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

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was approved as partial fulfillment of the requirements for
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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 active compound from *C. nutans* that responsible for anti-inflammatory, wound healing, 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 as low as 250 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, wound healing and anti-adherence activities.
Keywords: *Clinacanthus nutans,* purpurin-18 phytyl ester, anti-inflammatory, wound healing, anti-adherence activities.

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LIST OF ABBREVIATIONS AND SYMBOLS

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
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. Most of secondary metabolites, such as terpenes, phenolic compounds and alkaloids are classified based on their biosynthetic origin. The absence of secondary metabolites does not result in immediate death, but rather cause long-term 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.

Herbal medicine is the alternative to avoid the adverse effect of oral wound healing drug. 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] Wound healing and anti-adherence activity of *C. nutans* have never been investigated. Therefore, this study provides information on wound healing 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, wound healing 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 study inhibition effects of *C. nutans* extract and isolated constituent on nitric oxide production by RAW 264.7 macrophage cells.

2. To study wound healing effects of *C. nutans* extract and isolated constituent by human gingival fibroblast.
3. 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 wound healing effects in human gingival fibroblast.
3. *C. nutans* extract and isolated constituent can inhibit biofilm formation by *S. mutans.*

1.6 Study Design

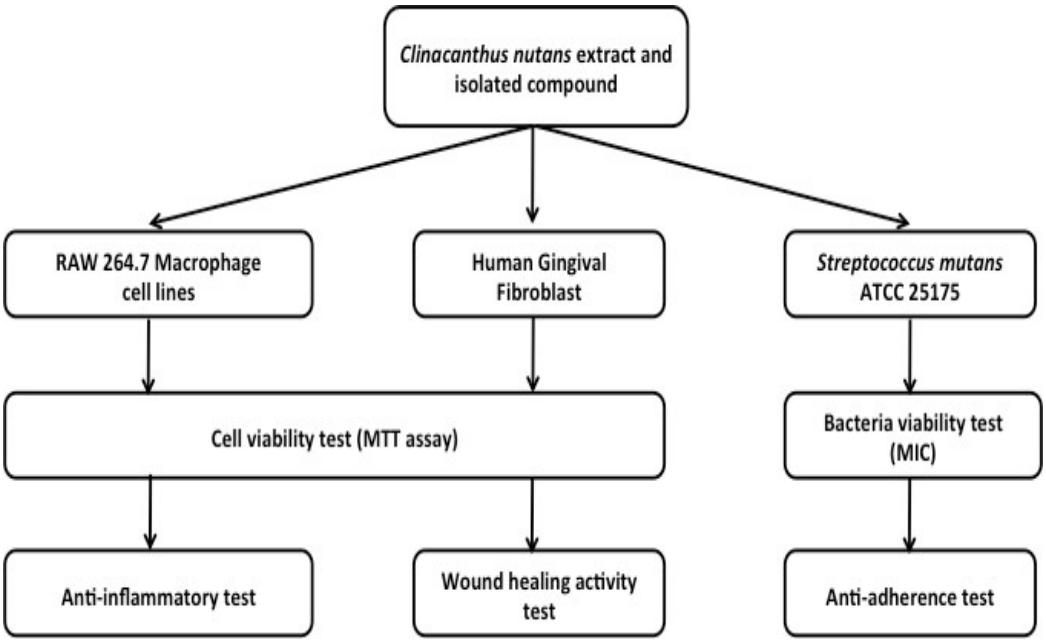


Figure 1.2 Study Design

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*

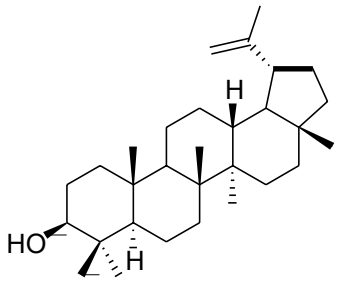
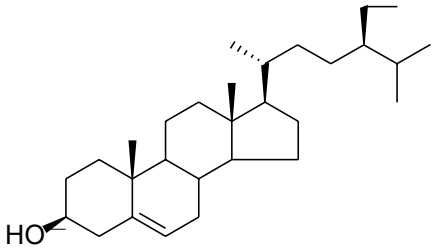
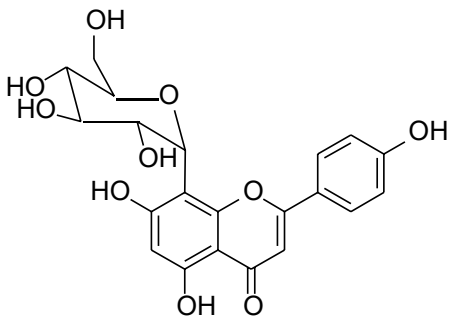
Chemical compound	Biological activity	Reference
Lupeol **[1]** 		[21]
β-sitosterol **[2]** 		[21]
Vitexin **[3]** 		[22]

Table 2.1 (continued)

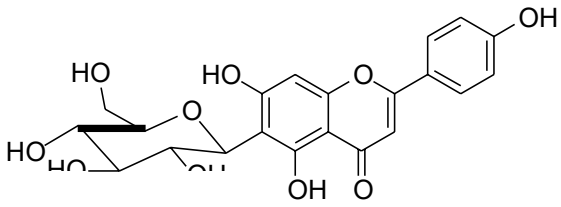
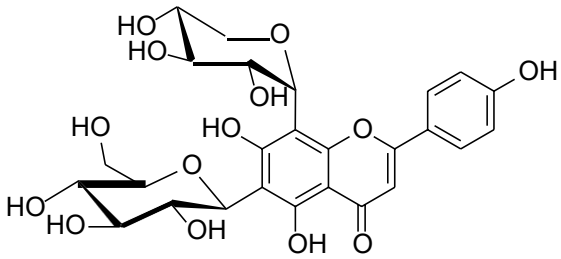
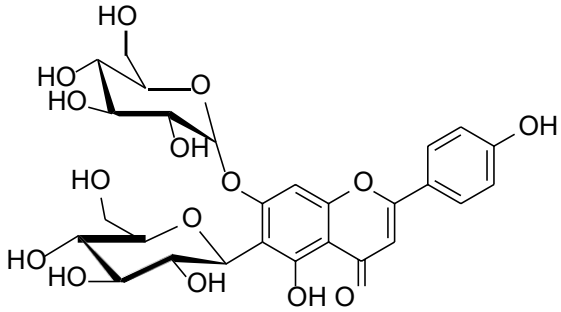
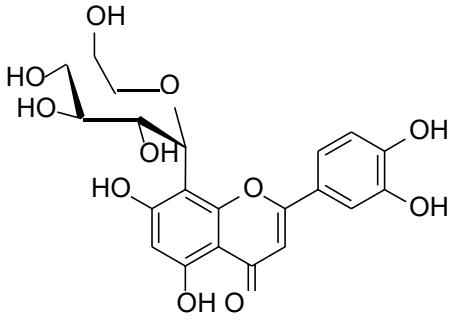
Chemical compound	Biological activity	Reference
Isovitexin **[4]** 		[22]
Shaftoside **[5]** 		[22]
Isomollupentin 7-*O*-β glucopyranoside **[6]** 		[22]
Orientin **[7]** 		[22]

Table 2.1 (continued)

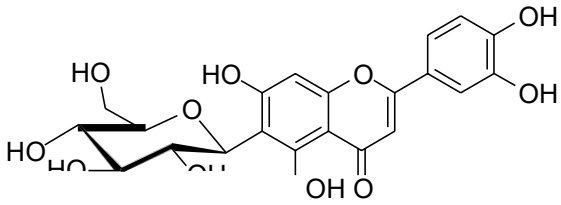
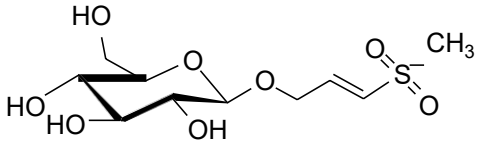
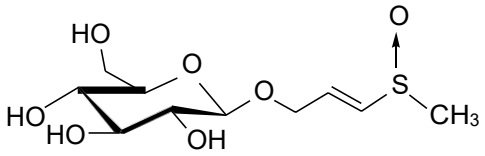
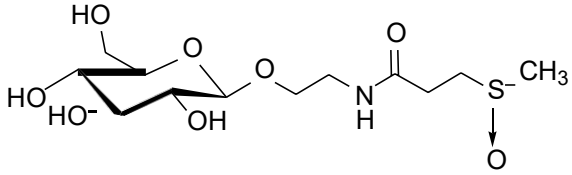
Chemical compound	Biological activity	Reference
Isoorientin **[8]** 		[22]
3-methylsulfonyl-2-propenyl β-D-glucoside/clinacoside A **[9]** 		[23]
3-methylsulfinyl-2-propenyl β-D-glucoside/clinacoside B **[10]** 		[23]
$C_{12}H_{21}NO_8S$/Clinacoside C **[11]** 		[23]

Table 2.1 (continued)

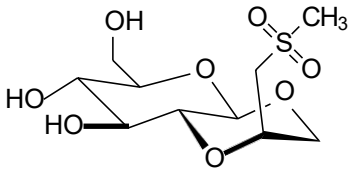
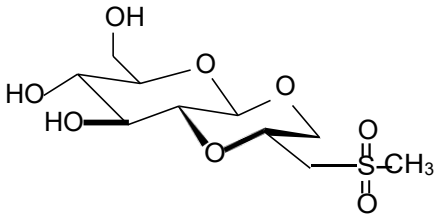
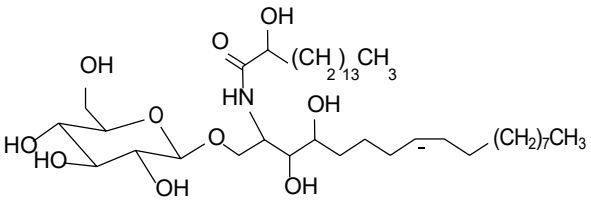
Chemical compound	Biological activity	Reference
$C_{10}H_{18}O_8S$/Cycloclinacoside A1 **[12]** 		[23]
Cycloclinacoside A2 **[13]** 		[23]
1-**O**-b -D-glucosides of phytosphingosines/cerebrosides **[14]** 		[24]

Table 2.1 (continued)

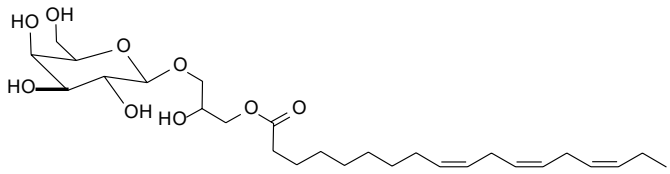
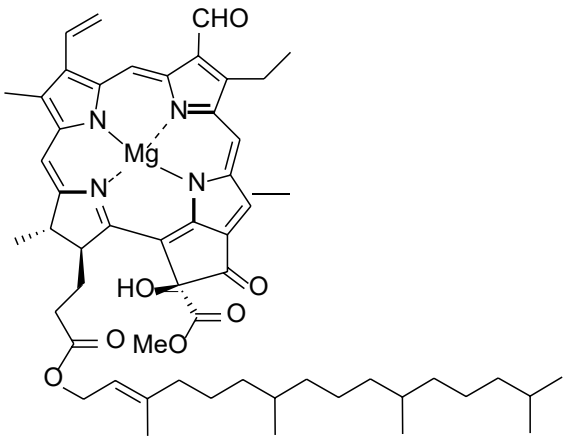
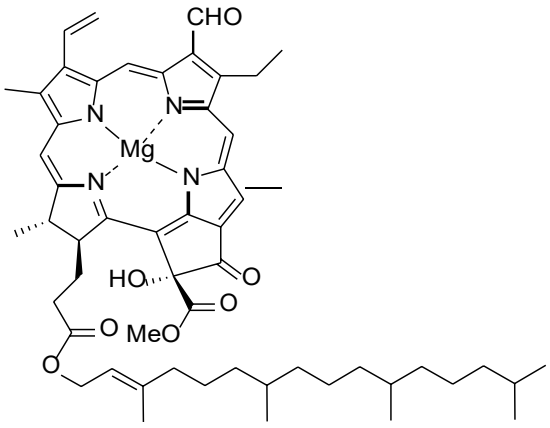
Chemical compound	Biological activity	Reference
(2S)-1-O-Linolenoyl-3-O-b -D-galactopyranosylglycerol **[15]** 		[24]
13^2-hydroxy-(13^2 -*S*)-chlorophyll b **[16]** 		[25]
13^2-hydroxy-(13^2 -*R*)-chlorophyll b **[17]** 		[25]

Table 2.1 (continued)

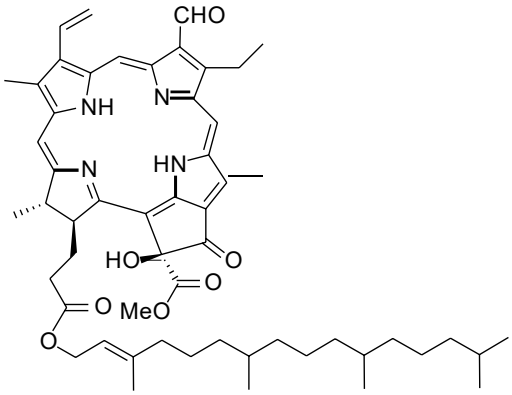
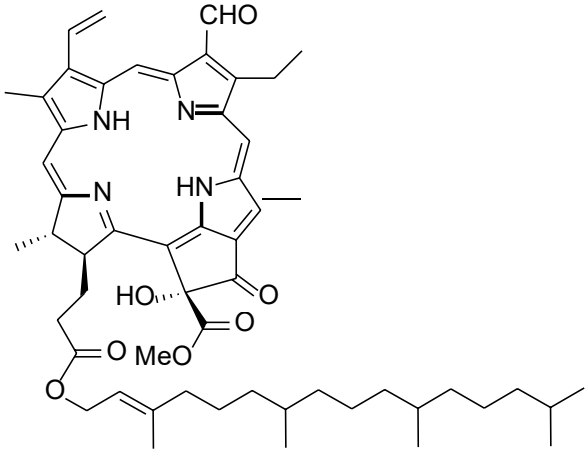
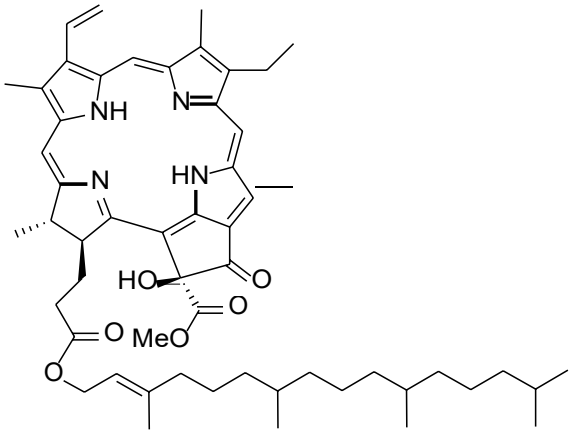
Chemical compound	Biological activity	Reference
13^2-hydroxy-(13^2 -*S*)-phaeophytin b **[18]** 		[25]
13^2-hydroxy-(13^2 -*R*)-phaeophytin b **[19]** 	Anti-herpes simplex activity	[25]
13^2-hydroxy-(13^2 -*S*)-phaeophytin a **[20]** 	Anti-herpes simplex activity	[25]

Table 2.1 (continued)

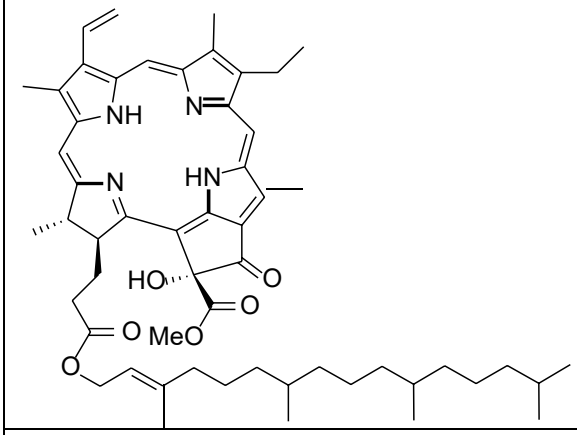
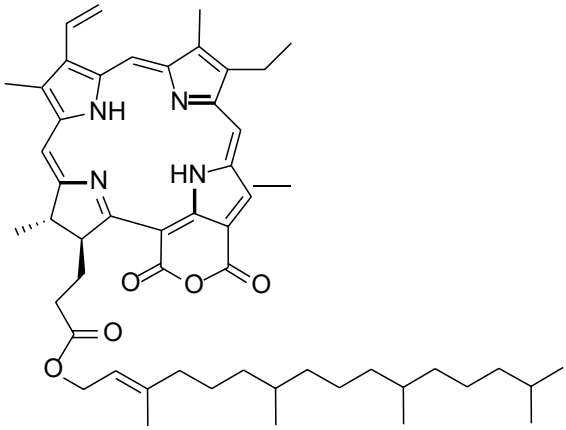
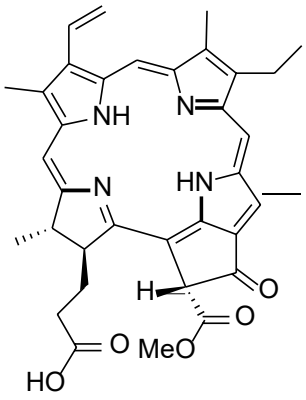
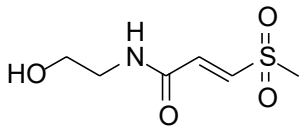
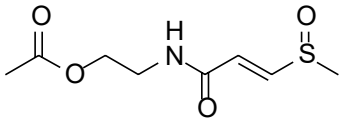
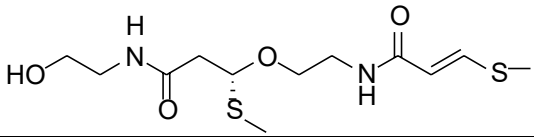
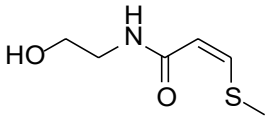
Chemical compound	Biological activity	Reference
13^2-hydroxy-(13^2 -*R*)-phaeophytin a **[21]** 	Anti-herpes simplex activity	[25]
Purpurin 18 phytyl ester **[22]** 		[25]
Phaeophorbide a **[23]** 		[25]

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] Inflammation is the first response of the immune system to infection or irritation. 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]

Injury to an organ or tissue results in progressive changes in damage area. The main signs are redness, heat, and swelling. These signs are the results of vascular alterations in the area of injury. The redness and heat result from an increase in blood flow, which in turn is the results of vasodilation, first involving arterioles, and then capillaries and venules. Swelling is the results of alterations in vascular permeability leading to exudation of fluid, plasma proteins, and white blood cells. 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]

Gram-negative bacteria are covered by outer membrane (cell wall), which is asymmetric. 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

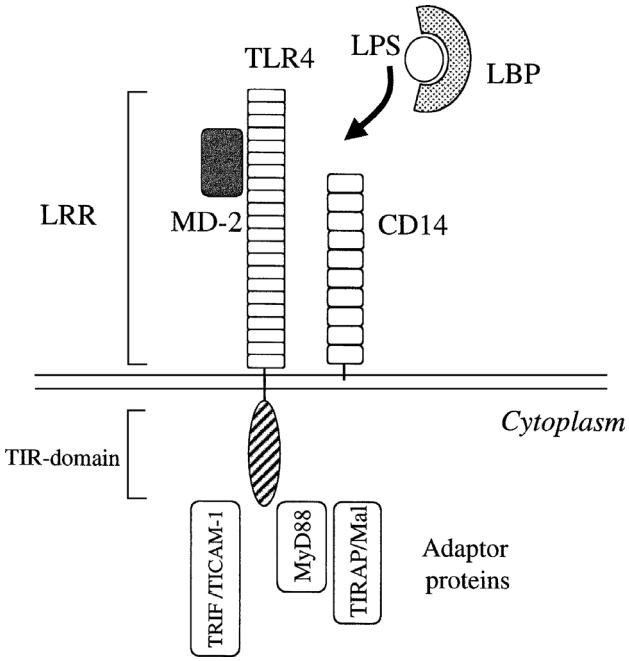


Figure 2.1 LPS receptor complex on macrophage [49]

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]

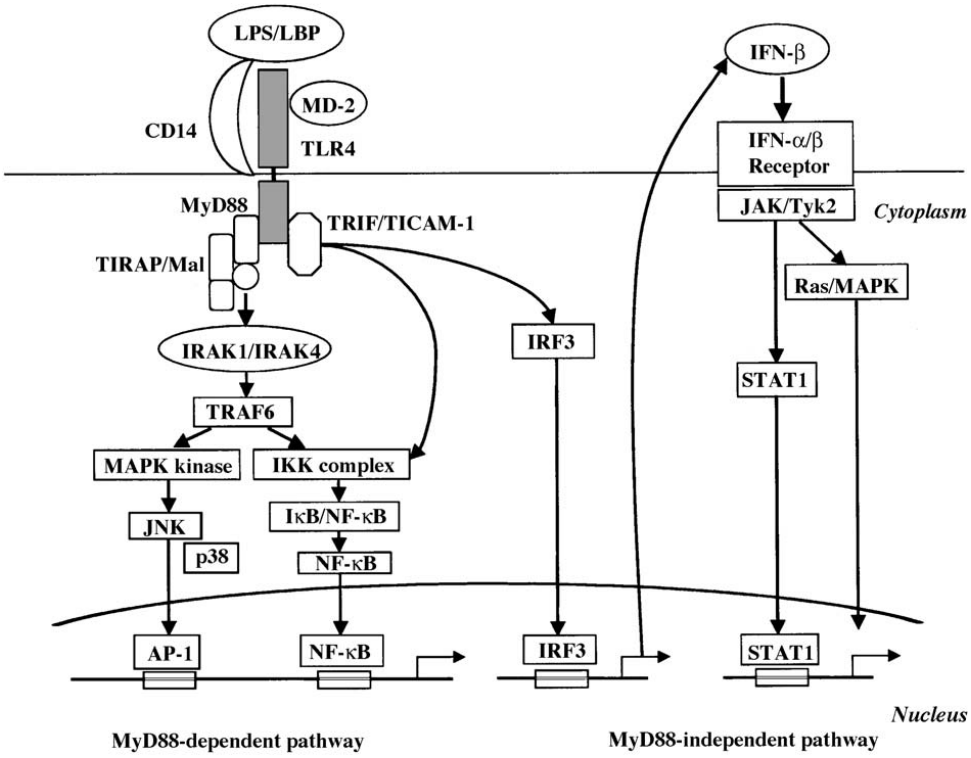


Figure 2.2 Macrophage's response to LPS mediated by TLR4 [49]

Macrophage function primarily as phagocytes during innate immune responses. During infection, detection of pathogen-associated molecular patterns (PAMPs) by macrophages leads to the production of cytokines and chemokines, promoting the recruitment of other cells and triggering an immune response. Macrophage can promote or perpetuate adaptive immunity by degrading pathogen-derived antigens and presenting them to T cells. By engulfing and degrading bacteria, macrophages sterilize tissue, resolve inflammation, and prevent further stimulation of the immune system.[50,51]

Macrophages have another important function, which is responsible for efferocytosis, the process of engulfing and eliminating apoptotic cells. Without the removal via efferocytosis, apoptotic bodies disintegrate and release their intracellular contents. This process, known as secondary necrosis, leads to inflammation and can be contribute to autoimmunity.[52] Although efferocytosis resembles phagocytosis, it is a distinct process, mediated by specific receptors, bridging molecules, and downstream signaling pathways.[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. There are both direct effects of NO that are mediated by the NO molecule itself, and indirect effects of NO that are mediated by reactive nitrogen species produced by the interaction of NO with superoxide anion or with oxygen. cGMP that is produced by the interaction of NO with soluble guanylate cyclase,

mediates many of the physiological effects of NO, and it is also an important example of the direct effects of NO.[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 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

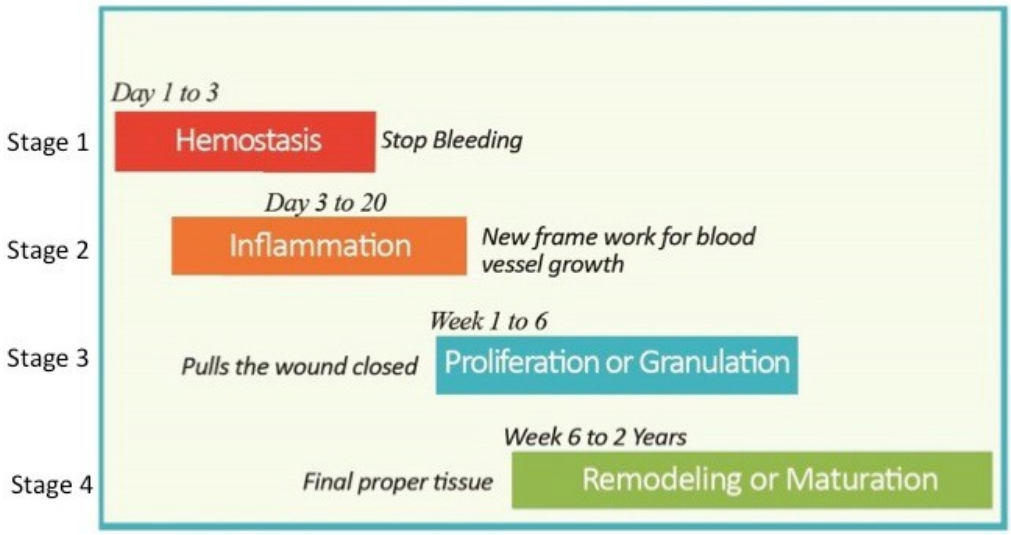


Figure 2.3 *Four stages of wound healing*

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 aimed to covering 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. Procollagen is then formed into fibers that are arranged in parallel fashion and cross-linked to form thicker and stronger 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 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, we have demonstrated that the chloroform extract from *C. nutans* and its isolated compound, purpurin-18 phytyl ester, have potential to inhibit inflammation by reducing NO production on RAW 264.7, potential to heal gingival wound by accelerating gingival fibroblast migration, and potential to inhibit *S. mutans* adherence.

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.