ผลของสารสกัดจากเหง้าไพล *(Zingiber cassumunar Roxb.)* ต่อระดับไฮยาลูโรแนน ไกลโคซามิโนไกลแคน และเอนไซม์แมทริกซ์เมทัลโลโปรตีเนส จากเซลล์สร้างเส้นใยและเซลล์เยื่อบุผิวจากช่องปาก

Effect of Plai *(Zingiber cassumunar Roxb.)* Extract on the Levels of Hyaluronan, Glycosaminoglycan and Matrix Metalloproteinases from Oral Fibroblast and Epithelial Cells

ศิริวรรณ องค์ไชย
สุทธิชัย กฤษณะประกรกิจ
ปรัชญา คงทวีเลิศ
ปรีณา โชติจำลอง

พ.ศ.2552
สนับสนุนโดยทุนอุดหนุนการวิจัยคณะแพทยศาสตร์
คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

ผลของสารสกัดจากเหง้าไพล *(Zingiber cassumunar Roxb.)* ต่อระดับไฮยาลูโรแนน ไกลโคซามิโนไกลแคน และเอนไซม์แมทริกซ์เมทัลโลโปรตีเนส จากเซลล์สร้างเส้นใยและเซลล์เยื่อบุผิวจากช่องปาก

Effect of Plai *(Zingiber cassumunar Roxb.)* Extract on the Levels of Hyaluronan, Glycosaminoglycan and Matrix Metalloproteinases from Oral Fibroblast and Epithelial Cells

ศิริวรรณ องค์ไชย
สุทธิชัย กฤษณะประกรกิจ
ปรัชญา คงทวีเลิศ
ปรีณา โชติจำลอง

พ.ศ.2552
สนับสนุนโดยทุนอุดหนุนการวิจัยคณะแพทยศาสตร์
คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

ACKNOWLEDGEMENT

This work was supported by Faculty of Medicine Research Fund,

Chiang Mai Univeristy, Chiang Mai, Thailand.

ผลของสารสกัดจากเหง้าไพล *(Zingiber cassumunar Roxb.)* ต่อระดับไฮยาลูโรแนน ไกลโคซามิโนไกลแคน และเอนไซม์แมทริกซ์เมทัลโลโปรตีเนส จากเซลล์สร้างเส้นใยและเซลล์เยื่อบุผิวจากช่องปาก

บทคัดย่อ

เมื่อมีการอักเสบของเนื้อเยื่อ จะพบการเปลี่ยนแปลงของสารชีวเคมีนอกเซลล์ เช่น ไฮยาลูโรแนน ไกลโคสะมิโนไกลแคน รวมทั้งเอนไซม์แมทริกซ์เมทัลโลโปรตีเนส มีรายงานผลการวิจัยที่ผ่านมาพบว่าสารสกัดจากไพล (*Zingiber cassumunar Roxb.*) มีฤทธิ์ต้านการเกิดอนุมูลอิสระและต้านการอักเสบปวดบวมได้ งานวิจัยครั้งนี้จึงมีจุดประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดไพลต่อการเปลี่ยนแปลงระดับสารประกอบนอกเซลล์ในน้ำเลี้ยงเซลล์สร้างเส้นใยและเซลล์เยื่อบุผิวจากช่องปาก โดยเลี้ยงเซลล์ในน้ำเลี้ยงแล้วทดสอบสารสกัดไพลที่สกัดได้จากน้ำ เฮกเซนและเอทานอล ในภาวะที่มี หรือไม่มีสารละลายกรดเรติโนอิก (RA) เป็นเวลา 24 ชั่วโมง น้ำเลี้ยงถูกนำมาวิเคราะห์ระดับไฮยาลูโรแนน (HA) ไกลโคซามิโนไกลแคน (GAG) และเอนไซม์แมทริกซ์เมทัลโลโปรตีเนส (MMP) ด้วยวิธีทดสอบ ELISA-based assay, ปฏิกิริยาของ Farndale, และ Gelatin Zymography ตามลำดับ ผลการทดลองพบว่าในน้ำเลี้ยงเซลล์สร้างเส้นใยที่มีสารสกัดไพลด้วยเฮกเซนและเอทานอลลดระดับ HA และ GAG รวมถึงสารละลายรวมระหว่าง RAที่ 10 ไมโครโมลาร์กับสารสกัดไพลที่ 12.5 ไมโครกรัมต่อมิลลิลิตรหรือมากกว่า สามารถลดระดับ HA ได้อย่างมีนัยสำคัญ ($p<0.05$) ขณะที่สารสกัดไพลด้วยน้ำไม่มีผลต่อการลดระดับ HA การทำงานของเอนไซม์ MMP-2 จะถูกยับยั้งที่ความเข้มข้นของสารสกัดไพลความเข้มข้นสูง แต่ RA เพิ่มการทำงานของ MMP-2 และเมื่อทดสอบด้วยสารละลายรวมระหว่าง 10 ไมโครโมลาร์ RA กับสารสกัดไพล พบว่าไพลมีผลในการลดฤทธิ์ของ RA ในทางตรงกันข้าม ในน้ำเลี้ยงเซลล์เยื่อบุผิวพบว่า ระดับของ HA และ GAG เพิ่มขึ้น การทำงานของเอนไซม์ MMP-9 ลดลง เมื่อมีสารสกัดไพล ขณะที่ RA จะเพิ่มการทำงานของ MMP-9 และเมื่อทดสอบด้วยสารละลายรวมระหว่าง RA กับสารสกัดไพล พบว่า สารสกัดไพลมีผลต่อการยับยั้ง RA จากข้อมูลที่ได้นี้บ่งชี้ถึงความสามารถของสารสกัดไพลในการยับยั้ง HA และ MMP ของเซลล์สร้างเส้นใยและพบผลตรงข้ามในเซลล์เยื่อบุผิว นำไปสู่การลดอาการบวมและการอักเสบของเนื้อเยื่อระหว่างการเกิดบาดแผลได้

Effect of Plai (*Zingiber cassumunar* Roxb.) Extract on the Levels of Hyaluronan, Glycosaminoglycan and Matrix Metalloproteinases from Oral Fibroblast and Epithelial Cells

ABSTRACT

Changes of etxtracellular matrix biomolecules such as hyaluronan, glycosaminoglycan and matrix metalloproteinases were found in tissue inflammation. Plai or *Zingiber cassumunar* Roxb. was previously shown to possess antioxidant and potent anti-inflammatory activity. The aim of this study was to investigate the effect of its extract on the levels of extracellular matrix (ECM) components in cultured human oral fibroblasts and epithelial cells. Cultured mediums were treated with various fractions of the solvents; water, hexane, and ethanol, or retinoic acid (RA) overnight, or left untreated. hyaluronan (HA), glycosaminoglycan (GAG), and matrix metalloproteinase (MMP), the major ECM components of wound healing process, were analyzed for the quantities by the ELISA-based assay, Farndale reaction, and Gelatin Zymography, respectively. Cultured fibroblasts medium, the hexane and ethanol extract of *Zingiber cassumunar* Roxb. decreased HA and GAG levels. In addition, combination between 10 μM of RA and 12.5 μg/ml or greater of the extract significantly decreased HA levels ($P < 0.05$). While treatment with water extract failed to affect HA levels. The activity of MMP-2 was inhibited by high concentration of the ethanol extract, whereas RA increased the activity of enzymes. The combination of 10 μM RA and the ethanol extract of *Zingiber cassumunar* Roxb. inhibited the effect of RA. In the opposite, HA and GAG levels were increased in cultured human oral epithelium medium. The activity of MMP-9 was inhibited by the ethanol extract, whereas RA increased the activity of the enzyme. The combination of RA and the ethanol extract of *Zingiber cassumunar* Roxb. inhibited the effect of RA. Collectively, these data indicated the inhibitory activity of *Zingiber cassumunar* on HA production and MMP activities in human oral fibroblasts, but showed the opposite effects in human oral epithelial cells. The ability of the ethanol extract to reduce tissue hydration and inflammation during wound healing was suggested.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURES **PAGE**

ABBREVIATIONS

B-HABPs	biotinylated hyaluronan binding proteins
BSA	bovine serum albumin
CS	chondroitin sulfate
CS-C	chondroitin 6-sulfate
DBA	dye binding assay
DMEM	Dulbecco's modified Eagle's medium
DMMB	1,9-dimethylmethylene blue
DMPBD	(E)-1-(3',4'-dimethoxyphenyl) butadiene
DS	dermatan sulfate
ELISA	enzyme-linked immunosorbent assay
GAGs	glycosaminoglycans
GalNAc	N-acetyl-galactosamine
GlcA	D-glucuronic acid
HA	hyaluronan
HABPs	hyaluronan binding proteins
hEGF	human Epidermal growth factor
HS	heparan sulfate
IL-1β	interleukin-1β
LDH	lactate dehydrogenase
MMPs	matrix metalloproteinases
RA	retinoic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S-GAG	sulfated glycosaminoglycan
TEMED	N,N,N',N'-tetra-methyl-ethylenediamine
TPA	12-O-tetradecanoyl phorbol-13-acetate

CHAPTER I

INTRODUCTION

1.1 Statement of the problems

When human tissue is injured, a complex variety of cellular and molecular interactions are induced in order to return the tissue to homeostasis. These interactions can be categorized into four phases; hemostasis, inflammation, granulation tissue formation, and tissue remodeling. These phases comprise a sequentially integrated and interdependent process (1). Wound healing shows spatial dependency across the wound site as well. When tissue is injured, cutaneous vasculature becomes severed, causing blood to fill the wound area. When platelets come into contact with extraluminal collagen and tissue procoagulant factors from injured cells, they become activated and begin to aggregate. The platelet aggregates release alpha granules that contain clotting inducers, such as von Willebrand factor, fibrinogen, fibronectin (FN), and thrombospondin (TN). The fibrin clot provides the initial matrix that is in the wound provisionally and through which inflammatory and reparative cells can migrate, while the platelets release growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-α and -β (TGF-α, TGF-β), which are chemoattractants for smooth muscle cells, inflammatory cells, and fibroblasts (2).

Inflammation occurs as leukocytes are attracted to the wound area by a host of chemoattractants. These include fibrin clot byproducts, such as fibrinopeptides and fibrin degradation products, activated neutrophil releasates, such as leukotriene B4 and platelet-activating factor (PAF), and platelet releasates, such as platelet factor 4 and PDGF. The macrophages release numerous cytokines that are important for the attraction and proliferation of fibroblasts. These include PDGF, fibroblast growth factor (FGF), TGF-β. Other inflammatory cells that interact within the wound area include lymphocytes, plasma cells, and mast cells. These cells also contribute to cytokine production that is important for the tissue formation phase, such as interleukin-4 (IL-4), which induces collagen production in fibroblasts.

Re-epithelialization of human wounds is the first of the two stages of tissue formation to begin within a few hours post injury. Keratinocytes, epidermal cells originating in the surrounding epidermal sheet and differentiated from stem cells, move over one another in a leapfrog fashion until the wound is closed. After a few days, the epithelial cells begin to proliferate, and a new basement membrane is formed under the re-epithelialized site, binding the cells with the basement membrane through new hemidesmosomes. Granulation tissue is the other component of tissue formation. This new tissue fills the wound space and revascularizes the injured site. Fibroblasts secrete their own proteases, as well as relying on serum proteases, to carve a way through the highly crosslinked clot. Once inside the wound, they produce large quantities of FN, hyaluronic acid (HA), and collagen types I and III, and organize these proteins into a structured matrix, while degrading

the fibrin matrix. The remodeling phase of wound is protracted and can last for over a year. During this phase, the hyaluronan and the FN that were previously laid down in the provisional matrix are degraded.

Hyaluronan or hyaluronic acid (HA) is a non-sulfated glycosaminoglycan that is consisted of repeating N-acetyl glucosamine-glucuronic acid disaccharides using β1,4 and β1,3 linkages (3). Uncrosslinked HA is produced in granulation tissue within skin wounds mainly by fibroblasts. The HA may promote cell migration in granulation tissue through a few mechanisms (4). The first is the facilitation of adhesion-disadhesion between cells and the extracellular matrix (ECM). High levels of HA in the ECM have been shown to weaken the adhesion of cells. The loss of tight adhesion between the cell and the ECM might allow for rapid migration (5). The second is that hydrated HA forms a large pore matrix that can accommodate more cell proliferation and invasion. The third is that there are specific HA receptors that contribute to cellular interactions with the ECM. Two major cell surface receptors for HA are CD44 and receptor for hyaluronan-mediated motility (RHAMM). CD44 mediates cell adhesion and migration on HA, as well as the cellular uptake and degradation of HA. RHAMM regulates cell migration in response to soluble HA. In addition to interacting with fibroblasts, HA interacts with endothelial cells, thereby playing an important role in wound process (6). HA is of particular interest in wound healing because of the discovery that fetal wounds, which are bathed in an HA-rich fluid, heal without scarring. HA is broken into fragments during injury and these fragments act as cytokines to induce repair processes that lead to scarring.

Proteoglycans (mucoproteins) are formed by glycosaminoglycans (GAGs) covalently attached to the core proteins. They are found in all connective tissues, extracellular matrix (ECM), and on the surfaces of many cell types. Proteoglycans are remarkable for their diversity (i.e. different types of core protein, different numbers of GAGs with various lengths and compositions). Glycosaminoglycans forming the proteoglycans are the most abundant heteropolysaccharides in the body. They are long unbranched molecules containing a repeating disaccharide unit. Usually, one sugar is a uronic acid (either *D-glucuronic* or *L-iduronic*), and the other is either *GlcNAc* or *GalNAc*. One or both sugars contain sulfate groups (the only exception is hyaluronic acid). GAGs are highly negatively charged, which is essential for their function. For example, the large quantities of chondroitin sulfate (CS) and keratan sulfate (KS) found on aggrecan play an important role in the hydration of cartilage. They give the cartilage its gel-like properties and resistance to deformation.

HA and CS are both GAG components of the ECM. CS is comprised of alternating units of β-1,3-linked glucuronic acid and (β-1,4) N-acetyl-galactosamine (GalNAc) and is sulfated on the 4- or 6- position of the GalNAc residues. CS is usually found bound to a core

protein forming a proteoglycan, e.g. aggrecan or versican. Aggregan is the primary proteoglycan in cartilage, and its primary function is to swell and hydrate the collagen fibril framework. Versican is believed to play a role in intracellular signaling, cell recognition, and connecting ECM components to cell surface glycoproteins. Finally, other CS proteoglycans like neurocan and phosphacan play important roles in axon growth and path finding. Both HA and CS are found in the ECM of skin, present in healing wound tissue, and have been used in wound healing materials.

The matrix metalloproteinases (MMP) are a group of zinc dependent enzymes (endopeptidases) which degrade varying components of the ECM in both normal and diseased tissue (7). MMPs are also vital to wound healing. These enzymes degrade collagen and other components of the ECM. MMPs are zinc-dependent enzymes secreted from cells in the healing wound and, through their ability to break down ECM, allow cells to move through the wound bed. Thus, MMPs are necessary in the debridement of the wound bed during inflammation, granulation tissue formation, re-epithelialization and maturation. The activity of MMPs is tightly controlled through synthesis, secretion, and inhibition. Poor regulation could result in abnormal healing: an excess of MMP leads to the destruction of newly synthesized ECM, including collagen, and is also destructive to growth factors in the healing wound. It is believed that this might be a contributing factor to delayed healing in some chronic wounds.

An ulcer is described as a localized shedding of an epithelium. It is critical to treat these ulcers, because as soon as the epidermal cells die, a major barrier to bacteria is breached, and it can cause further necrosis to the surrounding tissues. An ulcer that is considered chronic, or nonhealing, is one that does not heal in a timely fashion. Many treatment schemes have been developed and used to treat these non-healing ulcers. Recently, alternative treatments to relieve pain and symptoms other than pain with less side effects than prescribed medications have been studied. Several previous studies showed that active compounds from some herbs could relieve and/or solve these problems. One of these studies demonstrated the anti-inflammatory effect of *Zingiber cassumunar* Roxb. (also known as Plai in Thai) extracts by suppressing inflammation and edema in carrageenan-induced rat paw. Plai has long been regarded by Thai massage therapists as one of the essential oil necessary to have in their kit to combat joint and muscle problems, and it has an anti-inflammatory activity. The plant has been proven to be extremely

useful for human health and then developed into several products such as creams and massage oils for relieving muscle pain. Thus, the aims of this study were to investigate the effects of *Zingiber cassumunar* Roxb. or Plai extracts on the levels of HA, sulfated-glycosaminoglycan, and matrix metalloproteinases (MMPs) in the culture medium of oral fibroblasts and epithelial cells. The results of this study may lead to the development of alternative pharmacological agents in the management of oral inflammatory disorders.

1.2 Literature reviews

1.2.1 Wound Healing

Wound healing is a dynamic (8), interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells (Figure 1 and 2). Wound healing has three phases, i.e. inflammation, proliferation, and maturation (Figure 3), that overlap in time. Tissue injury causes the disruption of blood vessels and extravasation of blood constituents. Production of both kinins and prostaglandins leads to vasodilatation and increased small vessel permeability in the region of the wound. This results in edema in the area of the injury and is responsible for the pain and swelling which occur early after injury. Within 6 hours, circulating immune cells start to appear in the wound. The blood clot reestablishes hemostasis and provides a provisional extracellular matrix for cell migration. The proliferative phase is characterized by the formation of granulation tissue in the wound. Granulation tissue consists of a combination of cellular elements, including fibroblasts and inflammatory cells, along with new capillaries embedded in a loose extracellular matrix of collagen, FN, and HA. Fibroblasts first appear in significant numbers in the wound on the third day post-injury and achieve peak numbers around the seventh day. This rapid expansion in the fibroblast population at the wound site occurs via a combination of proliferation and migration. Fibroblasts produce large quantities of collagen, a family of triple-chain glycoproteins, which form the main constituent of the extracellular wound matrix and which are ultimately responsible for imparting tensile strength to the scar (9).

Re-epithelialization of wounds begins within hours after injury. Epidermal cells from skin appendages, such as hair follicles, quickly remove clotted blood and damaged stroma from the wound space. Epidermal and dermal cells no longer adhere to one another, because of

the dissolution of hemidesmosomal links between the epidermis and the basement membrane, which allows the lateral movement of epidermal cells. The expression of integrin receptors on epidermal cells allows them to interact with a variety of extracellular matrix proteins (e.g. FN and vitronectin) that are interspersed with stromal type I collagen at the margin of the wound and interwoven with the fibrin clot in the wound space.

One to two days after injury, epidermal cells at the wound margin begin to proliferate behind the actively migrating cells. New stroma, often called granulation tissue, begins to invade the wound space approximately four days after injury. Numerous new capillaries endow the new stroma with its granular appearance. Macrophages, fibroblasts, and blood vessels move into the wound space at the same time. The fibroblasts produce the new extracellular matrix necessary to support cell ingrowth; and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism.

In maturation phase (day 7 to 1 year), almost as soon as the extracellular matrix is laid down, its reorganization begins. Initially, the extracellular matrix is rich in FN, which forms a provisional fiber network. This serves not only as a substratum for migration and ingrowth of cells, but also as a template for collagen deposition by fibroblasts. There are also significant quantities of HA and large molecular weight proteoglycans present, which contribute to the gel-like consistency of the extracellular matrix and aid cellular infiltration. Collagen rapidly becomes the predominant constituent of the matrix. The initially randomly distributed collagen fibers become cross-linked and aggregated into fibrillar bundles, which gradually provide the healing tissue with increasing stiffness and tensile strength. After a 5-day lag period, which corresponds to early granulation tissue formation and a matrix largely composed of FN and HA, there is a rapid increase in wound breaking strength due to collagen fibrogenesis. The subsequent rate of gain in wound tensile strength is slow, with the wound having gained only 20% of its final strength after 3 weeks. The final strength of the wound remains less than that of uninjured skin, with the maximum breaking strength of the scar reaching only 70% of that of the intact skin. The extracellular matrix can have a positive or negative effect on the ability of fibroblasts to synthesize, deposit, remodel, and generally interact with the extracellular matrix. The regulation of wound contraction remains poorly defined. Information regarding the effects of specific cytokines on contraction is limited and often conflicting.

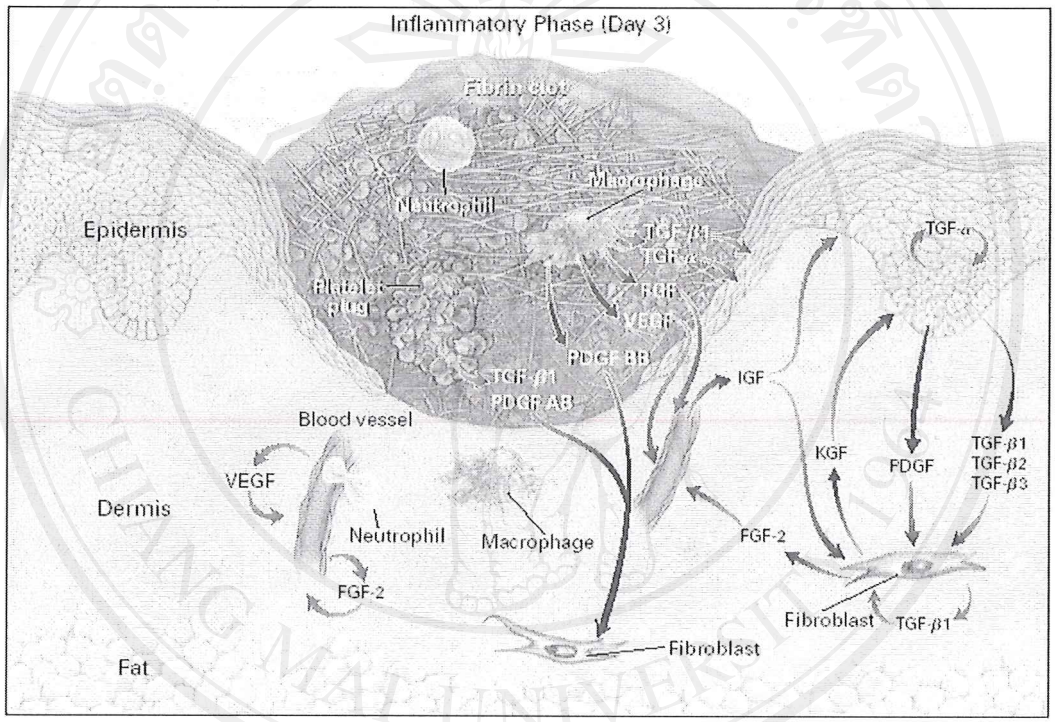


Figure 1. A cutaneous wound three days after injury (10). Growth factors thought to be necessary for cell movement into the wound are shown. TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor-β1, -β2, and -β3, respectively; TGF-α transforming growth factor-α; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF AB, and PDGF BB platelet-derived growth factor, platelet-derived growth factor AB, and platelet-derived growth factor BB, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth factor.

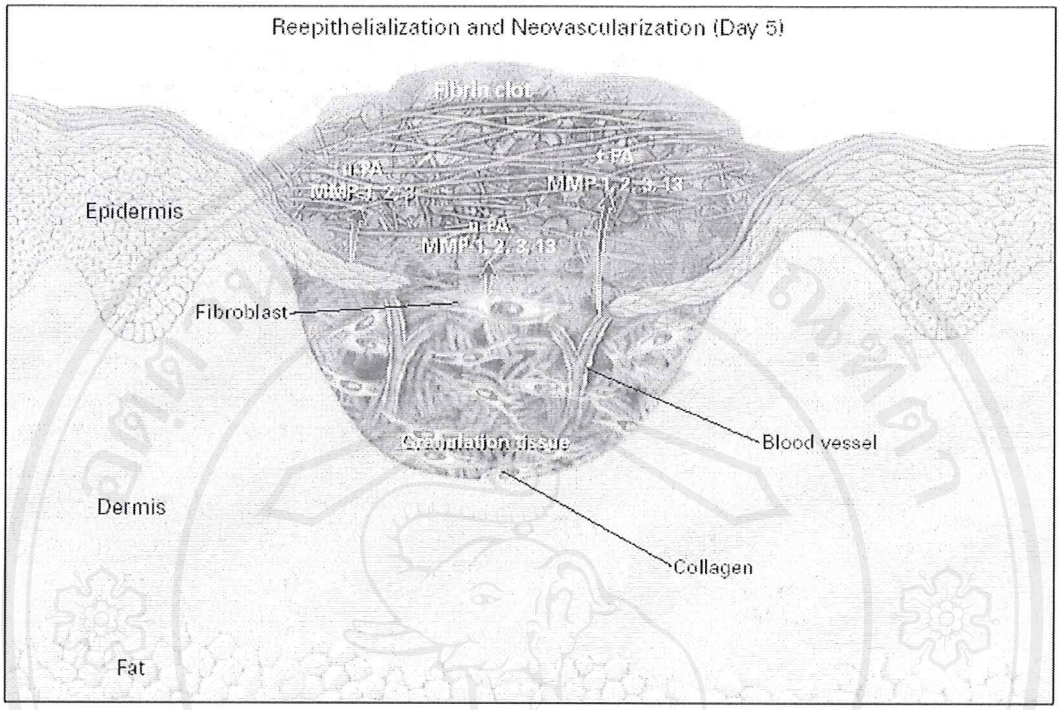


Figure 2. A cutaneous wound five days after injury (10). Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. The abbreviation u-PA denotes urokinase-type plasminogen activator; MMP-1, 2, 3, and 13 matrix metalloproteinases 1, 2, 3, and 13 (collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively); and t-PA tissue plasminogen activator.

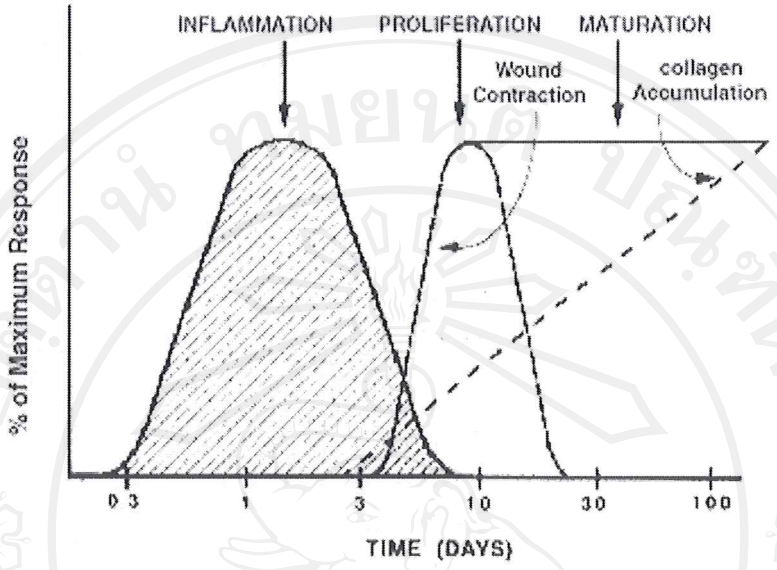


Figure 3. Phases of wound repair (11). Wound healing has been arbitrarily divided into three phases: inflammation, proliferation and maturation.

1.2.2 Hyaluronan

Hyaluronan (HA) is a polymer containing multiple copies of the disaccharide of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronate (GlcA) (Figure 4). HA is synthesized at the plasma membrane and is not associated with any core protein. It differs from other glycosaminoglycans in that it is non-sulfated; it also does not bind covalently with proteins to form proteoglycan monomers, serving instead as the backbone of proteoglycan aggregates. It is the only glycosaminoglycan that is not limited to animal tissues, being found also in bacteria. It serves as a lubricant and a shock absorber in the synovial fluid and is found in the vitreous humor of the eye.

HA is present in all soft tissues of higher organisms and in particularly high concentrations in the synovial fluid and vitreous humor of the eye. HA is an indispensable component of intact healthy gums and oral mucosal tissue. It is distributed in a selective and specific manner and it tends to concentrate particularly in those layers of the gingival epithelium closest to the surface where it acts as a barrier imparting stability and elasticity to the periodontal connective tissue (4). It plays a vital role in many biological processes, such as tissue hydration, proteoglycan organization, cell differentiation, angiogenesis, etc., and acts as a protective coating around the cell membrane. It plays a vital role in cell motility and cell-cell interactions. It binds to cells through three main classes of cell surface receptors, and the main cell surface receptor is CD44, which is most widely distributed in the body. Whether bound to cells or the extracellular matrix components, its hydrophilic nature creates an environment permissive for cellular migration to new tissue sites, while its free radical scavenging and protein exclusion properties offer protection to cells and extracellular matrix molecules against free radical and proteolytic damage.

High concentrations of HA, particularly in fetal skin, have long been noted to be associated with rapid healing with little scarring (13). It is postulated that HA is the extracellular matrix (fluid between skin cells) that is the natural transportation system for the events of wound healing (migrations of inflammatory, fibroblasts, and epithelial cells) to smoothly occur. The rapid production of HA by fibroblasts in the early stages of wound healing may be of crucial importance as HA stimulates the migration and mitosis of mesenchymal and epithelial cells (14). Increased levels of HA, as observed during fetal wound healing or as achieved by the topical

application of HA during wound dressing, are associated with brisker healing and reduced scarring. Glucosamine availability appears to be the rate-limiting factor for HA synthesis (3)(4). Thus, the administration of adequate amounts of glucosamine by mouth during the first few days after surgery or trauma can be expected to enhance HA production in the wound, promote swifter healing, and possibly diminish complications related to scarring.

The synthesis of HA occurs primarily in fibroblasts and is accomplished by the enzyme hyaluronan synthase (HAS). This enzyme, which is located on the inner face of the plasma membrane of the cell, is responsible for both the synthesis of HA and the transport of HA out of the cell (14). The HA turnover in the body is almost completely dependent on degradation. Virtually no HA is lost through excretion. The degradation is accomplished nearly entirely by the hyaluronidases, a group of endohexosaminidases broadly distributed throughout the body with variable specificities and optimal conditions.

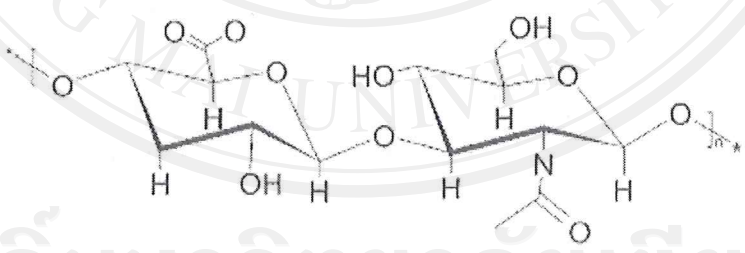


Figure 4. D-glucuronic acid and N-acetyl glucosamine, the disaccharide backbone of HA (12).

1.2.3 Glycosaminoglycans (GAGs) and Proteoglycans

Proteoglycans are a class of heterogeneous molecules consisting of specific types of polysaccharide chains attached covalently to a core protein. The polysaccharides found in proteoglycans typically contain acetylated amino sugars and are referred to as glycosaminoglycans (GAGs). GAGs in a proteoglycan are CS, dermatan sulfate (DS), heparan sulfate, heparin, keratan sulfate (KS). The protein component of proteoglycans is a core protein to which different molecular constructions and functions of proteoglycans are directed (15). The models of proteoglycans are shown in Figure 5.

The GAG chains are linear polymers of repeating disaccharide units containing an amino sugar consisting of N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlaNAc), or N-sulfonylglucosamine ($GlcNSO_3$) residues alternatively linked with glucuronic acid (GlcUA), iduronic acid (IdUA), or galactose (Gal) residues by glycosidic bonds to form the unbranched polysaccharide chain attached to the core protein through a specific oligosaccharide linkage (Figure 6) (15). They are invariably sulfated, leading to the generation of high degree of total negative charges in the molecule. In addition, the core proteins differ greatly, and the GAG chains vary widely in number, length, and structural complexity. Many of the proteoglycans are prominent in extracellular matrix for which a range of structural and metabolic function have been established in cartilage, bone, ligaments, tendon, skin, and blood vessel.

GAG-dependent functions can be divided into two classes: the biochemical and the biophysical. The biochemical function of GAGs is mediated by specific binding of GAGs to other macromolecules, mostly proteins. These molecules are called proteoglycans that participate in cell and tissue development and physiology. The biophysical function depends on the unique properties of GAGs, i.e. their ability to fill the extracellular space, bind and organize water molecules, and repel negatively charged molecules. Because of high viscosity and low compressibility, they are ideal for a lubricating fluid in the joints (15). On the other hand, their rigidity provides structural integrity to the cells and allows cell migration by providing the passageways between the cells. For example, the large quantities of chondroitin sulfate and keratan sulfate found in a proteoglycan, named "*aggrecan*", play an important role in the hydration of cartilage. They give the cartilage its gel-like properties and resistance to deformation. Aggrecan, one of the most important extracellular proteoglycans, forms very large

aggregates (a single aggregate is one of the largest known macromolecules and its length can be more than *4 microns*). A number of aggrecan molecules are non-covalently bound to the long molecule of hyaluronan (like bristles in a bottlebrush), which is facilitated by the linking proteins. For each aggrecan molecule, a core protein and multiple chains of chondroitin sulfate and keratan sulfate are covalently attached through the trisaccharide linker.

During the process of normal maintenance of tissues, i.e. synthesis, repair, and degradation, the proteoglycans are continually being broken down and released from the matrix (4). The synthesis is a process that begins with translation of the core protein and its transport into the lumen of the rough endoplasmic reticulum (RER) (16). Subsequent to the activation of sugar and the formation and translocation of the precursor sugar nucleotides, xylose (Xyl) is added to serine residues of the core protein. A tetrasaccharide linkage region is completed by sequential addition of two galactose residues, and followed by a glucuronic acid (GlcA) residue to produce Glc-Gal-Gal-Xyl-Ser. Finally, the nascent PG is transported to the Golgi where the repeating disaccharides of the GAG chain are individually added and subsequently sulfated. Several enzymes responsible for adding each of these sugars to the protein core are differentially confined within the specific membranes of these secretion pathways and function in different compartments of the ER and Golgi apparatus. The specific structure of both the core protein and the attached GAG chain is important for the function of the mature PG, and the outcome of this systematically regulated biosynthesis has therefore significant consequences in the physiological and pathological functions of proteoglycans.

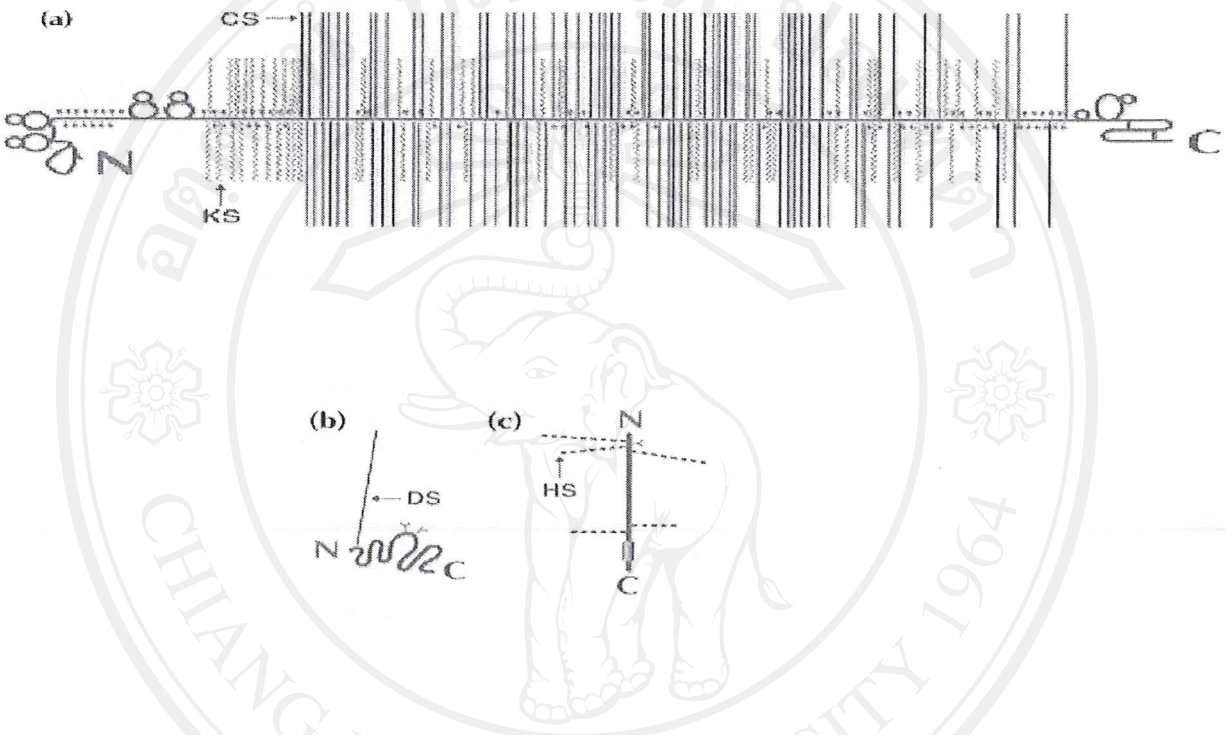


Figure 5. The models of typical proteoglycans; (a) aggrecan, (b) decorin, and (c) syndecan 1. The amino and carboxy termini of core proteins are indicated by N and C, respectively. Glycosaminoglycans are depicted by red lines [i.e., solid lines, chondroitin sulfate / dermatan sulfate (CS / DS); broken lines, heparan sulfate (HS); wavy lines, keratan sulfate (KS)]. The sizes of these three proteoglycans are approximately compared when they are stretched. A block in the syndecan 1 core protein represents the transmembrane hydrophobic domain. This figure is obtained from http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA00E.html.

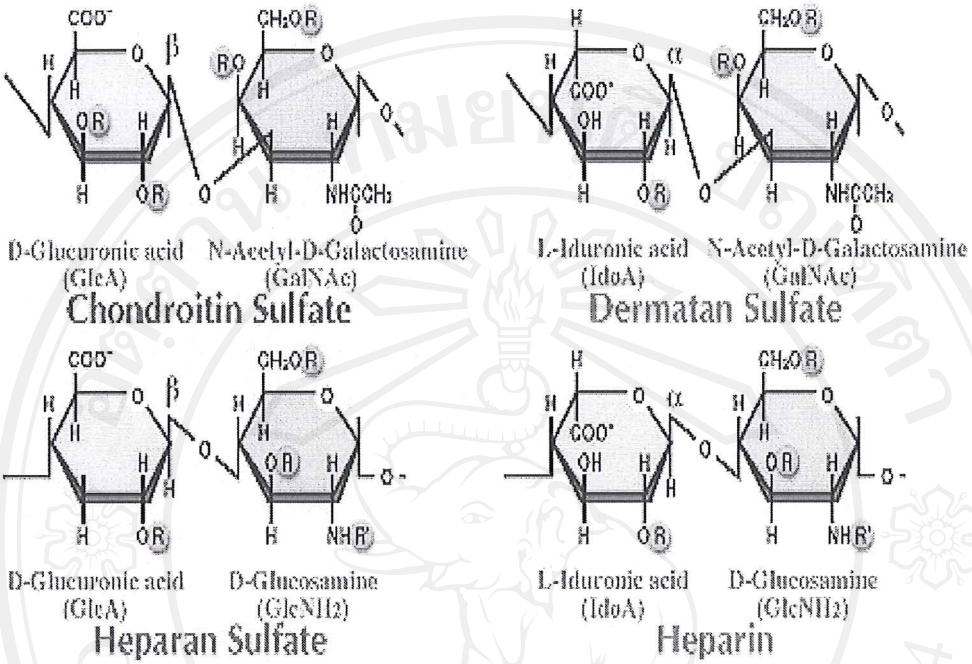


Figure 6. A schematic diagram demonstrating the molecular structure of glycosaminoglycans, including chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate, and heparin. This figure is obtained from http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA06E.html.

1.2.4 Matrix metalloproteinase (MMP)

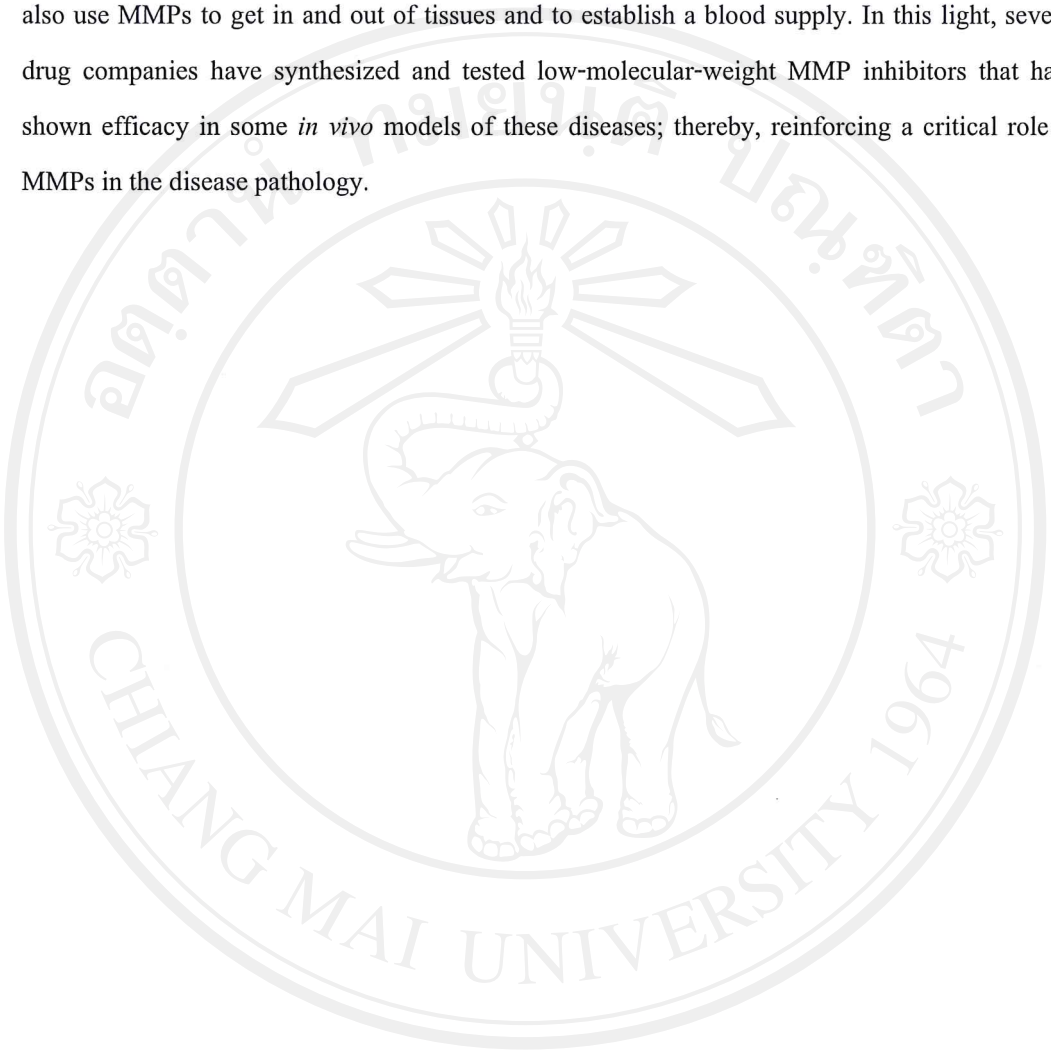
All MMPs are synthesized as inactive zymogens (or pro MMPs) and must be activated by proteolytic cleavage of the propeptide domain from the N- terminus of the enzyme. Generally, they are present as soluble forms, but some are membrane bound. They are composed of three distinct domains: an amino-terminal propeptide domain that is involved in the maintenance of enzyme latency; a catalytic domain that binds zinc and calcium ions which are required for the stability and enzymatic activity; and a hemopexin-like domain at the carboxy terminus (Figure 7) (17).

The turnover of MMPs is controlled by both physiological and pathological factors, such as pro-inflammatory cytokines, hormones, growth factors, and proteases. The MMPs are capable of degrading a variety of ECM biomolecules, including collagens, proteoglycans, fibronectin, and laminin. The members of the MMP family are largely distinguished by the substrates they degrade (Table 1).

MMPs constitute a multigene family of zinc- and calcium-dependent endopeptidases with extensive sequence homology. To date, at least 25 different MMPs have been identified that share significant sequence homology and a common multi-domain organization. According to their structural and functional properties, the MMP family can be subdivided into five major groups (I) the collagenases (MMP-1, -8, -13), (II) the gelatinases (MMP-2, -9), (III) the stromelysins (MMP-3, -10, -11), (IV) a heterogeneous subgroup including matrilysin (MMP-7), enamelysin (MMP-20), macrophage metalloelastase (MMP-12), and MMP-19, and (V) the membrane-type MMPs (MMP-14 to –17 and –24, -25 or MT1-6 MMP) (19).

Although the connection between a single MMP and its individual substrates is not as straightforward as once thought, it is clear that, as a whole family, the MMPs are capable of breaking down any extracellular matrix components. In normal physiology, MMPs produced by connective tissue cells are thought to contribute to tissue remodeling in the development, the menstrual cycle, and as part of repair processes following tissue damage (20). The obvious destructive capability of MMPs had initially drawn most researchers' attention to diseases that involve breakdown of the connective tissues (e.g., rheumatoid arthritis, cancer, and periodontal disease). Leukocytes, particularly macrophages, are major sources of MMP production. MMPs released by leukocytes play vital roles in allowing leukocytes to extravasate and penetrate tissues, a key event in inflammatory disease (16). The MMP action not only permits leukocyte emigration into tissues and causes tissue damage, but also generates immunogenic fragments of normal

proteins that may escalate autoimmune disease. In an analogous way, metastatic cancer cells can also use MMPs to get in and out of tissues and to establish a blood supply. In this light, several drug companies have synthesized and tested low-molecular-weight MMP inhibitors that have shown efficacy in some *in vivo* models of these diseases; thereby, reinforcing a critical role of MMPs in the disease pathology.



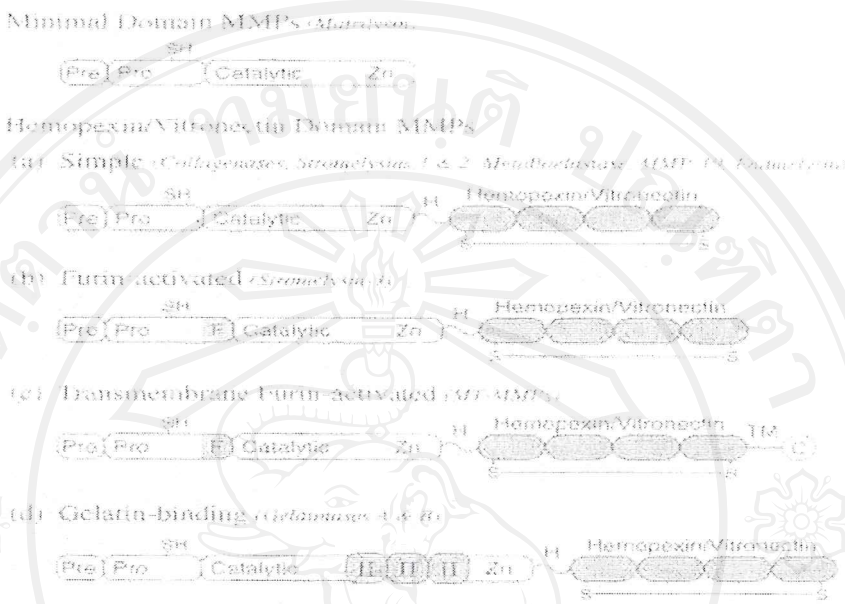


Figure 7. The domain structure of the MMPs (17). Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol group (SH); F, a furin-like enzyme-recognition motif; Zn, a zinc-binding site; II, collagen-binding fibronectin type II inserts; H, a hinge region; TM, a transmembrane domain; C, a cytoplasmic tail. The haemopexin/vitronectin-like C-terminal domain contains four repeats with the first and fourth being connected by a disulfide bridge.

Table 1. Matrix metalloproteinases (MMPs) and their substrates (18).

Matrix metalloproteinases (MMPs) and their substrates

MMP	Enzyme	M_r latent	M_r active	Known substrates
MMP-1	Interstitial collagenase (collagenase-1)	55,000	45,000	Collagens I, II, III, VII, VIII and X, gelatin, aggrecan, versican, proteoglycan link protein, casein, α_1-proteinase inhibitor, α_2-M, pregnancy zone protein, ovostatin, nidogen, MBP, proTNF, L-selectin, proMMP-2, proMMP-9
MMP-2	Gelatinase A	72,000	66,000	Collagens I, IV, V, VII, X, XI and XIV, gelatin, elastin, fibronectin, aggrecan, versican, proteoglycan link protein, MBP, proTNF, α_1-proteinase inhibitor, proMMP-9, proMMP-13
MMP-3	Stromelysin-1	57,000	45,000	Collagens III, IV, IX and X, gelatin, aggrecan, versican, perlecan, nidogen, proteoglycan link protein, fibronectin, laminin, elastin, casein, fibrinogen, antithrombin-III, α_2M, ovostatin, α_1-proteinase inhibitor, MBP, proTNF, proMMP-1, proMMP-7, proMMP-8, proMMP-9, proMMP-13
MMP-7	Matrilysin-1 (PUMP-1)	28,000	19,000	Collagens IV and X, gelatin, aggrecan, proteoglycan link protein, fibronectin, laminin, entactin, elastin, casein, transferrin, MBP, α_1-proteinase inhibitor, proTNF, proMMP-1, proMMP-2, proMMP-9
MMP-8	Neutrophil collagenase (collagenase-2)	75,000	58,000	Collagens I, II, III, V, VII, VIII and X, gelatin, aggrecan, α_1-proteinase inhibitor, α_2-antiplasmin, fibronectin
MMP-9	Gelatinase B	92,000	86,000	Collagens IV, V, VII, X and XIV, gelatin, elastin, aggrecan, versican, proteoglycan link protein, fibronectin, nidogen, α_1-proteinase inhibitor, MBP, proTNF
MMP-10	Stromelysin-2	57,000	44,000	Collagens III, IV and V, gelatin, casein, aggrecan, elastin, proteoglycan link protein, fibronectin, proMMP-1, proMMP-8
MMP-11	Stromelysin-3	51,000	44,000	α_1-proteinase inhibitor
MMP-12	Macrophage metalloelastase	54,000	45,000/ 22,000	Collagen IV, gelatin, elastin, α_1-proteinase inhibitor, fibronectin, vitronectin, laminin, proTNF, MBP
MMP-13	Collagenase-3	60,000	48,000	Collagens I, II, III and IV, gelatin, plasminogen activator inhibitor 2, aggrecan, perlecan, tenascin
MMP-14	MT1-MMP	66,000	56,000	Collagens I, II and III, gelatin, casein, elastin, fibronectin, laminin B chain, vitronectin, aggrecan, dermatan sulfate proteoglycan, MMP-2, MMP-13, proTNF
MMP-15	MT2-MMP	72,000	60,000	proMMP-2, gelatin, fibronectin, tenascin, nidogen, laminin
MMP-16	MT3-MMP	64,000	52,000	proMMP-2
MMP-17	MT4-MMP	57,000	53,000	
MMP-18	Xenopus collagenase	55,000	42,000	
MMP-19		54,000	45,000	Collagen IV, gelatin, laminin, nidogen, tenascin, fibronectin, aggrecan, COMP
MMP-20	Enamelysin	54,000	22,000	Amelogenin
MMP-21	XMMP (xenopus)	70,000	53,000	
MMP-22 (MMP-27)	CMMP (chicken)	52,000	43,000	Gelatin, casein
MMP-23	CA-MMP	?	?	
MMP-24	MT5-MMP	63,000	45,000	proMMP-2, proMMP-9, gelatin
MMP-25	MT6-MMP, leukolysin		56,000	Collagen IV, gelatin, fibronectin, fibrin
MMP-26	Matrilysin-2, endometase	28,000		Collagen IV, fibronectin, fibrinogen, gelatin, α_1-proteinase inhibitor, proMMP-9
MMP-28	Epilysin	59,000 (55,000)		Casein

α_2-M, α_2-macroglobulin; COMP, cartilage oligomeric matrix protein; MBP, myelin basic protein; M_r, relative molecular mass; TNF, tumour necrosis factor.

1.2.5 *Zingiber cassumunar* Roxb. or Plai

Zingiber cassumunar Roxb., a medicinal plant cultivated only in tropical Asian countries, belongs to the Zingiberaceae family (21). Its common names in Thailand are Plai; Puu loi, Puu loei (Northern), Waan fai (Central), and Min-sa-laang (Maehongson). It is propagated vegetatively by rhizomes. Its rhizome has a yellow to green color with fleshy thick texture containing multiple sessile tubers. Stored rhizomes are susceptible to pathogens causing limited supplies for high-quality rhizomes. Moreover, the quality of volatile oil obtained from the rhizomes varies with the plant age. Essential oil of *Plai* is steam distilled from the rhizome and has a pale amber color. The scent is cool and green peppery with a touch of a bite (22). Active chemicals containing in the essential oil of *Zingiber cassumunar* Roxb. are sabinene (27-34%), terpinene (6-8%), pinene (4-5%), terpinen-4-ol (30-35%), and (E)-1-3',4'- dimethoxyphenyl butadiene (DMPBD) (12-19%). The chemical structure of these chemicals is illustrated in Fig. 9. These ingredients are known for their efficacy in anti-inflammatory activity (23).

The rhizome of *Zingiber cassumunar* Roxb. is widely used in Thai traditional medicine for topical treatment of sprains, contusions, joint inflammations, muscular pain, abscesses, and similar inflammation-related disorders. DMPBD, the most active compound in the rhizome extract, was found to exert an anti-inflammatory activity whose potency was as twice as the reference drug diclofenac (=3 vs 6 mg/paw, respectively) (24). Interestingly, all five compounds isolated from the hexane extract of rhizome were found to possess equally or more potent anti-inflammatory activity than the reference drug diclofenac as shown in Table 2. In addition, an *in vivo* study revealed that the anti-inflammatory effects of DMPBD were via both cyclooxygenase (COX) and lipoxygenase (LOX) in arachidonic acid (AA) metabolism pathways (25). In addition to the anti-inflammatory activity of the five major components in the essential oil as mentioned above, DMPBD, terpinen-4-ol, and pinene significantly inhibited edema formation, whereas sabinene and terpinene were inactive up to 6 mg/paw.

The acute toxicity test of *Zingiber cassumunar* Roxb. showed no evidence of toxicity in mice when given 10 g/kg body weight. The safety of 50% alcohol extract from *Zingiber cassumunar* Roxb. administered via an oral or subcutaneous route was more than 20 g/kg and that via an intraperitoneal route was 14.8 g/kg (26). With regard to the chronic toxicity test conducted

during twelve months in 192 Wister rats, the results showed that male rats forced fed by 3.0 g/kg/day of *Zingiber cassumunar* Roxb. consumed less food than the control by 12%; therefore, less body weight gained in the forced fed rats. However, the hematological examination showed no significant differences in all rats, suggesting that all rats were normal. In conclusion, the findings from all of these studies have shown that the *Zingiber cassumunar* Roxb. is safe for both a short and long term use and can potentially be developed into a new pharmacological agent for the treatment of inflammatory disorders (27). In this study, to investigate a potent of the anti-inflammatory activity of *Zingiber cassumunar* Roxb. in the confluent primary oral fibroblasts and epithelial cells on the levels of ECM component, eg. HA, GAG and MMP activity, that be released in culture medium.

A

B

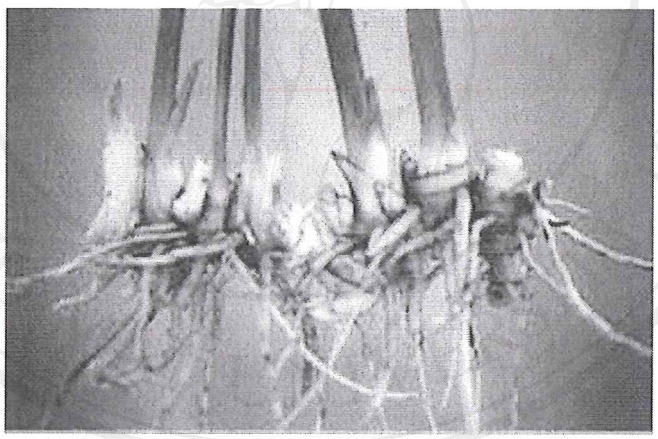


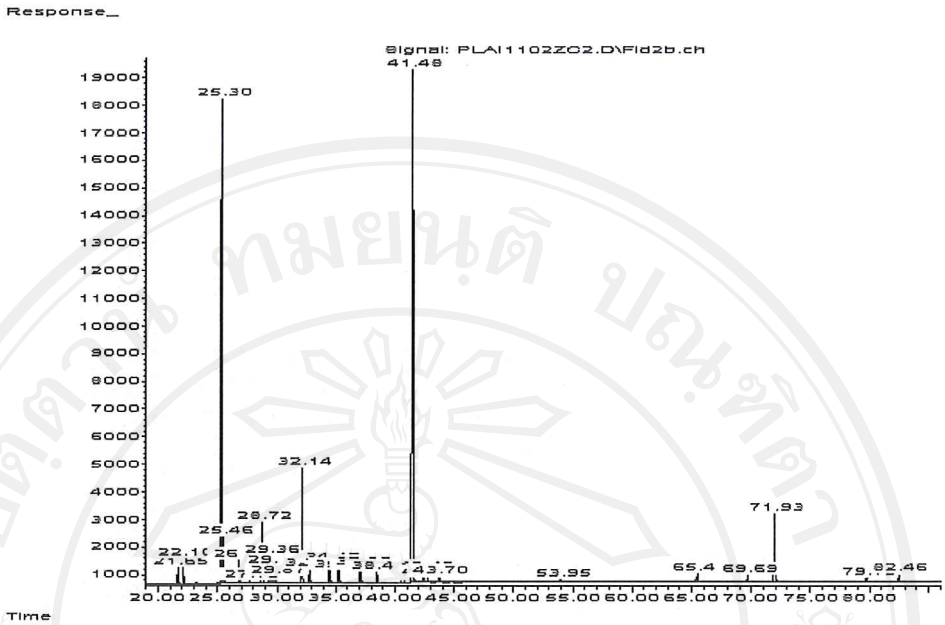
Figure 8. *Zingiber cassumunar* Roxb. and its rhizomes (28). *Zingiber cassumunar* Roxb. is a perennial herb that has bright yellow underground rhizomes. Its rhizomes are widely used in Thai traditional medicine for topical treatment of sprains, contusions, joint inflammations, muscular pain, abscesses, and similar inflammation-related disorders.

A= Obtained from http:// www.gpo.or.th/herbal/ phlai/phlai.htm

B= Obtained from http:// www.thaifitway.com/.../ n2db/question.asp QID=22

Table 2. All five compounds isolated from the hexane extract of rhizome were found to possess equally or more potent anti-inflammatory activity than the reference drug diclofenac (28).

Sample	ID_{50} (μg/ear)
Hexane extract	854
(E)-4-(3',4'-Dimethoxyphenyl)but-3-enyl acetate (4)	62
cis-3-(3',4'-Dimethoxyphenyl)-4-[(E)-3''',4'''-dimethoxystyryl]cyclohex-1-ene(5)	21
cis-3-(3',4'-Dimethoxyphenyl)-4-[(E)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (6)	20
cis-3-(2',4',5'-Trimethoxyphenyl)-4-[(E)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (7)	2
(E)-4-(3',4'-Dimethoxyphenyl)but-3-en-1-ol (8)	47
Diclofenac	61



Main Components

RT	Library/ID	FID%
21.65	alpha-thujene	0.84%
22.10	alpha-pinene	1.32%
25.31	sabinene	27.03%
25.46	beta-pinene	2.73%
26.81	beta-myrcene	1.33%
28.72	alpha-terpinene	3.49%
29.36	p-cymene	1.55%
29.68	beta-phellandrene	0.96%
29.87	1,8-cinelole	0.36%
32.14	gamma-terpinene	6.54%
32.78	trans-sabinene hydrate	0.78%
34.47	terpinolene	1.14%
35.24	cis-sabinene hydrate	0.70%
37.05	cis-p-menth-2-en-1-ol	0.90%
38.47	trans-p-menth-2-en-1-ol	0.67%
41.49	terpinen-4-ol	41.74%
42.42	alpha-terpineol	0.65%
42.78	trans-piperitol	0.20%
43.70	cis-piperitol	0.34%
65.48	beta-sesquiphellandrene	0.48%
71.93	3,4-dimethoxyphenyl)butadiene(DMPBD)	4.59%

Figure 9. Diagram and Main compound of active compounds in *Zingiber cassumunar* Roxb. (http:// www.gpo.or.th/herbal/ phlai/phlai.htm)

1.2.6 Objective

To compare the effects of *Zingiber cassumunar* Roxb. on the levels of extracellular matrix (ECM), i.e. HA and sulfated-GAG, and the enzymatic activities of the matrix metalloproteinases, i.e. gelatinase A and B (or MMP-2 and -9, respectively), in the culture media from both untreated and treated oral fibroblasts and epithelial cells.

1.2.7 Specific Objectives

1. To investigate the effect of the extract of *Zingiber cassumunar* Roxb. on the level of HA release (non sulfated-GAG)
2. To investigate the effect of the extract of *Zingiber cassumunar* Roxb. on the level of sulfated-GAG release
3. To investigate the effect of the extract of *Zingiber cassumunar* Roxb. on MMP2, 9 activity or gelatinase A and B, respectively, whose function is to degrade extracellular matrix components, are essentially involved in the pathogenesis of several inflammatory oral disorders, including gingivitis, periodontitis, and oral mucositis.

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 $p < 0.05$.

CHAPTER III

RESULTS

3.1 Effect of *Zingiber cassumunar* Roxb. extract on phenotype of oral fibroblast and epithelial cells

The rhizome of *Zingiber cassumunar* Roxb. (Plai) is widely used in Thai traditional medicine for topical treatment of joint inflammations, muscular pain, and similar inflammation-related disorders. In this study, three extract fractions were added to the medium of oral fibroblast and epithelial cells. Treatment of the extract for 24 hours, we investigated the morphology of both cells by microscopy. The result showed that *Zingiber cassumunar* Roxb extract did not change phenotype of both cells type as compare to untreated primary cells, the result shown in Figure 10.

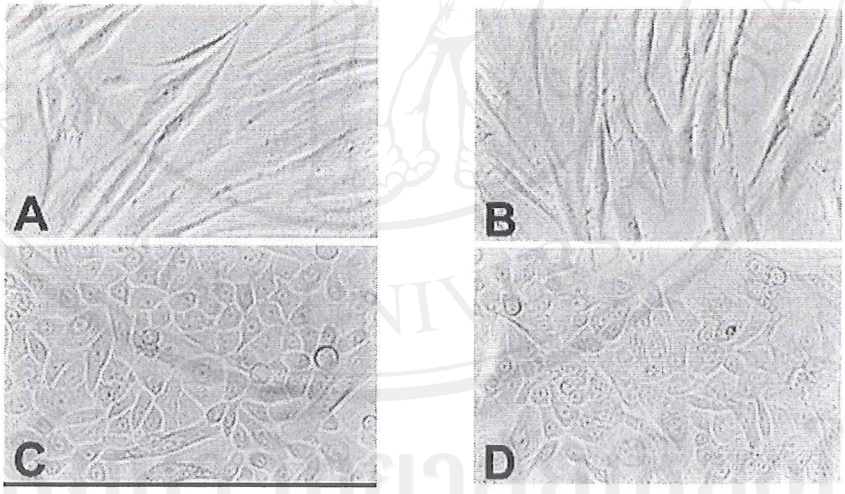


Figure 10. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral fibroblast medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml and control (untreated). Data are the mean values ± standard deviation of triplicate per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.2 Effects of *Zingiber cassumunar* Roxb. extract on the level of extracellular matrix (ECM); HA, sulfated-GAG, MMP-2, -9, and the cytotoxicity from oral cells cultured medium.

The rhizome of *Zingiber cassumunar* Roxb. (Plai) is widely used in Thai traditional medicine for topical treatment of joint inflammations, muscular pain, and similar inflammation-related disorders. Preliminary studies revealed the effect of (E)-1-(3-4-dimethoxyphynyl) butadiene (DMPBD) on anti-inflammatory activity by action on both cyclooxygenase (COX) and lipoxygenase (LOX) in arachidonic acid (AA) metabolic pathways. To study the effect of this extract on the level of HA, sulfated-GAG, MMP-2, 9 and the cytotoxicity, oral fibroblast and epithelial cells were treated with various concentrations of three extract fractions, ethanol, hexane, and water, overnight.

3.2.1 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of hyaluronic acid (HA) in oral fibroblast medium.

In this study, three extract fractions were added to the medium of oral fibroblasts. After 24 hours of treatment, the release of HA was investigated in the culture medium by ELISA assay. The results showed that the ethanol and hexane fractions were able to inhibit the release of HA into the culture media in dose dependent manner but not the water fraction. When compare between hexane and ethanol extracts, it was found that the hexane extract of Plai showed less HA in culture media than ethanol extract at the same concentration as shown in Table 3 and Figure 11.

Retinoic acid (RA) was used as inhibitor to inhibit hyaluronate synthesis *in vitro* model. RA was treated at doses 0.1, 1.0, 10.0, and 50.0 μM in the culture medium overnight. The culture media were collected for analysis. The results showed that the release of HA was partially decreased less than untreated control as shown in figure 12. In addition, oral fibroblasts were treated with the combination between 10.0 μM RA and ethanol fraction at doses 0, 0.1, 1.0, 6.3, 12.5, 25.0, and 50.0 μg/ml overnight. The release of HA was decreased as treated only with ethanol extract as showed in figure 13.

Table 3: Inhibitory effect of Plai extracts on the release of HA in oral fibroblast medium

Treatment	Relative change of HA release (%)
Untreated control	100 ± 0
0.1 µg/ml P-EtOH	128.43 ± 28.22
1.0 µg/ml P-EtOH	113.83 ± 43.55
6.3 µg/ml P-EtOH	88.82 ± 26.08
12.5 µg/ml P-EtOH	100.33 ± 18.00
25.0 µg/ml P-EtOH	67.05 ± 29.03*
50.0 µg/ml P-EtOH	59.40 ± 29.87*
0.1 µg/ml P-Hex	138.73 ± 11.47
1.0 µg/ml P-Hex	107.02 ± 9.75
6.3 µg/ml P-Hex	105.49 ± 75.73
12.5 µg/ml P-Hex	74.56 ± 59.43
25.0 µg/ml P-Hex	47.60 ± 37.67*
50.0 µg/ml P-Hex	31.51 ± 13.35*
0.1 µg/ml P-H_2O	140.00 ± 26.83
1.0 µg/ml P- H_2O	151.98 ± 45.55
6.3 µg/ml P- H_2O	141.24 ± 30.97
12.5 µg/ml P- H_2O	162.35 ± 20.21
25.0 µg/ml P- H_2O	152.22 ± 30.03
50.0 µg/ml P- H_2O	178.99 ± 33.03

Data shown are mean value ± standard deviation of triplicate assay per treatment.

* Denoted value that was significantly different from the untreated control ($p<0.05$).

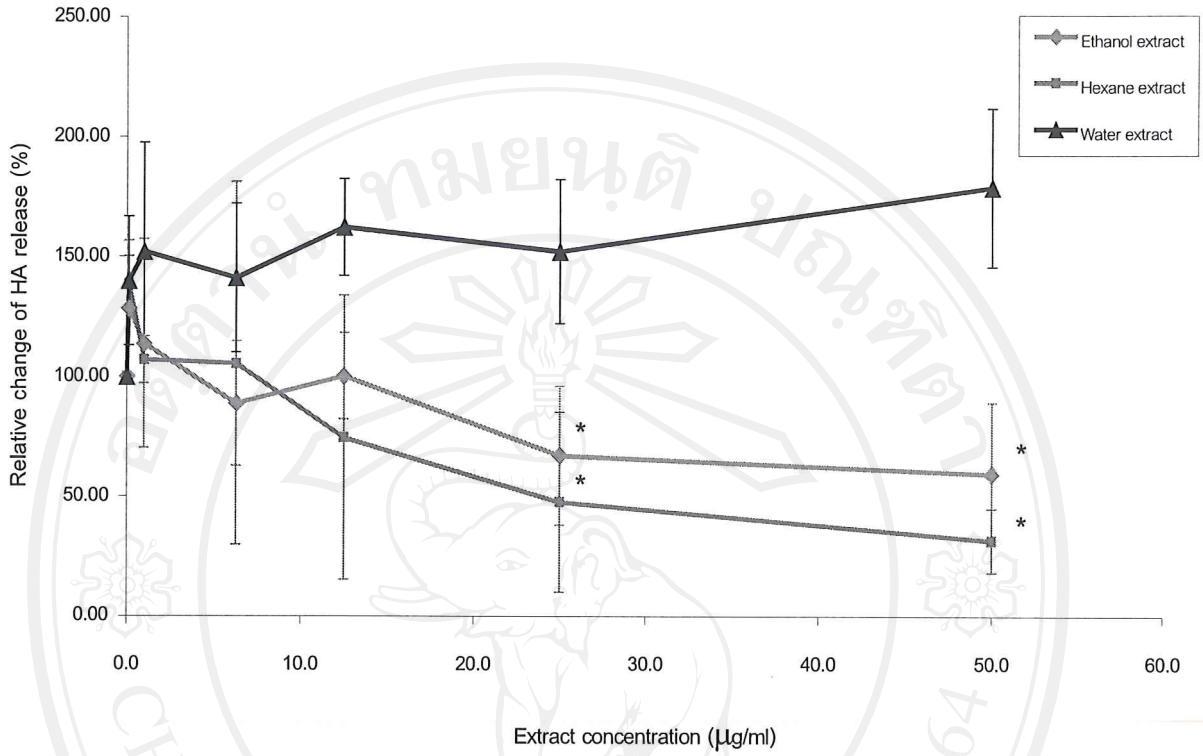


Figure 11. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral fibroblast medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 µg/ml and control (untreated). Data are the mean values ± standard deviation of triplicate per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

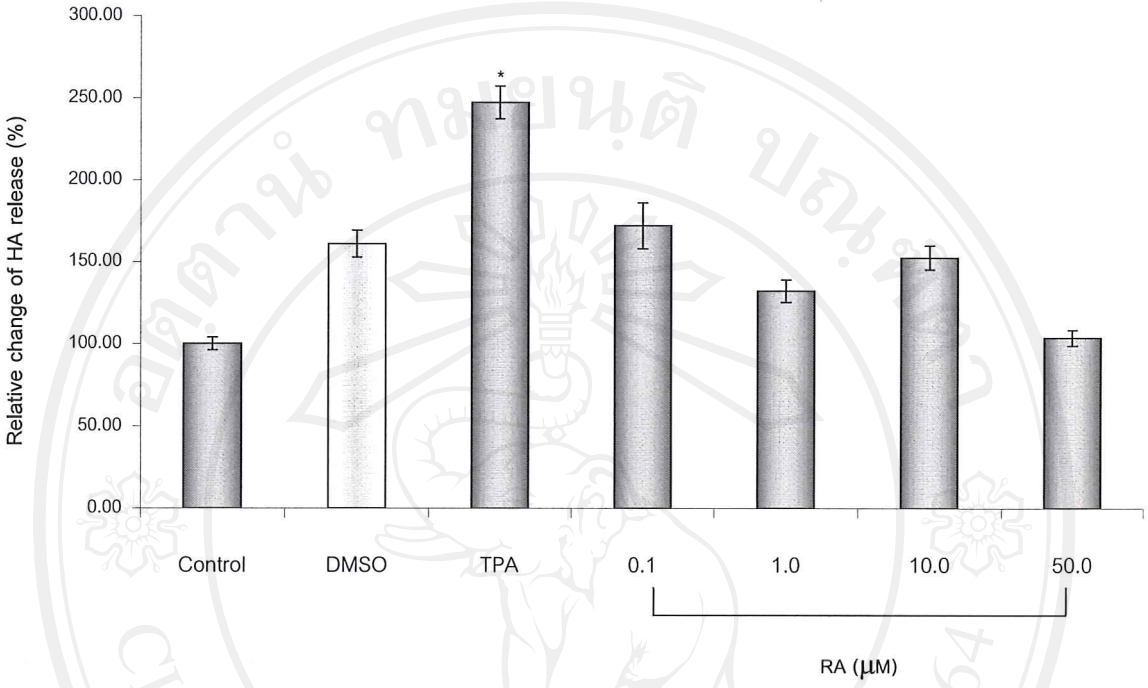


Figure 12. Effect of various concentrations of Retinoic acid (RA) on the releases of HA in oral fibroblast medium. Cells were treated at doses 0.1, 1, 10, 50 μM, control (untreated), solvent control (treated with DMSO) and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

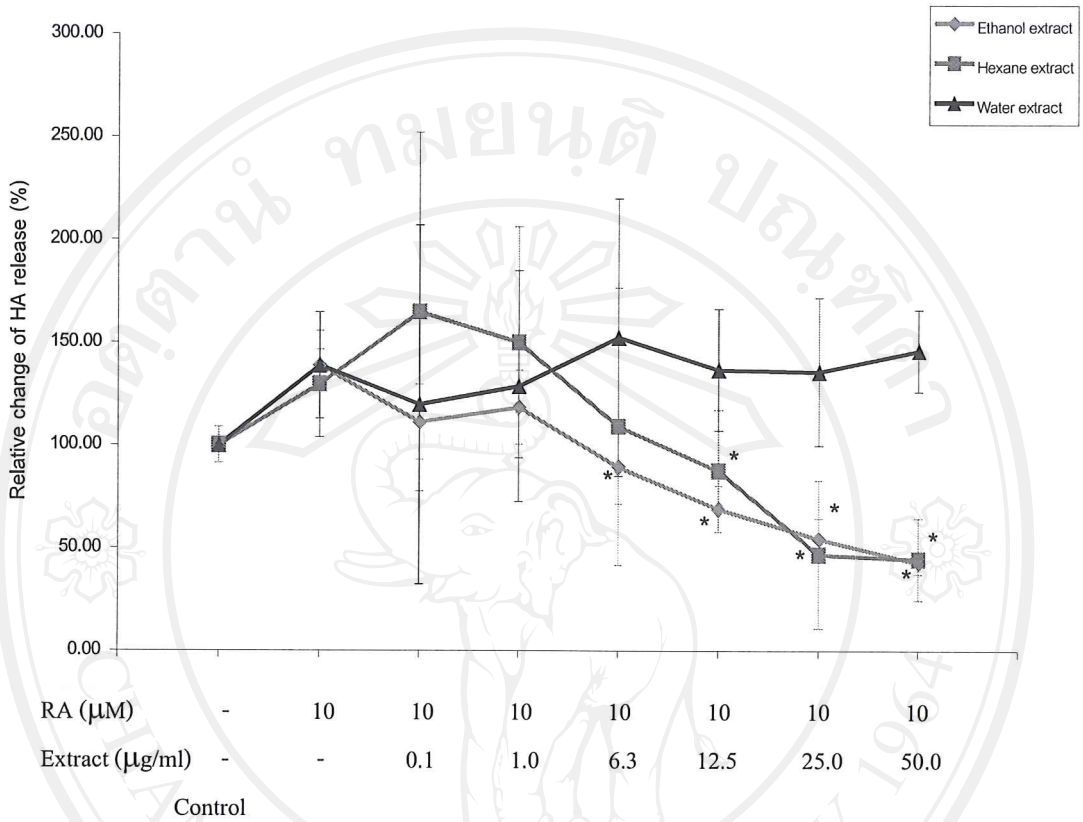


Figure 13. Inhibitory effect of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the release of HA in oral fibroblast medium. Cells were treated with 10 μM RA and the extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO) and 10 μM RA-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment. *Denoted values that were significantly different from RA-treated control (+RA/-Plai), ($p<0.05$).

3.2.2 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of HA from oral epithelial medium.

To study the effect of this extract on the level of HA, sulfated-GAG, MMP-2, 9 and the cytotoxicity, oral epithelia were treated with various concentrations of three extract fractions, which are ethanol, hexane and water, overnight.

In this study, the three extract fractions were added to the medium of oral epithelia. The release of HA was investigated in the culture medium by ELISA assay. The result showed that Plai extract from ethanol and hexane fractions were able to stimulate the release of HA into the culture media in the dose dependent manner but the water fraction was not. When compare between hexane and ethanol extracts, it was found that hexane extract of Plai showed more HA in culture media than ethanol extract at the same concentration as shown in Table 4. and Figure 14.

Retinoic acid (RA) at dose 1.0, 10.0, 25.0, 50.0 μM and 1 μg/ml TPA were added to the culture medium overnight. The culture media were collected for analysis. The release of HA seemed to be more than untreated control at doses 1.0 and 10.0 μM RA but not in treatment of TPA changed as shown in Figure 15. In addition, oral fibroblasts were treated with the combination of 10.0 μM RA and Plai extract at doses 0, 0.1, 1.0, 6.3, 12.5, 25.0, and 50.0 μg/ml overnight. The results showed that the release of HA was increased 2 fold as compare with treatment only Plai extract as shown in Figure 16.

Table 4: Inhibitory effects of Plai extracts on the release of HA in oral epithelial medium

Treatment	Relative change of HA release (%)
Untreated control	100 ± 0
0.1 μg/ml P-EtOH	90.44 ± 11.26
1.0 μg/ml P-EtOH	81.23 ± 15.38
6.3 μg/ml P-EtOH	160.66 ± 71.57
12.5 μg/ml P-EtOH	168.14 ± 36.57
25.0 μg/ml P-EtOH	264.86 ± 74.39*
50.0 μg/ml P-EtOH	312.49 ± 28.00*
0.1 μg/ml P-Hex	74.35 ± 11.47
1.0 μg/ml P-Hex	98.87 ± 19.75
6.3 μg/ml P-Hex	193.13 ± 65.73
12.5 μg/ml P-Hex	265.45 ± 32.43
25.0 μg/ml P-Hex	299.51 ± 74.67*
50.0 μg/ml P-Hex	352.84 ± 31.35*
0.1 μg/ml P-H_2O	74.35 ± 14.83
1.0 μg/ml P- H_2O	108.87 ± 15.55
6.3 μg/ml P- H_2O	83.13 ± 69.97
12.5 μg/ml P- H_2O	115.45 ± 35.23
25.0 μg/ml P- H_2O	99.51 ± 75.03
50.0 μg/ml P- H_2O	112.84 ± 29.03

Data shown are mean value ± standard deviation of triplicate assay per treatment.

* Denoted value that was significantly different from the untreated control ($p<0.05$).

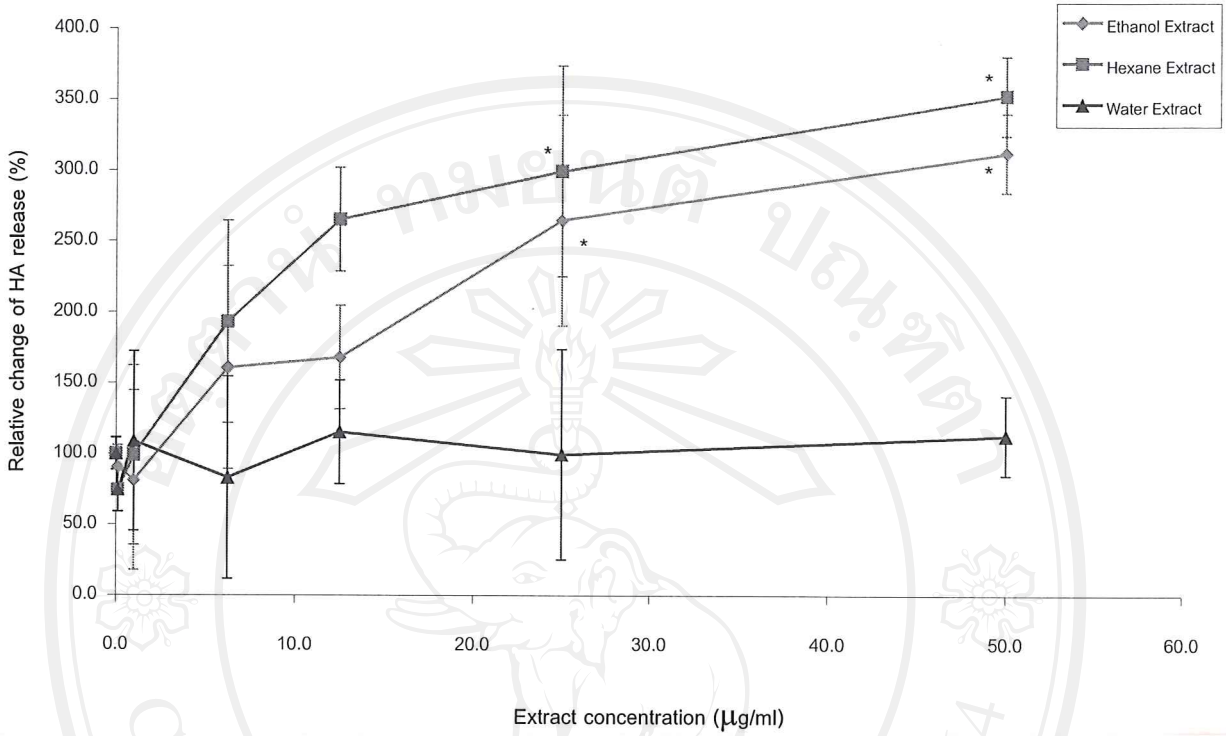


Figure 14. Stimulatory effects of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the release of HA in oral epithelial medium. Cells were treated with ethanol, hexane, and water extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 µg/ml and control (untreated). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

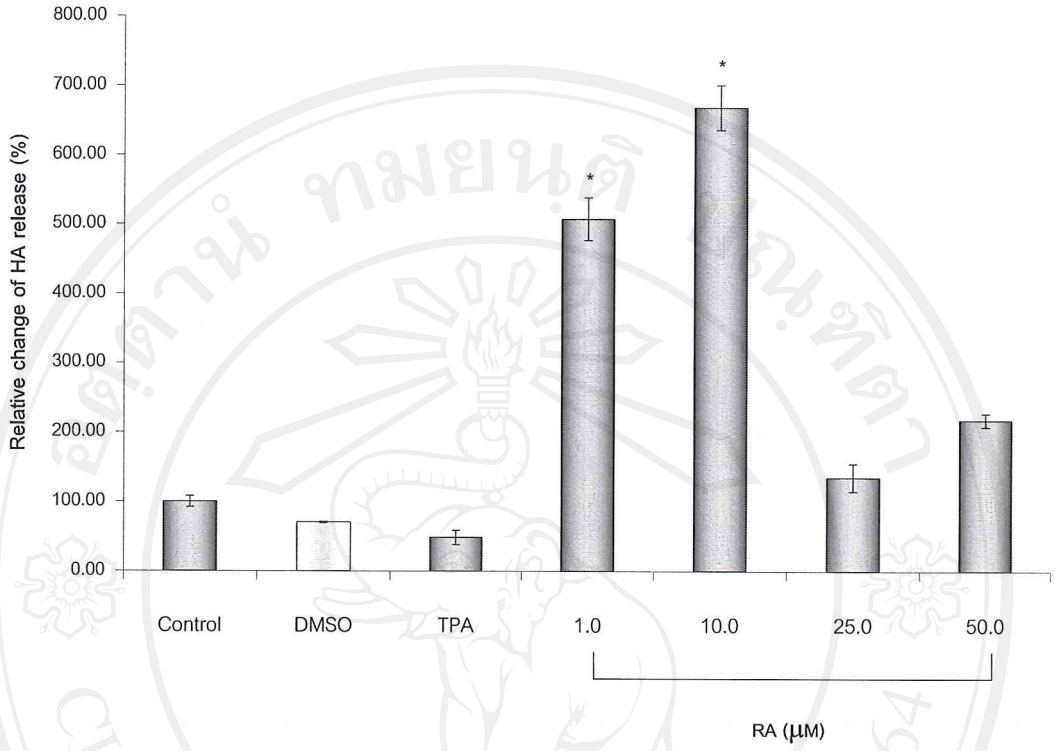


Figure 15. Effect of various concentrations of Retinoic acid (RA) on the release of HA in oral epithelial medium. Cells were treated at doses 1, 10, 25, 50 μM, control (untreated), solvent control (treated with DMSO) and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

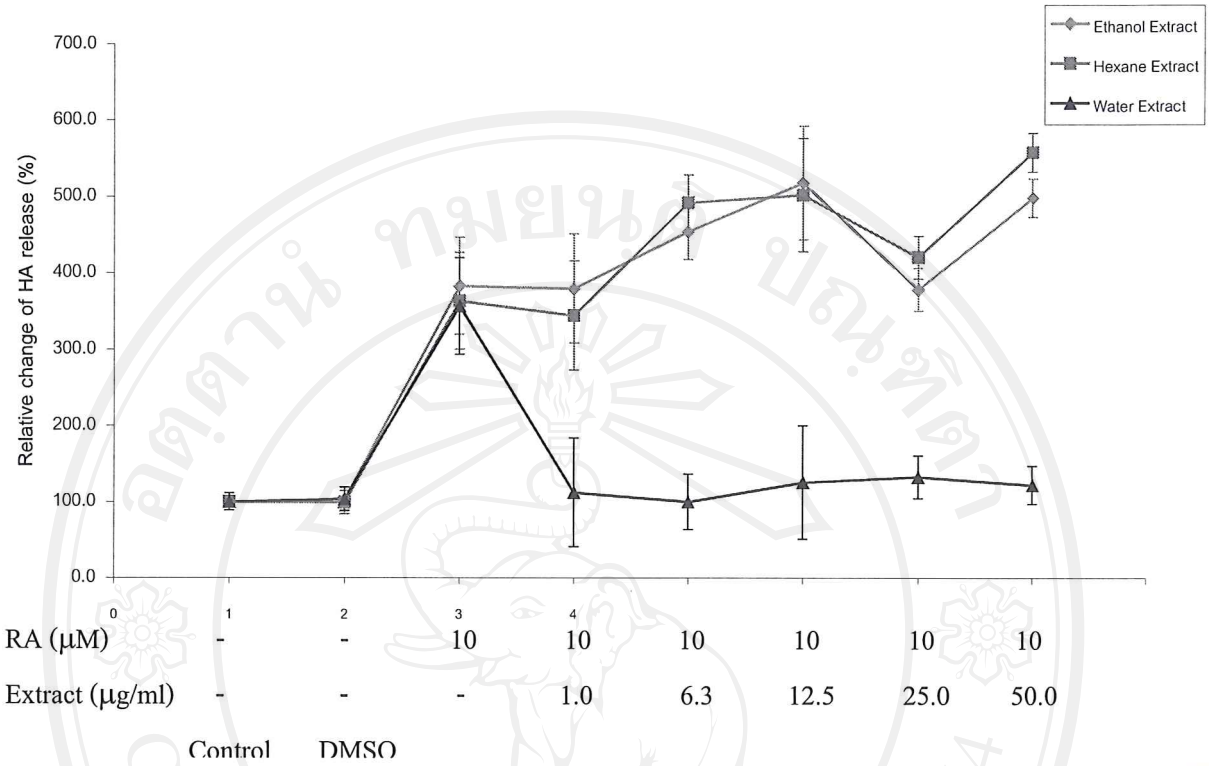


Figure 16. Stimulatory effects of various concentrations of the extracts of *Zingiber cassumunar* Roxb. on the releases of HA in oral epithelial medium. Cells were treated with 10 μM RA and the extract was added at doses 0.1, 1, 6.3, 12.5, 25, 50 μg/ml, control (untreated), solvent control (treated with DMSO) and 10 μM RA-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.2.3 Effect of *Zingiber cassumunar* Roxb. Extract and Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of S-GAG from oral fibroblast medium.

Oral fibroblasts were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and Plai extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and 1.0 μg/ml TPA overnight. The culture media were collected to determine S-GAG content by Farndale reaction assay (dye binding assay). The results showed that the releases of sulfated-GAG was inhibited by RA at doses 0.1-10.0 μM in dose dependent manner as by Plai extract at doses 0.1-25.0 μg/ml as showed in Table 5. and Figure 17. The high concentration of RA (50.0 μM), Plai extract (50.0 μg/ml) and 1 μg/ml TPA were not changed compared with untreated control.

Table 5: Effect of ethanol extracted Plai, RA and TPA on the release of S-GAG in oral fibroblast medium

Treatment	Relative change of S-GAG release (%)
Untreated control	100 ± 3.48
DMSO	159.07 ± 7.34
TPA	113.60 ± 3.83
0.1 μM RA	106.02 ± 2.94
1.0 μM RA	76.35 ± 2.67
10.0 μM RA	66.51 ± 2.23
50.0 μM RA	102.43 ± 3.51
0.1 μg/ml extract	101.64 ± 4.33
1.0 μg/ml extract	72.68 ± 1.55
6.3 μg/ml extract	68.79 ± 1.74
12.5 μg/ml extract	65.79 ± 2.26
25.0 μg/ml extract	59.63 ± 2.28
50.0 μg/ml extract	74.03 ± 2.05
10.0 μM RA + 0.1 μg/ml extract	87.68 ± 3.00
10.0 μM RA + 1.0 μg/ml extract	97.32 ± 4.52
10.0 μM RA + 6.3 μg/ml extract	67.70 ± 3.04
10.0 μM RA + 12.5 μg/ml extract	60.06 ± 2.07
10.0 μM RA + 25.0 μg/ml extract	57.29 ± 1.68
10.0 μM RA + 50.0 μg/ml extract	76.52 ± 1.48

Data shown are mean value ± standard deviation of triplicate assay per treatment.

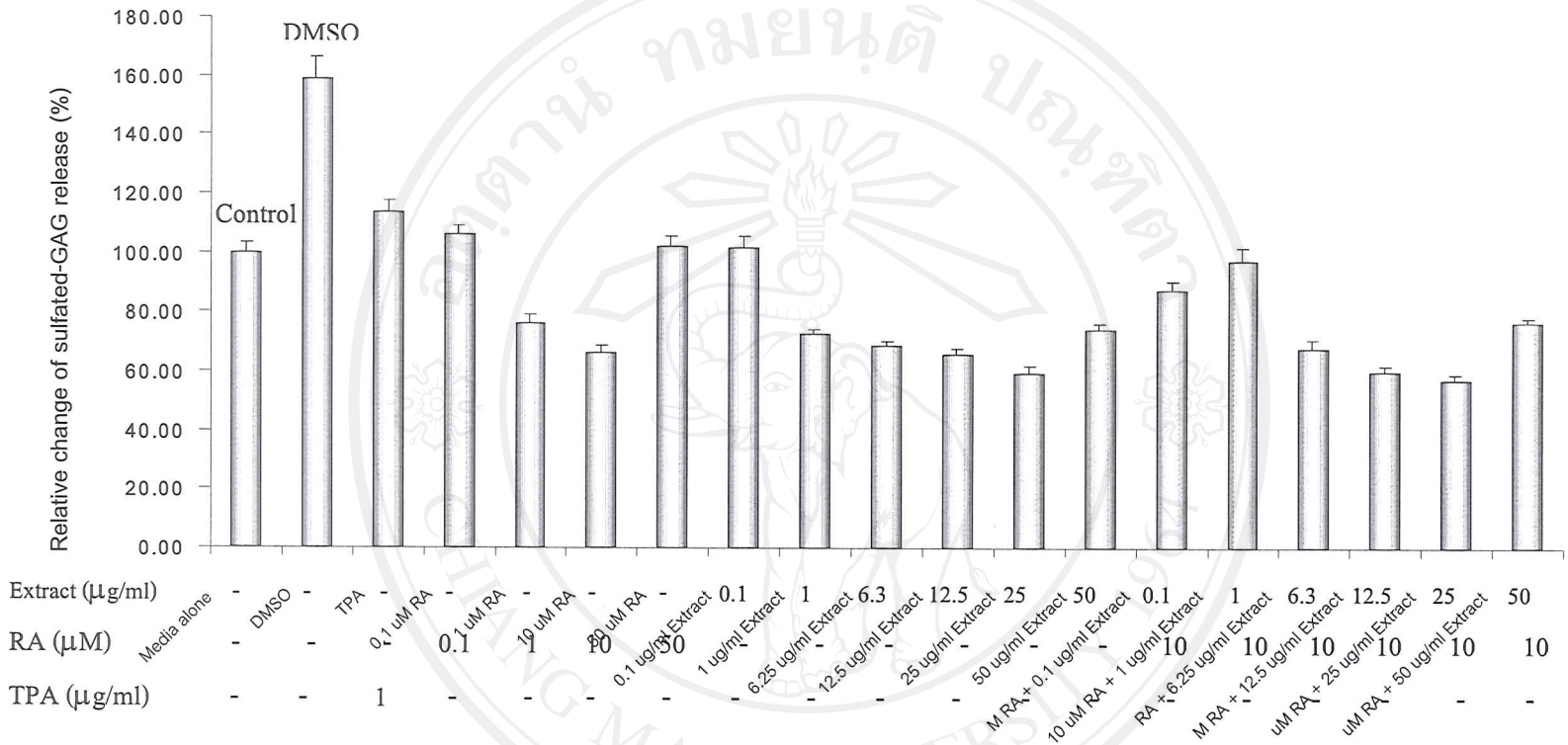


Figure 17. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on the releases of S-GAG from oral fibroblast medium. Cells were treated with 10 µM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 µg/ml, control (untreated), solvent control (treated with DMSO), 0.1, 1, 10, 50 µM RA, and 1 µg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.4 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of S-GAG from oral epithelial medium.

Oral epithelial cells were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and 1.0 μg/ml TPA overnight. The culture media were collected to determine S-GAG content by Farndale reaction assay. The results showed that the release of S-GAG was not changed by RA, Plai extract and TPA as shown in Table 6. and Figure 18.

Table 6: Effects of ethanol extracted Plai, RA and TPA on the release of S-GAG in oral epithelial medium

Treatment	Relative change of S-GAG release (%)
Untreated control	100 ± 0.74
DMSO	116.25 ± 0.27
1.0 μM RA	129.60 ± 1.02
10.0 μM RA	102.96 ± 1.16
25.0 μM RA	95.97 ± 1.46
50.0 μM RA	109.69 ± 1.00
1.0 μg/ml extract	83.23 ± 1.02
6.3 μg/ml extract	110.69 ± 2.76
12.5 μg/ml extract	140.01 ± 2.83
25.0 μg/ml extract	142.96 ± 1.93
50.0 μg/ml extract	132.84 ± 0.74
100.0 μg/ml extract	137.58 ± 1.34
10.0 μM RA + 1.0 μg/ml extract	95.29 ± 0.85
10.0 μM RA + 6.3 μg/ml extract	99.87 ± 0.31
10.0 μM RA + 12.5 μg/ml extract	107.03 ± 1.49
10.0 μM RA + 25.0 μg/ml extract	109.69 ± 2.78
10.0 μM RA + 50.0 μg/ml extract	100.00 ± 1.80
10.0 μM RA + 100.0 μg/ml extract	139.10 ± 0.37

Data shown are mean value ± standard deviation of triplicate assay per treatment.

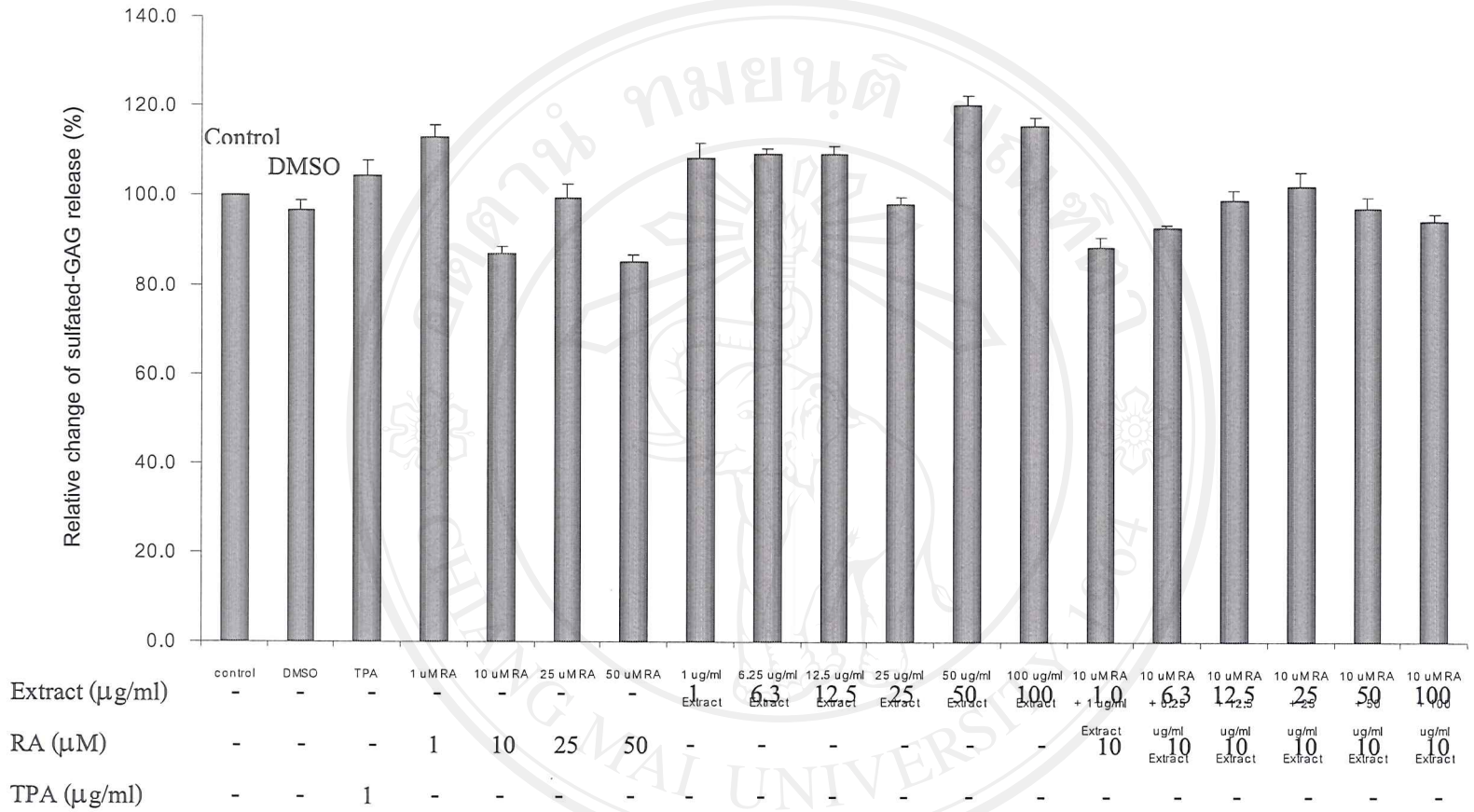


Figure 18. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on the releases of sulfated-GAG from oral epithelial medium. Cells were treated with 10 µM RA and the extract at doses 1, 6.25, 12.5, 25, 50, 100 µg/ml, control (untreated), solvent control (treated with DMSO), 0.1, 1, 10, 50 µM RA, and 1 µg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.5 Effect of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), and Interleukin-1β (IL-1β) on the level of MMP-2, 9 from oral fibroblast medium.

In this study, activities of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in the culture media were investigated by gelatin zymography. Culture media were electrophoresed on native, non-reducing, gelatin containing gels, which were subsequently stained with Coomassie blue; resolved gelatinolytic proteins were detected as unstained band. Oral fibroblast culture in serum-free medium secreted high levels of gelatinase, which are pro-MMP-2 and pro-MMP-9, when compared to standard enzyme and marker at 72 kDa and 92 kDa respectively.

The results showed that ethanol extracted Plai did not down regulate the production of MMP-2 in the oral fibroblast as compare with untreated control as did the treated-RA, TPA and IL-1β, as shown in Figures 19-21.

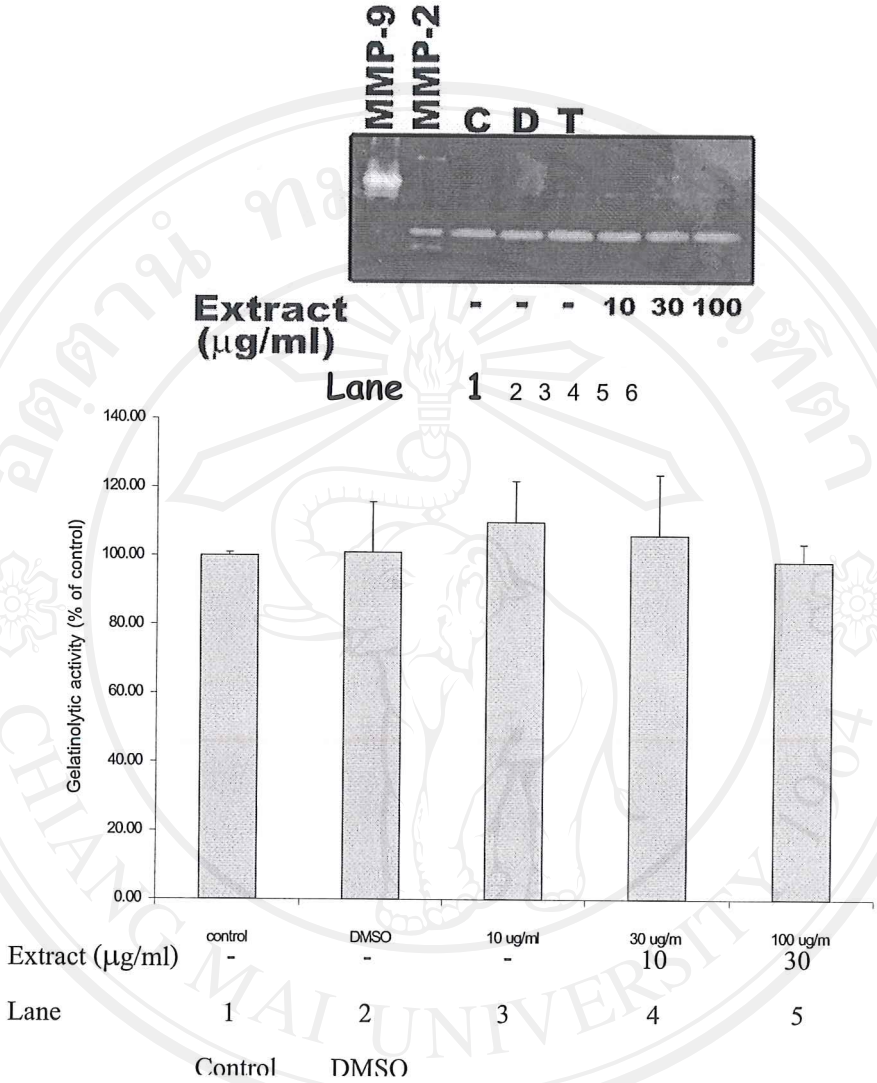
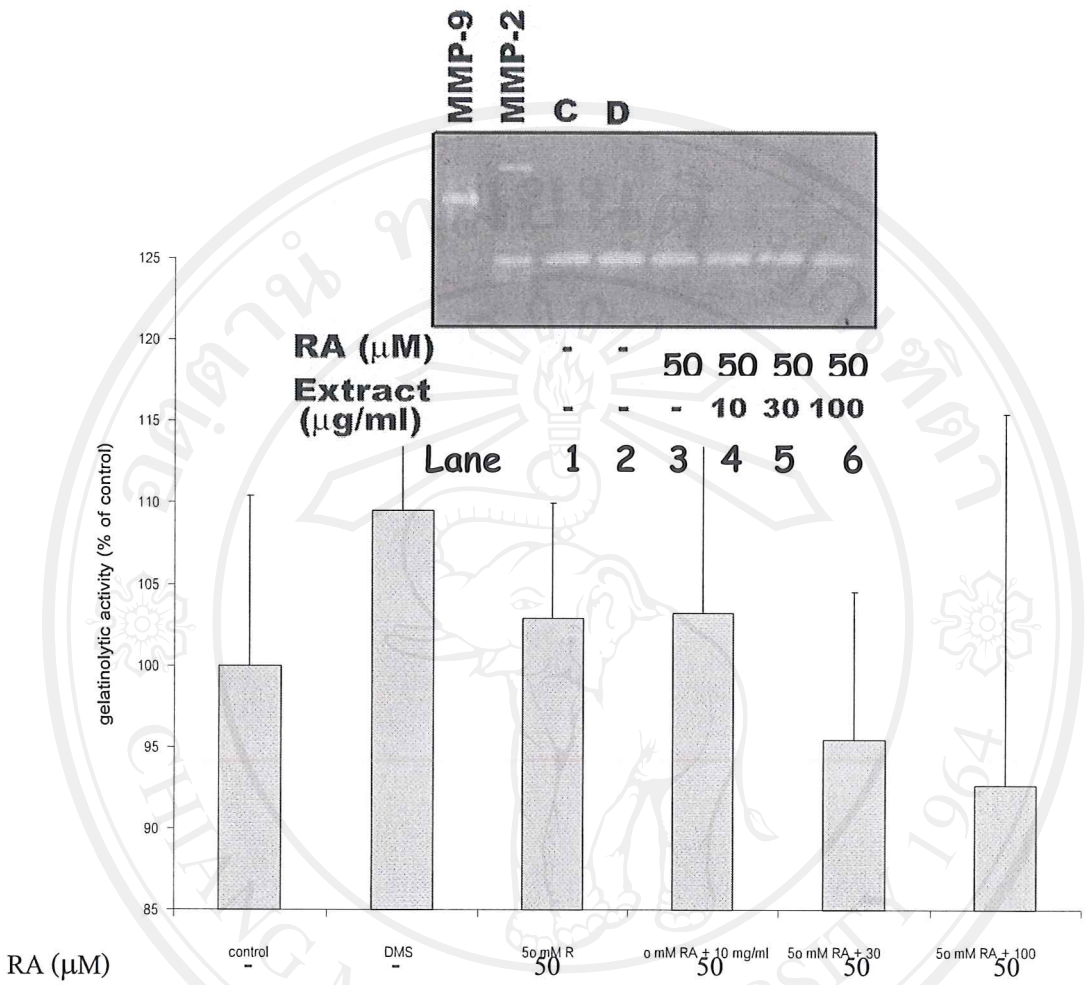
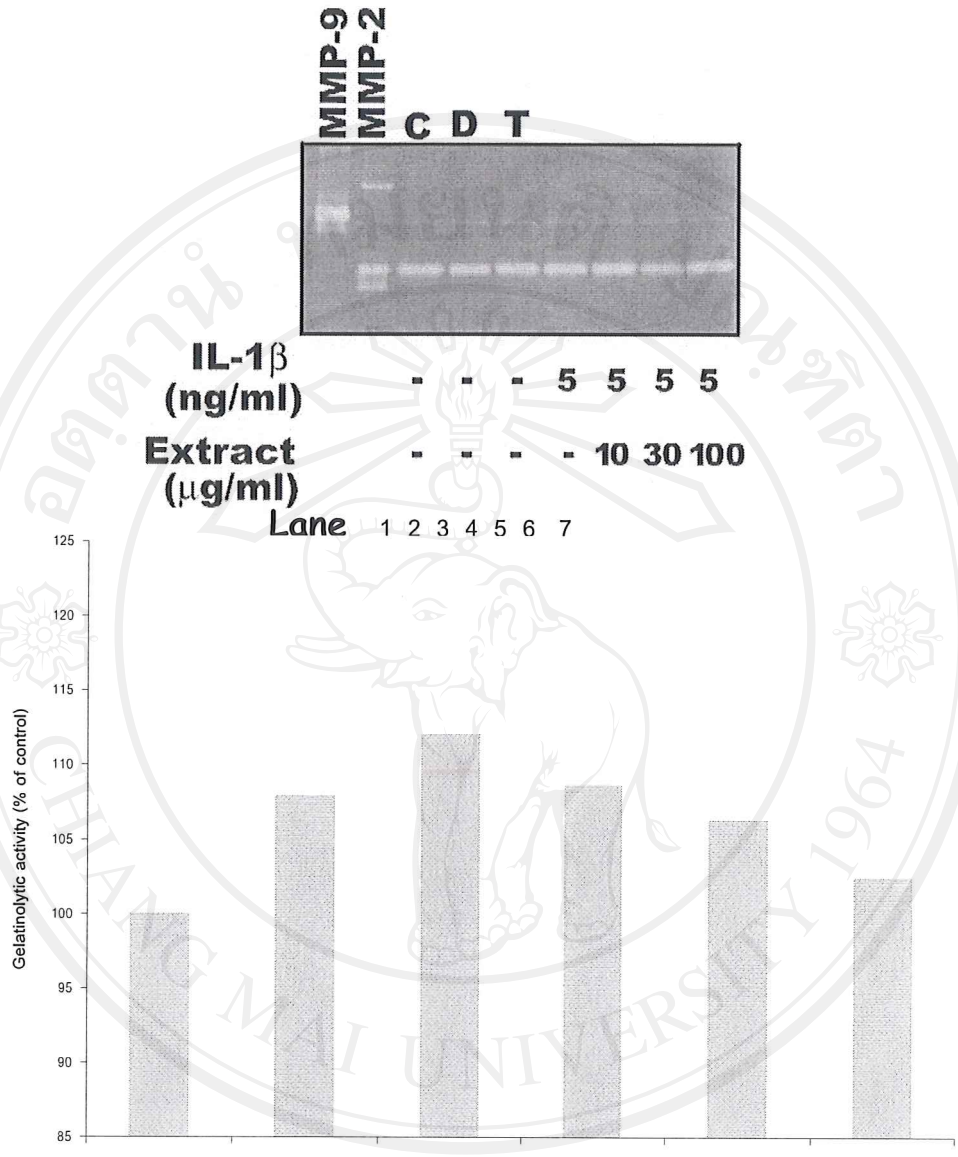


Figure 19. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with the extract was added at doses 10, 30, 100 μg/ml, control (untreated), solvent control (treated with DMSO), and 1 μg/ml TPA. Data shown are mean value ± standard deviation of triplicate assay per treatment.



RA (μM)	-	-	50	50	50	50
Extract(μg/ml)	-	-	-	10	30	100
Lane	1	2	3	4	5	6
	Control	DMSO				

Figure 20. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with 50 μM RA and the extract was added at doses 10, 30, 100 μg/ml, control (untreated), and solvent control (treated with DMSO), and 50 μM RA-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment.



IL-1β (ng/ml)	-	-	5	5	5	5
Extract(μg/ml)	-	-	-	10	30	100
Lane	1	2	3	4	5	6
	Control	DMSO				

Figure 21. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral fibroblast medium. Cells were treated with 5 ng/ml IL-1β and the extract was added at doses 10, 30, 100 μg/ml, control (untreated), and solvent control (treated with DMSO), and 5 ng/ml IL-1β-treated control. Data shown are mean value ± standard deviation of triplicate assay per treatment.

3.2.6 Effects of *Zingiber cassumunar* Roxb. extract, Retinoic acid (RA), Interleukin-1β (IL-1β), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the level of MMP-2, -9 from oral epithelial medium.

In this study, activity of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in the culture media were investigated by gelatin zymography. Culture media were electrophoresed on native, non-reducing, gelatin containing gels, which were subsequently stained with Coomassie blue; resolved gelatinolytic proteins were detected as unstained band. Oral epithelia culture in serum-free medium secreted high levels of gelatinase, which are pro-MMP-2 and pro-MMP-9, when compared to standard enzymes and markers at 72 kDa and 92 kDa respectively.

The results showed that ethanol extracted Plai significantly down regulated the production of MMP-2 and MMP-9 in the oral epithelia in dose dependent manner, as shown in Figure 22.

Treatment with RA, IL-1β, TPA showed significantly up regulation of the production of MMP-9 in oral epithelium media. Co-treatment of RA, IL-1β, TPA and ethanol extract exhibited down regulated the production of MMP-9 as compare with treated-RA, IL-1β, TPA and down regulated the production of MMP-2 as compare with untreated control as shown in Figures 23-25.

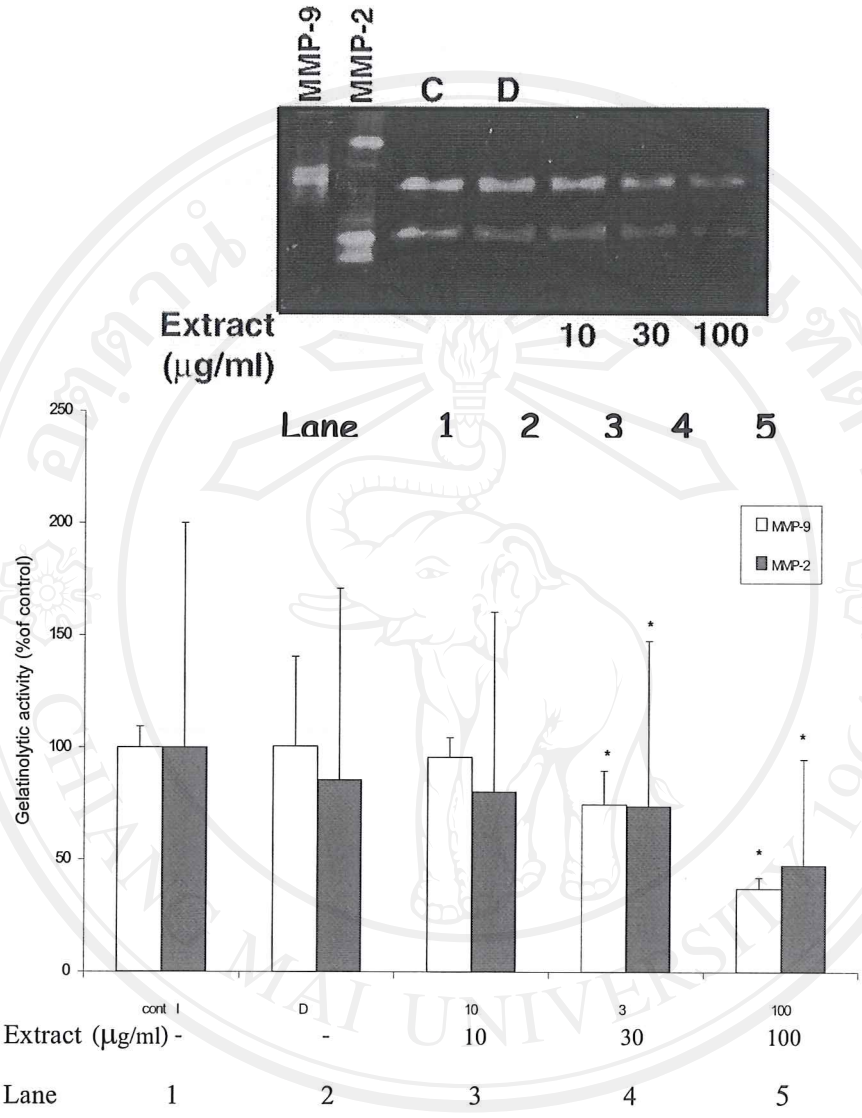


Figure 22. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with the extract at doses 10, 30, 100 μg/ml, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

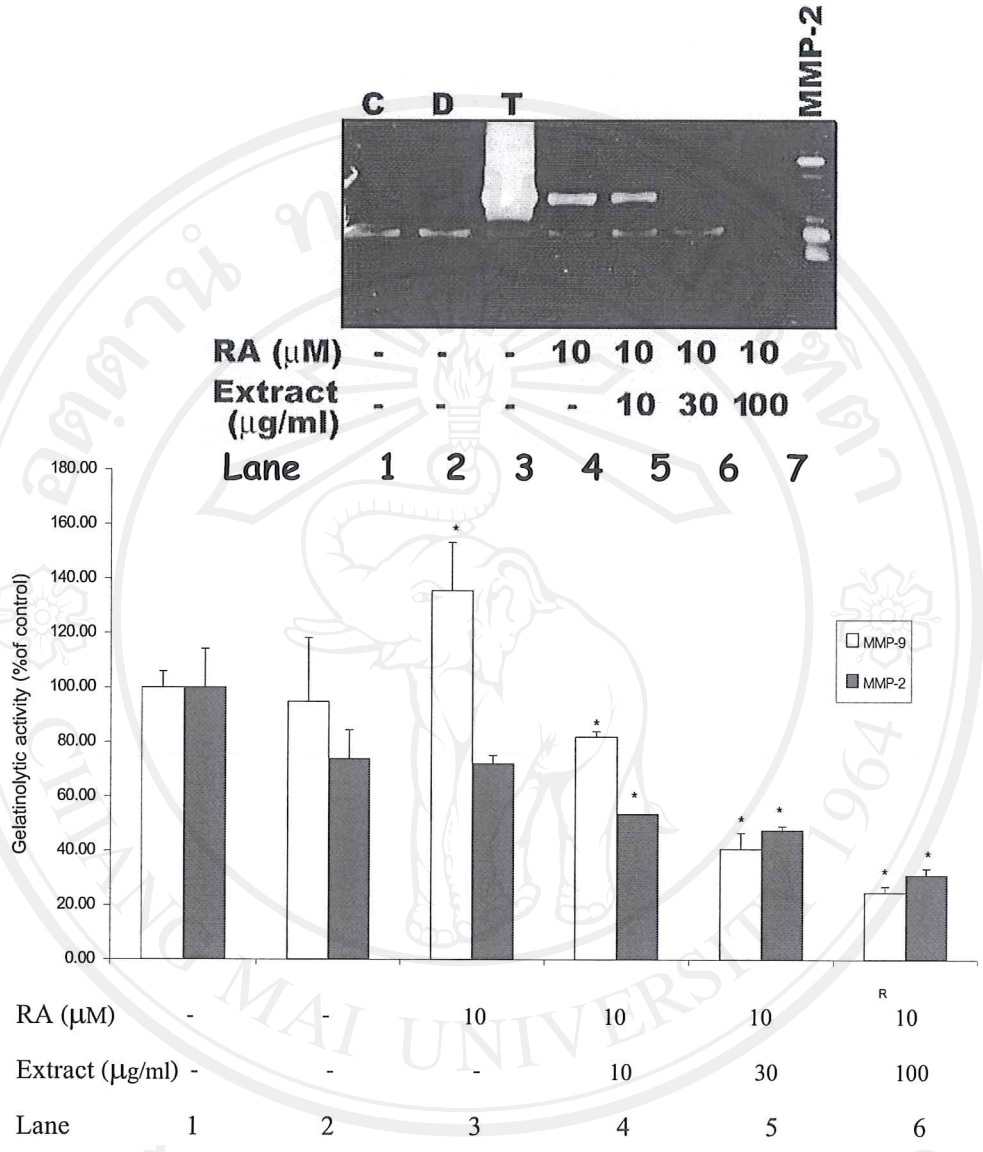


Figure 23. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 10 μM RA and the extract was added at doses 10, 30, 100 μg/ml, 10 μM RA, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

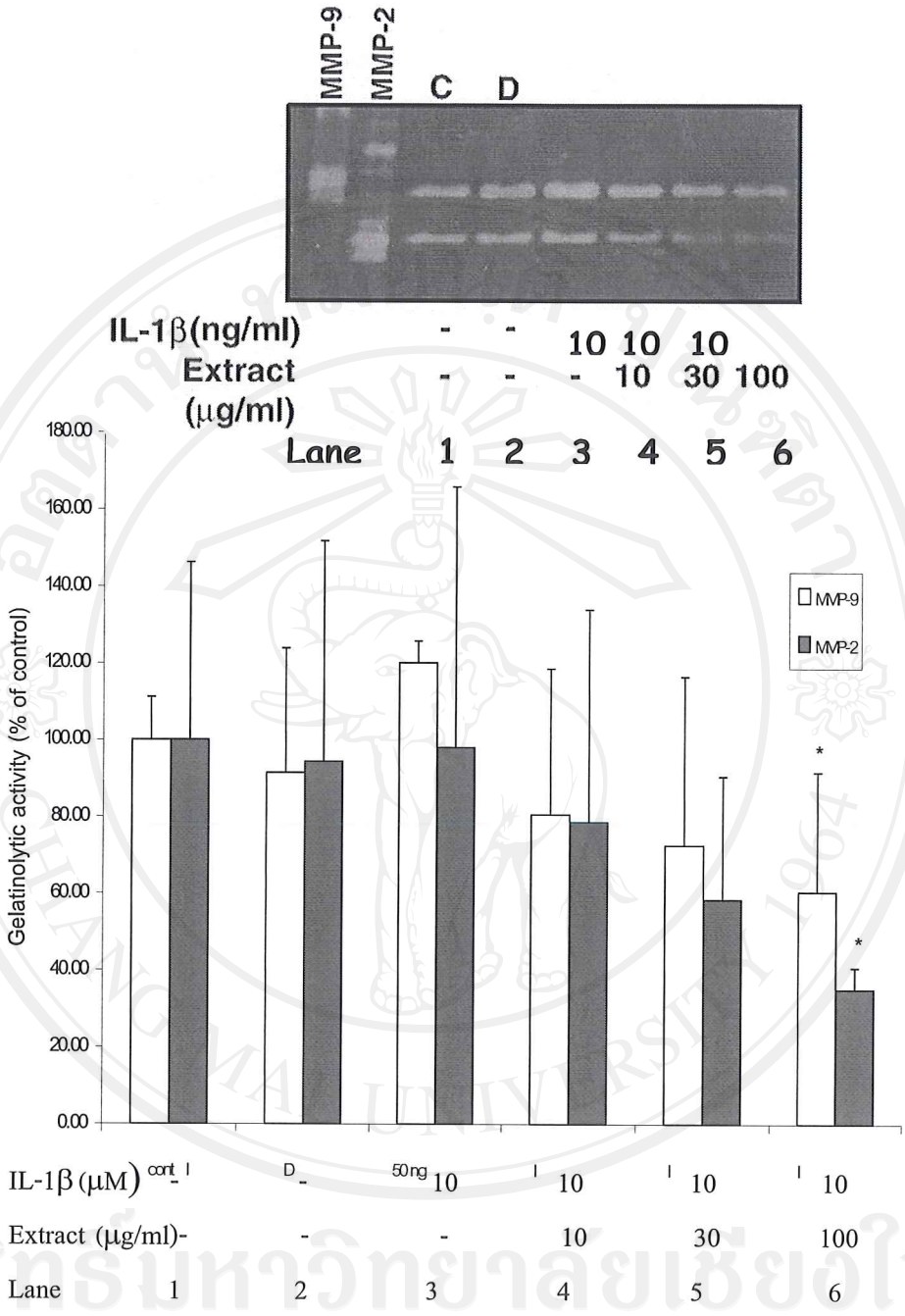


Figure 24. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 10 ng/ml IL-1β and the extract was added at doses 10, 30, 100 μg/ml, 10 ng/ml IL-1β-treated control, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

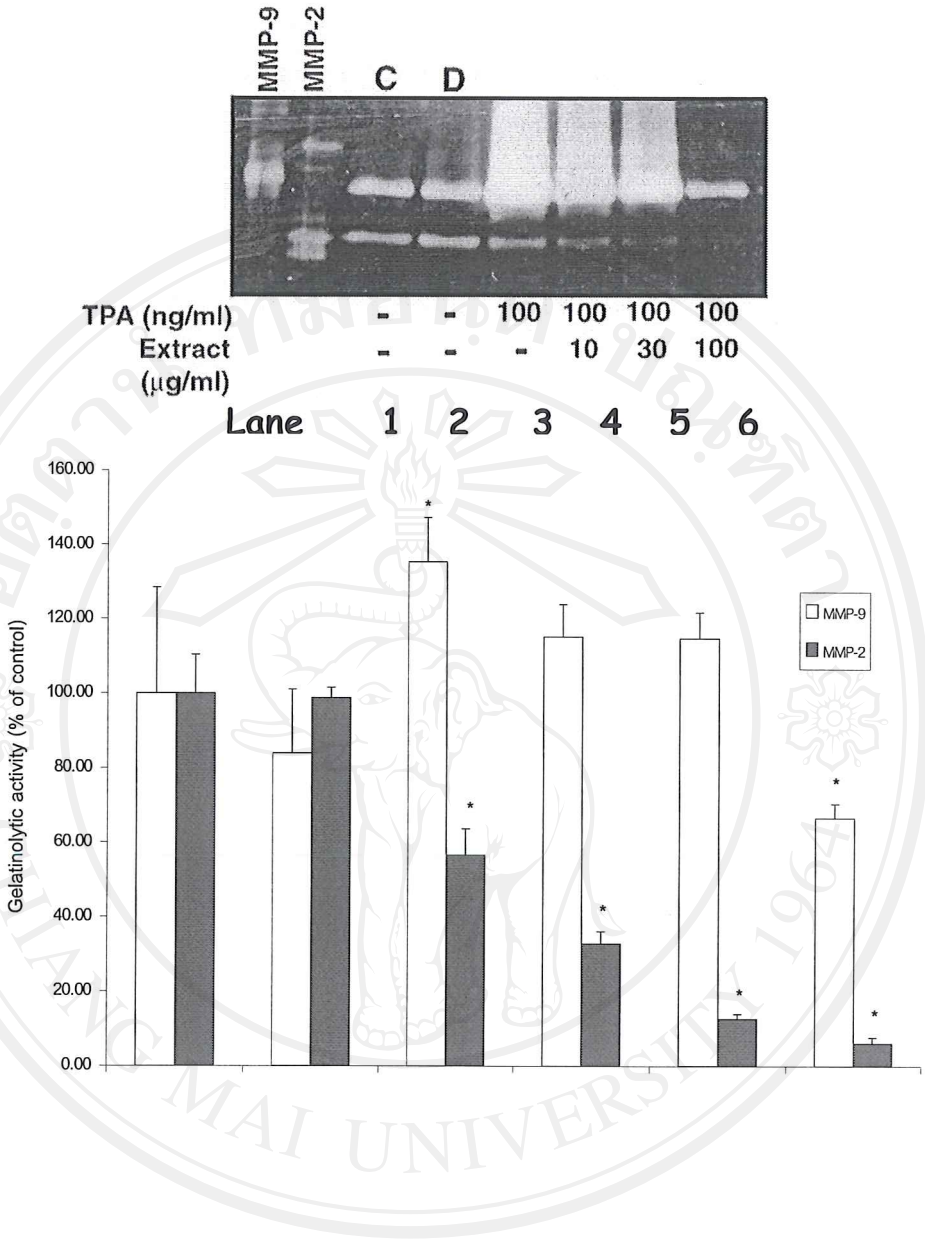


Figure 25. Inhibitory effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on gelatinolytic activity in oral epithelial medium. Cells were treated with 100 ng/ml TPA and the extract was added at doses 10, 30, 100 μg/ml, 100 ng/ml TPA-treated control, control (untreated), and solvent control (treated with DMSO). Data shown are mean value ± standard deviation of triplicate assay per treatment. * Denoted values that were significantly different from untreated control ($p < 0.05$).

3.3 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA) on cytotoxicity in oral fibroblast medium.

The cytotoxic of the extract to fibroblasts is base on the measurement of cytoplasmic enzyme activity releaesed by damaged cells. Oral fibroblasts were treated with RA at dose 0.1, 1.0, 10.0, 50.0 μM, or Plai ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, and/or combination between 10.0 μM RA and ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, 5.0 mM H_2O_2 overnight. The culture media were collected to determine the cytotoxicity by Berger-Broida assay. The results showed that the cytoplasmic enzyme activity released in treated culture media (RA, and Plai extract) were similar to the untreated control. The cytoplasmic enzyme activity released of the positive control was significantly increased by H_2O_2 (positive control) as showed in Figure 26.

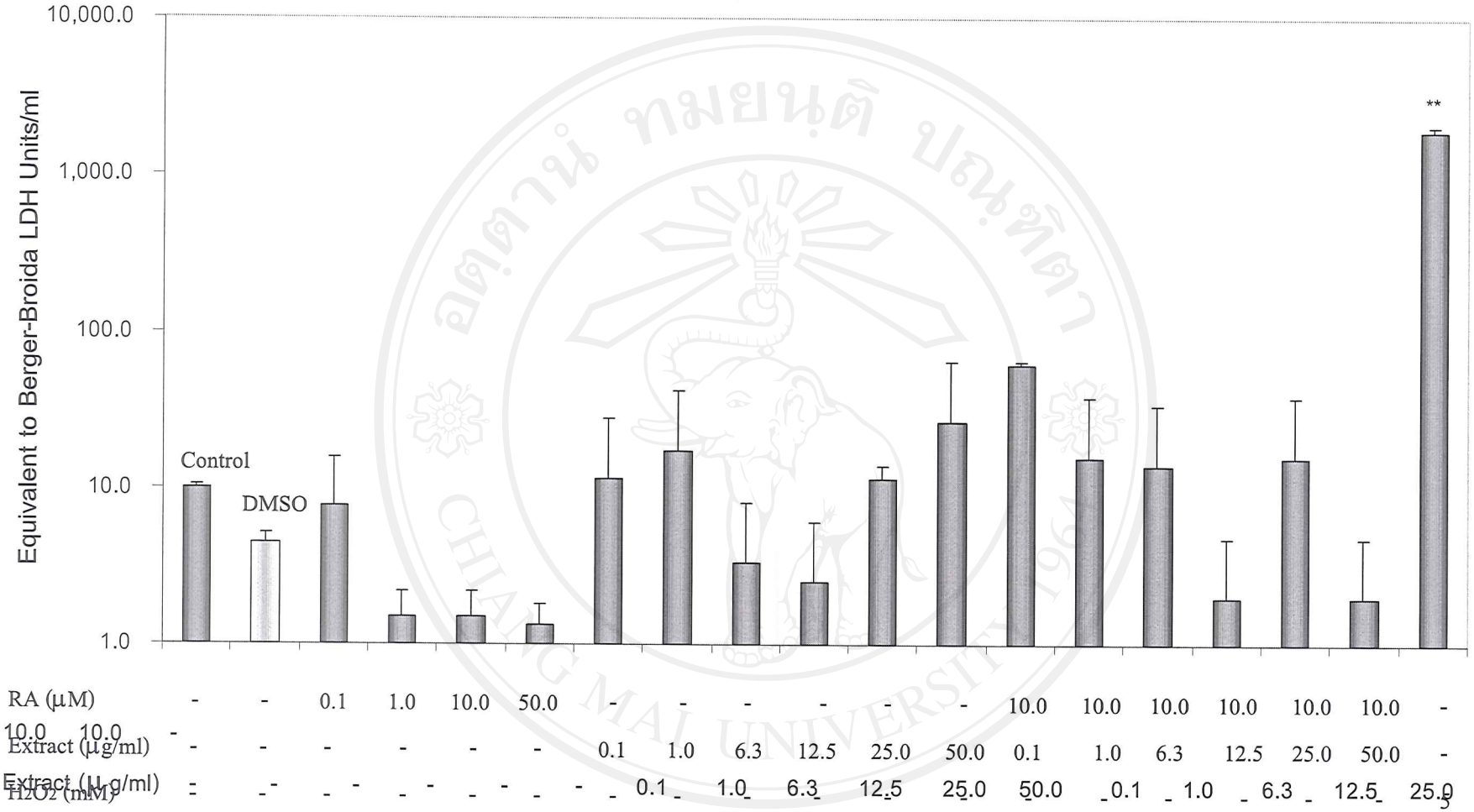


Figure 26. Effects of various concentrations of the ethanol extract of *Zingiber cassumunar* Roxb. on cytotoxicity in oral fibroblast medium. Cells were treated with 10 µM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 µg/ml, control (untreated), solvent control (treated with DMSO), and 0.1, 1, 10, 50 µM RA, 5.0 mM H_2O_2 (positive control). Data shown are mean value ± standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from untreated control, ($p<0.001$).

3.4 Effects of *Zingiber cassumunar* Roxb. extract and Retinoic acid (RA) on cytotoxicity in oral epithelial medium.

Oral epithelial cells were treated with RA at doses 0.1, 1.0, 10.0, 50.0 μM, ethanol extracted Plai at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, combination of 10.0 μM RA and ethanol extract at doses 0.1, 1.0, 6.3, 12.5, 25.0, 50.0 μg/ml, 5.0 mM H_2O_2 overnight. The culture media were collected to determine the cytotoxicity by Berger-Broida assay. The results showed that the cytoplasmic enzyme activity released of treated culture media (RA, and Plai extract) were similar to the untreated control. The cytoplasmic enzyme activity released in positive control was significantly increased by H_2O_2 (positive control) as shown in Figure 27.

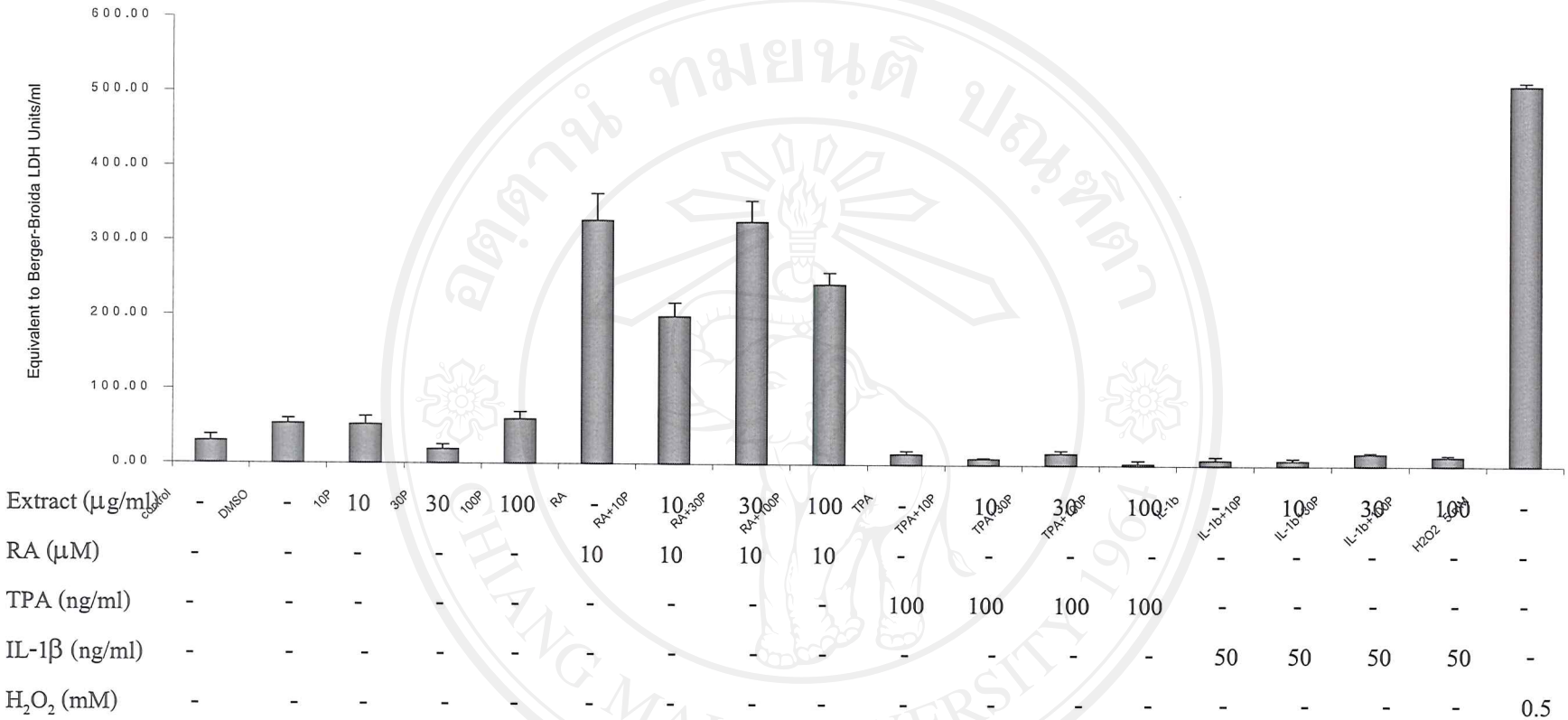


Figure 27. Effects of various concentrationsof the ethanol extract of *Zingiber cassumunar* Roxb. on cytotoxicity in oral epithelial medium. Cells were treated with 10 µM RA and the extract at doses 0.1, 1, 6.3, 12.5, 25, 50 µg/ml, control (untreated), solvent control (treated with DMSO), and 0.1, 1, 10, 50 µM RA. Data shown are mean value ± standard deviation of triplicate assay per treatment. ** Denoted values that were significantly different from untreated control, ($p<0.001$).

CHAPTER IV

Discussion

Wound healing is critical process for the organism. Numerous molecules appear to overlap each other's function. During wound healing, several molecules that are usually present only during embryonal development are found in the granulation tissue. Epithelial cells start to express extracellular matrix receptors that are not normally present in the resting epithelium. Fibroblastic cells with a special phenotype are found in the healing (29) (30). In this study, we found that phenotype of oral fibroblastic cells have different shape as compare to oral epithelial cells, and its produce HA high level. In previous studies found that periodonal fibroblasts produce HA (31) and six major proteoglycans, versican, a high-molecular-mass chondroitin sulphate proteoglycan (CSPG); decorin, a dermatan sulphate proteoglycan (DSPG); a membrane-associated heparan sulphate proteoglycan (HSPG); two medium- or matrix-associated HSPGs; and a 91 kDa membrane-associated CSPG (32). HA is a key component of chronic wound, also induces the production of a series of proinflammatory cytokines by fibroblasts, epithelium, cementoblasts and osteoblasts (33)(34).

The rapid production of HA by fibroblasts in the early stages of wound healing may be a crucial role as HA stimulates the migration and mitosis of mesenchymal and epithelial cells (4)(35). Increased levels of HA, as observed during fetal wound healing or as achieved by the topical application of HA during wound dressing, are associated with brisker healing and reduced scarring (36). Gingival epithelium contains hyaluronate, but there is little histochemical information about its localization (37), and the expression of extracellular matrix proteins and the metabolic activity of fibroblasts can be modulated by oral epithelial cells (38) (39)(40). Glucosamine availability appears to be rate-limiting for HA synthesis (41). Thus the administration of adequate amounts of glucosamine by mouth during the first few days after surgery or trauma can be expected to enhance HA production in the wound, promoting swifter healing and possibly diminishing complications related to scarring (42). HA has a multi-functional roles in the formation of some pathologic condition of connective tissue, such as inflammatory process and edema during wound healing. High concentrations of HA, particularly in fetal skin, have long been noted to be associated with rapid

healing with little scarring. It is postulated that HA is the extra cellular matrix (fluid between skin cells) that is the natural transportation system for the events of wound healing (inflammatory cell migration, fibroblast cell migration, cytokine migration and epithelial cell migration) to smoothly occur. HA always seems to surround proliferating and migrating cells in regenerating, remodeling, or healing tissues (43). HA found in ECM of oral connective tissue is mainly produced by oral fibroblasts. The wound tissue in the early inflammatory phase of repair is rich in HA within inflamed sites. HA production from oral fibroblasts were decreased by the ethanol extract of *Zingiber cassumunar* Roxb., this results indicate that the ethanol extract can inhibit the production of a series of proinflammatory cytokines by fibroblasts and epithelium by HA induction. This suggests that this extract may reduce tissue hydration (HA) and swelling during wound healing in oral inflammatory disorders, consistent with its effects found *in vivo*.

Zingiber cassumunar Roxb. (Plai) was traditionally used for relieving edema and inflammation at the wound healing site in alternative medicine. (E)-1-(3,4-Dimethoxyphenyl) butadiene (DMPBD, compound D) (43)(44) is an active ingredient of the essential oil derived from the rhizome of Plai by Thailand Institute of Scientific and Technological Research (TISTR) (23). Its potential anti-inflammatory and anti-edematous effects led to investigate its ability to the release of ECM biomolecules, such as HA, sulfated-GAG and MMP-2, 9 by using an *in vitro* culture model (45)(46). The result of three extract fractions of Plai, hexane, ethanol and water, we found that hexane extract seemed to potent effect to oral fibroblasts and epithelium than ethanol and water extracts. The major component isolated from the hexane extract is Compound D, showed a strong inhibitory activity on the edema formation in carrageenan-induced rat paw edema (47)(48).

In this study, the results showed the decreases of the HA release in culture media from oral fibroblasts by Plai extract. The Plai hexane extract showed less HA in culture medium than the ethanol extract in the same concentration. From oral epithelia, the hexane extract showed the more level of HA release than the ethanol extract. The hexane extract seemed to possess a potent anti-inflammatory activity which the major component is compound D, exhibited a strong inhibitory activity on the edema formation in carrageenan-induced rat paw edema. The decreasing of HA in oral

fibroblast culture media, exhibited the inhibition during inflammatory phase, and the increasing of HA in oral epithelium media showed the rapidly re-epithelialization and granulation tissue formation in wound healing process.

TPA or 12-O-tetradecanoyl-phorbol-13-acetate, as a well-known phorbol ester, induced cutaneous oxidative stress and toxicity in murine skin and the increasing of inflammation in skin. Retinoids or vitamin A have long been associated with wound healing. Vitamin A deficiency retards repair, and retinoids restore steroid-retarded repair toward normal. Because vitamin A tends to suppress fibroblasts in cell culture and stimulate steroid-treated macrophages to initiate reparative behavior in tissue, the researcher favor the hypothesis that retinoids are particularly important in macrophagic inflammation, which plays a central role in the control of wound healing (49). Probably all patients who take anti-inflammatory steroids should control their retinoid intake, but how they should control it is as yet unknown. Profound metabolic changes take place in keratinizing epithelia in the presence of retinoic acid. *In vivo* as well as *in vitro* the proliferative activity of epidermal cells is greatly enhanced. Together with the increased rate of new cell production cellular differentiation (keratinization and cornification) is also altered. The effects are species-unspecific, probably tissue-specific and dose-dependent. The precise action of retinoic acid, however, still remains unknown.

IL-1β, a pro-inflammatory cytokine produced by several cell types, including endothelial cell, synoviocyte, and chondrocyte. Stimulation of IL-1β has been shown the wide spread matrix degradation, including loss of tissue proteoglycan (PGs) and collagens. Increasing of the gelatinase activity in the culture media treated with IL-1β also indicated the effect of IL-1β on the elevation of MMPs activities (50). These results were in agreement with the previous studies reporting that IL-1β played a major role in the pathology of cartilage degeneration by stimulation of MMP activity and consecutive matrix component degradation.

The mechanism of IL-1β and RA induced ECM degradation involves the release of matrix metalloproteinase and other degradative products. In agreement with this study both IL-1β and RA induce the release of MMP, both MMP-2 and MMP-9 into culture media. MMPs are the key enzyme in various diseases that collectively degrade all the components of the ECM. The role of MMP in the pathological destruction of tissue is promoted by various pro-inflammatory cytokines

that perturb the balance between synthesis and degradation of ECM components to favor matrix breakdown. Proteoglycan loss is a rapid event following pro-inflammatory stimulation but it can be readily replaced once the stimulus is removed. Collagen is more resistant to degradation but is much more difficult to replace. The gelatinases (MMP-2 and MMP-9) is inhibited by the *Zingiber cassumunar* extract in oral epithelial cells. This result demonstrate that the *Zingiber cassumunar* extract may involve in periodontitis by suppression of degradation of MMPs activity

These results demonstrate the ability of *Zingiber cassumunar* Roxb. ethanol extract to inhibit HA production from oral fibroblasts, and inhibit the gelatinase activity from stimulated oral epithelial cells, corresponding with its potent anti-inflammatory activity. The findings from this *in vitro* study will be essential for future development of a new drug in the management of inflammatory oral diseases.

Further studies

The results show the high potent of anti-inflammation in Plai treatment so, the further study should be the purification and investigate the active compound in hexane and ethanol extract whether they contain the wound healing activity, including the investigation in animal model and clinical trial should be done.

CONCLUSION

Zingiber cassumunar Roxb. or Plai extract has a potent anti-inflammation activity in wound healing process. Therefore, it is possible to use this compound as a new pharmacological agent for the management of oral diseases. However, further investigation in animal model and clinical trial should be done, including the structural characterization of active compound.

REFERENCES

1. Clark RA. (1995) Wound repair——overview and general considerations. In: Clark RA, editor. The molecular and cellular biology of wound repair. New York: Plenum Press: 3—50.
2. Heldin C-H, Westermark B. (1996) Role of platelet-derived growth factor *in vivo*. In: Clark RAF, ed. The molecular and cellular biology of wound repair. 2nd ed. New York: Plenum Press: 249-73.
3. McCarty MF. (1996) Glucosamine for wound healing. *Med Hypotheses*. 47(4): 273-5.
4. Oksala O, Salo T, Tammi R, Hakkinen L, Jalkanen M, Inki P, et al. (1995) Expression of proteoglycans and hyaluronan during wound healing. *J Histochem Cytochem*. 43: 125-35.
5. Thomas N W. Extracellular Matrix. 1 ed. London: Harwood academic publishers, 1996.
6. Ehrlich HP, Krummel TM. (1996) Regulation of wound healing from a connective tissue perspective. *Wound Rep Reg*. 4: 203—10.
7. Agren MS. (1999) Matrix metalloproteinases (MMPs) are required for re-epithelialization of cutaneous wounds. *Arch Dermatol Res*. 291: 583—590.
8. Hakkinen L, Uitto V, and Larjava H. (2000) Cell biology of gingival wound healing. *Periodontology*. 24: 127-152.
9. Longaker M. T. and Adzick N. S. (1991) The biology of fetal wound healing: a review. *Plast. Reconstr. Surg.* 87: 788-798.
10. Adam J. Singer, M.D., and Richard A.F. Clark, M.D. (1999)Cutaneous wound healing: Review article. *The New England J of Medicine*. 431: 738-741.
11. Riches DWH (1996). Macrophage involvement in wound repair, remodeling, and fibrosis. In: Clark RAF, editor. The molecular and cellular biology of wound repair. 2nd ed. New York: Plenum Press: 95-141.
12. P. Daniel Ward, Susan L. Thibeault, and Steven D. Gray (2002). Hyaluronic Acid: Its Role in Voice. *Journal of Voice*. 16(3): 303-309.
13. Lovvorn HN 3rd, Cass DL, Sylvester KG, Yang EY, Crombleholme TM, Adzick NS, Savani RC. (1998) Hyaluronan receptor expression increases in fetal excisional skin wounds and correlates with fibroplasia. *J Pediatr Surg*. 33(7):1062-9; discussion 1069-70.

14. Pogrel MA, Lowe M, and Stern R. (1996) Hyaluronan (Hyaluronic acid) in Human Saliva. *Archs oral Biol*. 41(7): 667-671.

15. Plaas AH, West LA, Wong-Palms S, Nelson FR. (1998) Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* ; 273(20): 12642-12649.

16. Ravanti L, Hakkinen L, Larjava H, Saarialho-Kere U, Foschi M, Han J, and Kahari V. (1999) Transforming Growth Factor-b Induces Collagenase-3 Expression by Human Gingival Fibroblasts via p38 Mitogen-activated Protein Kinase. *J Biol Chem*. 274(52): 37292–37300.

17. Mark D S, Zena W (1999). Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins. 2 ed. New York: Oxford University Press Inc.

18. Martel-P J, Welsch DJ, Pelletier JP. (2001). Metalloproteinase and inhibitors in arthritic diseases. *Best Practic and Research Clinical Rheumatology*. 15(5), 805-829.

19. Nagase H. (1997) Activation mechanisms of matrix metalloproteinases. *Biological Chemistry*. 378, 151-160.

20. Matrix metalloproteinase in arthritic disease. *Arthritis Research*; 4 (l3); 39-49. 2002.

21. Faculty of Pharmacy Mahidol University. Specification of Thai Medicinal Plants. Bangkok: Aksornsampan Press, 2004.

22. Habsah M, Amran M, Mackeen MM, Lajis NH, Kikuzaki H, Nakatani N et al. (2000). Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. *Journal of Ethnopharmacology*. 72(3): 403-410.

23. Jeenapongsa R, Yoovathaworn K, Sriwatanakul KM, Pongprayoon U, and Sriwatanakul K. (2003) Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl)butadiene from *Zingiber cassumunar* Roxb. *J Ethnopharmacol*. 87: 143-148.

24. Ozaki, Y., N. Kawahara, et al. (1991). Anti-inflammatory effect of *Zingiber cassumunar* Roxb. and its active principles. *Chemical & Pharmaceutical Bulletin* 39(9): 2353-2356.

25. Jitoe, A., T. Masuda, et al. (1994). Novel antioxidants, cassumunarin A, B, and C, from *Zingiber cassumunar*. *Tetrahedron Letters* 35(7): 981-984.

26. Piromrat, K., M. Tuchinda, et al. (1986). Antihistaminic effect of "Plai" (*Zingiber cassumunar* Roxb.) on histamine skin test in asthmatic children. Siriraj Hospital Gazette. 38(4): 251-256.

27. Kishore, N. and R. S. Dwivedi. (1992) Zerumbone: A potential fungitoxic agent isolated from *Zingiber cassumunar* Roxb. *Mycopathologia*. 120(3): 155-159.

28. Lymprasertkul A. R&D Newsletter : Plai (*Zingiber cassumunar* Roxb.). 1999.

29. Embery, G., Oliver, W. M., Stanbury, J. B. (1979) The metabolism of proteoglycans and glycosaminoglycans in inflamed human gingiva. *J Peridont. Res*. 14: 512-519.

30. Bartold, P. M., Page, R. C. (1986) The effect of chronic inflammation on gingival connective tissue proteoglycans and hyaluronic acid. *J Oral Pathol*. 15: 367-374.

31. Tammi R, Tammi M, Hakkinen L, and Larjava H. (1990) Histochemical localization of hyaluronate in human oral epithelium using a specific hyaluronate-binding probe. *Arch Oral Biol*. 35(3): 219-224.

32. Hashimoto-Uoshima M, Hascall VC.MacCallum DK, and Yanagishita M. (1995) Biosynthesis of Proteoglycans and Hyaluronic Acid by Rat Oral Epithelial Cells (Keratinocytes) *in vitro*. *Arch Biochem Biophys*. 316(2): 724-732.

33. Van Beurden et al. (2003) Fibroblast subpopulations in intra-oral wound healing. *Wound Repair and Regeneration*. 11(1): 55-63.

34. Laurent, T. C. (ed.). In: The chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives (1998). *Wenner-Gren International Series*, volume 72. Portland Press, London.

35. Chen, W. Y., Abatangelo, G.(1999) Functions of hyaluronan in wound repair. *Wound Rep. Reg*. 7: 79-89.

36. Meyer K. (1958) Chemical structure of hyaluronic acid. *Fedn. Proc.* 17: 1075-1078.

37. Tammi R, Tammi M, Hakkinen L, Larjava H. (1990) Histochemical localization of hyaluronate in human oral epithelium using a specific hyaluronate-binding probe. *Arch Oral Biol*;35(3):219-24.

38. Hou LT, Kollar EJ, Yaeger JA. (1992) Epithelial cell-fibroblast interactions: modulation of extracellular matrix proteins in cultured oral cells. *Biochem J.* 15;284 (Pt 1):267-74.

39. Nakamura T, Sakamoto A, and Ishikawa T. (2001) Effects of hyaluronan on matrix metalloproteinase activity in skin fibroblasts. *Inter Congress Series*. 1223: 259-263.

40. Croce MA, Dyne K, Boraldi F, Quaglino Jr D, Cetta G, Tiozzo R, and I. Ronchetti IP. (2001) Hyaluronan affects protein and collagen synthesis by *in vitro* human skin fibroblasts. *Tissue & Cell.* 33(4): 326-331.

41. Isnard N, Legeais J-M, Renarb G, Robert L. (2001) Effect of Hyaluronan on MMP Expression and Activation. *Cell Biol Inter.* 25(8): 735-739.Paul H. Weigel. Ph. D. Bacterial Hyaluronan Synthases. Biochemistry and Molecular Biology. *Oklahoma Health Sciences Center University.* 1998.

42. Nagano, T., Y. Oyama, et al. (1997). New curcuminoids isolated from *Zingiber cassumunar* protect cells suffering from oxidative stress: A flow-cytometric study using rat thymocytes and H2O2. *Japanese Journal of Pharmacology.* 75(4): 363-370.

43. Amastayakul T, et al. (1979) Chemistry and crystal structures of some constituents of *Zingiber cassumunar*. *Aust J Chem.* 32: 71-88.

44. Kuroyanagi M, et al. (1980) Further characterization of the constituents of a Thai medicinal plant, *Zingiber cassumunar* Roxb. *Chem Pharm Bull.* 28(10): 2948-2959.

45. Masuda, T. and A. Jitoe (1995). Phenylbutenoid monomers from the rhizomes of *Zingiber cassumunar*. *Phytochemistry Oxford* 39(2): 459-461.

46. Isacke CM and Yarwood H. (2002) The hyaluronan receptor, CD44. *IJBCB.* 34: 718-721.

47. Pongprayoon U, Tuchinda P, Claeson P, et al. (1997) Topical antiinflammatory activity of DMPBD, a phenylbutanoid from Zingiber cassumunar. Part 2 hexane extractives. *Phytomedicine.* 3[4], 323-326.

48. Pongprayoon U, Soontornsaratune P, Jarikasem S, et al. Topical antiinflammatory activity of the major lipophilic constitients of the rhizome of Zingiber cassumunar. Part 1 the essential oils. *Phytomedicine.* 3[4], 319-322. 1997.

49. Dingle JT, Horsfield P, Fell HB, Barratt ME. (1975) Breakdown of proteoglycan and collagen induced in pig articular cartilage in organ culture. *Ann Rheum Dis.* 34(4):303-311.

50. Abramson SB, Amin A. Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. *Rheumatology* (Oxford).41(9):972-980.

51. J. Frederick Woessner, JR. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* 5: 2145-2154.

52. Farndale RW, Buttle DJ, Barrett AJ. (1986) Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. *Biochem Biophys Acta*. 883(2): 173-177.

53. Pothacharoen P. The Quantitative analysis of Chondroitin Sulfate Epitope and hyaluronan as Diagnostic Markers for Degenerative Joint diseases by ELISA technique. Chiangmai University, 2000.

54. Lindy O, Konttinen YT, Sorsa T, Ding Y, Santavirta S, C eponis A, *et al.* (1997) Matrix metalloproteinase 13 (collagenase-3) in human rheumatoid synovium. *Arthritis Rheum*. 40:1391—9.

55. Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J, Kinne RW, Santavirta S, Sorsa T, Lopez-Otin C, and Takagi M. (1999) Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. 58: 691-697.

56. Clegg PD, Burke RM, Coughlan AR, Riggs CM, Carter SD. (1997) Characterization of equine matrix metalloproteinase 2 and 9; and identification of the cellular sources of these enzymes in joints. *Equine Vet J*. 29(5): 335-342.

57. Sigma, Technical Bulletin No. 500. The colorimetric determination of Lactate Dehydrogenase by L. Berger and D. Broida, Reissue 1962.

58. Chiwongsa R. Effect of Plai (*Zingiber cassumunar* Roxb.) extracts on hyaluronan and proteoglycan degradation of cartilage explants. Chiangmai University, 2004.

APPENDIX

Preperation of Plai (*Zingiber cassumunar* Roxb.) extracts (57)

Fresh rhizome of Plai were cut into pieces, dried at 50-60° C and ground. Dried powder of Plai 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 evaporater, 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. However the HPLC fingerprint of each extract was recorded for further reference.

The HPLC system for isocratic elution

Column : Apollo C18 5 μ, 250 X 4.6 mm

Guard column : Apollo C18 5 μ, 7.5 X 4.6 mm

Mobile phase : 50% acetonitrile

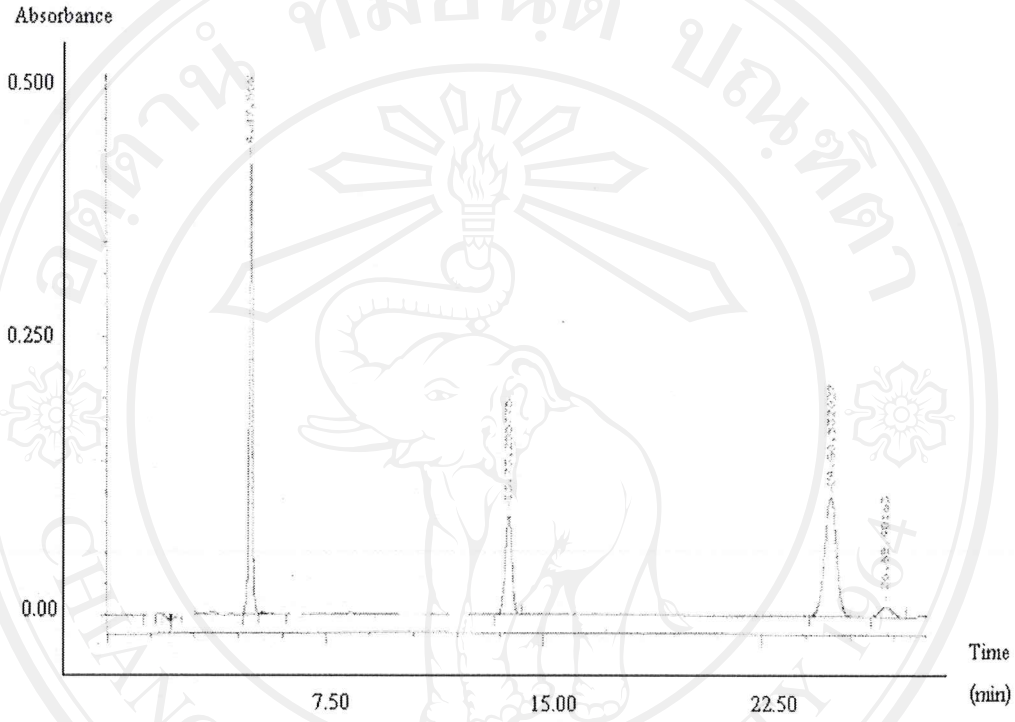
Flow rate: 1.0 ml/min

Injection volume: 10 μl

Run time : 30 min

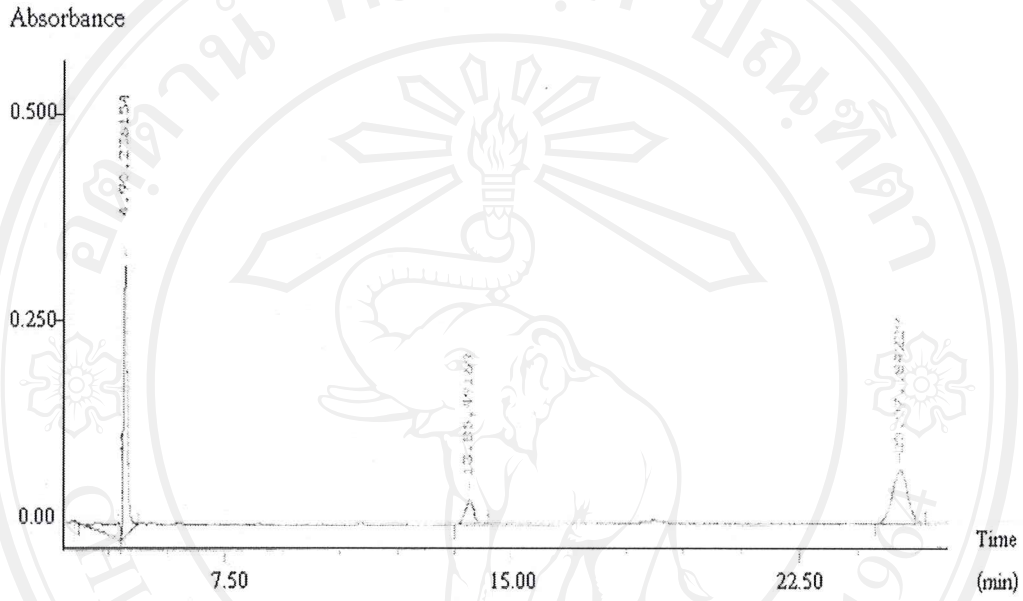
Detection : 267 nm

HPLC chromatogram of hexane extracted Plai. (57)



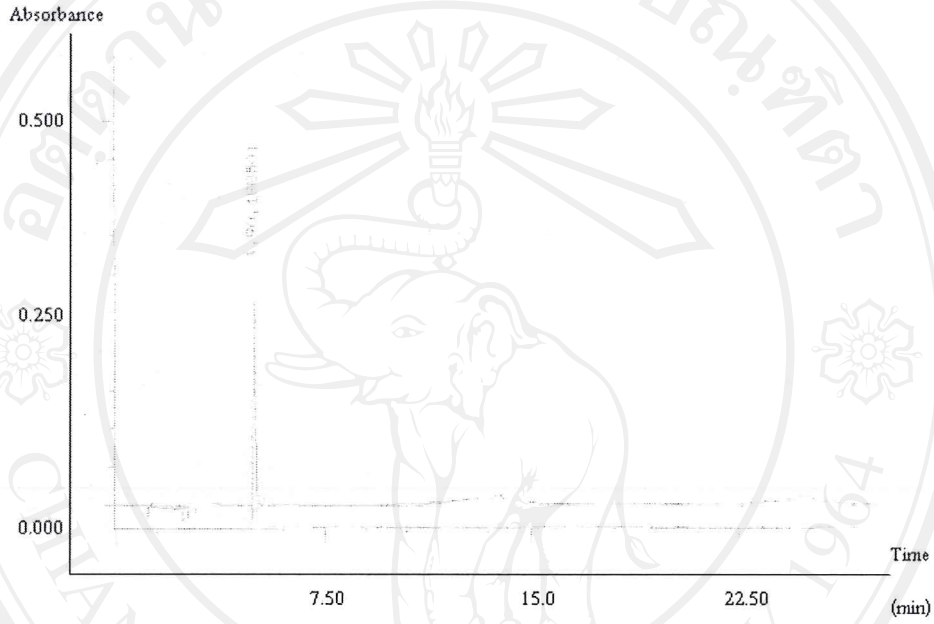
HPLC fingerprint of Hexane extrct analysed by the HPLC system for isocretic elution, with 50% acetonitrile mobile phase, Apollo C18 5 u, 250 X 4.6 mm column and 1.0 ml/min flow rate.

HPLC chromatogram of 70% ethanol extracted Plai. (57)



HPLC fingerprint of 70%Ethanol extrct analysed by the HPLC system for isocretic elution, with 50% acetonitrile mobile phase, Apollo C18 5 u, 250 X 4.6 mm column and 1.0 ml/min flow rate.

HPLC chromatogram of water extracted Plai. (57)



HPLC fingerprint of water extrct analysed by the HPLC system for isocretic elution, with 50% acetonitrile mobile phase, Apollo C18 5 u, 250 X 4.6 mm column and 1.0 ml/min flow rate.

Percentage of composition from Plai extracts analysed by HPLC analysis. (57)

	Peak 1 (RT= 4.9)	Peak 2 (RT= 13.7)	Peak 3 (RT= 24.8)	Peak 4 (RT= 26.7)
Hexane	40.41%	18.23%	37.92%	3.433%
70% Ethanol	50.29%	10.47%	39.23%	-
Water	100%	-	-	-

Reagents and buffers preparation

Enzyme-linked immunosorbent assay

1. **Phosphate buffer saline (PBS)**

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.44	g
$Na_2HPO_4.2H_2O$	0.24	g

All chemicals were dissolved in 900 ml of distilled water, adjusted to pH 7.4 and then added with distilled water to adjust to the volume 1 L. Stored at room temperature. For PBS-Tween, Tween-20 was added to 0.05%.

2. **Tris-Incubating buffer**

Tris-HCl	1.21	g
NaCl	8.77	g

All chemicals were dissolved in 900 ml of distilled water, adjusted to pH 7.4 and then added distilled water to adjust to the volume 1 L. Added 0.5 g BSA and 1,000 µl Tween-20. Stored the reagent at 4 ^{0}C.

3. **Coating buffer (for HA)**

$NaHCO_3$	8.4	g

The chemical was dissolved in 900 ml of distilled water, adjusted to pH 7.4 and then added with distilled water to adjust to the volume 1L.

4. **Coating buffer (foe WF6)**

$NaHCO_3$	1.0559	g

The chemical was dissolved in 400 ml of distilled water, adjusted to pH 9.6 and then added with distilled water to adjust to the volume 500 ml.

5. 1% Bovine serum albumin (BSA)

BSA	0.1	g
PBS	10	ml

6. Citrate phosphate buffer

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

The chemicals were dissolved in 900 ml of distilled water, adjusted to pH 5.0 and made up to the volume 1 L. Stored the reagent at 4^0C.

7. Substrate solution

OPD	8	mg
Citrate phosphate buffer	12	ml
35% H_2O_2	7	µl

Prepared the reagent fresh for 1 plate; kept in the dark before used.

Sulfated-GAG assay

1. DMMB Dye

Glycine	1.52	g
NaCl	1.1850	g

All chemicals were dissolved in 400 ml of deionized distilled water, adjusted to pH 3.0. Add 0.0080 g dimethylmethylene blue and made up to a volume 500 ml. Store the reagent at room temperature.

Gelatin Zymography

1. Acrylamide/Bis solution

Acrylamide	29.2	g
N'N'-bis-methylene acrylamide	0.8	g

All chemicals were dissolved in deionized distilled water, made up to the volume 100 ml. Filtered through a membrane filter pore size 0.45 µm, collected in a dark bottle. Stored the reagent at 4^0C.

2. Separating gel buffer 1.5 M Tris-HCl, pH 8.8

Tris-base	18.15	g

The chemical was dissolved in 80 ml of deionized distilled water, adjusted to pH 8.8 and made up to the volume 100 ml. Stored the reagent at 4^0C.

3. Stacking gel buffer 0.5 M Tris-HCl, pH 6.8

Tris-base	6.0	g

The chemical was dissolved in 80 ml of deionized distilled water, adjusted to pH 6.8 and made up to the volume 100 ml. Stored the reagent at 4^0C.

4. 10% SDS solution

SDS	10	g

The chemical was dissolved in deionized distilled water, made up to the volume 100 ml.

5. 20% Ammonium persulfate solution

Ammonium persulfate	0.2	g
Deionized distilled water	1.0	ml

6. 0.1% Gelatin solution

Gelatin type B	0.01	g
Deionized distilled water	1.0	ml

7. 2X sample buffer

0.5 M Tris-HCl, pH 6.8	2.5	ml
Glycerol	2.0	ml
10% SDS	4.0	ml
0.1% Bromophenol blue	0.5	ml
Deionized distilled water	1.0	ml

8. 10X Running Buffer, pH 8.3

Tris-base	30.3	g
Glycine	144	g
SDS	10	g

All chemicals were dissolved in deionized distilled water, made up volume to 1,000 ml. Stored reagent at 4 ^{0}C.

9. Running buffer

10X Running Buffer	100	ml
Deionize distilled water	900	ml

10. 2.5 % Triton X-100

Triton X-100	25	ml

The chemical was dissolved in deionized distilled water, made up to the volume 1 L. Filtered through a membrane filter pore size 0.45 μm. Stored the reagent at 4 ^{0}C.

11. Activating buffer

Tris-HCl	6.06	g
$CaCl_2$	1.47	g
NaCl	2.92	g

All chemicals were dissolved in 800 ml of deionized distilled water, adjusted to pH 7.6. Added with 500 μl of Brij35, and made up to the volume 1 L. Filtered through a membrane filter pore size 0.45 μm. Stored the reagent at 4 ^{0}C.

12. Coomassie Brilliant blue G250

Coomassie Brilliant blue G250	1	g
Methanol	50	ml
Acetic acid	10	ml
Deionized distilled water	40	ml

13. Coomassie Brilliant blue destaining solution

Methanol	50	ml
Acetic acid	10	ml
Deionized distilled water	40	ml

14. 10% Seperating gelatin gel

Deionized distilled water	1.95	ml
Acrylamide/Bis solution	3.3	ml
Separating gel buffer	3.75	ml
10%SDS	100	µl
20%APS	100	µl
Gelatin solution	1	ml
TEMED	20	µl

15. 4% Stacking gel

Deionized distilled water	6.10	ml
Acrylamide/Bis solution	1.3	ml
Separating gel buffer	2.5	ml
10%SDS	100	µl
20%APS	100	µl
TEMED	20	µl