PAGE.

Aliquots of lysates or concentrated media were heated for 5 minutes at 95°c and then subjected to 10%SDS-PAGE and transferred to nitrocellulose membrane with a glycine transferring buffer [20 mM Tris base, 0.2 M glycine, 20% v/v methanol (pH 8.5)]. After blocking nonspecific binding with 5% nonfat dried milk, the membrane was probed with rabbit mAnti-β-actin, mouse mAnti-MMP-1, mouse mAnti-MMP-3, mouse mAnti-MMP-13 (Calbiochem®), rabbit pAnti –phosphorylated-p44/42 MAPK

antibody, rabbit Anti-phosphorylated- SAPK/JNK antibody, rabbit pAnti-phosphorylated-p38 MAPK antibody, rabbit pAnti p44/42 MAPK antibody, rabbit mAnti - SAPK/JNK antibody and rabbit pAntip38 MAPK antibody and rabbit anti-phosphorylated-IKKβ, rabbit anti-IKKβ, mouse anti-phosphorylated IκBα, mouse anti-IκBα, rabbit anti-phosphorylated NFκB p65 and rabbit anti- NFκB p65 antibodies (Cell signaling technology®) at 4°c overnight. The primary antibodies (1:1000) and secondary antibody consisting of horse-radish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:2000) was used for the detection. The band were visualized by the ECL detection system (KPL® system). The integrated densities of band will be calculated using Scion image analysis software.

2.5.10 Western blot for aggrecanase activities

Conditioned media were digested with 10^{-3} U chondroitinase ABC, keratinase I and II overnight at 37°C overnight. After sample was dialyzed with water, it was subjected to 10%SDS-PAGE and transferred to nitrocellulose membrane with a glycine-transferring buffer [20 mM Tris base, 0.2 M glycine, 20% v/v methanol (pH 8.5)]. After blocking nonspecific binding with 5% nonfat dried milk, the membrane was probed with BC-3 antibody at 4°c overnight. BC-3 antibody can react with ^{374}ARGSVI sequence with appear on aggrecan-cleaved aggrecan core protein (243). The primary antibodies (1:100) and secondary antibody consisting of alkaline phosphatase conjugated anti-mouse IgG (1:7500) were used for the detection. The band will be visualized by Alkaline Phosphatase chromogen (BCIP/NBT) (Abcam®).

2.5.11 Immunohistochemical analysis

For type II collagen staining, after deparafinnization and dehydation, sections were digested with 0.25 U chondroitinase ABC, 1.45U testicular hyaluronidase and 0.25% trypsin for 15 min. Then sections were incubated in 3% H_2O_2 for 5 min and after washed with PBS, sections were blocked with 5% bovine serum albumin in PBS for 30 min. Anti-mouse mAnti-type II collagen antibody (Calbiochem®) (1:250 in PBS) was added into the sections and incubated at 4°c overnight. Horse-radish peroxidase-conjugated pAnti-mouse IgG antibody (1:250) was added and incubated at room temperature for 1 h. The color was developed using peroxidase substrate (DAB reagent set, KPL® system) following by rehydration. The sections were mounted with mounting medium and left at room temperature until dry.