ANTI-INFLAMMATORY, WOUND HEALING AND ANTI-ADHERENCE ACTIVITY OF CLINACANTHUS NUTANS AND ITS CONSTITUENT

BY

MOEHAMAD ORLIANDO ROESLAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (ORAL HEALTH SCIENCE)

FACULTY OF DENTISTRY

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ENTITLED

ANTI-INFLAMMATORY, WOUND HEALING, ANTI-ADHERENCE ACITIVITY OF *CLINACANTHUS NUTANS* AND ITS CONSTITUENT

was approved as partial fulfillment of the requirements for
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on

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ABSTRACT

Clinacanthus nutans (C. nutans) has been known to have anti-inflammatory activity, however the active compound of anti-inflammatory is still unknown. The aim of this study is to identify bioactive compound from *C. nutans* that responsible for anti-inflammatory, cell migration, and anti-adherence activities.

Materials and methods: The isolation of pure compound from the extract of *C. nutans* was performed by chromatographic techniques and bioassay-guided fractionation. The pure isolated compound was characterized by spectroscopic data. The study included cytotoxicity assay, nitric oxide assay, wound scratch assay, adherence assay, and visualization of biofilm by confocal laser scanning microscopy.

Results: The results showed that the chloroform extract from *C. nutans* and its pure isolated compound namely purpurin-18 phytyl ester significantly inhibited LPS-induced NO production at concentration 100 μg/mL for the chloroform extract, and 10 μg/mL for the isolated compound ($p<0.05$), significantly induced migration of gingival fibroblast at concentration 10 μg/mL ($p<0.05$), and significantly inhibited the adherence of *S. mutans* at concentration 12.5-1000 μg/mL for the chloroform extract, and 25 μg/mL for the isolated compound ($p<0.05$).

Conclusion: These findings suggest that purpurin-18 phytyl ester may be the bioactive compound from *C. nutans* that responsible for anti-inflammatory, cell migration, and anti-adherence activities.

Keywords: *Clinacanthus nutans,* purpurin-18 phytyl ester, anti-inflammatory, cell migration, anti-adherence activities.

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Mr. Moehamad Orliando Roeslan

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Symbols/Abbreviations	Terms
°C	Degree Celsius
^{1}H-NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	Carbon Nuclear Magnetic Resonance
µg	Microgram
µM	Micromolar
AEP	Acquired Enamel Pellicle
AcOEt	Ethyl Acetate
ATCC	American Type Culture Collection
aFGF	Acidic Fibroblast Growth Factor
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
BHI	Brain Heart Infusion
CI	Confidence Intervals
cm	Centimeter
CMIR	Cell-mediated Immune
COX	Cyclooxygenase
CO_2	Carbon dioxide
δ	Chemical Shift
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EPP	Ethyl Phenylpropiolate
FBS	Fetal Bovine Serum

FGF	Fibroblast Growth Factor
G	Gram
Gbp	Glucan Binding Protein
GTF	Glucosyltransferase
h	Hour
HA	Hydroxyapatite
HSV	Herpes Simplex Virus
Hz	Hertz
IGF	Insulin-like Growth Factor
IL	Interleukin
J	Coupling Constant
Kg	Kilogram
LPS	Lipopolysaccharide
MD	Myeloid Differentiation
mg	Milligram
min	Minute
ml	Milliliter
mM	Milimolar
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK	Natural Killer
NO	Nitric Oxide
P18PE	Purpurin-18 phytyl ester
PAF	Platelet Activating Factor
PAMPs	Pathogen-associated Molecular Patterns
PBS	Phosphate Buffered Saline
PBMCs	Peripheral Blood Mononuclear Cells
PDGF	Platelet-derived growth factor
PG	Percent of Growth
PMA	Phorbol Myristate Acetate

ppm	Part Per Million
PTLC	Preparative Thin Layer Chromatography
PVP	Polyvinylpyrrolidone
OD	Optical Density
RT	Room Temperature
SE	Standard Error
TLC	Thin Layer Chromatography
TLR	Toll-like Receptors
TGF	Transforming Growth Factor
TNF-α	Tumor Necrosis Factor-alpha

CHAPTER 1

INTRODUCTION

1.1 Natural products

Since early human history, human always rely on nature for their basic needs, such as for the production of clothing, food, shelters, and of course, medicines.[1] Natural product has been used as medicine throughout history in the form of traditional medicine, remedies, potions and oil.[2] Plants have formed to be the basic of traditional medicine systems that have been in exist for thousands of years and continue to provide mankind with new remedies.[1,2] The oldest records, date back to 2600 B. C., were it was written on clay tablets from Cuneiform in Mesopotamia. These records indicate that there were up to 1000 plant based medicine used at that time. The natural products they used were oils of *Cedrus* species (cedar) and *Cupressus sempervirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh), and *Papaver somniferum* (poppy juice), all of which are still in use today for the treatment of coughs, colds, parasitic infections and inflammation.[3]

Natural product is compound that derived from natural sources (plant, animals, and microorganism) and has biological activities. The term natural products is commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine.[4] Natural product also refers to substance that occurred naturally, but it is generally taken to mean a secondary metabolite, which is a small molecule that is not involved in the main life processes, such as primary metabolism of the cell.[5] Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. The lack of secondary metabolites more likely will cause impairment of the organism's survivability. While primary metabolites have a key role in survive of the species, playing an active function in the photosynthesis and respiration.[6]

Natural products will continue to play an important role as one of the major sources of new drugs in the years to come. The reasons are because of (i) their incomparable structural diversity, (ii) the relatively small dimensions of many of them (<2000 Da), and (iii) their ''drug like'' properties, that is their ability to be absorbed and metabolized.[7] Based on the information presented on source of new drugs from 1981 to 2007 indicate that almost half of the drug approved since 1994 are based on

natural products.[8] Approximately 10,000 to 15,000 of the world's plant have documented medicinal uses and roughly 150-200 has been incorporated in western medicine.[9,10]

Isolation of natural products from plants, marine organisms and microorganisms is still urgently needed, therefore state-of-the-art methods for separation and isolation procedures is required. Isolation of natural products usually combines various separation techniques, which depend on the solubility, volatility and stability of the compounds to be separated. Taking into consideration that a plant contains thousands of constituents, the separation and isolation process will take long time and tedious.[7]

1.2 *Clinacanthus nutans*

Clinacanthus nutans (Burm.f.) Lindau, commonly in Thai as Phaya Yo, belongs to the family Acanthaceae. This plant is a small shrub and can be found in Thailand, Vietnam, Indonesia, Malaysia, and parts of China. In Indonesia, known as Dandang Gendis and in Malaysia known as Belalai Gajah.[11,12] The common English name is snake grass or snake plant. The taxonomic classification and nomenclature of *C. nutans* is as follow:[13]

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Lamiales
Family : Acanthaceae
Genus : *Clinacanthus*
Species : *nutans* – Lindau

Scientific name: *Clinacanthus nutans* (Burm.f.) Lindau

C. nutans is small shrubs, about one meter tall and erect plant that can grow up to 1 meter in height. The stems are cylindric, striate, and yellow when dry. The leaf has the shape like lance head, narrow and tapering to a pointed apex (2.5- 13 cm long x 0.5 - 1.5 cm wide). Surfaces of leaves are pubescent when young then glabrescent. The length of petiole is 5-7 cm or more (Figure 1.1). The color of the flower is yellow or greenish yellow.[13]

C. nutans is a well-known medicinal plant widely used in Thai traditional medicine. Fresh leaves of *C. nutans* has long been used by traditional doctors to treat skin rashes, insect and snake bite as well as herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions.[14] In Indonesia, they use it for diabetes and dysentery treatment and in Malaysia, they use it as anti-cancer traditionally.[15] Chloroform extract of *C. nutans* possess anti-HSV and anti-oxidant activities. This extract also has anti-proliferative activity against some cancer cell lines.[16] Ethanol extract of this plant showed antioxidant and immunomodulatory activity.[14,16,17] This plant also has antibacterial activity against selected skin pathogen.[12] Investigation of inflammatory activity of *C. nutans* has been conducted by using leaves methanol extract. This extract possessed significant anti-inflammatory activity by inhibition of neutrophil responsiveness and had no effect on neutrophil apoptosis.[11]

1.3 Problem statement

Inflammatory is one of the stages in wound healing. This process is accompanied by the activation of various immune cells such as macrophages, neutrophils, and lymphocytes. Inflammation is a healthy process that can be look as an immunologic mechanism as protective response and produces local clinical and morphological changes.[18]

Wound healing is a complex and dynamic process that aims to restore the integrity and function of the injured tissue through several stages, which are hemostasis, inflammation, proliferation, and remodeling. All those four stages must occur in the proper sequence and time frame. Healing also requires collaborative tissues and cell lineages. Large numbers of cell types—including neutrophils, macrophages, lymphocytes, keratinocytes, fibroblasts, and endothelial cells are involved in this process.[19] Fibroblast plays a major role in proliferation to close the wound.

Researchers have been exploring herbal product for their potential to control biofilm. Biofilm adherence is the key role in the pathogenesis of dental caries and periodontal disease. *Streptococcus mutans* as the primary bacteria that involved in dental caries, inhibiting this microorganism in oral cavity would be the key to prevent the disease.[20] *C. nutans* is natural medicine candidate that could be used for preventing biofilm formation.

People have been using *C. nutans* as traditional medicine, but not for wound healing. *C. nutans* is a natural product that also has potency as an anti-inflammatory drug. In general, the study of *C. nutans* focused on antiviral, anti-inflammatory, antioxidant and anti microbial. Study on anti-inflammatory activity of *C. nutans* has already done by Wanikiat, et al. They used crude leaves methanol extract. Most of the scientist that investigated *C. nutans* used crude extract, except that group who investigated antivirus activity. It was found that chlorophyll related compound is the isolated constituent that responsible for the anti-HSV-1F activity.[14] Anti-microbial of *C. nutans* has been investigated against *S. aureus, E. coli, P. acnes, S. epidermis* and *B. cereus.*[12] Cell migration and anti-adherence activity of *C. nutans* have never been investigated. Therefore, this study provides information on cell migration effect and anti-adherence activity of this plant. For anti-inflammatory activity, the compound that responsible for the activity is still unknown. The present study also provides information on bioactive compound from *C. nutans* that has a role on anti-inflammatory, cell migration effect and anti-adherence activity of *C.nutans*.

A. B.

Figure 1.1 C. nutans (Burm.f.) Lindau. A. The plant, B. The leaves

1.4 Objectives

1. To isolate bioactive compound with anti-inflammatory activity from *C. nutans.*
2. To study inhibition effects of *C. nutans* extract and isolated constituent on nitric oxide production by RAW 264.7 macrophage cells.
3. To study *in vitro* migration effects of *C. nutans* extract and isolated constituent by human gingival fibroblast.

4. To study inhibition effects of *C. nutans* extract and isolated constituent on biofilm formation by *S. mutans*.

1.5 Hypothesis

1. *C. nutans* extract and isolated constituent can reduce nitric oxide production in RAW 264.7 macrophage cells.
2. *C. nutans* extract and isolated constituent have *in vitro* migration effects in human gingival fibroblast.
3. *C. nutans* extract and isolated constituent can inhibit biofilm formation by *S. mutans*.

1.6 Study Design

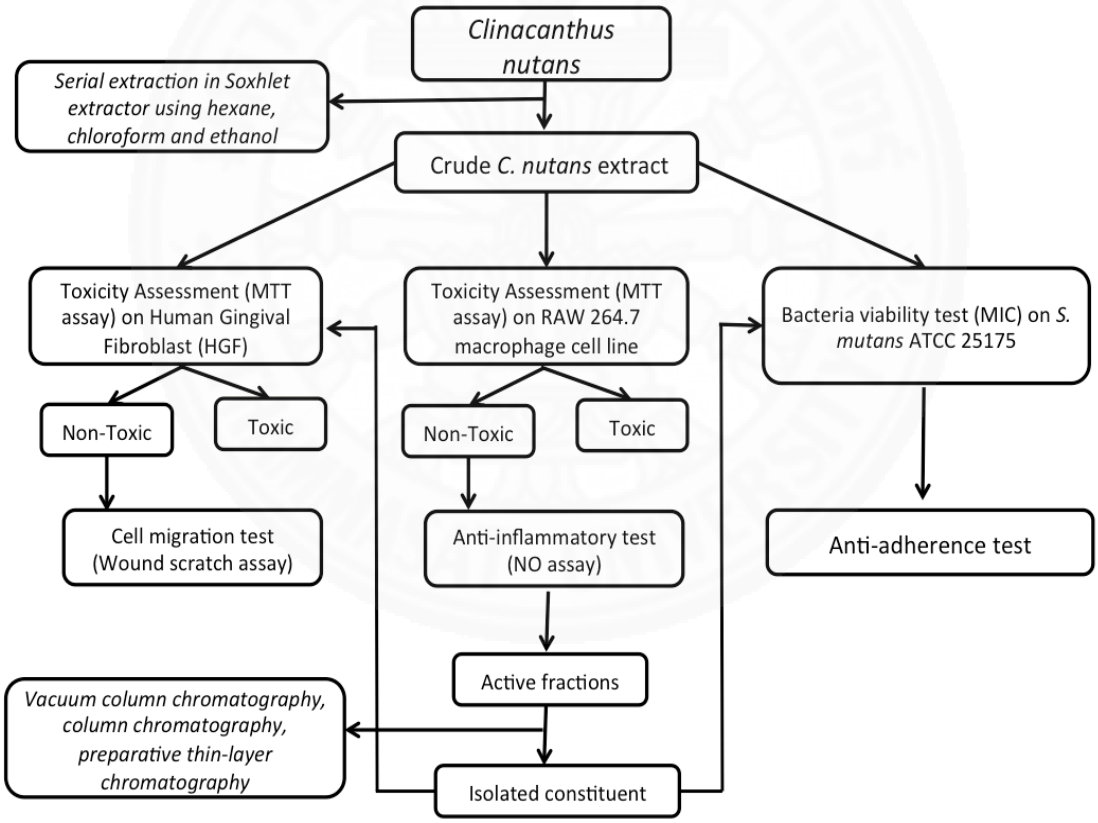


Figure 1.2 Study Design

CHAPTER 2
LITERATURE REVIEW

2.1 Chemical constituents of *C. nutans* (Burm. f.) Lindau.

Numbers of compounds have been isolated from the leaves of this plant. Some chemical constituents and their biological activities are shown in table 1.

Table 2.1 Compounds and activities of *C. nutans*

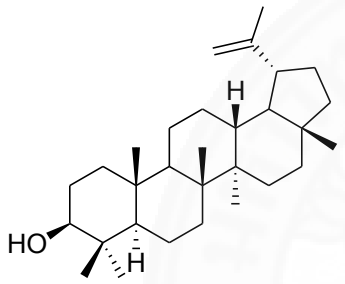
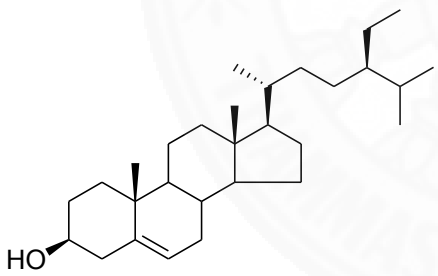
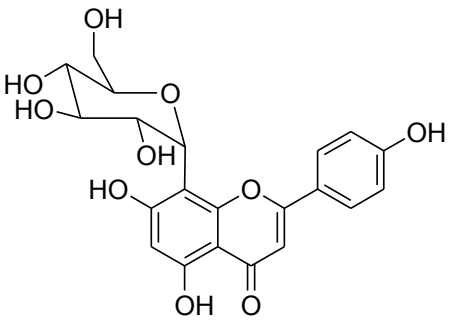
Chemical compound	Biological activity	Reference
Lupeol **[1]** 		[21]
β-sitosterol **[2]** 		[21]
Vitexin **[3]** 		[22]

Table 2.1 (continued)

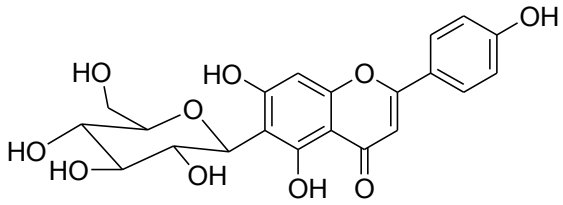
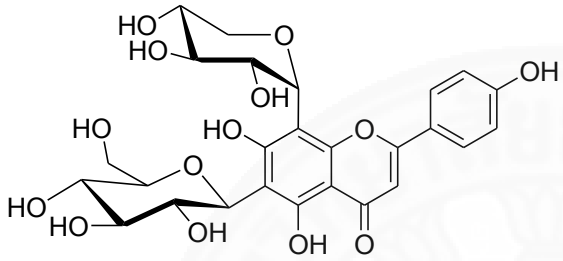
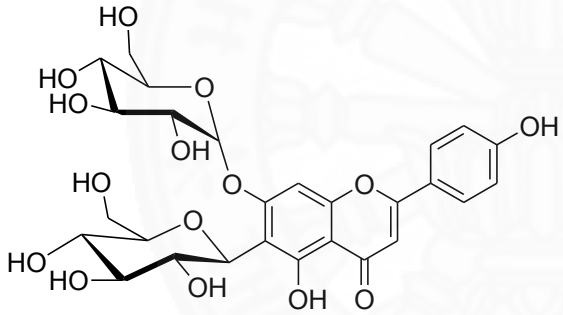
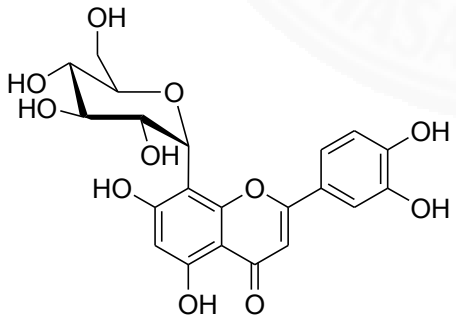
Chemical compound	Biological activity	Reference
Isovitexin **[4]** 		[22]
Shaftoside **[5]** 		[22]
Isomollupentin 7-*O*-β glucopyranoside **[6]** 		[22]
Orientin **[7]** 		[22]

Table 2.1 (continued)

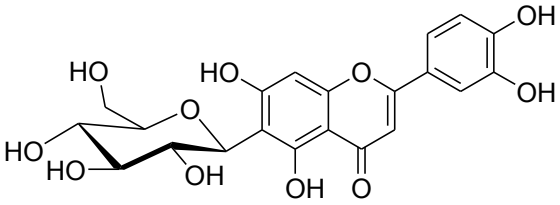
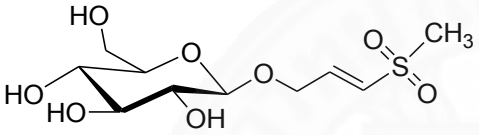
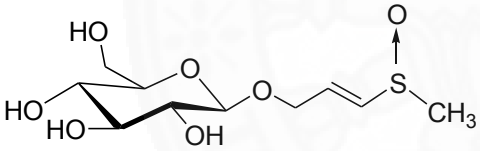
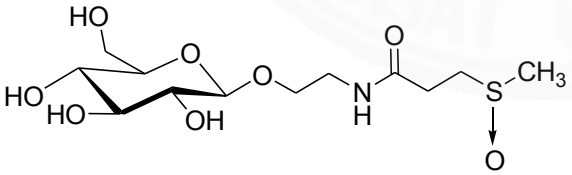
Chemical compound	Biological activity	Reference
Isoorientin **[8]** 		[22]
3-methylsulfonyl-2-propenyl β-D-glucoside/clinacoside A **[9]** 		[23]
3-methylsulfinyl-2-propenyl β-D-glucoside/clinacoside B **[10]** 		[23]
$C_{12}H_{21}NO_8S$/Clinacoside C **[11]** 		[23]

Table 2.1 (continued)

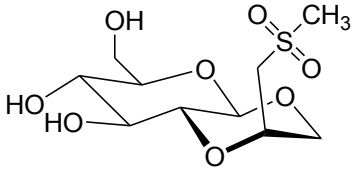
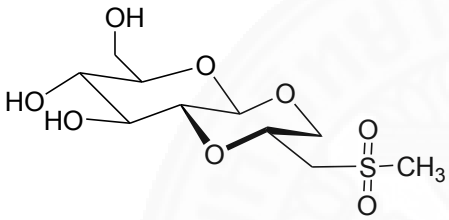
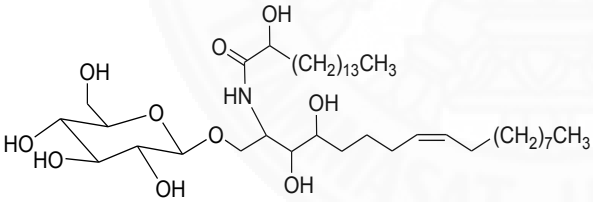
Chemical compound	Biological activity	Reference
$C_{10}H_{18}O_8S$/Cycloclinacoside A1 **[12]** 		[23]
Cycloclinacoside A2 **[13]** 		[23]
1-**O**-b -D-glucosides of phytosphingosines/cerebrosides **[14]** 		[24]

Table 2.1 (continued)

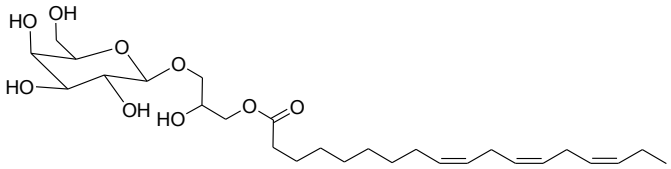
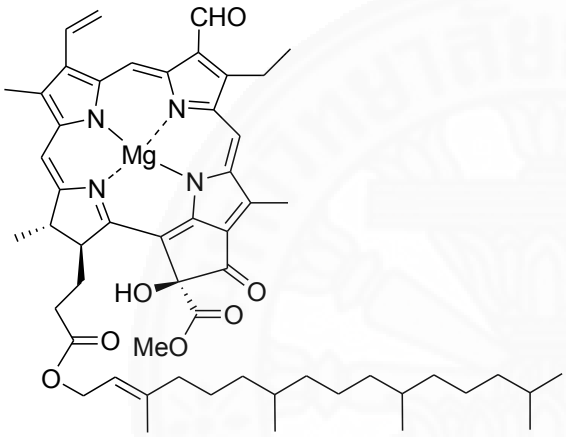
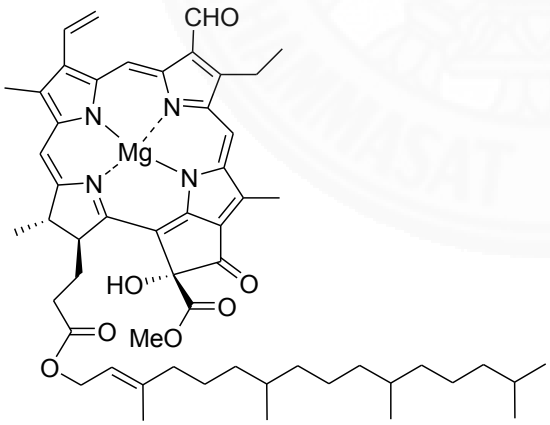
Chemical compound	Biological activity	Reference
(2S)-1-O-Linolenoyl-3-O-b -D-galactopyranosylglycerol **[15]** 		[24]
13^2-hydroxy-(13^2-*S*)-chlorophyll b **[16]** 		[25]
13^2-hydroxy-(13^2-*R*)-chlorophyll b **[17]** 		[25]

Table 2.1 (continued)

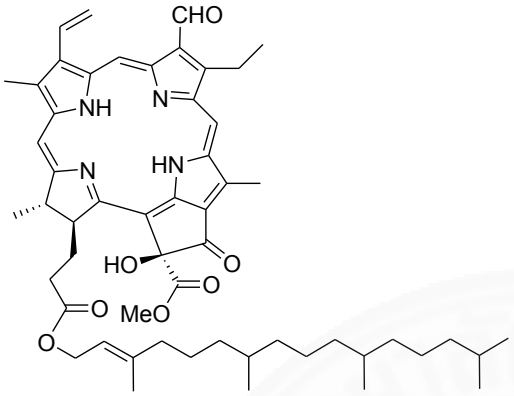
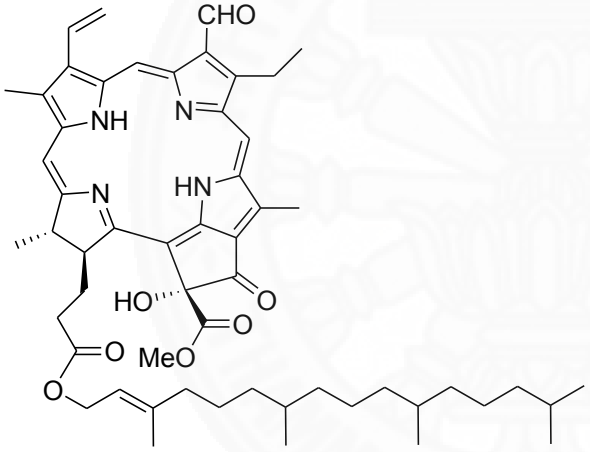
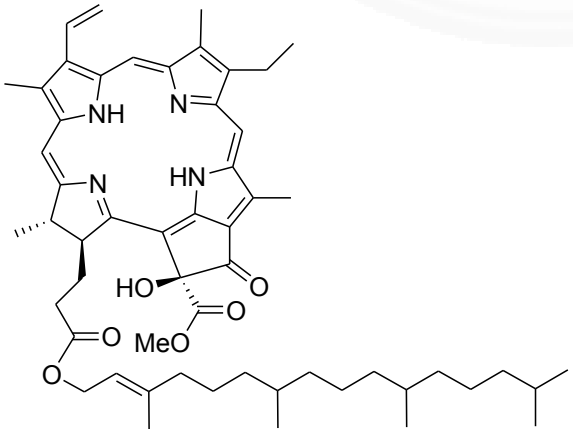
Chemical compound	Biological activity	Reference
13^2-hydroxy-(13^2-*S*)-phaeophytin b **[18]** 		[25]
13^2-hydroxy-(13^2-*R*)-phaeophytin b **[19]** 	Anti-herpes simplex activity	[25]
13^2-hydroxy-(13^2-*S*)-phaeophytin a **[20]** 	Anti-herpes simplex activity	[25]

Table 2.1 (continued)

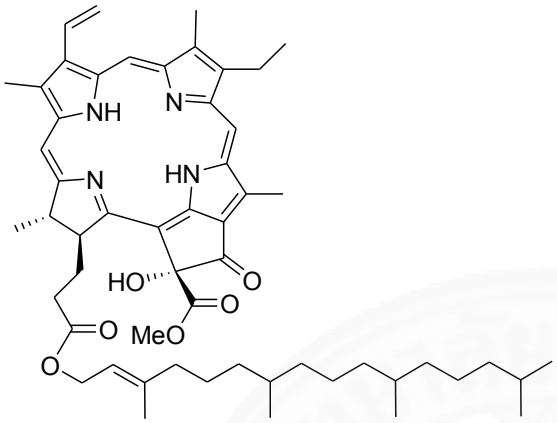
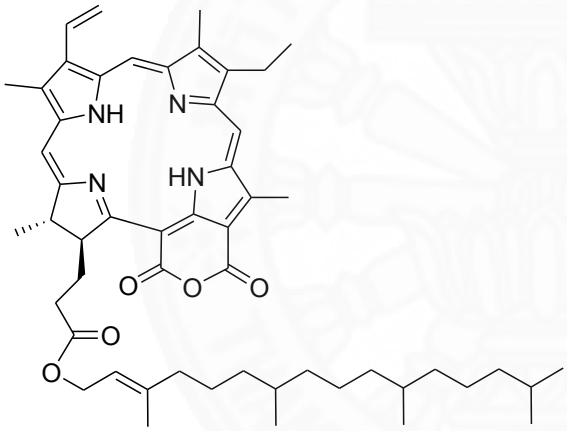
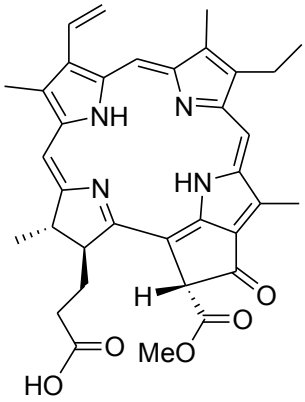
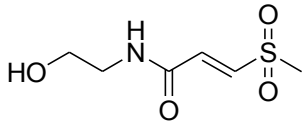
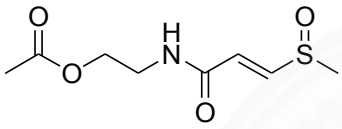
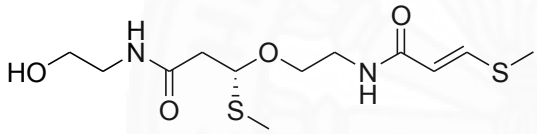
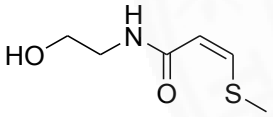
Chemical compound	Biological activity	Reference
13^2-hydroxy-(13^2-*R*)-phaeophytin a **[21]** 	Anti-herpes simplex activity	[25]
Purpurin 18 phytyl ester **[22]** 		[25]
Phaeophorbide a **[23]** 		[25]

Table 2.1 (continued)

Chemical compound	Biological activity	Reference
Clinamides A-C 1 **[24]** 		[26]
Clinamides A-C 2 **[25]** 		[26]
Clinamides A-C 3 **[26]** 		[26]
2-cis-entadamide A **[27]** 		[26]

2.2 Biological activities of *C. nutans*

The biological activities of *C. nutans* that have been investigated are as the following:

2.2.1 Antiviral activity

Crude extract of *C. nutans* leaves was found to inhibit varicella-zoster virus multiplication and herpes simplex virus type-2 *in vitro* and *in vivo*.[27-29] Compound [20], [21], and [22] that were found in chloroform extract of *C. nutans*, which were identified as chlorophyll a and chlorophyll b related compounds, showed inhibitory activities against HSV-1F in pre-viral entry step *in vitro*.[30] The extracellular activity of ethanol extract of *C. nutans* against HSV-2 infected on HEp-2 cells was

investigated in terms of its molecular aspects. The result showed that *C. nutans* extract highly inactivated or inhibit HSV-2 before infection.[15]

2.2.2 Antioxidant activity

Ethanol extract of *C. nutans* has *in vitro* antioxidant activity. The extract demonstrated an inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA). This extract also has protective effect against 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH)-induced rat red blood cell lysis.[31] On experiments on eight human cancer cell lines, including human liver hepatocellular carcinoma (HepG2), human neuroblastoma cell line (IMR-32), human lung cancer cell line (NCI-H23), human gastric cancer cell line (SNU-1), human colon adenocarcinoma cell line (LS-174T), human erythroleukemia cell line (K-562), human cervical cancer cell line (HeLa), human Burkitt's lymphoma cell line (Raji), among chloroform, methanol and aqueous extract of *C. nutans*, chloroform extract showed the most potent in scavenging free radicals and inhibiting the growth of cultural cancer cell line.[16]

2.2.3 Anti-inflammatory activity

Methanolic extract of *C. nutans* was evaluated using rat paw edema model induced by injection of carrageenan and ethyl phenylpropiolate (EPP)-induced rat ear edema model. This study showed that methanolic extract has anti-inflammatory activity by exerted *in vitro* inhibitory effects on neutrophil functional responsiveness without having a significant cytotoxic effect.[11]

2.2.4 Immunomodulatory activity

Ethanolic extract of *C. nutans* was evaluated on a cell-mediated immune response (CMIR) by studying its effects on lymphocyte proliferation, natural killer (NK) cell activity and cytokine production of human peripheral blood mononuclear cells (PBMCs). *C. nutans* was able to enhance lymphocyte stimulation by dose-dependent manner. This ethanol extract also induced IL-4 production of peripheral blood cells, therefore this extract showed a reduction in the function of NK cells since IL-4 has an inhibitory effect on cytotoxicity of NK cells.[17]

Ethanolic extract of *C. nutans* also showed antitumor and immunomodulatory properties on HepA tumor-bearing mouse model. This study revealed that *C. nutans* ethanolic extract could inhibit proliferation of hepatoma cells by underwent apoptosis after treated by a low or high dose of *C. nutans* ethanolic extract.[32]

2.2.5 Antibacterial activity

Antibacterial activity of *C. nutans* was investigated using leaves methanol extract against selected skin pathogens (*S. aureus, E. coli, P. acnes, S. epidermis and B. cereus*).[12] Its positive results may relate to the fact that *C. nutans* contain lupeol, β-sitosterol, flavonoid, and terpenoid. Those compounds reported to having shown antibacterial activity.[33-35]

2.2.6 Analgesic activity

Analgesic activity of *C. nutans* was investigated using leaves n-butanol extract. This group investigated *C. nutans*'s analgesic and anti-inflammatory activity in an animal model. The n-butanol extract reduced writhing and vascular permeability in a dose-dependent manner. The n-butanol extract (270 mg/kg) reduced edema in rat paw as much as aspirin (100 mg/kg). This study concluded that *C. nutans* possesses analgesic activity of the aspirin type instead of morphine type.[36]

2.3 Inflammation

Inflammation is a defense reaction of higher animals to the presence of any injurious stimulus, and the irritant can be physical in nature, such as heat, chemical or bacterial. According to the modern concept, inflammation is a healthy process resulting from some disturbance or disease. Inflammation can be look as an immunologic mechanism as protective response and produces local clinical and morphological changes.[37,38] Immune system's first reaction to irritation and infection is inflammation. The response consists of a vascular and a cellular reaction. These reactions are mediated by chemical factors derived from plasma proteins or cells.[38]

Based on the sequence of event, inflammation can be categorized into two phases, acute and chronic. Acute refers to the response that is abrupt in onset and in short duration, thus, acute inflammation can become chronic if the injurious agent persistent. The acute phase refers to physiological and metabolic alterations that ensue immediately after onset of infection or tissue injury. A variety of changes in the organism act in concert to neutralize the inflammatory agent and foster healing of damaged tissues. Whereas acute inflammation involves exudative reactions, in which fluid, plasma proteins, and cells leave the bloodstream and enter the tissues, chronic inflammation is characterized by infiltration of injured tissue by leukocytes, as well as by proliferative responses, where cells are stimulated to multiply. Chronic

inflammation is the type of inflammatory response that persists for more than a few days or weeks. It may develop in two ways, depending on the nature of the inflammatory stimulus or stimuli involved.[38,39]

Redness, heat and swelling are the main signs that caused by an injury to an organ or tissue. Vascular alteration in injury area is the caused of these signs. Increase in blood flow is the reason of redness and heat. This blood enhancement is the results of vasodilatation, which involving arterioles, capillaries and venules. Vascular permeability alterations is the caused of swelling that leads to fluid exudation, white blood cells and plasma protein. The events of the vascular response to injury are not necessarily in a precise sequence. Several events may be occurring simultaneously or even overlap each other.[38]

2.3.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a molecular component of Gram-negative bacteria that have biological activities. LPS has functioned as a barrier to protect bacteria from its surrounding. Immune system recognized lipopolysaccharide as a marker of bacterial invasion that may cause inflammatory response.[40] Lipopolysaccharide composed of lipid A or endotoxin (hydrophobic domain), core oligosaccharide and O-antigen (distal polysaccharide). Lipid A is the one that responsible for innate immunity activation. Lipid A that similar with the one that found in *E. coli*, is synthesized by many Gram-negative bacteria.[41]

Outer membrane part or cell wall, which is asymmetric, is covering gram-negative bacteria. Ninety percent of the cell surface in its outer leaflet covers by LPS, where the inner leaflet covered by phospholipids. This phospholipid has a resemblance to the composition of the cytoplasmic inner membrane. The function of this membrane is a physical barrier. The composition of LPS is difference between various strains, although they have similar phospholipid composition.[41,42]

In macrophage, toll-like receptors (TLRs), especially TLR4 play role as a receptor of LPS. TLR4 that activate by lipid A triggers synthesis of inflammation mediators.[41,43] The phagocytes of innate immunity are LPS primary cell target. They are monocytes, macrophages, neutrophils, which express the membrane-bound form of CD14 antigen as well as TLR4.[44,45] Toll-like receptors that expressed by macrophage, increased phagocytic activity, such as interleukin 6 (IL-6), interferon-**β**, tumor necrosis factor-alpha (TNF-α), and induction pro-inflammatory proteins synthesis, such as inducible NO synthase (iNOS).[46,47] *In vitro*, LPS activate

mononuclear cells to secrete endogenous mediators, including pro-inflammatory cytokines macrophage migration factor (MIF), IL-1β, IL-6, IL-8, IL-12, IL-15, and IL-18 the colony-stimulating factors M-CSF, GCSF, and GM-CSF, lipid-derived mediators like platelet-activating factor (PAF), prostaglandin E2 (PGE2), leukotrienes, reduced oxygen species like the superoxide anion (O_2^-), hydroxyl radicals (OH) or nitric oxide (NO). Lipid A and polysaccharide region of LPS also activate complement system (classical and alternate) by release of anaphylatoxins C3a and C5a.[48]

Inflammatory mediators and cytokines that produce by macrophage participate in control of the spread of pathogens. Abundant and uncontrolled inflammatory mediators and cytokines may cause systemic complication such as tissue damage, septic shock, and dysfunction of microcirculatory.[49]

Besides TLR4, there are two other proteins that play role in recognition of LPS on macrophage they are CD14 and MD-2 (myeloid differentiation-2). LPS transferred to CD14 by LBP serum, then CD14 presents it to TLR4-MD-2. Regulation of cellular distribution of LPS to TLR4 is mediated by MD-2. TLR4 is also responsible as a signal-transducing receptor for LPS. There are three other proteins that functioned as adaptor they are MyD88 (myeloid differentiation factor88), TIRAP (TIR domain-containing adaptor protein), and TRIF (TIR domain-containing adaptor inducing IFN-β) that play a role as TLR4 signaling mediator (Figure 2.1).[49]

After recruiting MyD88 and IRAK-1 (IL-1 receptor-associated kinase-1) and IRAK-4 to the membrane, TLR4 activates intracellular signaling cascade. Then IRAKs activates TRAF6 (TNF receptor-activated factor 6) that causes activation of IKK (IκB kinase) complex and MAPK (mitogen-activated protein) kinase. Through JNK (c-Jun N terminal) kinase and p38 MAPK, MAP kinase activates transcription factor AP-1 (activator protein-1). IKK complex that activated by TRAF6, will cause degradation of IκB and release transcription factor NF-κB (nuclear factor-κB). This NF-κB would

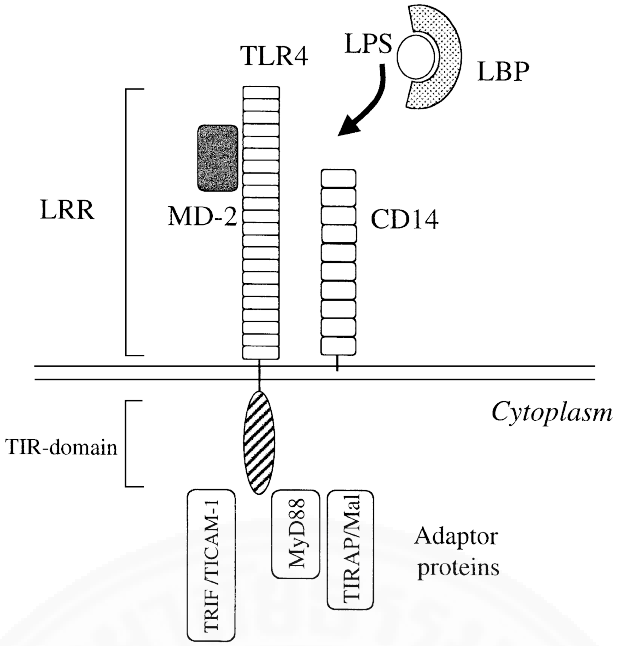


Figure 2.1 LPS receptor complex on macrophage [49]

transcript inflammatory cytokines. TLR4 activation by LPS also activates IRF3 (IFN regulatory factor) phosphorylation and nuclear translocation. This activation is independent of MyD88, and it up-regulates IFN-β (Interferon-β). This IFN-β activates STAT1 (signal transducer and activator of transcription-1) and effecting IFN-inducible genes. In this pathway, there is another protein that involves, which is TRIF (TIR domain-containing adaptor inducing IFN-β) or TICAM-1 (TIR domain-containing adaptor molecule) (Figure 2.2).[49]

2.3.2 Macrophages

Mononuclear phagocytes originate from bone marrow, and they remain in the bone marrow for only short period of time (<24 hours) and then migrate to the peripheral blood. After leaving the circulation, monocytes differentiate into macrophages in tissues and organ where they remain for days before being replaced by influx monocytes and to a less extent by locally dividing macrophages.[50]

During inflammation, the number of monocytes in circulation increases. The increase of monocyte production results from the temporal shortening of the cell cycle time in promonocytes in the bone marrow. There is also an increase in the local production of macrophages at the site of inflammation. Although, it depends on the kind of inflammatory stimulus.[50]

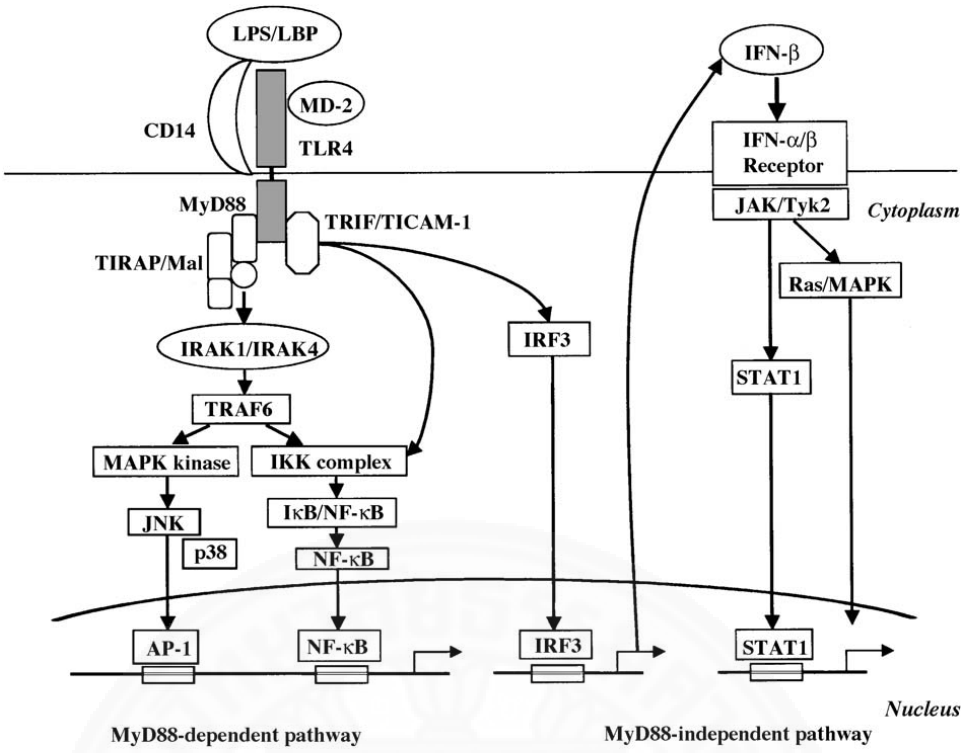


Figure 2.2 Macrophage's response to LPS mediated by TLR4 [49]

Phagocytosis is the main function of macrophage during innate immune responses. Macrophage's detection of pathogen-associated molecular patterns (PAMPs) during infection leads to cytokines and chemokines production, other cells recruitment and triggered an immune response. Macrophages can propagated or immortalized adaptive immunity by phagocytes antigens and presenting them to T cells. By engulfing and phagocytosis antigens or bacteria, macrophages prevent further stimulation of immune system, resolve inflammation and sterilize tissue.[50,51]

Macrophages have another important function, which is responsible for efferocytosis, the process of engulfing and eliminating apoptotic cells. If efferocytosis does not exist, apoptotic bodies destroyed and their intracellular contents will be released. This event called as secondary necrosis, which lead to another inflammation and contributed to autoimmunity.[52]

Although efferocytosis reflects phagocytosis, it is a different process, mediated by different receptors, downstream signaling pathways and bridging molecules.[50] Macrophages also promote the return to hemostasis by removal of apoptotic cells and cell debris, and by contributing to every stage of damage repair.[53]

Inflammation is accompanied by the activation of various immune cells such as macrophages, neutrophils and lymphocytes. Macrophages play an important role in control of inflammation and immune response and are involved in various diseases including autoimmune disease, inflammatory disorder and infections.[54-56] Macrophages are remarkably versatile cells, in inflammatory and cell-mediated immune responds, macrophages perpetuate inflammation and can cause destruction, loss of function and scarring. Besides that, macrophages are also essential for wound repair, angiogenesis, antigen presentation and defense against microorganisms and tumors.[54,57] Activated macrophages secrete many inflammatory mediators that essentials for host survival and are also required for tissue injury repair. [58]

Macrophage can kill pathogens directly by phagocytosis and indirectly via the secretion of various pro-inflammatory mediators, reactive oxygen species, metalloproteinases, cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6).[59,60] Overproduction of the inflammatory mediators by activated macrophages has been implicated in the pathophysiology of many inflammatory diseases.[61] Inflammatory mediators such as nitric oxide (NO), prostaglandin (PGE_2), which are produces by iNOS and cyclooxygenase (COX-)2 proteins, respectively, as well as inflammatory cytokines such as TNF-α and IL-6.[58,59,62,63]

2.3.3 Nitric Oxide

Nitric oxide (NO) is a pleitropic, short-lived free radical that participates in diverse biological processes such as the regulation of blood vessel and airway tone, inflammation, neurotransmission, and apoptosis. It is widely utilized as a signaling molecule in cells throughout the body, carrying out numerous roles. In general NO will cause local vasodilation and increasing oxygen delivery.[64-67]

Nitric oxide is synthesized from L-arginine and molecular oxygen by an enzymatic process that utilizes electrons donated by NADPH. The NO synthase (NOS) enzymes convert L-arginine to NO and L-citrulline via the intermediate N-hydroxy-L-arginine. One molecule of L-arginine produces one molecule of NO, the nitrogen atom of the latter deriving from a terminal guanidino group of the arginine side chain.[68]

There are three distinct enzyme isoforms that synthesize NO. First is the endothelial NO synthase (eNOS), which was the first to be identified in the vascular endothelium. Second is enzyme in the neuronal tissue, mainly in the brain, is known as neuronal NO synthase (nNOS). Third is an inducible NO synthase (iNOS) that was identified in macrophages and is not present in non-activated cells, can be generated de novo when white cells are incubated with lipopolysaccharide or with certain cytokines. The eNOS is encoded by gene present on chromosome 7, nNOS is encoded by gene present in chromosome 12, and the iNOS is encoded by gene on chromosome 17. Endothelial NO synthase relates to physiology and pathophysiology of the pathway in the cardiovascular system, nNOS has role in the central and peripheral nervous tissue, while iNOS has role in immunology and inflammation.[60,66]

Inducible NO synthase (iNOS) was originally identified in circulating active macrophages. To be active, macrophages have to be stimulated by lipopolysaccharide or certain cytokines. Nitric oxide that produced by macrophages is in large quantities and for very long periods. This iNOS in inflammatory cells uses NO as a cytostatic and cytotoxic agent.[69] Many cells express iNOS, including fibroblast, endothelial and epithelial cells, keratinocytes and chondrocytes, monocytes/macrophages, antigen-presenting cells, and natural killer (NK) cells.[68,70-72]

Nitric oxide is a short-lived gas that can diffuse freely through cells, and whose effects can be propagated via the interaction with thiol groups on cysteines and glutathione, or protein heme groups.[73,74] Nitric oxide has long been recognized as an important molecule involved simultaneously in the regulation of apoptotic death and cell viability by influencing on mitochondrial function.[61,75,76] Nitric oxide competes with oxygen for substrate binding sites in several enzyme components of the bioenergetics pathways, and also affects catalytic activity by forming complexes with heme and iron-sulfur clusters present in many mitochondrial proteins. Nitric oxide has the ability to directly diminish the mitochondrial inner membrane potential and to induce swelling in isolated mitochondrial.[77-79]

Nitric oxide is a reactive molecule that has a variety of effects depending on the relative concentrations of NO and the surrounding milieu in which NO is produced. Molecule of NO itself can mediate the direct effect. While interaction of NO with superoxide anion or with oxygen that produced reactive nitrogen species act as mediator of NO's indirect effect. Interaction of NO with

soluble guanylate cyclase that produced cGMP is the mediator of many NO's physiological effects.[80]

There are many evidences that NO is involved in several inflammatory disorders. It has been shown that NO can be pro-inflammatory (immunostimulatory, anti-apoptotic) or anti-inflammatory (immunosuppressive, pro-apoptotic), host-protective or host damaging during infections. For these reasons, NO has been described as "double edge sword mediator" and this phenomenon are often referred to as the NO paradox.[81] Nitric oxide is pro-inflammatory at low concentrations by inducing vasodilation and the recruitment of neutrophils, whereas at high concentration it down regulates adhesion molecules, suppresses activation and induces apoptosis of inflammatory cells.[68,82] Nitric oxide produced in controlled manner plays an important role in many aspects of mammalian physiology. However, excessive production of this highly reactive small molecule is potentially toxic. Nitric oxide has been implicated in number of pathophysiological conditions in human as well as in animal models. For example is the documentation of increased NO formation in a number of chronic infection or inflammation conditions in humans, including those which have been linked to higher cancer risk.[83]

The expression of iNOS has been shown in various mouse and human cells, there are marked cell type and species specific differences in the responsiveness of iNOS expression to different stimuli.[84] Responses in human cells seem to be different from those in mouse cells, which have been used widely in the studies on iNOS expression. Many mouse cells readily express iNOS in response to LPS or to a single cytokine, whereas human cells usually require a combination of different cytokines for detectable iNOS expression and NO synthesis. Furthermore, iNOS is expressed at high levels in activated murine macrophages, but it has been difficult to induce iNOS in human macrophages *in vitro*, although iNOS expression in macrophages in inflamed human tissue has been shown in *ex vivo* studies.[85-87]

Various signal transduction pathways have been suggested to regulate iNOS expression. The importance of pathways leading to the activation of transcription factors NF-κB and Stat1 have been proposed. cAMP activating compounds can both enhance and inhibit cytokine induced iNOS expression.[88-91] The role of mitogen-activating protein kinases in the regulation of iNOS expression has been investigated. Extracellular signal-regulated kinase 1 and 2 have been shown to

up-regulate[92,93] or to have no role in iNOS expression.[94,95] Also p38 MAP kinase has been reported to up-regulate,[96,97] down-regulate,[98,99] or to have no role in iNOS expression.[100,101] Activation of JNK up-regulates iNOS expression.[102,103]

2.4. Wound healing

Wounds have been with mankind from the prehistoric beginnings. It is often referred to as the world's oldest medical manuscript (written in 2100 BC), 3 gestures were recommended for the treatment of wounds: washing, making plasters, and bandaging.[104] Numbers of plants were discovered by primitive peoples in various parts of the world as a wound treatment. Herbs could either be applied to the wound in a balsam or given as a draught.[105]

Wounds come from pathologic processes that begin internally or externally to the involved organ that caused accidentally or intentionally or be the result of a disease process.[106] Wounding disrupts the local environment within the tissue, which causes in bleeding, contraction of vessels, coagulation, activation of tissue complement, and other inflammatory responses.[107] Wound, which is disturbed state of tissue can also cause by physical, chemical, microbial or immunological insults, or typically associated with loss function. According to the wound healing society, wounds are physical injuries, that results in an opening or break in the skin that causes disturbance in the normal skin anatomy and function.[108]

The wound healing process aims to restore the integrity and function of the injured tissue through several overlapping stages: hemostasis, inflammation, proliferation, and tissue remodeling.[109,110] Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin.[111] The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part.[112] Healing requires collaborative tissues and cell lineages also involve platelet aggregation and blood clotting, the formation of fibrin, an inflammatory response to injury, alteration it ground substances, angiogenesis, and re-epithelialization.[111]

Research on wound healing agents is one of the developing areas in modern biomedical sciences.[111] The progress in this field has allowed the synthesis of large numbers of molecules associated with wound repair process. Delivery of growth

factors in order to mimic the natural microenvironments of tissue formation and repair is believed to be therapeutically effective. Despite finding new methods of stimulation of the wound repair process, wound care has returned to the roots of medicine with some of the remedies used millennia ago. Plant-derived natural products are significant as sources of medicinal agents and models for the design of new remedies.[113] As plants are a source of many bioactive compounds and many plant ingredients are traditionally used to accelerate healing, scientists go back to traditional folk medicines as they are generally characterized by high acceptability and good

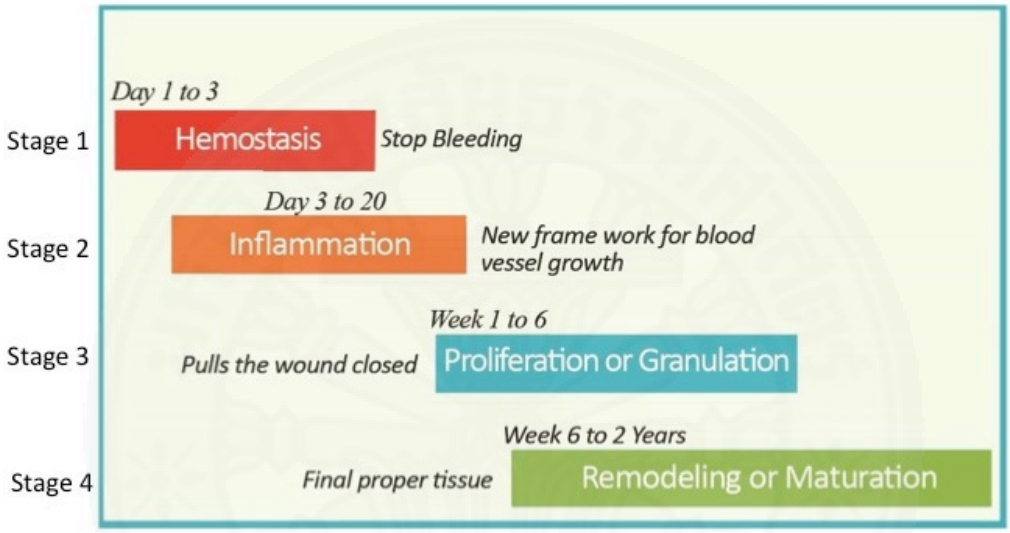


Figure 2.3 *Four stages of wound healing*

toleration.[114]

2.4.1 Process of wound healing

Skin wound healing is a highly regulated process of cellular, humoral and molecular mechanisms. The closure of the wound only can be managed by regeneration and repair. The process of wound healing depends on many factors, including cells, growth factors, and cytokines.[110] However, when there is disruption between cells and mediators, the deficiency of cell type or the absence of a mediator can be compensated by other cells or mediator that are involved in wound healing process, so that the repair can still occur.[115] There are several stages in the process of acute wound healing (Figure 2.3).

2.4.1.1 Hemostasis stage

This stage occurs immediately after injury, and dedicated to hemostasis and the formation of a provisional wound matrix.[110] The aim of these

mechanisms is to protect the vascular system, keeping it intact, so that the vital organs remain unharmed despite the injury. Another aim of these mechanisms is to provide a matrix for invading cells that are needed in the later stage of healing.[104,116] Bleeding that occurs when injury, not only serve as hemostasis, but also to flush microorganisms or antigen from the wound.[108] The clotting cascades are then initiated by clotting factors from injured skin, and thrombocytes get activated for aggregation by exposed collagen.

Within 24 hours, platelets start to aggregate by binding to collagen that exposed due to rupture of endothelial vessels. Active bleeding will be limited by fibrin clot forming. Fibrin clot also serve as scaffold for cells recruitment, such as; leukocytes, keratinocytes, fibroblasts and endothelial cells.[110] Additionally, it serves as reservoir of several growth factors and cytokines that are release as activated platelets degranulate.[113]

There are many molecules that involved as bleeding control and limit the extend of injury, such as Insulin-like and epidermal growth factors (IGF, EGF), Fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF), and Transforming growth factor alpha and beta (TGF-α,β). These molecules are also act as promoters of wound healing cascade by activation and attraction of neutrophils, macrophages, endothelial cells and fibroblast.[113]

2.4.1.2 Inflammation stage

Inflammation is the second stage of wound healing. This stage is triggered by mediator released from injured tissue and capillaries, activated platelets and their cytokines, and by products of hemostasis.[108] Besides that, due to the response of the activated complement pathway, neutrophils are recruited to the site of the injury and are present for 2–5 days unless the wound gets infected. Neutrophil's ability in phagocytosis and protease secretion kills local bacteria and helps to degrade necrotic tissue make this cell is crucial within the first day of after injury.[110]

Three days after injury, macrophages enter the wound site and performing phagocytosis of pathogens and cell debris as well as secreted growth factors, chemokines and cytokines.[110,117] Macrophages attracted by clotting factors, complement components, PDGF, TGF-β, leukotriene B_4, platelet factor IV, and elastin and collagen breakdown products appear in the wound and continue the

phagocytosis. Macrophages synthesizes a variety of cytokines including growth factors that involved in the migration, proliferation, and organization of new connective tissue and vascular beds within the wound.[108,118] Lymphocytes are the last cells to enter the wound site in inflammation stage. These cells arrive in wound site 72 hours after injury attracted by interleukin-1 (IL-1), complement component and immunoglobulin G (IgG) breakdown products.[119]

2.4.1.3 Proliferative stage

Third stage of wound healing is proliferative stage. It starts on the third day after injury and last for about 2 weeks.[116] In this stage, the mechanisms are purpose to cover the wound surface with new skin (re-epithelialization), restoring vascular integrity to the region (neovascularization), and repairing the structure integrity of the tissue defect by filling it with new connective tissue (granulation).[108] Fibroblasts constitute the predominant cell type in granulation tissue.

Keratinocyte is the cell that most responsible in re-epithelialization. After keratinocyte cover the surface of skin defect, it undergoes intense mitotic activity along the wound edges. Cells migrating across the wound attach to the provisional matrix below. Migration requires a fluid movement and involves complex series of steps controlled by a chemotactic gradient generated by various growth factors.[108,116]

Establishment of new blood vessels (angiogenesis) is critical in wound healing and takes place simultaneously with all stage of reparative process.[116] The first step in new vessel formation is the binding of growth factors to their receptors on the endothelial cells of existing vessels, which will activate intracellular signaling cascades. Activated endothelial cells secrete proteolyitic enzymes to dissolve the basal lamina. Thus, the endothelial cells are able to proliferate and migrate into the wound site. In order to open space for the proliferating cells, local degradation of the basement membrane and extracellular matrix is induced. This process is known as sprouting.[104,110]

Angiogenesis is stimulated by growth factor and tissue hypoxia.[120] A hypoxic wound environment is created following the closure of the wound surface by fibrin clot. Closure of the wound surface is necessary to create a hypoxic wound environment. The fibrin clot formed during hemostasis provides a temporary cover that creates a closed system in which angiogenesis can proceed. The hypoxic conditions are thought to induce macrophages to secrete angiogenetic factors

such as basic fibroblast growth factor (bFGF or FGF-2) and acidic FGF (aFGF or FGF-1) that are released immediately after cell disruption.[108,121]

Development of acute granulation tissue is the last step in proliferation stage. This granulation tissue development is characterized by high density of fibroblast, granulocytes, macrophages, capillaries and loosely organized collagen bundles. It called granulation tissue because of it has high amount of cellular compounds and highly vascular. That is the reason why it appears redness and easily traumatized.[110]

Fibroblasts migrate into the wound site attracted by factors such as TGF-β, PDGF, and Fibroblast growth factor (FGF), which are released by inflammatory cells and platelet.[122] Fibroblasts are responsible for the synthesize, deposition, and remodeling of the extracellular matrix.[123] At the wound site, fibroblasts proliferate profusely and produce the matrix protein hyaluronan, fibronectin, proteoglycans and type 1 and type 3 procollagen.[104,124] The influx of fibroblast causes the provisional matrix of fibrin/fibronectin to be degraded and replace with new matrix. This new matrix provide a scaffold for cell migration and organization.[108]

Subsequently, fibroblasts change into their myofibroblast phenotype, which involves formation pseudopodia capable of attaching to fibronectin and collagen in the extracellular matrix (ECM). Myofibroblast contain contractile protein such as actin, and these cells are arranged in densely packed group. With this arrangement, allows the myofibroblast to pull wound edges together through contraction process. Contraction decreases the size of the wound and reduces the amount of extracellular matrix that needed to repair the wounds, this is why contraction will decrease healing time.[108]

2.4.1.4 Remodeling stage

Remodeling or maturation of granulation tissue into mature connective tissue is the final stage of wound healing. This stage occurs from day 21 to up to 1 year after injury. During this stage, all of the processes activated after injury wind down and cease. Most of the endothelial cells, keratinocytes, macrophages and myofibroblast undergo apoptosis. These cells leaving a mass that contain few cells and consists mostly of collagen and other extracellular matrix proteins.[108,116,121]

Collagen is first released in precursor form as a triple helix

protein called procollagen. Then this procollagen will formed into fibers, which are in parallel fashion arrangements and cross-linked in order to form stronger and thicker strands. The new connective tissue is not as well anchored to the underlying connective tissue matrix and is thicker than normal skin.[108]

The angiogenic process diminishes, the wound blood flow declines, and the acute wound metabolic activity slows down and finally stops. Remodeling is regulated by fibroblast through the synthesis of new collagen and the degradation of old. Fibroblast regulates remodeling by synthesizing extracellular matrix component and matrix metalloproteinases that control cell differentiation.[108]

2.5 Biofilm in Oral Cavity

Dental caries is the most common disease in oral cavity, which caused by biofilms that formed by microorganism on the teeth and gum surface. *S. mutans* is the principal etiological agent of the disease.[125,126] It is important to understand the mechanism of *S. mutans* activity in forming a biofilm, which can be applied for prevention, early diagnostic and finding a compound that can inhibit the forming of biofilm.[125] *Streptococcus* has a long filamentous structure that shows adhesive properties that probably act as the important role in attached to host tissues. This structure also plays key role in the formation of microcolonies on host surface and aggregation of the bacteria itself, especially when influenced by human saliva. These are one of the reasons of *Streptococcus* pathogenicity.[127]

Biofilm formation starts with coating tooth surface through the salivary pellicle that formed by salivary components, such as amylase, histatin, mucin, peroxidase, lysozyme that specifically adsorbed to the Acquired Enamel Pellicle (AEP).[128] AEP is protein that attached to enamel surface and forming a thin protein layer. Microorganism used AEP as the basis of colonizing formation in oral cavity. Connection between hydroxyapatite on tooth surface and oral microbial biofilm is formed by AEP. This biofilm is one of the principal key of caries disease development.[129]

There are two mechanisms of *S. mutans* fuse with AEP, which are sucrose-dependent and sucrose-independent.[126] Sucrose-dependent mechanisms rely on glucosyltransferase (Gtf-B, -C, and –D) enzyme that produced by *S. mutans*. Gtf play important role in the establishment of glucan from sucrose. Glucan makes bacterial adhesion to tooth surface possible.[128] There are three genetically different

GTFs, and they all have important part in dental plaque formation. The three GTFs are GtfB, GtfC and GtfD. GtfB (GtfI) synthesizes insoluble glucan rich in α-1,3-linkages, which play role in interaction with other S. mutans. GtfC (GtfSI) synthesizes mixture of soluble (with mostly α-1,6-linkages) and insoluble glucans. This type of Gtf has a hydrophobic domain that enables the interaction with saliva protein in the pellicle. GtfD (GtfS) produces predominantly soluble, quickly metabolizable glucan.[130,131]

GtfB and GtfC expressed in response to glucose and sucrose situation in environment. Besides that, there are other factors that influence the expression. Such as RegM, luxS (AI-2 autoinducer-coding synthesis), ropA and VicRK signal transduction system. RegM is a protein that regulates *Streptococcus* catabolism. Deletion of this gene will decrease expression of GtfB and GtfC. While ropA gene play role in production of GtfB and GtfD regulation and VicRK influence the expression of Gtfs physiologically.[126,132,133] Carbohydrate availability, pH environmental and growth rate are also factors that affect Gtf gene expression.[134]

Bacteria to glucan binding is mediated by Gtf enzymes and glucan binding protein (Gbps). There are four different proteins, GbpA, GbpB, GbpC, and GbpD. They all support bacterial adhesion and forming a biofilm. GbpC play role as a receptor for glucan, which is why, it is related to bacterial cell wall.[128,132] The attachment of S. mutans to tooth surface is mediated by glucan and as time goes by, that attachment becomes stronger.[135] Glucan in pellicle also increases the binding of several oral microorganisms. Besides that, glucan also enhanced mechanical stability by binding bacteria together and to apatite surface, resulting them to stay adhere in tooth enamel for long period of time.[128]

The second mechanism is sucrose-independent. This mechanism involves agglutinins found in saliva. There are genes that play role as anchorage in the bacterial cell, which are AgB, SpaB and Pac1 adhesin (Antigen I/II family), which also known as multifactorial PI adhesin. These genes identified on the surface of *S. mutans* and other microorganism such as *Streptococcus pyogenes, Streptococcus agalactiae,* and *Streptococcus suis.*[136] Glycoprotein-340 (gp-340) in saliva interacts with these genes that caused bacteria aggregation. This gp-340 has the ability to be adsorbed on the teeth or gum surface, and act as initiator of bacterial adhesion process. Location of PI adhesin in *S. mutans* is important in interaction with saliva agglutinin. And not only that, interaction between bacteria also influence by Antigen

I/II protein. Saliva agglutinin and other salivary proteins, such as mucin and proline-rich protein, are the major component for adhesion and biofilm formation at early stage.[126,137] In addition, the environment of where the *S. mutans* growth and the existence and interaction with other bacteria influenced the virulence and biofilm formation of *S. mutans.*[132]

The composition of biofilm that formed on the tooth surface are glucan (10–20 % of dry weight), fructan (1–2 % of dry weight, depending on the last intake of food), proteins (40 % dry matter, mostly derived from bacteria and saliva) and variable amounts of lipid, Ca, P, Mg and F compared with surrounding saliva. Dental plaque in situ harbors approximately 80% water.[128,138] There is a physical and biochemical matrix in the biofilm structure that function as source of energy and provide adhesion and promote cohesion for microorganism. This membrane prevents incursion of substances from outside, such as antibiotics, and also provide ideal condition for bacterial survival, so that there is a limited diffusion to and from the biofilm.[125,128]

2.5.1 Anti-biofilm activity in natural product

Natural product as biofilm inhibitor has been extensively studied. The diversity of their molecule structure with specific activities made all the researchers find them attractive as new major drug discovery. Polyphenols are one of the secondary metabolite from natural products that have anti biofilm activity by several mechanisms. One of them is by inhibiting Gtf activity directly.[139-141] Without Gtf activity, plaque would be easily be cleaned by mechanical movement in oral cavity. Another advantage of targeting to inhibit Gtf is their potential to have anti-adherence activity without being anti-microbial, therefore it would prevent biofilm with minimum side effect on the ecological balance in terms of microbial community.[142] Fraction of oolong tea that has polymeric polyphenols showed inhibition of glucan that synthesis from GtfB and GtfD *S. mutans.* The mechanism they found that by inhibition of C-terminal glucan-binding domain of GtfB and GtfD.[143]

Another secondary metabolite from natural product that reported to have anti-adherence activity is flavonoids. Study showed that specific flavonoid such as myricetin and kaempferol, and flavones found in *Apis melifera* propolis have inhibitory effect of Gtf, especially GtfB and GtfC.[144] Proanthocyanidin oligomers from cranberry extracts also showed inhibition of glucan synthesis by Gtf. The

mechanisms that proposed from this study are disruption of acidogenic/aciduric properties and increase of *S. mutans* biofilm detachment.[145-147]

Active constituent of *Azadirachta indica*, which is gallotannin showed inhibition of adhesion to hydroxyapatite and production of insoluble glucan that synthesize by Gtf by *S. sanguis*.[148] Gallotannin from ethanolic extract of *Melaphis chinensis* also showed inhibition of Gtf by more than 91% at concentration as low as 7.9 μg/mL.[149]

Murata and co-workers studied isolated compound from *Rheedia gardneriana,* 7-epiclusianone. This compound showed properties to reduce biofilm formation and accumulation from *S. mutans*. The mechanism was by inhibition of glucan synthesis by GtfB and GtfC by up to 80% inhibition at concentration 100 μg/mL.[150]

One study showed that flavonoid found in apples, phloretin, has the ability to regulate formation of *E. coli* biofilm by inhibiting the production of fimbriae. Fimbriae required for biofilm formation. Phloretin was able to interfere biofilm formation without affecting the growth of planktonic cells and also without disturbing commensal flora.[151] Ding and co-workers studied emodin, which is anthraquinone that can be found in roots and barks of various plants. They reported that emodin have the properties to inhibit biofilm formation of *P. aeruginosa* and *S. maltophilia* at concentration 20 μg/mL.[152]

Herbal product has been investigated for their potential to control biofilm formation. Some toothpaste and mouthwashes have been added with plant extract to prevent biofilm formation in oral cavity. Therefore, natural medicine is a promising agent to investigate in the field of oral disease prevention.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

1. Aluminum Foil
2. Cell Scaper, Costar®, Corning, USA
3. Centrifuge Tube 15, 50 ml, Corning, USA
4. Chromatography Paper 3 mm CHR, Whatman®, UK
5. Culture Dish, 35Å~10, 100Å~20 mm, Corning, USA
6. Culture Flask 25, 75 Sq cm., Corning, USA
7. Culture Plate 6-well, Costar®, Corning, USA
8. Culture Plate 96-well, NUNC, Thermo, USA
9. Cyogenic Vial Non-pyrogen Polypropylene 2 ml, Costar®, Corning, USA
10. Disposable Syringe 5 ml, 10 ml, Nipro, Thailand
11. Disposable Polystyrene Pipette Tip 10, 200, 1000 µl, Costar®, Corning, USA
12. Dried *Clinacanthus nutans*
13. Filter 0.2 µm, Minisart, Sartorious Stedium, Germany
14. Filter Paper 55, 150 mm, Whatman®, UK
15. Microcentrifuge Tube, Rnase&Dnase Free 0.65, 1.7 ml, Costar®, Corning, USA
16. Parafilm®M Barrier Film, Structure Probe Inc., USA
17. Reagent Reservoir 50 ml, Costar®, Corning, USA
18. Silica gel 60 PF_{254} Merck®, Germany
19. Silica Gel 60 F_{254}, Precoated Plate, Aluminum Sheet, Merck®, Germany.
20. Silica Gel 60 Particle Size 63-200 µm (70-230 mesh ASTM) Merck®, Germany
21. Surgical Blade No. 15, Feather, Japan
22. Thimble Filter 73Å~100, Advanctec, MFS Inc., USA
23. Transfer Medium Pure Nitrocellulose Membrane 0.45 µm, Trans-blot®, Bio-Rad, USA

3.2 Chemicals

1. 0.25%Trypsin-EDTA, Gibco® Invitrigen™, USA
2. 28% Ammonium Hydroxide, BDH®, UK
3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma®, USA
4. Agarose, Low EEO, Research Organics, Inc., USA
5. Ammonium Persulfate (APS), BDH®), UK
6. Amphotericin-B, PAA, Austria
7. Anisaldehyde (4-Methoxybenzaldehyde), Sigma®, USA
8. Anti-bump, Fisher Chemicals, UK
9. Bovine Serum Albumin (BSA), PAA, Austria
10. Chloroform, BDH®, UK
11. Crystal violet, BDH®, UK
12. Dulbecco's Modified Eagle Medium (DMEM), Gibco®, USA
13. Ethanol, BDH®, UK
14. Ethyl Acetate, BDH®, UK
15. Ethylenediaminetetraacetic Acid (Na_2 EDTA.$2H_2O$, EDTA), Sigma®, USA
16. Fetal Bovine Serum (FBS), HyClone®, Thermo Scientific, USA
17. Genistein, Sigma®, USA
18. Glacial Acetic Acid, BDH®, UK
19. Glycerol, Ajax, Australia
20. Glycine, Research organics, Inc., USA
21. Hexane, BDH®, UK
22. Hydrochloric Acid 35.5-38%, J.T.Baker, USA
23. L-ascorbic Acid (AA), BDH®, UK
24. Lipopolysaccharide (LPS; Escherichia coli 0111:B4), Sigma®, USA
25. LIVE/DEAD® *Bac*Light Bacterial Viability Kit, Thermo Fisher, USA
26. Methanol, BDH®, UK
27. Penicillin Streptomycin, Gibco® Invitrogen™, USA
28. Polyvinylpyrrolidone K30 (PVP), Fluka®, Sigma®, USA
29. Sodium Chloride (NaCl), Merck®, Germany
30. Sodium Dihydrogen Phosphate Monohydrate, Merck®, Germany
31. Sodium Dodecyl Sulfate (SDS), Bio Basic, Inc.,Canada.
32. Sulfuric Acid, BDH®, UK

33. Tween® 20, Sigma®, USA

3.3 Equipments and Instruments

1. Blade Holder, CE, Germany
2. Centrifuge 5415R, Eppendrof, Germany
3. Centrifuge 5810R, Eppendrof, Germany
4. Coulter Cell Counter Machine, Beckman, USA
5. Column Chromatographic Apparatus
6. Culture Incubator, Binder, USA
7. Confocal Microscopes C2, Nikon, Japan
8. Filter funnel, Buchner with KIMFLOW® fritted disc fine porosity 125 ml, Robu®, Germany
9. Gel Documentation, Uvitec®, UK
10. Hot Air Oven, Memert, Germany
11. Inverted Microscope Eclipse Ti, Nikon, Japan
12. Light Microscope, Nikon, USA
13. Laminar Air Work Bench, ESCO®, USA
14. Hot Plate Magnetic Stirring Machine, HS-115, HL Instrument, Harikul Science, Thailand
15. Micro-pipette 2, 10, 20,100, 200, 1000, Gilson, France
16. Microplate Reader, Sunrise, Tecan, Austria
17. Microplate Shaker, Monoshake, Variomag®, USA
18. Microwave Oven, Whirpool
19. Minus 70º C and 150 º C Refrigerator, 4ºc Refrigerator, Sanyo, Japan
20. Multi-channel Micro-pipette, Transferpette®, Germany
21. pH Meter,Orion 2 Star, Thermo Scientific, USA
22. Power Supply, Power Pac™HC, Bio-Rad USA
23. Rotary Evaporator, BÜCHI, Swizerland
24. Shaker Machine, H+B Labortechnik AG, Germany
25. SmartSpec™ 3000 Spectrophotometer, Bio-Rad, USA
26. Soxhlet Apparatus: Heating Mantel, MTopo, Circulater, HAAKEDC10, Thermo, Germany
27. Surgical Scissors, CE, Germany
28. Washer, Columbus Washer, Tecan, Austria

29. Ultraviolet Light Lamp, 254, 366 nm, CAMAG, Switzerland
30. Vortexter, Fine Vortex, Fine PCR®, Korea
31. Warm Water Bath, Memmert, Germany

3.4 Phytochemical Study

3.4.1 Source of Plant Material

Several samples of *C. nutans* dried leaves were purchased from different sources in Thailand and Indonesia. The chemical identification of purchased samples was performed by thin-layer chromatographic technique for selection of good quality of plant material. The selected material was sent for pharmacognostic study to identify its scientific name. The identified specimen has been deposited at Dental Research Laboratory, Faculty of Dentistry, Thammasat University, Rangsit Campus, Pathum Thani, Thailand.

3.4.2 General Techniques

3.4.2.1 Analytical Thin-Layer Chromatography (TLC)

Technique	: One Dimension, Ascending
Adsorbent	: Silica Gel 60 F254, Precoated Plate, Aluminum Sheet
Layer thickness	: 0.2 mm
Distance	: 15 cm
Temperature	: Room Temperature (25-30° C)
Detection	: 1. Daylight 2. Ultraviolet Light at the wavelength of 254 and 366 nm 3. Anisaldehyde-sulfuric Acid Reagent Spray: Freshly prepared solution of 0.5 ml anisaldehyde in a mixture of 10 ml glacial acetic acid and 85 ml of methanol, with addition of 5 ml of sulfuric acid. Treatment: The chromatogram was heated at 110° C for 10-15 min after spraying.

3.4.2.2 Preparative Thin-Layer Chromatography (PTLC)

Technique : One Dimension, Ascending

Adsorbent : Silica Gel 60 PF_{254}

Layer thickness : 1 mm

Distance : 15 cm

Temperature : Room Temperature (25-30° C)

Detection : Ultraviolet Light at the Wavelength of 366 nm

3.4.2.3 Conventional Column Chromatography

Adsorbent : Silica Gel 60 (No.107754) Particle Size 63-200 μm (70-230 mesh ASTM)

Packing method : Wet Packing

Solvent : Gradient of n-Hexane and Ethyl Acetate System.

Sample loading : Sample was dissolved in small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, allowed to dry in air and then added gently to the top of column.

Detection : Fractions were examined by TLC observing under ultraviolet light at a wavelength of 254 and 366 nm followed by spraying with anisaldehyde-sulfuric acid reagent and heated at 110ºC for 10-15 min. Those fractions of similar pattern were combined.

3.4.2.4 Vacuum Column Chromatography

Apparatus : Sintered glass filter (size 5 x 5 cm), suction flask, vacuum pump

Adsorbent : Silica Gel 60 (No.107754) Particle Size 63-200 μm (70-230 mesh ASTM)

Packing method : Dry packing

Solvent	: Gradient of n-Hexane and Ethyl Acetate system
Sample loading	: Sample was dissolved in small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, allowed to dry in air and then added gently to the top of column.
Detection	: Fractions were examined by TLC observing under ultraviolet light at a wavelength of 254 and 366 nm followed by spraying with anisaldehyde-sulfuric acid reagent and heated at 110ºC for 10-15 min. Those fractions of similar pattern were combined.

3.4.2.5 Spectroscopy

IR spectra were recorded as neat on a Perkin–Elmer FT-IR spectrum 400 spectrometer (ATR). ^{1}H NMR spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 MHz. The chemical shifts (δ) are reported in ppm, and coupling constants (J) are given in Hz. For the spectra taken in C_5D_5N, the residual nondeuterated solvent signal at δ 8.71 ppm was used as references for ^{1}H NMR spectra. HR-ESI-MS of separated compound was obtained using a Bruker micrOTOF-II mass spectrometer.

3.5 Extraction and Phytochemical Identification

3.5.1 Screening of plant material

The dried coarsely powdered leaves of each plant material, purchased from different sources were refluxed with ethanol for 1 hour and filtered. The remaining residue continued for another reflux. This process was repeated 3 times. After that, the filtrates were combined and evaporated at temperature not exceeding

55°C to give the ethanolic extract. The extracts were identified by TLC.

3.5.2 Preparation of extracts for the isolation of active constituents

The leaves were dried under the sun and separated from the stem. The dried sample was ground to powder. Dried powdered leaves were sequentially extracted using hexane, chloroform and ethanol, respectively, in a Soxhlet. The liquid extracts were evaporated in rotary evaporator at 50°C to provide hexane, chloroform, ethanol extracts. All extracts were identified by TLC and examined for anti-inflammatory activity by NO assay.

3.6 Isolation Procedure

Based on NO assay, the active extract was chosen for further isolation procedure. It was vacuum chromatographed on a silica gel column using hexane-ethyl acetate (AcOEt) gradient system as eluting solvent. All fractions examined for anti-inflammatory activity by NO assay. The active fractions were further separated by using conventional column chromatography (hexane-AcOEt) gradient system as eluting solvent and further purified by preparative layer chromatography (PTLC) (hexane:AcOEt, 7:3).

3.7 Characterization of Isolated Constituents

The structure of isolated constituent was determined base on their IR spectra, Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR) spectra.

3.9 Investigation of Biological Activity

3.9.1 Preparation on Cell Culture Testing System

All extracts extract and constituents were separately combined with polyvinylpyrrolidone (PVP) at a ratio of 1:10 (extract or compound: PVP). Each residual was then dissolved in water and kept at -20°C for use as the stock solution

3.9.2 RAW 264.7 macrophage cell lines

RAW 264.7 macrophage cell lines cultured at 37°C in a humidified atmosphere (5% CO_2) in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% amphotericin B, 1% HEPES, and 1% sodium pyruvate.

3.9.3 Human Gingival Fibroblast Culture

Human gingival fibroblast obtained from gingival tissue that attach to impacted teeth that extracted from healthy individuals. Gingival tissue minced with blade into small fragments and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml of amphotericin B). The cultures maintained at 37°C in an atmosphere containing 5% CO_2. Confluent cells detached with 0.25% trypsin and 0.05% EDTA and aliquots of separated cellswere sub cultured. Cells cultures between passages 4 to 8 used in this study.

3.9.4 Cytotoxicity assay

Cytotoxicity for RAW 264.7 macrophage cell lines and human gingival fibroblast of the all extracts and isolated constituents was determined by MTT assay. MTT assay is a laboratory test and a standard colorimetric assay for measuring cellular proliferation. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to a purple formazan in the mitochondria of living cells. In this assay, only viable cells can reduce MTT to insoluble purple formazan. Thus, the intensity of purple color in turns represents the number of viable cells. For MTT assay, cells from passage 4 to 8 were used. The cells were cultured in 96-well micro titer plate at a density of 2×10^4 cells per 200μL for 24 hours. Then, the medium was changed to fresh D-MEM (control culture) and D-MEM supplemented with different concentration of *Clinacanthus nutans*. The final concentrations of the extracts were 10 μg/mL, 25 μg/mL, 50 μg/mL, 100μg/ml, and 200 μg/ml. The cells were then incubated in humidified atmosphere of 5% CO_2 at 37°C. MTT assay was performed on 48 hours post-treatment. To evaluate the number of viable cells, 200μL of MTT solution was added into each well and incubated for 4 hour at 37°C in dark. The formazan crystals that formed by living cells were solubilized with 200 μL MTT solvent and the absorbance were measured at 570 nm by ELISA reader. All experiments were repeated independent 3 samples. The absorbance at 570 nm were recorded immediately and calculated to percent of growth (PG).

$$PG = 100*(OD_{sample}/OD_{control})$$

3.9.5 Nitric oxide measurement

The RAW 264.7 cells were seeded in 6 well plates and incubated overnight at 37°C and 5% CO2. Then media of each well were aspirated and fresh media were replaced. Various concentrations of *C. nutans* extract, fractions or isolated compound were prepared in DMEM to give a total volume of 1 mL in each well of a microtiter plate. After 4 hours treatment, cells were stimulated with 100 ng/mL of LPS from *Escherichia coli* (0111:B4; Sigma) for 24 hours. Nitrite concentration was measured in cell culture media using commercial NO detection kit (Griess Reagents System, Promega). Briefly, 50 μL of supernatant was incubated with an equal volume of Griess reagent (1 % sulfanilamide and 0.1 % naphthyl-ethylenediamine dihydrochloride in 2.5 % phosphoric acid). After 10 minutes incubation at room temperature, the absorbance was measured at 540 nm. Nitrite concentrations were calculated by comparison with a standard calibration curve with sodium ($NaNO_2$: 1.26-100 mmol/L), with control baseline supernatant as the blank. Genistein (Sigma) was used as positive control.

3.9.6 Wound scratch assay

To evaluate the migration of human gingival fibroblast, wound healing assay performed by scratching the confluent culture. Human gingival fibroblast was cultured into 6-well plated in D-MEM. After reaching approximately 80% confluent, the medium was discarded and a scratch was made using micropipette tip, followed by washing with PBS to remove cell debris resulted from scratching. The cultures fed with D-MEM supplemented with various concentrations of *C. nutans* extracts or isolated compounds. The cell with D-MEM without supplement was used as control. The images of the wound healing area were captured at t0 (right after scratching) and t24 (24 hours after scratching). The migration rate of human gingival fibroblasts was calculated using “Image J” software by measuring the pixel gap that was closed by the cells. For each well and each time frame, 15 scratch areas were captured to get an average cell migration rate. Closure percentage of the scratch was calculated as follows,

(surface of the scratch at t24/surface of the scratch at t0) x 100

The method of this assay is according to paper that previously described with slight modification on cell culture dishes coating.[153]

3.9.7 Determination of bacterial viability

S. mutans ATCC 25175 used in this study were purchased from the National Institute of Health, Ministry of Public Health (NIH), Thailand. *S. mutans* were grown in 10 ml Brain Heart Infusion (BHI) at 37° C under anaerobic conditions for 24 hours. An aliquot of 100 μl (0.1 McFarland = 1.5 x 10^7 cfu/mL) was dispensed into each well of 96-well plates in the presence of 100 μl BHI containing various concentration of chloroform extract or isolated compound of *C. nutans*. The well containing BHI only served as control. Chlorhexidine (1 μg/mL) were used as positive control. Then the plates were incubated in anaerobic conditions at 37° C for 24 hours. After incubation, the absorbance was measured at wavelength 600 nm.

3.9.8 Determination of anti-adherence activity

3.9.8.1 Preparation of hydroxyapatite plate

Tissue-culture-treated, 96-well, polystyrene microtitre plates were filled with calcifying solution (390 μL per well) containing 2.5 mmol l^{-1} $CaCl_2$ $2H_2O$, 7.5 mmol l^{-1} KH_2PO_4, 250.0 mmol l^{-1} triethanolamine, at pH 7.4. The plates were incubated at 75° C for 1.5 hour with the lid off, after which they were emptied, refilled with calcifying solution and re-incubated. Following a total of four 1.5 h precipitation cycles, the plates were rinsed with distilled water and allowed to air dry at room temperature. A continuous mineral deposit formed in the wells; the outermost wells occasionally had an uneven coating and were usually eliminated from the assay.[154]

3.9.8.2 Anti-adherence assay

The anti-adherence properties of *C. nutans* on *S. mutan* were tested with HA-coated 96-well. *S. mutans* were grown in 10 ml Brain Heart Infusion (BHI) at 37° C under anaerobic conditions for 24 hours. An aliquot of 100 μl (0.1 McFaland = 1.5 x 10^7 cfu/mL) was dispensed into each well of 96-well plates in the presence of 100 μl BHI supplemented with 2% sucrose (w/v) containing various dose of chloroform extract of *C. nutans* (12.5, 25, 50, 100, 250, 500, and 1000 μg/mL). The well containing BHI supplemented with 2% sucrose only served as

control. Then the plates were incubated in anaerobic conditions at 37° C for 48 hours.[155]

Cell attachment was assessed using crystal violet assay. After incubation, the content of each well was aspirated and each well were washed three times with sterile distilled water to remove loosely attached cells. The remaining attached bacteria were fixed with 200 µl of methanol per well, and after 15 minutes plates were emptied and left to dry. Then, each well were stained with 2% crystal violet, after 15 minutes the crystal violet in each well were emptied and washed three times with sterile distilled water. After the plates were air dried, the dye bound to adherent cells was re-solubilized with 160 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using an automated ELISA reader.[156]

3.9.9. Visualization of biofilm

S. mutans were grown in 10 ml Brain Heart Infusion (BHI) at 37° C under anaerobic conditions for 24 hours. An aliquot of 100 µl (0.1 McFarland = 1.5 x 10^9 cfu/mL) was dispensed into each 8-well chamber in the presence of 100 µl BHI supplemented with 2% sucrose (w/v) containing various concentration of chloroform extract of *C. nutans* (12.5, 25, 50, 100, 250, 500, and 1000 µg/mL) and isolated compound (25, 50, 100, 250, and 500 µg/mL). The well containing BHI supplemented with 2% sucrose only served as control. Then the plates were incubated in anaerobic conditions at 37° C for 48 hours.[155]

After incubation, medium from each chamber were aspirated and washed the resident biofilm gently with sterile distilled water three times. Bacteria was fixed with 200 µL of methanol, incubate for 15 minutes, then methanol was aspirated from each well. Then, 200 µL of BacLight Live/Dead (mixture of component A and component B, 1.5 µL each per mL of sterile distilled water) solution was added to each well and incubated in room temperature for 15 minutes. Protect culture from light. The stain was aspirated and washed with sterile distilled water gently for three times. The plastic well was removed from the slide, then, 50 µL of 50% glycerol in water was added to each well so that a coverslip is placed on the gasket. The edges of the coverslip were sealed with nail polish, and wait to dry for 15 minutes prior to examining via microscope.[157]

3.10 Statistical Analysis

All experiments were performed in three independent samples. Significant differences were determined using One-way analysis of variance followed with Dunnett's multiple comparison (Prism5; GraphPad Software Inc., San Diego, CA, USA). Differences with p values ≤ 0.05 were considered significant.

CHAPTER 4
RESULTS AND DISCUSSION

4. 1 Results

4.1.1 Screening of plant material

The dried coarsely powdered leaves of each plant materials (10 g) was repeated refluxed with 250 ml of ethanol. Each combined filtrate was evaporated to give ethanol extract. The qualities of the extracts were compared by TLC identification (Figure 4.1). The sample from Indonesia was selected to use for this study.

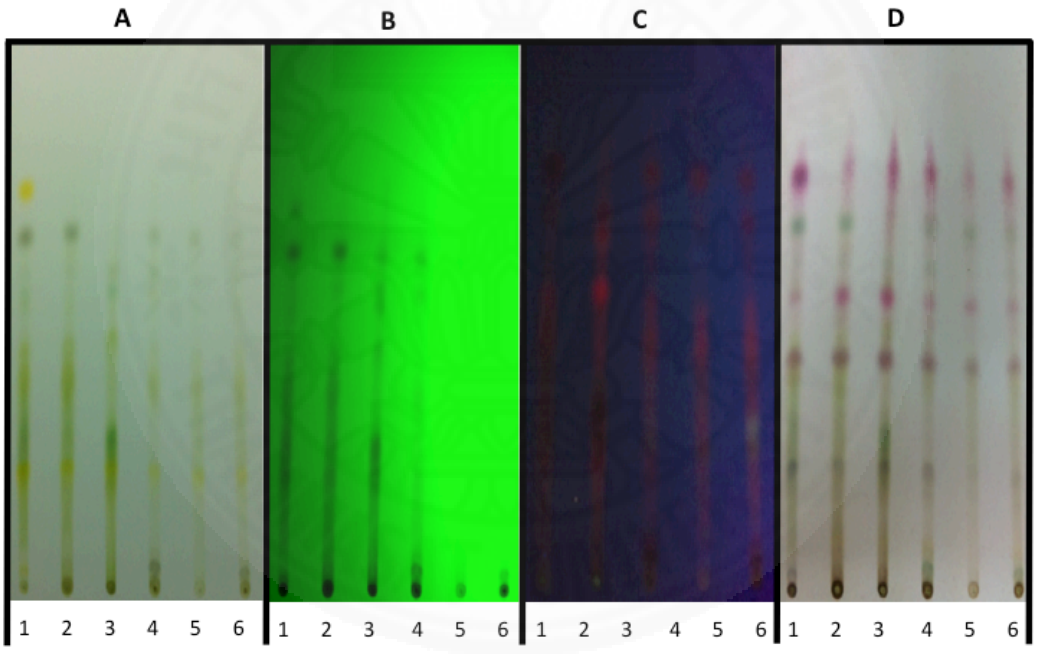


Figure 4.1. Thin-layer Chromatogram of Ethanol Extract of C. nutans. TLC sample form Thailand and Indonesia for screening of phytochemical compounds.

A = Detection under visible light
B = Detection under UV light (254 nm)
C = Detection under UV light (366 nm)
D = Detection with anisaldehyde-sulfuric acid solution
1 = Sample from Indonesia
2 = Sample from Thailand 1
3 = Sample from Thailand 2

4 = Sample from Thailand 3

5 = Sample from Thailand 4

6 = Sample from Thailand 5

4.1.2 Preparation of extracts for the isolation of active constituents

Dried coarsely powdered leaves of *C. nutans* (545 g) were sequentially extracted using hexane, chloroform, and ethanol, respectively, in a Soxhlet. The liquid extracts were evaporated to provide hexane (20.05 g), chloroform (16.2 g), and ethanol (38.34 g) extracts.

4.1.3 Identification of extracts

The thin layer chromatographic characteristics of the extracts are shown in Figure 4.2.

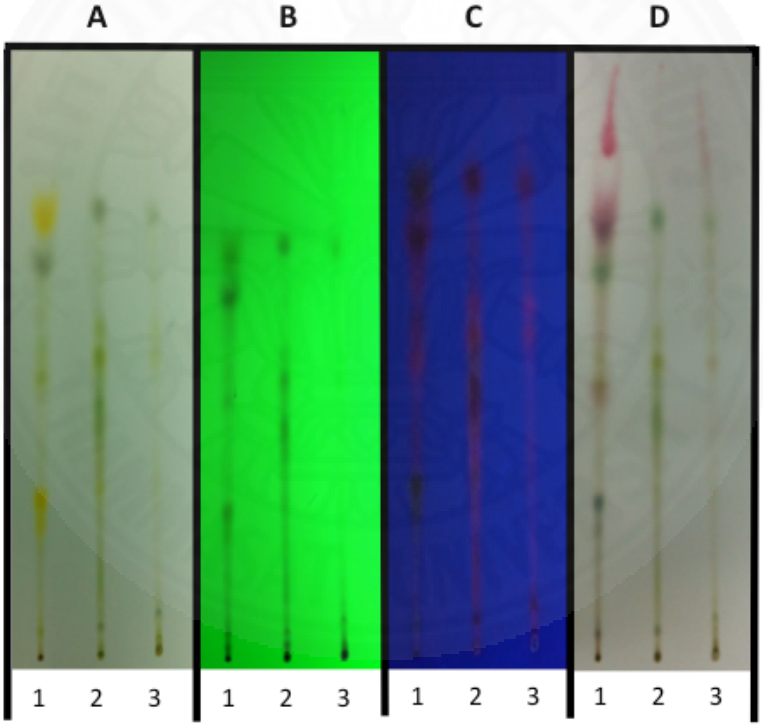


Figure 4.2 Thin-layer Chromatogram of Hexane, Chloroform and Ethanol Extract of C. nutans.

A = Detection under visible light

B = Detection under UV light (254 nm)

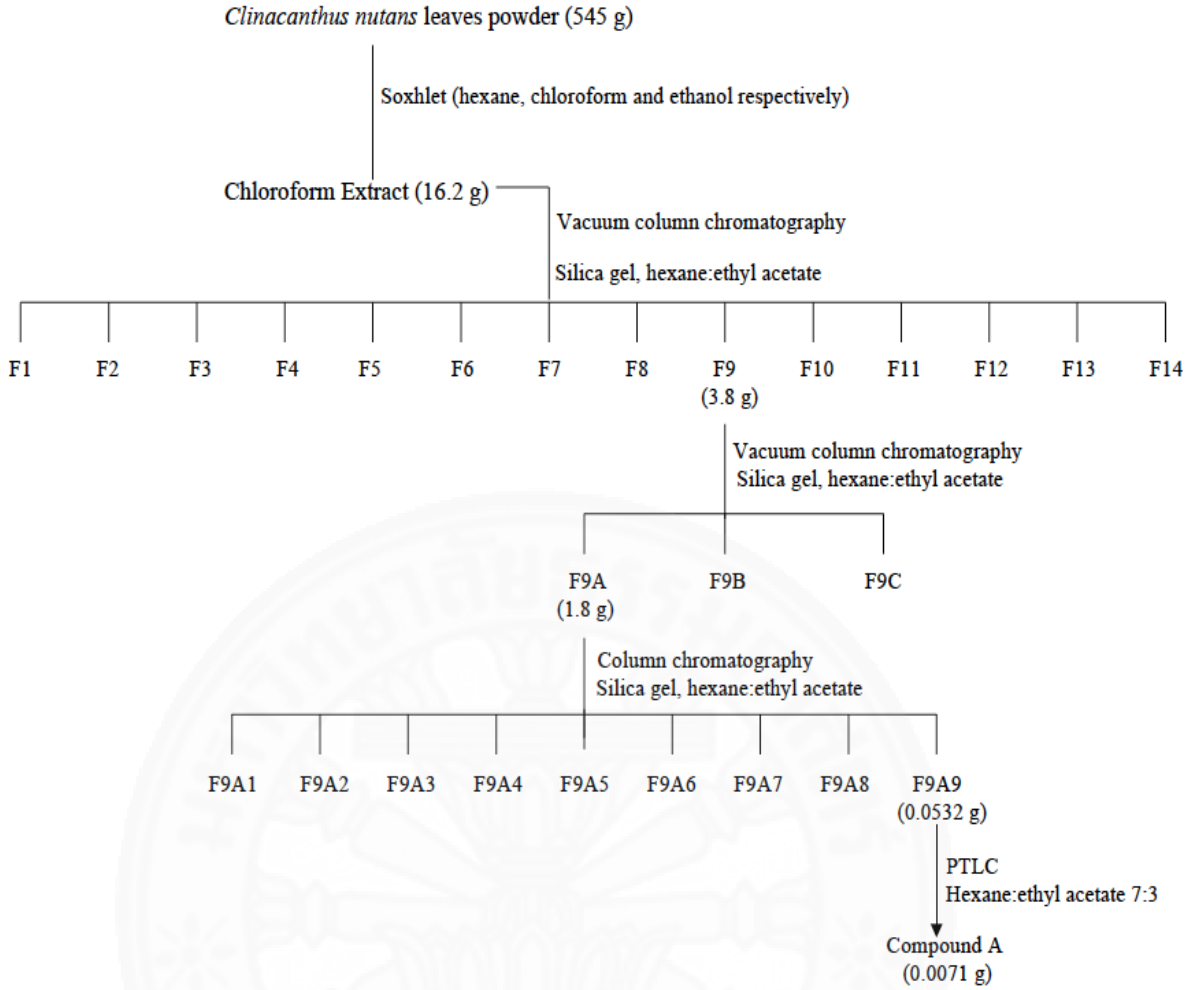
C = Detection under UV light (366 nm)

D = Detection under with anisaldehyde-sulfuric acid solution

1 = Hexane extract

2 = Chloroform extract

3 = Ethanol extract



Scheme 4.1. *Extraction and Isolation Steps of C. nutans Constituents*

4.1.4 Isolation of constituents from active extract

The chloroform extract (16.2 g) was separated by column chromatographic technique, and eluted fractions were examined by TLC to give fractions 1 to 14. All fractions were examined for anti-inflammatory activity by NO assay. Fraction 9 (3.8 g) was chosen for further separation by vacuum column chromatography to give 3 fractions Fraction 9A (1.8 g) was chosen for further separation using conventional column chromatography (hexane-ethyl acetate gradient system as eluting solvent) to give fraction 1 to 9. Fraction 9A9 (0.0532 g) was chosen for further separation by repeated preparative thin layer chromatography (PTLC) (hexane:AcOEt, 7:3), which afforded green colored **compound A** (0.0071 g) (Scheme 4.1).

4.1.5 Characterization of Isolated Constituents

Compound A was obtained as a green color. It was soluble in chloroform. From TLC, hR_f value = 58

Spectroscopy analysis:

IR: ν_{max} 3393, 2952, 2924, 2855, 1731, 1694, 1457, 1377, 1222, 1161, 1025, 980 cm^{-1}; ^{1}H-NMR: (400 MHz, C_5D_5N) δ 9.95 (*s*, 1H, H-10), 9.69 (*s*, 1H, H-5), 8.83 (*s*, 1H, H-20), 8.11 (*dd*, *J* = 17.7, 11.5 Hz, 1H, H-3^1), 6.39 (*d*, *J* = 17.8 Hz, 1H, H-3^2 (*E*)), 6.18 (*d*, *J* = 11.5 Hz, 1H, H-3^2 (*Z*)), 5.43 (*m*, 2H, OCH_2CH=C and H-17), 4.72 (*m*, 3H, OCH_2CH=C and H-18), 3.86 (*s*, 3H, H-12^1), 3.71 (q, *J* = 6.6 Hz, 2H, H-8^1), 3.30 (*s*, 3H, H-2^1), 3.17 (*s*, 3H, H-7^1), 1.80 (*d*, *J* = 7.2 Hz, 3H, H-18^1), 1.68 (t, *J* = 6.6 Hz, 3H, H-8^2), 0.7-0.9 (12H, 4xCH_3-phytyl), HR-ESI-TOFMS m/z 865.5232 $[M + Na]^+$ (calcd for $C_{53}H_{70}N_4O_5Na$, 865.5238).

The molecular formula of this compound was determined to be $C_{53}H_{70}N_4O_5$ by the pseudomolecular ion peak $[M + Na]^+$ at m/z 865.5232 in the HR-ESI-MS. The ^{1}H-NMR spectrum of this compound exhibited signals of three vinyl methyls on the chlorin ring at δ 3.17 (CH_3-7^1), 3.30 (CH_3-2^1) and 3.86 (CH_3-12^1) and seven olefinic protons at δ 5.43 (phytyl-H), 6.18 (H-3^2 (*Z*)), 6.39 (H-3^2(*E*)), 8.11 (H-3^1), 8.83 (H-20), 9.69 (H-5) and 9.95 (H-10). The isolated compound contained phytyl ester as the olefinic proton at δ 5.43 (phytyl-H) and methyl protons at δ 0.70-0.90 (m) were observed. The spectroscopic data of isolated compound were consistent with the reported values, thus it was characterized as purpurin-18 phytyl ester (P18PE). The structure of P18PE is shown below (Figure 4.3).[158]

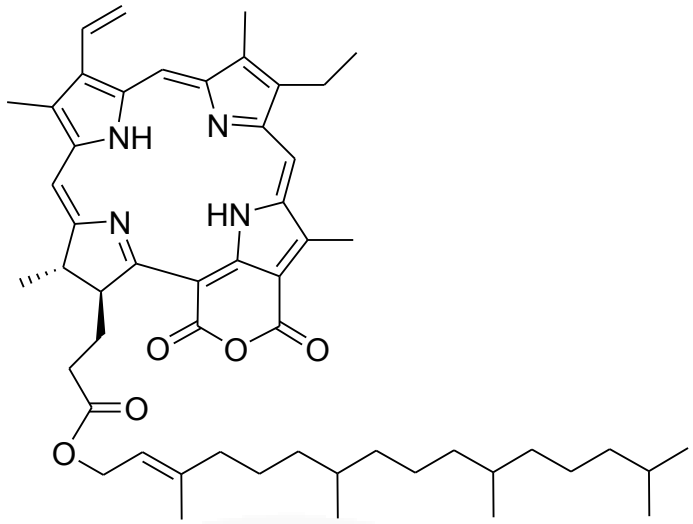


Figure 4.3 Chemical structure of C. nutans constituent, purpurin-18 phytyl ester.

4.1.6 Chromatographic Characteristic of Chloroform Extract

Analytical Thin-layer Chromatography (TLC) (Figure 4.4)

Test Solution (Solution A): Dissolved 15 mg of chloroform extract in 5 ml of chloroform

Marker Solution (Solution B): Dissolved 1 mg of P18PE in 1 ml of chloroform

Chromatographic Condition:

Absorbent	: Silica Gel GF_{254}
Layer Thickness	: 0.2 mm
Developing Solvent	: Hexane: Ethyl Acetate (7:3)
Distance	: 15 cm
Detection	: UV light (366 nm)

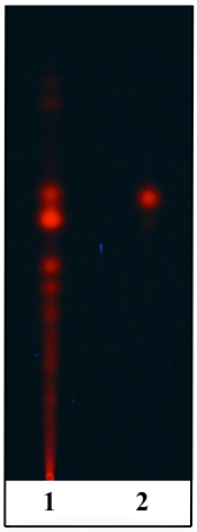


Figure 4.4 Thin-layer Chromatogram of Chloroform Extract of C. nutans detection under UV light (366 nm).

1 = chloroform extract

2 = P18PE

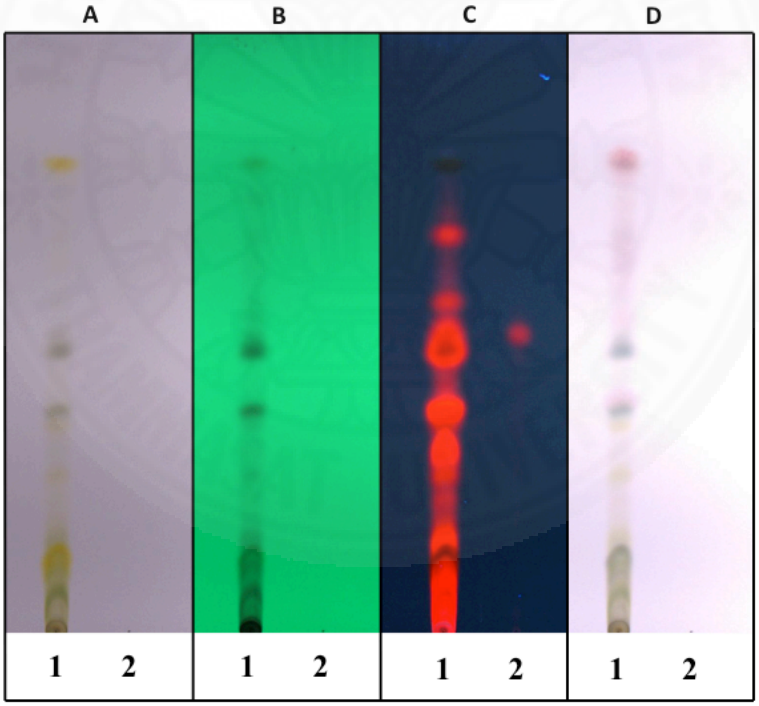


Figure 4.5 Thin-layer Chromatogram of Ethanol (reflux) Extract of C. nutans and P18PE.

A = Detection under visible light

B = Detection under UV light (254 nm)

C = Detection under UV light (366 nm)

D = Detection under with anisaldehyde-sulfuric acid solution

1 = Ethanol (reflux) Extract

2 = P18PE

4.1.7 Chromatographic Characteristic of Ethanol Extract

Analytical Thin-layer Chromatography (TLC) (Figure 4.5)

Test Solution (Solution A): Dissolved 15 mg of ethanol extract in 5 ml of ethanol

Marker Solution (Solution B): Dissolved 1 mg of P18PE in 1 ml of chloroform

Chromatographic Condition:

Absorbent	: Silica Gel GF_{254}
Layer Thickness	: 0.2 mm
Developing Solvent	: Hexane: Ethyl Acetate (7:3)
Distance	: 15 cm
Detection	: A. Visible light
	B. UV light (254 nm)
	C. UV light (366 nm)
	D. Spray with anisaldehyde-sulfuric acid solution

4.1.8 Cytotoxicity Assay

4.1.8.1 Cytotoxicity of Hexane Extract on Human Gingival Fibroblast

The cytotoxicity of hexane extract was tested on human gingival fibroblast (10-200 μg/mL). The result showed that hexane extract at concentration up to 200 μg/ml did not toxic to human gingival fibroblast (Figure 4.6).

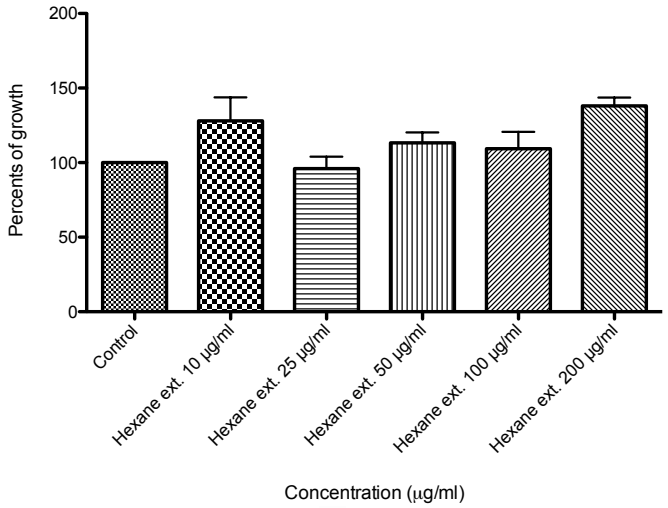


Figure 4.6 Graph of Cytotoxicity of Hexane Extract on Human Gingival Fibroblast after incubation for 48 h. Data represents mean ±SE of 3 independent samples.

4.1.8.2 Cytotoxicity of Chloroform Extract on Human Gingival Fibroblast

The cytotoxicity of chloroform extract was tested on human gingival fibroblast (10-200 µg/mL). The result showed that chloroform extract at the concentration up to 200 µg/ml did not toxic to human gingival fibroblast (Figure 4.7).

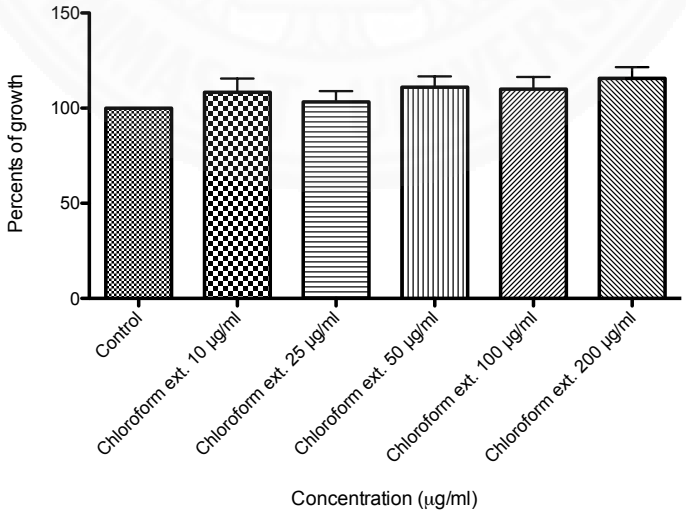


Figure 4.7 Graph of Cytotoxicity of Chloroform Extract on Human Gingival Fibroblast after incubation for 48 h. Data represents mean ±SE of 3 independent samples.

4.1.8.3 Cytotoxicity of Ethanol Extract on Human Gingival Fibroblast

The cytotoxicity of ethanol extract was tested on human gingival fibroblast (10-200 μg/mL). The result showed that ethanol extract at the concentration up to 200 μg/ml did not toxic to human gingival fibroblast (Figure 4.8).

4.1.8.4 Cytotoxicity of P18PE on Human Gingival Fibroblast

The cytotoxicity of P18PE was tested on human gingival fibroblast (10-100 μg/mL). The result showed that compound A at the concentration up to 100 μg/ml did not toxic to human gingival fibroblast (Figure 4.9).

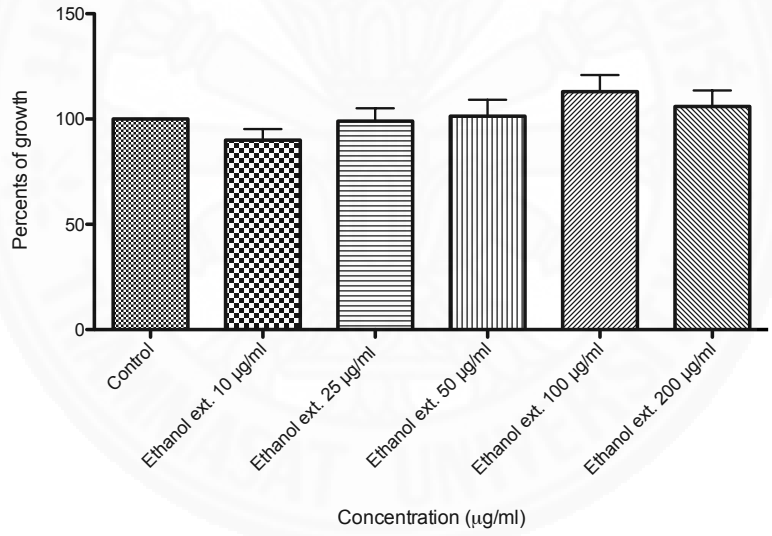


Figure 4.8 Graph of Cytotoxicity of Ethanol Extract on Human Gingival Fibroblast after incubation for 48 h. Data represents mean ±SE of 3 independent samples.

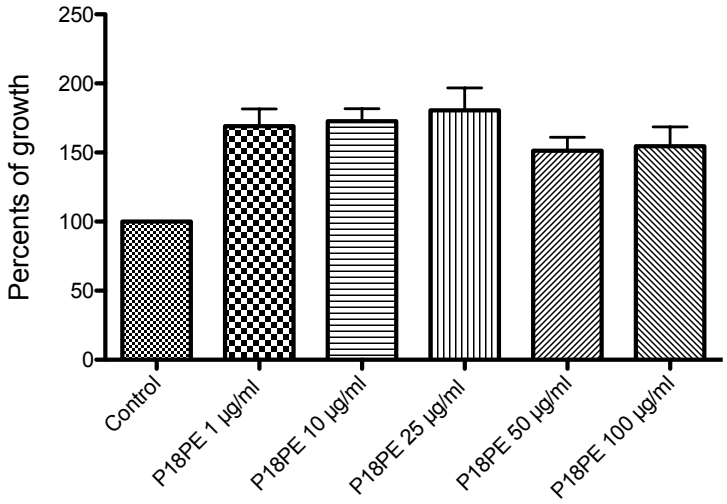


Figure 4.9 Graph of Cytotoxicity of P18PE on Human Gingival Fibroblast after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

4.1.8.5 Cytotoxicity of LPS on RAW 264.7 Macrophage Cell Lines

To determine non-toxic doses of LPS, cytotoxicity of LPS was tested on RAW 264.7 macrophage cell lines (1-1000 ng/mL). The result showed that LPS at the concentration up to 1000 ng/ml did not toxic to RAW 264.7 macrophage cell lines (Figure 4.10).

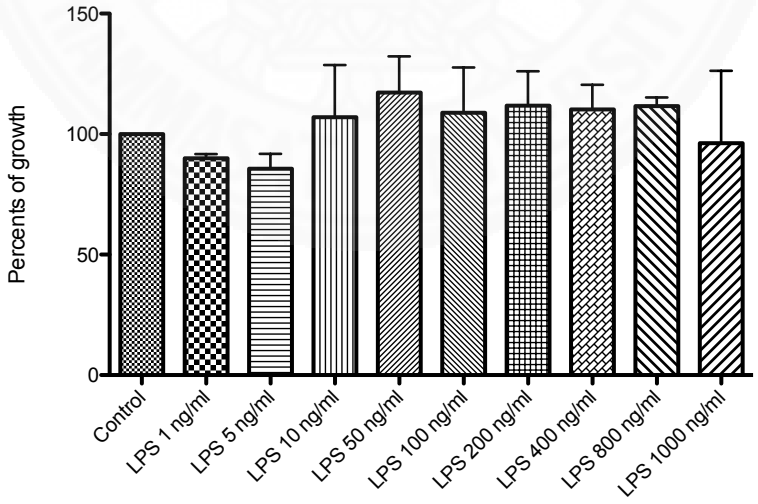


Figure 4.10 Graph of Cytotoxicity of LPS on RAW 264.7 Macrophage Cell Lines after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

4.1.8.6 Cytotoxicity of Hexane Extract on RAW 264.7 Macrophage Cell Lines

The cytotoxicity of hexane extract was tested on RAW 264.7 Macrophage Cell Lines (1-200 μg/mL). The result showed that hexane extract at the concentration up to 200 μg/mL did not toxic to RAW 264.7 macrophage cell lines (Figure 4.11).

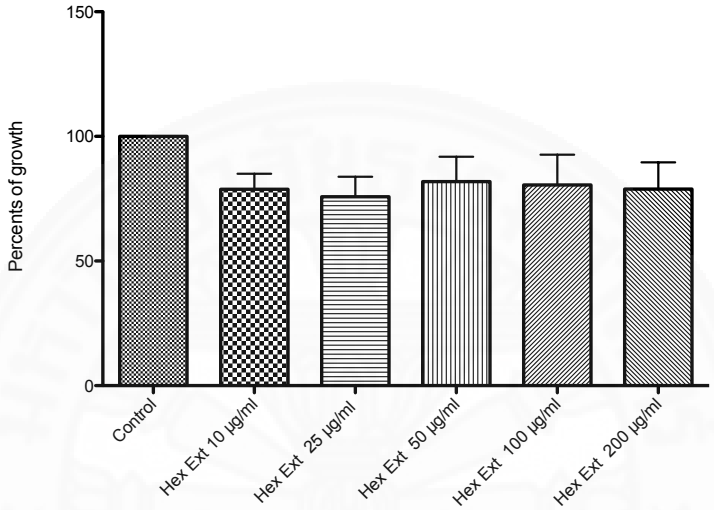


Figure 4.11 Graph of Cytotoxicity of Hexane Extract on RAW 264.7 Macrophage Cell Lines after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

4.1.8.7 Cytotoxicity of Chloroform Extract on RAW 264.7 Macrophage Cell Lines

The cytotoxicity of chloroform extract was tested on RAW 264.7 Macrophage Cell Lines (1-200 μg/mL). The result showed that chloroform extract at concentration up to 200 μg/mL did not toxic to RAW 264.7 macrophage cell lines (Figure 4.12).

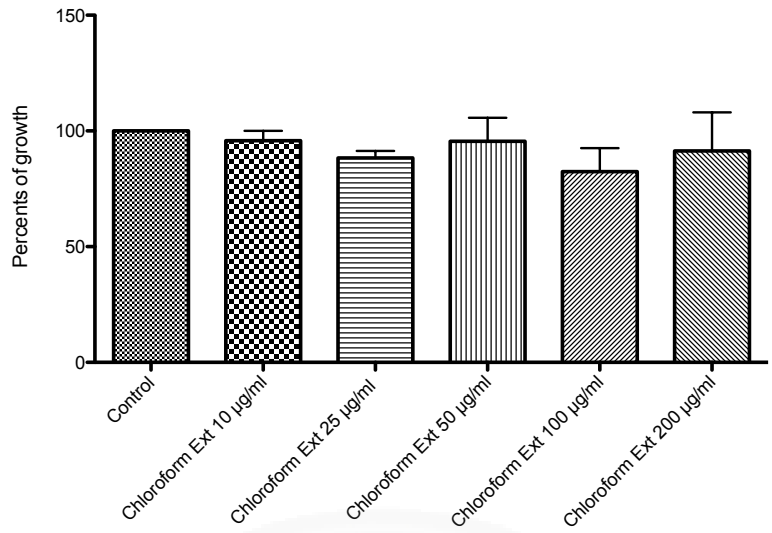


Figure 4.12 Graph of Cytotoxicity of Chloroform Extract on RAW 264.7 Macrophage Cell Lines after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

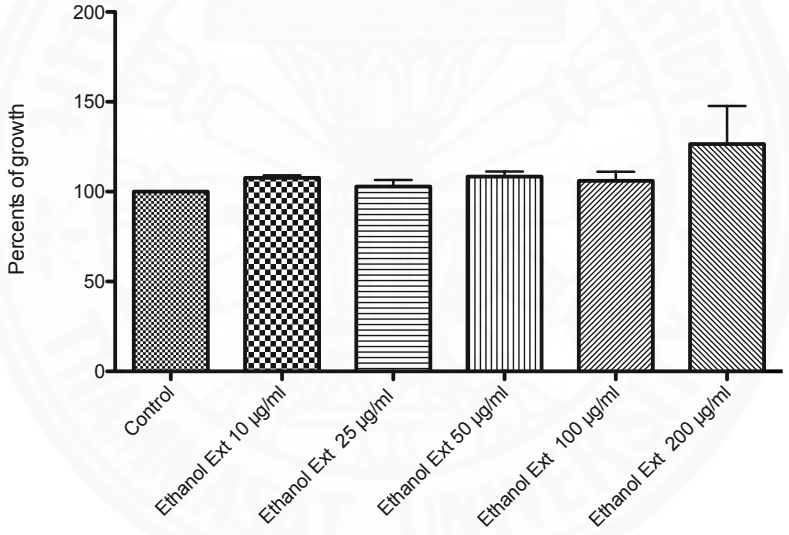


Figure 4.13 Graph of Cytotoxicity of Ethanol Extract on RAW 264.7 Macrophage Cell Lines after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

4.1.8.8 Cytotoxicity of Ethanol Extract on RAW 264.7 Macrophage Cell Lines

The cytotoxicity of ethanol extract was tested on RAW 264.7 Macrophage Cell Lines (1-200 µg/mL). The result showed that ethanol extract at concentration up to 200 µg

/mL did not toxic to RAW 264.7 macrophage cell lines (Figure 4.13).

4.1.8.9 Cytotoxicity of P18PE on RAW 264.7 Macrophage Cell Lines

The cytotoxicity of P18PE was tested on RAW 264.7 Macrophage Cell Lines (1-200 μg/mL). The result showed that P18PE at the concentration up to 200 μg/mL did not toxic to RAW 264.7 macrophage cell lines (Figure 4.14).

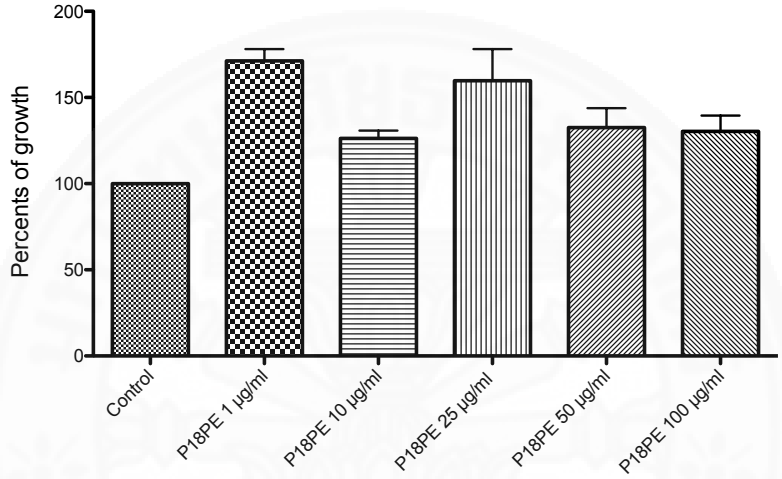


Figure 4.14 Graph of Cytotoxicity of P18PE on RAW 264.7 Macrophage Cell Lines after incubation for 48 h. Data represents mean ± SE of 3 independent samples.

4.1.9 Determination of Nitric Oxide Production

4.1.9.1 Nitric oxide assay of LPS-stimulated RAW 264.7 Macrophage Cell Lines

A preliminary study of LPS dosage showed that LPS could stimulate nitric oxide production on RAW 264.7 macrophage cell lines in dose-dependent manner. Based on this result, LPS 100 ng/mL used for next experiments (Figure 4.15).

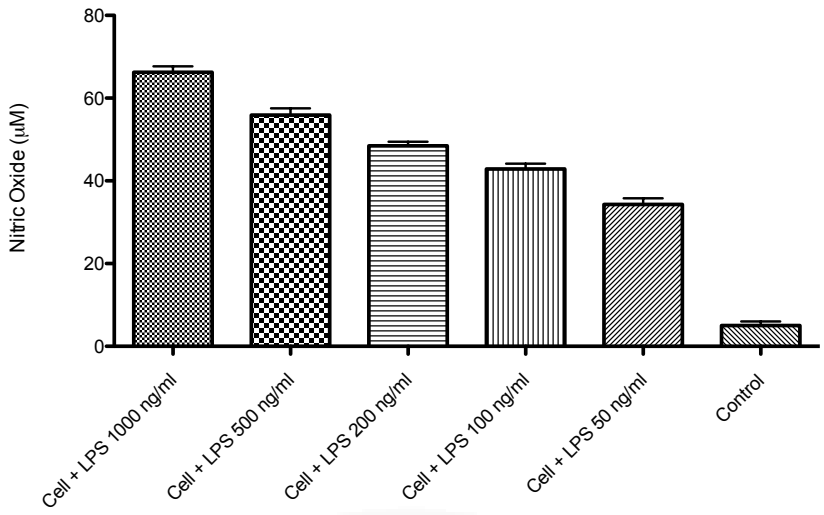


Figure 4.15 Nitric Oxide Assay of LPS-stimulated RAW 264.7 Macrophage Cell Lines at Concentration of 50, 100, 200, 500, and 1000 ng/ml. Data represents mean ± SE of 3 independent samples.

4.1.9.2 Nitric oxide assay of LPS-stimulated RAW 264.7 Macrophage Cell Lines pre-treated with *C. nutans* Hexane, Chloroform and Ethanol extracts (100 μg/mL).

A preliminary study to determine extracts that posses the highest activity to inhibit nitric oxide production. The results showed that *C. nutans* hexane, chloroform and ethanol extracts at concentration 100 μg/ml significantly (p<0.05) inhibit nitric oxide production compare to control. Chloroform extract showed the highest activity among other experiment group. Based on this result, chloroform extract was chosen for further isolation for bioactive compound. Group with LPS-induced and without *C. nutans* extracts treatment used as control. Genistein (50 ng/mL) used as positive control (Figure 4.16).

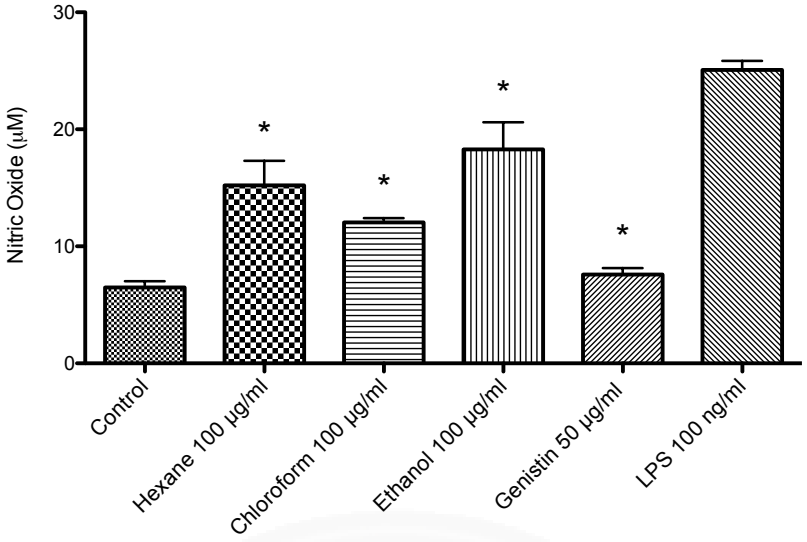


Figure 4.16 Nitric Oxide Assay of LPS-stimulated RAW 264.7 Macrophage Cell Lines Pre-treated with C. nutans Hexane, Chloroform, and Ethanol Extract (100 µg/ml). Data represents mean ± SE of 3 independent samples.

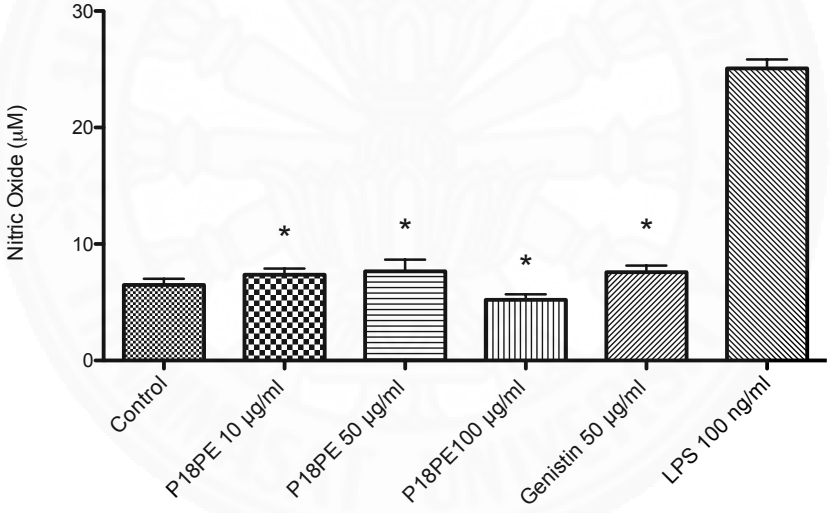


Figure 4.17 Nitric oxide assay of LPS-stimulated RAW 264.7 macrophage cell lines pre-treated with P18PE (10, 50 and 100 µg/ml). Data represents mean ± SE of 3 independent samples.

4.1.9.3 Nitric Oxide Assay of LPS-stimulated RAW 264.7 Macrophage Cell Lines Pre-treated with P18PE

Inhibition of nitric oxide production of P18PE was observed after incubation for 24 h. The study showed that P18PE significantly (p<0.05) could inhibit nitric oxide production. P18PE at 100 µg/mL possess the highest

activity. Group with LPS-induced and without P18PE treatment used as control. Genistein (50 ng/mL) used as positive control (Figure 4.17).

4.1.10 Determination of *In Vitro* Cell Migration Effect

4.1.10.1 Wound Scratch Assay of *C. nutans* Hexane, Chloroform, and Ethanol extract on Human Gingival Fibroblast

A preliminary study to determine extracts that possess the highest wound healing potential activity. The result showed that *C. nutans* chloroform and ethanol extracts at concentration 10 μg/mL significantly (p<0.05) could induce HGF migration after incubation for 24 hours. Group without *C. nutans* extract treatment served as control (Figure 4.18 and Figure 4.19).

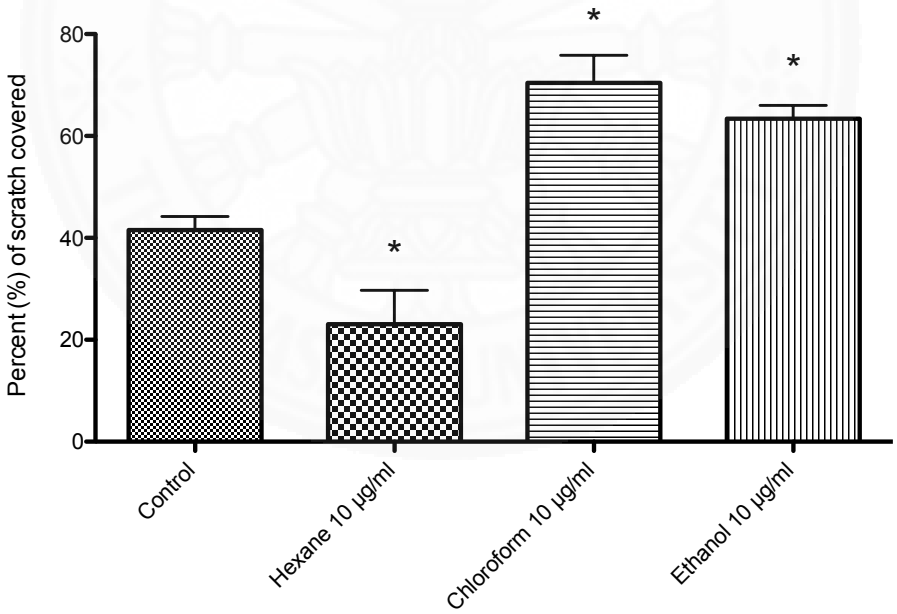


Figure 4.18 Graph of Wound Scratch Assay of C. nutans Hexane, Chloroform, and Ethanol Extract on Human Gingival Fibroblast After Incubation for 24 h. Data represents mean ± SE of 3 independent samples. An asterisk (*) indicates a significant difference in percentage of scratch covered compared to control.

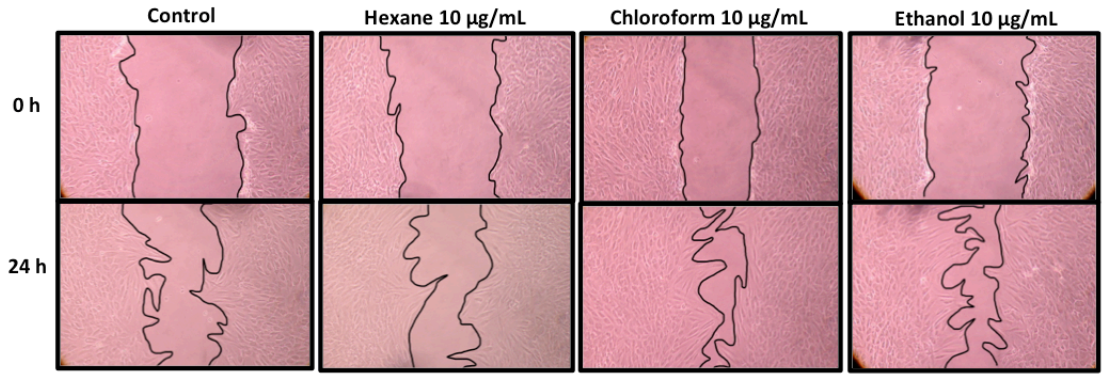


Figure 4.19. Micrograph of HGF on Monolayer Culture After Treatment with C. nutans Hexane, Chloroform and Ethanol Extract. Chloroform and ethanol extract could induce HGF migration.

4.1.10.2 Wound Scratch Assay of P18PE on Human Gingival Fibroblast

Wound healing potential activity of P18PE was evaluated by wound scratch assay. The result showed that P18PE at concentration 10, 50, 100 µg/mL significantly (p<0.05) could induce HGF migration after incubation for 24 hours in non-dose dependent manner. Group without P18PE treatment served as control (Figure 4.20 and Figure 4.21).

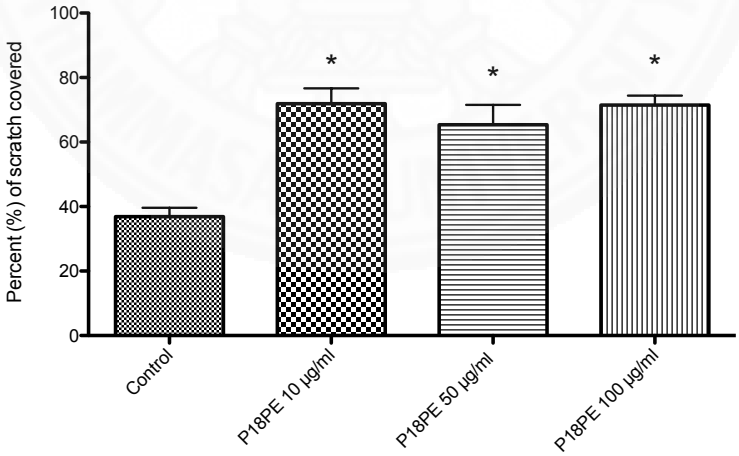


Figure 4.20 Graph of Wound Scratch Assay of P18PE on Human Gingival Fibroblast after incubation for 24 h. Data represents mean ± SE of 3 independent samples. An asterisk (*) indicates a significant difference in percentage of scratch covered compared to control.

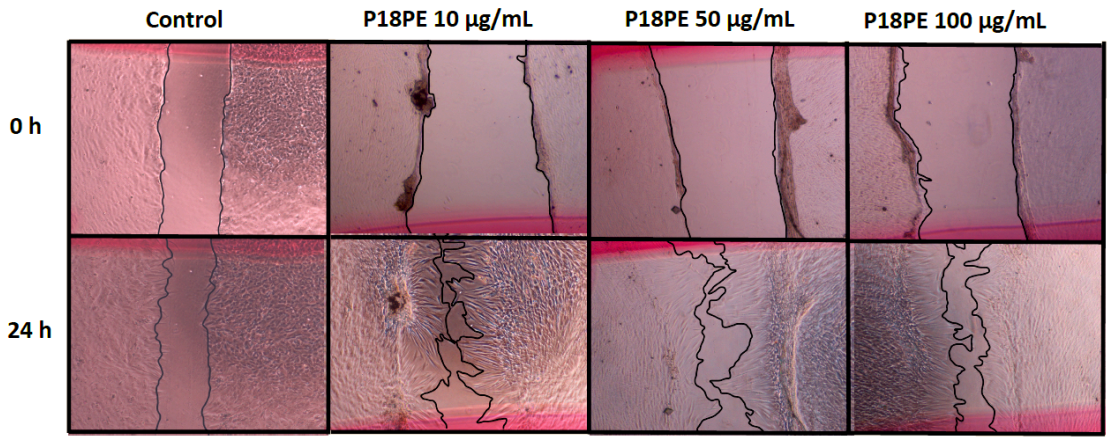


Figure 4.21 Micrograph of HGF on Monolayer Culture After Treatment with P18PE. P18PE could induce HGF migration

4.1.11 Determination of Bacteria Viability

4.1.11.1 Cytotoxicity of *C. nutans* Chloroform Extract on *S. mutans*

The cytotoxicity of chloroform extract was tested on *S. mutans* (100, 250, 500, and 1000 µg/mL). The result showed that chloroform extract at all concentration was not toxic to *S. mutans* (Fiure 4.22).

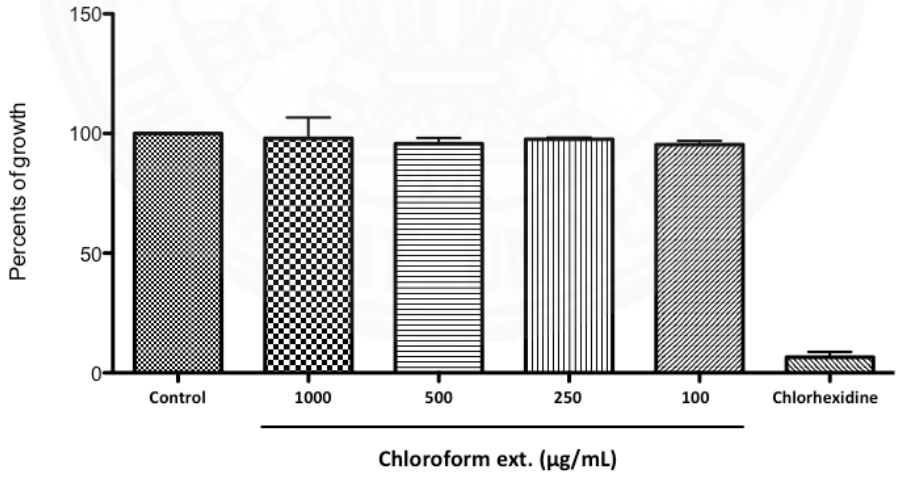


Figure 4.22 Bacteria Viability After Treatment with Various Concentration of Chloroform Extract for 24 hour. Data represents mean ± SE of 3 independent samples

4.1.11.2 Cytotoxicity of P18PE on *S. mutans*

The cytotoxicity of P18PE was tested on *S. mutans* (1-500 µg/mL). The result showed that P18PE at all concentration was not toxic to *S. mutans* (Figure 4.23).

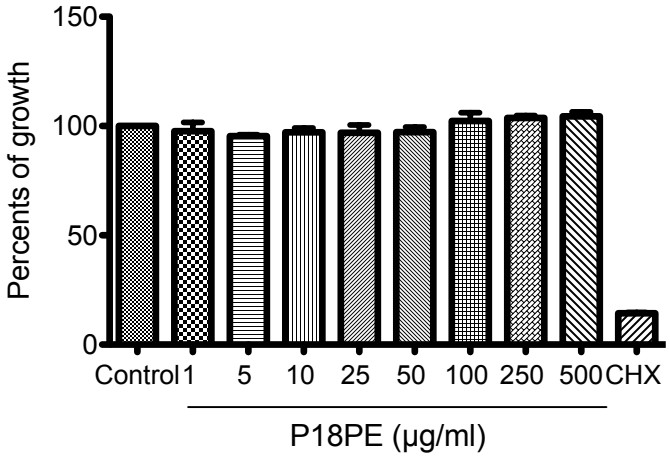


Figure 4.23 Bacteria Viability After Treatment with Various Concentration of P18PE for 24 hour (*n*=3). Data represents mean ± SE of 3 independent samples

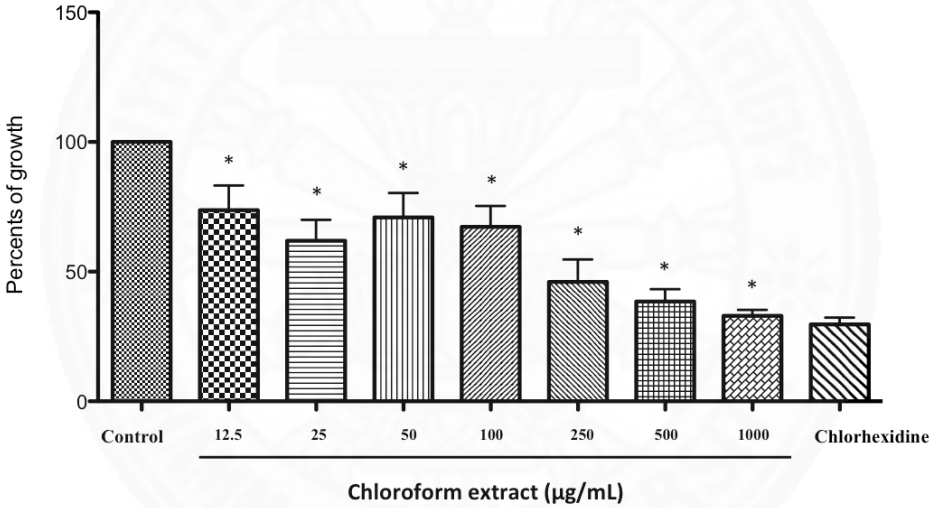


Figure 4.24 Anti-adherence Assay of C. nutans Chloroform Extract. Group without chloroform extract treatment served as control. Group with chlorhexidine treatment served as positive control (*n*=3). Data are expressed as mean ± SE. *: difference between experiment group and control group, ($p < 0.05$).

4.1.12 Determination of Anti-adherence Activity

4.1.12.1 Anti-adherence Assay of *C. nutans* Chloroform extract

Inhibition of *S. mutans* adherence by *C.nutans* chloroform extract was evaluated with anti-adherence assay. The result showed that *C. nutans* chloroform extract at concentration 12.5 - 1000 µg/mL could inhibit *S. mutans* adherence. Group without chloroform extract treatment used

as control. Chlorhexidine 1 μg/mL used as positive control. (Figure 4.24).

4.1.12.2 Anti-adherence Assay of P18PE

Inhibition of *S. mutans* adherence by P18PE was evaluated with anti-adherence assay. The result showed that P18PE significantly (p<0.05) could inhibit *S. mutans* adherence at concentration as low as 25 μg/mL. Group without P18PE treatment used as control. Chlorhexidine 1 μg/mL used as positive control (Figure 4.25).

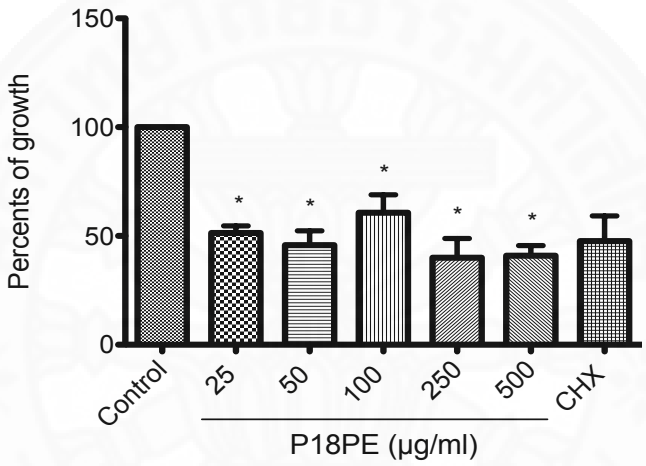


Figure 4.25 Anti-adherence Assay of P18PE. Group without P18PE treatment served as control. Group with chlorhexidine treatment served as positive control (n=3). Data are expressed as mean ± SE. *: difference between experiment group and control group, (p < 0.05).

4.1.13 Confocal laser scanning microscopy of biofilms

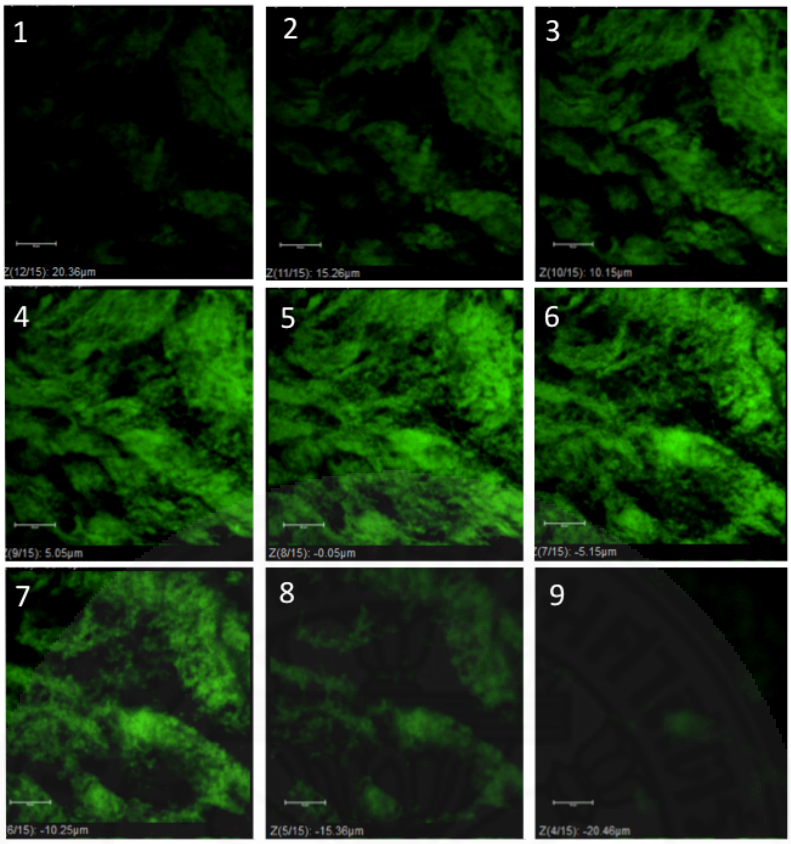
Confocal analysis of biofilms in the absence and presence of chloroform extract and P18PE was performed to examine its effect on biofilm. Bacteria were labeled with Live / Dead stain wherein live bacteria fluoresce green and dead bacteria fluoresce red. Chlorhexidine 1 μg/mL was used as positive control (Figure 4.26). Biofilm thickness in each experiment group is different. Number 1 to 9 indicates from outer to inner surface of biofilm. Control group and P18PE 10 μg/mL group showed no dead bacteria. Chlorhexidine group and P18PE 25-250 μg/mL group could

penetrate into biofilm and killed bacteria at certain depth. Complete biofilm thickness and P18PE penetration depth of each group are shown in Table 4.1.

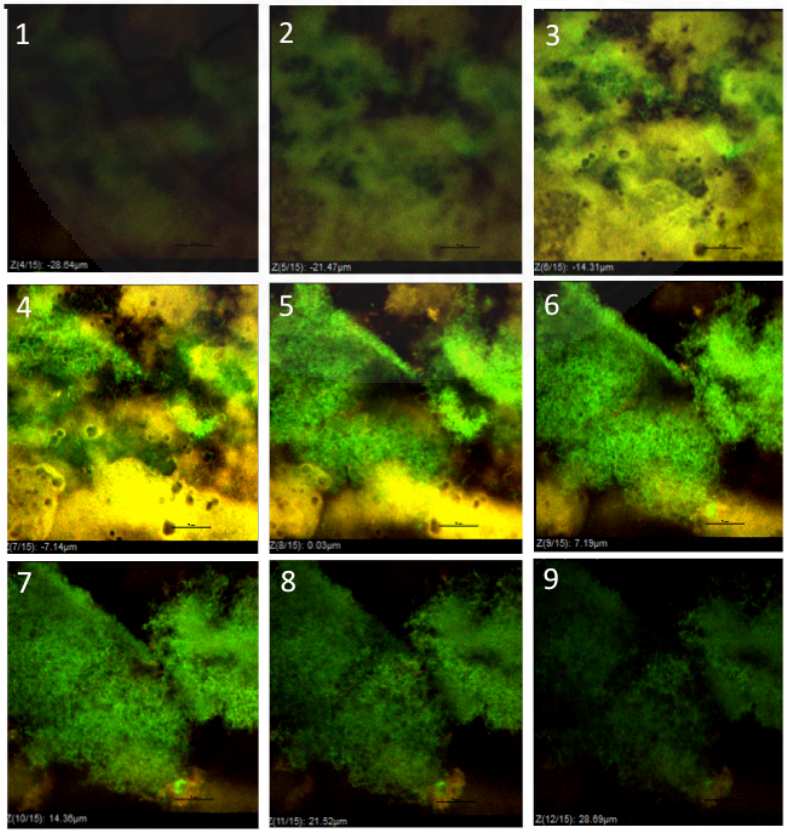
Table 4.1. Biofilm thickness, P18PE bactericidal penetration depth, and percentage bactericidal penetration of representative confocal laser scanning microscopy images.

	Biofilm thickness (μm)	**Bactericidal penetration depth (μm)**	**Percentage bacterial penetration**
Control	40.82	0	No penetration
P18PE 10 μg/mL	57.33	0	No penetration
P18PE 25 μg/mL	47.06	23.53	50%
P18PE 50 μg/mL	53.77	40.35	75%
P18PE 100 μg/mL	70.45	52.86	75%
P18PE 250 μg/mL	41.6	41.6	100%
Chlorhexidine 1μg/mL	49.36	30.85	62.5%

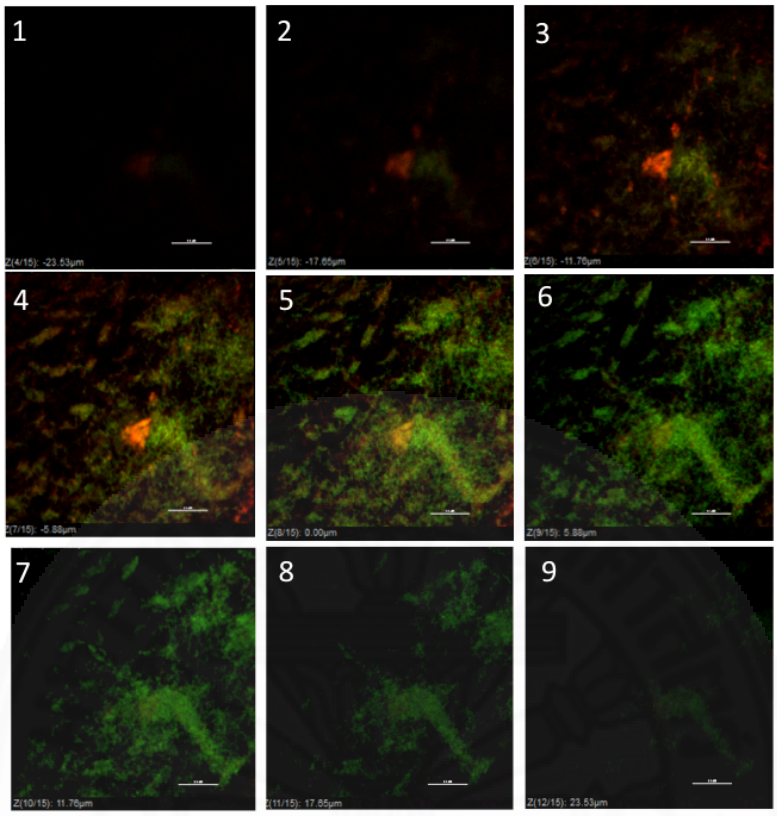
Control



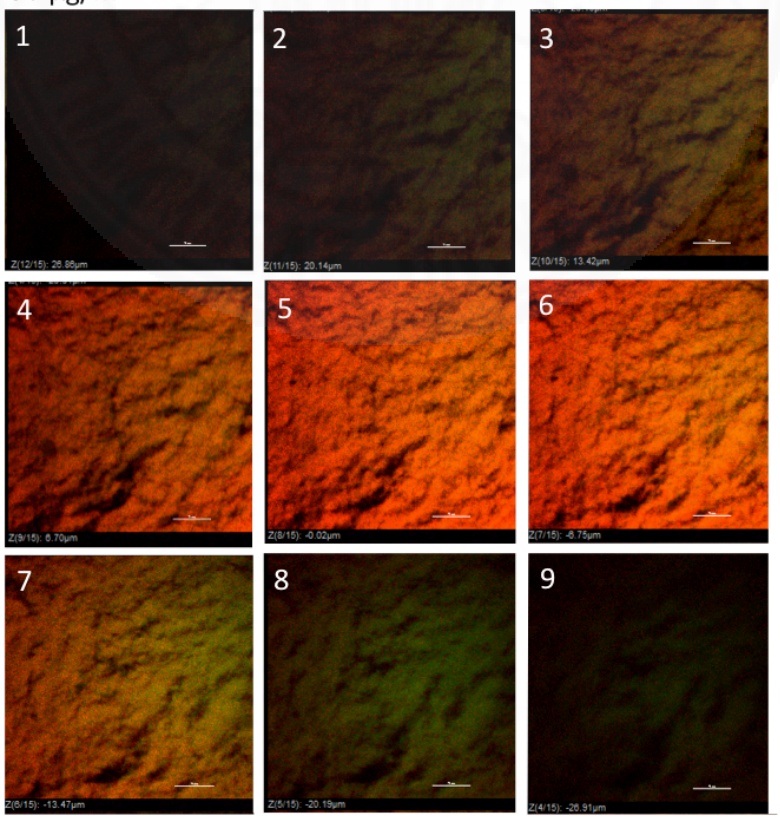
10 µg/mL



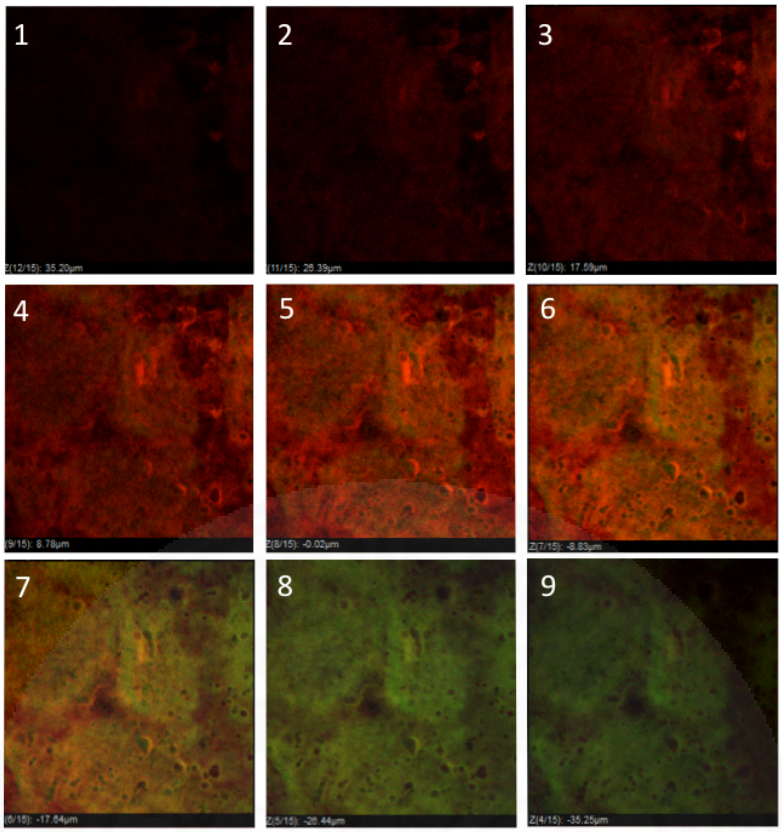
25 µg/mL



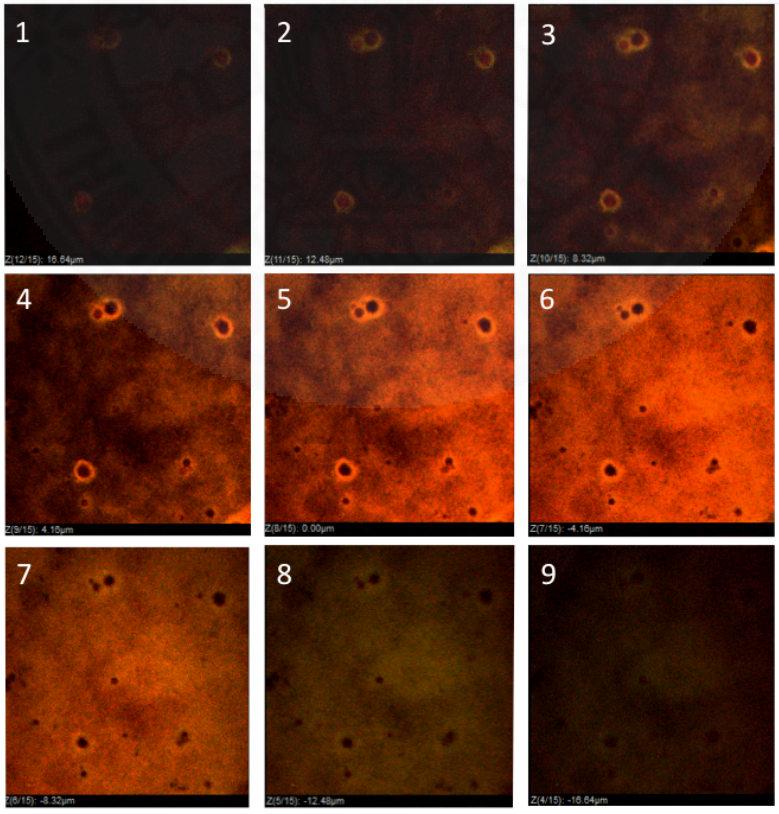
50 µg/mL



100 µg/mL



250 µg/mL



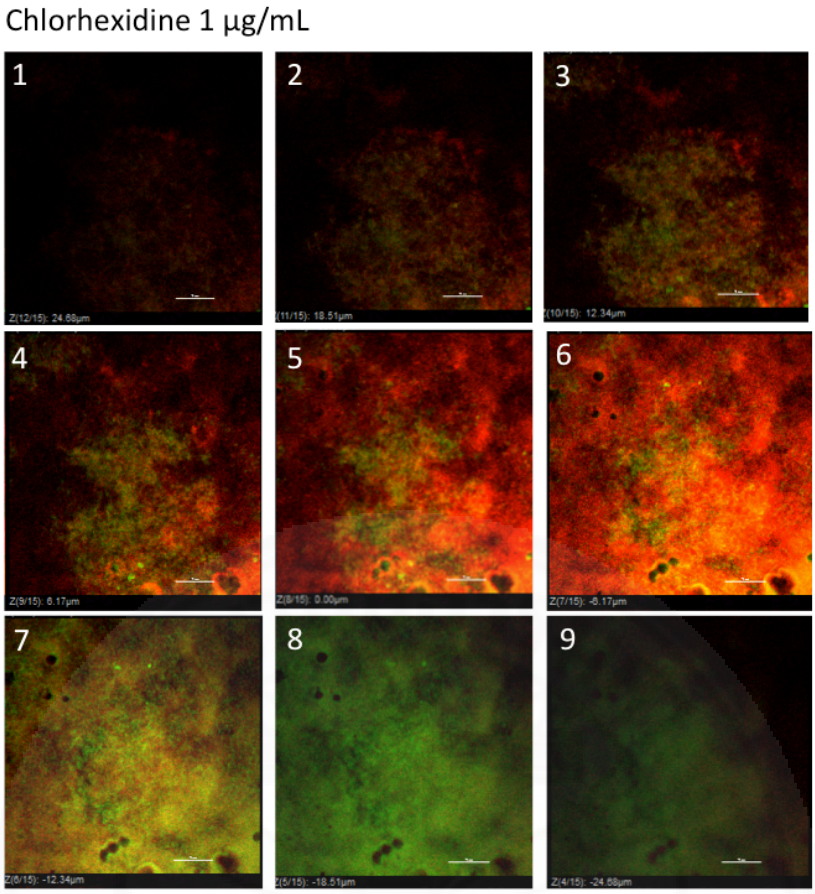


Figure 4.26 Representative Confocal Laser Scanning Microscopy Images of a Biofilm Formed by S.mutans in an 8-well Chamber Slide After Treatment with Various Concentration of P18PE. Group treated without P18PE treatment served as control. Group treated with chlorhexidine 1 µg/mL used as positive control. Bacteria were labeled with Live / Dead stain wherein live bacteria were stained green and dead bacteria were stained red. Number 1 to 9 indicates from upper to inner surface of biofilm. Scale bars, 50 µm.

4. 2 Discussion

Before this study begun, selection of good quality of *C. nutans* leaves was conducted. The method of extraction for screening purpose was reflux, using ethanol. The reason is to select the best sample of leaves that contain good quality of compounds inside because we do not know what season the plant was harvested, since the amount of the active constituents are not constant throughout the year.[159] In this study, the sample from Indonesia was chosen for the next step of isolation. The reason was that sample could be purchased from plantation in Bogor in lots of quantity. Considering that this study needed a lot of quantity of *C. nutans'*s leaves. Beside that, since it came from plantation, the sample from Indonesia was harvested

from the plantation, therefore the soil, time of harvest, post harvest treatment was paid attention really well. The quality of the herbal depends on (i) inter or intra-species variation that may be related to the country of origin, (ii) environmental factors like climate, altitude and other conditions under which it was cultivated, (iii) time of harvesting, (iv) plant part used, (v) post harvesting factors, such storage conditions, and processing treatment can greatly affect the quality of a herbal ingredient.[160]

After screening, the sample that was chosen, continue for further extraction, using Soxhlet extraction method. The solvents were hexane, chloroform, and ethanol respectively. The key advantage of this type of extraction; the only clean warm solvent is used to extract the solid in the thimble. This increases the efficiency of the extraction, compared with simply heating up the solid in a flask with the solvent. Besides that, Soxhlet extraction does not need filtration and this technique produces higher yield of extract with less volume of solvent. In contrary, the disadvantage of Soxhlet extraction methods is the compound that cannot stand to heat might be lost along the process and it is not possible to do agitation.

The active constituents of *C. nutans* have been extensively studied. Chlorophyll related compound is the only constituent from *C. nutans* that has been reported to have activity, which is antiviral activity.[30] This is the first study to investigate bioactive compound of *C. nutans* that has anti-inflammatory, cell migration effect, and anti-adherence activities. This is also the first study that investigated purpurin-18 phytyl ester (P18PE) activities. In the present study, the quantity of P18PE is low, which only 0.0071 g. It is because this study started only with 545 g of powder leaves, while in the previous study,[158] they started with 4.9 kg of powder leaves, which gave 0.0285 g P18PE. By percentage the present study has lower pecentage of P18PE (0.0013%) compare to previous study (0.005%). It might be because of different source of *C. nutans.*

LPS has the ability to activate mononuclear cells to secrete endogenous mediators, including NO.[48] In the present study, LPS from *E. coli* was used to stimulate NO production in macrophage RAW 264.7 cell lines. From the cytotoxicity assay of LPS on RAW 264.7 cell lines showed that concentration up to 1000 ng/mL did not toxic to the cell. To determine the dose of LPS to use in this study, preliminary NO assay of LPS on RAW 264.7 cell was being conducted. The result revealed that LPS could stimulate the production of NO in a dose-dependent manner (50-1000 ng/mL). Therefore, LPS at concentration 100 ng/mL was chosen in

this study. Genistein is isoflavones that have been studied to inhibit production of NO on LPS-induced RAW 264.7 cell lines.[161] Thus, in this study we chose genistein at concentration 50 μg/mL as positive control.

Methanolic extract of *C. nutans* has been reported to have anti-inflammatory activity.[11] The present study investigated the bioactive compound with anti-inflammatory activity. The result showed that P18PE is the compound responsible for the anti-inflammatory activity. In the present study, although all crude extract showed activity in NO production assay, chloroform extract showed the strongest activity, this is the reason why chloroform extract was chosen for further isolation. In the previous study, chlorophyll related compound also isolated from chloroform extract.[158] However, ethanol extract from refluxed extraction also showed the presence of P18PE (Figure 4.5).

In the present study showed that P18PE (mean = 5.2) activity in inhibiting nitric oxide higher than chloroform extract (mean = 12.6). It seems that there are other compound in crude *C. nutans* extract that disrupt the activity to inhibit nitric oxide. Hexane extract showed the lowest effect, so probably the compounds in hexane extract that works in opposite way higher than in other extracts. However, based on TLC of all crude extracts, hexane extract has low color intensity of P18PE compared to chloroform and ethanol extract. Probably this might be the reason why hexane extract showed low effect. The crude extract has one characteristic that its constituent may have opposite, moderating or enhancing effects.[113]

Mai et al. studied the anti-inflammatory mechanism of *C. nutans* leaves.[162] They compared extract from polar and non-polar solvent. The results showed that extract from polar solvents had the strongest effect in inhibiting NO. Polar solvent they used were methanol and dichloromethane, while in the present study, chloroform extract showed the strongest activity in inhibiting NO. There is slightly difference of polarity index between dichloromethane and chloroform. Polarity index of dichloromethane is 3.1, while chloroform is 4.1. This polarity difference between dichloromethane and chloroform that used as solvents for extracting plant still become controversy among researchers. Some researchers suggested there is no difference of compounds in a plant that extracted with dichloromethane or chloroform, and some researchers suggested the other way.

P18PE first reported in *C. nutans* as new compound related to chlorophyll a and chlorophyll b by Na Ayudhya et al.[158] In that study, this compound was found to related to purpurin 18 with an extra phytyl ester proton side chain. P18PE is one of the chlorophylls with magnesium-free degradation products. Chlorophyll a and phaeophytins, which also a chlorophyll degradation products, that comes from fresh leaves of *Streblus* asper, *Chromolaena. odorata, Synedrella nodiflora*, and *Amaranthus viridis* L. have been studied to have anti-inflammatory activity by inhibiting pro-inflammatory cytokine TNF-α gene expression on HEK293 cells.[163] Their study showed the anti-inflammatory activity of chlorophyll a is as good as diclofenac. Chlorophyllin, a chlorophyll derivative also has been studied to have anti-inflammatory activity by inhibiting NO production and iNOS gene expression on RAW 264.7 cell line.[164] These results give us hope that degradation products of chlorophyll a are promising materials for inexpensive phytomedicine development.[163]

To the best our knowledge, this is the first time *C. nutans* investigated for cell migration activity. In this study, chloroform and ethanol extract showed activity in accelerating HGF migration, while hexane extract was not. The activity of P18PE (mean = 71.9) in accelerating fibroblast migration is not much different as the activity of chloroform extract (mean = 70.4). It seems that P18PE is the main compound in *C. nutans* chloroform extracts to accelerate fibroblast migration.

Proliferation, differentiation, and migration of fibroblast are the key role for wound healing. However, fibroblast migration is the one event that critical in biological response in wound healing.[165,166] Based on this study, P18PE is the constituent of *C. nutans* that could accelerate migration of HGF to close the gap in monolayer cell culture. Considering *in vivo* wound healing is more complex than *in vitro*, this result still needs to be confirmed by further investigation such as expression of other wound healing related factors in gingival fibroblast and also in an animal model. However, based on this result, suggesting *C. nutans* as potential therapeutic drug for gingivitis and mouth ulcer.

Blepharis maderaspatensis (L.) B.Heyne ex Roth shares the same family with *C. nutans*, which is Acanthaceae. Rajasekaran et al. studied the ethanol extract of this plant on wound healing activity in animal model. The results showed that this plant accelerates wound healing by improving in all phases of wound repair.[167] Another plant from Acanthaceae family that has been studied for their

wound healing activity is *Justicia Flava.* Agyare et al. studied methanol extract of this plant in animal model and found that it has wound healing properties by significantly increase the rate of wound contraction. This plant also showed antimicrobial activity against *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa*. *C. nutans* methanol extract also has antimicrobial activity against *S. aureus* and *E. coli.*[168] Flavanoids, alkaloids, and tannins in this plant, might be the reason for *Justicia Flava*'s wound healing activity.[169,170] Although the bioactive compound that responsible for wound healing activity in this plant is still unknown.

Numerous traditional medicinal plants have been evaluated for their potential application in the prevention or treatment of oral diseases, including as mouth rinse. Plants from Acanthaceae family also have been studied to have anti-biofilm properties against gram-positive and gram-negative bacteria.[171,172] However there is no study of Acanthaceae family on anti-adherence against *S. mutans*. This is the first study of *C. nutans* on anti-adherence activity, particularly against *S. mutans*. Chlorhexidine has been studied to effective in killing all *S. mutans* isolate at concentration as low as 1 μg/mL.[173] Thus, in the present study, chlorhexidine at concentration 1 μg/mL was used as positive control on anti-adherence assay.

In this study, chloroform extract at concentration up to 1000 μg/mL did not show any antimicrobial activity. P18PE at concentration up to 500 μg/mL also did not show any antimicrobial activity. Compare to the previous study, methanolic extract exhibit antimicrobial activity against *S. aureus, E. coli, P. acnes, S. epidermis* and *B. cereus* at concentration MIC > 12.5 mg/mL, which is very high.[12] Another previous study, ethyl acetate extract *C. nutans* showed antimicrobial activity against *Bacillus cereus, Escherichia coli, Salmonella enterica* Typhimurium and *Candida albicans* at concentration 6.31mg/mL.[174] Those study showed that high concentration of *C. nutans* possesses antimicrobial activity. In oral cavity, high concentration cannot be use.

In the present study, the thickness of hydroxyapatite coating in each of the experiment group was not the same. Anti-adherence assay showed that P18PE at all concentrations had no significant difference compared to chlorhexidine. In contrary, based on representatives images of confocal laser scanning microscopy, P18PE 10 μg/mL could not kill bacteria. The penetration depth of P18PE 25

μg/mL was the lowest compared to other groups, but still showed dead bacteria. P18PE 50, 100

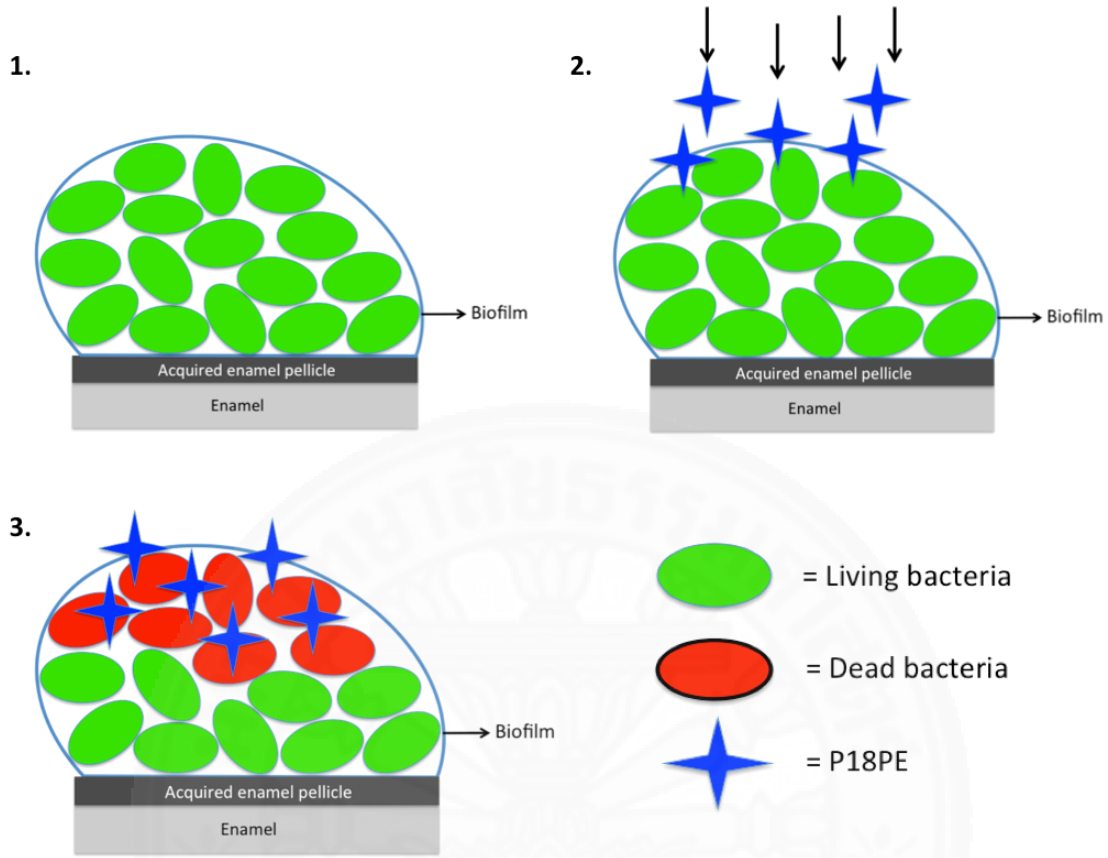


Figure 4.27 Illustration of P18PE mechanism as anti-adherence.

and 250 μg/mL had higher penetration depth than chlorhexidine. While the anti-adherence assay results showed that 25 – 250 μg/mL had anti-adherence activity and had no significant difference with chlorhexidine. The outer surface of biofilm layer consisted a higher quantity of dead bacteria than the inner layer of biofilm. This was likely because the outer surface of biofilm is in contact with culture medium, which contain P18PE. The higher P18PE concentration, the deeper P18PE could penetrated into biofilm to kill bacteria. P18PE 250 μg/mL could kill the entire bacteria in biofilm.

There are three mechanisms of how anti-adherence work, first, by the intervention of bacteria's adhesion to enamel surface. Second, by the intervention of bacteria co-aggregation which therefore stopping bacteria's growth. The last one is by removing the formed biofilm.[175] Oral streptococci able to form 60-90% of biofilm in

the first 24 hours of colonization.[176,177] In this study, *S. mutans* were grown 24 hours before P18PE added to the well. It means that in 24 hours biofilm already formed by *S. mutans*. The mechanism of P18PE's anti-adherence is by interfering biofilm that already formed by *S. mutans* (Figure 4.27). P18PE able to kill *S. mutans* that formed biofilm, as showed in the representatives images of confocal laser scanning microscopy (Figure 4.26).

Further investigations by *in vivo* studies are necessary to establish *C. nutans* effect on *S. mutans* adherence in oral cavity, because *in vitro* study always has limitation. Biofilm formed and structure in oral cavity is microbiologically and chemically different in comparison to biofilm that grown *in vitro*. Biofilm in oral cavity composed of mixed microbial with their products, and also there is glycoprotein from saliva. An interaction between plant extract or its constituents and salivary compound cannot be neglected. Future studies should explore the underlying cellular and molecular interaction between plant extract, bacteria and salivary compound *in vitro*, then continue to *in vivo* research.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

1. We have isolated bioactive compound, purpurin-18 phytyl ester from *C. nutans*
2. *C. nutans* extract and purpurin-18 phytyl ester have anti-inflammatory activity by reducing NO production on LPS-stimulated RAW 264.7 cell lines.
3. *C. nutans* extract and purpurin-18 phytyl ester have *in vitro* cell migration effect on human gingival fibroblast.
4. *C. nutans* extract and purpurin-18 phytyl ester have anti-adherence activity on *S. mutans*

5.2 Recommendation

For further investigation, we recommend to focus on cellular interaction and molecular mechanism of purpurin-18 phytyl ester on anti-inflammatory, wound healing and anti-adherence activity. By investigating in molecular level, we can confirm the activity of this compound and we also can understand the mechanism of this compound works. Besides that, there are still many unknown compounds in *C. nutans* that has not been isolated and investigated. Herbal medicine is always been a fascinating and wide field to explore.

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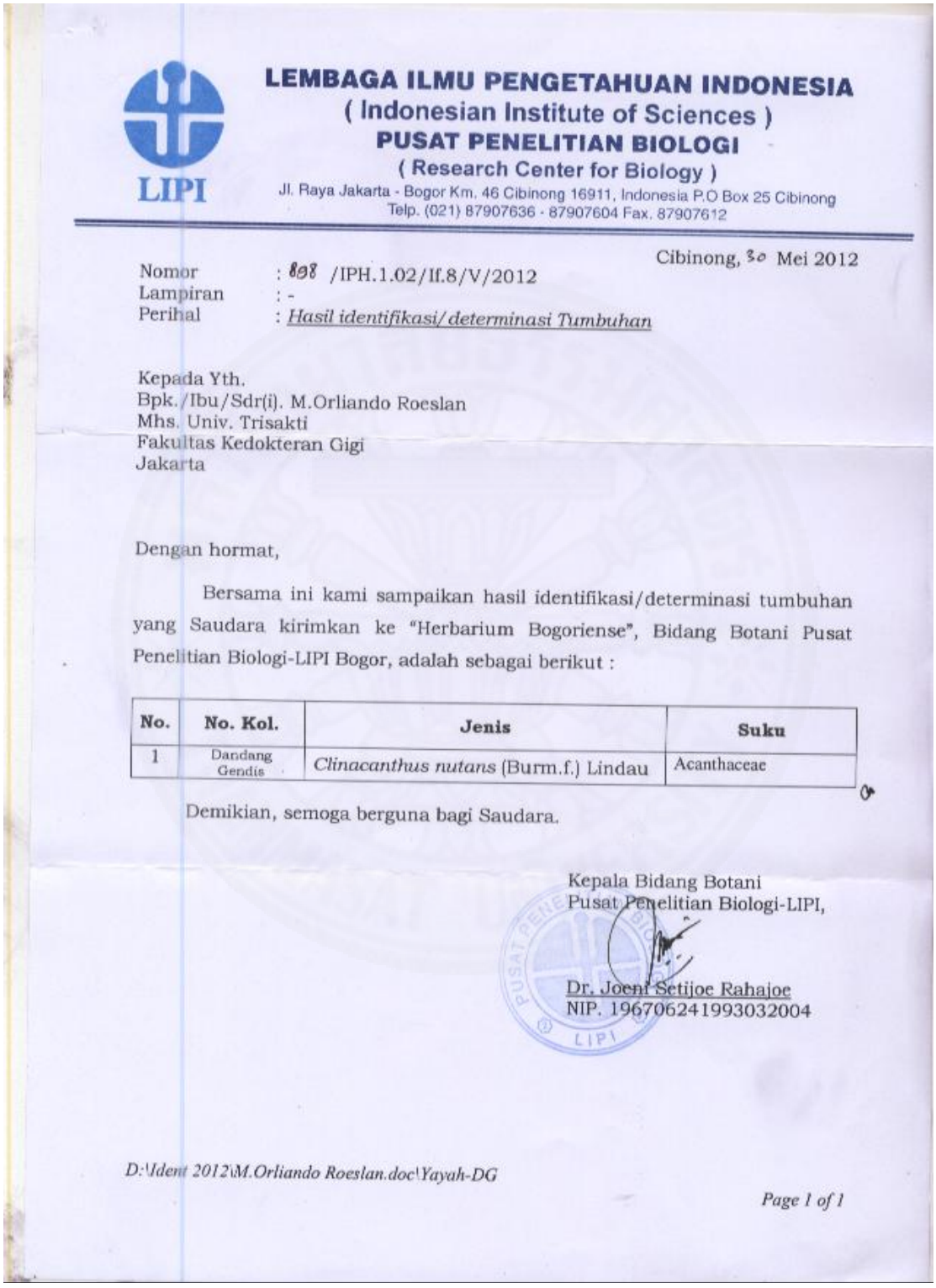
APPENDICES

APPENDIX A

IDENTIFICATION OF *CLINACANTHUS NUTANS*



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LIPI

Cibinong, 30 Mei 2012

Nomor : 898 /IPH.1.02/If.8/V/2012
Lampiran : -
Perihal : *Hasil identifikasi/ determinasi Tumbuhan*

Kepada Yth.
Bpk./Ibu/Sdr(i). M.Orliando Roeslan
Mhs. Univ. Trisakti
Fakultas Kedokteran Gigi
Jakarta

Dengan hormat,

Bersama ini kami sampaikan hasil identifikasi/determinasi tumbuhan yang Saudara kirimkan ke "Herbarium Bogoriense", Bidang Botani Pusat Penelitian Biologi-LIPI Bogor, adalah sebagai berikut :

No.	No. Kol.	Jenis	Suku
1	Dandang Gendis	*Clinacanthus nutans* (Burm.f.) Lindau	Acanthaceae

Demikian, semoga berguna bagi Saudara.

Kepala Bidang Botani
Pusat Penelitian Biologi-LIPI,

Dr. Joeni Setijoe Rahajoe
NIP. 196706241993032004

D:\Ident 2012\M.Orliando Roeslan.doc\Yayah-DG

Page 1 of 1

BIOGRAPHY

Name	Mr. Moehamad Orliando Roeslan
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Educational Attainment	Doctor of Dental Surgery, Trisakti University Master of Dental Science, Trisakti University
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Work Experiences	2006-Present Lecturer at Faculty of Dentistry, Trisakti University