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.

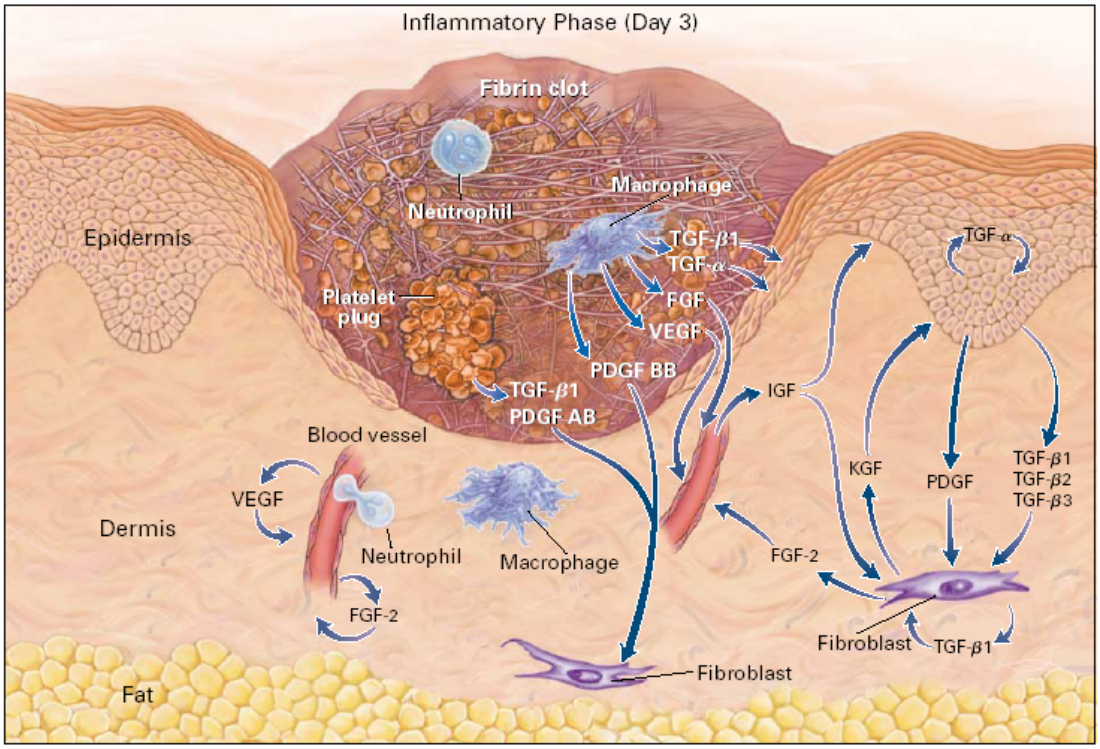


Figure 1. A cutaneous wound three days after injury (10). Growth factors thought to be necessary for cell movement into the wound are shown. TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor-β1, -β2, and -β3, respectively; TGF-α transforming growth factor-α; FGF fibroblast growth factor; VEGF 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.

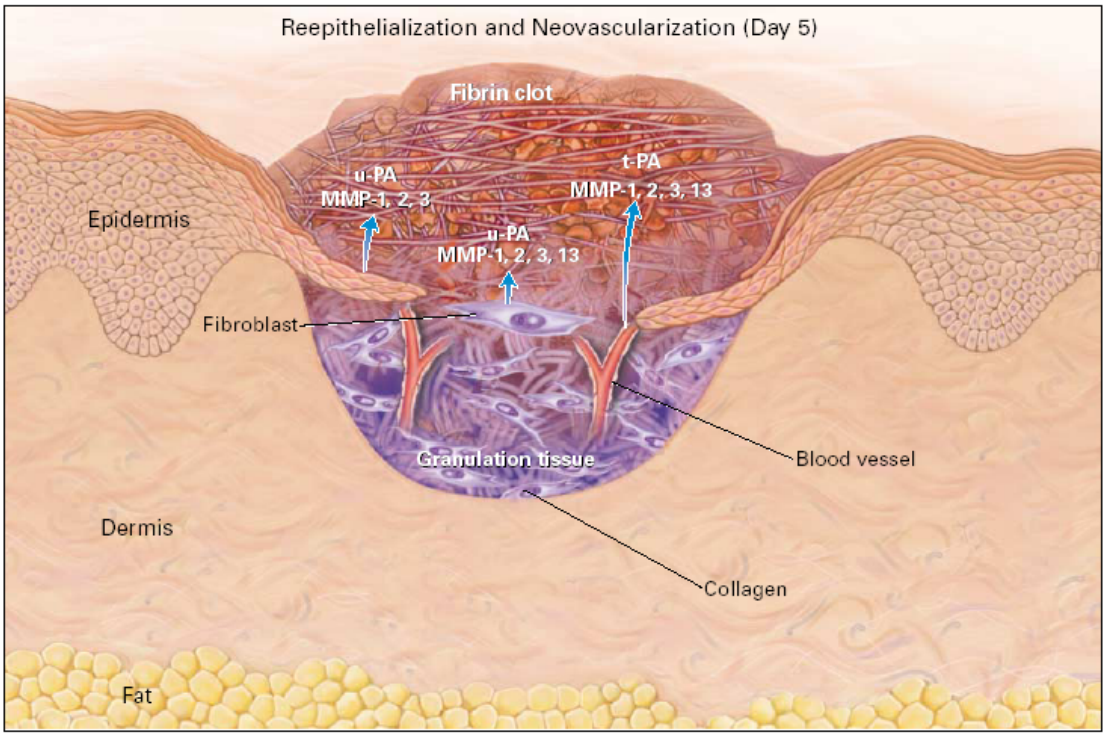


Figure 2. A cutaneous wound five days after injury (10). Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. The abbreviation u-PA denotes urokinase-type plasminogen activator; MMP-1, 2, 3, and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

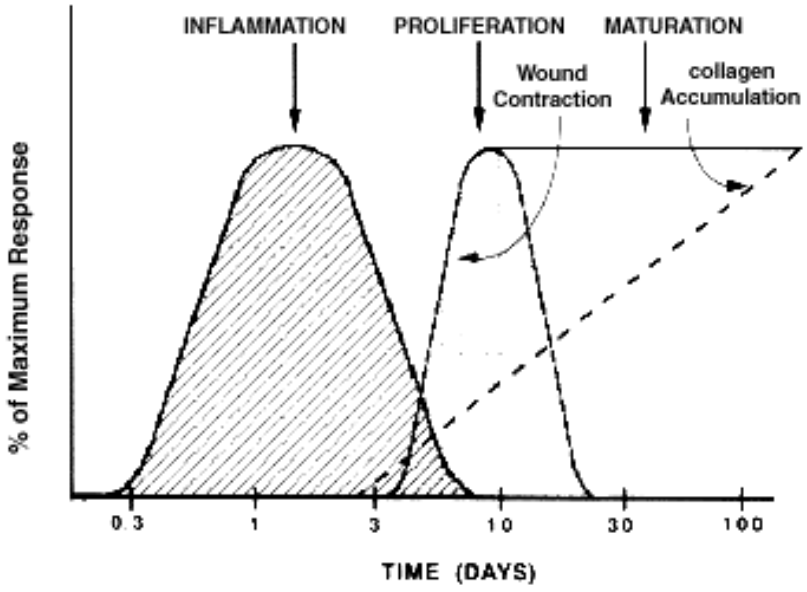


Figure 3. Phases of wound repair (11). Wound healing has been arbitrarily divided into three phases: inflammation, proliferation and maturation.

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.

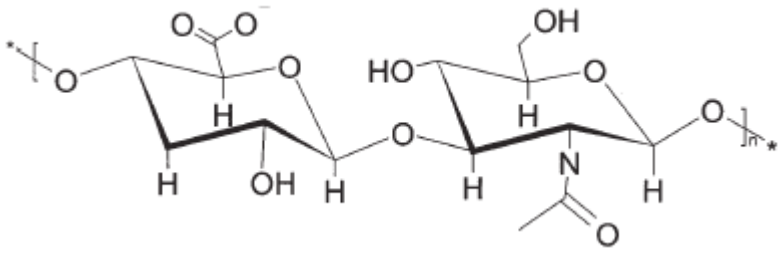


Figure 4. D-glucuronic acid and N-acetyl glucosamine, the disaccharide backbone of HA (12).

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.

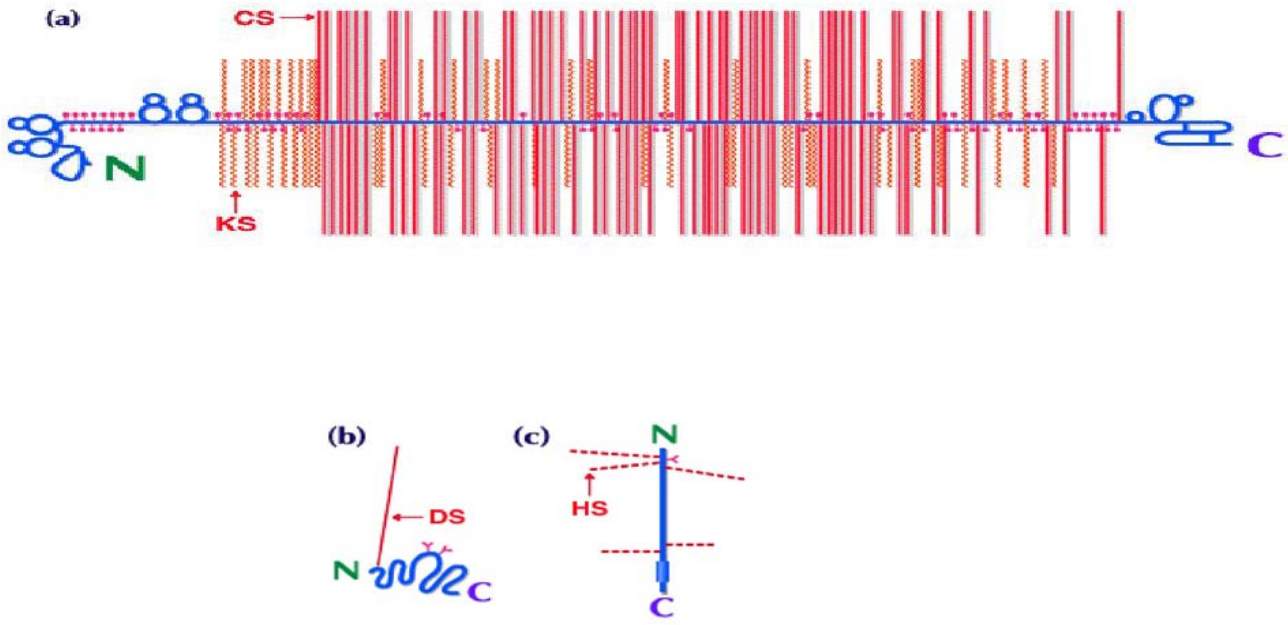


Figure 5. The models of typical proteoglycans; (a) aggrecan, (b) decorin, and (c) syndecan 1. The amino and carboxy termini of core proteins are indicated by N and C, respectively. Glycosaminoglycans are depicted by red lines [i.e., solid lines, chondroitin sulfate / dermatan sulfate (CS / DS); broken 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.

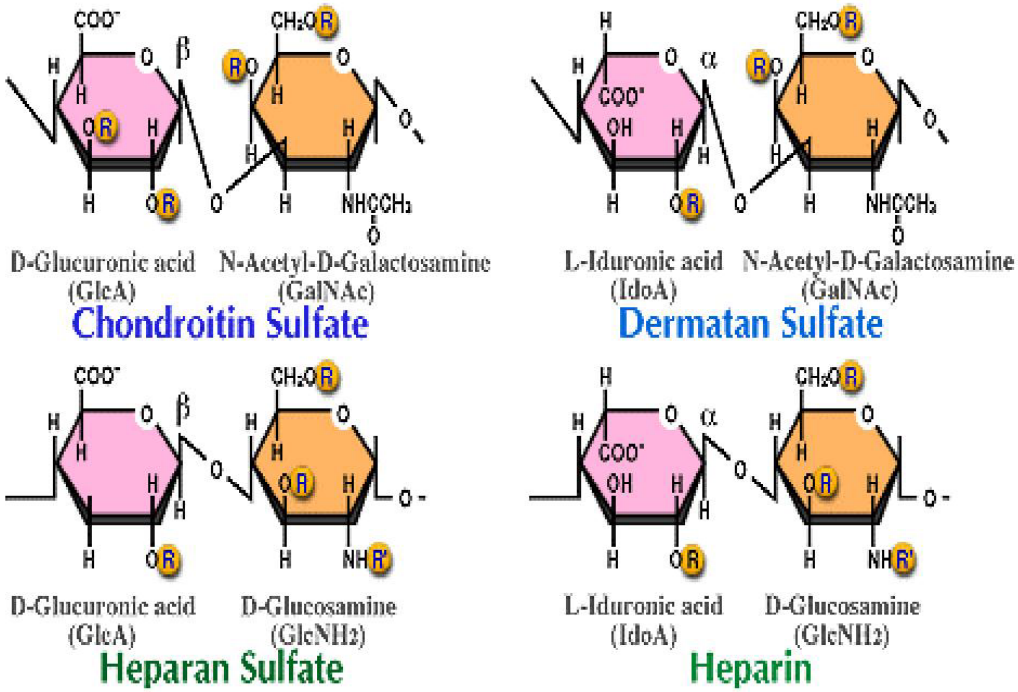


Figure 6. A schematic diagram demonstrating the molecular structure of glycosaminoglycans, including chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate, and heparin. This figure is obtained from http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA06E.html.

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.

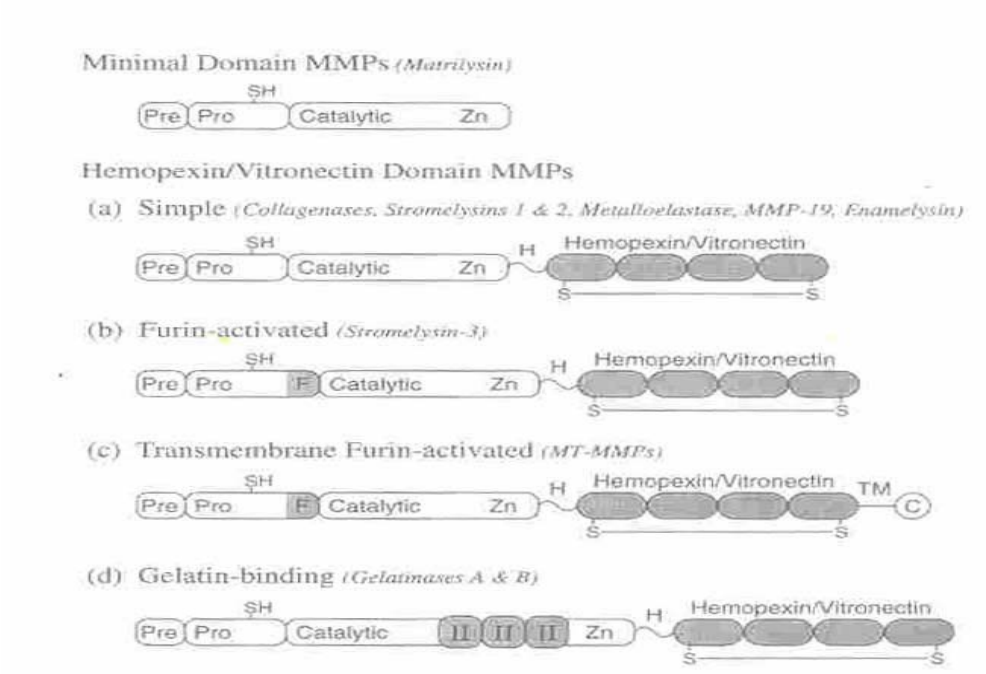


Figure 7. The domain structure of the MMPs (17). Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol group (SH); F, a furin-like enzyme-recognition motif; Zn, a zinc-binding site; II, collagen-binding fibronectin type II inserts; H, a hinge region; TM, a transmembrane domain; C, a cytoplasmic 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

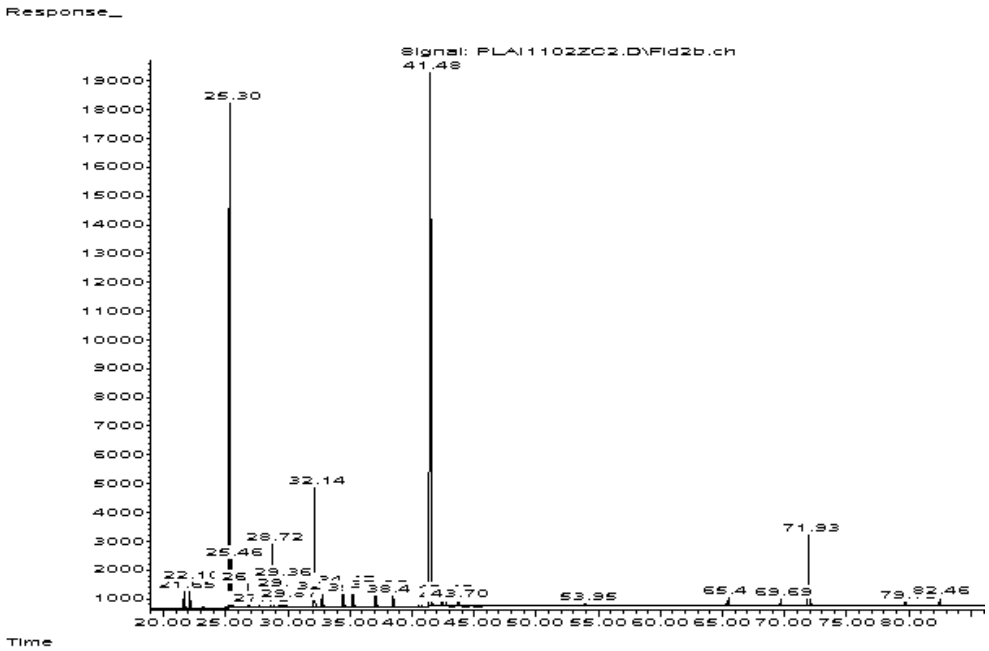
Figure 8. *Zingiber cassumunar* Roxb. and its rhizomes (28). *Zingiber cassumunar* Roxb. is a perennial herb that has bright yellow underground rhizomes. Its rhizomes are widely used in Thai traditional medicine for topical treatment of sprains, contusions, joint inflammations, muscular pain, abscesses,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