CHAPTER I

INTRODUCTION

1.1. Statement of Problems

Chronic inflammatory diseases, such as gingival and periodontal disease, are a major problem throughout the world. In the early stages of the disease, there are the accumulations of a dense infiltrate of inflammatory and lymphoid cells, marked alternation in the quality and quantity of connective tissue substance and pathologic pocket formation. Microbial plaque appears to play a key etiologic role in the initiation and progress of the disease. Various forms of periodontal disease with differing clinical manifestations, and responses to treatment result from differences in the microbial etiology among groups and individual factors that modify host response mechanism or alternate susceptibility.

Beside the microorganism, there is saliva that baths the soft and hard tissues in oral cavity. Human saliva is a complex fluid which secreted into the oral cavity by the parotid, submandibular, sublingual and minor salivary glands. It fulfills a number of important function in regulating oral health, with respect to both the volume produced and the constituents of it contains. Saliva contains a variety of enzymes including esterases and proteinase. Elevated levels of some oral fluid enzyme have been found in periodontal disease (Nieminer et al., 1993). The protease have been shown to promote inflammatory reactions, where as their inhibitors may act as immune suppressive agents. Thus, the aims of this study to evaluate the levels of the proteinases and their inhibitors in human saliva from normal subject and patients with gingivitis and periodontitis.

Many herbs are used for controlling the oral hygiene and interfering with the onset of dental caries and periodontal disease (Elven-Lewis M., 1980). These include inhibition of

acid production, *in vitro* plaque formation, attachment of microorganism to surface, glycosyltranferase activity and glucan formation (Homer et al., 1990). As it was known from other reports those proteinase inhibitors presented in plant, it would be very useful to know that which kinds of Thai-herbs contain proteinase inhibitor. In the present study, the amount of proteinase inhibitor was measured to define which kinds of Thai-herbs contained high proteinase inhibitor. In addition, the effect of Thai-herb extracts on the reduction of proteinase activity was examined in the saliva samples of periodontitis group.

1.2. Literature reviews

1.2.1. The Oral Environment

The oral cavity imitates the biological characteristics of the gut as a whole. The main problems confronting the dentist involve the relatively restricted regions of the tooth surface and its supporting tissues, since the bacterial populations in these areas modify their environment in a manner that may lead to disease. The relationship between the tooth surface and the rest of the mouth can be summarized as follows:

Enamel + environment { mixed saliva, diet, bacteria } → dental plaque { specific bacteria, matrix, fluid }

(Cole and Eastoe, 1988)

Dynamic interaction among the saliva, diet and oral bacteria leads to the accumulation of material on the tooth surface. Plaque (unlike food debris) is almost always found on the non-occluding surface of the teeth. It is composed of localized concentration of bacterial specific to this surface together with some degraded mammalian cells. A matrix containing both protein and polysaccharide surrounds these

and the whole is bathed in a fluid drive from the saliva. Plaque may be present in healthy mouths and does not necessarily lead to pathological changes. However, it is recognized as the common factor in two main dental diseases, namely dental caries and periodontal disease.

Dental caries is characterized by the loss of calcified material from the tooth while periodontal disease is a chronic inflammatory process that lead to the gradual destruction of the supporting tissue of the tooth and is often associated with the deposition of calculus.

Plaque can form in the absence of food and, perhaps surprisingly, the rate of plaque formation is greater during periods of fasting than immediately after meals. Newly formed pellicle is quickly invaded by bacteria (Figure 1.1) derived from the saliva, the adjacent soft tissues and any defects in the enamel surface, and their growth produce discrete colonies which eventually fuse to produce a bacterial mass.

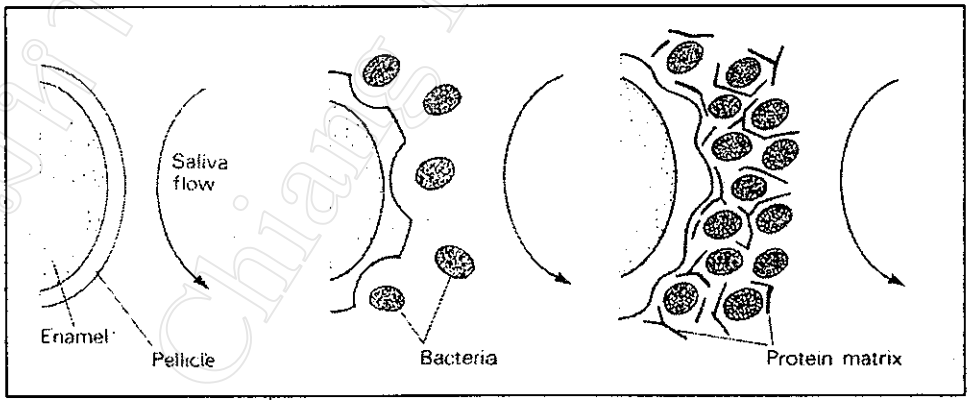


Figure 1.1 Three successive stages in the colonization of a pellicle-covered enamel surface by bacteria during early plaque formation (Cole, 1988).

1.2.1.1. Saliva

Saliva is a mixed secretion more than 90% of which is produced by the major salivary gland: parotid, submandibular and sublingual glands. The mixed oral fluid, are usually referred to as "whole saliva". A number of physiologic factors influence, the composition of whole saliva. Important among these are the source, collection method and degree of stimulation. In addition to fraction from each gland it contains food debris, plaque, bacterial, degraded mammalian cells and possibly some gingival sulcus fluid. Some idea of the composition of a normal average sample of mixed saliva can be obtained from Table 1.1.

Saliva is a unique mixture of electrolytes, proteins and other constituents that have specific roles in digestion and the maintenance of oral health (Table 1.2). Saliva also contains a variety of enzyme including esterase, transferase, glycosidase and proteases (Table 1.3) (Chauncey, 1961; Soder, 1972). Elevated levels of the activity of some salivary enzymes have been found in periodontal disease (Gibbons and Etherde, 1986) (Nakamura and Slots, 1983). Even through salivary enzymes have been the subjects of numerous studies, their function and origin remains unclear in many cases. Salivary glands, serum, leukocytes, epithelial cells and oral bacteria have all been shown to contribute to the production of these enzymes (Watanabe et al., 1981).

Table 1.1 The average composition of mixed human saliva and normal values for plasma (Cole and Eastoe, 1988)

Water	94.0-99.5%	
Solids	0.5% (stimulated) - 6.0% (unstimulated)	
Specific gravity	1.002-1.008	
pH (average)	6.7	
pH (range)	6.2-7.6	
Inorganic	*Saliva (mM)*	*Plasma (mM)*
Ca^{2+}	1-2	2.5
Mg^{2+}	0.2-0.5	1
Na^{+}	6-26	140
K^{+}	14-32	4
NH_4^{+}	1-7	0.03
$H_2PO_4^{-}$ + HPO_4^{2-}	2-23	2
Cl^{-}	17-29	103
HCO_3^{-}	2-30	27
F^{-}	0.001-0.005	0.01
SN^{-}	0.1-2.0	-
Organic		
Urea (adults)	2-6	5
Urea (children)	1-2	-
Uric acid	0.2	3
Amino acids (free)	1-2	2
Glucose (free)	0.05	5
Lactate	0.1	1
Fatty acids (mgl^{-1})	10	3000
Macromolecules (mgl^{-1})		
Proteins	1400-1600	70000
Glycoprotein sugars	110-300	1400
Amylase	380	-
Lysozyme	109	-
Peroxidase	3	-
IgA	194	1300
IgG	14	13000
IgM	2	1000
Lipid	20-30	5500

Table 1.2 The major functions of saliva (Cole and Eastoe, 1988)

Functions	Salivary components involved
1. Protective functions	
Lubrication	Mucins, proline-rich glycoproteins; water
Antimicrobial	Salivary proteins: lysozyme, lactoferrin, lactoperoxidase, mucins, cystatins, histatins, secretory IgA; proline-rich glycoproteins
Mucosal integrity	Mucins, electrolytes, water
Lavage / cleansing	Water
Buffering	Bicarbonate, phosphate ions
Remineralisation	Calcium, phosphate, statherin, anionic, proline-rich proteins
2. Food and speech related functions	
Food preparation	Water, mucins
Digestion	Amylases, lipase, ribonuclease, proteases, water, mucins
Taste	Water, gustin
Speech	Water, mucins

Table 1.3 Salivary constituents (Cole and Eastoe, 1988)

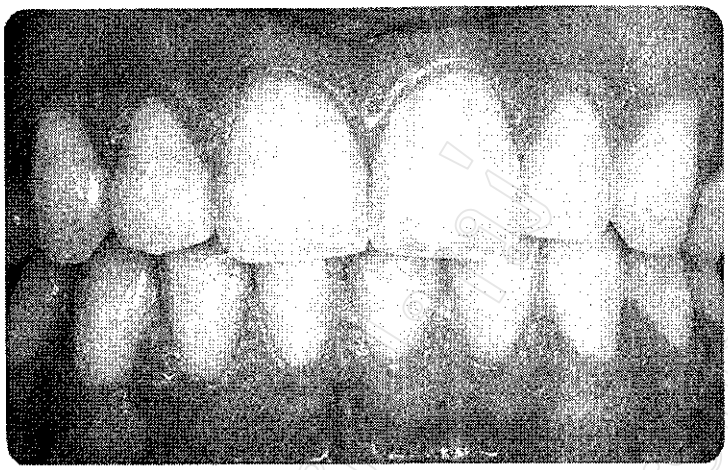
1. Proteins	2. Small organic molecules	3. Electrolytes
Albumin	Creatinine	Ammonia
Amylase	Glucose	Bicarbonate
β-glucuronidase	Lipids	Calcium
Carbohydrases	Nitrogen	Chloride
Cystatins	Sialic acid	Fluoride
Epidermal growth factor	Urea	Iodide
Esterases	Uric acid	Magnesium
Fibronectin		Non-specific buffer
Gustin		Phosphates buffer
Histatins		Potassium
Immunoglobulin A		Sodium
Immunoglobulin G		Sulphates
Immunoglobulin M		Thiocyanate
Kallikrein		
Lactoferrin		
Lipase		
Lactic dehydrogenase		
Lysozyme		
Mucins		
Nerve growth factor		
Parotid aggregins		
Peptidases		
Phosphatases		
Proline-rich proteins		
Ribonucleases		
Salivary peroxidases		
Secretory component		
Secretory IgA		
Serum proteins (trace)		
Tyrosine-rich proteins		
Vitamin-binding proteins		

1.2.2. Periodontium

The healthy periodontium provides the support necessary to maintain teeth in adequate function. The periodontium is comprised of four principal components: namely, the gingival, periodontal ligament, alveolar bone and cementum. Each of these periodontal components is distinct in its location, tissue architecture, biochemical and cellular composition and yet, they function together as a single unit. One periodontal component can influence the other; thus pathological changes occurring in one periodontal component may have significant ramifications for the maintenance, repair or regeneration other components of the periodontium (Curtis et al., 1989; Schluger et a;., 1990).

The gingival, in heath, normally covers the alveolar bone and attaches to the tooth at the level just coronal to the cemento-enamel junction. Histologically, it is composed of two distinct components the overlying epithelial structures and the underlying connective tissue. While the epithelium is predominantly cellular in nature, the connective tissue is less cellular and is composed primarily of an integrated network of fibrous and nonfibrous proteins, growth factors, minerals, lipids and water. Their two components are responsible for orchestrating the earliest responses associated with development of gingivitis and peridontitis (Figure 1.2)

A



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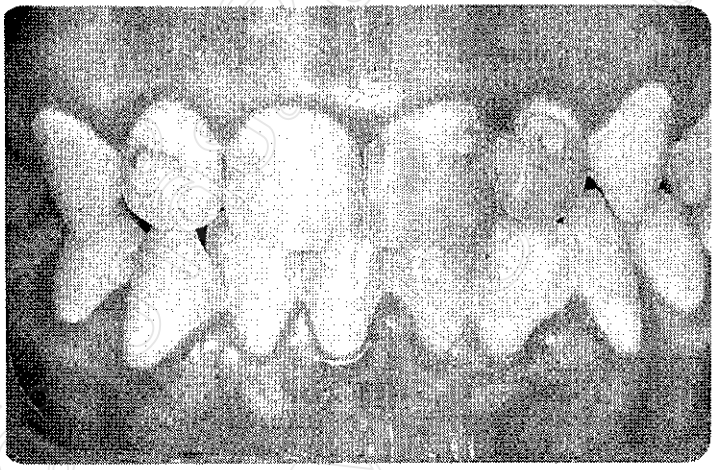

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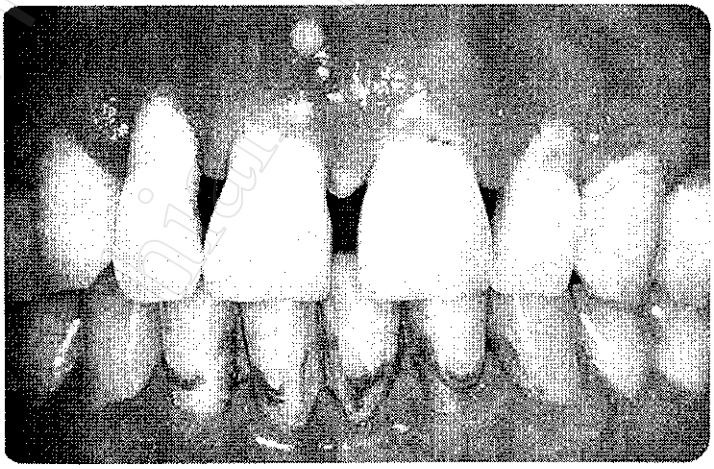

Figure 1.2 These pictures showed example of healthy normal gingiva (A), gingivitis (B), and periodontitis (C).

1.2.3. Gingivitis

Gingivitis and periodontitis are two of most common chronic inflammatory disease affecting humans as well as several, but not all, animal species. These diseases are the results of an induction of host inflammatory responses to the accumulation of bacterial plaque on tooth surfaces at supragingival and subgingival levels. Initially, gingivitis represents a generalized acute inflammatory of gingiva responded to the bacterial that colonizes on the tooth surface adjacent to the gingival. With time, gingivitis may become well established but still confined to the superficial gingival connective tissues and may manifest all the classic features of a chronic inflammatory lesion. If the inflammatory response contained within the gingivitis lesion spreads to the deeper periodontal tissues and alveolar bone is lost, then the resultant lesion is termed periodontitis. The precise mechanisms governing the progression of gingivitis to periodontitis are unclear. In some cases, gingivitis may represent the early stage in the evolution of periodontitis. However, in some individuals, gingivitis may exist as an independent clinical condition without progressing into periodontitis (Williams, 1990). Indeed, that gingivitis and periodontitis are quite separate diseases.

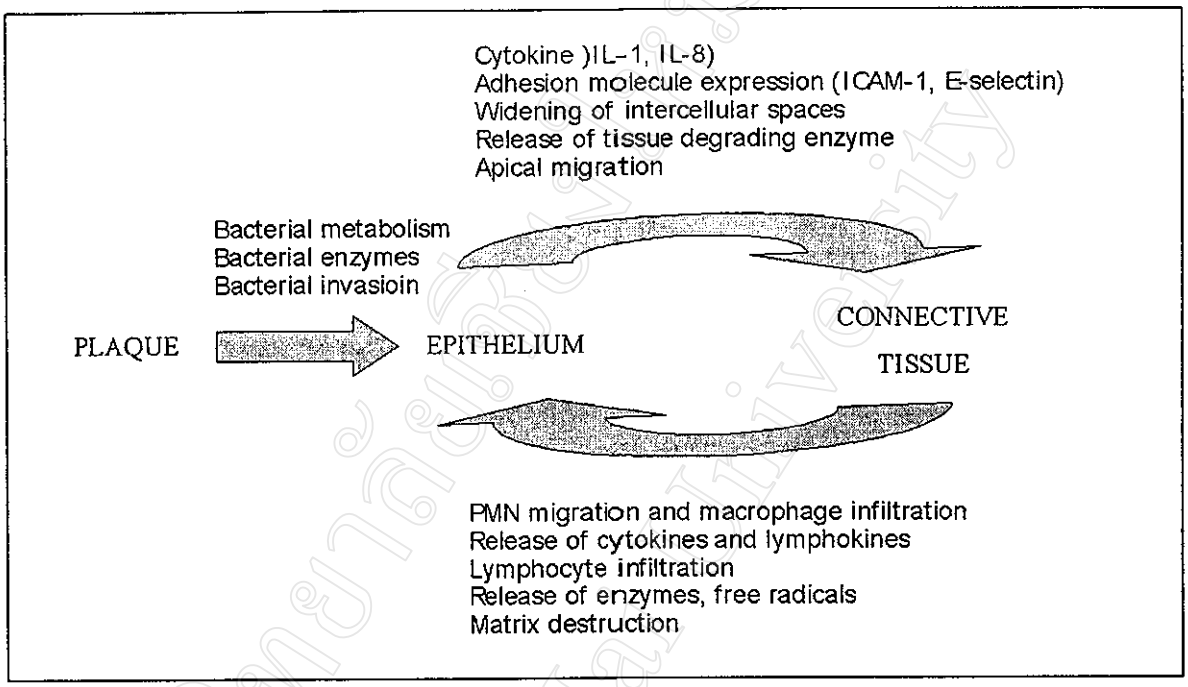

Figure 1.3 Schematic representation of interactive processes between gingival epithelium and connective during the initiation of gingival inflammation. PMN : polymorphonuclear lymphocytes. IL : interleukin. ICAM : intercellular adhesion molecule (Havemann and Janoff, 1978).

1.2.4. Periodontitis

Periodontitis is a destructive inflammatory disease of the supporting tissue of teeth. This disease is primarily related to chronic plaque accumulation. Putative periodontopathic bacteria such as *Porphyromonas gingivalis*, *Actinobaeillus actinomycetemcomitans* are suspected to play a role in the periodontal disease process. *P. gingivalis* is mostly found in deep periodontitis pockets and especially in active sites (Kojima et al., 1993). *A. actinomycetencomitans* is found in pockets from patients with localized juvenile periodontitis as well as advanced adult periodontitis (Genco et al., 1985). It is generally accepted that oral bacteria cause inflammatory responses, which can result in tissue destruction in various ways (Listgarten, 1987). Impaired regulation of proteolysis is an important biochemical aspect in the etiology of periodontal disease (Sandholm, 1986). In the first place, bacteria can directly contribute to periodontal disease by releasing proteolytic enzymes, which can damage to oral tissues. In addition, oral bacteria may induce tissue destruction indirectly by activating host defense cells, e.g. polymorphonuclear leukocytes (PMNs) which can release their lysosomal proteolytic enzymes at the inflamed sites. The intracellular proteases of PMNs are used for digestion of ingested bacteria, enhanced locomotion through connective tissue and for tissue degradation in remodeling. In inflammation, however, they can be released extracellulary in an uncontrolled manner. The PMNs proteases in extracellular matrix are capable of digesting tissue components: vascular basement membrane, collagen, elastin, glycosaminoglycans, and proteoglycans (Havemann and Janoff, 1978).

The health of the periodontal tissues is maintained in relatively stable state through the establishment of host-parasite equilibrium compatible with minimal tissue destruction. The ready regeneration may develop as a result of local or systemic changes that decrease host resistance or from quantitative and/or qualitative alteration of the periodontal microbiota that result in its increased virulence (Listgarten, 1986). Although indispensable for host defense against pathogens, this response may upset homeostasis within the periodontium, leading first to gingivitis and then to periodontitis. Clearly, the common feature of these two major forms of periodontal disease is the excessive

degradation of connective tissue proteins that may be considered to be the result of local, uncontrolled proteolysis.

1.2.5. Role of Bacteria

Although nearly 400 different bacterial taxa have been identified in dental plaque samples (Moore, 1987), only a few species have been implicated as periodontal pathogens. This group of bacterial includes *Porphyromonas gingivalis*, *Treponemia denticola*, Bacteroids for sythus and *Actinobacillus antinomycetemcomitas* (Potemapa et al., 1997) Bacteria can contribute to periodontal disease by direct injury of the host tissue via assorted toxins, enzyme, or toxic metabolic end products (Slots and Genca, 1984). They can also act indirectly by triggering host-mediated responses that may result in self-injury (Slots and Genca, 1984).

A variety of substances of bacterial origin are capable of causing injury to or death of individual cells or whole animals. They are usually of large molecular weight, antigenic, and may include some enzymes. Classically, toxins have been divided into exotoxins and endotoxins

Exotoxins are highly antigenic, nonpyrogenic thermolabile polypeptides actively secreted by living microorganisms that can be converted into toxoids. Leukotoxin is likely to be involved in the pathogenesis of periodontitis. It is known to be produced by species of *A. actinomycetemconmitans* (Taichman, 1980). Leukotoxin acts on cell membranes to produce small holes that result in lysis of leukocytes. Therefore, the action of the leukotoxin in promoting tissue injury is in part indirect to the extent that it is able to weaken the local host defense. The release of lysosomal enzymes from lysis PMNs may contribute furthers to local tissue destruction.

Endotoxins are highly pyrogenic, thermostable macromolecule later identified as lipopolysaccharide, which forms an integral part of the outer layer of gram-negative cell wall (Wang, 2000). Lipopolysaccharide is released during disintegration of bacteria after death. Endotoxin can exert a wide diversity of biological effects, yet they share a

common molecular structure. The linear molecule, which is orientated more or less perpendicularly to the bacterial cell surface, consists of 3 distinct regions. The lipid A region, which is innermost, forms part of the outer half of the outer membrane of the cell wall. The centrally located core polysaccharide, and the polysaccharide side chains, where the O-somatic antigens reside extend peripherally beyond the outer membrane (Figure 1.4). The lipid A is the region responsible for direct toxicity. The carbohydrate components provide the lipid A with hydrophilic properties, which enhances its pathogenically and may increase the resists of the bacteria to phagocylosis (Taussing, 1984). Endotoxins also are able to bind to the surface of phagocytes cells (PMNs and macrophages). Subsequent internalization of the endotoxin causes the cells to release their lysosomal enzymes extracellularly, with resulting damage to the local tissues and the generation of peptides that are vasoactive and chemotactic for PMNs by this mechanisms, endotoxins are able to amplify the inflammatory reaction (Roitt and Lehner, 1983). Endotoxins may activate complement through the indirect pathway with the consequent generation of complement – derived mediators of inflammation such as tumor necrosis factor-α (TNF-α) and interleukins (IL) (Figure 1.5) (Page, 1991)

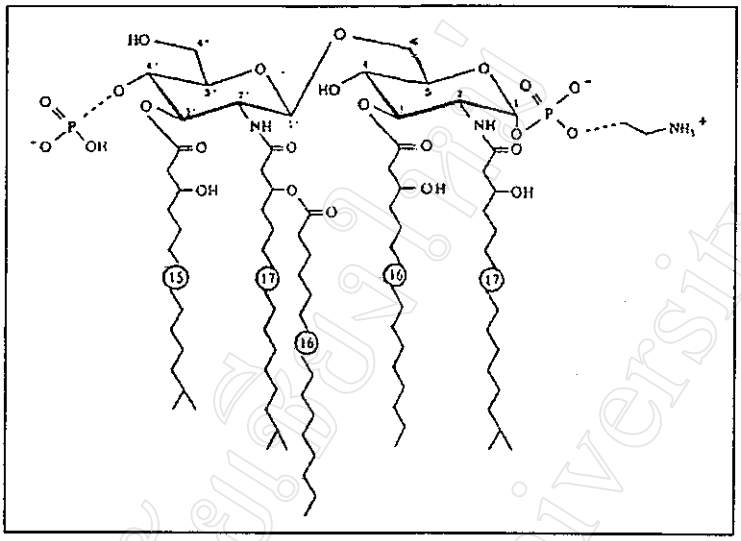

Figure 1.4 Proposed chemical structure of the lipid A molecule from *porphyromonas gingivalis.* (Kumasa et al, 1997)

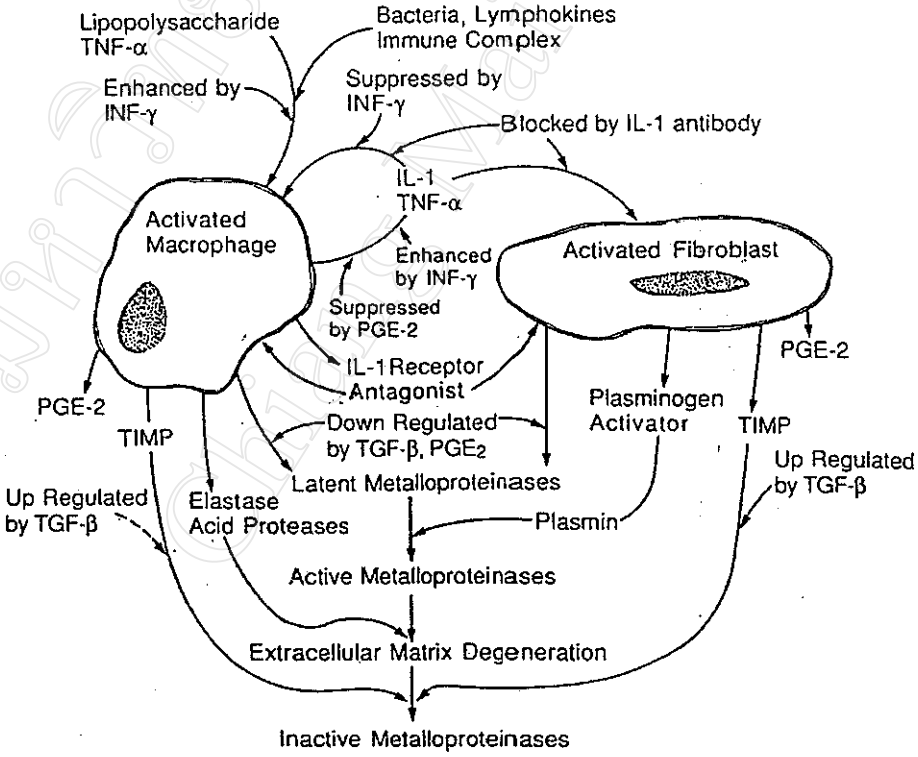

Figure 1.5 Schematic diagram of the enzymes pathways of host response (Page, 1991).

1.2.6. Proteinases and Matrix Degradation

The influx of inflammatory cells result in increases in cell proliferation, synthesis of extracellular matrix components, and degradation of extracellular matrix components. The net result of these processes is the destruction of normal tissue structures, replacement of these structures by inflammatory and fibrotic tissue, and, finally, loss of function. The degradation of extracellular matrix may be coupled of cell migration or to remodeling during synthesis of connective components, or it may be purely destructive. (Table 1.4)

Table 1.4 Extracellular matrix remodeling events involving proteolysis (Werb, 1989)

Emigration of inflammatory cells out of blood vessels, through the basement membrane and underlying loose connective tissue.
Migration and proliferation of microvascular endothelial cells and fibroblasts during neovascularization.
Editing of excess matrix components from migration, signaling, and assembly pathways.
Removal of obsolete matrix components from migration, signaling, and assembly pathways.
Breakdown of connective tissue components coupled to synthesis during tissue expansion and growth.
Destruction of extracellular matrix during inflammation, activation of connective tissue cells, and fibrosis.

1.2.7. Extracellular Matrix - Degrading Proteinase

The enzymes that are most important in the degradation of the extracellular matrix macromolecules in connective tissue are proteinase or endopeptidases that cleave internal peptide bond of proteins. They may be found intracellularly in lysosomes, where they act upon proteins take up by endocytosis, or extracellularly in the pericellular space and at a distance from the cell of origin. Numerous proteinase and peptidases are found intracellularly and extracellularly in various tissue fluids and plasma.

There are four classes of proteinases that can be classified by catalytic mechanism: the aspartic and cysteine proteinases, which are mostly active at acid pH, and the serine and metalloproteinases, which are active at neutral and slightly alkaline pH (Bond and Butler, 1987). The proteinases most likely to play a role in extracellular matrix degradation are listed in Table 1.5 and Table 1.6.

Table 1.5 Proteinases of connective tissues (Werb, 1989)

Class	Examples	Location*	pH Range	Protein
Aspartic	Cathepsin D	L, E	3-6	Inhibition
Cysteine	Cathepsin B	L, E	3-7	α2M
	Cathepsin L	L, E		α_2M
Serine	Plasmin	E	6-10	αCPI
	Kallikrein	E		α_2M, PN-1
	Thrombin	E		
	uPA	E, S		PN-1, PAI
	tPA			PAI-1
	PMN elastase	G, E		α_1-proteinase inhibitor
	Cathepsin G	G, E		αCPI
	Mast cell chymase	G, E		αCPI
	Mast cell tryptase	G, E		Aprotinin
	Granzymes	G, E		
Metallo	Collagenase	E	6-9	α_2M
	Stromelysin	E		TIMP
	PMN collagenase	G, E		IMP-1
	Macrophage elastase	E		IMP-2
	Gelatinase (92 kDa)	E, G		
	Gelatinase (68 kDa)	E		
	Gelatinase (60 kDa)	E		None
	Membrane neutral endopeptidase 22,11	S		None known

* E, extracellular; S, cell surface; C, cytoplasmic; L, lysosomal; G, granules.

Table 1.6 Proteinase susceptibility of extracellular matrix proteins (Werb, 1989)

Matrix Protein	Proteinases*
Cartilage	
Cartilage proteoglycans	Stromelysin, plasmin, mast cell chymase, cathepsin G, PMN elastase, cathepsin B, cathepsin L
Collagen type II	Collagenase
Collagen type X	Collagenase
Interstitial Connective Tissue	
Collagen type I	Collagenase, catherpsins, B, L, N
Collagen type III	Collagenase, plasmin
Collagen type V	92 kDa gelatinase, 68 kDa gelatinase
Fibronectin	Stromelysin, cathepsin G, uPA, plasmin, PMN elastase, macrophage elastase, stromelysin, cathepsin L
Elastin	PMN elastase, macrophage leastase, stromelysin, cathepsin D
Basement Membrane	
Collagen type IV	Stromelysin, 68 kDa gelatinase, 92 kDa gelatinase, plasmin, PMN elastase, mast cell chymase
Heparan sulfate proteoglycans	Mast cell chymase, PMN elastase
Laminin	Plasmin, stromelysin, PMN elastase
Denatured collagen	92 kDA gelatinase, 68 kDa gelatinase, PMN elastase, cathepsin B, cathepsin L

* Where known.

1.2.8. Role of Inflammation in Periodontal Disease

Inflammation leads to the accumulation of polymorphonnclear neutrophil leucocytes (PMNs), macrophages, lymphocytes and mast cells that are very important in protecting the body against infection. The inflammatory cells contain destructive enzymes within their lysosomes, which are normally used to degrade phagocyte material. These enzymes are, however, capable of degrading gingival tissue components if released such enzymes may be released by inflammatory cells during their function or when they degenerate or die. Cell and tissue in the vicinity of these cells will be damaged and this process is known as by stander damage. The main tissue damages in this process are the connective tissue components and the breakdowns of these tissues around the inflammatory cells help the spread of these cells through the tissue. The main connective components are collagen and proteoglycans. The collagen degradation is multiple stage process (Figure 1.6). Each collagen molecule consists of two distant regions. The larger region making up about 90% of the molecule is the triple helical region which can only be attacked by the metalloproteinase, collagenase. The smaller terminal regions consist of peptides know as the terminal peptide, which contain the sites of intra and inter molecular cross-link. These are resistant to attack by collagenases but can be attacked by number of other serine and cysteine proteinases acting in concert (Harris and Carlwright , 1977). Collagenases are present in the cells in the form of latent enzymes either as proenzymes or enzyme inhibitor complexes (Eley and Cox, 1998) and the released pro-collagenases need to be activated by other proteinases (Figure 1.6). When connective tissue is broken down the degradation of collagen is usually proceeded by that of the proteoglycan. They consist of a glycosaminoglycan (GAG) molecule linked to a protein core (Figure 1.7). The principle proteoglycans of the gingival and periodontal ligament are hyaluronic acid, heparin sulphate, dermatan sulphate and chondroitin sulphate 4. In proteoglycan degradation occurs first to release the GAGs from the protein core. A number of metallo-, serine and cysteine proteinases can carry out this function. The released GAGs may remain intact or be further degraded by others hydrolytic enzymes.

More specifically gingival proteoglycan degradation may be effected through a neutral proteinase secreted by human PMNs.

1.2.9. Proteolytic Enzymes within Inflammatory Cell

The granules of neutrophils contain several potent proteolytic enzymes as well as other hydrolases (e. g., phospholipases) that are capable of killing microorganisms, which can also induce damage to tissue. Over 20 different granule products with activity have been identified. Among them are proteolytic enzymes that function optimally at acid pH (cathepsin B and D) and neutral proteolytic enzymes such as elastase, cathepsin G, collagenase and gelatinases (Spitznage, 1984). During the process of microbial phagocytosis, granule fuse with the phagocytic vacuole and the granule contents are extruded into the vacuole and the release of granule contents outside of the cell is responsible for the tissue injury. These granule components are listed in Table 1.7.

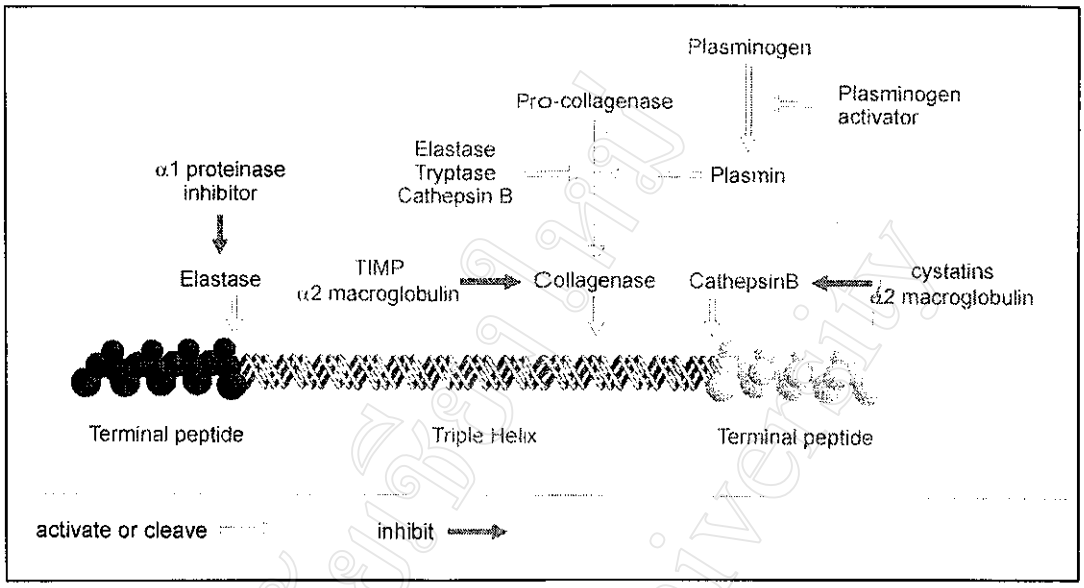

Figure 1.6 Diagram of collagen degradation showing the proteolytic enzymes and inhibitors involved (Eley, 1998).

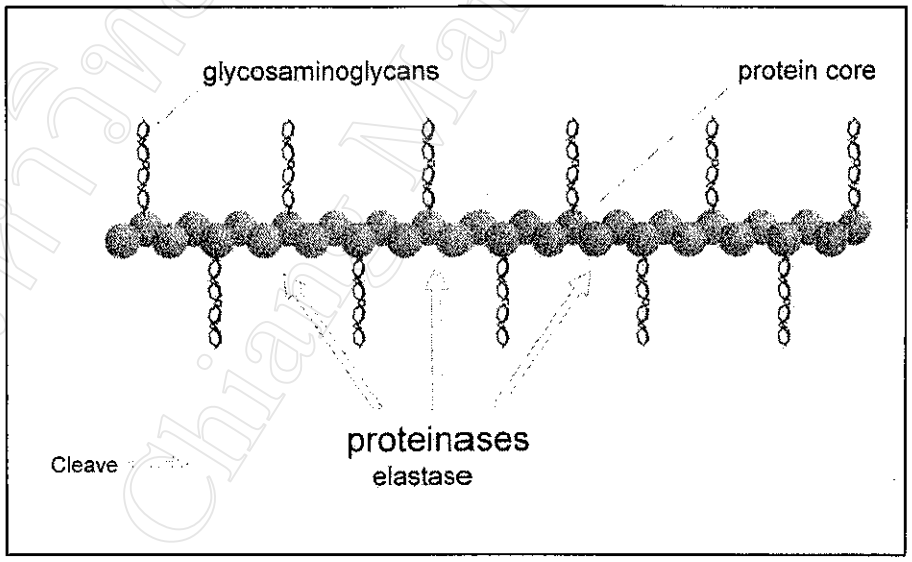

Figure 1.7 Diagram of proteoglycan degradation showing the proteolytic enzyme (Eley, 1998).

Table 1.7 Constituents of neutrophil granules (Spitznagel, 1984)

Constituents of neutrophil granules
Cathepsin B
Cathepsin D
Cathepsin G
Elastase
Collagenase
Gelatinase
B-Glucuronidase
N-Acetylglucosaminidase
a-Mannosidase
Lysozyme
Defensins
C5a cleaving enzyme
Plasminogen activator
Alkaline phosphatase

1.2.10. Cysteine Proteinase

Cysteine proteinases have been associated with inflammatory reaction cathepsin B and catherpsin L are the best known lysosomal cysteine proteinases. These enzymes are related to each other and, evolutionally, to papain and have catalytic sites that require cysteine and histidine residues (Barrett and Kirschke, 1981). Both cathepsin and cathepsin L are synthesized as proenzymes of molecular weight and sorted into lysosomes, where they are activated slowly, by limited proteolysis, to enzymes of molecular weight 25,000 (Troen et al., 1988). In human tissues, the cysteine proteinase with greatest activity against collagen and proteoglycan is cathepsin L, cathepsin B and L both cleave the N-terminal peptides of collagen that contain the covalent cross links-within and between molecules, but cathepsin L is the more active of the two. Cathepsin B cleaves the hyaluronid acid-binding region from cartilage proteoglycan and degrades

the glycosaminoglycans-attachment region to rather small fragments (Kirschke et al., 1982).

1.2.11. Serine Proteinases

The family of endopeptidases, with a catalytically essential serine residue at their active site, is the largest class of mammalian proteinase. These enzymes are most active at about neutral pH. The serine proteinases include many of the proteins of cascades of coagulation, fibrinolysis, and complement activation such as plasmin, and plasminogen activator. The serine proteinase from the neurophil granules of PMNs, elastase, and cathepsin G, may play a role in extracellular matrix degradation.

Polymorphonuclear leukocyte elastase (PMN elastase) is present in the zurophil granules of PMNs and monocytes. It is a protein of about molecular weight 28,000 that acts on cartilage proteoglycan to remove the hyaluronic acid-binding region and then to fragment the glycosaminoglycan attachment region (Kirschke, 1982). Elastase consists of 218 amino acid residue and joined together by four intramolecular disulfide bonds linking of eight half-cysteine residues (Sinha et al., 1987). Unlike the collagenases, the elastases are not specific for the substrate after which they are named but have very broad proteotylic activity.

PMN elastase degrades collagen fibers by first degrading the N-terminal peptides, with the elimination of the cross-links that play a crucial part in the stabilization of collagen fiber. The individual molecules then separate, if solubilized, denature in a few hours at 37oC and are degraded to small peptides and amino acids by further proteolytic activity. PMN elastase is several times more active against cartilage collagen (type II) than against type I collagen, where as the reverse is true of the specific collagenase (starkey et al., 1977) Extracellular activity of PMN elastase is controlled by α_1-proteinase inhibitor and α_2-macroglobulin. Inhibitors of PMN elastase include di-ispropyl flurophosphate and phenylmethy-sulfonylfluoride (general inhibitors of the serine proteinase) (Werb, 1989).

1.2.12. Endogenous Proteinase Inhibitors

The activities of proteinases *in vivo* are modulated by the naturally occurring inhibitors of the enzymes. The inhibitors are likely to be important in the control of extracellular matrix degradation that are secreted by tissue cells or that derive from the plasma (Table 1.8)

Table 1.8 Polypeptide inhibitors of matrix-degrading proteinases (Werb, 1989)

Inhibitor	Molecular Weight	Source	Specificity
α_2-M	750,000	Plasma, macrophages	All classes
α-CPI		Plasma	Cysteine proteinases
α_1Porteinase inhibitor	54,000	Plasma, macrophages	PMN elastase, other cysteine proteinases
Protease nexin	45,000	Fibroblasts	Trypsin-like serine proteinases
PAI-1	45,000	Fibroblasts, endothelial cells	TPA uPA
PAI-2	45,000	Macrophages	TPA, uPA
α_1-Antichymotropsin	50,000	Plasma	Chymotropsin-like proteinases
Aprotinin	7,000	Mast cells	Serine proteinases
TIMP	30,000	Fibroblasts, endothelial cells, macrophages, plasma	Metalloproteinases
IMP-1	20,000	Endothelial cells, fibroblasts	Metalloproteinases
IMP-2	18,000	Endothelia cells, fibroblasts	Metalloproteinases

1.2.13. Cysteine Proteinase Inhibitors

In human cells, there are two cytoplasmic inhibitors of cysteine proteinases called cystatins, molecular weight about 13 KDa, and inhibitory activity corresponding to these was extracted from human articular cartilage. The cystatin superfamily comprises a diverse group of cysteine protease inhibitors widely distributed in mammalian tissues and plasma. They protect the organism against the uncontrolled action of endogenous and/or exogenous cysteine proteinase (Lindhal et al., 1988). Originally, members of the cystatin superfamily were grouped into three families, family I or stefins, family II or cystatin (including salivary cystatins) and family III or kininogen (Barrett et al., 1986)

Human salivary cystatin genes are part of a multigene family composed of seven membrane segregated on chromosome 20 (Saitoh et al., 1987). Salivary cystatins S (SAP-I), SN (SA-I) and SA contain 121 amino acid and have ~90% sequence homology. In addition, salivary cystatins have a 54% sequence homology with serum cystatin C, which is present in the gingival crevicular fluid and consequently in the oral milieu (Aguirre et al., 1992)

1.2.14. Enzyme Mechanism

In adult organism, most intact connective tissue turn over slowly. However, the orderly degradation of interstitial extracellular matrix and basement membrane is one of the fundamental processes governing growth, development, morphogenesis, remodeling and repair under both normal and pathologic condition. The degradative mechanisms that operate under one set of conditions may not apply to different tissue or conditions. The differing primary structures of the genetically distinct collagen types, glycoproteins, and proteoglycans may require distinct enzymes. Various cells make different combinations of proteinases, proteinase inhibitors and activators of zymogens. The extent and type of intermolecular cross-links, and the assembly of the collagen types

and other connective tissue proteins into supramolecular structures, may also influence the susceptibility of the matrix to proteolysis. Degradation may take place at extracellular sites or in the lysosomal system with in cells. As an example, the complexity of the pathways for degradation of collagens is shown in Figure 1.7.

1.2.15. Plant Proteinase Inhibitors

Proteinase inhibitor from plants, animals and microganisms, as well as synthetic inhibitors, have been valuable pools in the study of enzymes. The biological importance of the granulocyte proteinase has led to survey a wide variety of plants as potential sources of their specific inhibitors. Various flower bulbs and vegetable and legume seeds were tested for inhibitors of polymorphonuclear leukocyte elastase, pancretic elastase, cathepsin E, cathepsin B, trypsin, α-chymotrypsin, Hageman factor fragment, plasma kallikren, and plasmin (Hojima et al., 1983) (Table 1.9).

There have been few reports of the effects of extracts from plants on the growth or physiological properties of the bacteria suspected of involvement in periodontal disease. Extracts prepared from several plants used in traditional folk medicine, impair a number of physiological function are of the cariogenic bacteria (Loesche, 1986). These include inhibition of acid production, *in vitro* plaque formation, attachment to surfaces, glucosyltransferase activity and glucan formation (Dzink and Socransky, 1985; Wu and Wu, 1988).

Table 1.9 Proteinase inhibitor by plant extracted (Hojima et al., 1983)

No.	Common name	Genus	IU/g tissue or seeds									
			PMN elastase*	Pancreatic elastase*	Cathepsin G	Cathepsin B (pH 7.5)	Cathepsin B (pH 5.5)	Trypsin	Chymotrypsin	HF_r	Plasma kallikrein	Plasmin
	Bulbs											
1	Calla	*Zantedeschia aethiopica*	27	0†	17	0.04	0	43	15	0.07	91	120
2	Lily	*Lilium sp.*	4.5	1.6	8.6	0.07	0	11		1.8	0.72	
3	Hyacinth	*Hyacinthus orientalis*	1.7	0.12	3.2	0	0	1.8		0.60	1.5	
4	Grape hyacinth	*Muscari botryoides*	4.5	0.14	6.0	0	0	0.65		0.18	0.36	
5	Tulip	*Tulipa greigii*	4.4	0	15	0	0	1.5		1.9	0.29	
6	Iris	*Iris reticulata*	1.6	0.06	9.2	0.04	0	4.8		2.4	0	
7	Ranunculus	*Ranunculus asiaticus*	11	0.55	15	0.06	0.12	4.1	0.82	0	0	
	Seeds											
8	Radish	*Raphanus sativus*	0.48	0	6.1	3.9	2.1	2.7	0.73	0.50	0.35	4.7
9	Broccoli	*Brassica oleracea italica*	7.7	0	6.5	0.42	0.32	3.7	0.11	0.58	0	8.7
10	Cauliflower	*Brassica oleracea botrytis*	0.60	0	4.3	0.28	0.22	2.6	0.26	1.6	0	6.0
11	Cabbage	*Brassica oleracea capitata*	0.72	0	11	0.66	0.44	14	0.55	5.1	0	31
12	Turnip	*Brassica rapa*	0.60	0	3.2	0.03	0.03	2.2	0.52	0.66	0	3.3
13	Okra	*Hibiscus esculentus*	9.1	0	36	0.05	0.04	9.8	4.4	0.72	0.08	4.8
14	Beet	*Beta vulgaris*	4.9	0.03	11	0.36	0.13	0.03	0.04	0	0	
15	Celery	*Apium graveolens*	7.9	0	8.6	0.30	0.21	0	0	0.11	0.12	
16	Parsley	*Petroselinum crispum*	2.5	0	4.6	0.26	0.12	0	0	0	0.05	
17	Lettuce	*Lactuca sativa*	20	0	34	0.26	0	0.11	0	0	0	
18	Asparagus	*Asparagus officinalis*	1.1	0	8.2	0.34	0.08	0.04	0	0	0	
19	Spinach	*Spinacia oleracea*	4.7	0	9.6	0.28	0.36	0.27	0.05	0.08	0.30	
20	Soybean	*Glycine soja*	56	1.4	140	0.09	0	46		0	35	
21	Lima bean	*Phaseolus limensis*	49	0.06	78	0.07	0.03	38		0.34	0	
22	Red kidney bean	*Phaseolus vulgaris*	19	0.34	89	0.13	0.04	19		0	0	
23	Adzuki bean	*Phaseolus angularis*	16	0.29	63	0.16	0.10	27		0	0	
24	Lentil	*Lens esculenta*	20	0.06	63	0.06	0.12	2.5		0	0	
25	Sweet pea	*Lathyrus odoratus*	14	0	37	0.22	0.20	7.8		0	0	
26	Peanut (albumen)	*Arachis hypogaea*	9.6	0	15	0.11	0.09	2.0		0.62	0.03	
27	Corn	*Zea mays*	4.5	0.04	9.6	0.05	0	0.67	0.08	0.40	0	
28	Pumpkin	*Cucurbita maxima*	2.7	0	6.9	0.05	0.04	0.68	0.03	0.35	0	
	Others											
29	Potato (tuber)	*Solanum tuberosum*	1.7	0.09	4.5	0.05	0.09	1.2		0.55	1.7	
30	Sweet potato (root)	*Ipomoea batatas*	1.9	0	13	0.07	0.03	0.73		1.6	0.06	

* Data from assays with synthetic substrates.
† 0 IU/g was less than 0.03 IU/g.

1.3. Objectives

The objectives of this study were:

1. To determine the concentration of total proteinase, cysteine proteinase, elastase, total proteinase inhibitor, cystatin and elastase inhibitor in saliva of normal, gingivitis and periodontitis groups.
2. To detect the total proteinase inhibitor in thirty-two Thai herbs.
3. To compare the percent of inhibition of total proteinase, cysteine proteinase, and elastase in the saliva of periodontitis group with and without adding with selected Thai herbs extract.