ANTI-INFLAMMATORY ACTIVITY OF *HARRISONIA PERFORATA* ROOT EXTRACT

MISS PATTAMA SOMSILL

A Thesis Submitted in Partial Fulfillment of the Requirements

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ฤทธิ์ต้านการอักเสบของสิ่งสกัดจากรากคนทา

นางสาวปัทมา สมศิลป์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปัทมา สมศิลป์: ฤทธิ์ต้านการอักเสบของสิ่งสกัดจากรากคนทา (ANTI-INFLAMMATORY ACTIVITY OF *HARRISONIA PERFORATA* ROOT EXTRACT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. จันทนี อิทธิพานิชพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.นิจศิริ เรืองรังษี, ผศ. ดร.วัชรี ลิมปนสิทธิกุล, 70 หน้า.

การอักเสบเป็นประโยชน์ต่อร่างกายในการกำจัดสิ่งแปลกปลอมและเนื้อเยื่อที่บาดเจ็บ การอักเสบมักเกี่ยวข้องกับการตอบสนองของเซลล์ในระบบภูมิคุ้มกัน โดยจะมีการหลั่งสารสื่อการอักเสบต่างๆเช่น tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and nitric oxide (NO) งานวิจัยนี้ได้ทำการศึกษาฤทธิ์ต้านการอักเสบของสิ่งสกัดด้วยเอทานอลจากรากคนทา ซึ่งเป็นส่วนประกอบจากรากทั้ง 5 ของตำรับยาไทยแผนโบราณเบญจโลกวิเชียร ที่ใช้ในการลดอาการไข้ โดยทำการศึกษาในเซลล์แมคโครฟาจ (J774A.1) ที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาร์ไรด์ (LPS) และในหนูแรทที่ทำให้เกิดการอักเสบที่อุ้งเท้าโดยการฉีดคาร์ราจีแนน (carrageenan) ผลการศึกษาพบว่าสิ่งสกัดจากรากคนทา 12.5 - 50 ไมโครกรัม/มิลลิลิตร ยับยั้งการหลั่งสารสื่ออักเสบ (pro-inflammatory cytokines) TNF-α และ IL-1β ได้อย่างมีนัยสำคัญและขึ้นกับความเข้มข้นที่ใช้ โดยสิ่งสกัดจากรากคนทา 50 ไมโครกรัม/มิลลิลิตร สามารถยับยั้งการแสดงออกของยีน TNF-α และ IL-1β ได้สูงสุดถึง 49.83% และ 47.27% ตามลำดับ สำหรับ IL-6 สิ่งสกัดจากรากคนทา 12.5 ไมโครกรัม/มิลลิลิตร ยับยั้งการแสดงออกของยีนสูงสุด 32.16% นอกจากนี้สิ่งสกัดจากรากคนทา 50 ไมโครกรัม/มิลลิลิตร ยังยับยั้งการแสดงออกของยีน iNOS และ COX-2 ซึ่งมีความสัมพันธ์กับการอักเสบได้ 88.11% และ 93.68% ตามลำดับ โดยไม่พบความเป็นพิษต่อเซลล์ที่ศึกษา การฉีดสิ่งสกัดจากรากคนทาเข้าทางช่องท้องหนูแรท ในขนาด 50, 100, 200 และ 400 มิลลิกรัม/กิโลกรัม สามารถลดการอักเสบแบบเฉียบพลันที่เวลา 2 ชั่วโมงภายหลังการเหนี่ยวนำให้เกิดอุ้งเท้าอักเสบได้ 28.48%, 31.18%, 47.85% และ 65.05% ตามลำดับ ผลการทดลองที่ได้บ่งชี้ว่าสิ่งสกัดจากรากคนทามีฤทธิ์ต้านการอักเสบ

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต……..

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PATTAMA SOMSILL: ANTI-INFLAMMATORY ACTIVITY OF *HARRISONIA PERFORATA* ROOT EXTRACT. THESIS ADVISOR: ASSOC.PROF. CHANDHANEE ITTHIPANICHPONG, THESIS CO-ADVISOR: ASSOC.PROF. NIJSIRI RUANGRUNGSI, Ph.D., ASST.PROF.WACHAREE LIMPANASITHIKUL, Ph.D., 70 pp.

Inflammation is a beneficial host response to pathogen or tissue injury. It stimulates immune cells to release a variety of inflammatory mediators and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandinE_2 (PGE_2) and nitric oxide (NO). *Harrisonia perforata* Merr. (*Simaroubaceae*) root is one of the five root compositions of a Thai traditional medicine named Bencha-Loga-Wichien which is used as a remedy for treating fever. In this study we investigated the potential anti-inflammatory property of the ethanolic extract from *H. perforata* root on LPS-activated macrophage,J774A.1 cells and in carrageenan-induced rat paw edema model. *H. perforata* significantly inhibited various pro-inflammatory cytokines expression. The inhibitory concentrations were observed at 12.5 - 50 µg/ml in concentration-dependent manner. The maximum inhibitory effects of *H. perforata* extract (50 µg/ml) were 49.83% and 47.27% for TNF-α and IL-1β respectively. For IL-6, the mamximum inhibitory effect (32.16%) was shown at concentration 12.5 µg/ml. *H. perforata* extract 50 µg/ml also effectively decreased mRNA expression of important inflammatory enzyme iNOS and COX-2 level up to 88.11% and 93.68% respectively. There was no cytotoxicity at the highest concentration used in this study. Intraperitoneal treatment of rat with *H. perforata* (50, 100, 200 and 400 mg/kg) reduced the effect on acute inflammation induced by carrageenan in paw edema model by 28.49% , 31.18%, 47.85% and 65.05% respectively at 2 hour after stimulation. All the results obtained from this study indicated that *H. perforata* possessed anti-inflammatory action.

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Student's Signature……..……
Advisor's Signature……..……
Co-Advisor's Signature……
Co-Advisor's Signature……..……..…..

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LIST OF ABBREVIATIONS

Ca^{2+}	Calcium
cDNA	Complementary DNA
CNS	Central nervous system
CO_2	Carbon dioxide
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
IC_{50}	Inhibition concentration 50%
ICAM-1	Intercellular adhesion molecule 1
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LFA-1	Leukocyte function-associated antigen 1
LPS	Lipopolysaccharide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
M	Molarities (mole per liter)
MBL	Mannose-binding lectin
mg	Milligram (s)
ml	Milliliter(s)
NO	Nitric oxide
NK	Natural killer
NSAIDs	Non steroidal anti-inflammatory drugs

LIST OF ABBREVIATIONS (con.)

O_2	Oxygen
OD	Optical density
P	Probability
PRRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecule patters
STAT3	Signal transducer and activator of transcription 3
SOCS3	Suppressor of cytokine signaling 3
TLRs	Toll-like receptors
VCAM	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
°C	Degree Celsius
µg	Microgram (s)
µl	Microliter (s)
µM	Micromolar
ng	Nanogram (s)
%	Percent
<	Less than
/	Per

CHAPTER I
INTRODUCTION

Background and Rationale

Inflammation is a host response to harmful stimuli (pathogens, damaged cells, or irritants). Therefore inflammation is a processed of immune system to destroy foreign invader or cells and tissue damage. Inflammation involves the local vascular system, the immune system, and various cells within the injured tissue. Dominant features of inflammation are pain, edema, redeness and fever. Inflammation response feature of vascular change is initiated by chemical mediators which are vasoative and chemotactic factor (such as histamine and nitric oxide). They cause vasodilation, increasing vascular permeability and blood flow stasis. The leukocytes such as macrophages insert to endothelial and transmigrate into intercellular space. . Macrophages are activated and released a number of cytokines including tumor necrotic factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and chemokines (such as interleukin 8, IL-8) .IL-8, a chemotractant, triggers other inflammatory or immune cells and let them get into the area of inflammation. In addition macrophages are able to stimulate production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide, all of which play role in inflammatory process. (Abbas *et.al.*, 2005; Ma *et.al.*, 2003)

The inflammatory effect still remains until the adulterated things are eliminated. Chronic inflammation is resulted from the remaining of adulterated thing which can not be destroyed and the function disorder or tissue damage may occur. Abnormalities associated with inflammation comprise a large, officially unrelated group of disorders which underlie a variety of human diseases such as inflammation related tumor, inflammation related asthma and inflammation related rheumatoid arthritis. (Haworth *et.al.*, 2008; Hinz *et.al.*, 2002; Mantovani *et.al.*, 2008)

Several countries try to develop anti-inflammatory drugs from herbal medicine. Thailand is plenty of herbal plants. They are used as food as well as traditional drugs. Evidence-based researches are required to assure their pharmacological properties. Root of *Harrisonia perforata* (*H. perforata*) which is one of the herbal root in five roots combination of Bencha-Loga-Wichien remedy used for treating fever in Thai traditional medicine. The drug formula is composed of five herbal roots in an equal part by weight including roots of *Capparis micracantha* (Ching-chee), *Tiliacora triandra* (Ya-nang), *Harrisonia perforata* (Khon-thaa), *Clerodendrum petasites* (Mai-tao-yai-mom) and *Ficus racemosa* (Ma-dueo-chumporn).Our interest is focusing on the anti-inflammatory effect of *Harrisonia perforata* root extract which hasn't been reported before.

Objective of the study

1. To study the effect of *Harrisonia perforata* ethanolic root extract on pro-inflammatory mediators and pro-inflammatory cytokines in LPS-stimulated macrophages.

2. To study anti-inflammatory activity of *Harrisonia perforata* ethanolic root extract in carrageenan-induced rat paw edema model.

Hypothesis

1. The *Harrisonia perforata* ethanolic root extract is able to inhibit pro-inflammatory mediators and pro-inflammatory cytokines in LPS-stimulated macrophages.

2. The *Harrisonia perforata* ethanolic root extract is able to reduce acute inflammation in carrageenan induced rat paw edema.

Research design

This study is experimental research.

Expected benefits and applications

If the evidence from this study indicates that *Harrisonia perforata* root extract possesses anti-inflammatory effect in molecular level and in vivo anti-inflammatory model, it will in part support the use of this Thai traditional medicine and also support further study in detail according to the methods required to investigate the efficacy and safety of herbal medicine.

Keywords

Macrophage/ Anti-inflammatory effect/ *Harrisonia perforata*

CHAPTER II
REVIEW OF LITERATURE

Immunity System

The immunity system is a biological system to defend the host from pathogen. Body defense mechanisms consist of innate immunity and adaptive immunity. The innate immunity is initial protection against pathogen but it is non-specific response. An adaptive immunity is timely develops and mediates the later but more effective defense against pathogen. Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules. The immune response dependent with activity of cells types in blood circulation.

The innate response is usually triggered when microbes are identified by pattern recognition receptors (PRRs, e.g. mannose binding lectin, macrophages mannose receptor, Toll-like receptor) which is interact to pathogen-associated molecule patters (PAMPs) on pathogen types. The types of cells involved are macrophages, neutrophils, natural killer cells and mast cells. The elements of innate immunity compose of the following events.

- Differences in susceptibility to certain pathogens
- Anatomical defenses
- Tissue bactericides, including complement
- Inflammation

When the innate immunity is inadequate to cope with infection, the adaptive immunity is mobilized by cues from the innate response. The adaptive immune response has ability to response to a variety of antigen, to discriminate between non-self (foreign) antigen and self antigen of the host, to response to a previously encountered antigen in a learned way by initiating a vigorous memory response. (Alberts *et.al.,* 2002; Tosi, 2005)

Table1 Comparison of innate and adaptive immunity (Alberts *et.al.,* 2002)

	Innate	Adaptive
Response time	Hours	Days
Specificity	Limited and fixed	Highly diverse, improves during the course of immune response
Response to repeat infection	Identical to primary response	Much more rapid than primary response

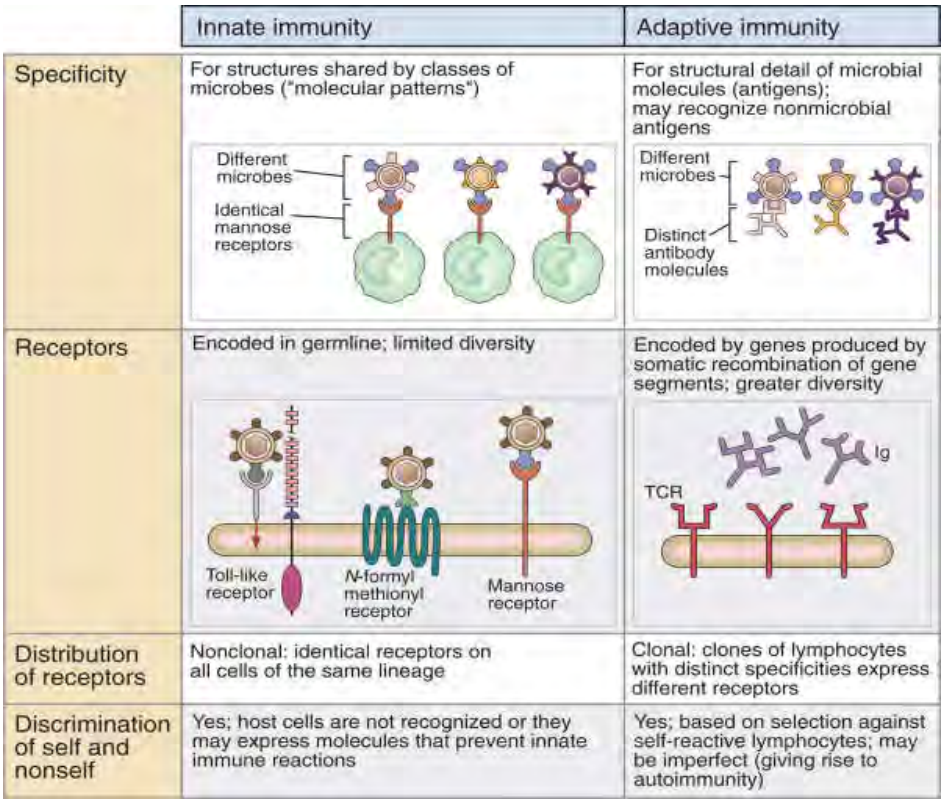

Figure1 The important features of the specificity and receptors of innate and adaptive immunity are summarized, with selected examples, some of which are illustrated in the *boxed panels.* (Abbas and Lichtman, 2006)

Inflammation (Abbas *et.al*., 2005; Huang *et.al*., 2004; Kumar *et.al*., 2007)

Inflammation is a protective mechanism of host to foreign invaders and tissue injury or necrotic tissue and is one of the first responses of the immune system. It is critical for both innate and adaptive immunity. The inflammatory process involves a vascular reaction and a cellular response, both are activated by mediators that are derived from plasma protein and various immune cells. Inflammation is related to a resolution process by tissue regeneration or replace with connective tissue or both accompany. There are five components in the inflammatory response.

1). Recognition of the foreign invaders

2). Recruitment of leukocytes

3). Removal of the agent

4). Regulation of the response

5). Resolution (repair)

Inflammation is related to several diseases such as cancer which is evidenced by inflammatory cells, chemokines and cytokines presenting in the microenvironment of all tumors in experimental animal model. Asthma is also one of the inflammatory disease of respiratory tract and the release of inflammatory mediators affect on smooth muscle contraction and stimulates mucus in the respiratory tract. (Haworth *et.al*., 2008; Hinz *et.al*., 2002; Mantovani *et.al*., 2008)

Although the inflammation and resolution are beneficial for elimination of foreign invaders and repairing tissue, it may be a punishment on host if it initiates hypersensitivity or the injurious agents can not be quickly eliminated. They will lead to chronic inflammation. The inflammatory response is characterized by the following symptoms redness (rubor) heat (calor) swelling (tumor) pain (dolor) and possible dysfunction of the organs or tissues involved. There are two types of inflammation. They are acute inflammation and chronic inflammation.

Acute inflammation

Acute inflammation is a rapid response to foreign invaders. This acute inflammatory response is characterized by the arrival of polymorphonuclear granulocytes (neutrophils), to phagocyte that engulfs and eliminates microbes. Blood monocytes are also recruited and differentiate into macrophages and dendritic cells at the site of inflammation. Macrophages share the role of phagocytosis with neutrophils but they have a much longer life span and important roles in orchestrating immune responses (Lawrence *et.al*. 2010). Reactions of acute inflammation may be induced by several of stimuli. e.g.

- Infections (bacterial, viral, fungal, and parasitic) and a microbial toxin.
- Trauma, physical and chemical agents
- Tissue necrosis including ischemia (as in a myocardial infract).
- Foreign bodies (splinter, dirt, sutures)
- Immune reactions; against environmental substances or against self tissue.

Acute inflammation has two major components including vascular changes and cellular events.

Vascular changes

1. Changes in vascular caliber and blood flow. After infection or injury, the arteriolar vasoconstriction should be short-lived and lasting only for seconds before the vasodilatation occurs. Tissue is redness and warmth because it's locally increases blood flow and the engorgement of down-stream capillary beds happens. It becomes congestion, slow flowing of blood and increased hypertension. The vascular permeability increases and plasma leaks out to vascular tissue. Red blood cells become more concentration, packing and slowing the circulation. The groups of red blood cells are stayed in central of vascular. They cause leukocytes accumulate along the vascular endothelial surface, a process is called margination.

2. Increased vascular permeability. Changes in vascular structure by mediators, arteriolar vasodilation and the increased volume of blood flow lead to a modulated of hydrostatic and osmotic pressure. Change of hydrostatic or osmotic pressure has effect on vascular permeability. Mechanisms contributions to increased vascular permeability in acute inflammatory reaction are of the following steps.

- Endothelial cell contraction leading to increased intracellular gaps.
- Direct endothelial injury (endothelial cells necrosis, burn or some infection)
- Leukocyte-mediated endothelial injury (leukocytes produce mediators that may cause endothelial injury).
- Increased transcytosis across the endothelial cytoplasm
- Leakage from new blood vessels

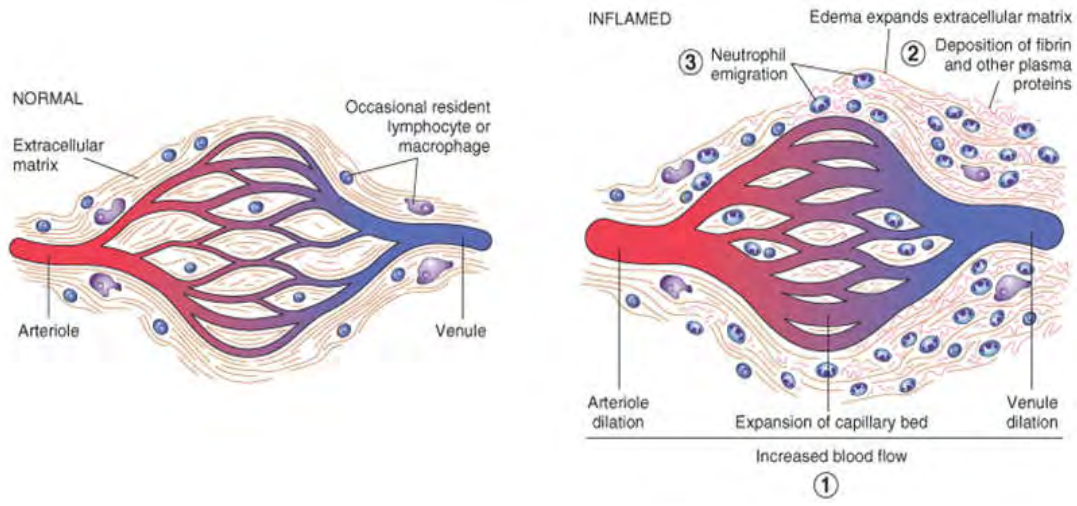

Figure 2 The major local manifestations of vascular change in acute inflammation, compared to normal (Kumar *et.al.,* 2005)

Cellular event

1. Leukocytes recruitment to inflammatory sites

Leukocytes pass through the vascular wall into the site of injury. The following consequences are contributed to its mechanisms.

- Margination and Rolling. Normally, laminar flow is a pattern of blood flow which plasma is stay nearest to endothelial vascular, next to is red blood cell and the leukocytes live in central of vascular. In inflammation, the red blood cells are packed and slowing blood flow. As a result, leukocytes are pushed out of the central axial column and interact with lining endothelial cell. Finally, leukocytes move and roll on the endothelial surface.

- Adhesion and Transmigration. The leukocytes are firm adhesion with endothelial surfaces. This adhesion is interaction between a adhesion molecule on leukocyte cells surfaces and receptor at endothelial cell. Inflammatory process involves with 4 adhesion receptor including the selectin, the immunoglobulin super family, the integrin and mucin-like glycoproteins.

Selectin associated to sugar-binding mammalian lectin, including E-selectin (ELAM-1) which is finding at the endothelial cell, P-selectin (GMP140) which is present in endothelial and platelets, and L-selectin (LAM-1) which is express on most leukocyte types. E-selectin and P-selectin use lectin domain binding with sialylated form of oligosaccharide on leukocyte surfaces and L-selectin is bound to various mucin-like glycoproteins on endothelial cells.

Immunoglobulins are adhesion molecules on endothelial cells, there are two types of endothelial adhesion molecule: intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1), both molecules bound to integrin.

Integrin are transmembrane heterodimeric glycoprotein. They are composed of α and β chain. The β2 integrin LFA-1 (CD11a/ CD18) and Mac-1 (CD11b/CD18) which is covalent bound with the ICAM-1, the β1 integrin (such as VLA-4) which binding to VCAM-1.

Mucin-like glycoproteins, are found in the extracellular matrix and on cell surfaces and which binding to L-selectin.

Table 2 Endothelial and leukocyte adhesion molecules (Kumar *et.al.,* 2007)

Endothelial molecules	Leukocyte molecule	Major role
P-selectin	Sialyl-Lewis X-modified proteins	Rolling (neutrophils, monocytes, lymphocytes)
E-selectin	Sialyl-Lewis X-modified proteins	Rolling and adhesion (neutrophils, monocytes, T-lymphocytes)
GlyCam-1, CD34	L-selectin	Rolling (neutrophils, monocytes*)
ICAM-1 (Immunoglobulin family)	CD11/CD18 integrins (LFA-1, Mac-1)	Adhesion, arrest, transmigration(neutrophils, monocytes, lymphocytes)
VCAM-1 (Immunoglobulin family)	VLA-4 integrin	Adhesion (eosinophils, monocytes, lymphocytes)
CD31	CD31	Trandmigration (all leukocytes)

*L-selectin-CD34 interactionsare also involved in the “homing” of circulation lymphocytes to the high endothelial venules in lymph nodes. ICAM-1 (Intercellular adhesion molecule 1), LFA-1 (leukocyte function-associated antigen 1), VCAM (vascular cell adhesion molecule 1), VLA-4 (very late antigen 4)

Various cytokines (TNF-α, IL-1) increase expression of selectin and integrin ligands on endothelium and the various chemokines promote a high-affinity of integrins for ligands; many of these cytokines are produced by tissue macrophages and other cells in responding to the invaders or damage tissue. After leukocytes are firm adhesion to endothelail surfaces, Leukocytes migrate through the endothelium by squeezing through endothelial cells and basement membrane. Leukocytes is changed the position from the blood vessel into extravascular tissue which different rate of speed. Neutrophils predominate in the early inflammatory infiltrate and are later replaced by macrophages.

- **Chemotaxis** is a migration of leukocyte from extravascular to inflammatory sites by chemical inducing substances (chemoattractant). Chemortactants for leukocyte include components of the complement system, particularly C5a; products of the lipoxygenase pathway, mainly leukotriene B4 (LTB_4); and cytokines especially those of the chemokine family (e.g. IL-8)

When chemotactic molecule binds to a specific receptor on the cell surface, phospholipase C is activated through the G-protein coupled receptor family on the surface of leukocytes. Some signal transductions lead to increase cytosolic cacium, which triggers the assembly of cytoskeletal contractile elements necessary for movement. The direction of such movement is specified by a higher density of receptor-chemotactic ligand interaction at the leading edge of cell.

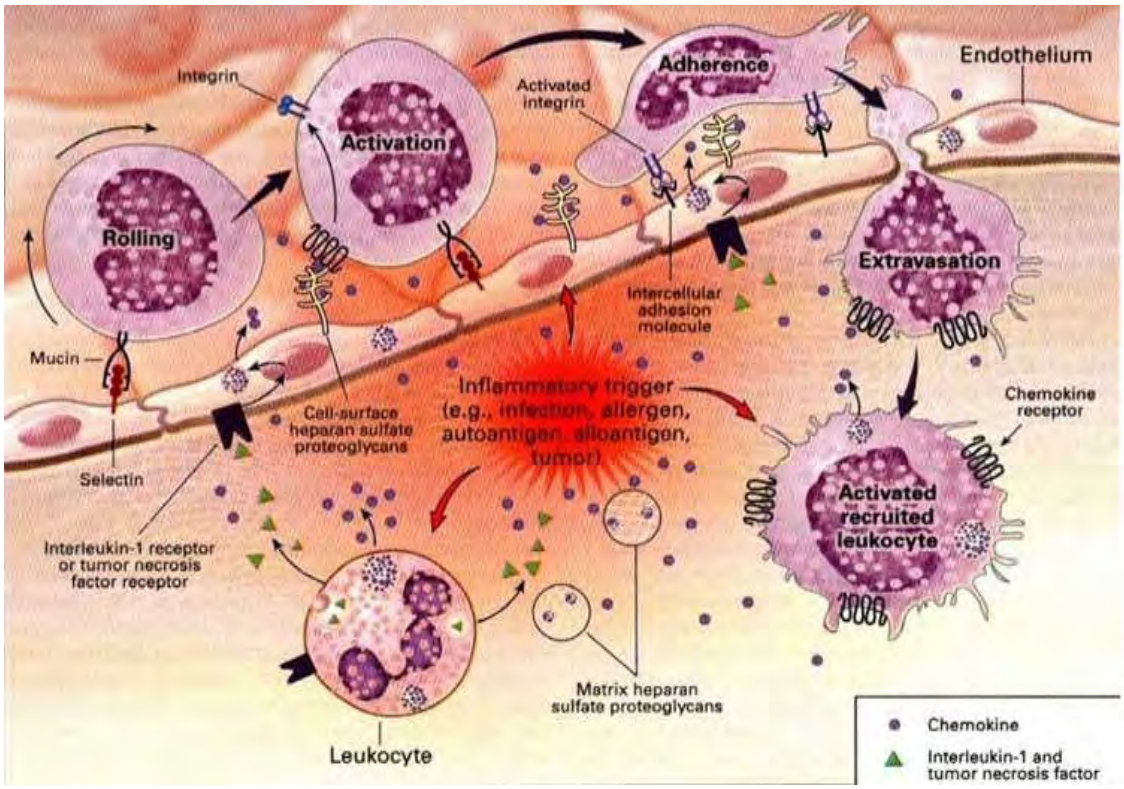

Figure 3 Cellular mechanism of leukocytes recruitment to inflammatory sites.

(http://www.bio.davidson.edu/Courses/immunology/chemokinespeech/chemo2.html)

2. Leukocyte activation

The functions of responses that are induced on leukocyte activation consist of the following mechanisms.

- **Production of mediators** e.g. arachidonic acid metabolites and cytokines.

- **Production of substances** e.g. lysosomal enzyme, reactive oxygen species (ROS) and reactive nitrogen species (RNS).

- **Leukocytes express a number of surface receptors** e.g. Toll-like receptors, seven-transmembrane G-protein coupled receptors and Mannose receptors.

- **Phagocytosis of particles:** phagocytes recognize pathogen by certain types of receptor and lead to the activation of the phagocytes to kill the ingested pathogen.

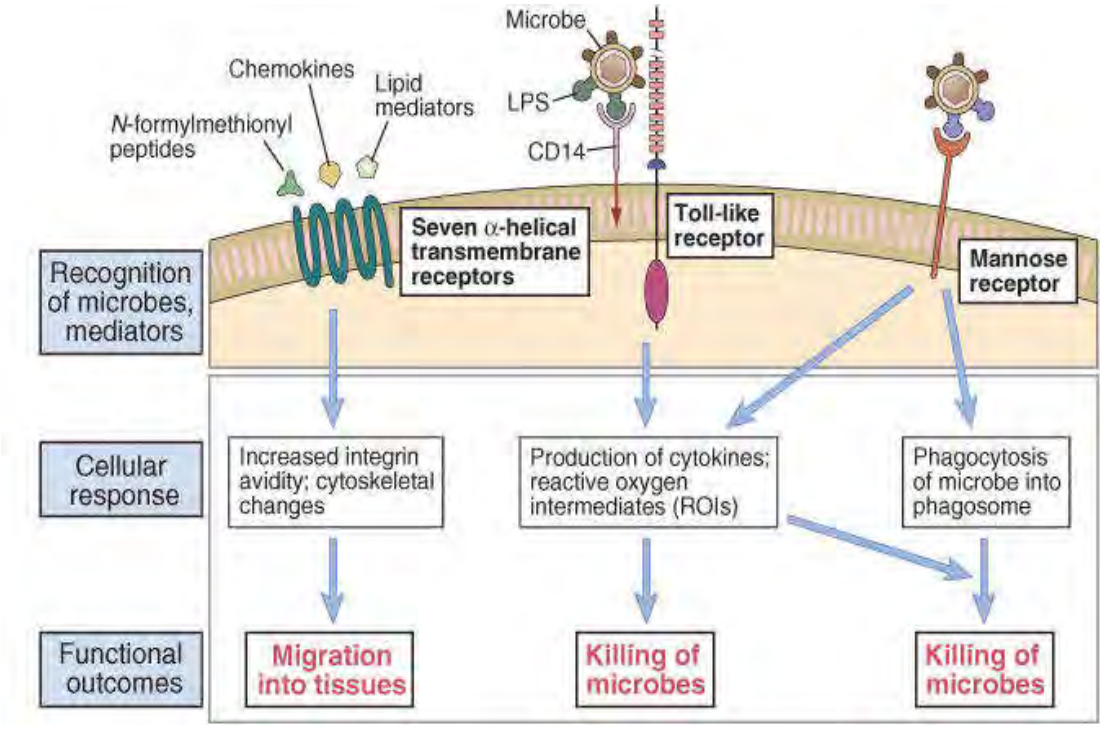

Figure 4 Different classes of cell surface receptors of neutrophils and macrophages recognize different stimuli. The receptors initiate responses that mediate the functions of neutrophils and macrophages (Kumar *et al.,* 2007).

Phagocytosis

A phagocyte has left the blood vessel and migrates to inflammation site and eats the microbes. When chemotacxis and adherence of pathogen to phagocyte occur, the pathogen is coated with opsonins (to increase the phagocytosis activity). Pathogens become attached to membrane evaginations called pseudopodia, the membranes close up and pinch off. Pseudopodia are forming to vesicle (called phagosome) and fuse with the membrane of a lysosome granule to form a phagolysosome. Several enzymes in the phagolysosomes are activated to generate a number of chemical mediators and cytotoxic substances required for killing pathogen via oxygen-dependent and oxygen–independent

mechanisms. The chemical mediators used to killing pathogen may be release into the extracellular space and may injure normal tissues which contribute to inflammation.

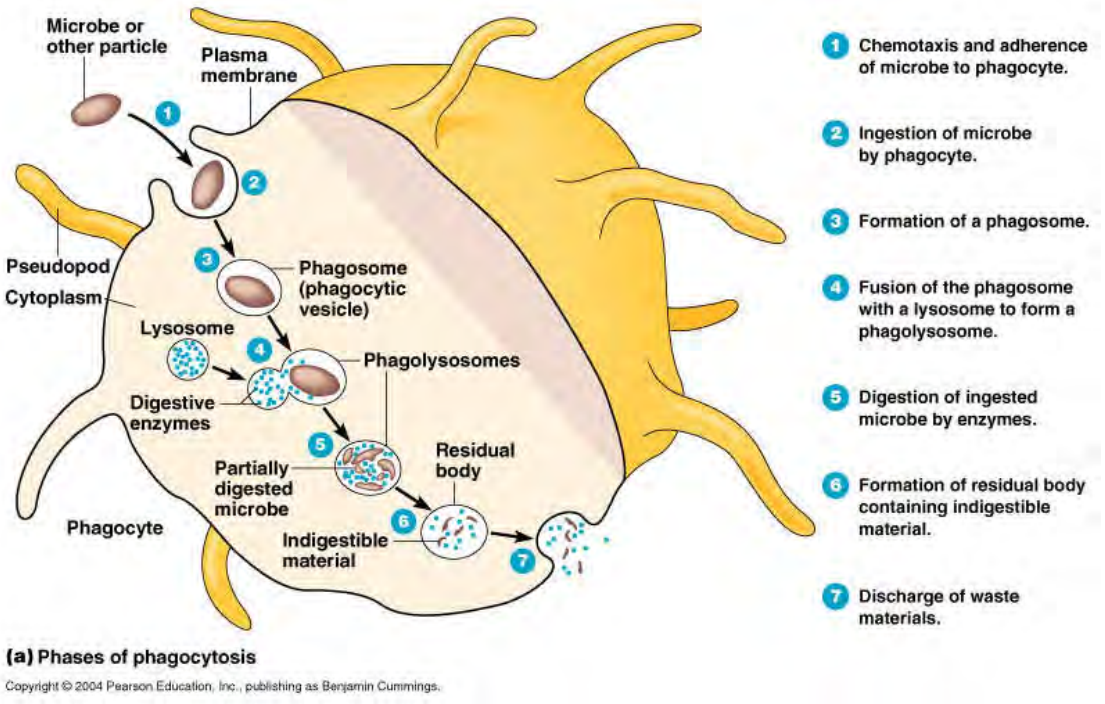

Figure 5 Phagocytosis of a particle by macrophages

Chronic inflammation

Chronic inflammation is associated with acute inflammation. Chronic inflammation is mediated by activation of inflammatory or immune cells and the duration of inflammation lasts for months, or years. Chronic inflammation is infiltration of mononuclear cells including macrophages, lymphocytes and plasma cells. The macrophages have ability to activate T-cells and T-cells have ability to activate macrophages which contribute to inflammatory response. Consequently, chronic inflammation is almost always accompanied by tissue destruction, the repair involved new vessel proliferation and fibrosis.

However, there are active anti-inflammatory mechanisms in the body that serve to control the response and prevent the host from causing excessive damage to the host.

Chemical mediators of inflammation

Cytokines

Tumor necrosis factor-alpha (TNF-α) TNF-α is a small polypeptide, which molecular weight is approximately 17 kd. TNF-α is secreted by macrophages, monocytes, lymphocytes and mast cells following their stimulation by lipopolysaccharides (a component in cell wall of Gram negative bacterial) and various pathogens. TNF-α is able to induce apoptotic cell death and inflammation. It's in turn activate monocytes or macrophages lead to production of other cytokines and chemokines, lipid mediators (e.g. platelet-activating factor , prostaglandins) and reactive oxygen species. The production of TNF-α is inhibited by IL-6, TGF-β, prostaglandin E_2 and dexamethasone. TNF-α also induces expression of adhesion molecules on endothelial cells, resulting in increase leukocyte binding with endothelial cells, which is the most important step for cellular event in the recruitment of leukocytes to inflammatory sites. (Abbas *et.al*., 2005; Dinarellot, 2000; Kumar *et.al.,* 2007)

Interleukin 1 (IL-1) IL-1 is produced by activated macrophages, as well as endothelial cells and mast cells. It has two isoforms which are IL-1α (MW 33 kd) and IL-1β (MW 17 kd). IL-1 originally is described as ''endogenous pyrogen,'' referring to its ability to produce fever in experimental animals and have showed early promise in vitro and in animal models of septic shock. The IL-1 stimulates the expression of adhesion molecules on endothelial cells. Both IL-1 and TNF-α play roles in immune response of the inflammation and metabolism and hematopoiesis. (Dinarellot, 1997; Dinarellot, 2000; Kumar *et.al,.* 2007)

Interleukin 6 (IL-6) IL-6 is produced by monocytes, macrophages, lymphocytes, endothelial cells, fibroblasts and other cells following the activation of microbes or cytokines. IL-6 acts at hepatocyte to induce the acute-phase protein and contribute to inflammation. In infection, excessive IL-6 is found since it is stimulated by IL-1 and TNF-α. IL-6 has the ability to induce the final maturation of B cells and stimulates IgG, IgM and IgA synthesis. (Abbas *et.al.,* 2005; Möller and Villiger, 2006; Tosi, 2005)

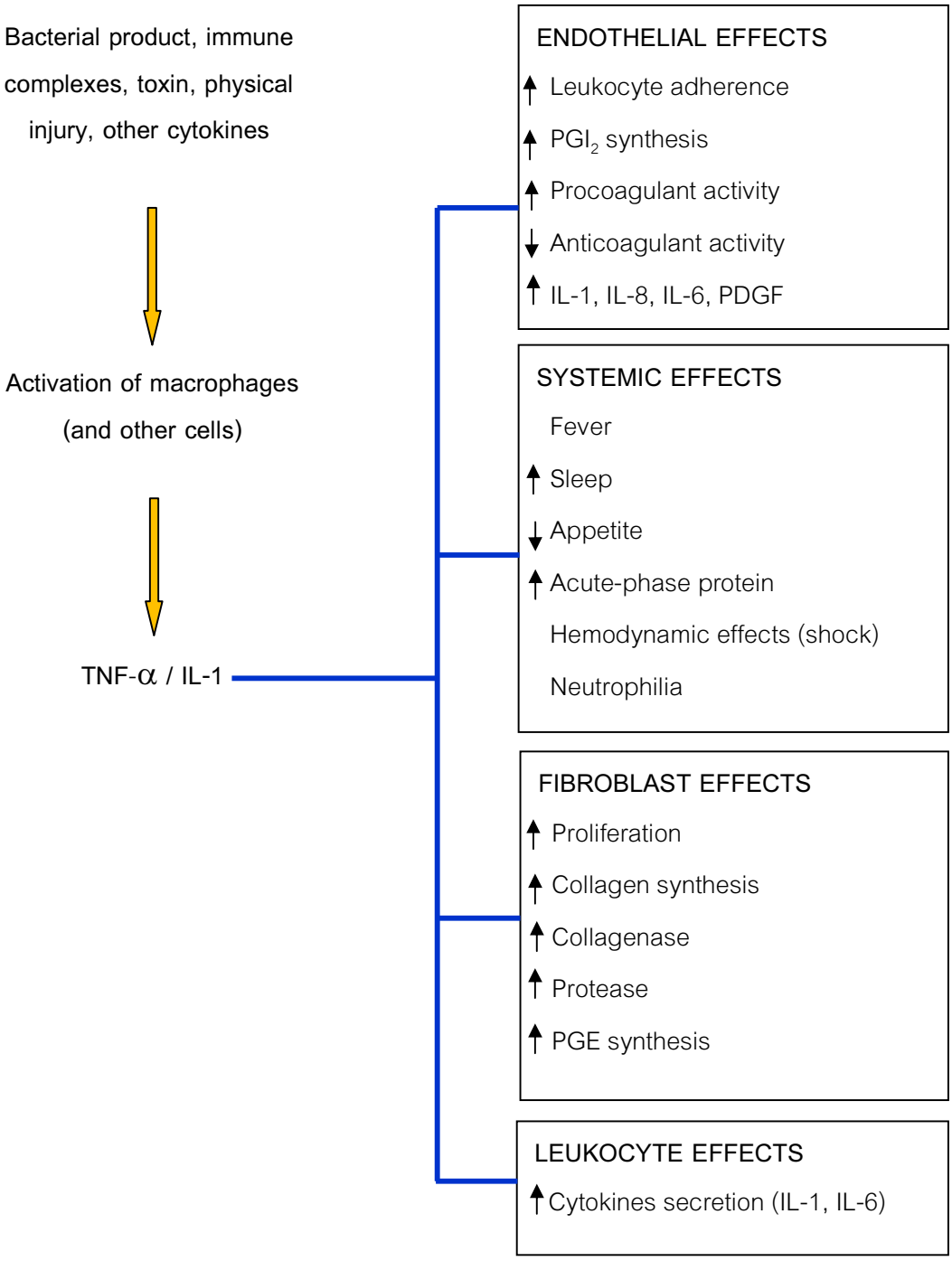

Figure 6 Major effect of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α.) in inflammation. (Kumar *et al.,* 2007)

Nitric Oxide (NO)

NO is a soluble gas which is generated by phagocytes (monocytes, macrophages and neutrophils) as part of the human immune response. NO plays role in inflammatory process, promoting blood vessels relaxation, regulation of blood pressure and vascular tone, neurotransmission, carcinogenesis and control of cellular growth. In normal physiological condition, NO exhibits an anti-inflammation but in abnormal state, NO over production together with other pro-inflammatory mediators induce inflammation. When an excessive amount of NO is generated, it reacts with superoxide to produce the damaging oxidant peroxynitrite which leading to tissue destruction as demonstrated in inflammatory autoimmune diseases. In contrast, inhaled nitric oxide has been shown to help patient survival and recovery from paraquat poisoning, which produces lung tissue-damaging. Thus NO can process pro- or anti- inflammatory effects depending on the concentration of NO level.

NO is synthesized by nitric oxide synthase enzyme (NOS) which catalyzed the L-arginine, molecular oxygen and NADPH into L-cirtrullin and producing NO. Nitric oxide synthase consist of three different types. They are endothelial nitric oxide synthase enzyme (eNOS), neuronal nitric oxide synthase enzyme (nNOS) and inducible nitric oxide synthase enzyme (iNOS). eNOS and nNOS are expressed constitutively in mammalian cells and synthesis NO which is dependent of enhance intracellular calcium (Ca^{2+}) levels. iNOS is induced after macrophages are activated by cytokines (INF-γ, TNF-α) and other agents. iNOS activity is independent of the level of calcium in the cell. As a result, the production of NO by iNOS lasts much longer than from the constitutive NOS. (Bogdan, 2001; Clancy, 1998; Coleman, 2001; Garcia *et.al.,* 2006; Kleinert *et.al*., 2004; Sharma *et.al*., 2007; Toda *et.al.,* 2009)

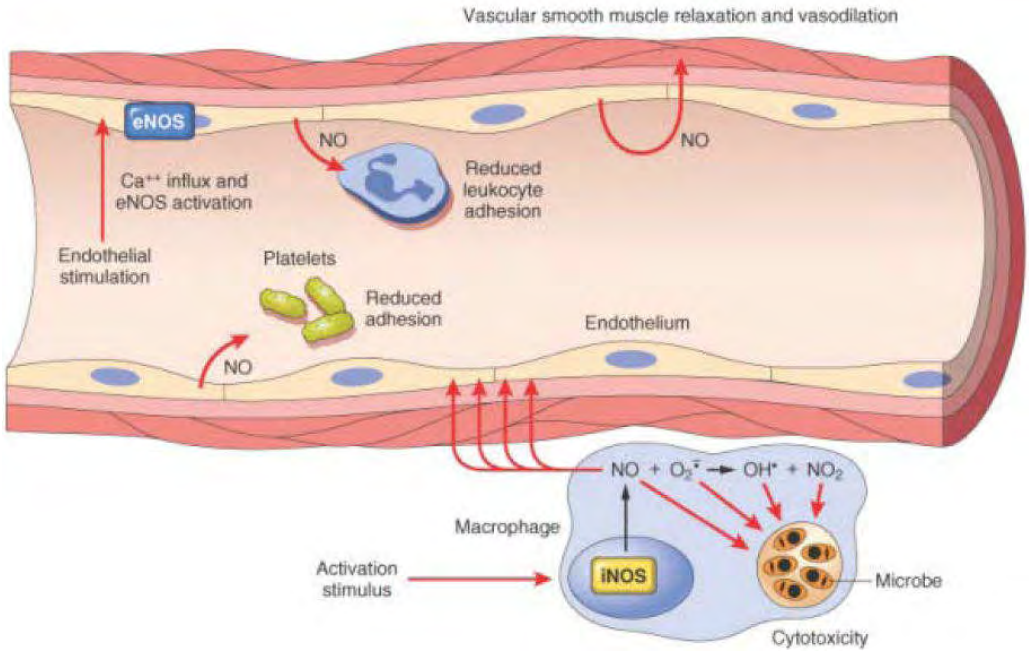

Figure7 Functions of nitric oxide (NO) produced by two nitric oxide synthase enzymes (eNOS and iNOS) in blood vessels and macrophages. NO cause's vasodilation and NO free radicals are toxic to microbial and mammalian cells (Kumar *et al.,* 2007).

Prostaglandins (PGs)

Arachidonic acid conversions to prostaglandin endoperoxide (PGG_2) by catalization of cycloxygenase (COX) enzyme, and a peroxidase reaction which PGG2 undergoes a two-electron reduction to PGH_2. PGH_2 is a precursor for bioactive lipids and prostaglandins including PGE_2, PGD_2, $PGF_2\alpha$, PGI_2 (prostacyclin) and TxA_2 (thromboxane A_2). Prostaglandins are involved in pathogenesis of pain and fever in inflammation. PGE_2 is hyperalgesic mediators that make the skin hypersensitive to painful stimuli. It is involved in cytokine-induced fever during infection. PGE_2 and $PGF_2\alpha$ cause vasodilation and increase the permeability of postcapillary venules, potentiating edema formation. PGD_2 is the major metabolite of the cyclooxygenase pathway in mast cells.

There are two isoforms of cyclooxygenase enzyme. Cyclooxygenase-1 (COX-1) is a constitutive enzyme in numerous cells. Its functions are important for platelet aggregation,

renal blood flow and gastrointestinal mucosa protection. Cyclooxygenase-2 (COX-2) is expressed by cells that are involved in inflammation such as macrophages. COX-2 is inducible by number of factors including IL-1, TNF-α, LPS, IL-2, GM-CSF, G-CSF and TGF-β. COX-2 expression and it is inhibited by glucocorticoid and IL-10. Administration of drugs that inhibit COX reduces swelling, pain and inflammation. (Hinz and Brune, 2002; Marco andRaymond, 2002; Turini and Dubois, 2002; Weinberg, 2000)

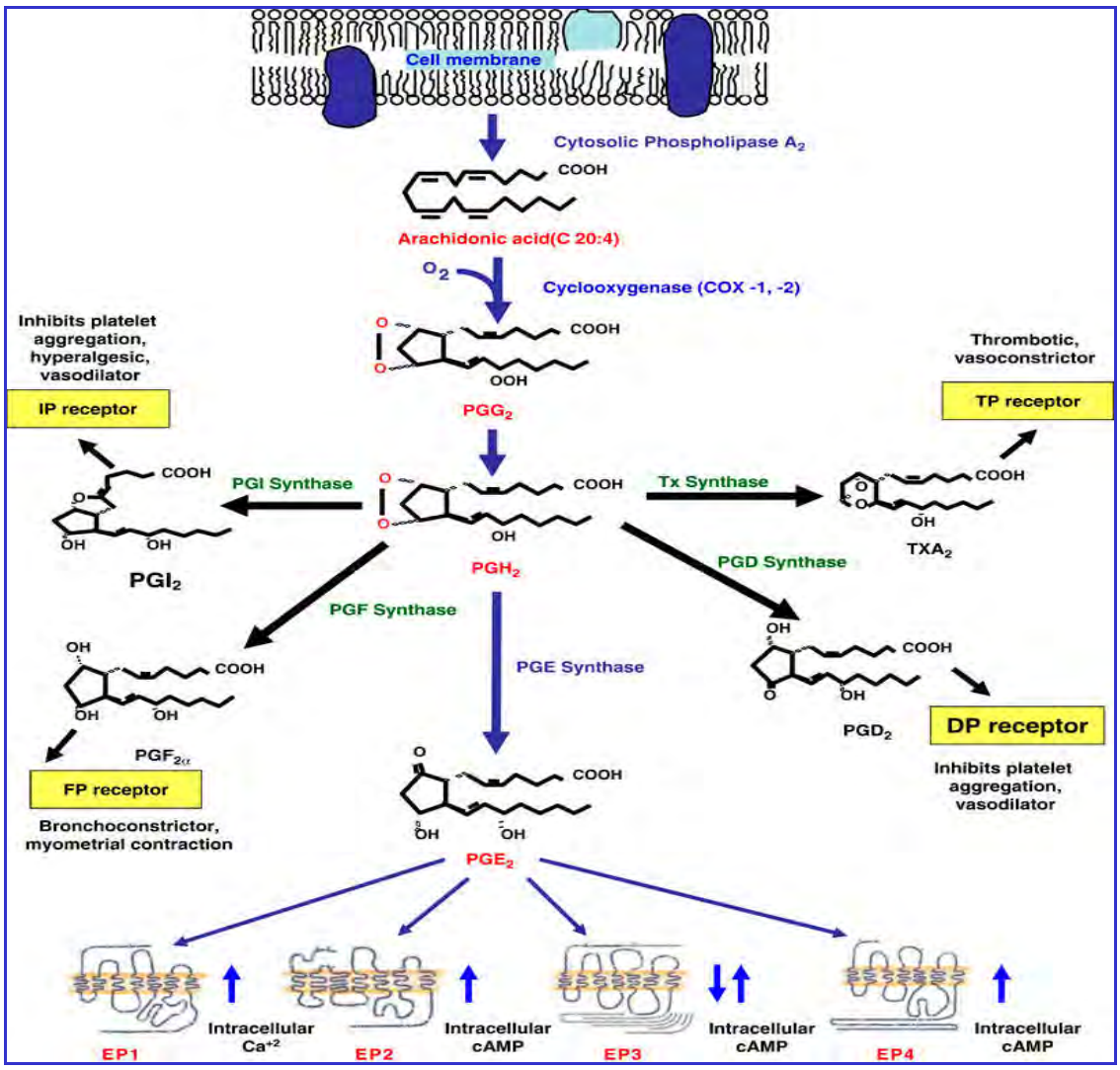

Figure 8 Biosynthesis and the pharmacological effect of prostaglandins

Macrophages

Macrophages are a major component of the mononuclear phagocyte systems that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. From the blood, monocytes migrate into various tissues and differentiate to macrophages. Macrophage has a specific name at location, dust cells/alveolar macrophages of the lungs; histiocytes of the connective tissue; Kupffer cells of the liver; microglia of the neural tissue; epithelioid cells of the granulomas; osteoclasts of the bone and sinusoidal lining cells of the spleen. Macrophages play a dual role in innate immunity and adaptive immunity in the host defence. They play a central role in many pathological processes during inflammation including overproduction of inflammatory mediators (e.g. NO, PGE_2), enhance expression of cell surface molecule. Macrophages can recognize a wild range of pathogen through pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs, such as toll-like receptors-4; TLR-4) on cell surface. When TLR-4 is activated by inflammatory stimuli such as lipopolysaccarides (LPS), it leads to signaling through pathways (e.g. mitogen-activated protein kinases; MAPKs) which maintain the inflammation state. In adaptive immunity, macrophages play role in an antigen presenting cell (APC) present pathogen to T cell. Products of macrophages have been feedback and stimulate them to up or down regulate inflammation state. (Abbas et.al., 2005; Gorden *et.al.*, 2005; Jeon *et.al.*, 2008; Lee *et.al.*, 2006; Ma *et.al.*, 2003; Mosser, 2003; Stow *et.al.*, 2009; Zhang and Mosser, 2008)

Table3 Products of macrophages and their potential roles in their autocrine regulation. (Ma *et.al.,* 2003)

Mediators	Effect
IFN-γ	Stimulation and priming of macrophages
TNF	Pro-inflammatory; regulation of apoptosis; priming
IL-1β	↑ of production of G-CSF and IL-1β;regulation of apoptosis
IL-6	↓ of GM-CSF and TNF gene expression
IL-8	↑ of production of IL-8 through CXCR1/MAPK
IL-10	↓ production of GM-CSF; regulation of IL-12 and TNF production; regulatory effect on dendritic cells
IL-12	Regulation of TNF,IL-1β,IL-6 and NO production; synergy with IL-8 in stimulation of INF-γproduction by BM-derived macrophages
IL-18	Augmentation of IL-12 induced INF-γ production by dendritic cells; synergy with IL-12 in stimulating INF-γ production by BM-derived macrophages
IL-15	Regulatory effect on other cytokines production
MIF	Regulatory effect of TNF and Toll-like receptor 4 production; inhibition of p53
HMGB1	Pro-inflammatory mediator
M-CSF	Sensitization of EMC protein(e.g. fibronectin and several integrins)
GM-CSF	Regulation of apoptosis
TGF-β	Regulation of pro-inflammatory cytokine production and macrophage differentiation
NO	Inhibition of pro-inflammatory cytokine production
PGE_2	Regulation of pro-inflammatory cytokine production
TXA_2	Regulation of TNF and IL-1β synthesis

Carrageenan-Induced Paw Edema in the Rat

Carrageenan-induced rat paw edema is used widely as a working model of inflammation in the search for new anti-inflammatory drug. This model has long been used to assess the anti-inflammatory effect of agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandins production. The development of edema in the paw of the rat after the injection of carrageenan is due to release of specific molecules within the inflammatory cascade. A solution of carrageenan in saline injected into the hind footpad of rats induces an acute swelling of the paw that becomes maximal about 3 hr after the injection. This model is a method to elicit and measure carrageenan-induced foot pad edema. Edema or swelling, one of the cardinal signs of acute inflammation, is an important parameter to be considered when evaluating compounds for their potential anti-inflammatory activity. (Ratheesh and Helen, 2007; Whiteley and Dalrymple, 1998)

Figure 9 Plethysmometer, an apparatus for the measurement of rat paw volume

Harrisonia perforata (Blanco) Merr.

Figure 10 *Harrisonia perforata* (Blanco) Merr.(Khon-thaa) and *Harrisonia perforata* root.

Family: Simaroubaceae

Synonyms: *Harrisonia paucijuga* Oliv. (1868), *Harrisonia bennettii* Benn. (1875).

Names at location: Thailand; khon-thaa (Central), naam chee (Northern). Indonesia; sesepang (Lampung), garut (Sundanese), ri kengkeng (Javanese). Malaysia; kait-kait (Murut, Sabah). Philippines; asimau, mamikil (Tagalog), muntani (Bisaya). Laos; dok kin ta. Vietnam; s[aa]n, da da, h[ar]i s[ow]n.

Distributed: in the southeast of Asia, Africa, and Oceania

Phytochemicals : Root; 2-hydroxymethyl-3-methylalloptaeroxylin, perforatic acid, lupeol, 5-hydroxy-6-7-dimethoxycoumin, β-sitosterol, campesterol, stigmasterol, β-sitosteryl-3-0-glucopyranoside, stigmasteryl-3-0-glucopyranoside and chloresteryl-3-0-glucopyranoside (Stitmannaitham, 1992), Bark of root; compound of saturated and unsaturated hydrocarbon, compound of campesterol, stigmasterol, β-sitosterol, harrisonin and obacunone (Singtothong, 1994), Wood; perforatin C, perforatin D, perforatin E, perforatin F, perforatin G, heteropeucenin-7-methyl ether, perforatin A, perforatic acid, perforatic acid methyl,

heteropeucenig-S-methoxy-7-methyl ether, 2-hydroxymethylallopataeroxylin-5-methyl ether, scopoletin, cedrelopsin, xanthoxyletin and coniferyl aldehyde (Tanaka *et al*., 1995), Stems and leaves; harrisotone A, harrisotone B, harrisotone C, harrisotone D, harrisotone E and harrisonol A (Yin *et.al.,* 2008)

Pharmacological Activities

Anti-hepatitis B Virus activity

The 95% ethyl alcohol extract of *Harrisonia perforata* dried root were screened For anti-hepatitis B virus by enzyme-linked immunosorbent assay (ELISA). At the concentration of 2 mg/ml, it could inhibit HbsAg secretion by PLC/PFR/5 cells up to 32%. (Sirotamarat *et.al.,* 2002)

Anti-infective effect

The 50% of ethanol extract from branch of *Harrisonia perforata* could inhibit adherence of *S. mutans* ATCC 25175 (the pathogen that causes the tooth decay) in vitro. *H. perforata* possessed inhibitory effect on bacterial adherence to glass surface and hydroxyapatite. The extract may inhibit bacterial growth by inhibiting any enzyme activity associated with the growth and/or glycolyzing systems of *S. mutans.* (Limsong *et.al.,* 2004)

Antipyretic effect

The powder root of *Harrisonia perforata* at dose of 100-400 mg/kg showed significant antipyretic effect in yeast's-induced fever model. The highest efficacy was observed at the dose of 200 mg/kg. Each herb at the dose of 40 mg/kg also showed antipyritc effect with different potency. No obvious toxic effect was observed at the concentration used. (Konsue *et al*., 2008)

Antimalarial effect

The extract of *Harrisonia perforata* leaf against *Plasmodium falciparum* was demonstrated with an IC_{50} value of 5.1 μg/ml on the MRC5 cells for the leaf methanol extract. (Nguyen-Pouplin *et al*., 2007)

CHAPTER III
MATERIALS AND METHODS

1. Materials

Plant extract

The ethanolic roots extract of *Harrisonia perforata* was prepared by Ruangrungsi, the plant was collected from Nongkhai province of Thailand and authenticated. The voucher and number of specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. The roots of *H. perforata* were dried under shade and grinded to coarse powders. The powder of roots was macerated in absolute ethanol in closed conical flask for 24 hours. Then the extracts were evaporated to dryness under vacuum and identified by TLC and stored in a solid stage at -4°C until used.

Preparation of the extract solution for in vitro study

The ethanolic roots extract of *H. perforata* was dissolved in 100% DMSO and stored at –20°C until used. The stock solution was diluted with sterile double distilled to the constant final concentration of DMSO at 0.2%.

Preparation of the extract solution for in vivo study

The ethanolic roots extract of *Harrisonia perforata* was dissolved in 5%Tween 80 solution.

Cells

J774A.1 cells are murine macrophages obtained from ATCC. The cells were maintained in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 97% humidity, 5% CO_2. They were subcultured 3 timed weekly during used.

Animals

Male Wistar rats weighing 100-150g were obtained from National Laboratory Animal Centre, Mahidol University, Salaya, Nakhorn Pathom, Thailand. The animals were house in animal care facility at the Faculty of Medicine, Chulalongkorn University, for at least 7 days before the experiment was performed. They were taken care with standard diet and water in a room under controlled environment; room temperature 25±2^{o}C with 12-h light/dark cycle. All animal handling and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Ethic Committee, Faculty of Medicine, Chulalongkorn University.

Equipments and instruments

The equipments and instruments used in the study were in the followings; 24 multi-well plates (Corning, USA), 96 multi-well plates (Corning, USA), T25 tissue culture flasks (Corning, USA), disposable cell scrapers (Greiner bio-one, USA), eppenddorf (corning, USA), sterile polypropylene centrifuge tubes : 15 ml, 50 ml (Corning, USA) , analytical balance (GMPH Satorius Germany and UMT2, Mettler Toledo, Switzerland), auclave (Hiclave TM , HVE-50, Hirayama, Japan), autopipette (Gilson, USA), centrifuge 5810R (Eppendorf, Germany), CO_2 incubator (Thermo, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), sterile laminar flow hood (ESSCO, USA), thermocycler machine (Eppendorf, USA), vortex mixer (Scientific industries, USA), Plethysmometer (UGO Basile 7140)

Chemicals and reagents

The chemicals and reagents used in this study were in the followings; Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), penicillin/streptomycin (Hyclone, USA), fetal bovine serum (Gibco, USA), sodium bicarbonate (Baker, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), trypan blue dye (Sigma, USA), lipopolysaccharide (LPS) (Sigma,

USA), nitric oxide assay kit (Promega, USA), rezasurin (Sigma, USA), TRiZol reagent (Invitrogen, UK), chloroform (Sigma, USA), isopropanol (Bio Basic,Canada), DEPC (Molekula, UK), ImProm-II™ Reverse Transcription system (Promega, USA), agarose gel (Vivantis, Malaysia), primers (Bio Basic,Canada), taq polymerase (Invitrogen, UK), tween 80 (Labchem, New Zealand), indomethacin (Parma, Italy), carrageenan (Sigma, USA), dexamethasone (T.P. drug, Thai)

2. Methods

2.1 Effect of the ethanol extract on LPS-activated NO production in J774A.1 cells

1. Incubate $2x10^5$ cell/well J774A.1 cells in 96-well plate for 24 h at 37°C, 97% humidity, 5% CO_2.
2. Treat the cells with the ethanol extract at the concentrations 3.125-50 µg/ml (5 concentrations) for 24 h at 37°C, 0.2% DMSO and 10 µM dexamethasone were used as the negative and the positive control, respectively.
3. Stimulate the cells with 100 ng/ml LPS for 24 h at 37°C.
4. Collect the supernatant of the treated cell for determining NO concentration and determine the viability of the treated cells left in the plate by the following procedure

4.1 Determination of NO concentration

The amount of NO production was determined by using Griess reagents as in the following steps;

- Pipette 100 µl supernatant from each well into 96-well plate.
- Add and mix with Griess reagents as in the following; 20 µl sulfanilamide for 10 min and then 20 µl N-1-napthylenediamine dihydrochloride (NED) for the other 10 min.
- Measure the absorbance of the reaction in each well with microplate reader at 540 nm.
- Calculate the concentrations of NO in each well from nitrite standard curve.

- Calculate the percentage of NO inhibition compared to LPS-activated condition without the extract.
- Determine the inhibitory concentration at 50% of the ethanol extract from the percentage of NO inhibition

$$\% \text{ NO Inhibition} = \left[\frac{[NO_2^-]_{LPS} - [NO_2^-]_{sample}}{[NO_2^-]_{LPS}} \right] X\ 100$$

4.2 Determination of cell viability

Effect of the ethanol extract on cell viability was determined by resazurin assay. This assay is based on the principle that the enzyme in viable cells can reduce blue color agent resazurin to red color resorufin.

- Remove all the supernatant from the treated cells.
- Add 100 µl the complete DMEM medium containing 50 µg/ml resazurin in each well and incubate for 3 h at 37°C.
- Measure the absorbance of each well by microplate reader at 570 and 600 nm.
- Calculate the percentage of cell viability compared to 0.2% DMSO treated condition.

$$\%\text{cells viability} = \left[\frac{OD_{0.2\%\ DMSO} - OD_{sample}}{OD_{0.2\%DMSO}} \right] X100$$

2.3 Effects of the ethanol extract on the mRNA expression of inflammatory mediators in LPS-activated macrophages

2.3.1 Isolation of Total RNA

1. Incubate J774A.1 cells at the density $2x10^5$ cell/well in 24-well plate for 24 h at 37°C, 97%humidity, 5% CO_2.
2. Treat the cells with the ethanol extract of *H. perforata* roots at concentration 12.5 - 50 µg/ml (3 concentrations) for 24 h at 37°C. 2% DMSO and 10µM dexamethasone were used as the negative and positive controls, respectively.
3. Stimulate the treated cells with 100 ng/ml LPS for 4 h for determining cytokine expression and for 24 h for iNOS and COX-2 expression.
4. Isolate total RNA from the cells by the following procedure;
    - Add 1 ml Trizol reagent to lyses and homogenize the cells in the fume hood.
    - Transfer the cell homogenate to 1.5 ml eppenddorf tubes and incubate at room temperature for 15 minutes.
    - Add 0.2 ml of chloroform to the eppenddorf tubes, securely cap the tube, and quickly vortex the tubes for 15 seconds.
    - Stand the tubes for 15 minutes at room temperature.
    - Centrifuge the tubes at 12,000 x g for 15 minutes at 4^0C.
    - Collect the aqueous phase which is the top layer to new 1.5 ml eppendorf tubes.
    - Add 0.5 ml isopropanol to precipitate total RNA, mix, and stand for 10 minutes at room temperature.
    - Centrifuge the tubes at 12,000 x g for 10 minutes at 4°C. Total RNA precipitate forms a gel-like pellet on the bottom of the tube.
    - Add 1ml 75% ethanol to wash the total RNA pellet, mix by vortex, and centrifuge at 7,500 x g for 5 minutes at 4°C.

- Remove the supernatant and air-dry the pellet for 5-10 minutes.
- Dissolve the total RNA pellet in DEPC-treated water.
- Determine the concentration and contamination of the total RNA at 260 and 280 nm by nanadrop
- Store at -70°C until use.

2.3.2 cDNA synthesis by reverse transcription

1. Add 1 µl Total RNA of each sample and 1 µl oligo dT15 primer into 0.2 ml PCR tube and mix.
2. Heat the tubes at 70°C for 5 min and then immediately cool the tubes on ice for 5 min.
3. Prepare reverse transcription mixture solution containing 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor, and reverse transcriptase. Add
4. Add 15 µl of the mixture solution into each tube.
5. Put the tubes in a thermocycler machine and set up the following condition to generate cDNA; set at 25 °C for 5 min, then 42 °C for 1 hour and 30 min, and finally 70 °C for 15 min.
6. Store the cDNA samples at -20 °C until use.

2.3.3 Inflammatory mediator genes amplification by polymerase chain reaction (PCR)

1. Add 1 µl cDNA of each sample and 24 µl PCR mixture solution containing primer, mixed dNTP, Taq polymerase and buffer in 0.2 PCR tube and mix.
2. Put the PCR tubes in the thermocycler and set up the PCR condition as in the followings; denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72 °C for 60 sec, and final extension at 72°C for 7 min at the end of 20-25th cycles.
3. Run 6 µl the PCR products mixed with 2 µl loading dye on 1.5% agarose gel electrophoresis at 100 volt for 1 h. in TBE buffer.

4. Stain the gel was with 0.5 µg/ml ethidium bromide solution for 4 min and then de-stains with TBE buffer for 30 min.
5. Identify and determine density of the PCR products on the gel by gel documentation

2.4 Effect of the extract on carrageenan-induced paw edema in rats

The carrageenan-induced paw edema model has long been used to evaluate anti-inflammatory activity of agents (Winter *et al*., 1962). Carrageenan is used as the irritant to induce acute inflammation. Test compounds are evaluated for acute anti-inflammatory activity by determining their ability to inhibit or prevent the development of carrageenan-induced paw swelling. Animal were starved overnight before performing experiments as in the following procedure;

1. Weigh body weight of rats for calculating doses of drug and the ethanol extract.
2. Mark with ink at a ground of lateral malleolus and measure the basal paw volume of animals by volume displacement method using Plethysmometer.
3. Randomly divide these animals into six groups with 6 animals in each group (n=6).
4. Intraperitoneally inject animals with agents as in the following;

    group I : 2%tween 80 (the negative control)
    group II : 5 mg/kg indomethacin (the positive control)
    group III : 50 mg/kg ethanol extract of *H. perforata* roots
    group IV : 100 mg/kg ethanol extract of *H. perforata* roots
    group V : 200 mg/kg ethanol extract of *H. perforata* roots
    group VI : 400 mg/kg ethanol extract of *H. perforata* roots

5. One hour after *H.Perforata* or drug dosing, each animal was subcutaneously injected into the sub-plantar side of the right hind paw with 0.05 ml of 1% carrageenan suspension.
6. Measures paw volume again at 1, 2, 3, 4, 5 and 6 h after carrageenan injection.

7. Calculate the increase in paw volume compared with the base line volume
8. Calculate the percentage of paw edema inhibition compared with the negative control animals, as in the following;

$$\%\text{inhibition} = \left[\frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}} \right] X100$$

V_0 : basal volume of paw before carrageenan injection.

V_t : volume of edema paw after carrageenan injection at each time point.

3. Statistical analysis

Data analysis was performed on SPSS 17.0. All data were presented as means.±.S.E.M. One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was used to determine the statistical significance analysis. The p-value<0.05 was considered as statistically significance.

CHAPTER IV

RESULTS

1. Inhibitory effects of *H. perforata* on LPS-stimulate NO release from J774A.1 cells

After pre-treatment with *H. perforata* at concentration 3.125, 6.25, 12.5, 25 and 50 µg/ml in LPS-treated J774A.1 macrophage cells. The level of NO was measured by Griess assay and the percentages of nitric oxide inhibition of the extracts were calculated comparing with negative control. *H. perforata* significantly decreased the LPS- stimulated NO production in a concentration-dependent manner as demonstrated in figure 11 and table 4. The IC_{50} value was 23.14 µg/ml (Fig 12). Cell viability was not significantly altered by the presence of *H. perforata* at the concentration used. (Fig 13)

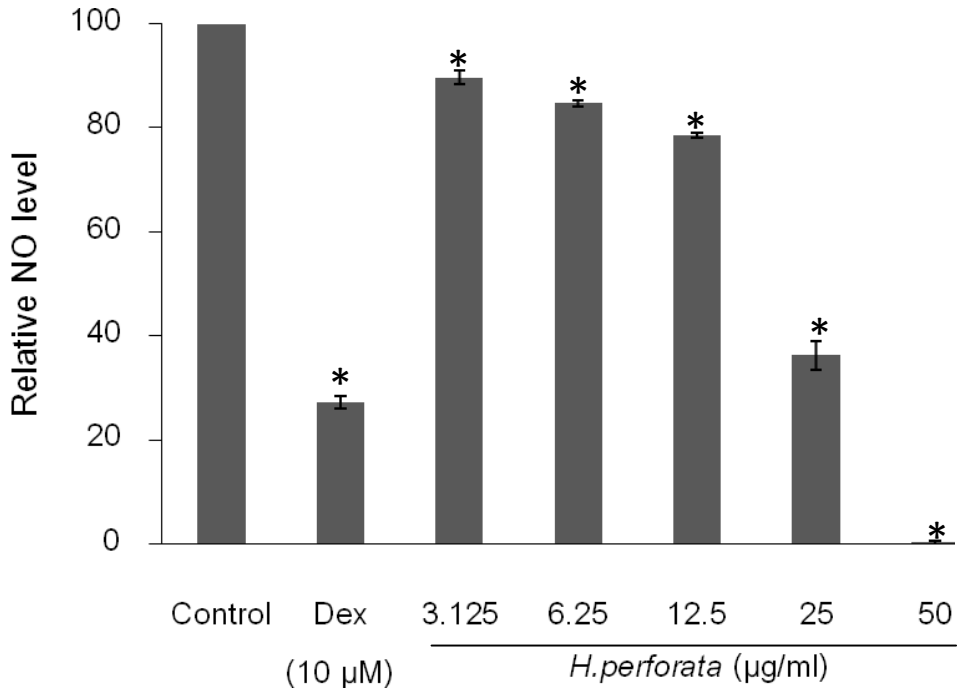

Figure 11 Level of NO production in LPS-stimulated J774A.1 macrophage cells pretreated with *H. perforata* 3.125 - 50 µg/ml and 10 µM dexamethasone (Dex). The data are expressed as mean ± S.E.M from 4 independent experiments (n=4). *$P < 0.05$ compared to untreated cells.

Table 4 Effect of *H. perforata* 3.125 - 50µg/ml and 10 µM dexamethasone on NO inhibition in LPS-stimulated J774A.1 cells. The data are expressed as mean ± S.E.M from 4 independent experiments. (n=4) *$P < 0.05$ compared to untreated cells.

	final concentration	% inhibition
Dexamathasone	10 µM	72.78± 1.15*
H. perforata	3.125 µg/ml	10.32± 1.29*
	6.25 µg/ml	15.27± 0.61*
	12.5 µg/ml	21.45± 0.52*
	25 µg/ml	63.71± 2.78*
	50 µg/ml	100± 0.32*

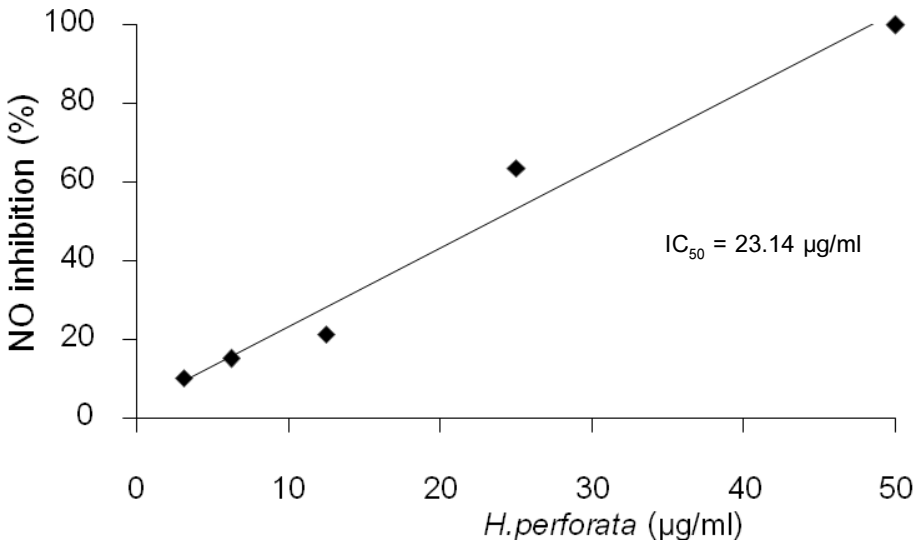

Figure 12 Inhibitory effect of *H. perforata* 3.125 - 50 µg/ml on NO production in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 4 independent experiments. (n=4)

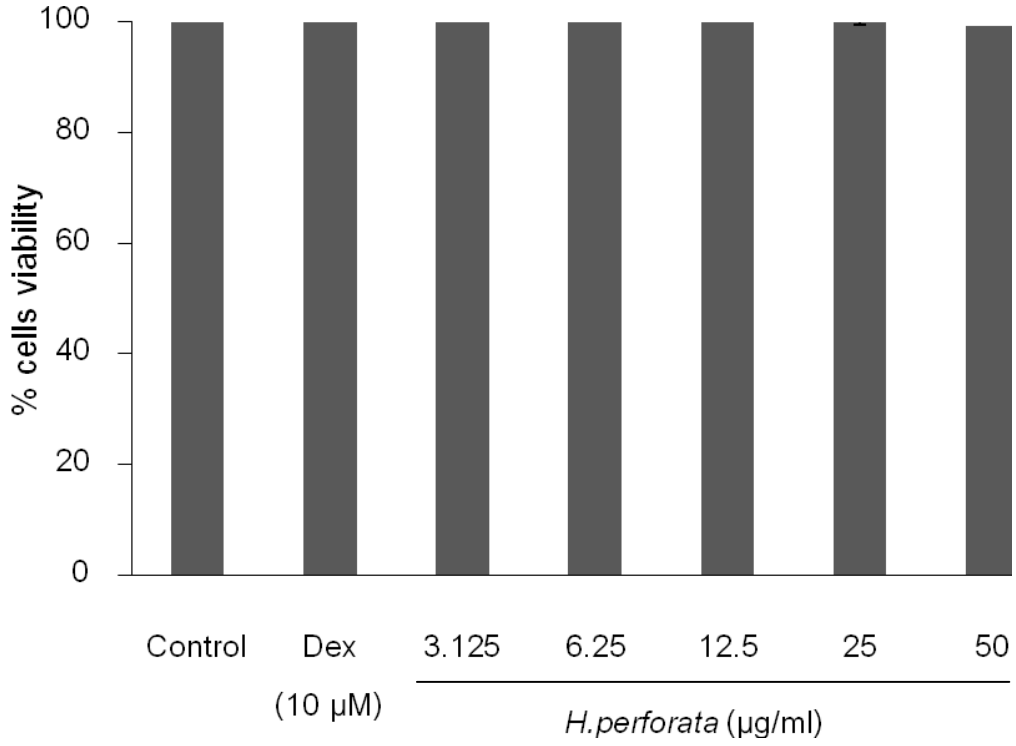

Figure 13 Effect of *H. perforata* 3.125 - 50 µg/ml and 10 µM dexamethasone (Dex) on cells viability in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 4 independent experiments (n=4). *$P < 0.05$ compared to untreated cells.

2. Effects of *H. perforata* on pro-inflammatory cytokine mRNA expression

The stimulation of J774A.1 with LPS leads to the increase in the mRNA expression of pro-inflammatory cytokines. Pre-treated with *H. perforata* 12.5 - 50 µg/ml reduced mRNA expression of TNF-α and IL-1β in dose dependent manner. The highest inhibition was found to be 49.83±2.71% and 47.27±3.77% for TNF-α and IL-1β respectively at the concentration 50µg/ml (Fig 14, 15 and table 5). It was found that *H. perforata* 50 µg/ml significantly increased mRNA expression of IL-6 by 43.93±5.65%, however the expression of IL-6 was inhibited at lowers concentration (12.5 and 25 µg/ml) (Fig 16, table 5).

Table 5 Effect of *H. perforata* 12.5 - 50 µg/ml and 10 µM dexamethasone on pro-inflammatory cytokine mRNA expression in LPS-stimulated J774A.1 cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells.

	final concentration	% Inhibition		
		TNF-α	IL-1β	IL-6
Dexamethasone	10 µM	30.06±4.09*	77.96±2.09*	89.44±0.54*
H. perforata	12.5 µg/ml	2.54±0.30	11.43±2.53*	32.16±2.82*
	25 µg/ml	20.49±1.61*	22.54±0.41*	18.13±0.52*
	50 µg/ml	49.83±2.71*	47.27±3.77*	-43.93±5.65*

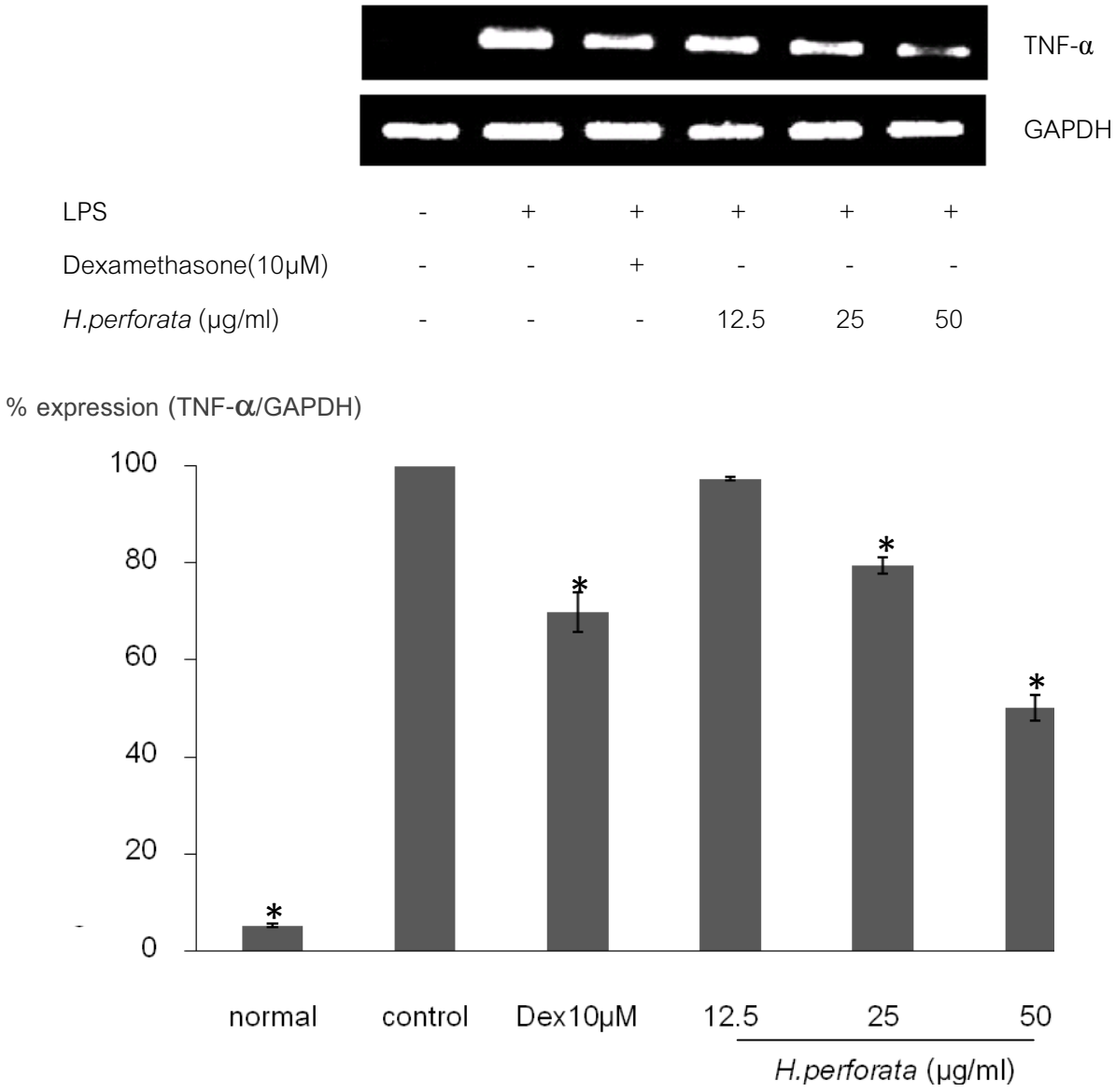

Figure14 Inhibitory effect of *H. perforata* at the concentrations 12.5 - 50 μg/ml and 10 μM dexamethasone (Dex) on TNF-α mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

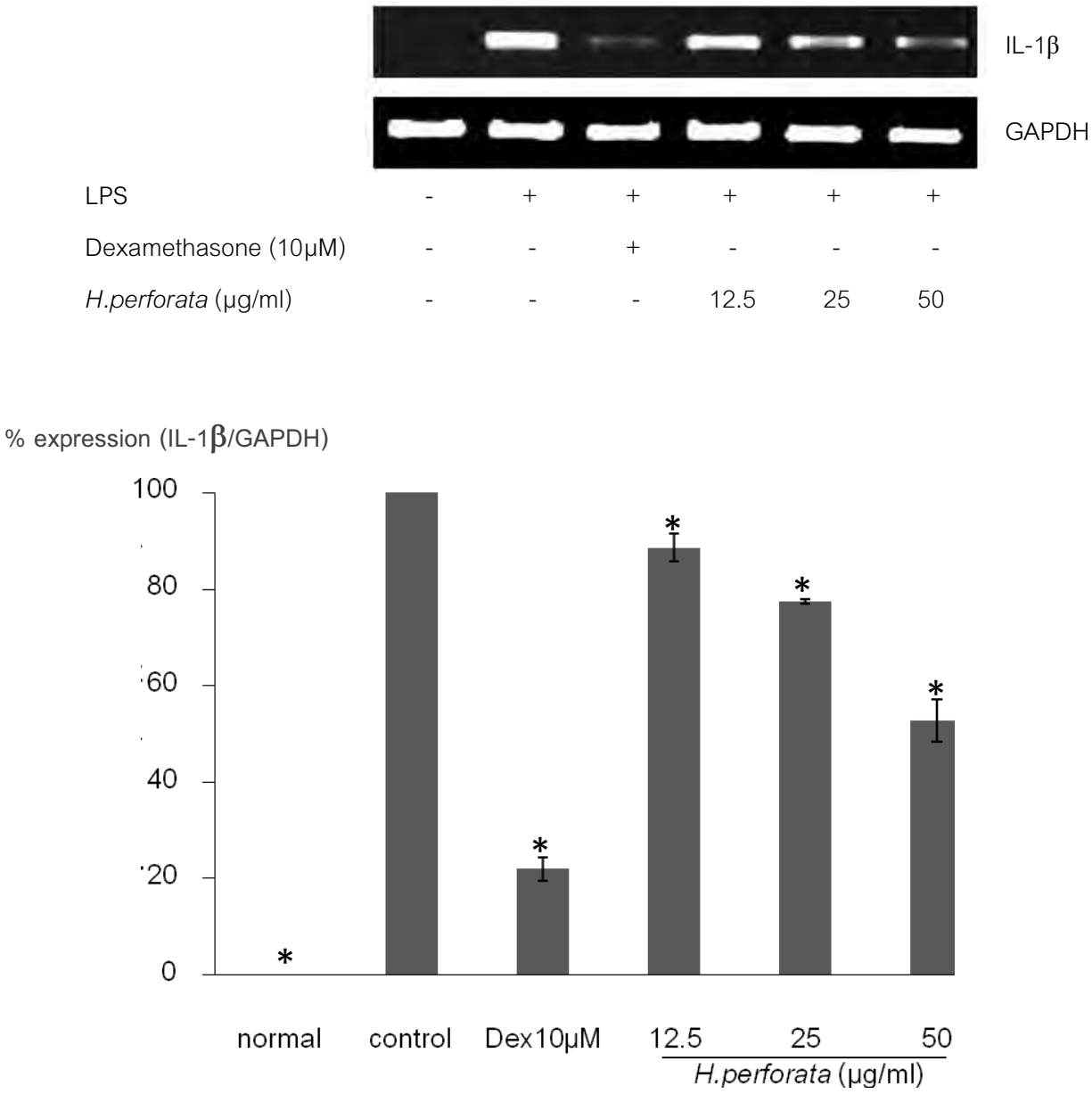

Figure15 Inhibitory effect of *H. perforata* at the concentrations 12.5 - 50 μg/ml and 10 μM dexamethasone (Dex) on IL-1β mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

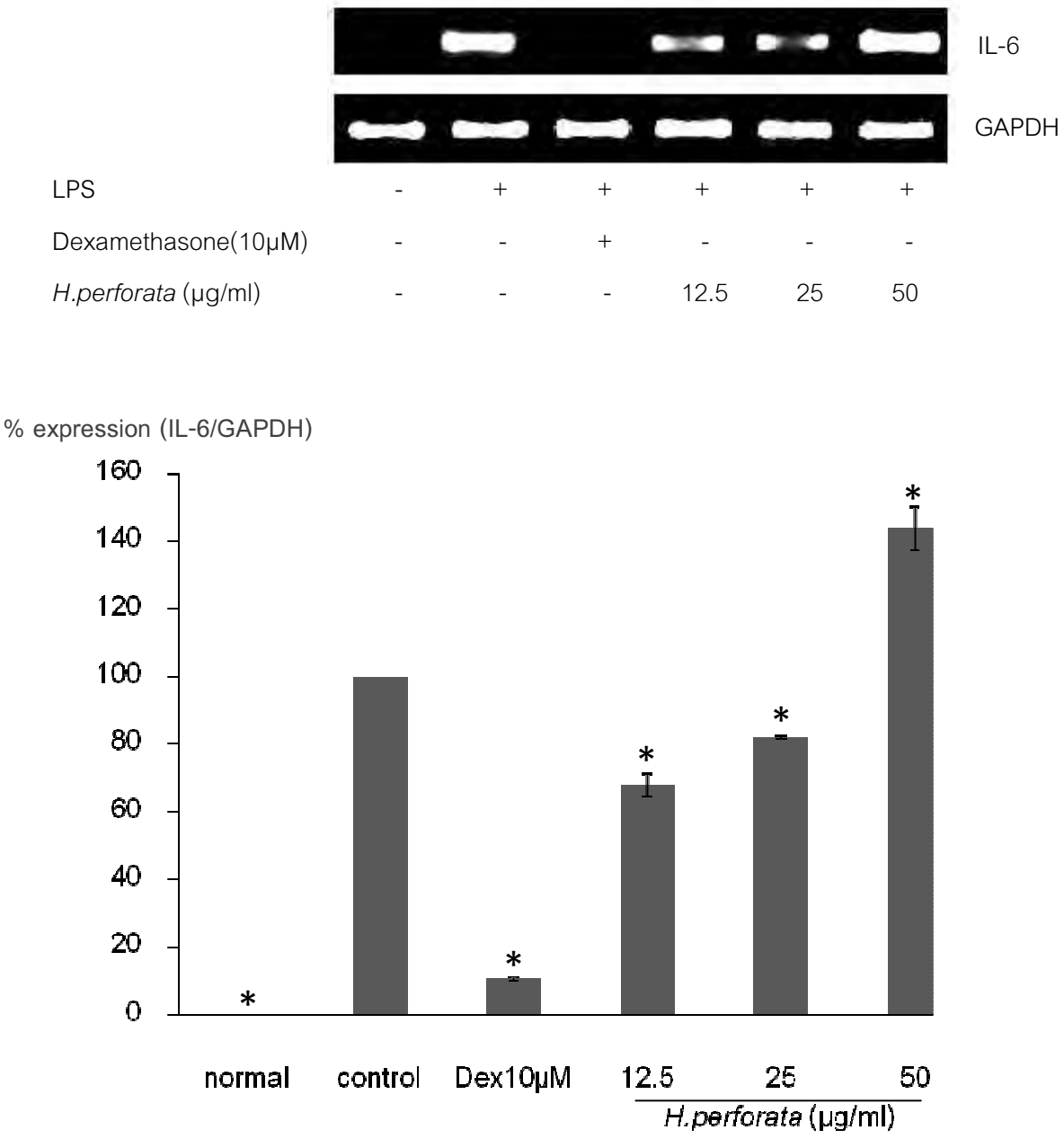

Figure 16 Effects of *H. perforata* at the concentrations 12.5 - 50 μg/ml and 10 μM dexamethasone (Dex) on IL-6 mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

3. Inhibitory effects of *H.perforata* on COX-2 and iNOS mRNA expression

Inhibitory effect of *H. perforata* was determined on LPS-induced COX-2 and iNOS expression in J774A.1 cells when the macrophage cells were pretreated with 12.5, 25 and 50 µg/ml of *H. perforata* root extract and stimulated with LPS. The RT-PCR was performed to determined COX-2 and iNOS mRNA expression. The results indicated that at *H. perforata* 50 µg/ml was significantly inhibited COX-2 and iNOS expression by 88.11±2.58% and 93.68±2.48% respectively (Table 6) Dexamethasone, the positive control was also significantly inhibited COX-2 and iNOS up to 65.18±1.78% and 29.81±0.81% respectively.

Table 6 Inhibition effects of *H. perforata* 12.5-50 µg/ml and 10 µM dexamethasone(DEX) on COX-2 and iNOS mRNA expression in LPS-stimulated J774A.1 cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

	final concentration	%Inhibition	
		COX-2	iNOS
Dexamathasone	10 µM	65.18±1.78*	29.81±0.81*
H.perforata	12.5 µg/ml	20.88±2.60*	32.58±5.79*
	25 µg/ml	70.3±1.18*	62.37±2.18*
	50 µg/ml	88.11±2.58*	93.68±2.48*

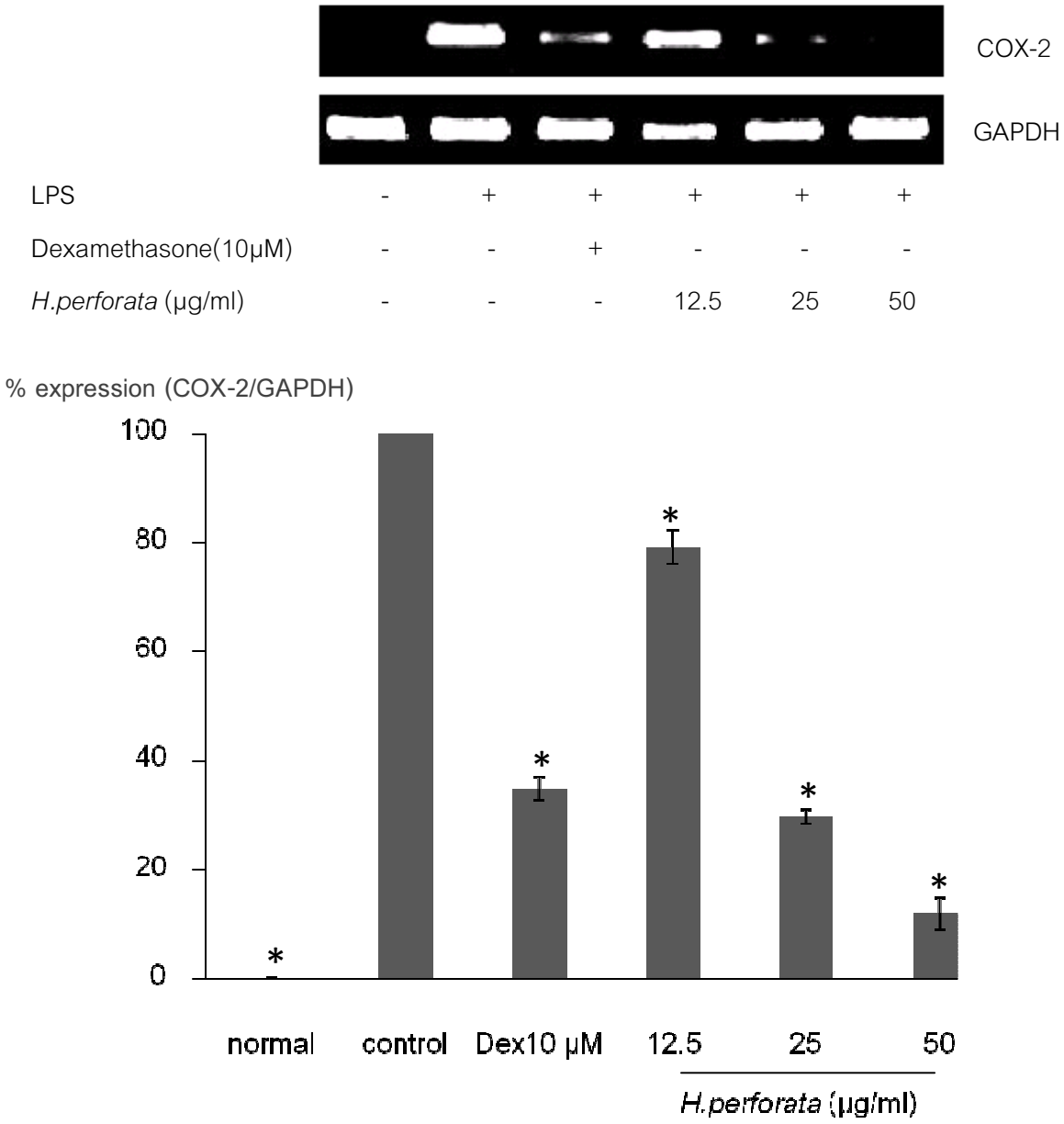

Figure 17 Inhibitory effects of *H. perforata* at the concentrations 12.5 - 50 μg/ml and 10 μM dexamethasone (Dex) on COX-2 mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

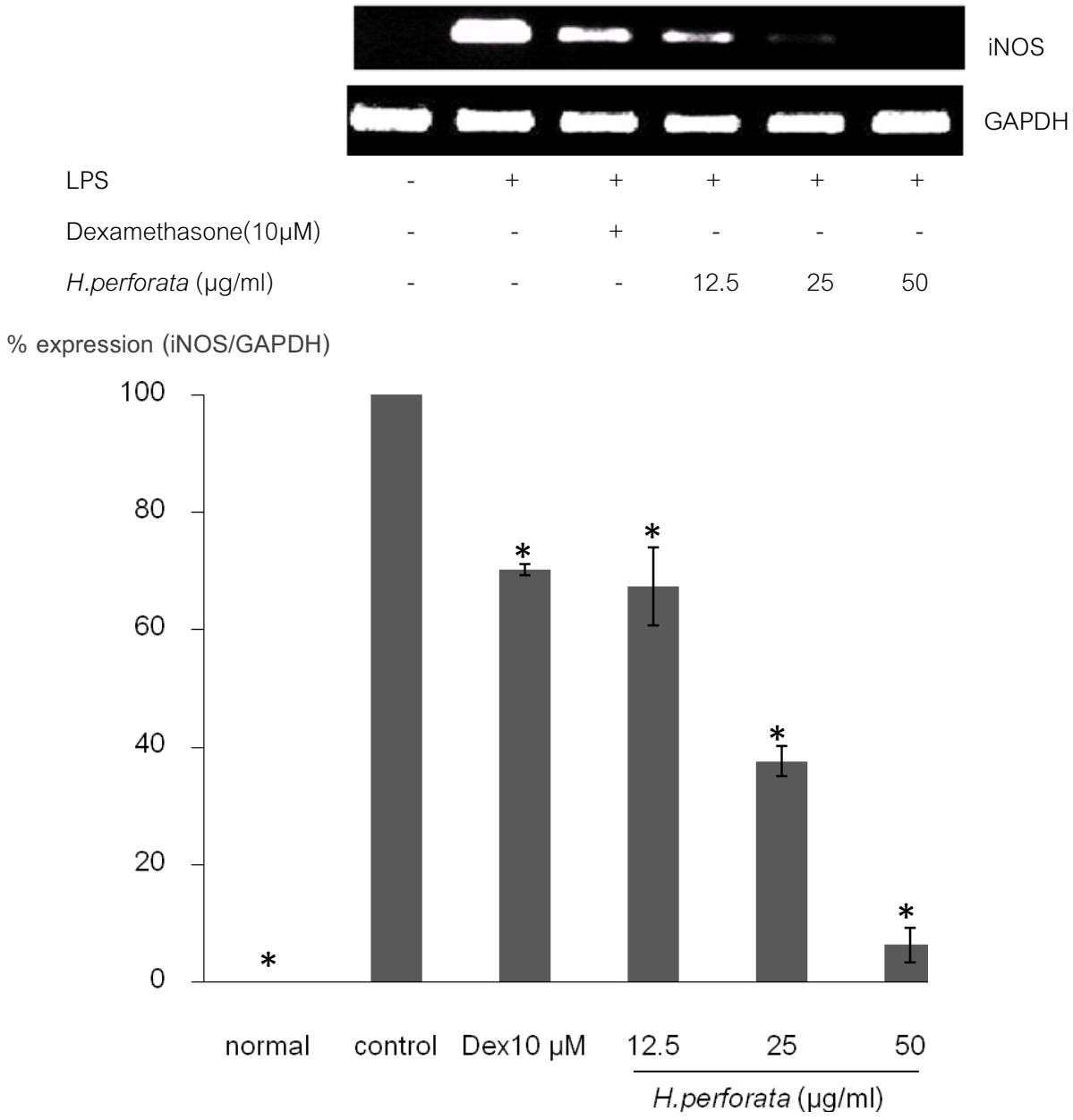

Figure 18 Inhibitory effects of *H. perforata* at the concentrations 12.5 - 50 μg/ml and 10 μM dexamethasone (Dex) on iNOS mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells (control).

4. Anti-inflammatory effects of *H.perforata* on carrageenan-induced paw edema in rats.

Table 7 summarized the effect of *H. perforata* at concentration 50 - 400 mg/kg and indomethacin 5 mg/kg given by intraperitoneal injection one hour before induction of rat hind paw edema by carrageenan. The peak of response to carrageenan at the second hour of *H. perforata* 50, 100, 200, 400 mg/kg were inhibited up to 28.49%, 31.18%, 47.85% and 65.05% respectively, where as indomethacin 5 mg/kg caused 37.10% inhibition of rat paw edema at the corresponding time. The highest inhibition of indomethacin (47.44%) was seen at 5 hour after carrageenan injection.

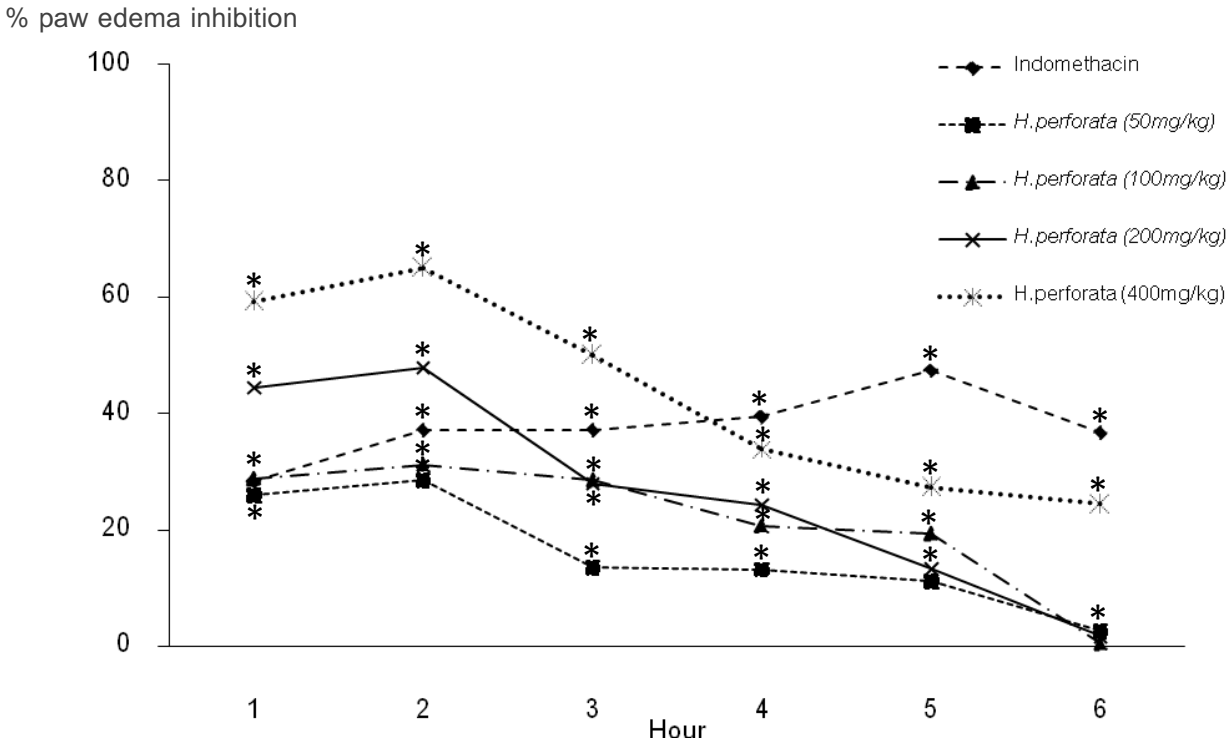

Figure 19 Anti-inflammatory effects of *H. perforata* 50 - 400 mg/kg and indomethacin 5 mg/kg on carrageenan-induced rats paw edema. Results are presented as the mean ± S.E.M from six rats (n=6). *$P < 0.05$ compared to the control group at the corresponding time.

Table 7 Anti-inflammatory effects of *H. perforata* 50 - 400 mg/kg and indomethacin 5 mg/kg on carrageenan-induced rats paw edema. Results are presented as the mean ± S.E.M from six rats (n=6). *$P < 0.05$ compared to the control group at the corresponding time.

Time (h)	Paw edema inhibition (%)					
	Control (2% Tween 80)	Indomethacin (5mg/kg)	*H.perforata* (mg/kg)			
			50	100	200	400
1	0.36±0.04	28.24* (0.26±0.05)	25.93* (0.27±0.04)	28.70* (0.26±0.04)	44.44* (0.20±0.01)	59.26* (0.15±0.01)
2	0.31±0.04	37.10* (0.20±0.04)	28.49* (0.22±0.03)	31.18* (0.21±0.03)	47.85* (0.16±0.01)	65.05* (0.11±0.01)
3	0.31±0.01	37.10* (0.20±0.04)	13.44* (0.27±0.05)	28.49* (0.22±0.05)	27.96* (0.22±0.03)	50.00* (0.16±0.03)
4	0.33±0.03	39.39* (0.20±0.05)	13.13* (0.29±0.06)	20.71* (0.26±0.06)	24.24* (0.25±0.03)	33.84* (0.22±0.02)
5	0.39±0.03	47.44* (0.21±0.04)	11.11* (0.35±0.05)	19.23* (0.32±0.06)	13.25* (0.34±0.04)	27.35* (0.28±0.03)
6	0.39±0.03	36.75* (0.25±0.04)	2.56* (0.38±0.06)	0.43* (0.39±0.05)	1.71* (0.38±0.03)	24.36* (0.30±0.04)

CHAPTER V
DISCUSSION, CONCLUSION AND SUGGESTION

Discussion

Harrisonia perforata root is one of the five root components in Bencha-Loga-Wichien, a Thai remedy used for the treatment of fever. Phytochemical study revealed that there was variety of compounds found in the root extract e.g. 2-hydroxymethyl-3-methylalloptaeroxylin, perforatic acid, lupeol, 5-hydroxy-6-7-dimethoxycoumin, β-sitosteryl-3-0-glucopyranoside, stigmasteryl-3-0-glucopyranoside, chloresteryl-3-0-glucopyranoside. β-sitosterol, campesterol and stigmasterol, (Stitmannaitham, 1992). Macrophages activation by bacterial lipopolysaccaride stimulates secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6). LPS also induced iNOS and COX-2 gene expression and causes inflammation. Acute inflammation is part of the body defense response but chronic inflammation links to a variety of diseases e.g. cardiovascular diseases, arthritis, tumor, Alzheimer's diseases. Thus suppression of these pro-inflammatory cytokines will contribute to anti-inflammatory effects.

In the present study, it was found that *H. perforata* extract 3.125 - 50 µg/ml possessed anti-oxidation activity in LPS-stimulated J774A.1 macrophage cells, since it inhibited NO production in concentration-dependent manner. The IC_{50} value was 23.14 µg/ml. Nitric oxide represents one of key mediators in inflammatory process. After formation, NO diffuses into vascular smooth muscle and it activates soluble gualylate cyclase (Huang *et.al.*, 2005) which results in vasodilatation, increasing volume of exudates. Decreasing NO level in macrophage as a result of *H. perforata* pretreatment might changed vascular permeability and ameliorate inflammation.

Macrophage activation plays a critical role in lymphocyte activation and up-regulates secretion of pro-inflammatory cytokines e.g. TNF-α, IL-1β and IL-6 these substances are important regulators of both innate and adaptive immunity. However over expression of them can cause acute and chronic inflammatory disease as previous mentioned. (Kao *et.al.,* 2009) The levels of TNF-α and IL-1β mRNA expression were suppressed in dose dependent manner following *H. perforata* pretreatment in the macrophage J774A.1 cells. *H. perforata* at the highest dose (50 µg/ml) failed to decrease mRNA expression of IL-6. This concentration is exceed the effective inhibitory concentration. However, at the lower concentration, *H. perforata* 12.5 and 25 µg/ml are able to inhibit IL-6 expression by 32.16 and18.13%. There were evidences showed that the LPS and the pro-inflammatory cytokine, TNF-α, have been shown to inhibit IL-6-mediated STAT3. This inhibition is most likely due to induction of the *de novo* synthesis of the JAK-inhibitor SOCS3. (Helnrich *et.al.,* 2003)

TNF-α is pro-inflammatory cytokine. It activates macrophage and lead to production of other cytokines, lipid mediators e.g. prostaglandins and reactive oxygen species. The production of TNF-α is inhibited by IL-6, TGF-β, Prostagladin E_2 and dexamethasone. It is well known that LPS and pro-inflammatory cytokines such as IL-1 promote gene expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) in macrophages resulting in increases secreting of pathological prostaglandins and nitric oxide (NO). (Hori *et.al.,* 2001) Prostaglandins formed impaired immune surveillance and modulate proliferation in a variety of cell types. During infection and inflammation, over production of NO has been found to cause DNA damage and mutation in vivo. The peroxidase function of NO also contributes to the activation of procarcinogen. (Lim *et.al*., 2008; Zhou *et.al*., 2007) It was also found that over expression of COX-2 and iNOS might be involved in the pathogenesis of many chronic inflammatory diseases e.g. cancer, atherosclerosis, chronic asthma. Our results demonstrated that *H. perforata* inhibited expression of COX-2 and iNOS mRNA expression in dose-dependent manner. The highest inhibition of *H. perforata* 50 µg/ml was found to be

88.11±2.58% and 93.68±2.48% for COX-2 and iNOS respectively, while dexamethasone 10 μM inhibited COX-2 and iNOS mRNA expression 65.28±1.78% and 29.81±0.81%. .Therefore decrease NO production which data obtained from Griess assay due to *H. perforata* could be related to the inhibitory effect on iNOS expression and the decrease of macrophage activation. All of these results indicated that *H. perforata* exerted anti-inflammatory effect.

Carrageenan-induced rat paw edema has been widely used model for evaluation of anti-inflammatory drugs especially the drugs which are sensitive to cyclooxygenase inhibitors or corticorsteroids. Carrageenan-induced rat paw edema model provided evidence that *H. perforata* was an anti-inflammatory agents in acute inflammation. It was effectively inhibited rat paw edema 2 hours after carrageenan injection. The dose that produced maximum inhibition of *H. perforata* was 400 mg/kg which found to be almost two fold more potent than indomethacin 5mg/kg (65.05% vs 37.10%). Indomethacin, a non-steroidal anti-inflammatory drug exhibits its anti-inflammatory effect by reversible blocking cyclooxygenase enzyme (especially COX-2). It leads to suppression of prostaglandins (PGS) biosynthesis. The inhibitory effect on rat paw edema was correlated with the inhibitory effect of *H. perforata* in mRNA expression of several pro-inflammatory cytokines (TNF-α , IL-1β, IL-1, iNOS and COX-2) expression obtained from the in vitro study.

Carrageenin stimulates the release of TNF-α, which in turn induces IL-1β and IL-6, which ultimately lead to the release of COX products. Besides TNF-α and PGE_2, NO is a key mediator in the early and late phases of carrageenan-induced paw inflammation and when demonstrated that NO activates COX-1 and up-regulates COX-2, resulting in production of PGE_2 and PGI_2 at the site of carrageenan inflammation. Therefore, the impairment of TNF-α synthesis/release, NO production, and of other pro-inflammatory cytokines of *H. perforata* represents an interesting alternative to the inhibition of PGE_2 production and consequently of edema. (Chein *et.al.,* 2008; Zhou *et.al.* 2007)

In relation to the constituents found in the root extract of *H. perforata*; lupeol, 2-hydroxymethyl-3-methylalloptaeroxylin, β-sitosterol, campesterol, stigmasterol, β-sitosteryl-3-0-glucopyranoside, stigmasteryl-3-0-glucopyranoside, chloresteryl-3-0-glucopyranoside perforatic acid and 5-hydroxy-6-7-dimethoxycoumin. (Stitmannaitham 1992) There were some evidences supported that sterol compound e.g. β-sitosterol, campesterol, stigmasterol,exhibited anti-inflammatory activity in vivo murine models of inflammation. (Navarro *et.al*., 2000; Prieto *et.al.,* 2006) However it was not able to conclude in this study since it couldn't be found the quantitative amount of each constituent of the extract.

Conclusion

The result obtained from the in vitro study demonstrated that *H. perforata* root extract possessed immunomodulatory action by inhibiting the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in macrophage J774A.1 cells. It also inhibited inflammatory mediator (NO) and mRNA expression of iNOS and COX-2. The in vivo study results supported the in vitro anti-inflammatory action since the extract reduced rat paw edema induced by carrageenan, which represents an acute inflammation model.

Suggestions

The ethanolic extract from the root of *H. perforata* demonstrated anti-inflammatory action both in vivo and in vitro model. However several key issues should be identified:

1. The active compounds of *H. perforata* which exhibit anti-inflammatory effect.

2. Molecular signaling pathway which explained anti-inflammatory effect of these compounds.

3. Further investigation in other inflammatory disease models e.g. cardiovascular (atherosclerosis), respiratory disease (asthma).

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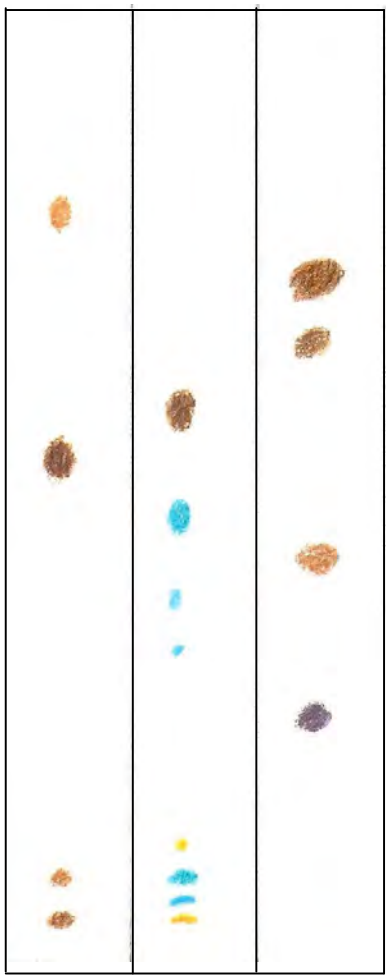
Appendices

Appendix A

TLC fingerprint from *H. perforata* extracts



I II III

Solvent system; Chloroform Stationary phase; SiO2: GF254

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with vanillin-sulfuric acid

*Vanillin-sulfuric acid regent

Preparation: vanillin (15 g) in ethanol (250 ml) and conc. sulfuric acid (2.5 ml)

**Spot color Development

Heat the plate after sprayed at 120 °C for 10 minutes.

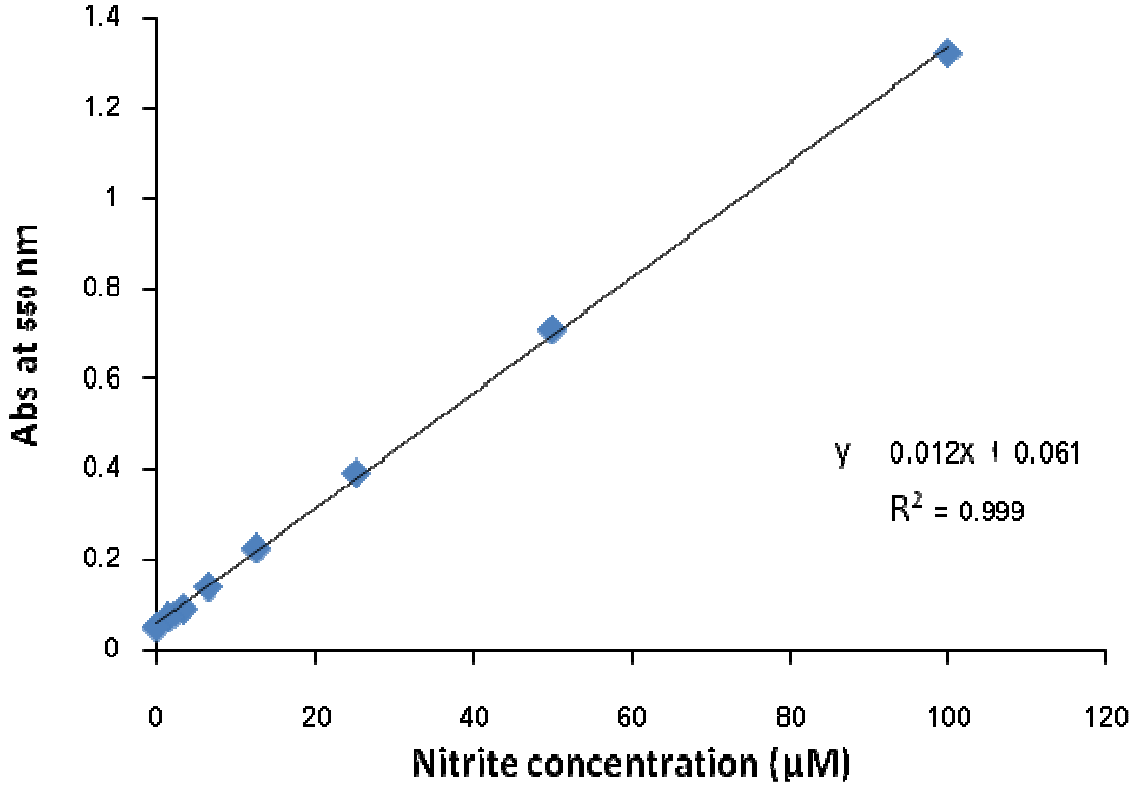
Appendix A-1: Thin-layer chromatogram of the ethanolic extract of *Harrisonia perforata* root

Appendix B

Appendix B-1: Data for linearity of nitrite standard calibration lines.

Concentration (μM)	Absorbance at 550 nm		
	1	2	Average
100.00	1.310	1.338	1.324
50.00	0.722	0.702	0.712
25.00	0.397	0.394	0.396
12.50	0.229	0.224	0.227
6.25	0.139	0.14	0.140
3.13	0.095	0.094	0.095
1.56	0.075	0.073	0.074
0.00	0.053	0.052	0.053

Appendix B-2: A representation of nitrite concentration calibration line by Griess reaction



Appendix B-3: The effect of *H. perforata* and dexamethasone on nitric oxide production in LPS stimulated-macrophage J774A.1 (as nitrite concentration)

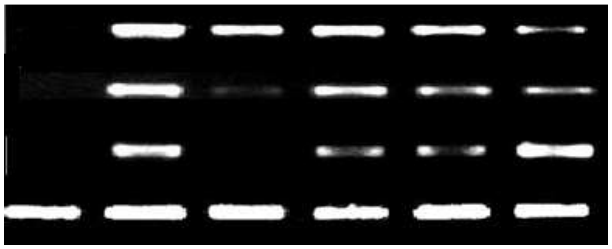
	final concentration	Concentration of nitrite (μM)				
		1	2	3	4	mean±S.E.M.
0.2% DMSO (control)		18.79	17.76	21.18	18.13	18.96±0.77
Dexamathasone	10 μM	4.59	5.31	5.64	5.06	5.15±0.22
H. perforata	3.125 μg/ml	16.65	16.40	18.34	16.53	16.98±0.46
	6.25 μg/ml	15.93	15.34	17.84	15.14	16.06±0.62
	12.5 μg/ml	15.01	13.79	16.48	14.30	14.89±0.58
	25 μg/ml	8.12	5.26	7.66	6.56	6.90±0.64
	50 μg/ml	0.30	0.30	0.00	0.00	0.15±0.09

Appendix B-4: Cytotoxicity *of H. perforata* (µg/ml) and dexamethasone (10 µM) in LPS stimulated-macrophage J774A.1

	final concentration	%Cytotoxic				
		1	2	3	4	mean±S.E.M.
0.2% DMSO (control)		0.00	0.00	0.00	0.00	0.00±0.00
Dexamathasone	10 µM	0.00	0.00	0.00	0.00	0.00±0.00
H. perforata	3.125 µg/ml	0.00	0.00	0.00	0.00	0.00±0.00
	6.25 µg/ml	0.00	0.00	0.00	0.00	0.00±0.00
	12.5 µg/ml	0.00	0.00	0.00	0.00	0.00±0.00
	25 µg/ml	0.00	0.00	0.00	0.00	0.00±0.00
	50 µg/ml	0.00	0.00	0.89	1.99	0.95±0.47

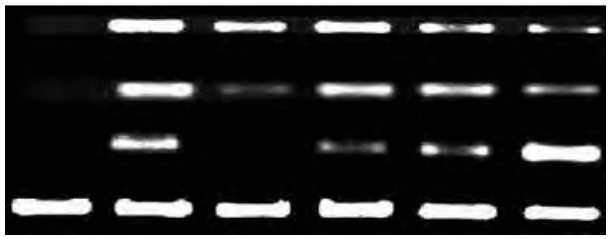
Appendix B-5: Effect of *H. perforata* and dexamethasone (10 μM) on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

N1



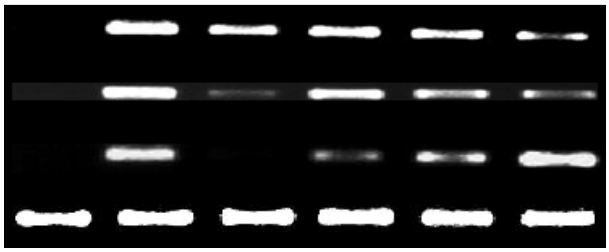
LPS	-	+	+	+	+	+
Dexamethasone (10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

N2



LPS	-	+	+	+	+	+
Dexamethasone (10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

N3



LPS	-	+	+	+	+	+
Dexamethasone (10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

Appendix B-6: Effect of *H. perforata* and dexamethasone (10 μM) on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

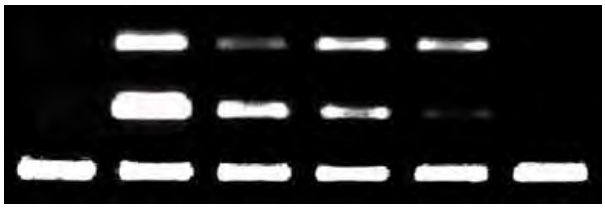
	final concentration	% Expression											
		TNF-α				IL-1β				IL-6			
		1	2	3	mean±S.E.M	1	2	3	mean±S.E.M	1	2	3	mean±S.E.M
0.2%DMSO		5.00	6.04	4.53	5.191±0.44	0.00	0.00	0.00	0.00±0.00	0.00	0.00	0.00	0.00±0.00
0.2%DMSO+ LPS		100.00	100.00	100.00	100.00±0.00	100.00	100.00	100.00	100.00±0.00	100.05	100.01	100.03	0.00±0.01
Dexamathasone+ LPS	10 μM	69.96	78.11	61.76	69.95±4.72	17.24	24.95	23.93	22.04±2.426	10.56	11.65	9.48	10.56±0.62
H. perforata+ LPS	12.5 μg/ml	96.78	97.72	97.89	97.46±0.35	82.83	90.55	92.34	88.57±2.92	69.82	72.23	61.48	67.84±3.26
	25 μg/ml	81.21	75.80	81.52	79.51±1.86	77.18	76.81	78.38	77.46±0.47	82.58	80.67	82.37	81.87±0.60
	50 μg/ml	44.35	51.12	55.05	50.18±3.13	46.55	50.49	61.14	52.73±4.36	149.21	151.63	130.97	143.93±6.52

Appendix B-7: Effect of *H. perforata* and dexamethasone (10 μM) on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells.

N1

LPS	-	+	+	+	+	+
Dexamethasone (10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

N2



LPS	-	+	+	+	+	+
Dexamethasone (10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

N3

LPS	-	+	+	+	+	+
Dexamethasone(10μM)	-	-	+	-	-	-
H. perforata (μg/ml)	-	-	-	12.5	25	50

Appendix B-8: Effect of *H. perforata* and dexamethasone (10 µM) on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells.

	final concentration	% Expression							
		COX-2				iNOS			
		1	2	3	mean±S.E.M	1	2	3	mean±S.E.M
0.2%DMSO		-0.94	-0.58	0.60	-0.31±0.46	0.33	-0.94	-0.46	-0.36±0.37
0.2%DMSO+ LPS		100.00	100.00	100.00	100.00±0.00	100.00	100.00	100.00	100.00±0.00
Dexamathasone+ LPS	10 µM	31.10	38.20	35.16	34.82±2.06	71.71	70.36	68.50	70.19±0.93
H. perforata+ LPS	12.5 µg/ml	79.86	83.90	73.59	79.12±3.00	77.89	69.38	54.99	67.42±6.68
	25 µg/ml	29.88	31.97	27.25	29.70±1.36	40.93	32.69	39.27	37.63±2.52
	50 µg/ml	8.39	9.46	17.81	11.89±2.98	11.68	5.41	1.87	6.32±2.87

Appendix B-9: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: 2%tween 80)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.17	1.20	1.42	1.45	1.34	1.37	1.33	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.66	1.55	1.77	1.77	1.67	1.66	1.68	
	Volume of edema	0.49	0.35	0.35	0.32	0.33	0.29	0.36	-
h(2)	Paw volume	1.55	1.56	1.69	1.72	1.54	1.73	1.63	
	Volume of edema	0.38	0.36	0.27	0.27	0.20	0.36	0.31	-
h(3)	Paw volume	1.48	1.52	1.70	1.77	1.63	1.69	1.63	
	Volume of edema	0.31	0.32	0.28	0.32	0.29	0.32	0.31	-
h(4)	Paw volume	1.50	1.53	1.79	1.81	1.56	1.71	1.65	
	Volume of edema	0.33	0.33	0.37	0.36	0.22	0.34	0.33	-
h(5)	Paw volume	1.63	1.57	1.84	1.86	1.61	1.77	1.71	
	Volume of edema	0.46	0.37	0.42	0.41	0.27	0.40	0.39	-
h(6)	Paw volume	1.56	1.66	1.84	1.78	1.66	1.8	1.72	
	Volume of edema	0.39	0.46	0.42	0.33	0.32	0.43	0.39	-

Appendix B-10: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: indomethacin 5 mg/kg)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.34	1.25	1.27	1.55	1.46	1.31	1.36	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.46	1.66	1.53	1.86	1.68	1.54	1.62	
	Volume of edema	0.12	0.41	0.26	0.31	0.22	0.23	0.26	28.24
h(2)	Paw volume	1.45	1.44	1.41	1.72	1.71	1.62	1.56	
	Volume of edema	0.11	0.19	0.14	0.17	0.25	0.31	0.20	37.1
h(3)	Paw volume	1.43	1.54	1.43	1.77	1.67	1.51	1.56	
	Volume of edema	0.09	0.29	0.16	0.22	0.21	0.20	0.20	37.1
h(4)	Paw volume	1.48	1.41	1.48	1.76	1.62	1.66	1.57	
	Volume of edema	0.14	0.16	0.21	0.21	0.16	0.35	0.21	39.39
h(5)	Paw volume	1.48	1.41	1.48	1.76	1.62	1.66	1.57	
	Volume of edema	0.14	0.16	0.21	0.21	0.16	0.35	0.21	47.44
h(6)	Paw volume	1.50	1.47	1.55	1.78	1.69	1.67	1.61	
	Volume of edema	0.16	0.22	0.28	0.23	0.23	0.36	0.25	36.75

Appendix B-11: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: *H. perforata* 50 mg/kg)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.21	1.40	1.31	1.24	1.39	1.40	1.33	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.42	1.57	1.52	1.57	1.68	1.79	1.59	
	Volume of edema	0.21	0.17	0.21	0.33	0.29	0.39	0.27	25.93
h(2)	Paw volume	1.35	1.59	1.53	1.54	1.66	1.61	1.55	
	Volume of edema	0.14	0.19	0.22	0.30	0.27	0.21	0.22	28.49
h(3)	Paw volume	1.41	1.54	1.52	1.62	1.76	1.71	1.59	
	Volume of edema	0.20	0.14	0.21	0.38	0.37	0.31	0.27	13.44
h(4)	Paw volume	1.45	1.54	1.48	1.62	1.77	1.81	1.61	
	Volume of edema	0.24	0.14	0.17	0.38	0.38	0.41	0.29	13.13
h(5)	Paw volume	1.48	1.69	1.53	1.70	1.80	1.83	1.67	
	Volume of edema	0.27	0.29	0.22	0.46	0.41	0.43	0.35	11.11
h(6)	Paw volume	1.49	1.62	1.69	1.70	1.87	1.86	1.71	
	Volume of edema	0.28	0.22	0.38	0.46	0.48	0.46	0.38	2.56

Appendix B-12: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: *H. perforata* 100 mg/kg)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.16	1.26	1.38	1.56	1.34	1.44	1.36	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.52	1.46	1.60	1.83	1.53	1.74	1.61	
	Volume of edema	0.36	0.20	0.22	0.27	0.19	0.30	0.26	28.7
h(2)	Paw volume	1.34	1.43	1.61	1.82	1.51	1.71	1.57	
	Volume of edema	0.18	0.17	0.23	0.26	0.17	0.27	0.21	31.18
h(3)	Paw volume	1.34	1.42	1.69	1.67	1.56	1.79	1.58	
	Volume of edema	0.18	0.16	0.31	0.11	0.22	0.35	0.22	28.49
h(4)	Paw volume	1.29	1.42	1.77	1.73	1.70	1.8	1.62	
	Volume of edema	0.13	0.16	0.39	0.17	0.36	0.36	0.26	20.71
h(5)	Paw volume	1.33	1.45	1.81	1.88	1.70	1.86	1.67	
	Volume of edema	0.17	0.19	0.43	0.32	0.36	0.42	0.32	19.23
h(6)	Paw volume	1.56	1.49	1.83	1.89	1.80	1.90	1.75	
	Volume of edema	0.40	0.23	0.45	0.33	0.46	0.46	0.39	0.43

Appendix B-13: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: *H. perforata* 200 mg/kg)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.13	1.14	1.25	1.41	1.31	1.28	1.25	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.36	1.34	1.46	1.61	1.5	1.45	1.45	
	Volume of edema	0.23	0.20	0.21	0.20	0.19	0.17	0.20	44.44
h(2)	Paw volume	1.30	1.29	1.43	1.60	1.47	1.40	1.42	
	Volume of edema	0.17	0.15	0.18	0.19	0.16	0.12	0.16	47.85
h(3)	Paw volume	1.32	1.37	1.42	1.64	1.49	1.62	1.48	
	Volume of edema	0.19	0.23	0.17	0.23	0.18	0.34	0.22	27.96
h(4)	Paw volume	1.42	1.40	1.50	1.73	1.45	1.52	1.50	
	Volume of edema	0.29	0.26	0.25	0.32	0.14	0.24	0.25	24.24
h(5)	Paw volume	1.48	1.37	1.55	1.83	1.72	1.60	1.59	
	Volume of edema	0.35	0.23	0.30	0.42	0.41	0.32	0.34	19.23
h(6)	Paw volume	1.52	1.41	1.61	1.85	1.73	1.70	1.64	
	Volume of edema	0.39	0.27	0.36	0.44	0.42	0.42	0.38	1.71

Appendix B-14: Effect of *H. perforata* on carrageenan-induced rats paw edema. (Group: *H. perforata* 400 mg/kg)

		n1	n2	n3	n4	n5	n6	mean	%inhibition
h(0)	Paw volume	1.36	1.39	1.58	1.13	1.53	1.25	1.37	
	Volume of edema	-	-	-	-	-	-	-	-
h(1)	Paw volume	1.53	1.52	1.74	1.25	1.67	1.41	1.52	
	Volume of edema	0.17	0.13	0.16	0.12	0.14	0.16	0.15	59.26
h(2)	Paw volume	1.48	1.46	1.72	1.25	1.62	1.36	1.48	
	Volume of edema	0.12	0.07	0.14	0.12	0.09	0.11	0.11	65.05
h(3)	Paw volume	1.59	1.52	1.71	1.34	1.63	1.38	1.53	
	Volume of edema	0.23	0.13	0.13	0.21	0.10	0.13	0.16	50
h(4)	Paw volume	1.58	1.56	1.77	1.39	1.76	1.49	1.59	
	Volume of edema	0.22	0.17	0.19	0.26	0.23	0.24	0.22	33.84
h(5)	Paw volume	1.60	1.62	1.85	1.50	1.77	1.60	1.66	
	Volume of edema	0.24	0.23	0.27	0.37	0.24	0.35	0.28	27.35
h(6)	Paw volume	1.62	1.66	1.87	1.56	1.73	1.57	1.67	
	Volume of edema	0.26	0.27	0.29	0.43	0.20	0.32	0.30	24.36

BIOGRAPHY

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