CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

All chemicals used were analytical grade or equivalent. The chemicals shown below are listed in groups according to suppliers.

Aldrich Chem. (USA)

1,9-Dimethyl-Methylene Blue

Bio-Rad Laboratories (Hercules, CA)

Acrylamide: N,N'-Methylenebisacrylamide Electrophoresis Purity Reagent, N,N,N',N'-tetra-methyl-ethylenediamine (TEMED), Protein assay (Dye reagent concentrate), Sodium dodecyl sulphate (SDS)

Carlo Erba reagent (Rodano (Mi), Italy)

di-Sodium hydrogen phosphate anhydrous, Potassium sulfate, Sodium chloride, Sodium dihydrogen phosphate monohydrate

Fisher Chemicals (UK)

Triton X-100, Acetonitrile HPLC grade

Fluka (Buchs, Swizerland)

Sodium hydroxide, tri-Sodium citrate dihydrate

Life Technologies (Life Technologies, Inc., Rockville, MD, USA)

Dulbecco's Modified Eagle Medium (DMEM; Cat. No.12100), Penicillin/Streptomycin, fetal bovine serum

Merck (Darmstadt, F.R. Germany)

Acetic acid, Citric acid monohydrate, Ethanol (absolute), Hydrogen peroxide, Potassium chloride, Sodium acetate trihydrate, Sodium chloride, Sodium hydrogen carbonate, Sodium carbonate anhydrous, Methanol

LAB-SCAN

n-Hexane

Pharmacia, Biotechnology (Uppsala, Sweden)

Coomassie brilliant blue R-250

Sigma (Sigma Aldrich, St.Louis, MO, USA)

Bovine serum albumin, Cesium chloride, Chondroitin sulfate C, Gelatin type B, Glycerol, Hyaluronic acid (from human umbilical cord), Matrix metalloproteinase-2 (MMP-2), Matrix metalloproteinase-9 (MMP-9), Polyoxyethylene sorbitan monolaurate (Tween-20), Sodium phosphate, Thermolysin, Retinoic acid, 12-O-tetradecanoyl phorbol-13-acetate

USB (OHIO,USA)

Tris (Hydroxymethyl) aminomethane hydrochloride, Glycine

Corning (Corning, Inc., NY, USA)

6-well culture plates

2.1.2 Lists of antibodies used in this study

Peroxidase conjugated anti-biotin monoclonal antibody (Zymed Laboratory, Inc. Ca, USA)

2.2 Methods

2.2.1 Preparation of *Zingiber cassumunar* Roxb. (Plai) extracts (58)

Fresh rhizome of *Zingiber cassumunar* Roxb. were cut into pieces, dried at 50 - 60° C and ground. Dried powder of *Zingiber cassumunar* Roxb. samples were extracted with hexane, 70% ethanol and distilled water. Dried ethanolic and water extracts were obtained after removing the solvent by evaporation under reduced pressure in evaporator, then lyophilized. Dried hexane extract was obtained after removing the solvent by evaporation and dry at 37°C. Dried residue was weight and stored at −20 ° C (22). The extracts were used in all experiments were from the same plant materials.

2.2.2 Oral cell culture

Normal gingival tissue overlying an impacted third molar was obtained from the Department of Oral Surgery, Faculty of Dentistry, Chiang Mai University. Primary oral epithelial cells were isolated from gingival tissue by incubating tissue with 0.5 mg/ml of thermolysin at 37° C for 90 min. Oral epithelial cells were cultured in Keratinocyte Basal Medium (KBM) with Bovine pituitary extract (BPE), human Epidermal growth factor (hEGF), Hydrocortisone, Insulin and Gentamicin sulfate Amphotericin-B (GA-100). Primary oral fibroblasts in connective tissue were immersed in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin until a sufficient number of fibroblasts spread from the tissue. After 80% confluence, cells were further passaged and seeded in 6-well culture plates according to experimental conditions. The cells were maintained in culture in a humidified incubator with 5% CO_2 at 37°C.

To evaluate the effects of *Zingiber cassumunar* Roxb. extract (extract with hexane, 70% ethanol or water), with varying concentration 0-100 μg/ml (dissolved in 10%DMSO), 0-50 μM of retinoic acid (RA), and 1 μg/ml of 12-O-tetradecanoyl phorbol-13-acetate (TPA) were added to the cultures, or left untreated as a control. Each experiment was performed independently four times with cell lines derived from different donors.

2.3 Analytical methods

2.3.1 Dye binding assay (52)

The sulfated glycosaminoglycan (S-GAG) concentrations were determined using a colorimetric dye binding assay modified by Farndale *et al*. The assay is based on a metachromatic shift in absorption maximal from 690 nm to 535 nm as a complex compound is formed in a mixture of 1,9-dimethylmethylene blue (DMMB) and the sulfated-GAG in the sample and standard. The dye solution was made by adding 16 mg of 1,9-dimethylmethylene blue to 5 ml ethanol to 2 g of sodium formate and 2 ml of formic acid in a total volume of 1 liter at pH 3.5. The maximum absorbance of the dye solution was at 620 nm. This solution was stored at 4^0C in dark bottle. Chondroitin 6-sulfate (CS-C) standards (0-40 μg/ml:50 μl) or samples (50 μl) were transferred to a microtitre plate. The dye solution (200 μl) was added immediately to each well and absorbance was measured at 620 nm, a precipitate might form on standing. A standard curve of CS-C concentration and absorbance 620 nm was plotted. The concentration of CS-C in the samples were calculated from the standard curve. (53).

2.3.2 Enzyme-linked immunosorbent assay

A competitive inhibition based ELISA for HA (53)

Microtiter plates (Maxisorp, Nunc) were coated at 4^0C overnight with umbilical cord HA (100 μl/well) in the coating buffer. Uncoated area was then blocked with 150 μl/well of 1% (w/v) BSA in the incubating buffer for 60 min at 25^0C. After washing, 100 μl of the mixture, sample or standard competitor (HA Healon:range 39.06-10,000 ng/ml) in B-HABPs (1:100), were added. After incubation for 60 min at 25^0C, plates were washed and then the peroxidase-mouse monoclonal anti-biotin (100 μl/well; 1:4,000) was added and incubated for 60 min at 25^0C. The plates were washed again and then the peroxidase substrate (100 μl/well) was added and incubated at 37^0C for 20 min to allow the color to develop. The reaction was stopped by addition of 50 μl of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

2.3.3 Gelatin Zymography (56)

Gelatinolytic activity of proteins from tissue culture media was assayed by electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a vertical gel apparatus according to the method of Laemmli (53), with modification that gelatin was included in the resolving gel . Gelatin type B (Sigma-Aldrich) was co-polymerized at a final concentration of 1 mg/ml into 0.75 mm. thickness, 10% polyacrylamide gel. Samples were mixed with an equal volume of 2X sample buffer, incubated at room temperature for 30 min. Each sample was loaded to a well and the samples were electrophoresed for 200 min at 90 V. After electrophoresis SDS was removed from the gel by washing 2 x 15 min in 2.5% Triton X-100 at room temperature and incubated for 16 hr at 37^0C in activating buffer (54). The gels were subsequently stained with 0.2% Coomassie Brilliant Blue R-250 for 5 hr. at room temperature, destained with 50% methanol and 10% acetic acid to reveal zone of lysis within the gelatin matrix. The gel was dried on a Whatman paper. Molecular weights of standard markers were run on each gel. Gelatinolytic activity was detected as clear band against a background of stained, intact gelatin-impregnated acrylamide gel.

Quantification of the gelatinolytic band on the zymogram was performed by densitometry. The image acquisition was done with an Agfa scanner (SNAPscan 1212), by using Adole Photoshop Elements 2.0 program. The zymogram densitometry was achieved with a Acion Image software for PC (Scion Corporation, Frederick, Maryland, USA), working in the Gel Plot 2 mode.

2.3.4 Cytotoxicity detection (57)

Quantification of cell death and cell lysis was determined by a colorimetric assay, based on the measurement of lactate dehydrogenase (LDH) activity in culture medium. The analyses were conducted according to the manufacturer's instruction by comparing the amount of LDH in the samples with the positive control, i.e. the culture medium from oral fibroblasts treated with 0.5 mM H_2O_2 for 10 hrs.

2.3.5 Protein assay

The protein content of each sample was determined by the Bio-Rad Protein Microassay. A portion (10 μl) of medium sample was added to 200 μl of concentrated dye reagent in a 96-well microtitre plate and mixed thoroughly. After 5 min the absorbance of samples was measured at 620 nm in an automated plate reader.

2.3.6 Statistical method

The release of extracellular matrixs (ECMs) biomolecules from culture medium of both cells type were estimated using the calculation:

$$\% \text{ Change} = \frac{\text{Sample medium / Protein content}}{\text{Untreated control / Protein content}} \times 100$$

The significance of the differences between groups of data was analyzed by a two-tail ANOVA analysis using the statistical package for social sciences (SPSS) version 10.0 for Windows. Statistical significance was considered when $p < 0.05$.

The release of matrix metalloproteinase (MMP) from culture medium was quantified by densitometry and was estimated by the calculation.

$$\% \text{ of Control} = \frac{\text{Density of sample medium}}{\text{Density of untreated control}} \times 100$$

The significance of the differences between groups of data was analyzed by a two-tail ANOVA analysis using the statistical package for social sciences (SPSS) version 10.0 for Windows. Statistical significance was considered when $p < 0.05$.