ACTIVITY OF THE EXTRACTS FROM *GLYCOSMIS PARVA* CRAIB IN MACROPHAGE CELL J774A.1

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ฤทธิ์ของสิ่งสกัดจาก *Glycosmis parva* ต่อการทำงานของ macrophage cell J774A.1

นางสาวสุจินตนา ชุมแสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุจินตนา ชุมแสง : ฤทธิ์ของสิ่งสกัดจาก *Glycosmis parva* ต่อการทำงานของ macrophage cell J774A.1 (ACTIVITY OF *GLYCOSMIS PARVA* CRAIB IN MACROPHAGE CELL J774A.1.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.จันทนี อิทธิพานิชพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. นิจศิริ เรืองรังษี, 98 หน้า.

การทดสอบสิ่งสกัดจาก *G. parva* ต่อแมคโครฟาจ J774A.1 ที่ถูกกระตุ้นด้วยไลโปโปลี่แซคคาไรด์ ในการศึกษานี้พบว่าสิ่งสกัดจากกิ่งและใบของ *G. parva* ด้วย hexane และ ethyl acetate (G1, G2, G5 และ G6) ในความเข้มข้นที่นำมาศึกษาสามารถยับยั้งการสร้างไนตริกซ์ออกไซด์และไม่เป็นพิษต่อเซลล์แมคโครฟาจ ผลการศึกษาพบว่าค่า IC_{50} ของการสร้างไนตริกซ์ออกไซด์ อยู่ระหว่าง 11.12 – 44.70 µg/ml และเมื่อศึกษาการแสดงออกของ mRNA โดยวิธี RT-PCR พบว่าสิ่งสกัดจาก *G. parva* สามารถยับยั้งการแสดงออกของ TNF-α, IL-1β และ IL-6 mRNA ได้แตกต่างกันโดยพบว่า สิ่งสกัดจากกิ่งด้วย hexane และ ethyl acetate (G5 และ G6) ที่ความเข้มข้น 25 µg/ml สามารถยับยั้งการแสดงออกของ TNF-α ได้อย่างเด่นชัดโดยมีฤทธิ์ยับยั้งได้ 94.2% และ 95% ตามลำดับเมื่อพิจารณาการแสดงออกของ IL-1β พบว่า G2 ที่ความเข้มข้น 20 µg/ml ยับยั้งการแสดงออกของ IL-1β ได้สูงสุดคือ 69.8% สำหรับ IL-6 พบว่า G2 และ G5 ไม่มีผลต่อการยับยั้งการแสดงออกของIL-6 นอกจากนี้ยังพบว่าสิ่งสกัดจาก *G. parva* (G1, G2, G5 และ G6) สามารถยับยั้งการแสดงออกของ COX-2 และ iNOS ได้โดยพบว่า G6 ที่ความเข้มข้น 25 µg/ml ยับยั้งการแสดงออกของ iNOS ได้สูงสุด 82.5% และยับยั้งการแสดงออกของ COX-2 ได้สูงสุด 93% ข้อมูลที่ได้จากการศึกษานี้แสดงให้เห็นว่าสิ่งสกัดจาก *G. parva* สามารถควบคุมการทำงานของ pro-inflammatory cytokines และเอ็นไซม์ที่เกี่ยวข้องกับการอักเสบได้ ดังนั้นสิ่งสกัดจาก *G. parva* จึงน่าจะมีฤทธิ์ต้านการอักเสบได้

สาขาวิชา เภสัชวิทยา ..... ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : MACROPHAGE/ ANTI-INFLAMMATORY / *GLYCOSMIS PARVA* CRAIB

SUCHINTANA CHUMSENG : ACTIVITY OF *GLYCOSMIS PARVA* CRAIB IN MACROPHAGE CELL J774A.1. THESIS ADVISOR : ASSOC.PROF CHANDHANEE ITTHIPANICHPONG, THESIS COADVISOR : ASSOC.PROF. NIJSIRI RUANGRUNGSI ,Ph.D., 98 pp

The activity of *Glycosmis prava* extracts were investigated in LPS-treated macrophage J77A.1 cell. It was found that the fractions of branches and leaves obtained from hexane, ethyl acetate (G1, G2, G5 and G6) suppressed nitric oxide production without cytotoxicity in the study dose. Their IC_{50} for NO production varied from 11.12 – 44.70 µg/ml. The RT-PCR showed that *G. parva* fractions significantly inhibited pro-inflammatory expression of cytokines, TNF-α, IL-1β and IL-6 in different manner. The prominent effect on TNF-α generation were found in ethyl acetate and hexane extract from leaves (G5 and G6) which caused about 94.2% and 95% at the concentration of 25 µg/ml. For IL-1β expression, G2 at the concentration of 20 µg/ml demonstrated highest inhibition effect (69.8%). However, inhibition effect on IL-6 expression were not evidenced in G2 and G5 from this present study. Moreover the expression of COX-2 and iNOS mRNA were also prohibited by *G. parva* extract (G1, G2, G5 and G6). The greatest inhibition of iNOS expression was seen in G6 at 25 µg/ml concentration (82.5%). The expression of COX-2 was pronounced inhibited by G6 at 25 µg/ml concentration (93%).The results obtained from this study indicated the potential of *G. parva* in down regulation of pro-inflammatory cytokines and enzymes production which may consider them as the leading compounds for anti-inflammatory action.

Field of study: Pharmacology..... Student's Signature.....

Academic year: 2009..... Advisor's Signature.....

Co-advisor's Signature.....

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ABBREVIATIONS

Ca^{2+}	Calcium
CBG	Corticosteroid-binding globulin
cDNA	Complementary DNA
CNS	Central nervous system
CO_2	Carbon dioxide
Dexa	Dexamethazone
DMEM	Dulbeco's Modified Eagle"s Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
GDP	Guanosine diphosphate
GM-CFU	Granulocyte/macrophage colony-forming unit
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
FBS	Fetal bovine serum
HSC	Haematopoietic stem cell
HPA	Hypothalamic-pituitary –adrenal
IC_{50}	Inhibition concentration 50%
IgG	Immunoglobulin G
M-CFU	Macrophage colony-forming unit
mg	Milligram (s)
ml	Milliliter(s)
NK	Natural killer
TLRs	Toll-like receptors
LPS	Lipopolysaccharide
M	Molarities (mole per liter)
MBL	Mannose-binding lectin
NO	Nitric oxide
NSAIDs	Non steroidal anti-inflammatory drugs

O_2	Oxygen
OD	Optical density
P	Probability
S.D.	Standard deviation
°C	Degree Celsius
µg	Microgram (s)
µl	Microliter (s)
µM	Micromolar
ng	Nanogram (s)
%	Percent
<	Less than
/	Per

CHAPTER I
INTRODUCTION

Macrophages play import roles in initiation, maintenance and resolution of inflammation. Macrophages have 3 major functions. They are antigen presentation, phagocytosis and immunomodulation.

Macrophage is antigen presentation for T cell to recognized antigen, these antigen must be processed and presented on the surface of an antigen-presenting cells such as dendritic cell, macrophage and B cells. Macrophage does its function by engulfing the antigen, then processing it into small pieces and combining the antigen fragment with special membrane proteins. The antigen complexes are then displayed on the cell surfaces where T lymphocytes (T helper cells) are recognized and become activated by them.

Phagocytosis occurs when inflammatory process is triggered by injurious stimulus (e.g. infection, antibodies, physical injuries). Macrophages are early responders to an acute inflammation. They are phagocytes that engulf and degrade microorganism. Macrophages are covered with a variety of receptors protein on their cell surfaces. Fc receptor help macrophages locate antigen that have been coated by antibodies, integrins and selectins receptor help macrophages stick to capillary wall and move to tissue.

Apart from their phagocytic function, macrophages possess important secretory function. They secret NO and various cytokines including IL-1β, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α). These cytokines promote inflammation and the activity of other white blood cells e.g. neutrophil and lymphocytes. Macrophages also secret a number of proteins which are important in inflammation and wound healing e.g. COX, collagenase, elastase, and fibroblast- growth factor (Copsted lee-Ellen C, 2005).

At present, most of new anti-inflammatory drugs are focussing on the macrophages function since they are involved and play key roles in inflammation process. *Glycosmis parva*, a Thai herbal plants found in all parts of Thailand, is the plant

of our interest since the constituents in which mostly found have been investigated for their pharmacological effects e.g. antimalarial effect, anti-proliferative effect, and anti-inflammatory effect. The potential of this plant on the secretory function of the macrophages in key inflammatory cytokines and proteins are performed in this study.

Objectives of the study

To investigate the effect of *Glycosmis parva* solvent extraction on NO production, expressions of pro-inflammatory cytokines, iNOS and COX-2 in LPS stimulated-macrophages.

Expected benefits and applications

The results obtained from this experiment would clarify the effect of *G. parva* on LPS – stimulated macrophages which may be benefit for scientists to do further researches on its potential for anti-inflammatory actions. In addition, investigation of herbal medicines would bring about the evidence to support development of drugs from natural substances which is one of the national drug development policy.

Research design

Experimental research

Keywords

Glycosmis parva

TNF-α

IL-1β

IL-6

iNOS

COX-2

CHAPTER II

Review of Literature

Inflammation is fundamentally a protective response, the goal of which is to get rid the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the consequences of such injury (e.g. necrotic cells and tissues). Cell injury induces releases of pro-inflammatory cytokines including tumor necrosis factor alpha(TNF-α), interleukin-1 (IL-1) from leukocytes, monocytes, and macrophages. These cytokines further trigger other pro-inflammatory cytokines and increase the expression of many cellular adhesion molecules (CAMs), selectins, integrins, and immunoglobulins. On the other hand, phagocytosis of bacteria or foreign particles is occurred. During this phase, high amounts of reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydroxyl radical(HO·), and hydrogen peroxide (H_2O_2) are produced and an increase in the expression of phospholipase A_2, 5-lipoxygenase (5-LOX), and cyclooxygenase -2 (COX-2), inducible nitric oxide synthase (iNOS) (Huang *et al*., 2004; Kumar *et al.* 2007).

Function of leukocytes (neutrophils and macrophages) are to deliver to the site of injury and to activate the leukocytes to perform their normal functions in inflammatory in host defense. Leukocytes ingest offending agents, kill bacteria and other microbes, and get rid of necrotic tissue and foreign substances. A pitfall of the defensive potency of leukocytes is that they may induce tissue damage and prolong inflammation, since leukocyte products that destroy microbes and necrotic tissues can also injure normal host tissues.

The process that host to elimination microbes that is functional responses of phagocytes in host defense consist of sequential steps-active recruitment of the cells to the sites of infection, recognition of microbes, phagocytosis, and destruction of ingested microbes.

Recruitment of the cells to the sites of infection

The recruitment of leukocytes to sites of injury and infection is a multistep process involving attachment of circulating leukocytes to endothelial cells and migration through the endothelium (Figure 1). The first events are the induction of adhesion molecules on endothelial cells, by a number of mechanisms. Mediators such as histamine, thrombin, and platelet activating factor (PAF) stimulate the redistribution of P-selectin from its normal intracellular stores in granules to the cell surface. Resident tissue macrophages, mast cells, and endothelial cells respond to injurious agents by secreting the cytokines TNF, IL-1, and chemokines (chemoattractant cytokines). Within 1 to 2 hours, the endothelial cells which are activated by TNF and IL-1 begin to express E-selectin. Leukocytes express at carbohydrate ligands for the selectins, and bind to the endothelial selectins. Then the bound leukocytes detach and bind again, and begin to roll along the endothelial surface. TNF and IL-1 also induce endothelial expression of ligands for integrins, mainly VCAM-1 (the ligand for the VLA-4 integrin) and ICAM-1 (the ligand for the LFA-1 and Mac-1 integrins). Meanwhile, chemokines that are produced at the site of injury enter the blood vessel, bind to endothelial cell heparan sulfate glycosaminoglycans and are displayed at high concentrations on the endothelial surface. These chemokines act on the rolling leukocytes and activate the leukocytes. The combination of induced expression of integrin ligands on the endothelium and activation of integrins on the leukocytes causes the firm integrin-mediated binding of the leukocytes to the endothelium at the site of infection. The leukocytes stop rolling, their cytoskeleton is reorganized, and they spread out on the endothelial surface. The next step in the process is migration of the leukocytes through the endothelium, called transmigration or diapedesis. Chemokines act on the adherent leukocytes and stimulate the cells to migrate through inter endothelial to the site of injury or infection. The net result of this process is that leukocytes, neutrophils and monocytes, rapidly accumulate around the infectious microbes. This reaction is typically elicited by microbes, but it may be seen in response to a variety of noninfectious stimuli as well. Figure 1

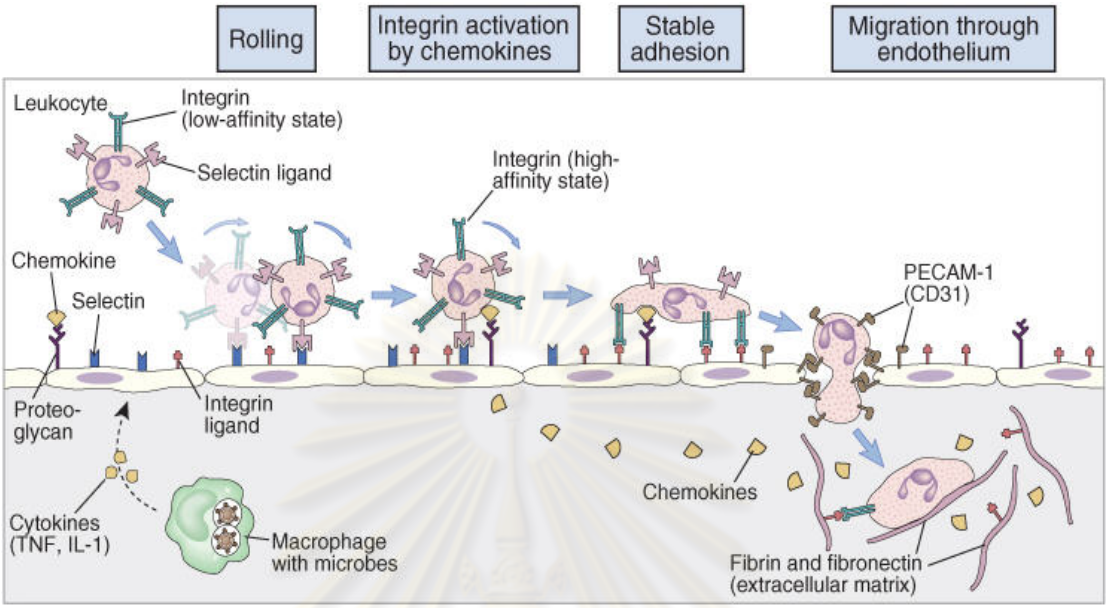


Figure 1: At sites of infection, macrophages that have encountered microbes produce cytokines (such as TNF and IL-1) that activate the endothelial cells of nearby venules to produce selectins, ligands for integrins, and chemokines. Selectins mediate weak tethering and rolling of blood leukocytes, such as neutrophils, on the endothelium; integrins mediate firm adhesion of neutrophils; and chemokines increase the affinity of neutrophil integrins and stimulate the migration of the cells through the endothelium to the site of infection (Abul *et al.*, 2005).

Recognition of microbes

In the blood and tissues neutrophils and macrophages express surface receptors that recognize microbes and stimulate the phagocytosis and killing of the microbes. These receptors include the following:

1. Different seven-transmembrane G-protein-coupled receptors recognize microbes and some mediators that are produced in response to infections and tissue injury. These receptors are found on neutrophils, macrophages, and most other types of leukocytes; and are specific for diverse ligands. Receptors of this class recognize short peptides containing *N*-formylmethionyl residues, as well as chemokines, chemotactic breakdown products of complement such as C5a, and lipid mediators of inflammation, including platelet-activating factor, prostaglandin E, and leukotriene B_4 (LTB_4). Since all

bacterial proteins are initiated by *N*-formylmethionine, this receptor allows neutrophils to detect and respond to bacterial proteins. Binding of ligands, such as microbial products and chemokines, to the G-protein-coupled receptors induces migration of the cells from the blood through the endothelium and production of microbicidal substances by activation of the respiratory burst. In a resting cell, the receptor-associated G-proteins form a stable inactive complex containing guanosine diphosphate (GDP) bound to Gα subunits. Occupancy of the receptor by ligand results in an exchange of GTP for GDP. The GTP-bound form of the G-protein activates numerous cellular enzymes, including an isoform of phosphatidylinositol-specific phospholipase C which functions to degrade inositol phospholipids and ultimately to increase intracellular $Ca^{2+}$and activate protein kinase C. The G-proteins also stimulate cytoskeletal changes, resulting in increased cell motility (Abul *et al*., 2005; Kumar *et al,*. 2007).

2. Mannose receptors and scavenger receptors function are to bind and ingest microbes. The mannose receptor is a macrophage lectin that binds terminal mannose and fucose residues of glycoproteins and glycolipids. Macrophage scavenger receptors bind a variety of microbes as well as modified LDL particles. Macrophage integrins, notably Mac-1 (CD11bCD18), may also bind microbes for phagocytosis (Linehan *et al.,*2000; Kumar *et al*., 2004).

3. Phagocytes express receptors for cytokines that are produced during immune responses. One of the most important of these cytokines is IFN-γ, which is secreted by natural killer (NK) cells during innate immune responses and by antigen-activated T lymphocytes during adaptive immune responses. IFN-γ is the major macrophage-activating cytokine.

4. Receptors for opsonins promote phagocytosis of microbes coated with various proteins and deliver signals that activate the phagocytes. The process of coating a particle, such as a microbe, to target it for phagocytosis is called opsonization, and substances that do this are opsonins. These substances are antibodies, complement proteins, and lectins. One of the most efficient systems for opsonizing particles is coating the particles with IgG antibodies, which are termed specific opsonins and are

recognized by the high-affinity Fcγ receptor of phagocytes, called FcγRI. Components of the complement system, especially fragments of the complement protein C3, are also potent opsonins, because these fragments bind to microbes and phagocytes express a receptor, named the type 1 complement receptor (CR1), which recognizes breakdown products of C3. These complement fragments are produced when complement is activated by either the classical (antibody-dependent) or the alternative (antibody-independent) pathway. A number of plasma proteins, including mannose-binding lectin (MBL), fibronectin, fibrinogen, and C-reactive protein, can coat microbes and are recognized by receptors on phagocytes. For example, a macrophage cell surface receptor called the C1q receptor binds microbes opsonized with plasma MBL, and integrins bind fibrinogen-coated particles (Abul *et al*., 2005; Kumar *et al*., 2007).

5. Toll-like receptors (TLRs), which are homologous to a *Drosophila* protein called Toll, are function to activate leukocytes in response to different types and components of microbes. There are 10 mammalian TLRs have been identified. Different TLRs play essential roles in cellular responses to bacterial lipopolysaccharide (LPS, or endotoxin), other bacterial proteoglycans, and unmethylated CpG nucleotides, all of which are found only in bacteria, as well as double-stranded RNA, which is produced only by some viruses. These receptors function are mediated through receptor-associated kinases to stimulate the production of microbicidal substances and cytokines in the leukocytes (Han *et al*., 2005; Lee *et al*., 2008).

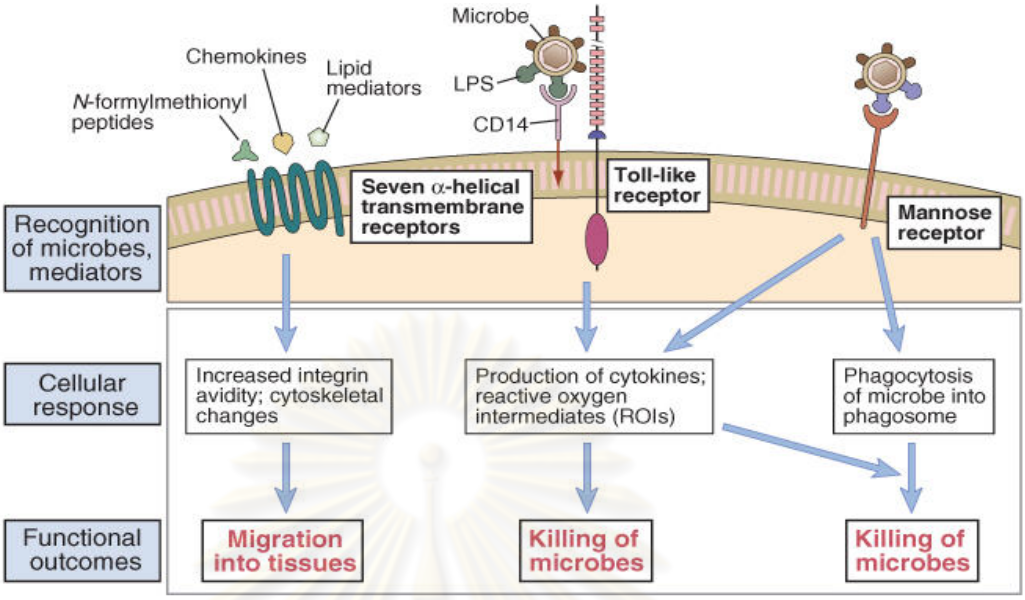


Figure 2: Different classes of cell surface receptors of neutrophils and macrophages recognize different stimuli. The receptors initiate responses that mediate the functions of neutrophils and macrophages (Kumar *et al.,* 2007).

Phagocytosis and destruction of ingested microbes

Phagocytosis is a cytoskeleton-dependent process of engulfment of large particles (>0.5 μm in diameter) and the release of enzymes by neutrophils and macrophages are responsible for eliminating the injurious agents. Phagocytosis involves three interrelated steps (Figure 4): (1) recognition and attachment of the particle to be ingested by the leukocyte; (2) its engulfment, with subsequent formation of a phagocytic vacuole; and (3) killing or degradation of the ingested material. Bacterial lipopolysaccharide (LPS or endotoxin) is a gram-negative bacteria product that is a mixture of fragments of the outer cell walls of gram-negative bacteria and contains both lipid components and polysaccharide moieties. LPS is a potent stimulator of innate immune responses that enhance killing of the bacteria, but it may also cause significant pathologic changes in the host. In innate immunity LPS is a potent activator of macrophages which lead to release cytokines such as IL-1 and TNF (called endogenous pyrogens) result in increase the enzymes, especially inducible nitric oxide synthase, cyclooxygenase-2 and initiate inflammation (Abul *et al.*, 2005; Kumar *et al.,* 2007).

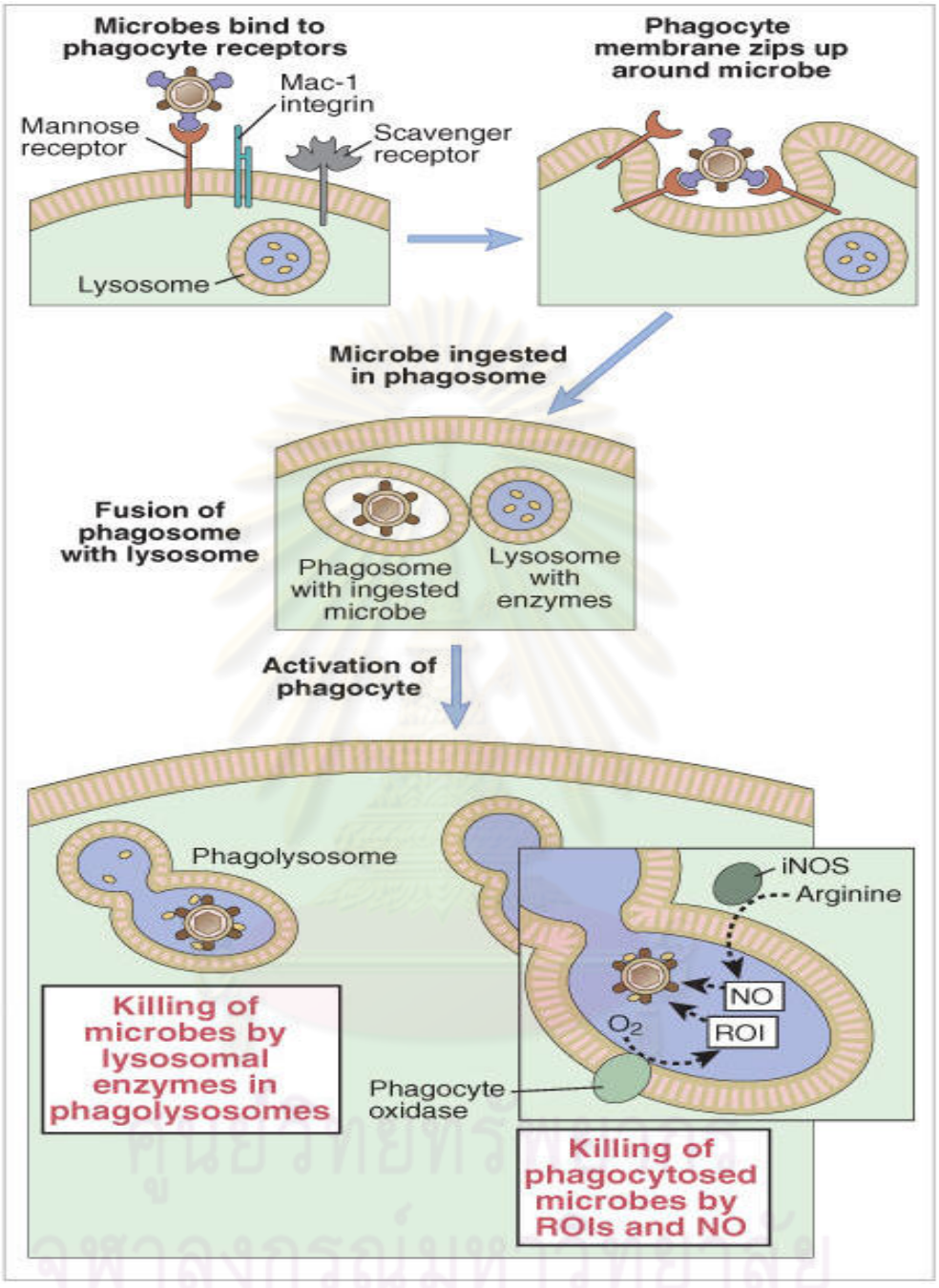


Figure 3: Phagocytosis of a particle by macrophages (Abul *et al*., 2005)

Macrophages

Macrophages are mononuclear phagocytes originate in the bone marrow from a common haematopoietic stem cells (HSC). In response to macrophage colony-stimulating factor, they divide and differentiate into monoblasts and pro-monocytes before becoming monocytes, which exit from the bone marrow then enter the bloodstream. Monocytes undergo a series of change to become a macrophage in the body tissue. Macrophage is classified into two major groups: free macrophages and fixed macrophages. Fixed macrophages are found in organs and connective tissues. They have special names to designate specific location for instance in pulmonary airways, they are called alveolar macrophage, in connective tissue are histiocytes, in neural tissue are microglia, in liver are kupffer cells, in granulomas are epithelioid cells, in bone are osteclasts, in spleen called sinusoidal cells

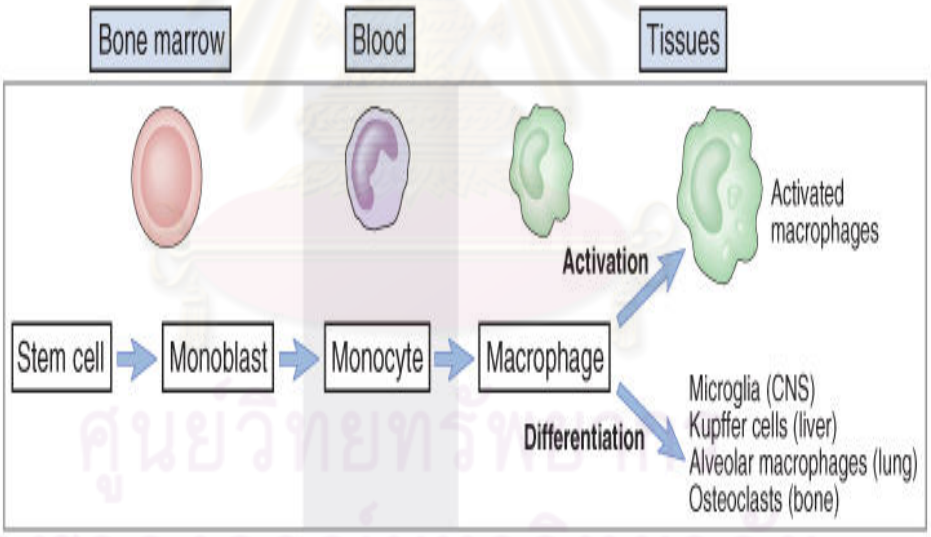


Figure4 : Mononuclear phagocytes develop in the bone marrow, circulate in the blood as monocytes, and are resident in all tissues of the body as macrophages. They may differentiate into specialized forms in particular tissues. (Abul *et al*., 2005)

Macrophages play central role in innate and adaptive immunity and play a key role in host defence against parasitic bateria, pathogenic protozoa, fungi and helminthes as well as against tumers. In innate immunity, macrophages response to microbes by secreting cytokines that activate phagocyte and stimulate cellular reaction of innate immunity leading to inflammation (Ma *et al*., 2003; Mosser 2003; Gorden *et al*., 2005; Zhang *et al*., 2008).

Cytokines

Cytokines are protein produced and secreted by cells of the immunity system (activated macrophage and lymphocytes). They are produced in response to antigens and microbes which stimulate diverse responses of cells involved in immunity and inflammation. Some cytokines promote inflammation are called pro-inflammatory cytokines. They are produced by activated macrophages and are involved in the up-regulation of inflammatory reactions. These cytokines are tumor necrosis factor (TNF-α), interleukin-1β (IL-1 β) and interleukin-6 (IL-6) (Dinarello CA. 2000; Stow *et al*., 2009).

Tumor necrosis factor-α (TNF-α)

TNF-α is a major cytokine that mediates inflammation. It is produced mainly by activated macrophages. The secretion of TNF-α can be stimulated by endotoxin and other microbial products, immune complexes, physical injury, and a variety of inflammatory stimuli. The principal physiologic function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes. These effects are mediated through several actions on vascular endothelial cells and leukocytes. It induces the expression of adhesion molecules (selectins and ligands for leukocyte integrins) that make the endothelial surface adhesive for leukocytes, initially for neutrophils and subsequently for monocytes and lymphocytes which is the most important event in the recruitment of leukocytes to sites of infection. It also stimulates endothelial cells and macrophages to secrete chemokines, cytokines (IL-6. IL-1β), eicosanoids, and nitric oxide (NO).

In infections, TNF-α is produced in large amounts and causes systemic and pathologic abnormalities as show in Fig. 5.

- TNF-α induces fever by increase synthesis of prostaglandins (PGE_2) then it acts on the hypothalamus, which generates a systemic response back to the rest of the body, causing heat-creating effects to match a new temperature level.

- TNF-α increases synthesis of certain serum proteins, such as serum amyloid A protein and fibrinogen that effect on hepatocytes and induce systemic acute-phase reactions.

- When serum concentration of TNF-α reachs 10^{-7} M or more, it results in a marked fall in blood pressure, or shock caused by decrease myocardial contractility and vascular smooth muscle tone. Furthermore TNF-α causes intravascular thrombosis, mainly as a result of loss of the normal anticoagulant properties of the endothelium and causes severe metabolic disturbances, such as a fall in blood glucose (Abul *et al.,* 2005; Kumar *et al*., 2007).

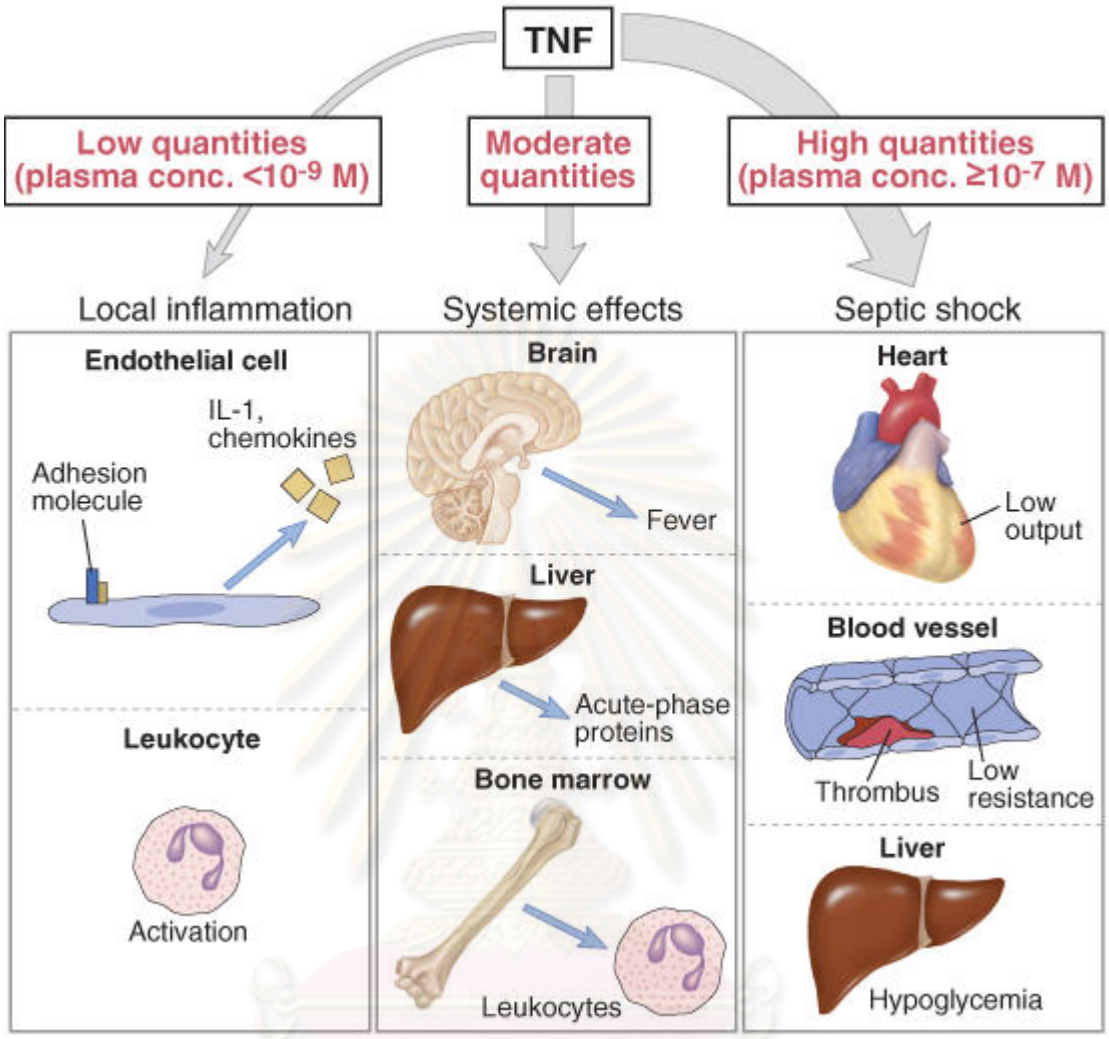


Figure 5: Biologic actions of TNF (Kumar *et al.*, 2007)

Interleukin-1 (IL-1)

IL-1 is produced by macrophages, neutrophils, epithelial cells and endothelial cells which are induced by bacterial products (such as LPS) or some cytokines (such as TNF-α). There are two forms of IL-1: IL-1α and IL-1β. IL-1β is most found in circulation. IL-1 possesses biologic effects quite similar to TNF as shown in Figure 6.

- It increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes, that the cells fight to pathogens in sites of infection and reset the hypothalamus leading to an increased body temperature which expresses as fever.

- It stimulates endothelial cells and macrophages to secrete IL-6 and nitric oxide (NO) and causes inflammation (Dinarellot 1997).

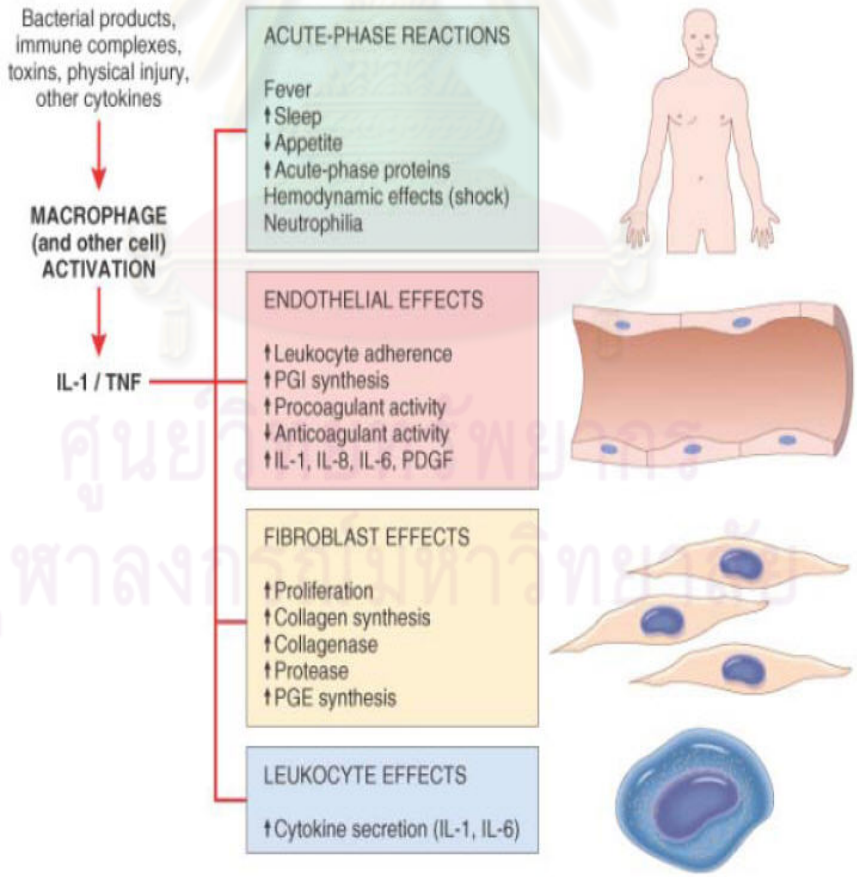


Figure 6: Major effect of interleukin-1 (IL-1) and tumor necrosis factor (TNF) in inflammation (Kumar *et al.,* 2007).

Interleukin-6 (IL-6)

IL-6 is produce by a variety of cell in immune system the most important sources are macrophages and monocytes at inflammatory site. IL-6 stimulates production of acute phase response This response consists of increased production of leukocytes; fever, which increases resistance to infection and changes in the levels of several plasma proteins, complement proteins, fibrinogen, C-reactive protein, and serum amyloid A protein. All these proteins play direct role in host defense. In extreme cases of severe infection, leads to shock, disseminated coagulation with multiorgan failure, and even death. IL-6 has biologic effects similar to TNF and IL-1. (Abul *et al.,* 2005; Kumar *et al.,* 2007)

Nitric oxide (NO)

NO is a soluble gas released from the endothelial cells and macrophages. It is synthesized from L-arginine catalysed by nitric oxide synthase enzyme (NOS). In mammals, three isoforms of NOS are discovered and named according to the activity or tissue types : endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), eNOS and nNOS are constitutively expressed, Ca^{2+} - dependent and low output. In contrast, inducible NOS (iNOS) is induced when macrophages and other cells are activated by cytokines (e.g. TNF, IFN-γ) or LPS and the enzyme is Ca^{2+} - independent.

NO plays important roles in body functions, including host defense, nonspecific immune response to infection (innate immunity), cytotoxicity and tissue damage, vasodilatation of smooth muscle cells (Figure 7 and tabel 1) (Kumar *et al.,* 2007).

In the inflammatory reaction, NO is an important inflammatory mediator working together with other pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) lead to expression of iNOS in monocytes, macrophages and neutrophil. Over production Of NO by iNOS has been implicated in various pathophysiology of human diseases such as multiple sclerosis, septic shock, tumor development, asthma and neurodegenerative diseases (Clancy *et al*., 1998; Bogdan 2001; Kleinert *et al*., 2004; Sharma *et al*., 2007; Toda *et al.,* 2009).

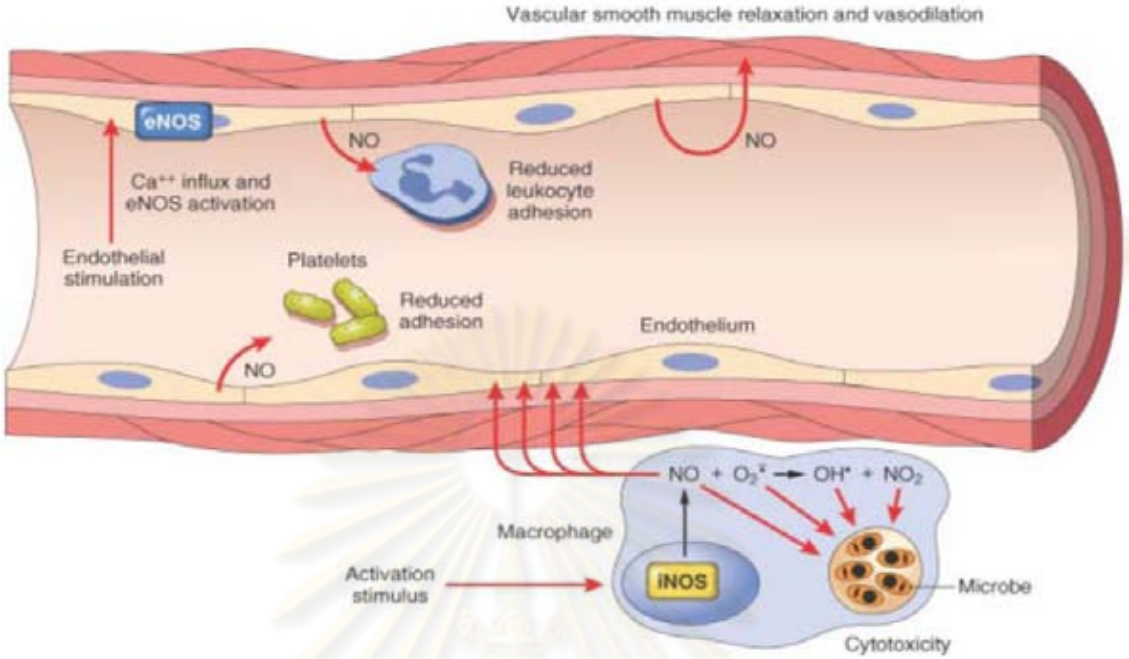


Figure 7: Functions of nitric oxide (NO) in blood vessels and macrophages, produced by two nitric oxide synthase enzymes (eNOS and iNOS). NO causes vasodilation, and NO free radicals are toxic to microbial and mammalian cells (Kumar *et al.,* 2007).

Table 1: Function of NO in immune system (Kumar *et al*., 2007)

Category	Producers of NO (examples)	Phenotypic effect of NO	Examples of underlying molecular mechanisms
		Effector functions	
Antimicrobial activity	Macrophages, microglia, neutrophils, eosinophils, fibroblasts, endothelial cells, epithelial cells, astroglia	Killing or reduced replication of infectious agents (viruses, bacteria, protozoa, fungi, helminths)	•Direct effect of NO on the pathogen •Indirect effects of the NOS pathway (*e.g.*, reaction of NO with other effector molecules, arginine depletion; see text)
Anti-tumor activity	Macrophages, eosinophils	Killing or growth inhibition of tumor cells	•Inhibition of enzymes essential for tumor growth (*e.g.*, enzymes of the respiratory chain, *cis*-aconitase, ribonucleotide reductase arginase, ornithine decarboxylase) •Growth inhibition *via* iNOS-dependent depletion of arginine •Cell-cycle arrest (downregulation of cyclin D1) •Induction of apoptosis (by activation of caspases and accumulation of p53) •Sensitization of tumor cells for TNF-induced cytotoxicity
Tissue-damaging effect (immunopathology)	Macrophages, microglia, astroglia, keratinocytes, mesangial cells	Necrosis or fibrosis of the parenchyma	•Apoptosis of parenchymal cells •Degradation of extracellular matrix •Deposition of matrix, proliferation of mesenchymal cells •Influx of inflammatory cells via chemokine regulation
		Immunoregulatory functions	
Anti-inflammatory–immunosuppressive effect	Macrophages ('suppressor phenotype')	Inhibition of: •T cell proliferation •B cell proliferation •Antibody production by $CD5^+$ B cells •Autoreactive T and B cell diversification Inhibition of leukocyte recruitment (adhesion, extravasation, chemotaxis)	•Apoptosis of T cells or APCs •Downregulation of MHC class II, costimulatory molecules or cytokines •Disruption of signaling cascades and transcription factors •Inhibition of DNA synthesis •Downregulation of adhesion molecules or chemokines
Modulation of the production and function of cytokines, chemokines, and growth factors (pro- or anti-inflammatory effects)	Macrophages T cells endothelial cells fibroblasts	Up- and downregulation, *e.g.*, of: •IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, TNF •TGF-β, G-CSF, M-CSF, VEGF, •MIP-1α, MIP-2, MCP-1	Modulation of •Signaling cascades (*e.g.* G-proteins, Jak, MAP kinases, caspases, protein phosphatases) •Transcription factors (*e.g.* NF-κB, Sp1, AP-1) •Proteins regulating mRNA stability or mRNA translation •Latent cytokine precursor complexes •Enzymes that process cytokine precursors
T helper cell deviation	*e.g.*, macrophages	•Induction and differentiation of T_H1 cells •Suppression of T_H1 (and T_H2) cell responses •Suppression of tolerogenic T cell responses	1. Possible stimulation of IL-12-mediated signaling 2. Suppression of IL-12 production

Prostaglandins

Prostaglandins are potent bioactive lipid messengers derived from arachidonic acid (AA). Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid (5, 8, 11, 14-eicosatetraenoic acid) which is derived from dietary sources or by conversion from the essential fatty acid linoleic acid. It is released from membrane phospholipids through the action of cellular phospholipases (e.g. phospholipase A_2) AA metabolites, also called eicosanoids, are synthesized by two major classes of enzymes: cyclooxygenases or COX results in production of prostaglandins and thromboxanes and lipoxygenases or LOX which results in production of leukotrienes. COX have two isoforms ; COX-1 and COX-2. COX-1 is constitutively expressed in all cell types and is involved in normal kidney, gastrointestinal and reproductive functions whereas COX-2 is inducible by a wide variety of mitogens, hormones, cytokines and other stimuli and is thus associated with inflammation and diseases.

Prostaglandins are divided into series based on their structural features as PGD, PGE, PGF, PGG, and PGH. The most important ones in inflammation are PGE_2, PGD_2, $PGF_2\alpha$, PGI_2 (prostacyclin), and TXA_2 (thromboxaneA_2) (table 2). Prostaglandins are also involved in the pathogenesis of pain and fever. In inflammation, PGE_2 is hyperalgesic that makes the skin hypersensitive to painful stimuli. PGD_2 is the major metabolite of the cyclooxygenase pathway in mast cells; along with PGE_2 and $PGF_2\alpha$, they cause vasodilation and increase the permeability of postcapillary venules, potentiating edema formation. Ferver is induced by pyrogens which subsequently stimulate the production of PGE_2 and increase body temperature through heat regulating center in hypothalamus. Bacterial LPS from infecting organisms, or circulating IL-1, stimulate the expression of COX-2 and of PGE synthase in endothelial cells or macrophages that constitute the blood-brain PGE_2 which is generated by PGE synthase diffuses out of the endothelial cells or macrophages into the organum vasculosum lamina terminalis (OVLT) region of the hypothalamus which is responsible for controlling fever.(Vane *et al*., 1998; Hinz and Brune 2002;Turini and Dubois. 2002; Marco *et al*., 2002)

Table2: Inflammatory actions of eicosanoids (Kumar *et al*., 2007)

Action	Metabolite
Vasoconstriction	Thromboxane A_2 , leukotrienes C_4, D_4, E_4
Vasodilation	PGI_2, PGE_1, PGE_2, PGD_2
Increased vascular permeability	Leukotrienes C_4, D_4, E_4
Chemotaxis, leukocyte adhesion	Leukotriene B_4, HETE, lipoxins

In state of over stimulation of the macrophags lead to over expression of mRNA of cytokines including TNF-α, IL-1β , IL-6 and over expression iNOS, COX-2. All these cytokines and enzymes are involved in the inflammatory diseases such as rheumatoid arthritis, atherosclerosis, lung fibrosis, septic shock and tissues damage. At present there are two general classes of drugs commonly used in the treatment of inflammatory diseases. They are steroid and nonsteroidal anti-inflammatory agents (NSAIDs)

Steroid

Steroidal compound structure and efficacy are similar to glucocorticoid hormone from adrenal cortex. Their effects are on entire body systems including carbohydrate, protein and lipid metabolism, electrolyte and water balance, cardiovascular system, skeletal muscle, central nervous system, forming elements of blood, anti-inflammatory and immunosuppressive action. Example of drugs in this group are dexamethasone, betamethasone, fludrocortisone, triamcinolone and prednisolone.

Mechanism of action of steroid for anti-inflammatory effect

Steroid is present in the blood in bound form on the corticosteroid-binding globulin (CBG). The intracellular receptor is bound to stabilizing proteins, including two molecules of heat shock protein 90 (Hsp90) and others. When the complex binds a molecule of cortisol, an unstable complex is created and the Hsp90 and associated molecules are released. The steroid-receptor complex is dimerize, enter the nucleus, bind to a glucocorticoid response element (GRE) on the regulatory region of the gene,

and regulate transcription by RNA polymerase II and associated transcription factors. The resulting mRNA is edited and exported to the cytoplasm for the production of protein that brings about the corresponding hormone response. The steroid-receptor complex interaction with a GRE is an interaction with and altering the function of other transcription factors, such as NF-ĸB in the nucleus of cells which result to reduce expression of pro-inflammatory cytokines, COX-2 and iNOS.

In the nucleus Glucocorticoid receptors (GR) can bind as a dimer onto the glucocorticoid response element (GRE), and regulate steroid-responsive genes regulating metabolic homeostasis. Recognized important function of activated GR is the inhibition of transcription of several cytokines and chemokines that are in inflammatory diseases. Another way steroid binding to glucocorticoid receptor (GR) activate to produce lipocortin which inhibits the activity of phospholipase A_2 that results in reducing the prostaglandins and reduction the number of lymphocytes, monocytes, eosinophils, and basophils at the site of inflammation.

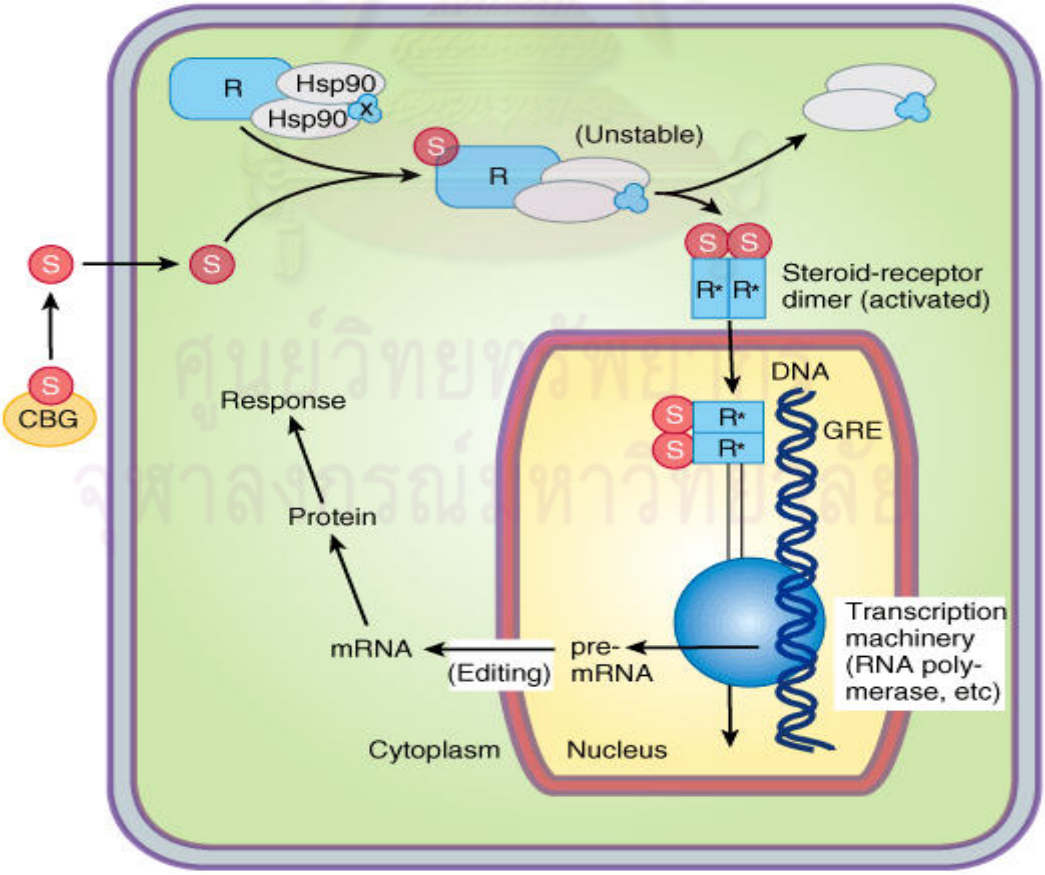


Figure 8: Mechanism of action of steroid (Schimmer *et al.*, 2006)

Adverse effects of steroid

Steroid is valued for its therapeutic application but it also causes many systemic side effects in long term administration. Since it suppresses the hypothalamic - pituitary –adrenal (HPA) axis and bring about iatrogenic Cushing's Syndrome. Fat tends to be redistributed from the extremities to the trunk, the back of the neck, and the supraclavicular fossae. There is an increase growth of fine hair over the face, thighs and trunk. Steroid-induced punctate acne may appear. Furthermore it causes insomnia, increased appetite, bone loss, peptic ulcers, myopathy, psychoses and glaucoma, increased susceptibility to infection and a risk for reactivation of latent tuberculosis (Chrousos *et al.,* 2007; Schimmer *et al.,* 2006).

Nonsteroidal anti-inflammation drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs are drugs with anti-inflammatory effects. Two major groups of NSAIDs are classified on the basic of their selectivity on COX enzyme. There are non-selective COX inhibitors (e.g. indometacin, ibuprofen, naproxen, piroxicam) and the selective COX-2 inhibitors (celecoxib, etoricoxib,).

Mechanism of action of NSAIDs

NSAIDs are inhibitors of the enzyme cyclooxygenase (COX-1 and COX-2) which catalyze arachidonic acid to form prostaglandins and thromboxanes. Prostaglandins act as messenger molecules in the process of inflammation especially PGE_2, PGI_2 and $PGF_{2\alpha}$. Suppression of these inflammatory prostaglandins result in alleviation of pain, fever, and inflammation.

- **Non-selective COX inhibitors**

  Non-selective COX inhibitors inhibit both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 COX-2) enzymes. COX-1 is primarily constitutive isoform found in most normal cell and tissue while COX-2 production is induced by inflammation, shock, tumor etc.

- Selective COX-2 inhibitors

Selective COX-2 inhibitors selectively inhibit COX-2 enzyme and prostaglandins production. While COX-1 but not COX-2 is expressed predominantly in gastric epithelial cells and is the major source of cytoprotective prostaglandins formation. Inhibition of COX-1 at this site is account for gastric adverse events.

NSAIDs are also known to reduce production of superoxide radicals, inhibit the expression of adhesion molecules, decrease nitric oxide synthase and decrease pro-inflammatory cytokines.

Adverse effects of NSAIDs

1. **Gastrointestinal system**: GI side effect is the most common symptoms associated with these drugs. The adverse effect is account for inhibition of cytoprotective prostaglandins (PGE_2 and PGI_2) which result in abdominal pain, nausea, diarrhea, anorexia, gastric erosions/ulcers, anemia, GI hemorrhage and perforation.
2. **Central nervous system**: Headaches, tinnitus, dizziness, confusion, depression, lowering of seizure threshold, hyperventilation (salicylates).
3. **Renal system**: Salt and water retention, edema, worsening of renal function in renal/cardiac and cirrhotic patients, decreased effectiveness of antihypertensive medications, decreased effectiveness of diuretic medications, decreased urate excretion (especially with aspirin), hyperkalemia.
4. **Platelets**: Inhibited platelet activation.
5. **Uterus** : Prolongation of gestation, inhibit labo.
6. **Cardiovascular system**: Fluid retention, hypertension, congestive heart failure, risk of myocardial infarction are associated with COX-2 inhibitors more frequently than non selective COX inhibitors.
7. **Hematologic system**: Rare thrombocytopenia, neutropenia, or even aplastic anemia (Furst *et al.,* 2006; Barke *et al.,* 2007).

Although a variety of nonsteroidal anti-inflammatory drugs (NSAIDs) are employed for the treatment of inflammatory diseases, the adverse effects of these drugs limited their therapeutic use both for non selective COX inhibitor and selective COX-2 inhibitors.

Research and development of the new drugs in these classes are still in need. The goal for development of new anti-inflammatory drugs are to lessen the adverse effect and to maximize the therapeutic effect. New drugs from plants are interesting source for the scientists.

Glycosmis parva, a plant in Thailand, are composed of different kinds of alkaloids and steroidal compounds . At present there is no report up on its pharmacological actions. However there are several reports are found in the compounds related to constituents found *G. parva* especially β-sitosterol. Thus it is our interest to investigate the chemical constituents of this plant for its effect on the macrophage, which are targeted cells responsible for inflammatory process.

Glycosmis parva Craib

G. parva Craib are plant belonged to the family Rutaceae. It is commonly know as Som-chuen, Prayon-kluean for its local name in Thailand. This plants is evergreen shrubs or undertrees. Leaves alternate, 1-5 foliate. Flowers usually small, axillary panicles, calyx 4 or 5 partial imbricate. Petals 4 or 5 imbricate, stamen 8 to 10 free, filaments dilated below. Ovary 2 to 5 celled, the style very short, not jointed ovule 1 in each cell. Fruits globose, freshy, berry. Seed 1 to 3 oblong, testa membranous.

At least two major groups of compounds are identified from the hexane extraction of the branches and leaves of this plant. They are acridone alkaloids (N-methylataphilline and 5-hydroxy-N-methylseverifoline) and steroidal compounds (β-sitosterol and stigmasterol) (Kongsubsopa 2000). Several new compounds are identified from *G. parva* extracts as demonstrated in Appendix B-31 (Reungrungsi N. and Chansriniyom C.)

Pharmacological effect of compounds related to the chemical constituents found in *G. parva*

1. Antimalarial effect

5-hydroxy-N-methylseverifoline isolated from Citrus, Glycosmis, or Severinia plants (members of the family Rutaceae) have shown antimalarial activities in vitro and in vivo. 5-hydroxy-N-methylseverifoline suppressed 92% of *Plasmodium yoelii* at a concentration of 10 ug/ml in vitro(Fujioka *et al.,* 1989).

2. Antiproliferative effect

5-hydroxy-N-methylseverifoline possesses antiproliferative activity toward monolayers and suspension of several types of cancer cell lines including :human lung carcinoma (A-549), melanin pigment producing mouse melanoma (B-16 melanoma 4A5), T-cell leukemia (CCRF-HSB-2); human gastric cancer cell, and lymph-node metastasized (TGBC11TKB) (Kawaii *et al.,* 1999).

3. Anti-inflammatory effect

β-sitosterol isolated from n-hexane extract of *Euphorbia hirta* reduced ear edema when test in TPA-induced ear model in mice (Vazquez *et al.,* 1999).

β-sitosterol isolated from the root of *Dystaenia takeshimona* showed inhibitory activity of COX-2 by 98.2%and 5-LOX by 77.3% and reduced production of protaglandinD_2(PGD_2) , leukotrienC_4 (LTC_4) in mouse bone marrow- derived mast cells (Kim *et al.,* 2006).

β-sitosterol has been shown to inhibit the oedema in oxazolone-induced contact-delayed-type hypersensitivity model (Prieto *et al.,*2006).

β-sitosterol isolated from *Radix Adenophorae* reduced airway inflammatory and airway hyperresponsiveness (AHR) in murine model of asthma through suppression of IL-13, IL-5, IgE, eosinophils, CCR3 expression (Roh *et al.,* 2008).

β-sitosterol isolated from *Rhus sylvestris* has been reduced secretion of IL-6 and TNF-α in RAW 264.7 macrophage cell line stimulated with LPS (Ding *et al.,* 2009).

Fractionation of the acetone extract of *Sideritis foetens* composed of campesterol 7.6%, stigmasterol 28.4%, β-sitosterol 61.1% decreased carrageenan paw

oedema, inhibition of mouse ear oedema induced by TPA and decreased neutrophil infiltration into inflamed tissue (Navarro *et al.,* 2001).

A mixture of β-sitosterol and stigmasterol were isolated from *Buddlleja globosa* reduced TPA – induced inflammatory in mice by 78.2% at the concentration of 1 mg/20ul/ear (Backhouse *et al.,* 2008).

CHAPTER III

MATERIALS AND METHODS

1.Materials

1.1 Extracts of *Glycosmis parva*

The hexane, ethyl acetate, butanol and water extracts of branches and leafs from *Glycosmis parva* were prepared and identified by Associate Professor Dr. Nijsiri Ruangrungsi and Mr. Chaisak Chansriniyom Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The TLC fingerprints of the extracts used in study are in Appendix A-1 which represent G1, G2, G5 and G6 respectively.

All extracts, except the water extracts, were dissolved in DMSO at 50 mg/ml as the stock solutions. These solutions were stored at –20°C until use. When they were used, they were diluted in a sterile double-distilled water to 2% DMSO solutions before treating cells at 1:10 ratio. These made the final solution of the extracts, at required concentrations, to be in 0.2% DMSO.

The stock solutions of the water extracts were prepared in double distilled water, sterilized through 0.22 um filters, and stored at –20°C until use.

1.2 Macrophages

The murine macrophage cells J774A.1, were purchased from the American Type Culture Collection (ATCC). The cells were grown in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a CO_2 incubator at 37°C. They were subcultured by scraping when the cells were 80% confluence. They were used in this study with their viability more than 85%.

1.3 Chemicals and reagents

The following reagents were used in this study:Chloroform (Sigma, USA), DEPC (Molekula, UK), disposable cell scraper (Greiner bio-one, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), hydrochloric acid (Merck,Germany), ImProm-IITM

Reverse Transcription system (Promega, USA), lipopolysaccharide (Sigma, USA), nitric oxide assay kit (Promega, USA), penicillin/streptomycin (Gibco, USA), primer (Bio Basic, Canada), Taq polymerase (Invitrogen, UK) , trypan blue dye (Sigma, USA), TRiZol reagent (Invitrogen, UK)

1.4 Equipment and Instruments

The followings equipments and instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hiclave ^{TM} , HVE-50, Hirayama, Japan), autopipette (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge machine (Hettich, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), 96 and 24 muti-well plate (Corning, USA), spectrophotometer (Shimadzu, Japan), thermocycle machine (Eppendorf, USA) , vortex mixer (Scientific industries, USA)

2.Methods

2.1 Effect of *Glycosmis prava* solvent extracts on NO production in LPS-stimulated J774A.1 cells

J774A.1 cells, at a density of 2 x 10^5 cells/ml were grown in a 96 well plate at 37 $^{\circ}$C for 24 h. The cells were pre-treated with the hexane, ethyl acetate, butanol and water extracts of branches and leaves from *G. prava* at concentration 3.125-100 µg/mL, for 24 h before being stimulated with 100 ng /ml LPS for the next 24 h. The non pre-treated LPS –stimulated cells and the 0.2% DMSO-treated cells were used as the control and the untreated control, respectively. The supernatants of the treated cells was collected for nitric oxide content determination and the cells were assessed for cytotoxic of the extracts.

The assay for nitric oxide content was perform in the dark at room temperature by using Griess reagents as in the following procedures. In 96-well plate, 100 µl of supernatants were reacted with 20 µl of sulfanilamide reagent for 10 min, then 20 µl of N-1-napthylenediamine dihydrochloride (NED) reagent was added. The plate was incubate further for 10 min and measured by microplate reader at 540 nm. The nitric

oxide content in each well was determined as nitrite content (μM) by using nitrite standard curve. The percentages of nitric oxide inhibition of the extracts were calculated by comparing with the non pre-treated LPS-stimulated condition.

$$\% \text{ NO inhibition} = \left(\frac{\text{NO conc. of Negative Control - NO conc. of Treatment}}{\text{NO conc. of Negative Control}} \right) \text{ X 100}$$

The 50% inhibitory concentration (IC_{50}) on NO production of the extracts was also calculated. These concentrations of the extracts were used in the next experiments.

The cytotoxicity of the extractes was performed by incubating the treated cells in 96 wells plate with 50 μg/ml resazurin at 37°C for 2 h. The amount of resorufin, the product from resazurin reduction in viable cells, was determined using microplate reader by subtracting the OD at 570 from the OD at 600 nm. The percentage of cytotoxicity of the extracts was calculated by using the following formular ;

$$\% \text{ cytotoxic} = \left(\frac{\text{delta OD (negative control) - delta OD}(\text{sample})}{\text{delta OD (negative control)}} \right) \text{ X 100}$$

The extracts that inhibit NO production at the non - cytotoxic concentrations (2 or 3 concentrations/extract) were assessed for their effects on mRNA expression of interested genes involved in macrophage stimulation.

2.2 Effects of the extracts on mRNA expression of cytokines, iNOS and COX-2 in LPS-stimulated J774A.1 cells

J774A.1 cells, at the density of 2 x 10^5 cells/ml, were grown in a 24 well plate at 37°C for 24 h. The cells were pretreated with the extracts (2 or 3 concentrations) at 37°C for 24 h, then treated with LPS 100 ng/ml at 37°C for 4 h for assessing cytokine expression. The same procedure was performed as above but the cells were stimulated with LPS for 24 h for iNOS and COX-2 expression. The non pre-treated LPS-stimulated cells and the 0.2% DMSO-treated cells were used as the control and the untreated control, respectively.

The treated cells were collected for total RNA preparation, cDNA production, and TNF-α, Il-1β, IL-6, iNOS and COX-2 expression determination.

Total RNA preparation

The cells were lysed and homogenized in 1 ml of TRIzol® Reagent at room temperature sample for 5 min. The homogenized samples were transfer to eppendorf tube. Two hundred µl chloroform was added into each tube. The tubes were vigorously shaked by hand for 15 seconds , incubated further at room temperature for 2-3 min, and separated for supernatant by centrifugation 12,000g for 15 min. at 4 °C. The supernatants were carefully collected into fresh eppendorf tubes. 0.5 ml of isopropyl alcohol was added into each tube. The tubes were incubated at room temperature for 10 min. The RNA pellets were separated by centrifugation 12,000g for 10 min. at 4 °C. The supernatant were discarded. The pellets were washed with 75% ethanol. Each wash the pellets were separated by centrifugation at 7,500g for 5 min. at 4°C. After washing, the pellets were air-dried and dissolved in RNase free-water. The RNA content was determined by spectrophotometer at 260 nm and calculated by the following formular

RNA(µg) = Absorbance at 260 nm X 40 X dilution factor

The RNA sample were store at -70 °C until use.

cDNA synthesis by reverse transcription

For each sample tube, 1.5 µg total RNA was pre-heat with Oligo dT_{15} primer in Nuclerse – Free Water at 5 µl final volume at 70 °C for 5 min. The tube were immediately chilled on ice for 5 min. Fifteen µl reverse transcription mixture containing; 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor and reverse transcriptase were added into each tubes. The tubes were incubated at 25 °C for 5 min, then 42 °C for 1 hour 30 min. and finally at 70 °C for 15 min. The samples were stored at -20 °C until use.

Amplification of interested cytokines, iNOS and COX-2 cDNA by polymerase Chain Reaction (PCR)

For each PCR tube, 1 µl of cDNA sample was mixed with PCR reaction mixture containing primer, mixed dNTP, Taq polymerase in PCR buffer. The PCR was performed by the following conditions; denaturation for 30 sec at 94 ˚C, annealing for 45 sec at 55˚C, extension for 1 min at 72 ˚C and final extension for 7 min at 72 ˚C at the end of 25th cycles. The PCR products were run by 1.5 % agarose gel electrophoresis at 100 volt for 45 min in TBE buffer , using 6 µl plus 2 µl loading dye for each sample. The gel was stained with ethidium bromide for 4 min and destained with TBE buffer for 30 min. The PCR products were analyzed and semicontitated by a gel documentation.

7. Statistical analysis

All data were presented as mean and standard deviation or standard error of means. Data analysis was performed on SPSS 17.0. If the test of homogeneity of variances showed that there was no significant deviation of variances in the data, the analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) post hoc test was used. The p-value of less than 0.05 was considered statistically significant.

CHAPTER IV
RESULTS

1. Effect of *Glycosmis parva* solvent extracts on LPS stimulated-macrophages

All solvent extracts from branches and leaves of *G. pava* were identified for their inhibitory activities on LPS-stimulated J774A.1. Inhibition of nitric oxide synthesis from the stimulated cells was used to evaluate these extracts. J774A.1 cells were pretreated with 10 - 100 μg/ml of each extract for 24 h and then treated with 100 ng/ml LPS for the next 24 h. The supernatant from the treated cells was collected for determining NO content by Griess reaction. The hexane and the ethyl acetate extracts from both branches (G1 and G2) and leaves (G5 and G6) of *G. pava* clearly inhibited NO production in LPS-stimulated J774A.1 cells (Fig.9a). However, all these extracts, except the hexane extract from branches (G1), at 100 μg/ml concentration also had highly cytotoxic effect on the treated cells (Fig.9b). These extracts were employed in the subsequent studies. The butanol and the water extracts of branches (G3 and G4) and leaves (G7 and G8) didn't have both the inhibitory and the cytotoxic effects to the cells. These extracts were not studied further in the subsequent studies.

The hexane and the ethyl acetate extracts from branches and leaves of *G. pava* (G1, G2, G5, and G6, respectively) were determined for their 50% inhibitory concentrations (IC_{50}'s) on NO production from LPS-stimulated J774A.1. The cells were pretreated with G1 (6.25-100 μg/ml), G2 (3.13-50 μg/ml), G5 and G6 (1.56-50 μg/ml) for 24 h and then treated with 100 ng/ml LPS for 24 h. The supernatant from the treated cells was collected for determining NO content by Griess reaction and the cells were used to determine the cytotoxicity of the extracts by resazurin staining assay. All extracts inhibited NO production in LPS-stimulated cells in a concentration-dependent manner (Fig.10a-13c). Their IC_{50} values for the NO production were 44.70, 16.70, 11.76 and 11.19 μg/ml in G1, G2, G5 and G6, respectively. The potencies of these extract were in the following order; G5 = G6 > G2 > G1. These IC_{50} values were used for

selecting concentrations of the extracts to study their molecular activities in the following studies.

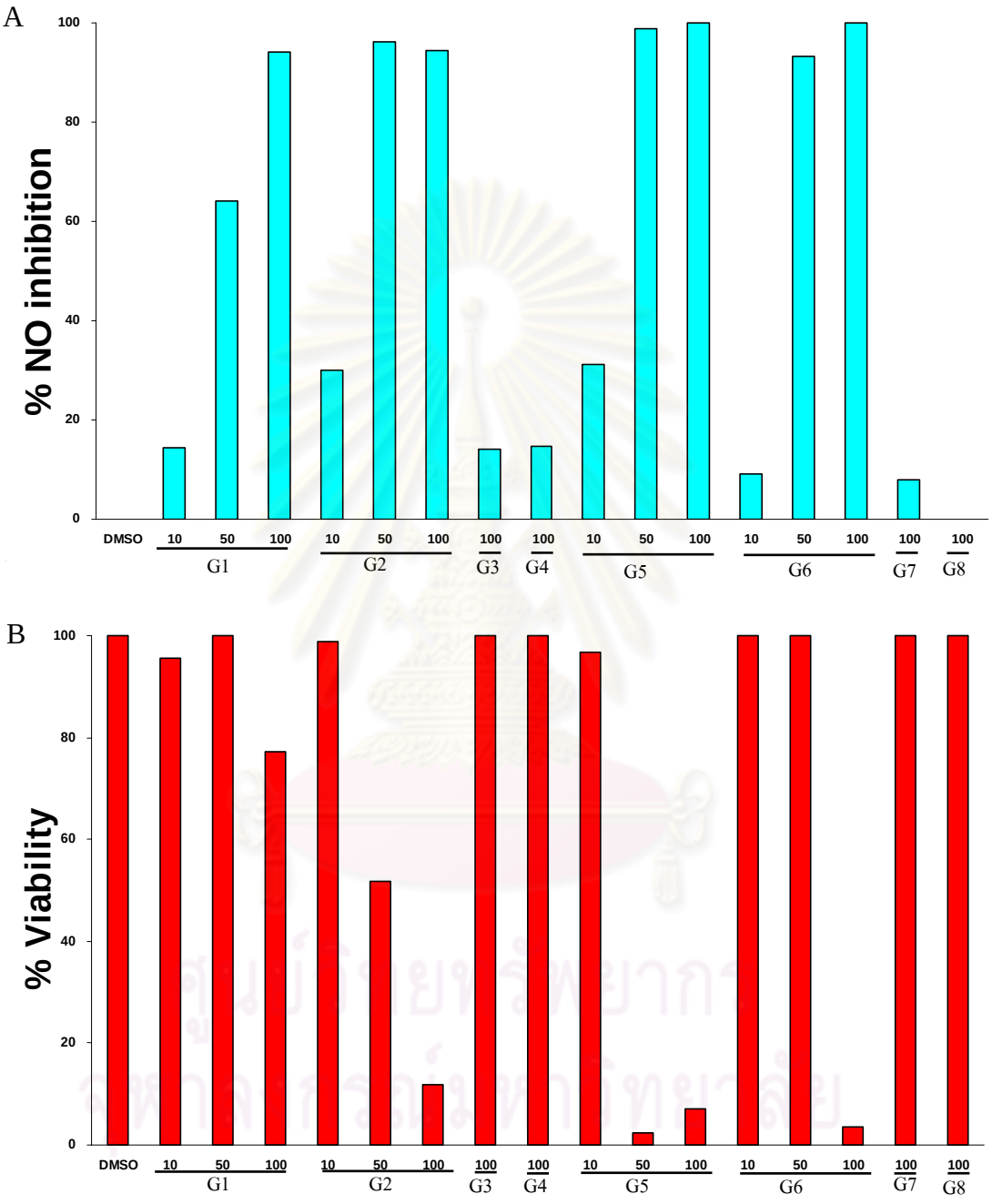


Figure 9: (A) Inhibition effect *G. parva* on NO production in LPS stimulated-macrophage J774A.1 at dose 10 -100 µg/ml of extracts. (B) Cytotoxic effect of *G. parva* in LPS stimulated-macrophage J774A.1 at dose 10 -100 µg/ml of extracts [The extracts from branches: G1: hexane, G2: ethyl acetate, G3: butanol, G4: water; The extracts from leaves: G5: hexane, G6: ethyl acetate, G7: butanol, G8: water]. Results are means ± S.D. (N=2).

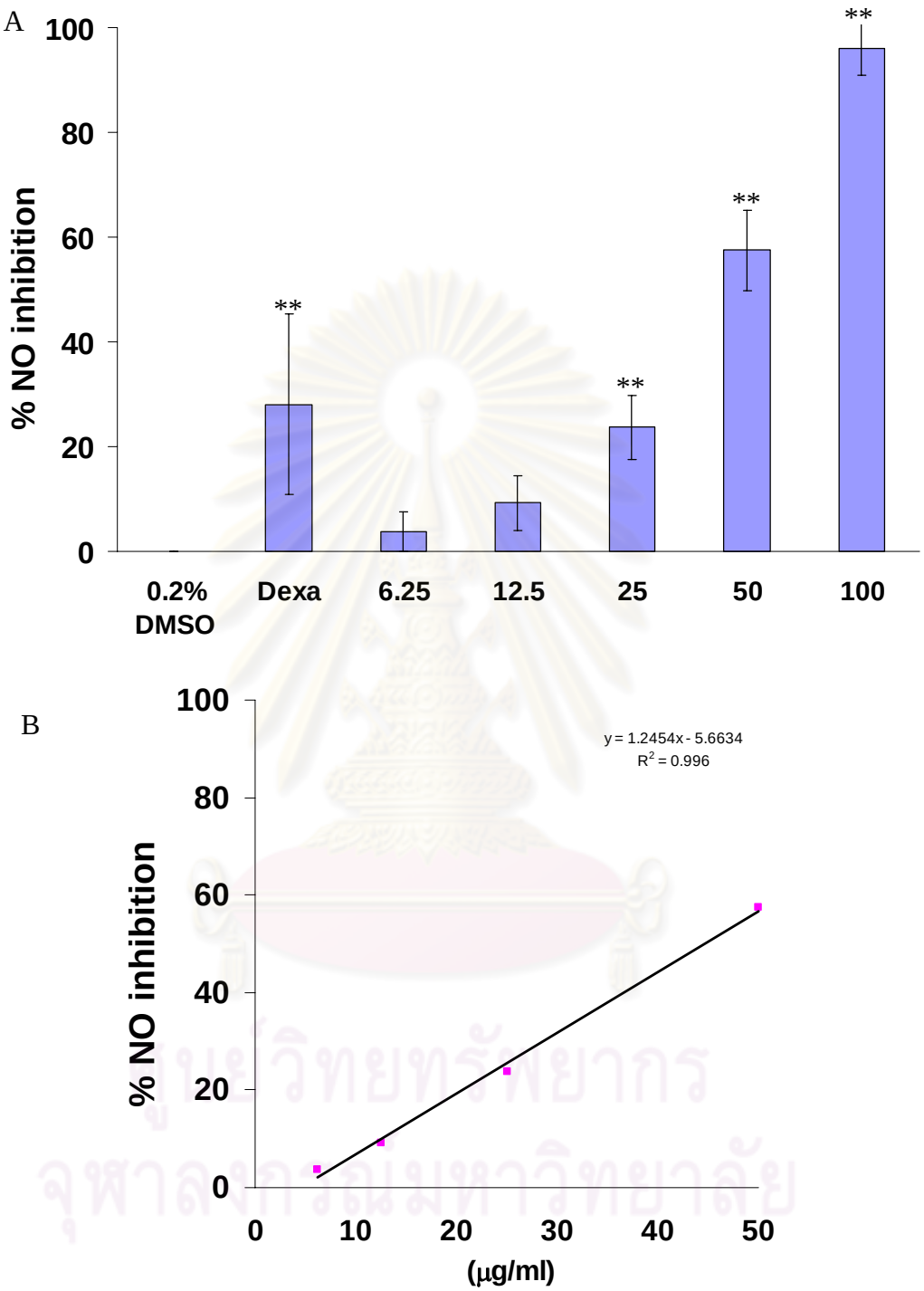


Figure 10: (A) Inhibition effect of G1 on NO production in LPS stimulated-macrophage J774A.1 at dose 6.25-100 µg/ml of extracts. (B) IC_{50} of G1 (44.69 µg/ml). ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

C

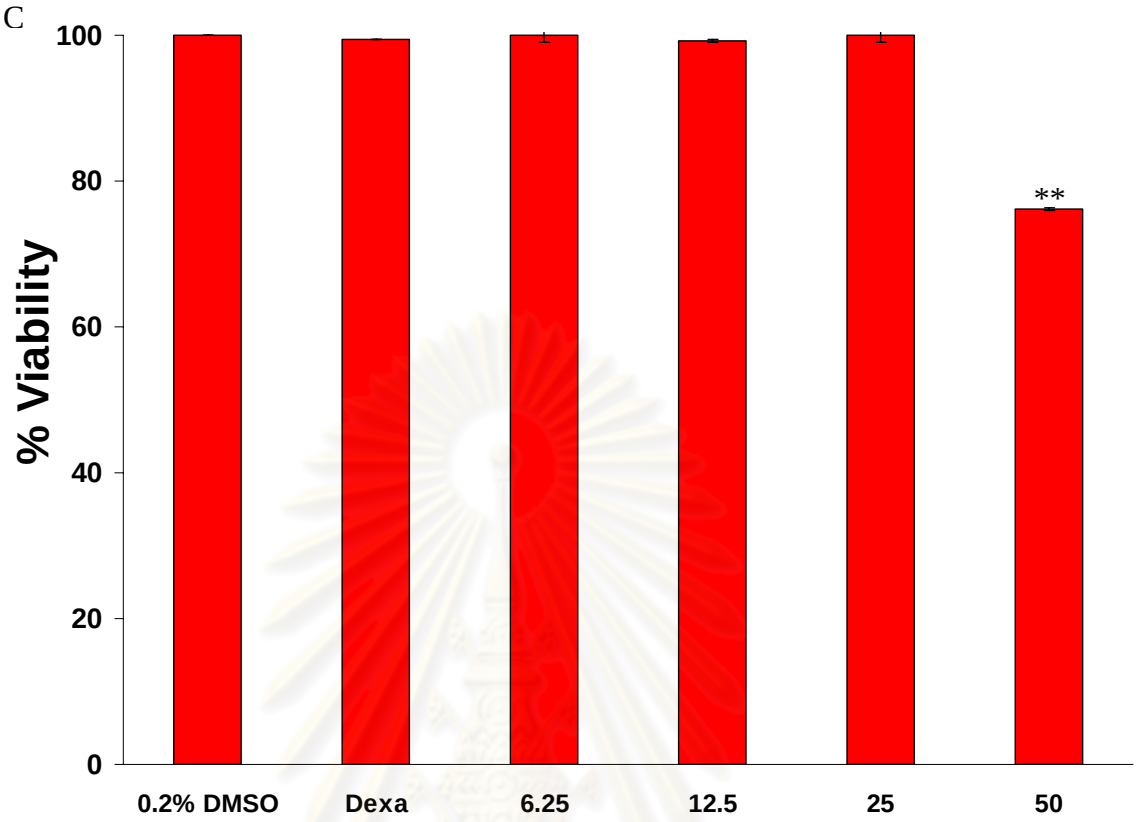


Figure 10: (C) Cytotoxic effect of G1 in LPS stimulated-macrophage J774A.1 at dose 6.25-100 µg/ml of extracts. ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

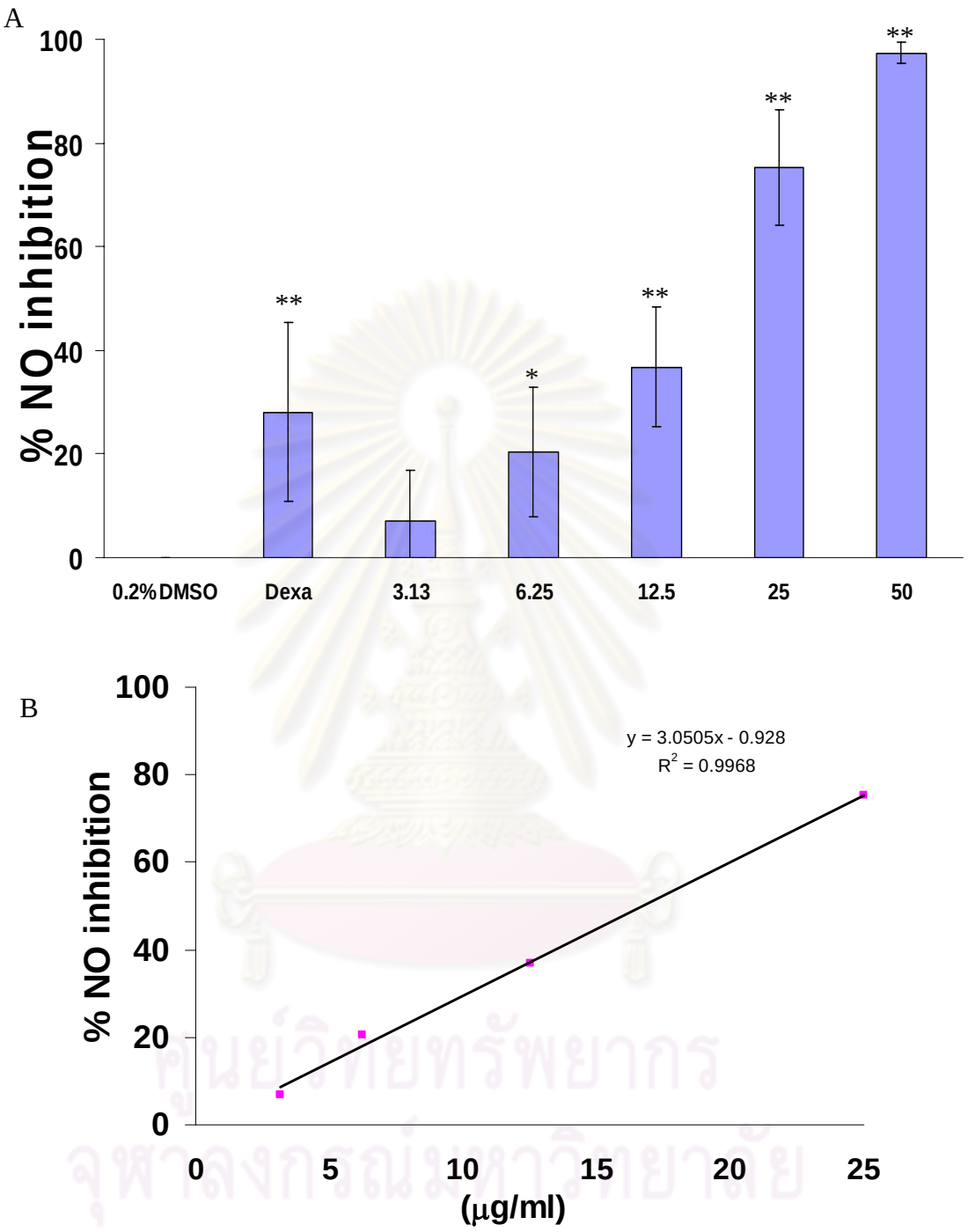


Figure 11: (A) Inhibition effect of G2 on NO production in LPS stimulated-macrophage J774A.1 at dose 3.13 - 50 µg/ml of extracts. (B) IC_{50} of G2 (16.69 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

C

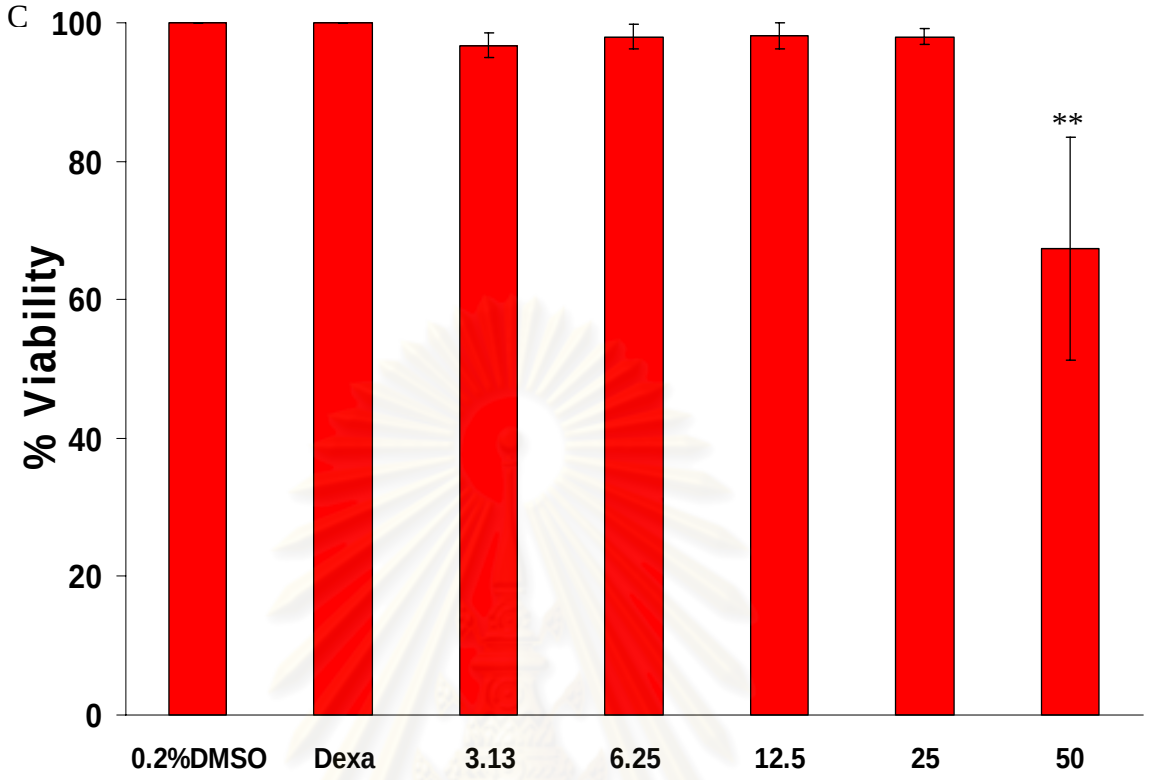


Figure 11: (C) Cytotoxic effect of G2 in LPS stimulated-macrophage J774A.1 at dose 3.13 - 50 µg/ml of extracts. ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

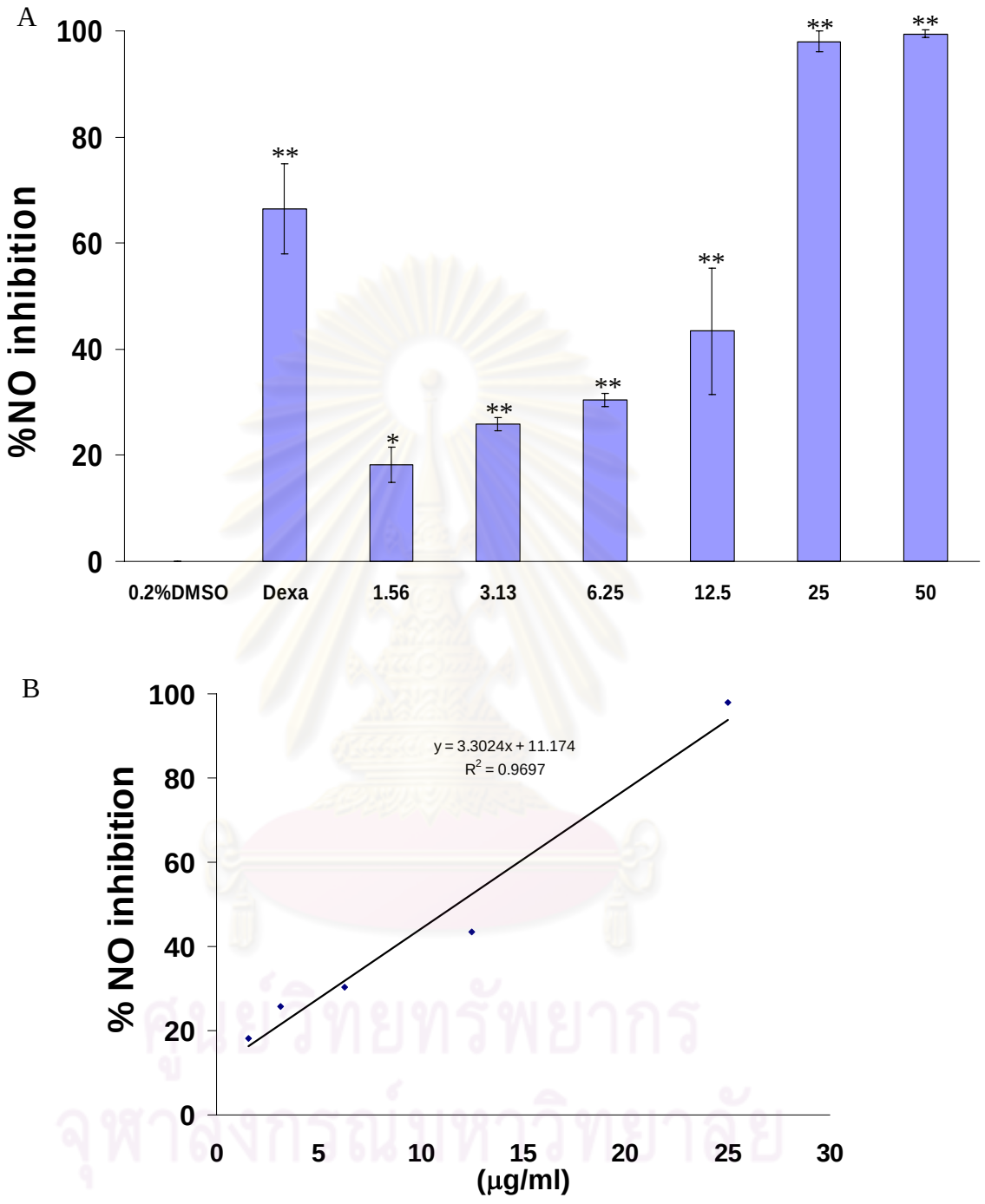


Figure 12: (A) Inhibition effect of G5 on NO production in LPS stimulated-macrophage J774A.1 at dose 1.56- 50 µg/ml of extracts. (B) IC_{50} of G5 (11.57 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$) Results are means ± S.D. (N=3).

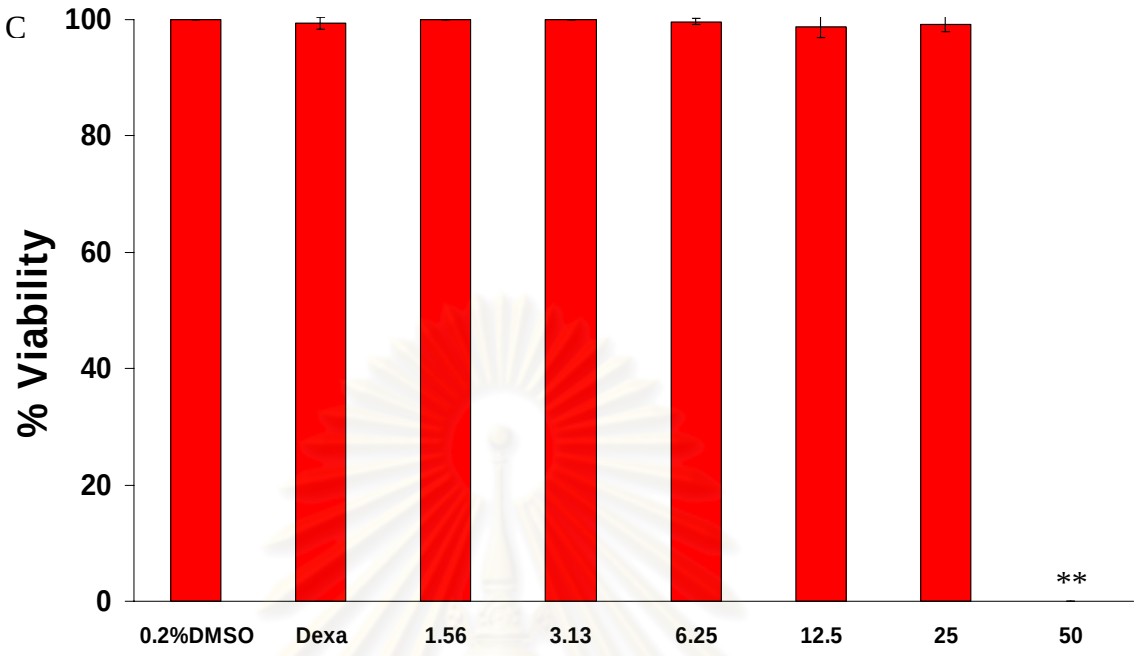


Figure 12: (C) Cytotoxic effect of G5 in LPS stimulated-macrophage J774A.1 at dose 1.56 - 50 µg/ml of extracts. * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

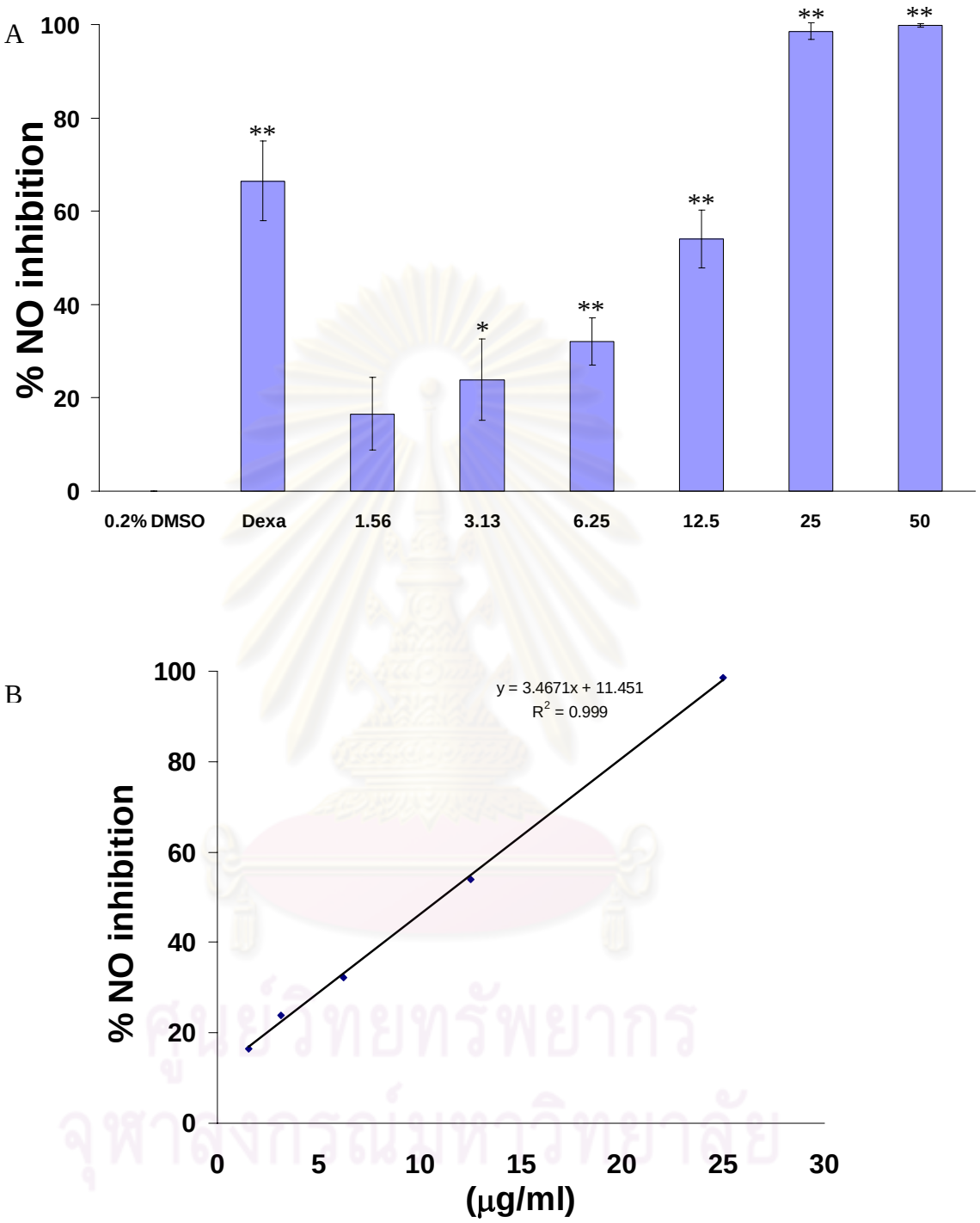


Figure 13: (A) Inhibition effect of G6 on NO production in LPS stimulated-macrophage J774A.1 at dose 1.56 - 50 µg/ml of extracts. (B) IC_{50} of G6 (11.12 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

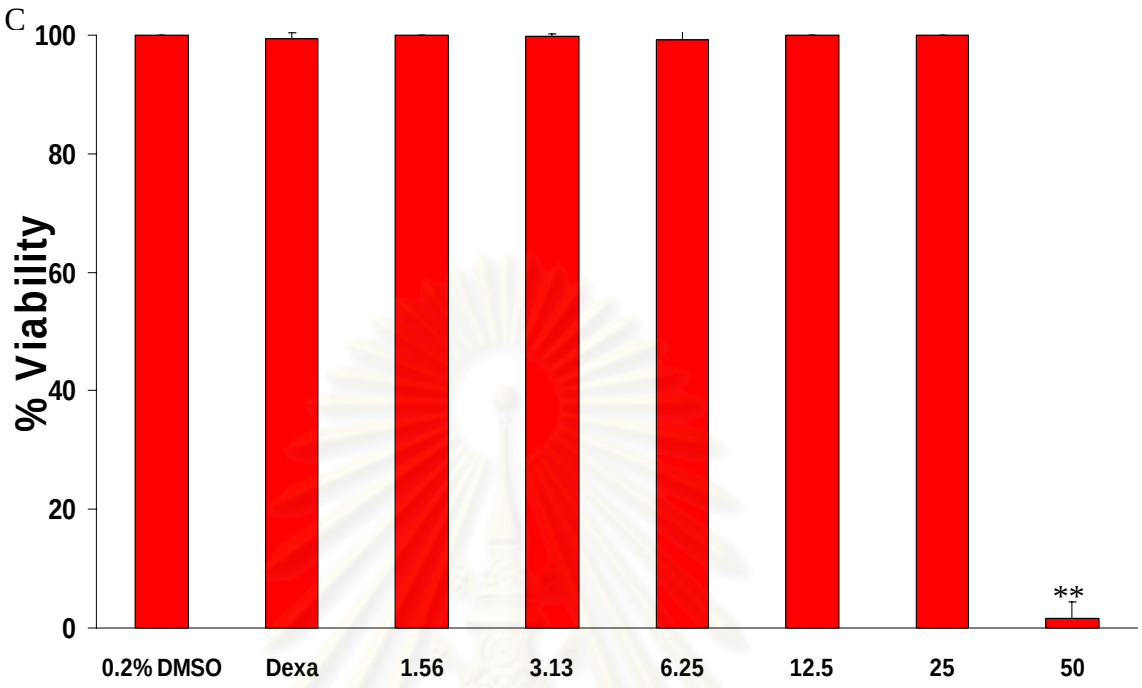


Figure 13: (C) Cytotoxic effect of G6 in LPS stimulated-macrophage J774A.1 at dose 1.56 -50 µg/ml of extracts. * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

2. Effect of *Glycosmis parva* extracts on the expressions of pro-inflammatory cytokines in LPS stimulated-macrophages.

The effects of the hexane and the ethyl acetate extracts from branches (G1 and G2) and leaves (G5 and G6) of *G. parva* on TNF-α, IL-1β, and IL-6 in LPS-stimulated J774A.1 were evaluated. The cells were pretreated with 2- or 3 concentrations of the extracts for 24 h and then treated with 100 ng/ml LPS for 4 h. The total RNA was isolated from the treated cells and used to determine the expression of TNF-α, IL-1β, and IL-6 by RT-PCR. All extracts were used at the IC_{50} for NO inhibition from the previous study plus one or two concentrations which were 2-folds lower and higher than the IC_{50}.

G1 at the concentrations of 25 and 50 μg/ml was used in these experiments. Its IC_{50} was 44.70 μg/ml. It inhibited the expression of the pro-inflammatory cytokines, TNF-α (23.4, 54.3% respectively), IL-1β (13.0%, 15.9%), and IL-6 (9.4, 56.1%) (Appendix B-12) in LPS-stimulated J774A.1. It had inhibitory effect on the expression of TNF-α and IL-6 expression higher than on the expression of IL-1β at 50 μg/ml (Fig. 14).

Ten and 20 μg/ml G2 was used in the study. Its IC_{50} was 11.70 μg/ml. It significantly inhibited the expression of TNF-α (57.8%) and IL-1β (69.85%) (Appendix B-14) at 20 μg/ml, but it didn't have effect on the expression of IL-6 at this concentration (Fig.15).

The IC_{50} of NO inhibition of G5 was 11.76 μg/ml. It was used in this study at 6.25, 12.5 and 25 μg/ml. It profoundly inhibited the expression of TNF-α (91.1, 93.02, 94.27%) (Appendix B-16) in all concentrations used (Fig.16). The inhibition effect of G5 on IL-1β was found only at high concentration (25 μg/ml). It didn't have effect on IL-6 expression.

The IC_{50} of NO inhibition of G6 was 11.20 μg/ml. It was used in this study at 6.25, 12.5 and 25 μg/ml. The results in Fig.17 demonstrated that it significantly decreased the expression of the pro-inflammatory cytokines at its IC_{50}. At 25 μg/ml, it greatly reduced the expression of TNF-α by 30.2- 98.6 %. For IL-1β only the concentration at 25 μg/ml of G6 significantly inhibited its expression by 35.2%. While the concentration of G6 at 12.5, 25 μg/ml significantly inhibited IL-6 by 55.48 and 97.94%. (Appendix B-18) These results suggest that these solvent extracts of *G. parva* have different patterns of pro-inflammatory cytokine inhibition. They may contain different constituents.

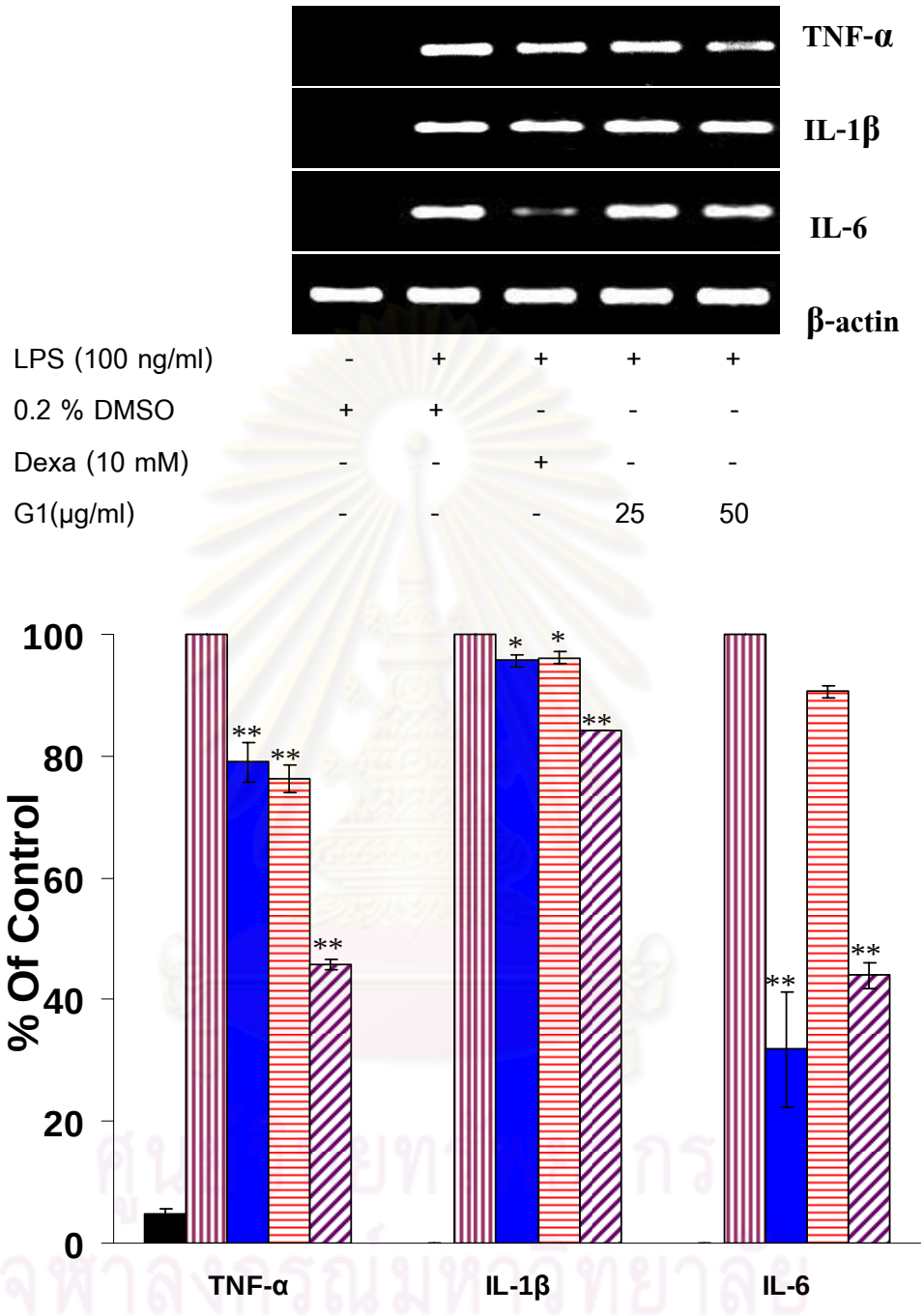


Figure 14 : Effect of G1 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexa-methasone 10μM+LPS , ▤ G1 25 μg/ml +LPS, ▨ G1 50 μg/ml + LPS.

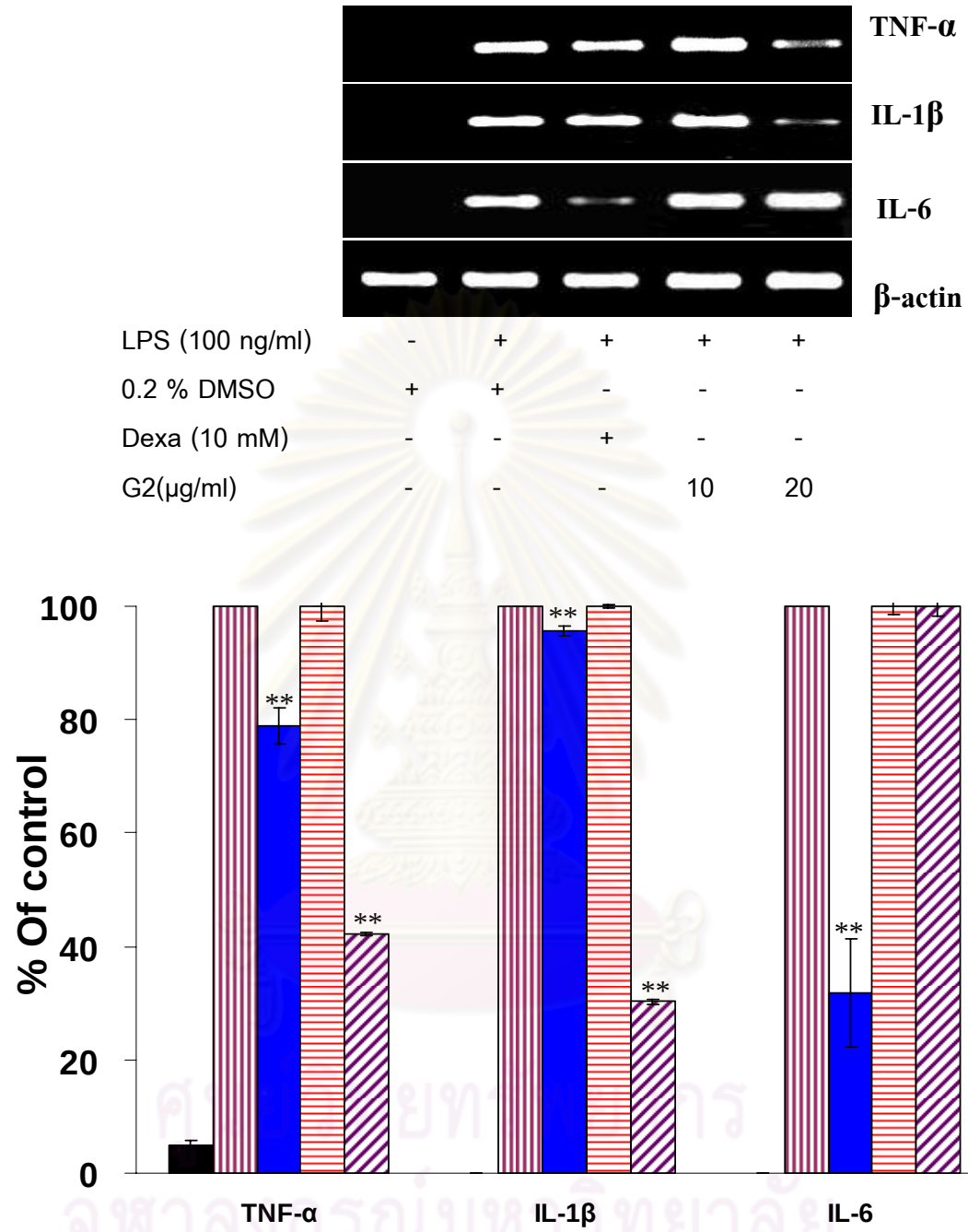
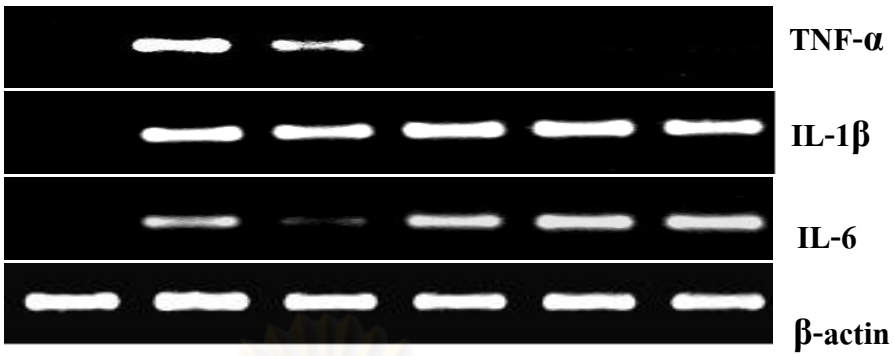


Figure 15 : Effect of G2 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO + LPS and test compounds($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexamethasone 10 μM+LPS, ▤ G2 10 μg/ml +LPS, ▨ G2 20 μg/ml +LPS.



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25

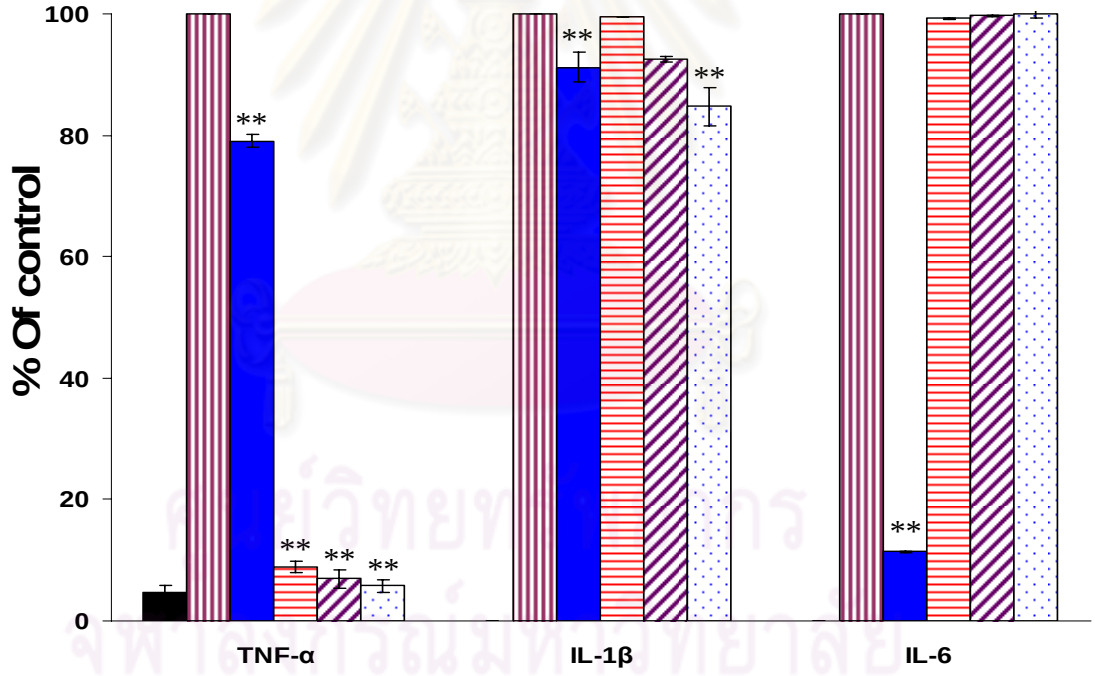
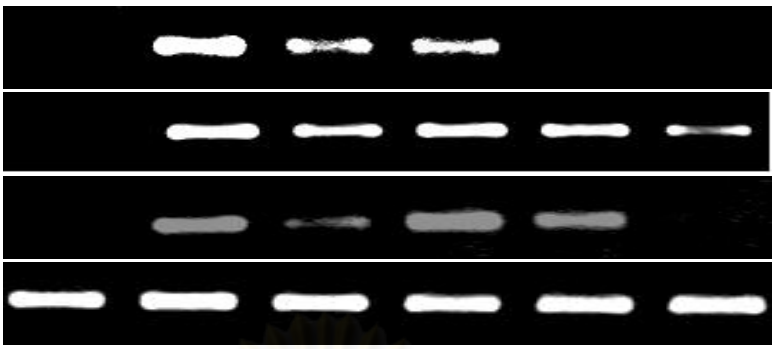


Figure 16 : Effect of G5 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexamethasone 10 μM+LPS , ▤ G5 6.25 μg/ml +LPS, ▨ G5 12.5 μg/ml +LPS, ⬚ G5 25 μg/ml +LPS.



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G6 (μg/ml)	-	-	-	6.25	12.5	25

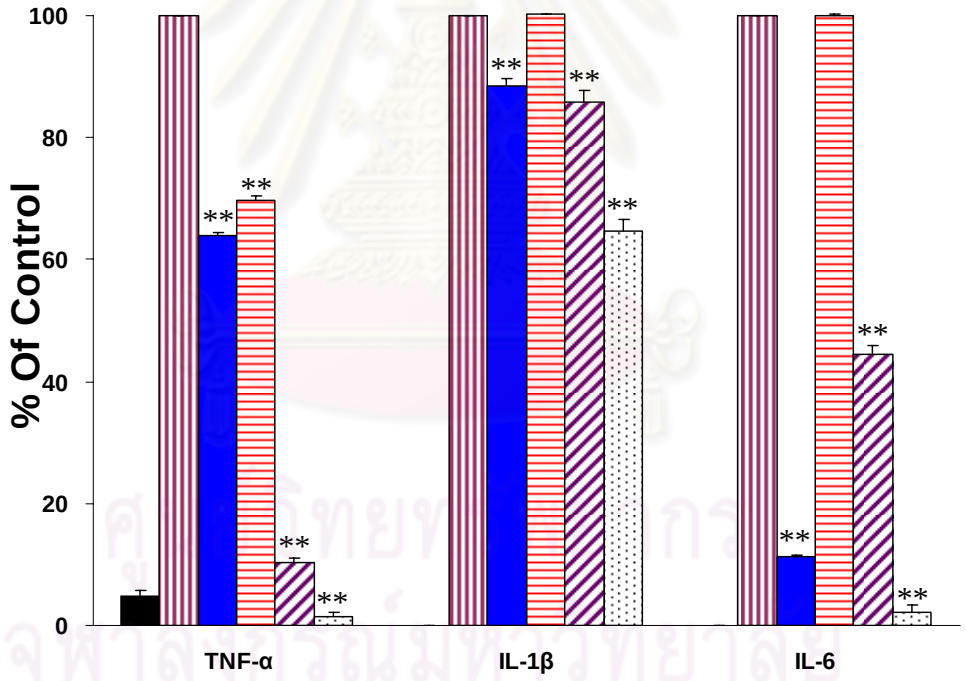
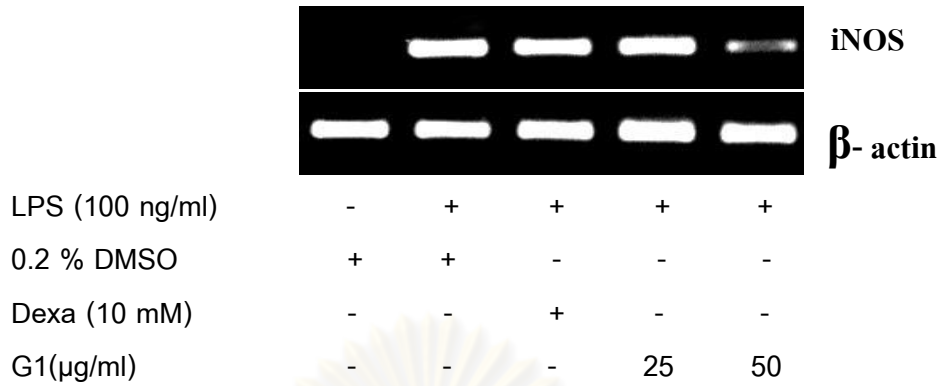


Figure 17 : Effect of G6 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO +LPS, ■ Dexamethasone 10 μM+LPS, ▤ G6 6.25 μg/ml +LPS, ▨ G6 12.5 μg/ml +LPS, ⁘ G6 25 μg/ml +LPS.

3. Effect of *Glycosmis prava* extracts on the expressions of iNOS in LPS stimulated-macrophages.

The inhibitory effects of G1, G2, G5 and G6 of *G. parva* on the activity of iNOS enzyme in J774A.1 were examined whether they correlated with their activity on NO production. All extracts were used at their IC_{50} for NO inhibition from the previous study plus one or two concentrations in 2-fold dilution. The cells were pretreated with the extracts for 24 h and then treated with 100 ng/ml LPS for 24 h. The total RNA was isolated from the treated cells and used to determine the expression of iNOS by RT-PCR. The results in Fig.20-21 demonstrated that the extracts inhibited iNOS expression in a concentration-dependent manner for G5 and G6. Fraction of G6 (25 µg/ml) seemed to produce most pronounced effect (82.52%) (Fig. 21, Appendix B-25 and B-30). Only high concentration of G2 (20 µg/ml) could significantly inhibit the mRNA expression of iNOS(Fig. 19, Appendix B-22 and B-28). These results correlated with the effects of these extracts on NO production.



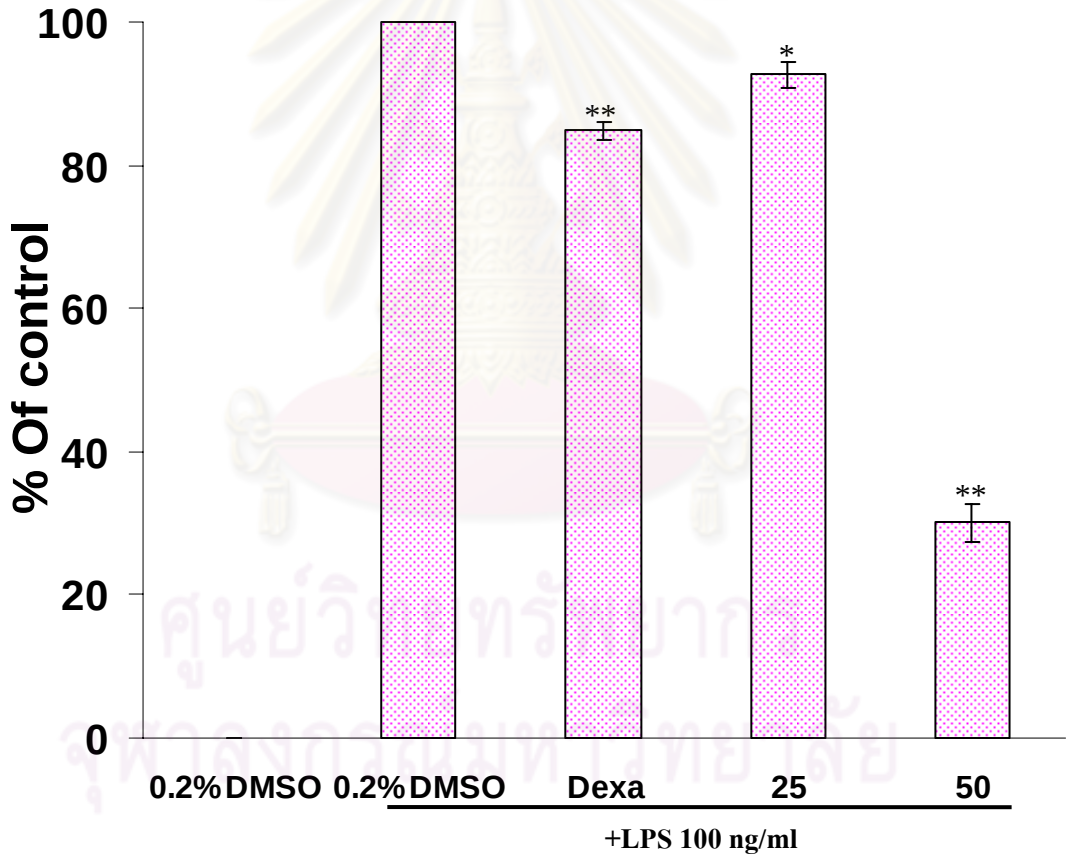
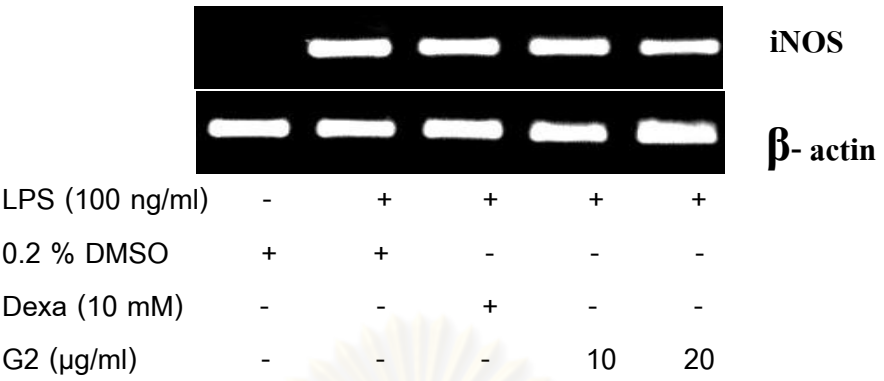


Figure 18 : Effect of G1 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



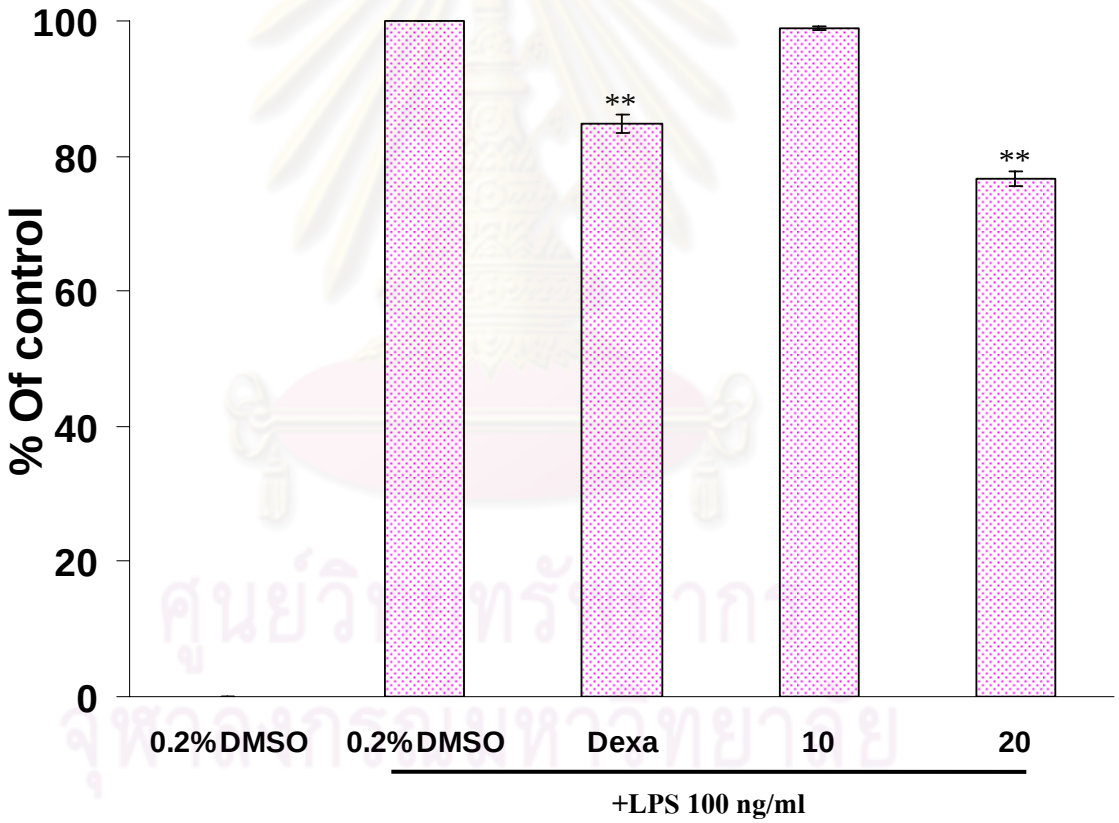
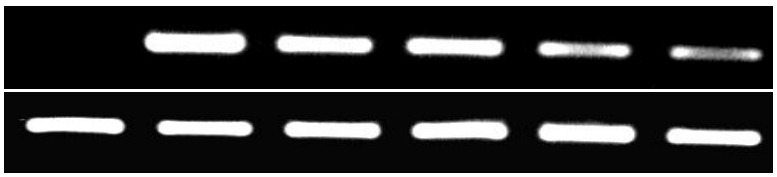


Figure 19 : Effect of G2 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25

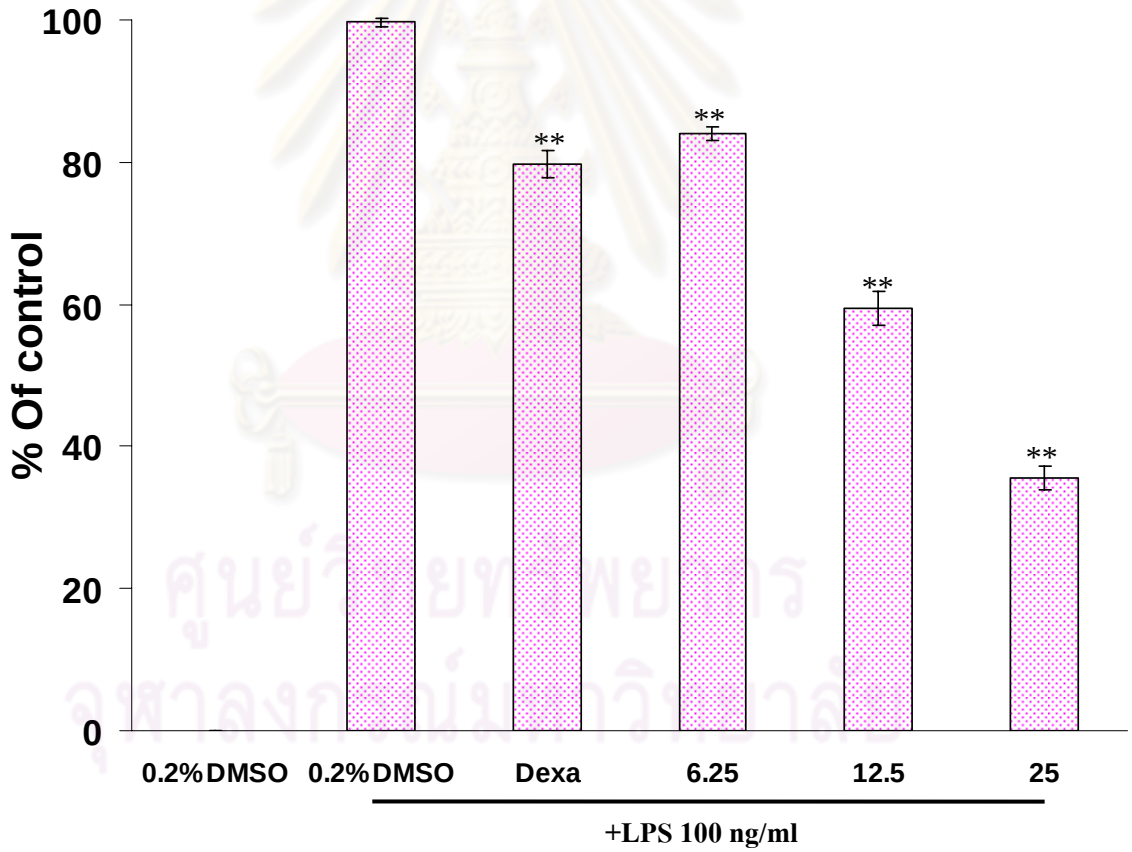


Figure 20 : Effect of G5 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).

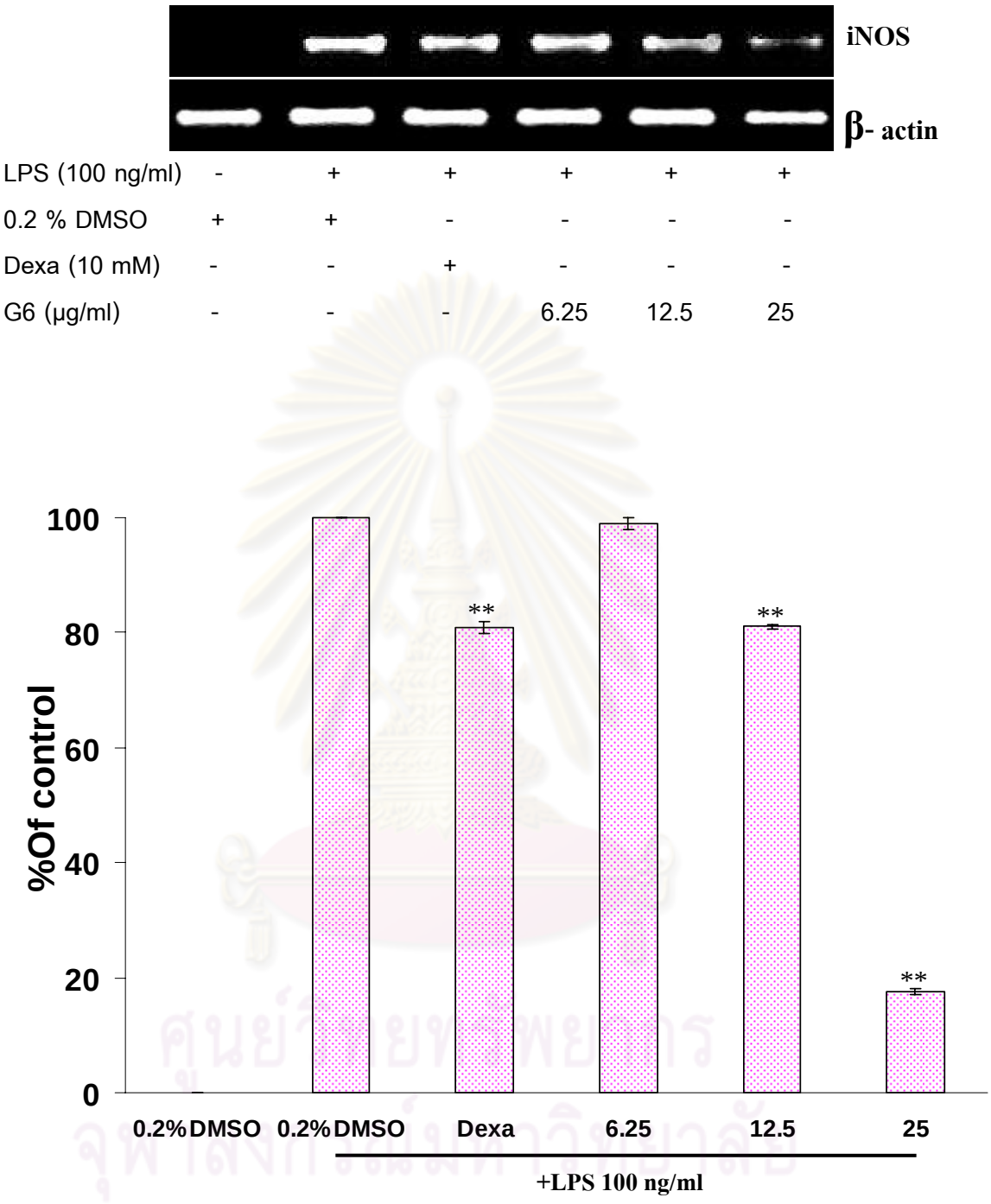


Figure 21 : Effect of G6 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).

4. Effect of *Glycosmis parva* extracts on the expressions of COX-2 in LPS stimulated-macrophages.

The inhibitory effects of the extracts on inflammatory mediator, prostaglandins, were also indirectly investigated by determining the expression of COX-2. J774A.1 cells were treated in the same way as the above study for iNOS expression. The total RNA was isolated from the treated cells and used to determine the expression of COX-2 by RT-PCR. All extracts significantly inhibited COX-2 expression except for the low concentration of G5 and G6 (6.25 µg/ml) (Fig.22-25). The prominent inhibition effect on COX-2 expression not only found in hexane and ethyl acetate extracts from the leaves of *G. parva* (G5 and G6) at concentration 25 µg/ml (82.1% and 93% respectively, Appendix B-29 and B-30) but also in hexane extract from branches (G1) at concentration 50 µg/ml (69.92%, Appendix B-27)

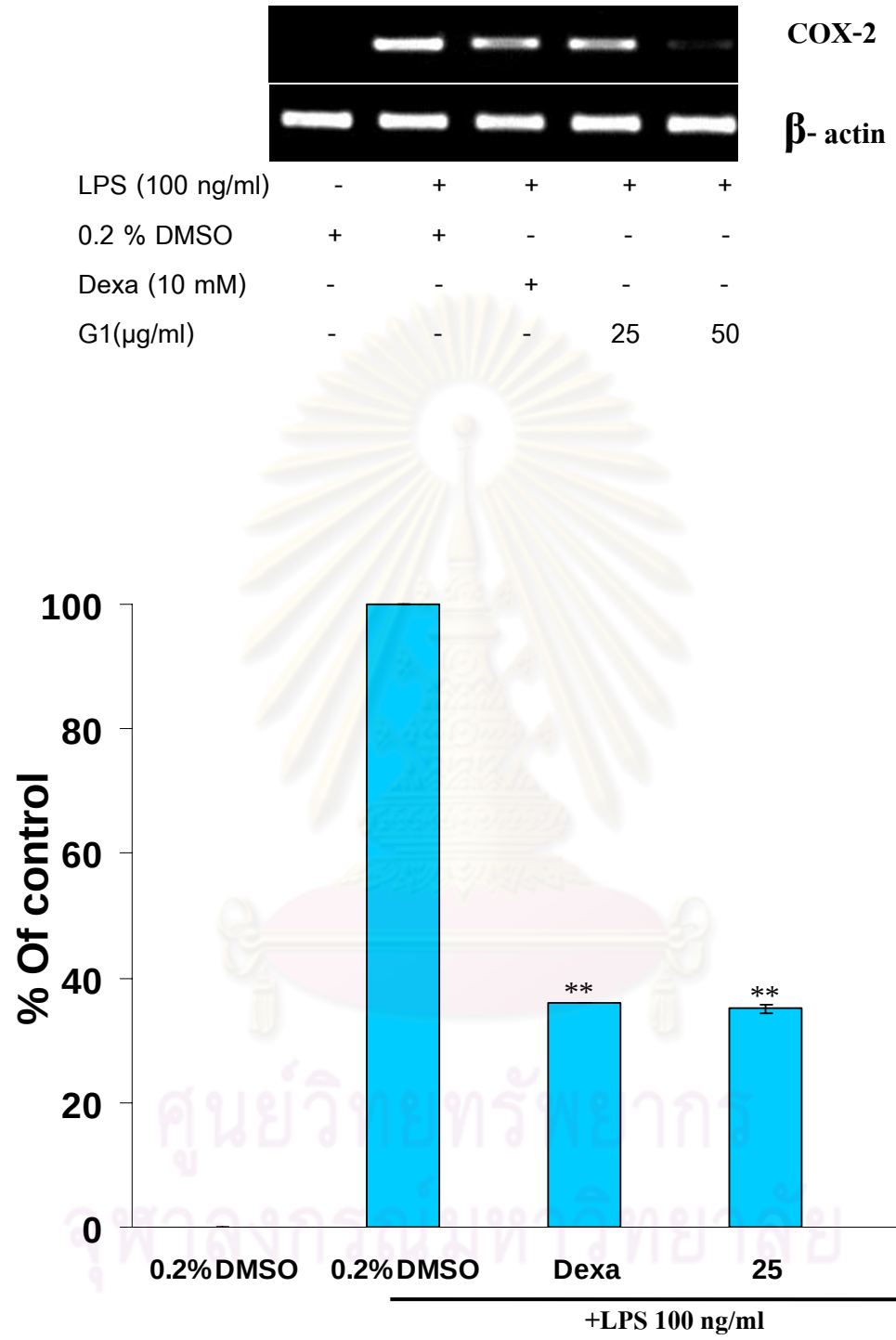


Figure 22 : Effect of G1 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).

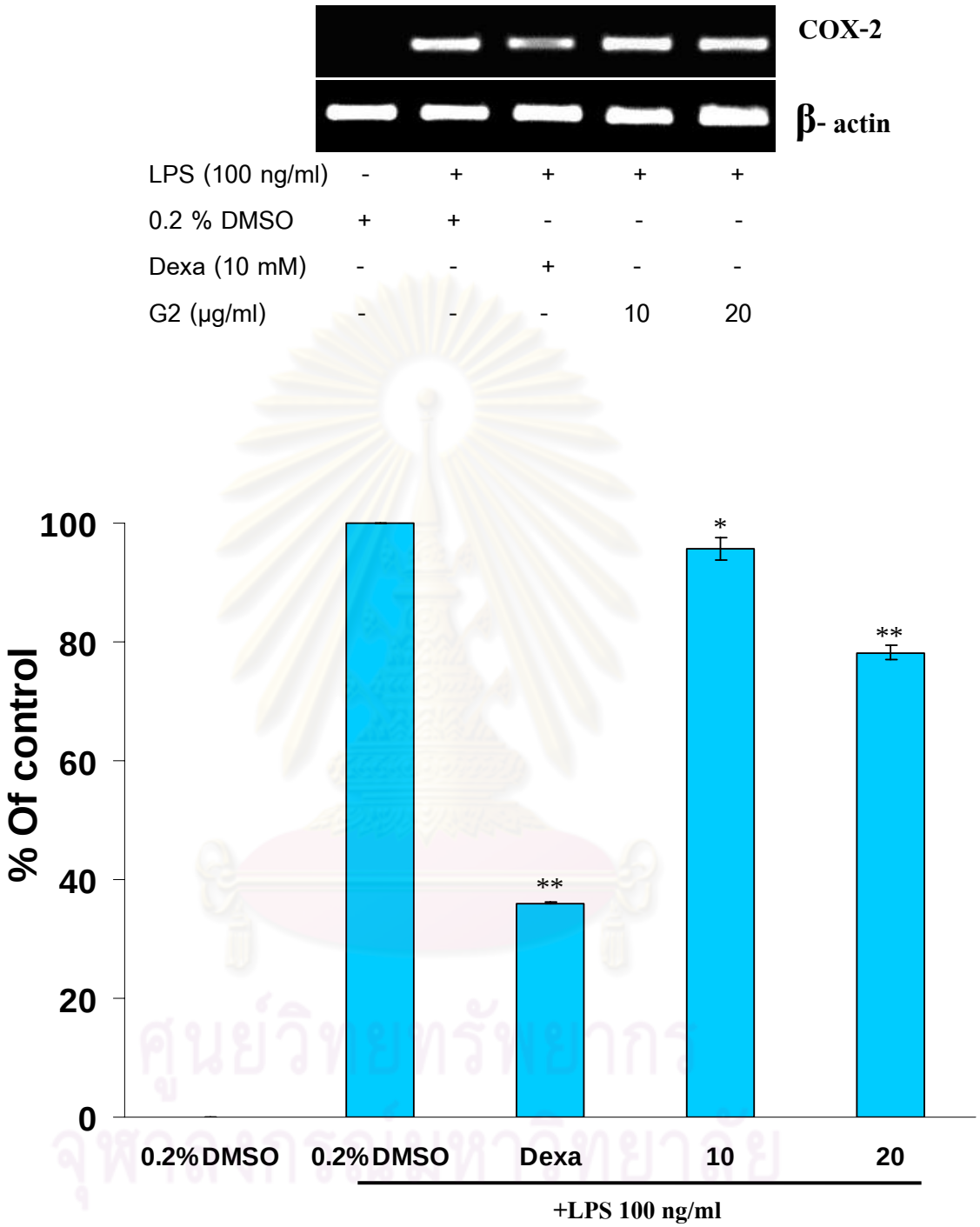
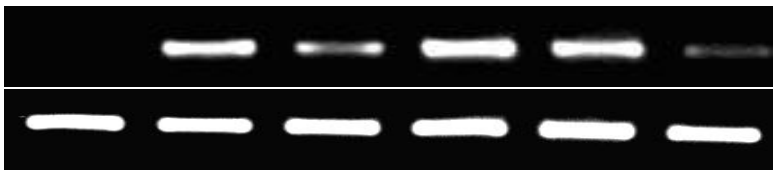


Figure 23 : Effect of G2 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells . * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (µg/ml)	-	-	-	6.25	12.5	25

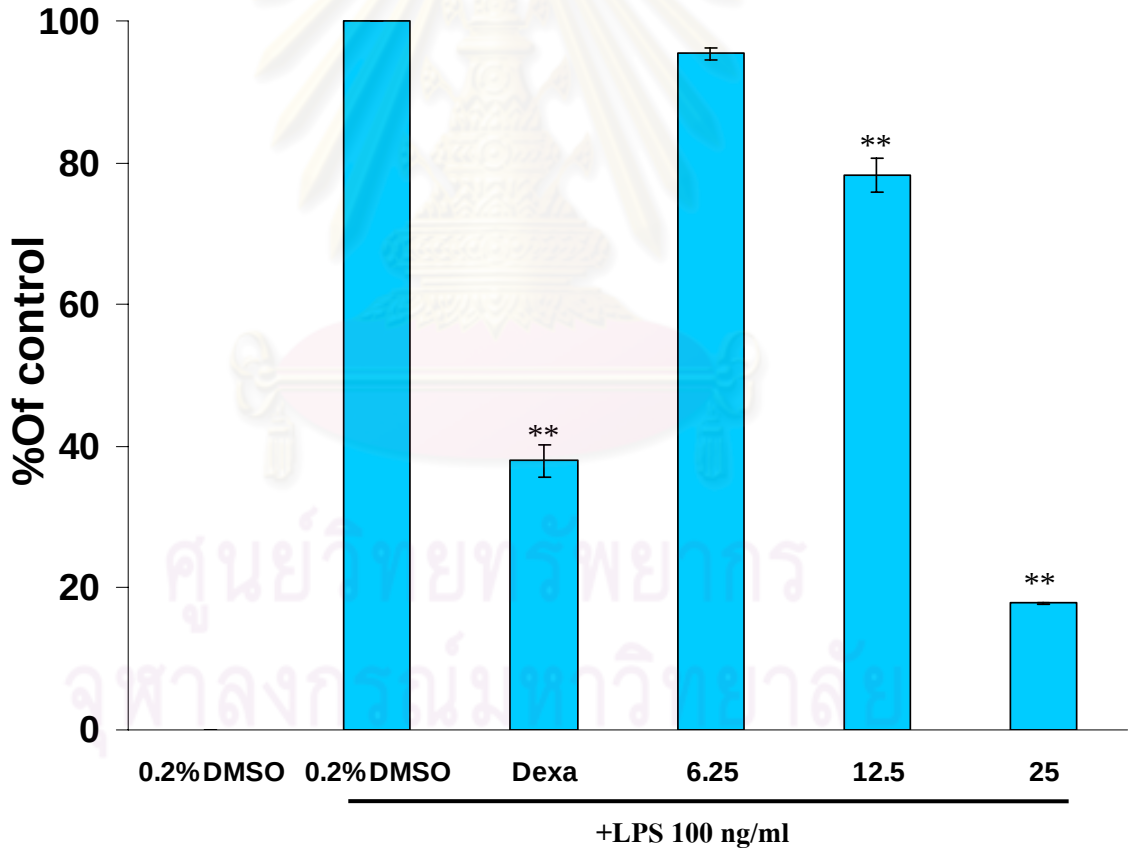


Figure 24 : Effect of G5 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells ; ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).

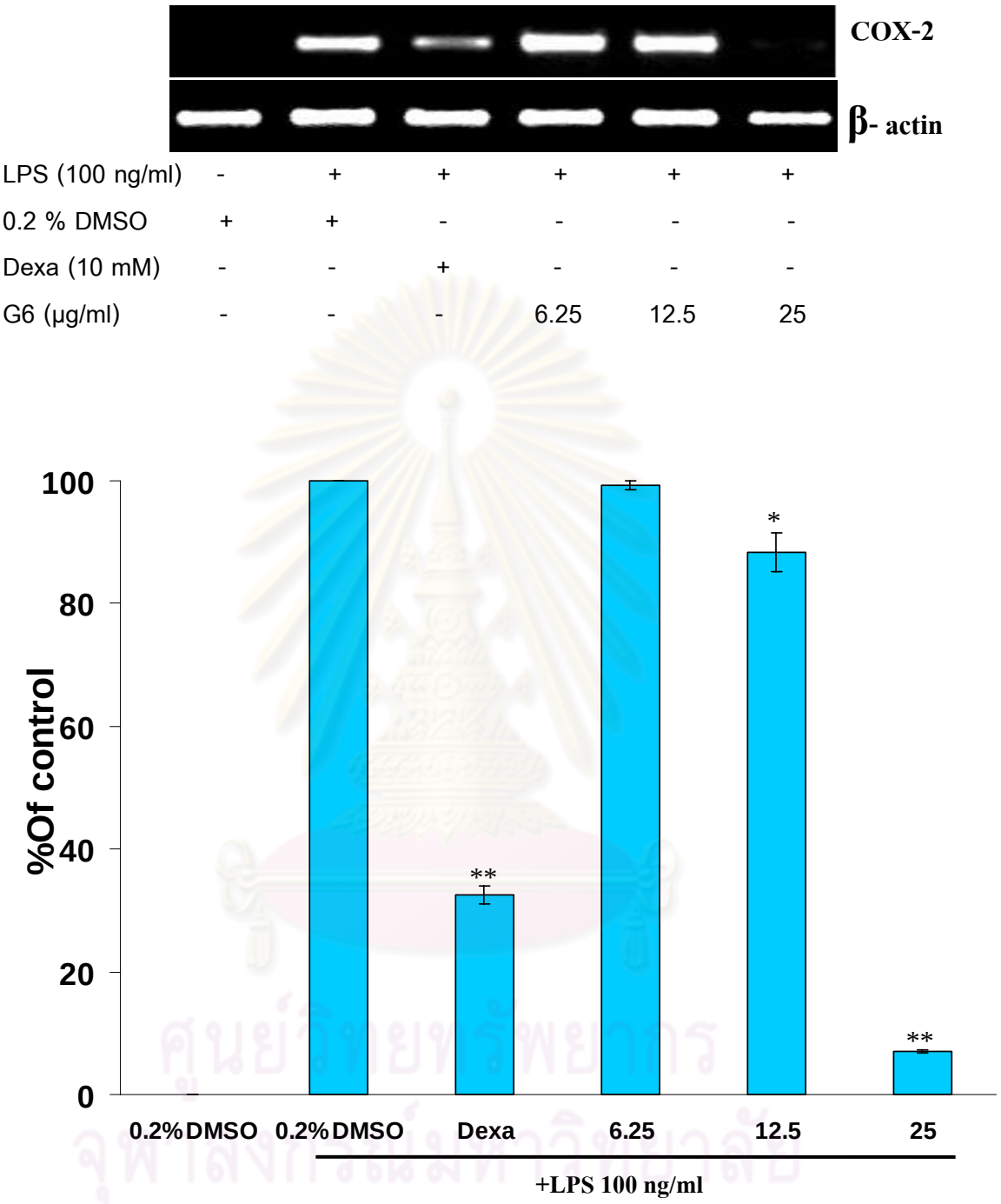


Figure 25 : Effect of G6 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$) Results are means ± S.D. (N=2).

CHAPTER V
DISCUSSION CONCLUSION AND SUGGESTION

Discussion and conclusion

Macrophage and monocyte are activated in response to component of pathogenic bacteria invading the host and a cascade intracellular response or tissue injury are initiated. Immune cells are stimulated by adhesion molecule activation signal in order to enhance the migration capacity to inflamed tissue. Lipopolysaccharide is one of the inflammatory stimuli which activated immune cells to up-regulate the inflammatory state. Nitric oxide, IL-1β, IL-6 , TNF-α and other pro-inflammatory cytokines are produced by immune cells during the inflammatory process .They possess a variety of biological activities in response to the immunopathology of acute and chronic inflammatory diseases for examples osteoarthritis, rheumatoid arthritis, Crohn's disease etc.

The hexane, ethyl acetate, butanol and water extracts (G1-G8) from leaves and branches of *G.parva*, a Thai herbal plant were investigated (G1-hexane extract from branches, G2-ethyl acetate extract from branches, G3-butanol extract from branches, G-4water extract from branches, G5-hexane extract from leaves, G6-ethyl acetate extract from leaves, G7-butanol extract from leaves, G8-water extract from leaves). Since their effects have not been investigated for the immunopathological view point in response to anti-inflammatory process. *G.parva* in difference fractions of solvent extract demonstrated a magnitude responses in cytokines secretory effect of the LPS-stimulated macrophage J774A.1 cells used in this study. Among the fractions that produced nitric oxide inhibition, (G1, G2, G5 and G6). G6 demonstrated the most potent effect (IC_{50} = 11.12 µg/ml) while cytotoxic effect occured at dose of 50 µg/ml. G5, G2 and G1 were less potent than G6 ,their IC_{50} for NO inhibition were 11.76, 16.70 and 44.96 µg/ml respectively. G3, G4, G7 and G8 exhibited minimal NO production in macrophage J 774A.1 stimulated with LPS, so they got no further investigation in this study. NO generation is stimulated during nitric oxide synthase (NOS) catalyse the

conversion of L-arginine to citrulline. The excess production of NO especially in macrophage can bring about inflammation , cytotoxicity, carcinogenicity and autoimmune diseases.(Liu RH, 1995,Nguyen *et al* 1992) Thus suppression of NO production is important for anti-inflammatory action. The extraction of *G parva,* G1, G2, G5 and G6 inhibited NO generation in dose dependence manner (Figure 10a, 11a, 12a, 13a) which are related to their capability on iNOS mRNA expression. (Figure 18, 19, 20, 21)

In this present study, fraction from branches and leaves of *G.parva* extracted by different polarity of solvents vary from hexane, ethylacetate (G1, G2, G5, G6) demonstrated inhibitory effect on the expression of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in difference magnitude. Their inhibitory activity didn't correlate with their activity on NO production, for example., the hexane extract of *G.parva* branches (G1) at near IC_{50} concentration of NO production (50 µg/ml) exhibited 54.3% inhibition of TNF-α expression by the LPS-treated macrophage while the hexane extract from the leaves of *G.parva* (G5) at the near its IC_{50} for NO production(12.5 µg/ml) demonstrated much more potent inhibition of the expression of TNF-α (95% inhibition). The inhibition effect on TNF-α, IL-1β and IL-6 might be associated with the reduction in pain and inflammation. In addition, all *G.parva* extract used in this study also significantly inhibited the expression of COX-2 and iNOS mRNA in LPS-treated macrophage for 24 h except for G5 and G6 at 6-25 µg/ml. The maximum inhibition effect of COX-2 expression was found in G6 at high concentration (25µg/ml) (93%, Appendix B-30). Both NO and prostaglandin (PGs) are known to be important mediators involve in acute and chronic inflammation. They are produced by nitric oxide synthase (NOS) and cyclooxygenase enzyme (COX) activation of their corresponding precursor , L-arginine and arachidonic acid (AA) respectively. Increase generation of NO is known to activate COX exzyme which in turn converts arachidonic acid to prostaglandins leading to pain and inflammation. In consideration of each *G. parva* extract ,it was found that G6 which was the leaves extracts with ethyl acetate at the concentration of 25 µg/ml predominantly inhibited the expression of TNF-α for about 98.6%(Appendix B-30) and its inhibitory

effect was greater than dexamethasone 10μM which caused only about 21% inhibition. The same results also obtained when the hexane extract from the leaves (G5) was tested for it's inhibitory effect on TNF-α generation in the LPS-stimulated macrophages (Appendix B-29). Dexamethasone, a steroidal anti-inflammatory drugs, showed 88.6% inhibition of IL-1β mRNA expression. Only 69.8% inhibition of IL-1β was demonstrated in G2, the branch extract with ethyl acetate and it was found at the concentration of 20 μg/ml. Surprisingly, the ethyl acetate extract from branches (G2) and hexane extract from leaves (G5) failed to demonstrate the inhibition effect on IL-6 expression (Fig. 15 and Fig.16) As mentioned before, acridone alkaloids and mixture of β –sitosterol/stigmasterol are among the major compounds found in hexane extract of *G. parva*. The results obtained from this study were in accordance with the previous study which demonstrated that β –sitosterol posses inhibitory activity of COX-2, IL-6 and TNF-α in macrophages treated with LPS (Ding *et al*., 2009). The down regulation of the expression of TNF-α, IL-1β and IL-6 mRNA expression may be useful for improvement of inflammatory disorder. However, other constituents of the *G. parva* extract e.g. acridone alkaloids (N-methylataphilline and 5-hydroxy-N methylseverifoline) might participate in immune regulation of these inflammatory cells. Difference constituents in each *G. prava* extract might possess difference role in modulation of inflammatory cytokines expression. Although the molecular mechanism associated with the inhibition of LPS-induced macrophage J774A.1 expression of TNF-α, IL-1β, IL-6, COX-2 and iNOS by the fractions of *G. parva* extracts in this study were not investigated, it is well established that several common pathways are known to linked the transcripton factor, nuclear factor (NF)-kB ,because it controls the expression of pro-inflammatory genes such as adhesion molecule and cytotoxic molecular generating enzyme including iNOS and COX-2 (Harmut *et al* 2004., Hong *et al* 2002.) The association of NF-kB and inhibitory effect of *G.parva* extract are needed to be elucidated. At present, investigators are focussing on the development of potent inhibitor of NF-kB for a novel anti-inflammatory drugs.

In summary , production of NO and the expression of iNOS and COX-2 mRNA as well as TNF-α , IL-1β and IL-6 by LPS-stimulated macrophage J774A.1 were measured in response to *G.parva* extracts (G1, G2, G5, G6). All different fractions of the extract were found to significantly inhibit COX-2 and iNOS gene expression in different magnitude and subsequent decrease production of prostaglandins and NO. Furthermore TNF-α , IL-1β and IL-6 were decrease in their mRNA expression due to the inhibitory effect of *G.prava* extract. The implication of these compounds for their anti-inflammatory effects were suggested.

Suggestion

Four fractions of *G.parva* extracts (G1, G2, G5 and G6) are screened on their activity toward pro-inflammatory cytokines generations on the LPS-stimulated macrophage J774A.1. Further studies needed to clarify the mechanism of action of their inhibiting effect on these pro-inflammatory cytokines production and enzyme generation. The in vivo anti-inflammatory potential of these extracts should be confirmed.

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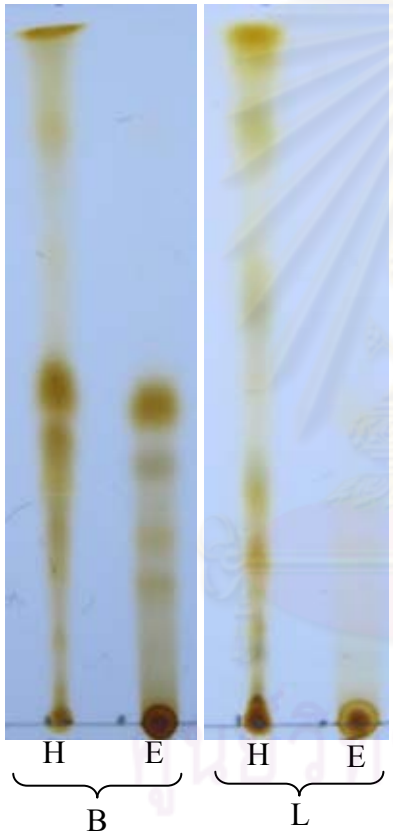
Appendices

A : TLC fingerprint from *G.parva* extracts

B : Data of study

Appendix A

Solvent system 2% MeOH in $CHCl_3$/ SiO_2 GF_{254}



H = Hexane extracts

E = Ethyl acetate

L = Leaves

B =Branches

Detector 10% H_2SO_4 in EtOH as spraying reagent

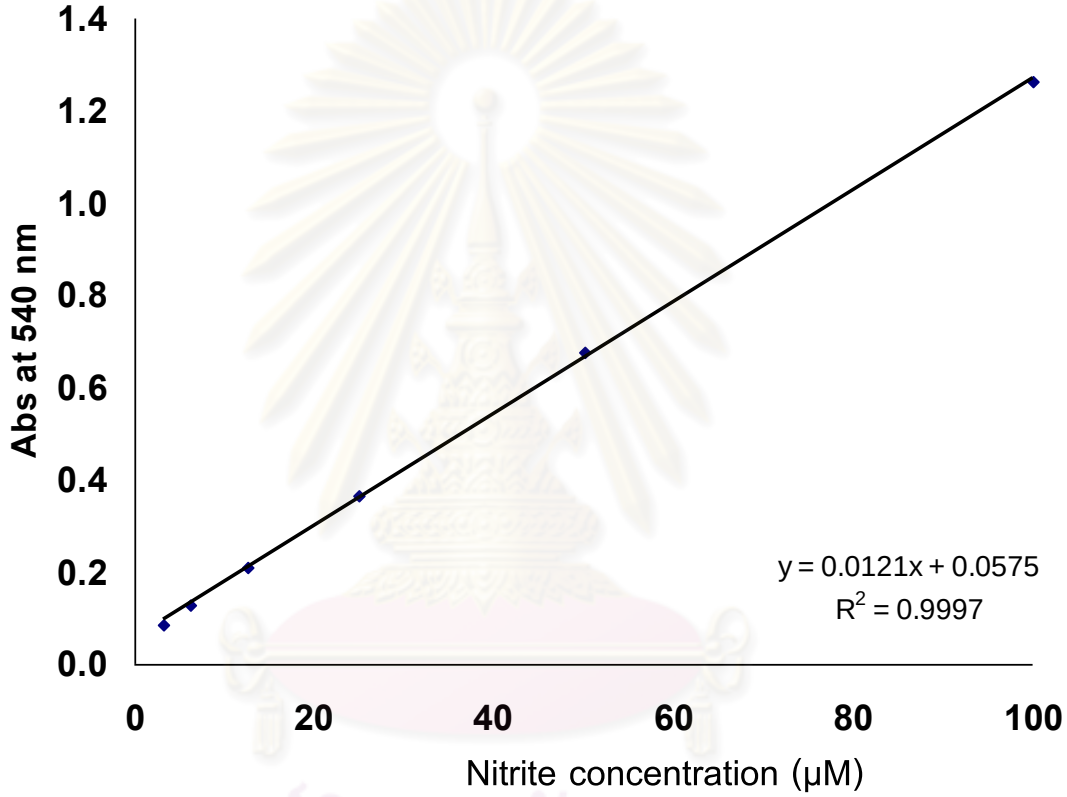
Appendix A-1:TLC fringerprint of *G. parva*

Appendix B

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

Concentration (μM)	Absorbance at 540 nm.		Mean
	1	2	
3.125	0.087	0.088	0.088
6.25	0.128	0.131	0.130
12.5	0.208	0.212	0.210
25	0.361	0.371	0.366
50	0.665	0.690	0.678
100	1.258	1.269	1.264

67



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

Appendix B-3: The effect of G1 on nitric oxide production in LPS stimulated-macrophage J774A.1 (as nitrite concentration)

Test Compounds	Concentration of nitrite (μM)								Mean ± S.D.
	1	2	3	4	5	6	7	8	
0.2% DMSO	29.068	29.449	22.678	24.373	23.229	23.554	24.959	22.756	25.008 ± 2.736
Dexa (10μM)	23.347	21.568	13.441	12.339	12.890	22.934	23.554	13.681	17.969 ± 5.266
6.25	26.186	30.127	21.449	22.424	21.831	23.678	24.752	20.866	23.914 ± 3.081
12.5	23.178	26.992	20.686	21.110	21.195	22.231	23.802	19.941	22.392 ± 2.261
25	19.280	21.356	15.602	18.695	18.356	18.099	20.372	18.597	18.795 ±1.699
50	9.025	12.458	6.703	11.280	11.068	10.992	11.488	10.613	10.453 ±1.796
100	0.000	0.424	0.347	1.831	3.314	0.826	0.744	0.000	0.936 ± 1.125

Appendix B-4: The effect of G2 on nitric oxide production in LPS stimulated-macrophage J774A.1 (as nitrite concentration)

Test Compounds	Concentration of nitrite (μM)								Mean ± S.D.
	1	2	3	4	5	6	7	8	
0.2% DMSO	29.068	29.449	22.678	24.373	23.229	23.554	24.959	22.756	25.008 ± 2.736
Dexa (10μM)	23.347	21.568	13.441	12.339	12.890	22.934	23.554	13.681	17.969 ± 5.266
3.15	28.941	29.237	21.661	21.280	22.720	21.777	23.678	16.034	23.166 ± 4.298
6.25	24.958	25.339	18.229	18.695	19.712	18.843	21.322	11.328	19.803 ± 4.408
12.5	21.017	20.127	12.763	15.347	16.703	15.413	16.157	8.555	15.760 ± 3.945
25	5.424	8.941	1.661	4.669	10.263	5.372	5.868	6.832	6.129 ± 2.638
50	0.720	0.805	0.686	0.644	1.534	0.207	0.537	0.000	0.642 ± 0.454

Appendix B-5: The effect of G5 on nitric oxide production in LPS stimulated-macrophage J774A.1 (as nitrite concentration)

Test Compounds	Concentration of nitrite (μM)			Mean ± S.D.
	1	2	3	
0.2% DMSO	13.588	17.642	18.208	16.479 ± 2.520
Dexa (10μM)	5.806	4.560	5.818	5.395 ± 2.520
1.56	11.056	13.868	15.535	13.486 ± 2.264
3.13	9.900	13.270	13.553	12.241 ± 2.032
6.25	9.400	12.107	12.925	11.477 ± 1.845
12.5	5.994	10.220	12.327	9.514 ± 3.225
25	0.000	0.692	0.409	0.367 ± 0.348
50	0.000	0.031	0.252	0.094 ± 0.137

Appendix B-6: The effect of G6 on nitric oxide production in LPS stimulated-macrophage J774A.1 (as nitrite concentration)

Test Compounds	Concentration of nitrite (μM)			Mean ± S.D.
	1	2	3	
0.2% DMSO	13.588	17.642	18.208	16.479 ± 2.520
Dexa (10μM)	5.806	4.560	5.818	5.395 ± 2.520
1.56	11.463	13.270	16.509	13.747 ± 2.557
3.13	10.869	11.667	14.969	12.501 ± 2.174
6.25	9.056	11.226	13.365	11.216 ± 2.155
12.5	6.056	7.170	9.591	7.606 ± 1.807
25	0.087	0.031	0.629	0.249 ± 0.330
50	0.000	0.000	0.126	0.042 ± 0.073

Appendix B-7: Cytotoxicity of G1 (µg/ml) in LPS stimulated-macrophage J774A.1

Test Compounds	% cytotoxicity								Mean ± S.D.
	1	2	3	4	5	6	7	8	
0.2% DMSO	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000 ± 0.000
Dexa (10µM)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000 ± 0.000
6.25	1.979	1.809	0.000	0.000	0.000	1.140	0.000	0.000	0.616 ± 0.883
12.5	0.427	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053 ± 0.151
25	1.732	1.146	0.000	0.000	0.000	2.371	1.130	0.000	0.797 ± 0.935
50	0.000	0.000	0.000	0.000	0.000	0.684	0.000	0.000	0.086 ± 0.242
100	29.487	28.427	34.584	23.196	24.914	16.256	16.275	18.000	23.892 ± 6.748

Appendix B-8: Cytotoxicity of G2 (µg/ml) in LPS stimulated-macrophage J774A.1

Test Compounds	% cytotoxicity								Mean ± S.D.
	1	2	3	4	5	6	7	8	
0.2% DMSO	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000 ± 0.000
Dexa (10µM)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000 ± 0.000
3.15	3.711	4.303	4.394	3.751	0.885	5.267	3.711	0.000	3.253 ± 1.825
6.25	1.462	1.989	2.043	2.184	0.000	5.381	3.504	0.000	2.070 ± 1.772
12.5	0.922	1.000	2.827	1.804	0.000	5.381	3.757	0.000	1.961 ± 1.905
25	2.969	2.663	2.518	1.235	3.317	2.554	0.738	0.000	1.999 ± 1.187
50	19.456	24.258	52.803	55.556	41.671	22.595	33.933	11.271	32.693 ± 16.109

Appendix B-9: Cytotoxicity of G5 (µg/ml) in LPS stimulated-macrophage J774A.1

Test Compounds	% cytotoxicity			Mean ± S.D.
	1	2	3	
0.2% DMSO	0.000	0.000	0.000	0.000 ± 0.000
Dexa (10µM)	1.805	0.000	0.000	0.602 ± 1.042
1.56	0.000	0.000	0.000	0.000 ± 0.000
3.13	0.000	0.000	0.000	0.000 ± 0.000
6.25	0.952	0.000	0.000	0.317 ± 0.550
12.5	3.459	0.000	0.000	1.153 ± 1.997
25	2.281	0.000	0.000	0.760 ± 1.317
50	100.000	100.000	100.000	100.000 ± 0.000

Appendix B-10: Cytotoxicity of G6 (µg/ml) in LPS stimulated-macrophage J774A.1

Test Compounds	% cytotoxicity			Mean ± S.D.
	1	2	3	
0.2% DMSO	0.000	0.000	0.000	0.000 ± 0.000
Dexa (10µM)	1.805	0.000	0.000	0.602 ± 1.042
1.56	0.000	0.000	0.000	0.000 ± 0.000
3.13	0.852	0.000	0.000	0.284 ± 0.492
6.25	2.456	0.000	0.000	0.819 ± 1.418
12.5	0.000	0.000	0.000	0.000 ± 0.000
25	0.000	0.000	0.000	0.000 ± 0.000
50	95.163	100.000	100.000	98.388 ± 2.793

LPS (100 ng/ml)	-	+	+	+	+	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	+	-	-
G1(μg/ml)	-	-	-	25	50	-	-	-	25	50

Appendix B-11 : Effect of branches extracted with hexane (G1) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

Appendix B-12: Effect of branches extracted with hexane (G1) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

Test Compounds	% Of control TNF-α		Mean ± S.D.	% Of control IL-1β		Mean ± S.D.	% Of control IL-6		Mean ± S.D.
	1	2		1	2		1	2	
0.2% DMSO	4.095	5.469	4.782 ± 0.971	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± .000
0.2% DMSO+LPS	100.026	100.032	100.029 ± 0.004	99.982	99.991	99.986 ± 0.006	99.923	99.997	99.960 ± 0.052
Dexa (10μM)+LPS	81.218	76.763	78.991 ± 3.150	94.991	96.276	95.633 ± 0.909	25.158	38.557	31.857 ± 9.475
25(μg/ml)+LPS	77.900	74.647	76.273 ± 2.300	96.823	95.400	96.111 ± 1.007	89.889	91.213	90.551 ± 0.936
50(μg/ml)+LPS	46.285	45.107	45.696 ± 0.833	84.182	84.077	84.129 ± 0.074	45.495	42.371	43.933 ± 2.209

LPS (100 ng/ml)	-	+	+	+	+	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	+	-	-
G2(μg/ml)	-	-	-	10	20	-	-	-	10	20

Appendix B-13: Effect of branches extracted with ethyl acetate (G2) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

Appendix B-14 : Effect of branches extracted with ethyl acetate (G2) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

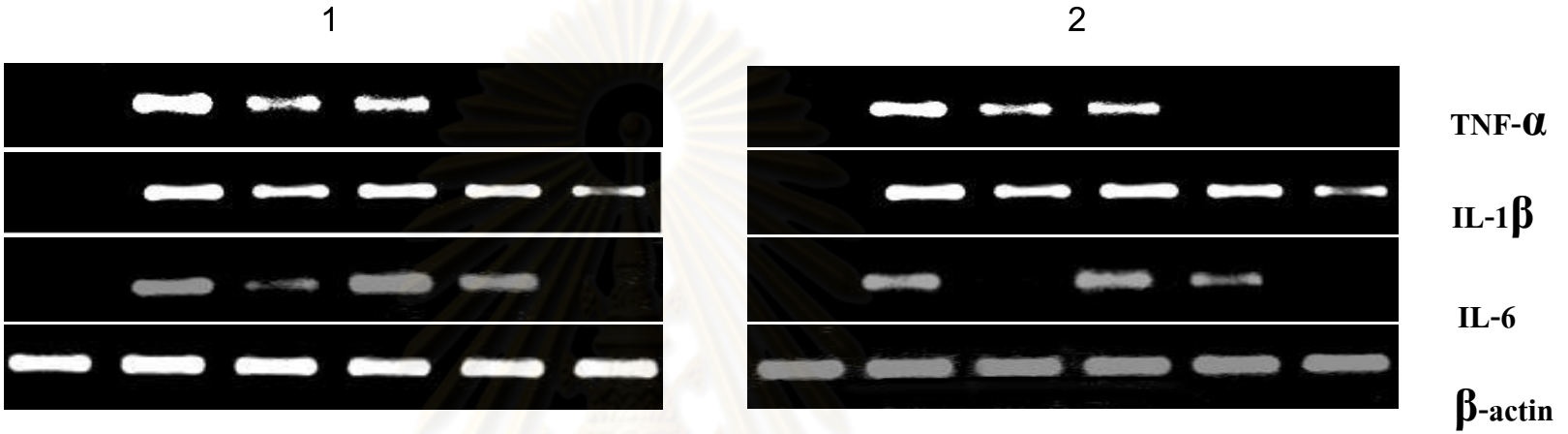
Test Compounds	% Of control TNF-α		Mean ± S.D.	% Of control IL-1β		Mean ± S.D.	% Of control IL-6		Mean ± S.D.
	1	2		1	2		1	2	
0.2% DMSO	4.095	5.469	4.782 ± 0.971	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	100.026	100.032	100.029 ± 0.004	99.982	99.991	99.986 ± 0.006	99.923	99.997	99.960 ± 0.052
Dexa (10μM)+LPS	81.218	76.763	78.991 ± 3.150	94.991	96.276	95.633 ± 0.909	25.158	38.557	31.857 ± 9.475
10(μg/ml)+LPS	98.135	101.643	99.889 ± 2.481	101.528	101.242	101.385 ± 0.202	100.643	102.672	101.657 ± 1.435
20(μg/ml)+LPS	42.014	42.343	42.179 ± 0.232	29.990	30.433	30.212 ± 0.313	100.740	103.028	101.884 ± 1.618

LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25	-	-	-	6.25	12.5	25

Appendix B-15: Effect of leaves extracted with hexane (G5) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

Appendix B-16 : Effect of leaves extracted with hexane (G5) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells.

Test Compounds	% Of control TNF-α		Mean ± S.D.	% Of control IL-1β		Mean ± S.D.	% Of control IL-6		Mean ± S.D.
	1	2		1	2		1	2	
0.2% DMSO	4.095	5.469	4.782 ± 0.971	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	100.020	99.998	100.009 ± 0.015	100.009	100.012	100.010 ± 0.002	100.009	100.024	100.016 ± 0.010
Dexa (10μM)+LPS	79.826	78.323	79.074 ± 1.063	89.418	92.968	91.193 ± 2.511	11.340	11.467	11.403 ± 0.090
6.25(μg/ml)+LPS	9.572	8.214	8.893 ± 0.960	99.564	99.559	99.561 ± 0.003	99.207	99.247	99.227 ± 0.060
12.5(μg/ml)+LPS	8.047	5.906	6.977 ± 1.514	92.254	92.933	92.593 ± 0.480	99.623	99.707	99.665 ± 0.028
25(μg/ml)+LPS	6.464	4.990	5.727 ± 1.042	86.987	82.507	84.747 ± 3.168	101.090	101.166	101.628 ± 0.690



LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	-	+	-	-	-
G6 (μg/ml)	-	-	-	6.25	12.5	25	-	-	-	6.25	12.5	25

Appendix B-17: Effect of leaves extracted with ethyl acetate (G6) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells

Appendix B-18: Effect of leaves extracted with ethyl acetate (G6) from *Glycosmis parva* on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Of control TNF-α		Mean ± S.D.	% Of control IL-1β		Mean ± S.D.	% Of control IL-6		Mean ± S.D.
	1	2		1	2		1	2	
0.2% DMSO	4.095	5.469	4.782 ± 0.971	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	100.020	99.998	100.009 ± 0.015	100.009	100.012	100.010 ± 0.002	100.009	100.024	100.016 ± 0.010
Dexa (10μM)+LPS	79.826	78.323	79.074 ± 1.063	89.418	92.968	91.193 ± 2.511	11.340	11.467	11.403 ± 0.090
6.25(μg/ml)+LPS	70.319	69.266	69.793 ± 0.745	100.214	100.287	100.251 ± 0.052	105.650	106.089	105.870 ± 0.310
12.5(μg/ml)+LPS	9.152	11.331	10.241 ± 1.541	86.424	85.446	85.935 ± 0.692	41.548	47.491	44.520 ± 4.202
25(μg/ml)+LPS	0.888	1.928	1.408 ± 0.736	63.397	66.048	64.723 ± 1.874	1.103	3.025	2.064 ± 1.359

LPS (100 ng/ml)	-	+	+	+	+	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	+	-	-
G1(μg/ml)	-	-	-	25	50	-	-	-	25	50

Appendix B-19: Effect of branches extracted with hexane (G1) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells.

Appendix B-20 : Effect of branches extracted with hexane (G1) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

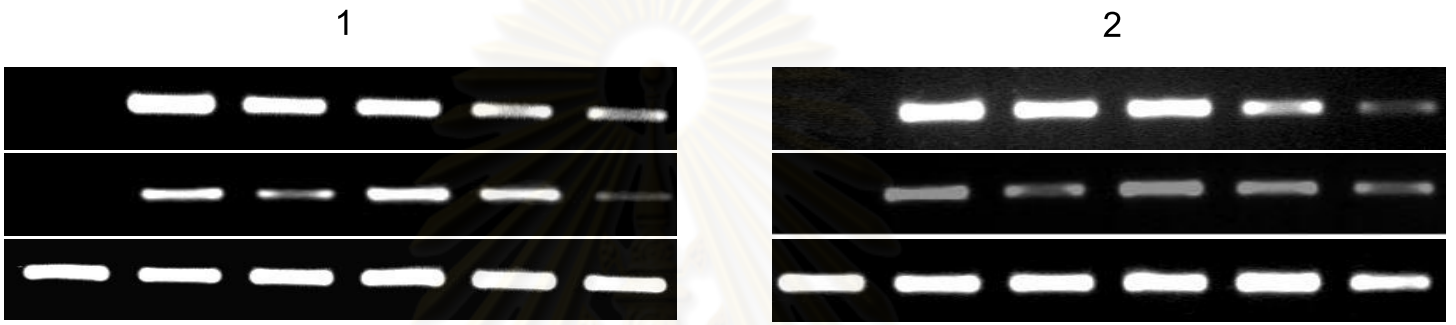
Test Compounds	% Of control iNOS		Mean ± S.D.	% Of control COX-2		Mean ± S.D.
	1	2		1	2	
0.2% DMSO	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	100.047	100.016	100.031 ± 0.022	99.985	100.000	99.992 ± 0.010
Dexa (10μM)+LPS	85.753	83.818	84.785 ± 1.368	35.935	36.109	36.022 ± 0.123
25(μg/ml)+LPS	93.894	91.346	92.620 ± 1.801	34.597	35.524	35.061 ± 0.655
50(μg/ml)+LPS	31.921	28.237	30.079 ± 2.604	16.273	15.643	15.958 ± 0.445

LPS (100 ng/ml)	-	+	+	+	+	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	+	-	-
G2(μg/ml)	-	-	-	10	20	-	-	-	10	20

Appendix B-21: Effect of branches extracted with ethyl acetate (G2) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Appendix B-22 : Effect of branches extracted with ethyl acetate (G2) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells.

Test Compounds	% Of control iNOS		Mean ± S.D.	% Of control COX-2		Mean ± S.D.
	1	2		1	2	
0.2% DMSO	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	100.047	100.016	100.031 ± 0.022	99.985	100.000	99.992 ± 0.010
Dexa (10μM)+LPS	85.753	83.818	84.785 ± 1.368	35.935	36.109	36.022 ± 0.123
10(μg/ml)+LPS	98.686	99.087	98.887 ± 0.284	94.274	97.040	95.657 ± 1.956
20(μg/ml)+LPS	75.914	77.427	76.670 ± 1.070	77.278	79.134	78.206 ± 1.312



LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25	-	-	-	6.25	12.5	25

Appendix B-23: Effect of leaves extracted with hexane (G5) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Appendix B-24 : Effect of leaves extracted with hexane (G5) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Of control iNOS		Mean ± S.D.	% Of control COX-2		Mean ± S.D.
	1	2		1	2	
0.2% DMSO	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	99.275	100.012	99.643 ± 0.521	99.953	100.047	100.000 ± 0.066
Dexa (10μM)+LPS	78.449	81.105	79.777 ± 1.878	36.260	39.613	37.936 ± 2.371
6.25(μg/ml)+LPS	83.330	84.740	84.035 ± 0.997	95.986	94.706	95.346 ± 0.905
12.5(μg/ml)+LPS	57.854	61.211	59.533 ± 2.374	79.963	76.563	78.263 ± 2.404
25(μg/ml)+LPS	34.375	36.737	35.556 ± 1.670	17.808	18.012	17.910 ± 0.145

LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-	-	-	+	-	-	-
G6 (µg/ml)	-	-	-	6.25	12.5	25	-	-	-	6.25	12.5	25

Appendix B-25 : Effect of leaves extracted with ethyl acetate (G6) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Appendix B-26 : Effect of leaves extracted with ethyl acetate (G6) from *Glycosmis parva* on mRNA expressions of iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells.

Test Compounds	% Of control iNOS		Mean ± S.D.	% Of control COX-2		Mean ± S.D.
	1	2		1	2	
0.2% DMSO	0.000	0.000	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000
0.2% DMSO+LPS	99.979	99.995	99.987 ± 0.012	99.931	100.018	99.975 ± 0.061
Dexa (10μM)+LPS	80.203	81.613	80.908 ± 0.997	33.541	31.546	32.544 ± 1.410
6.25(μg/ml)+LPS	99.704	98.229	98.966 ± 1.043	99.786	98.664	99.225 ± 0.793
12.5(μg/ml)+LPS	81.332	80.754	81.043 ± 0.409	86.069	90.608	88.338 ± 3.209
25(μg/ml)+LPS	17.094	17.859	17.476 ± 0.541	6.827	7.209	7.018 ± 0.270

Appendix B-27 : % inhibition of branches extracted with hexane (G1) from *Glycosmis parva* on mRNA expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Inhibition of mRNA expression				
	TNF-α	IL-1β	IL-6	iNOS	COX-2
0.2% DMSO	0.000	0.000	0.000	0.000	0.000
0.2% DMSO+LPS	0.000	0.014	0.040	0.000	0.000
Dexa (10μM)+LPS	21.009	4.367	68.143	15.215	15.215
1250(μg/ml)+LPS	23.727	3.889	9.449	7.380	7.380
50(μg/ml)+LPS	54.304	15.871	56.067	69.921	69.921

Appendix B-28 : % inhibition of branches extracted with ethyl acetate (G2) from *Glycosmis parva* on mRNA expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Inhibition of mRNA expression				
	TNF-α	IL-1β	IL-6	iNOS	COX-2
0.2% DMSO	0.000	0.000	0.000	0.000	0.000
0.2% DMSO+LPS	0.000	0.014	0.040	0.000	0.008
Dexa (10μM)+LPS	21.009	4.367	68.143	15.215	63.978
10(μg/ml)+LPS	0.111	-1.385	0.000	1.113	4.343
20(μg/ml)+LPS	57.821	69.788	0.000	23.330	21.794

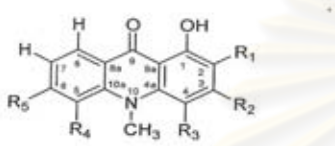
Appendix B-29 : % Inhibition of leaves extracted with hexane (G5) from *Glycosmis parva* on mRNA expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Inhibition of mRNA expression				
	TNF-α	IL-1β	IL-6	iNOS	COX-2
0.2% DMSO	0.000	0.000	0.000	0.000	0.000
0.2% DMSO+LPS	0.000	0.000	0.000	0.357	0.000
Dexa (10μM)+LPS	20.926	8.807	88.597	20.223	62.064
6.25(μg/ml)+LPS	91.107	0.439	0.773	15.965	4.654
12.5(μg/ml)+LPS	93.023	7.407	0.335	40.467	21.737
25(μg/ml)+LPS	94.273	15.253	-1.628	64.444	82.090

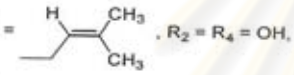
Appendix B-30 : % Inhibition of leaves extracted with ethyl acetate (G6) from *Glycosmis parva* on mRNA expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2 in LPS stimulated-macrophage J774A.1 cells

Test Compounds	% Inhibition of mRNA expression				
	TNF-α	IL-1β	IL-6	iNOS	COX-2
0.2% DMSO	0.000	0.000	0.000	0.000	0.000
0.2% DMSO+LPS	0.000	0.000	0.000	0.013	0.025
Dexa (10μM)+LPS	36.019	11.575	88.597	19.092	67.456
6.25(μg/ml)+LPS	30.207	0.000	0.000	1.034	0.775
12.5(μg/ml)+LPS	89.759	14.065	55.480	18.957	11.662
25(μg/ml)+LPS	98.592	35.277	97.936	82.524	92.982

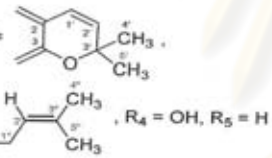
Appendix B-31 : Compounds are identified from *G. prava*



1; $R_1 = OCH_3$, $R_2 = R_4 = OH$, $R_3 = R_5 = H$



2; $R_1 = R_3 =$, $R_2 = R_4 = OH$, $R_5 = H$

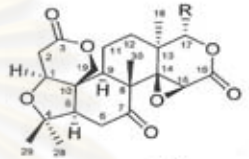


3; $R_1, R_2 =$, $R_3 =$, $R_4 = OH$, $R_5 = H$

4; $R_1 = H$, $R_2 = R_3 = R_4 = OCH_3$, $R_5 = OH$

5; $R_1 = R_4 = OCH_3$, $R_2 = R_5 = OH$, $R_3 = H$

6; $R_1 = R_2 = OCH_3$, $R_3 = R_4 = R_5 = H$



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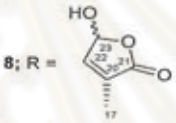

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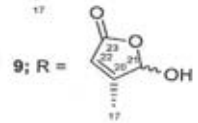

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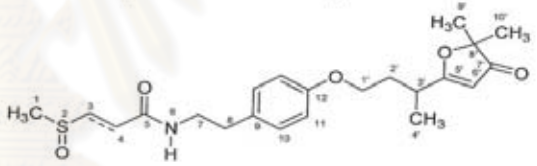
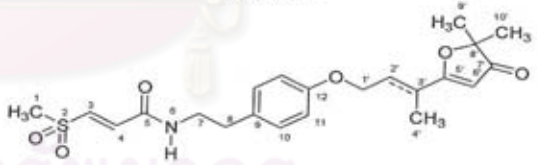


10; unsaturated (double bond on C-3 and C-4)

11; saturated



12; unsaturated (double bond on C-2' and C-3')

13; saturated

BIOGRAPHY

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