Inhibitory effects of compounds isolated from ethyl acetate extracts of *Glycosmis parva* branches and leaves on LPS-induced RAW 264.7 macrophage activation

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ฤทธิ์ยับยั้งการกระตุ้นเซลล์แมคโครฟาจ RAW 264.7 ด้วยแอลพีเอสของสารที่แยกได้จากสารสกัดเอทิลอะซิเตทจากใบและกิ่งของ *Glycosmis parva*

น.ส.ชัญญานุช ละประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ชัญญานุช ละประเสริฐ : ฤทธิ์ยับยั้งการกระตุ้นเซลล์แมคโครฟาจ RAW 264.7 ด้วยแอลพีเอสของสารที่แยกได้จากสารสกัดเอทิลอะซิเตทจากใบและกิ่งของ *Glycosmis parva* . (Inhibitory effects of compounds isolated from ethyl acetate extracts of *Glycosmis parva* branches and leaves on LPS-induced RAW 264.7 macrophage activation) อ.ที่ปรึกษาหลัก : ผศ. ดร.วัชรี ลิมปนสิทธิกุล, อ. ที่ปรึกษาร่วม : ผศ. ดร.ชัยศักดิ์ จันศรีนิยม

แมคโครฟาจเป็นเซลล์ที่สร้างสารต่างๆหลายชนิดที่เกี่ยวข้องกับการอักเสบในโรคที่มีการอักเสบแบบเรื้อรัง สารเหล่านี้เป็นเป้าหมายการออกฤทธิ์เพื่อบรรเทาอาการอักเสบของยาต้านอักเสบที่มีใช้ในปัจจุบัน ได้แก่ ยากลุ่มสเตียรอยด์ ยาต้านอักเสบที่ไม่ใช่สเตียรอย์ และยาต้านไซโตไคน์ แต่จากปัญหาจากการใช้ยาเหล่านี้ทำให้ยังมีความจำเป็นที่ต้องพัฒนายาต้านอักเสบตัวใหม่อย่างต่อเนื่อง งานวิจัยนี้มีเป้าหมายเพื่อศึกษาศักยภาพการเป็นสารต้านอักเสบของสารประกอบหลักสามตัว คือ arborinine, *N*-methylatalaphylline and *S*-deoxydihydroglyparvin (DDGP) ที่แยกได้จากสารสกัดเอทธิลอะซีเตตของกิ่งและใบจากพืช *Glycosmis parva* ต่อเซลล์แมคโครฟาจ RAW 264.7 ที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ (แอลพีเอส) ผลการศึกษามีเพียงสาร DDGP เท่านั้นที่มีความแรงสูงในการเป็นสารต้านอักเสบ พบว่า ในเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยแอลพีเอส DDGP ยับยั้งการแสดงออกของเอนไซม์ไนตริกออกไซด์ซินเตสและยับยั้งการสร้างไนตริกออกไซด์โดยมีค่า IC50 เท่ากับ 3.47 ± 0.1 μM สารนี้ยับยั้งการแสดงออกในระดับ mRNA และยับยั้งการสร้างระดับโปรตีนของไซโตไคน์ tumor necrosis factor-α, interleukin (IL)-1β, IL-6 โดยแอลพีเอสกระตุ้นการสร้างไซโตไคน์เหล่านี้ได้ในระยะแรกของการกระตุ้นเซลล์แมคโครฟาจ นอกจากนี้ DDGP ยังออกฤทธิ์ยับยั้งการแสดงออกของไซโตไคน์ monocyte chemoattractant protein-1 และ macrophage inflammatory protein-1α ยับยั้งการแสดงออกของเอนไซม์ cyclooxygenase-2 และ mitochondrial prostaglandin (PG) E synthase ที่ใช้สร้าง PGE2 และยับยั้งการสร้าง PGE2 สารนี้มีผลเพียงเล็กน้อยหรือไม่มีผลต่อการแสดงออกของโมเลกุลในกลุ่มยับยั้งการอักเสบ ผลของ DDGP ต่อโมเลกุลในการสื่อสัญญาณของแอลพีเอสเพื่อกระตุ้นเซลล์ พบว่า DDGP ยับยั้งการกระตุ้น p38 MAP kinase โดยยับยั้ง p38 phosphorylation ได้เกือบหมด มีผลเพียงเล็กน้อยหรือไม่มีผลต่อ phosphorylation ของ c-Jun N-terminal kinase และ extracellular-signal-regulated kinase และไม่มีผลเปลี่ยนแปลงการกระตุ้น nuclear factor kappa B ผลจากการศึกษานี้แสดงว่า *S*-deoxydihydroglyparvin ยับยั้งการสร้างโมเลกุลต่างๆที่เกี่ยวข้องกับการอักเสบผ่านทางการยับยั้งการการกระตุ้น p38 ในเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยแอลพีเอส สารนี้อาจเป็นสารต้านอักเสบได้

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

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Chanyanuch Laprasert : Inhibitory effects of compounds isolated from ethyl acetate extracts of *Glycosmis parva* branches and leaves on LPS-induced RAW 264.7 macrophage activation. Advisor: Asst. Prof. WACHAREE LIMPANASITHIKUL Co-advisor: Asst. Prof. CHAISAK CHANSRINIYOM

Macrophages play major roles to produce several inflammatory mediators in many chronic inflammatory diseases. Current anti-inflammatory drugs target these mediators to alleviate inflammation symptoms. Searching for new anti-inflammatory agents is always needed due to problems from currently used anti-inflammatory drugs. This study aimed to investigate the anti-inflammatory potential of three main compounds, arborinine, *N*-methylatalaphylline and *S*-deoxydihydroglyparvin (DDGP), isolated from *Glycosmis parva* leaves and branches on lipopolysaccharide (LPS)-activated macrophage RAW 264.7 cells. Only DDGP demonstrated a potent inhibitor of LPS-activated macrophages. It inhibited expression of inducible nitric oxide synthase and decreased nitric oxide production with IC50 3.47 ± 0.1 μM. It suppressed the mRNA expression and the protein production of pro-inflammatory cytokines, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6. LPS activates these cytokine productions at the early step of LPS-induced macrophage activation. DDGP also decreased the mRNA expression of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α, and two enzymes, cyclooxygenase-2 and mitochondrial prostaglandin (PG) E synthase, for PGE2 synthesis. Additionally, it inhibited PGE2 production. It had little or no effect on modulating anti-inflammatory molecules. Effects of DDGP on LPS signaling pathways were also evaluated. DDGP profoundly decreased phosphorylated p38 MAP kinase. It had little or no effect on the phosphorylation of c-Jun N-terminal kinase and extracellular-signal-regulated kinase, and on nuclear factor-κB activation. These results suggested that DDGP inhibited expression and production of inflammatory molecules in LPS-activated macrophages via suppressing p38 MAPK activation. DDGP may be a good candidate anti-inflammatory agent.

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

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Chanyanuch Laprasert

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LIST OF ABBREVIATIONS

µg	= Microgram
µl	= Microliter
µM	= Micromolar
AP-1	= Activator protein-1
cDNA	= Complementary deoxyribonucleic acid
COX	= Cyclooxygenase
DDGP	= *S*-deoxydihydroglyparvin
DEPC	= Diethyl pyrocarbonate
DMEM	= Dulbecco's modified eagle medium
DMSO	= Dimethyl sulfoxide
DNA	= Deoxyribonucleic acid
ELISA	= Enzyme-linked immunosorbent assay
eNOS	= Endothelial NOS
ERK	= Extracellular-signal-regulated kinase
EtOAc	= Ethyl acetate
GI	= Gastrointestinal
GP	= *Glycosmis parva*
GR	= Glucocorticoid receptors
H	= Hour

HRP	= Horseradish peroxidase
IC_{50}	= The half maximum inhibitory concentration
IFN	= Interferon
IKK	= Inhibitory kappa B kinases
IL	= Interleukin
iNOS	= Inducible nitric oxide synthase
IκB	= Inhibitory NF-κB
JAK	= Janus kinase
JNK	= c-Jun N- terminal kinases
LPS	= Lipopolysaccharide
MAPK	= Mitogen-activated protein kinase
MAPKK	= Mitogen-activated protein kinase kinase
MAPKKK	= Mitogen-activated protein kinase kinase kinase
MCP-1	= Monocyte chemoattractant protein-1
min	= Minute
MIP-1α	= Macrophage inflammatory protein-1α
Ml	= Mililier
mPGES	= Microsomal prostaglandin E synthase
NF-κB	= Nuclear transcription factor kappa-B

nm	= Nanometer
nNOS	= Neuronal NOS
NO	= Nitric oxide
NOS	= Nitric oxide synthase
NSAIDs	= Non-steroid anti-inflammatory drugs
OD	= Optical density
PCR	= Polymerase chain reaction
PGs	= Prostaglandins
PLA	= Phospholipase A
qPCR	= Quantitative polymerase chain reaction
S.E.M.	= Standard error of mean
SDS	= Sodium dodecyl sulfate
Sec.	= Second
STAT	= Signal transducer and activator of transcription
TBS	=Tris-buffered saline
TGF	= Transforming growth factor
TLR	= Toll-like receptor
TMB	= 3, 3', 5, 5'-tetramethylbenzidine
TNF	= Tumor necrosis factor
TXA2	=Thromboxane A2

CHAPTER I

INTRODUCTION

1.1. Background and rationale

Inflammation is a defense mechanism of the body in response to infection or harmful stimuli. However, long-term or chronic inflammation associates with a variety of diseases such as atherosclerosis, cancer, and autoimmune diseases as rheumatoid arthritis (1-3). Many of these diseases need anti-inflammatory drugs to alleviate signs and symptoms of inflammation (4, 5). Since it is well known that macrophages at inflamed tissues are key players for inflammatory process during chronic inflammation, inhibition of activated macrophages is a crucial strategy for clinically used anti-inflammatory drugs. Activated macrophages are the main sources of pro-inflammatory cytokines, chemokines, inflammatory mediators, and other several molecules involve in chronic inflammation (6, 7). Syntheses and activities of these cytokines, mediators, and molecules of activated macrophages become the targets of several anti-inflammatory drugs including corticosteroids, non-steroid anti-inflammatory drugs (NSAIDs), and anti-cytokines. However, side effects or high cost are burden for using these drugs. Corticosteroids are very potent anti-inflammatory agents with numerous side effects in long term use. NSAIDs which are non-selective cyclooxygenase (COX) inhibitors have gastrointestinal (GI) side effects. Those which are selective COX-2 inhibitors have cardiovascular and renal side effects (4, 5). Most of anti-cytokines such as inhibitors of tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 are biologics with very high cost and have many side effects (7).

Development of new anti-inflammatory agents are still needed. Medicinal plants are rich sources for searching new pharmacological agents, as well as anti-inflammatory agents. *Glycosmis parva* (GP) is a wild small shrub in the Rutaceae family. It has been used for appetite stimulant and anti-inflammation in Thai traditional medicine (8). Ethyl acetate (EtOAc) extracts from the leaves and the branches of *G. parva* demonstrated potent inhibitory effects on lipopolysaccharide (LPS)-activated macrophage J774A.1 cells. The extracts decreased the expression of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and COX-2 in LPS-activated J774A.1 cells (9). Acridone alkaloids and sulfur-containing propanamides are main compounds found in plants of *Glycosmis* genus. These compounds have many pharmacological activities including anti-malarial, anti- viral, anti-tumor, and anti-fungal activities. Several compounds were isolated from the EtOAc extracts from the leaves and the branches of *G. parva*. Three major compounds are a sulfur-containing propanamide; *S*-deoxydihydroglyparvin (DDGP) from leaves, and two acridone alkaloids; arborinine from the leaves; and N-methylatalaphylline from the branches (10). Anti-inflammatory activities of these compounds have not been reported.

1.2. Research objectives

1. To study inhibitory potential of active compounds isolated from EtOAc extracts of *G. parva* leaves and branches on LPS-activated RAW 264.7 macrophages.

2. To investigate the cellular and molecular effects of the potential compounds on inflammatory and anti-inflammatory enzymes and mediators in LPS-activated RAW 264.7 macrophages.
3. To investigate effect of the potential compounds on mian signaling pathways involved in LPS-induced macrophage activation.

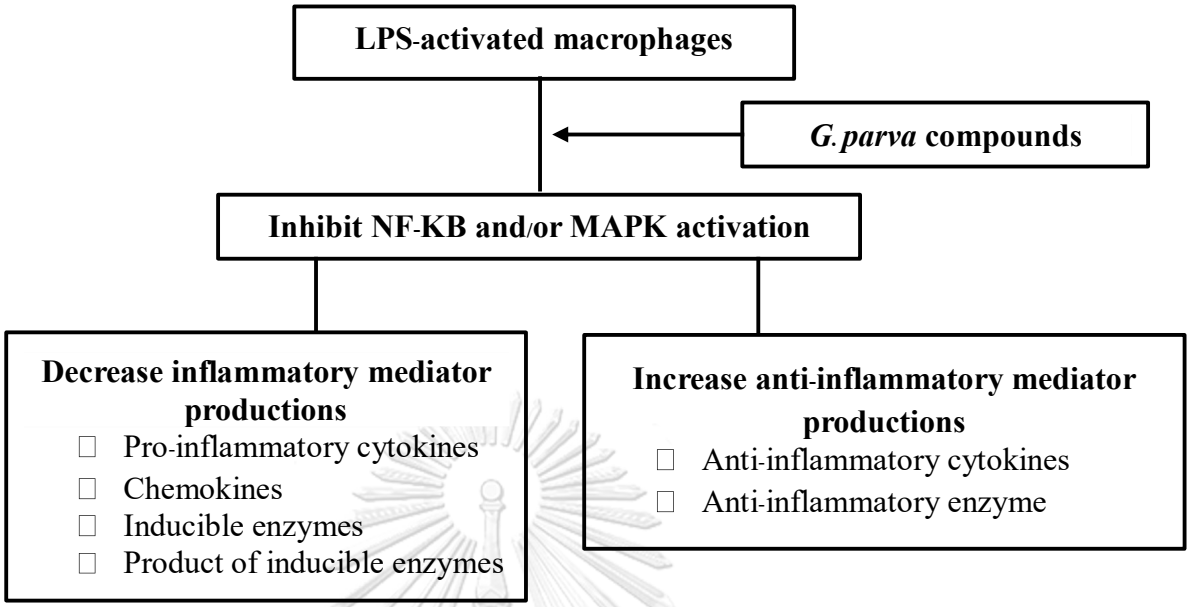
1.3. Research questions

1. Can active compounds isolated from EtOAc extracts of *G. parva* leaves and branches inhibit LPS-activated RAW 264.7 macrophages?
2. What are the cellular and molecular effects of the potential compounds on inflammatory and anti-inflammatory enzymes and mediators in LPS-activated RAW 264.7 macrophages?
3. How do the potential compounds modulate signaling molecules involved in LPS-induced macrophage activation?

1.4. Hypothesis

Active compounds from EtOAc extracts of *G. parva* leaves and branches have inhibitory effects on LPS-induced macrophage activation.

1.5. Conceptual framework



1.6. Expected benefit and application

1) This study may reveal active compounds from *G. parva* which inhibit LPS activated macrophages and have potential to be anti-inflammatory agents in the future
2) This study may confirm the use of *G. parva* as an herbal plant for treating inflammation.

1.7. Keywords

Glycosmis parva, *S*-deoxydihydroglyparvin, anti-inflammation, lipopolysaccharide, macrophage

CHAPTER II

LITERATURE REVIEW

2.1. Inflammation

Inflammation is a defense mechanism of the body against infectious and/or noninfectious harmful stimuli that cause vascularized tissue damages (1-3). It is a complex process that recruits leukocytes and plasma proteins from the blood into the site of damaged tissues for removing the harmful stimuli and damaged cells, and for repairing the tissues. The cells and mediators involve in inflammatory process include phagocytic leukocytes, complement proteins, and vascular endothelial cells. During inflammation, immune cells and the endothelial cells release pro-inflammatory cytokines and inflammatory mediators to amplify inflammatory reactions. Inflammation can be categorized into acute and chronic inflammation based on timing and characteristics (1).

2.1.1. Acute inflammation

Acute inflammation begins within seconds to minutes after tissues encountering harmful stimuli. It typically lasts in a short time (hours to days) (1, 3). Both vascular and cellular changes at the injured tissues cause the cardinal signs of inflammation including redness, increased heat, swelling, and pain (1).

- **Vascular changes**: Vasodilation and increased vascular permeability are two major events of vascular changes at injured or infected tissues. Mediators from immune cells at damaged tissues especially histamine and serotonin from activated mast cells are important for vascular changes. These mediators

induce vasodilation of arterioles at the injured tissues which causes more blood flow to this area. The tissues become red and warm. Dilation of blood vessel causes slower blood flow and the increase in vascular permeability which leads to the leakage of plasma proteins and the migration of plasma leukocytes into the injured area. This causes tissue edema. Vascular endothelial cells are stimulated to express several cell surface molecules as well as adhesion molecules and chemokines to play roles during inflammatory process for recruiting leukocytes into the injured area.

- **Cellular changes**: Leukocyte recruitment and activation are the cellular changes at injured tissues. In acute inflammation, leukocytes in the blood circulation migrate into the injured tissues. Neutrophils are recruited first followed by monocytes which develop to tissue macrophages. Adhesion molecules on these immune cells and on vascular endothelial cells as well as chemokines from both cell types play important roles on the leukocyte recruitment. Neutrophils are major phagocytes in injured tissues in acute inflammation. These cells become activated cells which can eliminate pathogens or harmful stimuli as well as damaged cells or cell debris by phagocytosis. These activated cells also produce several enzymes and mediators that involve in inflammatory process. These mediators can amplify leukocytes recruitment and activation. They also cause cardinal signs of inflammation by several mechanisms. Neutrophils are short-lifespan. Activated neutrophils in inflammatory tissues survive for 1-2 days. Activated

macrophages derived from blood monocytes play roles in inflammation after activated neutrophils decrease. They also produce several mediators to inhibit inflammation and induce tissue repair after eliminating the pathogens or harmful stimuli.

After removing infected pathogens or harmful stimuli by activated phagocytes at the injured tissues, these phagocytes are inhibited by anti-inflammatory mediators produced from these activated cells or undergo apoptosis. Acute inflammatory process decreases and tissue repair occurs (1, 11).

2.1.2. Chronic inflammation

Chronic inflammation is long-term inflammation lasting for several months to years. The harmful effects of chronic inflammation to the body are vary and depend on the cause of the injury and the ability of the body to repair the damage (1, 2). Chronic inflammation results from several causes, as follow:

1) Failure of removing harmful agent which causes acute inflammation.
2) Autoimmunity against self-tissue which lead to autoimmune disease such as rheumatoid arthritis.
3) Long-term exposure to a low level of irritant or foreign particles which can persist in the body such as silica.
4) Exposure to inflammatory or biochemical inducers which cause oxidative stress or mitochondrial dysfunction lead to increase reactive oxygen species, advanced glycation end products, uric acid crystals, oxidized lipoproteins, homocysteine, and others.

The uncontrolled chronic inflammation is a major cause of inflammatory pathologies and chronic inflammatory diseases. Examples of chronic inflammation associated diseases are rheumatoid arthritis, diabetes mellitus, atherosclerosis, and chronic obstructive pulmonary disease (2).

The features of chronic inflammation include vasodilation, increase vascular permeability, and migration of leukocytes into the damaged tissue. However, the composition of the leukocytes in the chronic tissue damage is changed. Macrophages and lymphocytes replace short-lived neutrophils. Thus the hallmarks of chronic inflammation are the infiltration of the primary inflammatory cells such as macrophages and lymphocytes in the tissue site, producing inflammatory cytokines, growth factors, enzymes and hence contributing to the progression of tissue damage, fibrosis and granuloma formation (12).

2.2. Macrophages

Macrophages are mononuclear phagocytes derived from two sources. The first lineage, macrophages are originated from the bone marrow hematopoietic stem cells which differentiate to blood circulating monocytes and migrate into tissues, especially during inflammatory process, to become macrophages. the second lineage, these cells are from fetal liver during fetal development and differentiate to tissue resident macrophages with specialized phenotypes depend on tissues or organs they reside (Figure 1) (1, 13). Macrophages along with lymphocytes play important roles in chronic inflammation (1, 14, 15).

There are 4 functions of macrophages during process of inflammation and tissue resolution including

1) Ingest microbes and dead host cells.

   In infected tissue, macrophages produce reactive oxygen and nitrogen species to kill microbes. In addition, dead host cells in tissues caused by trauma or lack of blood supply are engulfed and cleaned up by macrophages.

2) Secrete several pro-inflammatory cytokines, chemokines, inflammatory and anti-inflammatory mediators to amplify inflammatory response.

3) Display antigens to activate T lymphocytes.

   Macrophages play as antigen-presenting cell to detects, engulfs, digest pathogens and initiate adaptive immune response.

4) Repair damaged tissues

   Macrophages promote the repair of damaged tissues by stimulating angiogenesis and synthesis of fibrosis. These functions are mediated by anti-inflammatory cytokines secreted by the macrophages (1, 16).

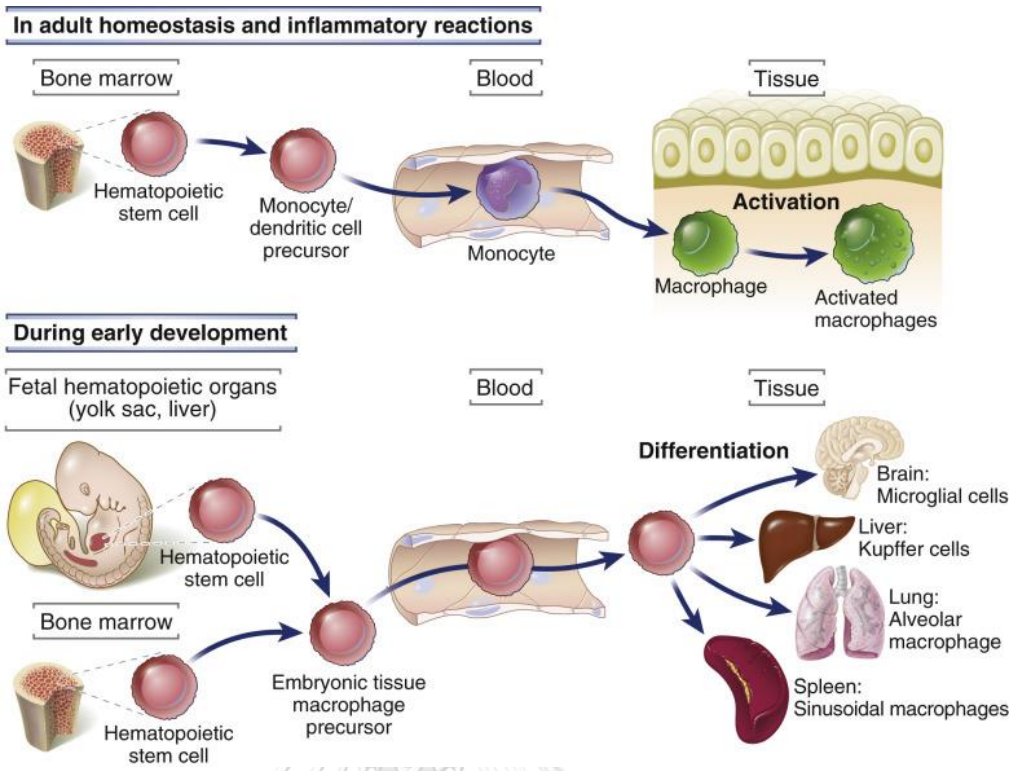


Figure 1 Maturation of macrophages (1)

2.3. Lipopolysaccharide-induced macrophage activation model

LPS is a component of the outer membrane of gram-negative bacteria. It is a very potent stimulators of innate immune system. LPS consists of a polysaccharide region and a lipid moiety termed lipid A. Lipid A causes LPS toxicity. Polysaccharide part involves in activation of the immune system (17, 18). LPS binds to toll-like receptor (TLR) 4 on macrophages, activate signaling pathways, both MyD88-dependent and MyD88-independent pathways induce the expression of pro-inflammatory cytokines and other inflammatory mediators through upregulation of mitogen-activated protein kinase (MAPK) signaling cascades and nuclear transcription factor kappa-B (NF-κB) activation (Figure 2) (18-21). LPS-induced macrophage activation is commonly used as

a model for studying inflammatory response because it activates the expression and production of almost all molecules involve in inflammatory process (18). These molecules can be used as the biomarkers of inflammation.

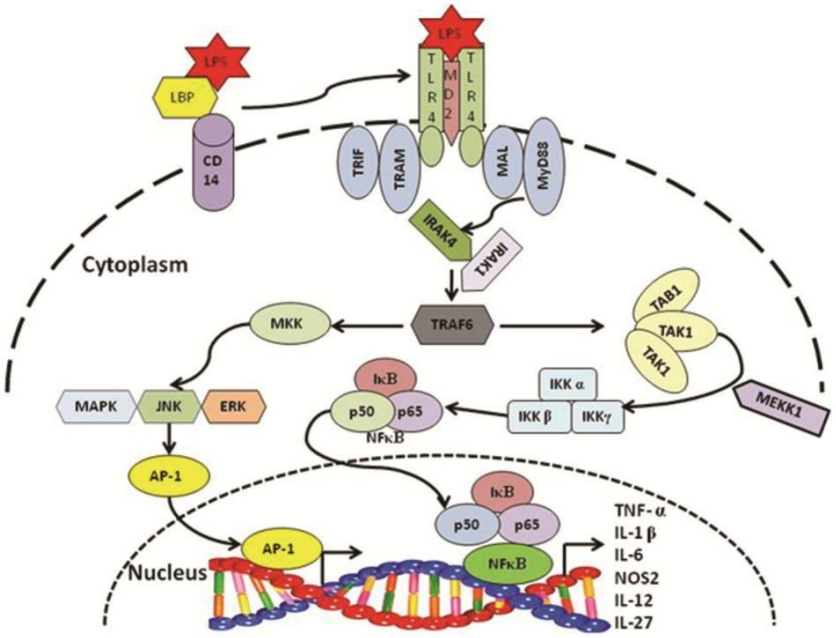


Figure 2 LPS induced macrophage activation pathway and expression of inflammatory cytokines (22)

2.4. Mediators involve in chronic inflammation

Macrophages are main inflammatory cells in chronic inflammation. Activated macrophages at an inflamed tissue continuously produce numerous enzymes and mediators which play roles in inflammation, anti-inflammation, and fibrosis.

2.4.1. Proinflammatory cytokines

These cytokines play roles in promoting inflammation. The most important proinflammatory cytokines from activated macrophages are TNF-α, IL-1, and IL-6 (23, 24).

- **Tumor necrosis factor α**: This cytokine is produced by activated leukocytes including macrophages, dendritic cells, T-lymphocytes. It is rapidly expressed and produced after macrophages are activated by stimuli as well as bacterial-derived LPS. LPS induces TNF-α gene expression through the activation of TLR4-mediated NF-κB transcription factor (1, 25). TNF-α interacts with TNF receptor on several immune cells, activates NF-κB and MAPK signaling pathway, and induces the expression of several enzymes and cytokines including pro-inflammatory cytokines, chemokines, inducible COX-2 which generates large amount prostaglandins (PGs), iNOS which generate large amount nitric oxide (NO). Products of TNF-α activation results in inflammatory process such as increase of leukocyte adhesion and trans-endothelial migration, and vascular leak. TNF-α is a well-known pro-inflammatory cytokine in many chronic inflammatory diseases (25, 26).
- **Interleukin-1**: IL-1 is produced from activated macrophages and other nonimmune cells such as fibroblasts and endothelial cells (1, 23). IL-1 has 2 forms, IL-1α and IL-1β. IL-1β is the main biologically active isoform. IL-1β production is activated by several stimuli as well as LPS and TNF- α. IL-1 acts

by binding to IL-1 receptor. This leads to the activation of NF-κB and activator protein-1 (AP-1) transcription factors. IL-1 has biological activities similar to TNF-α which induces the production of pro-inflammatory cytokines, increases the expression and production of adhesion molecules, several enzymes and inflammatory mediators (1, 27).

- **Interleukin-6**: IL-6 is also a pro-inflammatory cytokine which is produced by various activated immune cells, vascular endothelial cells and fibroblasts. Its production is induced by several stimuli as well as LPS and pro-inflammatory cytokines. IL-6 binds to IL-6 receptor, activates Janus kinase (JAK)1-signal transducer and activator of transcription (STAT)3 signaling pathway. This leads to activation the transcription factor STAT3 resulting in expression and production inflammatory mediator as well as acute-phase protein from hepatocytes (1, 28, 29).

2.4.2. Chemokines

Chemokines are cytokines that induce leukocyte migration from the blood circulation and recruitment the cells at an injured tissue. They are produced by several immune and non-immune cells. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are the chemokines from activated macrophages which play roles in leukocyte migration and recruitment (1, 30). They are produced in activated leukocytes and other cell types after being stimulated by pro-inflammatory cytokines (1).

- **Monocyte chemoattractant protein-1**: MCP-1 is a chemokine that induces migration and infiltration of monocytes or macrophages. It is constitutively produced in many cell types, monocytes and macrophages are the major source of this cytokine. MCP-1 is increasingly produced by several stimulators such as oxidative stress, cytokines, growth factors or LPS. Increase expression of MCP-1 and its receptor C-C chemokine receptor type 2 associates with in various chronic inflammatory diseases including multiple sclerosis, rheumatoid arthritis, atherosclerosis, and insulin-resistant diabetes (31).
- **Macrophage inflammatory protein-1α**: MIP-1 α is the chemokine which plays a major role in the recruitment of leukocytes to sites of injured tissue. It is produced by many immune cells, endothelial cells, fibroblasts, and osteoblasts. This chemokine weakly expressed in resting cells. It expression is upregulated by LPS, pro-inflammatory cytokines, and interferon (IFN)-γ (32).

2.4.3. Inducible nitric oxide synthase and nitric oxide

NO is a soluble gas which acts as vasodilator and a pro-inflammatory mediator. It synthesized from L-arginine by nitric oxide synthase (NOS) (Figure 3) (33). There are three isoforms of NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. eNOS and nNOS are constitutively produced in many cell types for physiological activities. LPS and pro-inflammatory cytokines can up-regulated iNOS expression leading to the production of larger amount of NO than NO generated by eNOS and nNOS. Within phagolysosomes of activated macrophages,

NO reacts with hydrogen peroxide or superoxide to become highly reactive peroxynitrite radicals that can kill microbes (1, 34). Excessive production of NO by iNOS has been shown to be associated with various chronic inflammatory diseases (35).

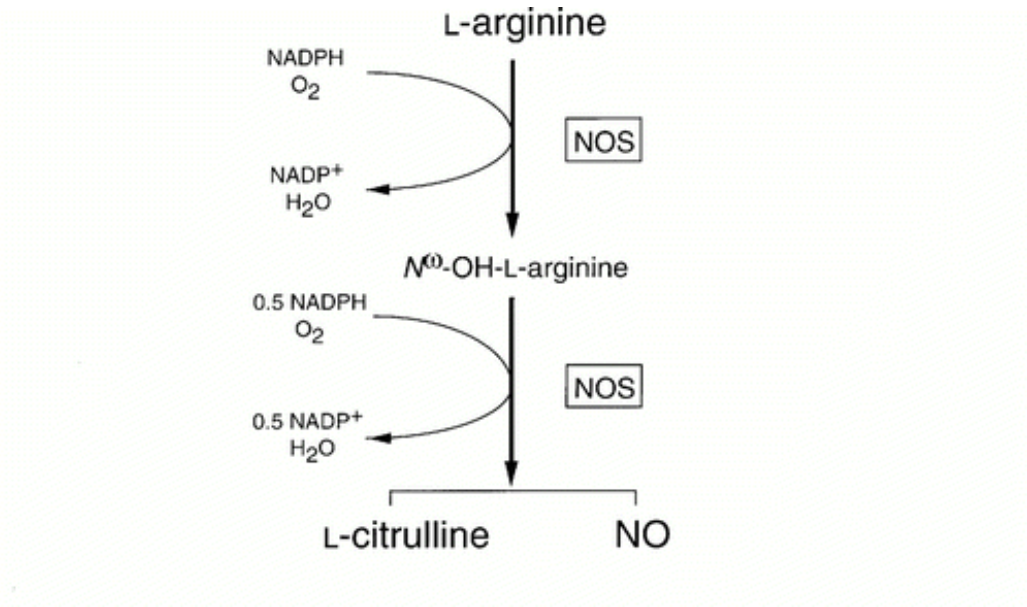


Figure 3 Biochemical pathway of NO production in mammalian cells (33).

2.4.4. Cyclooxygenase and prostaglandin E2

PGs are lipid-derived mediators generated from phospholipid of the plasma membrane by phospholipase A (PLA)2 to become arachidonic acid followed by COX enzymes to become PGH2 (Figure 4). COX has 2 isoforms, COX-1 and COX-2. COX-1 is constitutively expressed enzyme that generates PGs in various tissues, including the platelets, endothelial cells, kidneys and stomach to maintain physiological functions such as gastric mucosal integrity, normal renal function, and platelet aggregation. COX-2 is an inducible enzyme that can be upregulated by many inflammatory stimuli in activated macrophages, synovial cells, and

fibroblasts. COX-2 generates large amount of PGE2 which is an important mediator associated with inflammation and pain. PGE2 is synthesized from PGH_2 by microsomal prostaglandin E synthase (mPGES)-1 which is markedly upregulated by cytokines in activated macrophages. Large amount of PGE2 causes pain and fever and associates with variety of pathological conditions (36, 37).

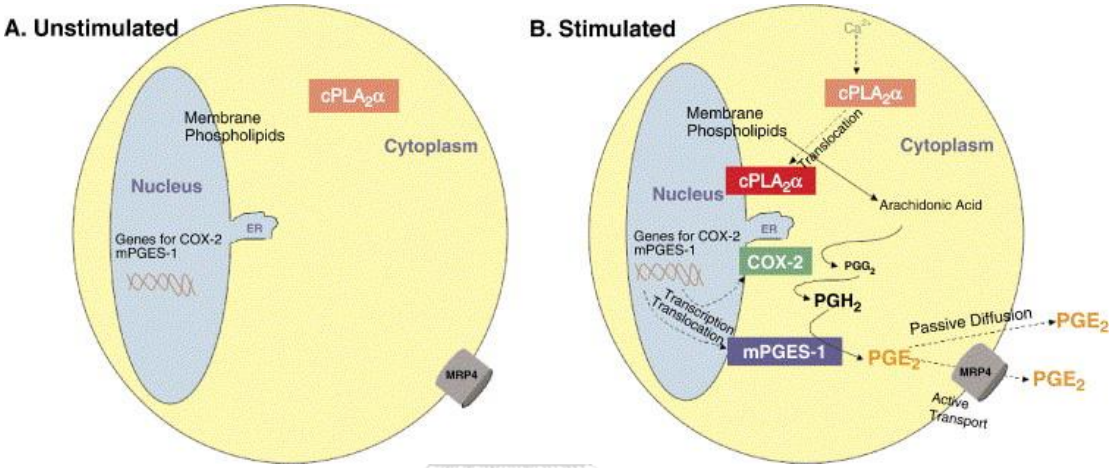


Figure 4 Production of PGE2 by PLA2, COX-2, and mPGES-1 (37)

2.4.5. Anti-inflammatory cytokines

During inflammatory process, not only inflammatory mediators but also anti-inflammatory enzymes and mediators are produced from activated macrophages. The major anti-inflammatory cytokines are IL-10 and transforming growth factor (TGF)-β. The main anti-inflammatory enzyme is arginase (38).

- **Interleukin 10**: IL-10 is a potent anti-inflammatory cytokine produced by macrophages, dendritic cells, regulatory T cells, B cells. It induces the inhibition of many pro-inflammatory cytokines and chemokines including TNF-α, IL-1, IL-

12, and MIP-1α. It also induces the inhibition of NF-κB nuclear translocation after LPS stimulation (1, 38). It is an important inhibitory cytokine for controlling the inflammatory response in many inflammatory and autoimmune diseases (39).

- **Transforming growth factor β**: TGF-β is produced and functions in similar manner to IL-10. TGF-β is less potent than IL-10. It has no effect on IL-1 production. It plays role on resolution of inflammation and repairment of the injured tissues by regulating cell proliferation, differentiation and formation of the extracellular matrix (1, 38). It has been reported that TGF-β knockout mouse demonstrated severe inflammatory reactions than normal mouse (40).

2.4.6. Arginase

Arginase is an enzyme that catalyzes L-arginine to L-ornithine and urea (Figure 5). L-ornithine is the precursor for the production of polyamines and proline. Polyamines involve in cell proliferation and differentiation. Proline is used to produce collagen for wound healing (41, 42). Arginase has two isoforms, arginase-1 in cytosol and arginase-2 in mitochondria. These isoforms are encoded by different genes. They have the same mechanisms of actions and generate the same metabolites. They are different in localization and predominant expression in various organs and tissues. Arginase-1 is constitutively and abundantly expressed in the liver but no arginase-2 is detected in hepatocytes. In macrophages, arginase-1 and arginase-2 expression is inducible by many stimuli (43, 44). This enzyme generates L-ornithine which is crucial for wound healing. Tissue injury could up-

regulate iNOS and arginase at different time point (45). iNOS was induced first followed by arginase. It is evidenced that expression of arginase-1 in macrophages could reduce NO production by iNOS via competing intracellular L-arginine leading to the decrease of iNOS expression (46).

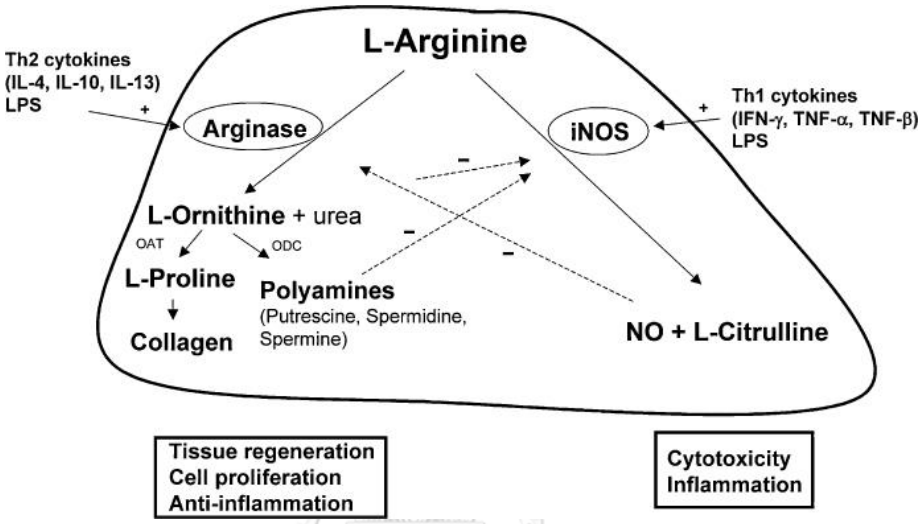


Figure 5 Regulation of arginase and iNOS in macrophage cells (42)

2.5. Signaling pathways in inflammatory process

Key events in inflammatory processes are: 1) cell surface pattern receptors recognize harmful stimuli; 2) inflammatory pathways are activated; 3) inflammatory markers are released; and 4) inflammatory cells are recruited and activated. These processes cause inflammatory response via the coordinate activation of inflammatory signaling pathways that regulate inflammatory mediator production and leukocyte recruitment.

Inflammatory stimuli, such as microbial products and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), bind to their specific receptors (TLRs and cytokine receptors)

and activate intracellular signaling pathways in leukocytes such as neutrophil and macrophages to produce inflammatory mediators. Receptor activation triggers important intracellular signaling pathways, including the MAPK, NF-κB, and JAK-STAT pathways. These inflammatory signaling pathways activate multiple transcription factors to regulate a variety of inflammatory genes, such as IL-1, TNF-α, IL-6, colony stimulating factor, IFN, TGF, and chemokines.

- **Nuclear transcription factor kappa-B**: The NF-κB transcription factor plays important roles in inflammatory, immune response, survival, and apoptosis processes (47). In inflammatory processes, NF-κB activity is activated by microbial products (such as LPS) and pro-inflammatory cytokines by stimulating inhibitory kappa B kinases (IKK) to phosphorylate NF-κB inhibitor named IκB protein. IκB phosphorylation results in its degradation by the proteasome and the subsequent release of NF-κB for nuclear translocation and gene transcription activation (Figure 6) (47, 48). This pathway regulates pro-inflammatory cytokine production and inflammatory cell recruitment, which contribute to the inflammatory response (47).

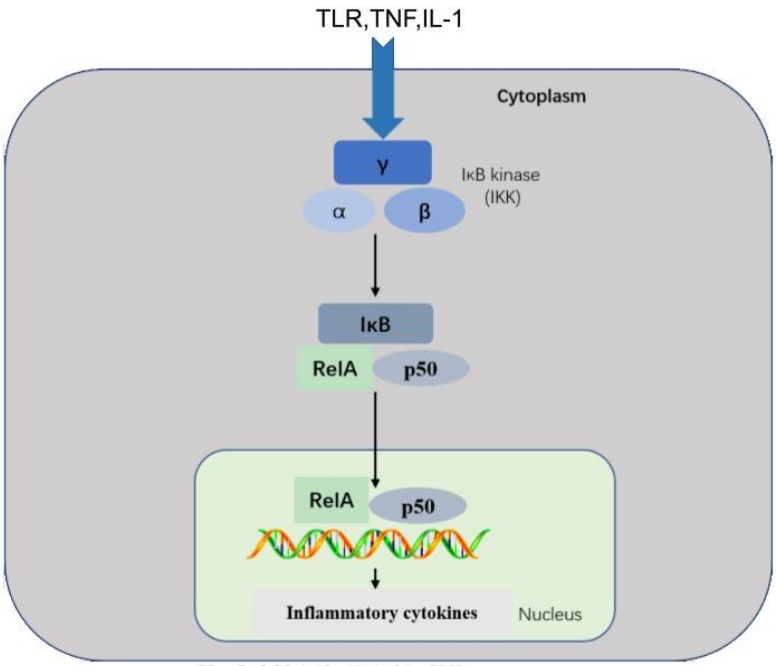


Figure 6 NF-κB pathway (48).

- **Mitogen-activated protein kinase pathway**: MAPKs are a groups of serine/threonine protein kinases that induce cellular responses to numerous stimuli as well as microbial products (such as LPS) and pro-inflammatory cytokines. The members of MAPKs include extracellular-signal-regulated kinase (ERK)1/2, p38 MAP Kinase, and c-Jun N-terminal kinases (JNK). There are at least three kinases in MAPK signaling pathway, a MAPK, a MAPK kinase (MAPKK), and a MAPK kinase kinase (MAPKKK). MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs. ERKs are generally activated by mitogens and differentiation signals, while inflammatory stimuli and stress activate JNK and p38. MKK1 and MKK2 activate ERK1/2, MKK4 and MKK7 activate JNK, and MKK3 and MKK6 activate p38. Activation of the MAPKs (ERK1/2, JNK, and p38) lead to phosphorylation and activation of transcription factors in the cytoplasm or nucleus, which initiates the inflammatory response (Figure 7) (48, 49).

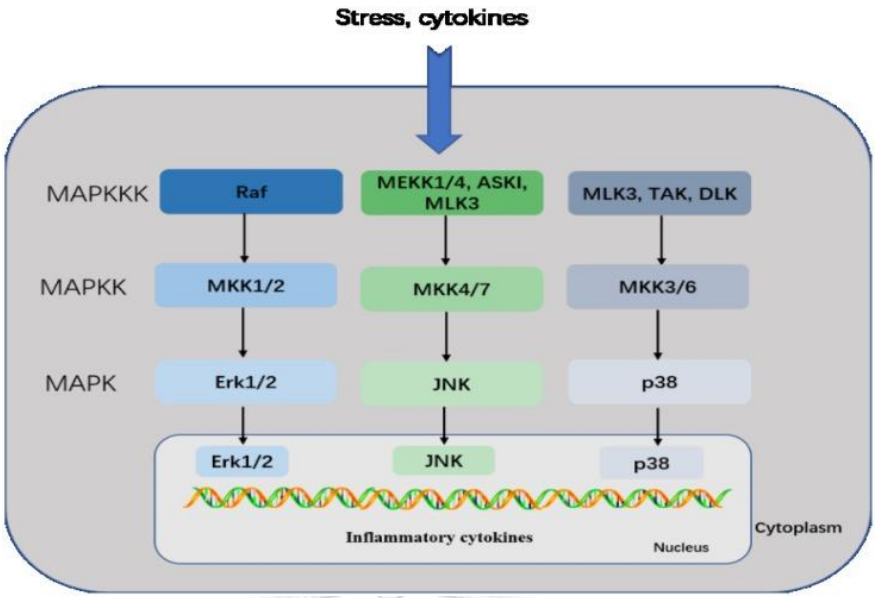


Figure 7 MAPK pathway (48).

- **Janus kinase-signal transducer and activator of transcription pathways**: JAK-STAT signaling pathway involves many cytokines (as well as IL-6), growth factors, leptin, and growth hormone. Receptor-associated JAKs are activated by ligands and tyrosine phosphorylate one other to create docking sites for STATs, which are latent, cytoplasmic transcription factors. Cytoplasmic STATs are recruited to phosphorylated JAKs, undergo phosphorylation, form dimer by dimerization, and translocate to the nucleus. Tyrosine phosphorylation is essential for STAT dimerization and DNA binding (Figure 8) (50). For example, binding of IL-6 to IL-6 receptor activates the JAK-STAT proteins. STAT proteins translocated into the nucleus bind target gene promoter regions to regulate transcription of inflammatory genes (48).

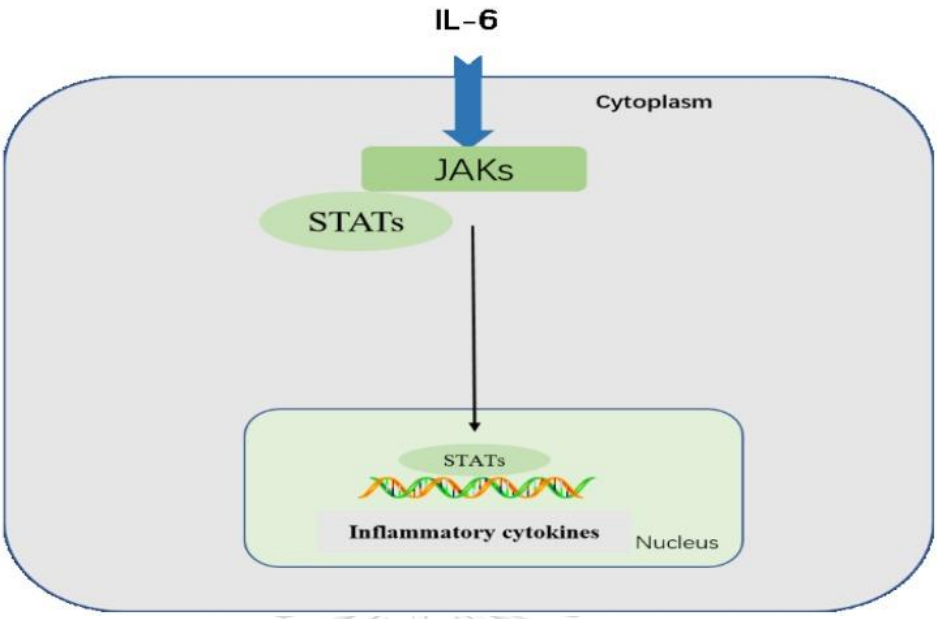


Figure 8 JAK-STAT pathway (48).

2.6. Anti-inflammatory Drugs

Anti-inflammatory agents are used to treat patients with inflammation. Goals of the treatment are the relief of symptoms, the maintenance of tissue function, and the slowing or arrest of the tissue damaging process. Commonly used anti-inflammatory agents are glucocorticoids, nonsteroidal anti-inflammatory agents, and anti-cytokines.

2.6.1. Glucocorticoids

Glucocorticoids are very potent anti-inflammatory drugs. Examples of clinically used glucocorticoids are dexamethasone, betamethasone, fludrocortisone, triamcinolone and prednisolone. They are used widely on a chronic basis to treat several chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease, rheumatoid arthritis and inflammatory bowel disease.

- Mechanisms of anti-inflammatory actions of glucocorticoids are divided into genomic and non-genomic actions. For classical or genomic actions, glucocorticoids act as inducers or repressors to modulate the expression of many genes involve in inflammation at the transcriptional level. Onset of these actions takes hours or days. Once glucocorticoids enter the cells, they bind to glucocorticoid receptors (GR) in the cytoplasm. The steroid-GR complexes then translocate to the nucleus, bind to specific sites on DNA and suppress the expression of almost all pro-inflammatory cytokines, chemokines, COX-2, and iNOS. The complexes also upregulate the expression of anti-inflammatory genes such as IL-10 and annexin-1. Annexin-1 binds to and inhibits activity of phospholipase A2 (PLA_2) which generate arachidonic acid for prostaglandin production. In addition, the drug receptor complexs can directly bind and inhibit AP-1 or NF-kB which upregulate many inflammatory genes (Figure 9). Non-classical or non-genomic actions of glucocorticoids occur within seconds to minutes after binding to GR without gene expression. Several evidences have been suggested that GR mediates nongenomic activity by directly interacting with second messengers or signaling molecules in several cell signaling pathways (51, 52).

- Anti-inflammatory activities of glucocorticoids: Glucocorticoids dramatically reduce inflammatory manifestations. They have profound inhibitory effects on leukocyte migration and infiltration and on the expression and production of

inflammatory enzymes and mediators. They increase the production of anti-inflammatory mediators.

- Side effects: Long-term use of glucocorticoid drugs causes numerous side effects. These include immunosuppression, impair wound healing, metabolic effects or Cushing's syndrome (fluid retention, increased appetite, osteoporosis, hypertension, and peptic ulcer), and adrenal suppression which needs to slowly reduce dose of these drug before stopping use.

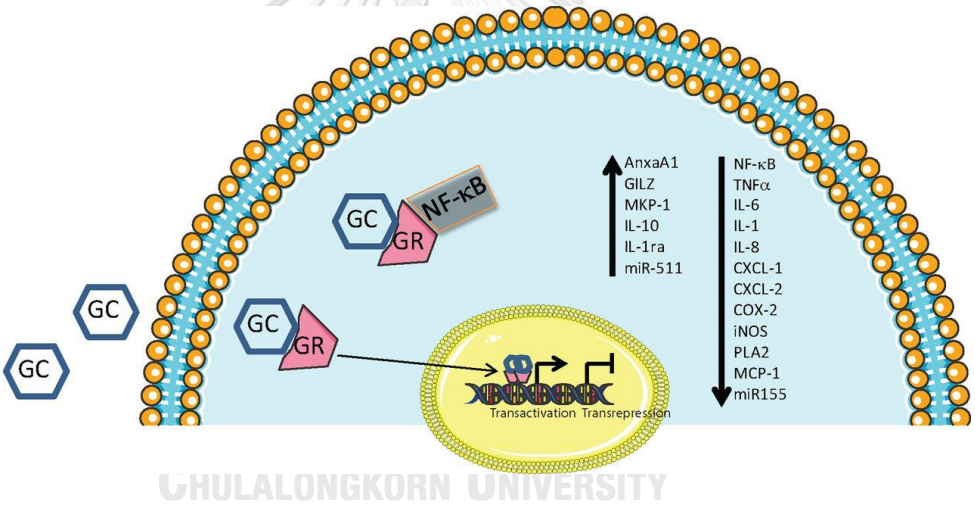


Figure 9 Classical or genomic pathway of glucocorticoids (52)

2.6.2. Non-steroidal anti-inflammatory drug

NSAIDs are the most widely used anti-inflammatory agents. Their anti-inflammatory activity is mediated mainly by inhibiting prostaglandin, especially PGE2, biosynthesis.

- Mechanism of action: NSAIDs bind to and inhibit COX which catalyze arachidonic acid to the unstable intermediates PGG_2 and PGH_2 and leads to the

production of prostaglandins and thromboxane A_2 (TXA_2) Suppression of PG synthesis results to relief of pain, fever and inflammation.

There are 2 major groups of NSAIDs based on their selectivity with COX enzyme, non-selective COX-inhibitors and selective COX-2 inhibitors.

1) Non-selective COX-inhibitors: These NSIADs inhibit both of protective COX-1 and inflammatory COX-2 enzymes. COX-1 is expressed constitutively in most cells for generating low amount of PGs which have several physiological functions such as gastric epithelial cytoprotection and hemostasis. COX-2 is an inducible enzyme produced mainly in activated leukocytes during inflammation. COX-2 produces large amount of PGE2 which is one of main inflammatory mediators in chronic inflammation. Non-selective COX-inhibitors which are commonly used are aspirin, diclofenac, indomethacin, ibuprofen, and naproxen. They are used for treating acute or chronic inflammatory diseases such as rheumatoid arthritis, osteoarthritis, and acute gout. Major side effects of these NSIADs are gastrointestinal irritation, peptic ulcer, and renal toxicity.

2) Selective COX_2-inhibitors: These NSIADs selectively bind to and inhibit COX-2 enzyme in order to reduce the common side effects from COX-1 inhibition. They have less GI side effects than nonselective NSIADs. However, they have similar renal side effects similar to nonselective COX inhibitors because COX-2 also functions in keeping

homeostasis of renal. The main side effects of selective COX-2 inhibitors are cardiovascular side effects which include heart attack, thrombosis and stroke. Inhibition of COX-2 activity causes the imbalance between TXA_2 and prostacyclin leading to increase of vasoconstriction and platelet aggregation. Selective COX_2-inhibitor which are commonly used is celecoxib (4, 5).

2.6.3. Cytokine inhibitors

Cytokine inhibitors used for treatment of chronic inflammatory diseases target on pro-inflammatory cytokines, their receptors, or signaling molecules of their receptor activation. Most of them are biologic drugs either therapeutic monoclonal antibodies or fusion proteins produced by genetic engineering technology. They are TNF- α inhibitors (adalimumab, certolizumab, etanercept, golimumab, and infliximab); IL-1 inhibitors (anakinra, canakinumab, rilonacept); and IL-6 inhibitor (toclilizumab, siltuximab). These biologic drugs have to parenterally administer. They are used as alternative anti-inflammatory drugs in patients with rheumatoid arthritis who do not response well to the standard treatment. These drugs have very high cost. Their long term efficacies and side effects are not fully clarified. The common side effects of these drugs are infection, hypersensitivity and immune disorders (7, 53). Tofacitinib and baricitinib are inhibitors of JAKs which are crucial tyrosine kinases involve in signaling pathways of many pro-inflammatory cytokine receptors. They were approved in for treating rheumatoid arthritis and are

under clinical trials for treatment of other autoimmune diseases (54). Inhibitors of p38 MAPK, losmapimod and talmapimod are under clinical trialed as anti-inflammatory agents (42, 43).

Due to limitation and side effects of current inflammatory drugs, development of new anti-inflammatory agents with more effective and less toxic is still needed. Medicinal plants are rich sources for searching new pharmacological agents, as well as anti-inflammatory agents.

2.7. *Glycosmis parva* Craib

Glycosmis parva Craib is a medicinal plant in the Rutaceae family. Its local names in Thai are Som-Chuen and Prayong-Kluean. *G. parva* is a wild small shrub. The leaves are simple leaves. The inflorescence is small, axillary panicles. The flower is composed of 5 calyxes, 5 white-yellowish petals, and glabrous stamens. The ovary is smooth and glabrous. The fruits are oval, pinkish-orange berries (Figure 10) (8, 55).

Figure 10 *Glycosmis parva* Craib

2.8. Pharmacological activities of *G. parva*

GP has been used for appetite stimulant and anti-inflammation in Thai traditional medicine. Previous studies demonstrated that the ethyl acetate extract of GP has some pharmacological activities. These activities included antiviral activity against herpes simplex virus type 1 and 2, inhibitory activity on LPS-activated on macrophage J774A.1, and anticancer activity against human colorectal cancer HT-29 cells (10, 56, 57). Chumsang et al. demonstrated that ethyl acetate extracts from branches and leaves of GP potently inhibited NO production in LPS-activated J774A.1 macrophages, with IC 50 values 16.69 µg/ml (the branch extract) and 11.12 µg/ml (the leaf extract). These extracts inhibited the expression of pro-inflammatory cytokines, iNOS, and COX-2 in LPS-activated macrophages (57).

Plants in the genus *Glycosmis* are the rich source of acridone alkaloids and sulfur-containing propanamides especially in branches and leaves. Pharmacological activities of these compounds have been reported, as shown in Table 1. Most of them had antimicrobial and anticancer activities. No report on their anti-inflammatory activities.

Table 1 pharmacological activities of acridone alkaloids and sulfur-containing propanamides from *Glycosmis* species

Activities	**References**
Acridone alkaloids	
1) Anti- viral activity	
- Suppressed herpes simplex virus type 1 and type 2 in infected cells	(10, 58)
- Inhibited human immunodeficiency virus -1 replication in chronically infected cells	(59)
2) Anti-cancer activity	
- Inhibited proliferation of human promyelocytic leukemia cells (HL-60)	(60)
- Inhibited promotion of skin tumor in mouse	(61)
- Decreased proliferation and migration of HeLa cervical cancer cells	
3) Antioxidant	(62)
- Showed moderate free radical scavenging activity against in DPPH assay	(63)
4) Anti-protozoal activity	
- Suppressed proliferation of *Plasmodium* species *in vitro* and *in vivo*	(64)
- Suppressed proliferation of *Leishmania* species *in vitro*	(65)

Sulfur-containing propanamides

5) Anti-fungal activity

- Inhibited growth of fungal *Cladosporium cladosporioides* (66)

6) Anti-viral activity

- Suppressed herpes simplex virus type 1 and type 2 in infected cells (10)

7) Anti-protozoal activity

- Suppressed proliferation of *Leishmania* species *in vitro* (65)
- Inhibited growth of *Trypanosoma cruzi in vitro* (67)

Chansriniyom et al. isolated active compounds from ethyl acetate extracts of *G. parva* leaves and branches, and characterized these compounds, as shown in Table 2 (10). They found that acridone alkaloids and sulfur-containing propanamide derivatives were main active compounds in the extracts similar to the previous studies on *Glycosmis* spp. However, anti-inflammatory activities of these compounds have not been reported.

Table 2 Active compounds isolated from the ethyl acetate extracts of *G. parva* leaves and branches

	Compound groups	**Active compounds**
Branches	Acridone alkaloids	Glycosparvarine *N*-methylatalaphylline *N*-methylcyclo-atalaphylline-A Glycofolinine Citramine
	Limonoids	limonin Limonexic acid Isolimonexic acid
Leaves	Sulfur-containing propanamide	*S*-deoxydihydroglyparvin *S*-deoxytetrahydroglyparvin Glyparvin-A Dihydroglyparvin
	Acridone alkaloids	arborinine

CHAPTER III

METERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and reagents

The following chemicals, reagents and reagent kits were used in this study: Dulbecco's modified eagle medium (DMEM) (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), penicillin and streptomycin (Gibco, USA), 0.4% trypan blue dye (Sigma, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), resazurin (Sigma, USA), LPS (Sigma, USA), Griess reagent system (Promega, USA), TRIzol™ RNA isolation solution (Biotechrabbit, Germany), diethyl pyrocarbonate (DEPC) (Molekula, UK), absolute ethanol (Merck, Germany), chloroform (Merck, USA), isopropanol (Merck, USA), ImProm-II™ reverse transcription system (Promega, USA), primer (Bio Basic, Canada), qPCR (quantitative PCR) green master mix (Biotechrabbit, Germany), mouse TNF-α and IL-6 ELISA (enzyme-linked immunosorbent assay) kits (Immuno Tools, Germany), mouse IL-1β ELISA kit (Thermo, USA), mouse PGE2 ELISA kit (R&D Systems, USA), Bio-Rad Protein assay (Bio-Rad, USA), RIPA lysis buffer (Abcam, USA), Cell Fractionation Kit (Cell Signaling, USA), proteinase inhibitor (Abcam, USA), bromophenol blue (Sigma, USA), β-mercaptoethanol (Sigma, USA), protein ladder (Bio-Helix, Taiwan), Rabbit monoclonal antibodies against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180-

Tyr182), NF-κB p65, IκB-α, GAPDH and PCNA (Cell Signaling, USA), rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling, USA), sodium dodecyl sulfate (SDS) (Merck, Germany), Tween 20 (Merck, Germany), glycerol (Merck, Germany), tetramethylethylenediamine (EM Science, USA), Tris-base (EM Science, USA).

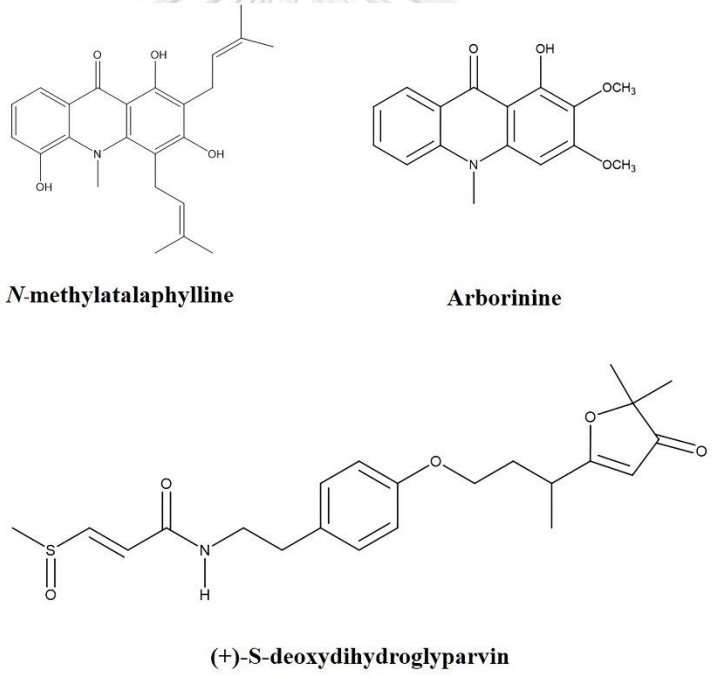
3.1.2. Equipment and instruments

The following equipment and instruments were used in this study:

analytical balance (Satorius, Germany and Mettler Toledo, Switzerland), autopipette (Brand, Germany), pipette controller (Eppendorf, Germany), biohazard laminar flow hood (Labconco, USA), centrifuge (Hettich, Germany), microplate reader (Thermo, Finland), hemacytometer (Brand, Germany), autoclave (Hirayama, Japan), pH meter (Mettler Tuledo, Switzerland), refrigerator 4ºC and -20ºC (Sanyo, Japan), incubator (Thermo, USA), light microscope (Nikon, Japan), vortex mixer (Scientific Industries, USA), Nanodrop™ One spectrophotometer (Thermo, USA), Master cycler (Eppendorf, Germany), StepOnePlus Real-Time PCR system (Thermo, USA), gel electrophoresis (Biorad, USA), TE 22 mini tank transfer unit (Biorad, USA), T25 tissue culture flasks (Corning, USA), 96- and 6-well plates (Corning, USA), 15, 50 ml polyethylene tubes (Corning, USA), cell scrappers (SLP, Korea), sterile syringe filters (Minisart), PCR strip tubes (Corning, USA), microcentrifuge tubes (Corning, USA), nitrocellulose membranes (Merck, Germany), C-DiGit® Blot Scanner (LI-COR, USA).

3.1.3. Test compounds

S-deoxydihydroglyparvin (DDGP) and arborinine (ABN) from the leaves, and N-methylatalaphylline (MPL) from the branches of *G. parva* (Figure 11) were isolated from ethyl acetate extracts of the plant and well characterized by Chaisak Chansriniyom, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (10). The compounds were prepared as 50 µM stock solution in DMSO. This stock solution was diluted to required final concentrations at constant 0.2% DMSO.



***N*-methylatalaphylline** **Arborinine**

(+)-S-deoxydihydroglyparvin

Figure 11 Structures of compounds isolated from ethyl acetate extracts of *G. parva* leaves and branches.

3.1.4. Cells

RAW 264.7 murine macrophages from American Type Culture Collection (ATCC) were grown and maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin under a humidified air atmosphere of 5% CO_2 at 37 °C. The cells with more than 90% viability determined by 0.4% trypan blue staining were used in all experiments in this study. The cells at $4x10^5$ cells/ml were cultured in 6- or 96-well plates for 24 h before being activated with 100 ng/ml LPS and treated with the test compounds.

3.2. Methods

All assays were performed in at least three independent experiments. LPS at 100 ng/ml was used to generate LPS-activated RAW 264.7 macrophages. Ten µM dexamethasone and 0.2% DMSO solution were used as the positive and the negative control, respectively.

3.2.1. Screening the inhibitory effects of MPL, ABN, DDGP on LPS-activated macrophages by determining NO production using Griess assay

Effects of the compounds on NO production by Griess assay were used to evaluate their inhibitory activities on LPS-activated macrophages. LPS-activated RAW 264.7 cells were treated with MPL, ABN and DDGP at 3 and 30 µM at 37°C for 24 h. A hundred µl supernatant of the treated cells in each well was collected to react with Griess reagents, 20 µl of sulfanilamide for 10 min followed by 20 µl N-1-napthylethylenediamine dihydrochloride for 10 min at room temperature in the dark. The azo dye product was measured at 540 nm by a

microplate reader. The concentration of nitrite in each well was determined from a standard curve of standard nitrite. The inhibitory effects on NO production of the test compounds were calculated as the percentage of inhibition as follow:

$$\%\ \text{NO inhibition} = \left\{\frac{\left[(\text{NO}_{\text{control}}) - (\text{NO}_{\text{test compound}})\right]}{(\text{NO}_{\text{control}})}\right\} \times 100$$

When control was the LPS-activated control.

The tested compound DDGP which inhibited NO production at non-cytotoxic concentrations was selected for determining its IC50 value on NO production in LPS-activated RAW 264.7 cells.

3.2.2. Determination of effect of MPL, ABN, DDGP on cell viability by resazurin reduction assay

In living cells, resazurin (dark-blue) is reduced by mitochondrial reductase enzyme to resorufin (pink). The remaining treated cells from NO production determination were incubated with 100 µl DMEM medium containing 50 µg/ml resazurin at 37°C for 4 h. the resorufin product in each well was measured at 570 nm and 600 nm by a microplate reader assay (68). The percentage of cell viability of the treated cells was determined by comparing to the 0.2% DMSO solvent control as follow,

$$\%\ \text{cell viability} = \left\{\frac{\left[(\Delta\text{OD}_{\text{test compound}})\right]}{(\Delta\text{OD}_{\text{control}})}\right\} \times 100$$

Δ OD = OD_{570}-OD_{600}

When control was 0.2% DMSO solvent control

The tested compound DDGP which inhibited NO production at non-cytotoxic concentrations was selected for evaluating its inhibitory effect on LPS-activated RAW 264.7 cells in detail.

3.2.3. Determination of effects of DDGP on the expression of inflammatory and anti-inflammatory mediators & involving enzymes in LPS-activated macrophages by qPCR

LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5 and 10 µM DDGP at 37°C for 4h and/or 24 h. Total RNA was isolated from the treated cells, reverse transcribed to complementary deoxyribonucleic acid (cDNA), and amplified genes of interest including pro-inflammatory cytokines (TNF- α, IL-1β and IL-6), inducible enzymes (iNOS, COX-2 and mPGES-1), chemokines (MCP-1 and MIP-1α), anti-inflammatory cytokines (IL-10, TGF-β) and anti-inflammatory mediator (arginase-1).

1) Isolation of total RNA

The treated cells were lysed and homogenized in 1000 µl/well TRIzol™ RNA isolation solution. The lysate samples were incubated at room temperature for 5 min, vigorously mixed with 200 µl chloroform 15 sec., incubated at room temperature for 3 min, and centrifuged at 12,000g, 4°C for 15 min. The total RNA in aqueous phase was precipitated by adding 500 µl isopropanol, incubated at -20°C for 1h, and centrifuged at 12,000g, 4°C for 10 min. The pellets were washed with 1 ml of 75% ethanol in DEPC treated water

and centrifuged at 7,500 g, 4°C for 10 min. The pellets were air-dried and dissolved in RNase-free water. The total RNA concentration of each sample was determined using Nanodrop^{TM}. The total RNA samples were stored at -80°C until used.

2) Synthesis of cDNA from total RNA samples by reverse transcription

cDNA was reversed transcribed from the total RNA sample using Impromt II reverse transcription kit by the following procedures. One µg of each total RNA sample was mixed with 1 µl oligo dT (15) primer. The mixture samples were heated at 70°C for 5 min, immediately chilled on ice for 5 min, and added with 15 µl transcription mixture solution containing 25 mM $MgCl_2$, 10 mM mixed dNTP, ribonuclease inhibitor and reverse transcriptase for synthesizing cDNA in a master cycler using the following conditions: 25ºC for 5 min, 42ºC for 90 min, and 70ºC for 15 min. The cDNA samples were stored at -20ºC until used.

3) Determination of gene expression by real time PCR

Expression of the genes of interest was determined from the cDNA samples as follow; One µl cDNA sample was mixed with 19 µl PCR reaction mixture solution containing specific primers for genes of interest (Table 3) and QPCR green master mix. The real-time PCR reaction was performed in StepOnePlus Real-Time PCR System with the following conditions; 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 30 sec., 60°C for 30 sec., and 72°C for 30 s. The expression of β-actin gene was used as a housekeeping gene

control. Expression of the genes was calculated using the $2^{-\Delta\Delta ct}$ method and indicated as the percentage changes from LPS-activated control.

Table 3 Primer sequences of the investigated genes in qPCR

Gene		Primer sequences	Length
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'	374
	R	5'-CCTGTAGCCCACGTCGTAGC-3'	
IL-1β	F	5'-CAGGATGAGGACATGAGCACC-3'	447
	R	5'-CTCTGCAGACTCAAACTCCAC-3'	
IL-6	F	5'-AGGACCAAGACCATCCAATTCA-3'	97
	R	5'-GCTTAGGCATAACGCACTAGG-3'	
MCP-1	F	5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'	274
	R	5'-TTCCTTCTTGGGGTCAGCACAGAC-3'	
MIP-1α	F	5'-GCCCTTGCTGTTCTTCTCTGT-3'	258
	R	5'-GCCCTTGCTGTTCTTCTCTGT-3'	
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAG-3'	496
	R	5'-GGTGTCAGAGCCTCGTGGCT-3'	
COX-2	F	5'-CACTACATCCTGACCCACTT-3'	696
	R	5'-ATGCTCCTGCTTGAGTATGT-3'	
mPGES-1	F	5'-CAGCCTAATGTTCAGCGACA-3'	565
	R	5'-CCTTGAGCTGACAGCCTACC-3'	

IL-10	F	5'-CATTTTGATCATCATGTATGCTTCT-3'	313
	R	5'-GGACTTTAAGGGTTACTTGGGTTGCC-3'	
TGF-β	F	5'-TGGACCGCAACAACGCCATCTATGAG-3'	525
	R	5'-TGGAGCTGAAGCAATAGTTGGTATCCA-3'	
Arginase-1	F	5'-GGGATTGGCAAGGTGATGGA-3'	287
	R	5'-GCCAAGGTTAAAGCCACTGC-3'	
B-actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

3.2.4. Determination of effects of DDGP on TNF-α, IL-1β, IL-6 and PGE2 production in LPS-activated macrophages by ELISA

LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 µM DDGP at 37°C for 24 h. The supernatants of the treated cells were collected for determining TNF-α, IL-1β, IL-6 and PGE2 concentrations by ELISA according to the manufacturer's instructions.

1) Sandwich ELISA for determining TNF-α, IL-1β and IL-6 production

An ELISA 96-well plate was coated with 100 µL/well captured antibodies against TNF-α, IL-1β or IL-6 solution overnight at 4°C. The coated wells were washed 3 times with washing buffer solution and blocked with 200 µL/well blocking solution at room temperature for 1 h. After washing the wells, 100 µl/well of the supernatants of treated cells and standard solutions of cytokines

of interest were added and incubated at room temperature for 2 h. After washing the wells 5 times, 100 µl/well detection antibody was added and incubated at room temperature for 1 h. After washing the wells 5 times, 100 µl/well streptavidin-HRP solution was added and incubated at room temperature for 30 min. After washing the wells 5 times, 100 µl µL/well 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for 15 min, then 50 µl/well 2 N H_2SO_4 was added to stop the reaction. The plate was measured at 450 nm by a microplate reader. TNF-α, IL-1β, or IL-6 concentration of each sample was determined from a standard curve.

2) Competitive ELISA for determining PGE2 production

By using pre-coated PGE2 captured antibody 96-well ELISA plate, 150 µl/well supernatant samples or PGE2 standard solutions were added, followed by 50 µl/well primary antibody solution. The plate was incubated at room temperature for 1 h. Fifty µl/well HRP-conjugated PGE2 was added and incubated for 2 h at room temperature. After washing the wells 4 times, 200 µl/well of TMB substrate solution was added and incubated at room temperature in the dark for 30 min and then 100 µl/well stop solution was added. The optical density of each well was determined at 540 nm using a microplate reader. PGE2 concentration of each sample was calculated from a PGE2 standard curve.

3.2.5. Determination of effects of DDGP on JNK/ERK, p38, p-JNK/p-ERK, p-p38, IKB and NF-κB p65 levels in LPS-activated macrophages by Western blot assay

LPS-activated RAW 264.7 cells were treated with 2.5, 5, and 10 μM DDGP at 37°C for 30 min. The treated cells were collected for isolating cellular proteins using RIPA lysis buffer, and for fractionating cytosolic and nuclear and proteins using cell fractionation kit. Protein concentration of each sample was determined using a protein assay reagent. For Western blot analysis, 20 μg protein of each sample was separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk in tris-buffered saline (TBS), incubated with primary rabbit monoclonal antibodies against JNK (dilution 1:1000), ERK (dilution 1:1000), p38 (dilution 1:1000), p-JNK (dilution 1:1000, p-ERK (dilution 1:1000), p-p38 (dilution 1:1000), IKB (dilution 1:1000), NF-κB p65 (dilution 1:1000) , β-actin (dilution 1:1000) and PCNA (dilution 1:1000) at 4 °C overnight, washed with TBS-Tween 20, incubated with HRP-conjugated secondary antibodies (dilution 1:2000) at room temperature for 2 h, and determined the density of proteins of interest using a chemiluminescence detector.

3.2.6. Data analysis

Data from at least three independent experiments were expressed as mean with standard error of mean (mean ± S.E.M.). The data of tested compounds were compared to the suitable control by using one-way analysis of variance followed

by Turkey's post hoc test for multiple comparisons. SPSS program version 22 was used to analyze all statistical analysis. The *p*-value less than 0.05 was considered as statistically significant.

CHAPTER IV

RESULTS

4.1. Searching for the compounds from GP extracts containing inhibitory activities on NO production in LPS-activated RAW 264.7 macrophages

Inhibition of NO production in LPS-activated RAW 264.7 cells was used for screening inhibitory potential of MPL, ABN, and DDGP. LPS-activated RAW 264.7 cells were treated with 3 and 10 µM of these compounds for 24 h and then the supernatant of the treated cells was collected for determining NO level by Griess reaction. The cytotoxic effects of the compounds were determined using resazurin reduction assay.

By comparing to the LPS control, all three test compounds, at 10 µM, significantly decreased NO production in LPS-activated macrophages (Figure 12). MPL, ABN, and DDGP suppressed NO level to 52%, 78%, and 19%, respectively. Ten µM dexamethasone decreased NO level to 42%. These test compounds inhibited NO production without cytotoxic effects on activated macrophages (Figure 13). Among the test compounds, DDGP demonstrated potent inhibitory effect on LPS-activated RAW264.7 cells. DDGP was selected for further study to assess its anti-inflammatory activity.

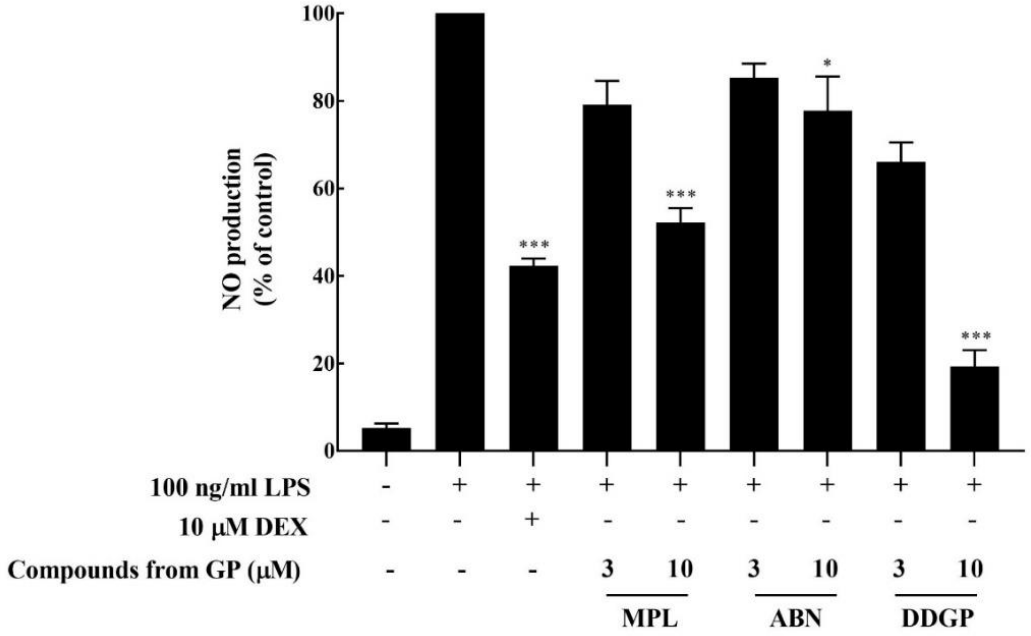


Figure 12 Effects of MPL, ABN, and DDGP on NO production in LPS-activated RAW 264.7 macrophages. The cells were treated with 3 and 30 μM of the test compounds. Level of NO in culture media was determined by Griess reaction assay. The data are presented as means ± S.E.M. of three independent experiments (n = 3). *p < 0.05 and ***p < 0.001 indicate statistical difference compared to the LPS control.

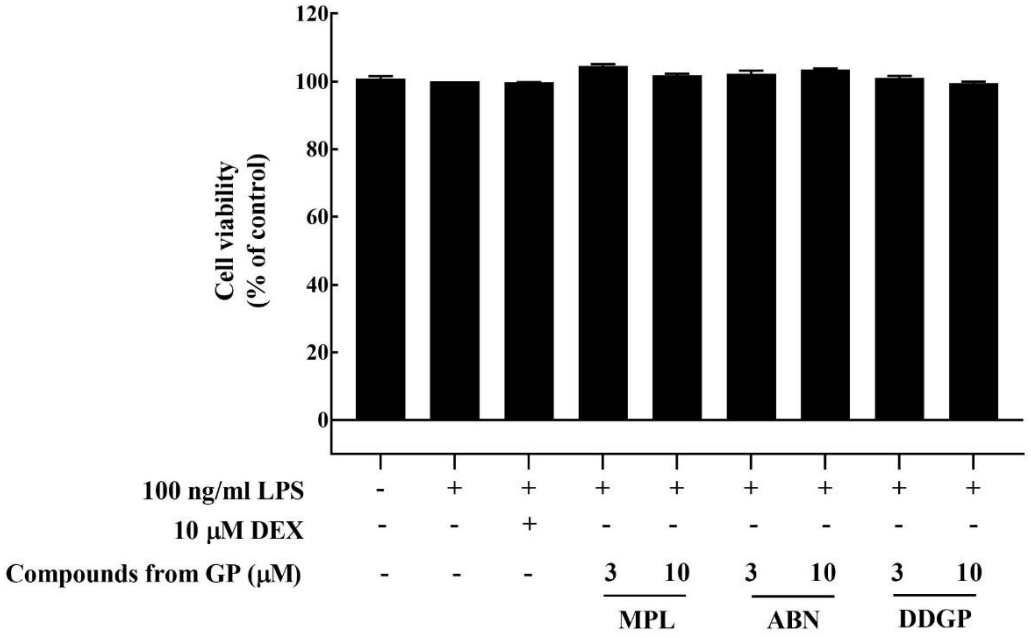


Figure 13 Effects of MPL, ABN, and DDGP on cell viability in LPS-activated RAW 264.7 macrophages. The cells were treated with 3 and 30 μM of the test compounds. Viability of the treated cells was measured by resazurin assay. The data are presented as means ± S.E.M. of three independent experiments (n = 3).

4.2. DDGP inhibited NO production and iNOS expression in LPS-activated RAW 264.7 macrophages

To determine anti-inflammatory activity of DDGP in detail, effect of this compound on NO production and iNOS expression in LPS-activated RAW 264.7 cells was evaluated. The LPS-activated cells were treated with 0.3125, 0.625, 1.25, 5, and10 µM DDGP for 24 h. NO production of the treated cells was determined. DDGP at 1.25-10 uM significantly inhibited NO production with its IC50 value at 3.47 ± 0.1 µM (Figure 14). The effect of DDGP on iNOS expression was also evaluated for revealing whether the suppression of NO production of DDGP was due to its effect on iNOS or not. LPS-activated RAW264.7 cells were treated with 1.25, 2.5, 5, and10 uM of DDGP for 24 h and the iNOS mRNA expression of the treated cells was determined by qPCR. As shown in Figure 15, DDGP at all concentrations significantly decreased the expression of iNOS when compared to the LPS control. The suppression of iNOS expression by DDGP was correlated to its inhibitory effect on NO production. These results indicate that DDGP suppresses iNOS expression leading to the reduction of NO production in LPS-activated RAW264.7 cells.

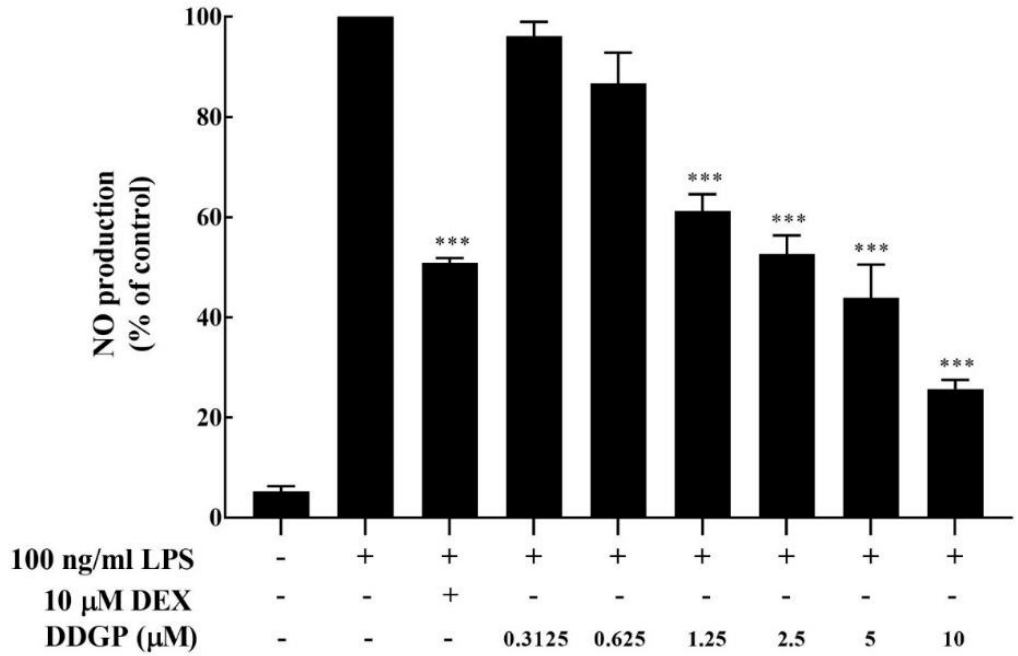


Figure 14 Effects of DDGP on NO production in LPS-activated RAW 264.7 macrophages. LPS-activated cells were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. Levels of NO in the cell culture media were determined by Griess assay. The data are presented as means ± S.E.M. of three independent experiments (n = 3). ***p < 0.001 indicates statistical difference compared to the LPS control.

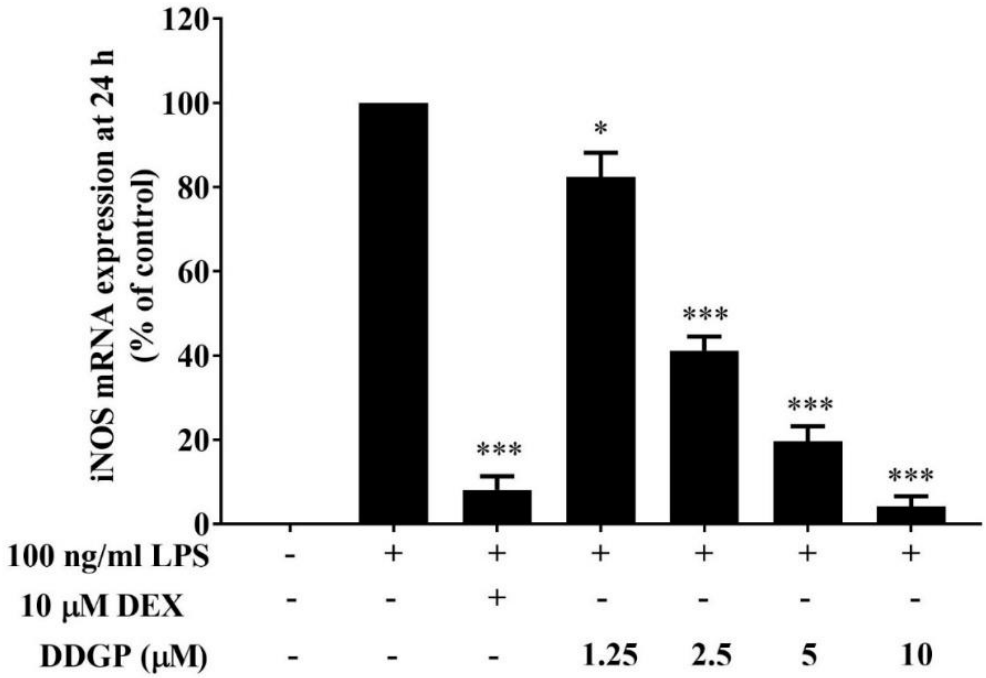


Figure 15 Effects of DDGP on iNOS expression in LPS-activated RAW 264.7 macrophages. LPS-activated cells were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. iNOS expression of the treated cells was determined by qPCR. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

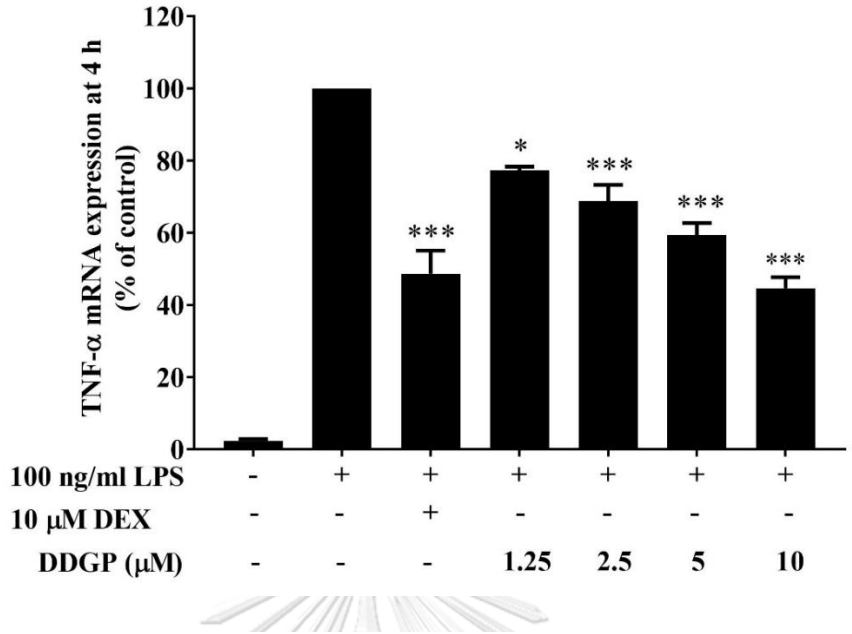
4.3. Effect of DDGP on the expression and the production of pro-inflammatory cytokines in LPS-activated RAW 264.7 macrophages

In chronic inflammation, pro-inflammatory cytokines from activated macrophages play important roles by inducing the production of many inflammatory mediators such as chemokines, PGs, and NO. LPS can induce these cytokine expression and production in the early event of LPS-induced macrophage activation. The effect of DDGP on these cytokines, including TNF-α, IL-6, and IL-1β were evaluated. LPS-activated RAW264.7 cells were treated with 1.25, 2.5, 5, and10 µM DDGP for 4 or 24 h for evaluating their mRNA expression by qPCR, and for 24 h for determining their protein levels in the supernatant of the treated cells by ELISA. The expression and the production of these inflammatory cytokines was almost undetectable in solvent-treated RAW264.7 cells without LPS stimulation (Figure 16-21).

1) **Effect of DDGP on TNF-α**: After 4 h of exposure, DDGP at 1.25, 2.5, 5, and 10 µM significantly suppressed the mRNA expression of TNF-α to 77.39±0.96%, 68.91±4.42%, 59.35±3.41%, and 44.62±3.13% respectively, when compared to the LPS control (Figure 16A). After 24 h of exposure, DDGP at 2.5, 5, and 10 µM significantly decreased the mRNA expression of TNF-α to 83.24±3.04%, 79.18±4.26%, and 70.11±4.18% respectively (Figure 16B). The suppression of TNF-α expression was correlated to the decrease of TNF-α level in the supernatant of the treated cells. DDGP at 1.25, 2.5, 5, and 10 µM significantly decreased the level of TNF-α to 90.17±3.02%, 65.54±0.64%,

47.40±1.27, and 37.13±1.46% respectively when compared to the LPS control (Figure 17). These results suggest that DDGP inhibits this cytokine at the transcription level leading to the decrease in the cytokine production in LPS-activated RAW264.7 cells.

(A)



(B)

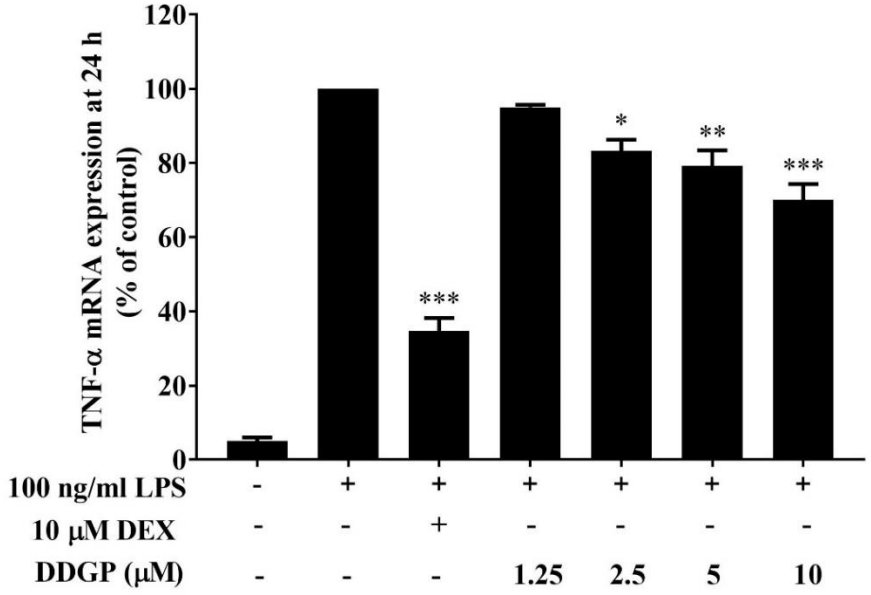


Figure 16 Effects of DDGP on TNF-α expression. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP. The expression of TNF-α was determined after 4h (A) and 24 h (B) of treatment by qPCR. The data are presented as means ± S.E.M. (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.001 indicate statistical difference compared to the LPS control.

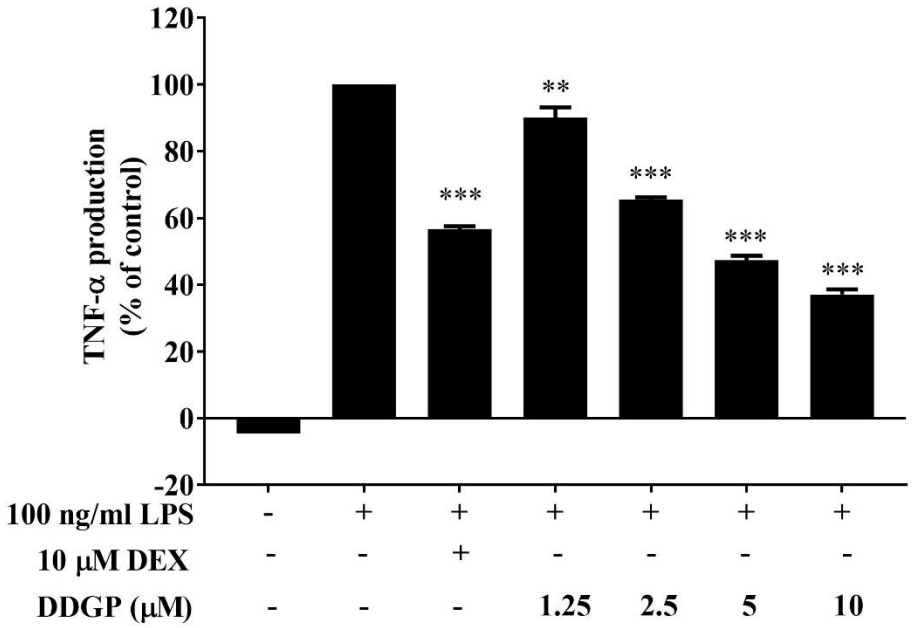


Figure 17 Effects of DDGP on TNF-α levels. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. Level of TNF-α in cultured media was determined by ELISA. The data are presented as means ± S.E.M. (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

2) **Effect of DDGP on IL-1**: After 4 h of exposure, DDGP at 1.25, 2.5, 5, and 10 µM significantly suppressed the mRNA expression of IL-1 to 71.35±5.30%, 58.61±5.71%, 49.11±3.49, and 18.04±5.15%, respectively, when compared to the LPS control (Figure 18A). After 24 h of exposure, DDGP at 2.5, 5, and 10 µM significantly decreased the mRNA expression of IL-1 to 56.84±5.76%, 18.46±4.89, and 2.01±0.75, respectively (Figure 18B). DDGP at 5 and 10 µM significantly decreased the level of IL-1 in the supernatant of the treated cells to 57.45±11.04% and 26.43±4.95%, respectively when compared to the LPS control (Figure 19). These results suggest that DDGP also inhibits IL-1 at the transcription level in LPS-activated RAW264.7 cells.

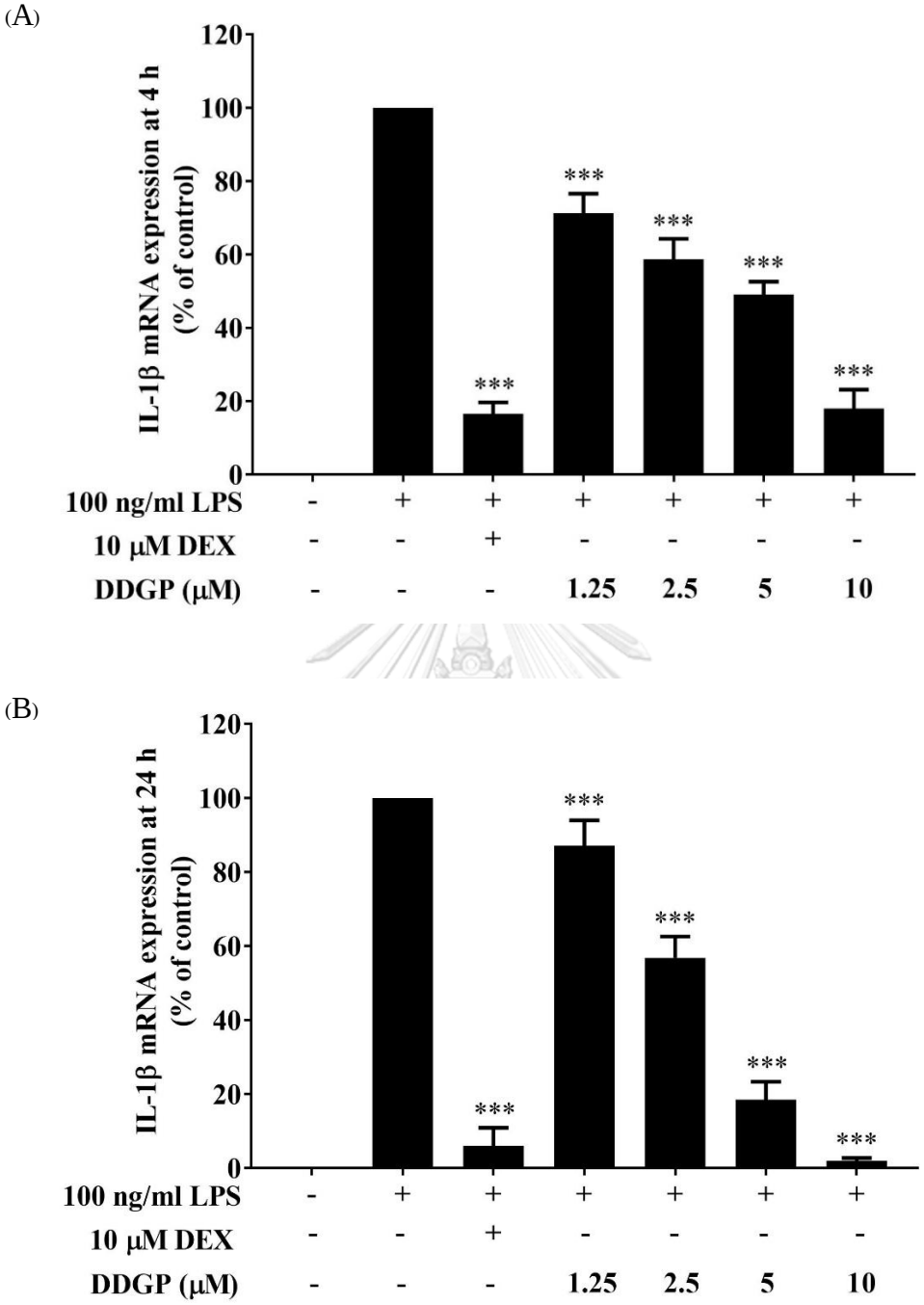


Figure 18 Effects of DDGP on IL-1β expression. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP. The expression of IL-1β was determined after 4h (A) and 24 h (B) of treatment by qPCR. The data are presented as mean ±

S.E.M. (n = 3). ***p < 0.001 indicates statistical difference compared to the LPS control.

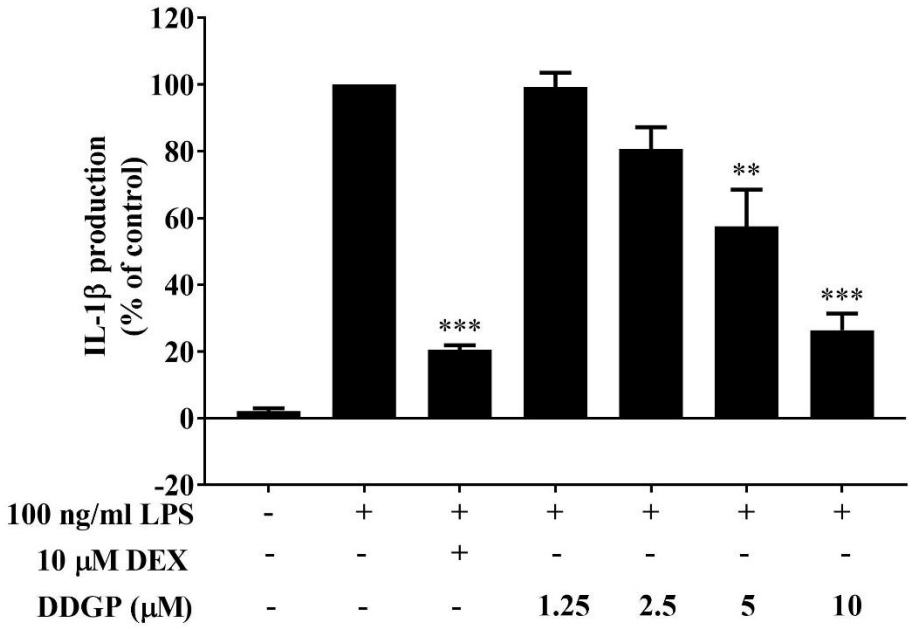
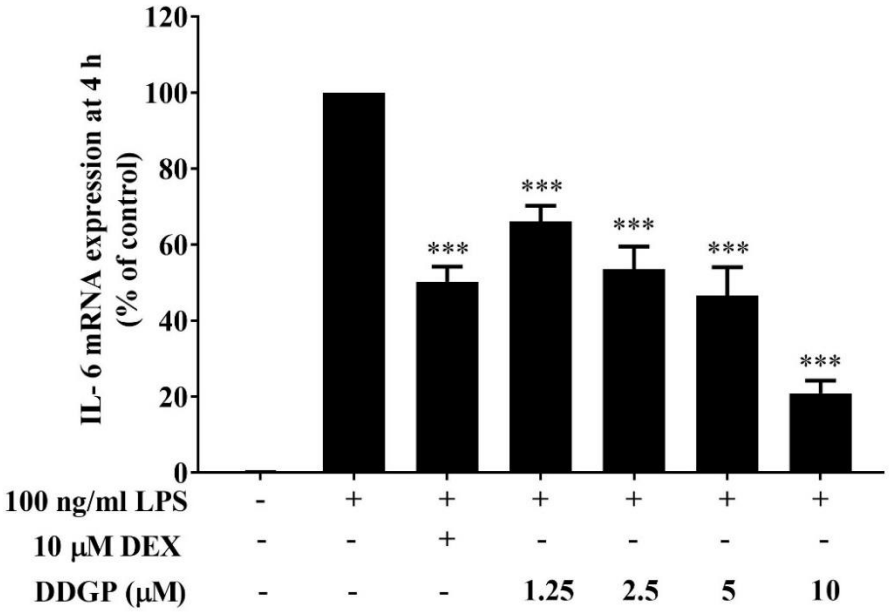


Figure 19 Effects of DDGP on IL-1β levels. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. Level of IL-1β in cultured media was determined by ELISA. Data are presented as means ± S.E.M. (n = 3). **p < 0.01 and ***p < 0.001 indicate statistical difference compared to the LPS control.

3) **Effect of DDGP on IL-6**: After 4 h of exposure, DDGP at 1.25, 2.5, 5, and 10 µM significantly suppressed the mRNA expression of IL-6 to 66.04±4.23%, 53.51±5.97%, 46.59±7.45%, and 20.87±3.36%, respectively, when compared to the LPS control (Figure 20A). After 24 h of exposure, DDGP at 1.25, 2.5, 5, and 10 µM significantly decreased the mRNA expression of IL-6 to 65.32±1.93, 52.40±5.64%, 38.22±3.44%, and 23.97±4.22%, respectively (Figure 20B). DDGP at 5 and 10 µM significantly decreased the level of IL-6 in the supernatant of the treated cells to 59.80±7.78% and 33.85±5.29%, respectively when compared to the LPS control (Figure 21). These results also suggest that DDGP inhibits IL-6 at the transcription level in LPS-activated RAW264.7 cells.

(A)



(B)

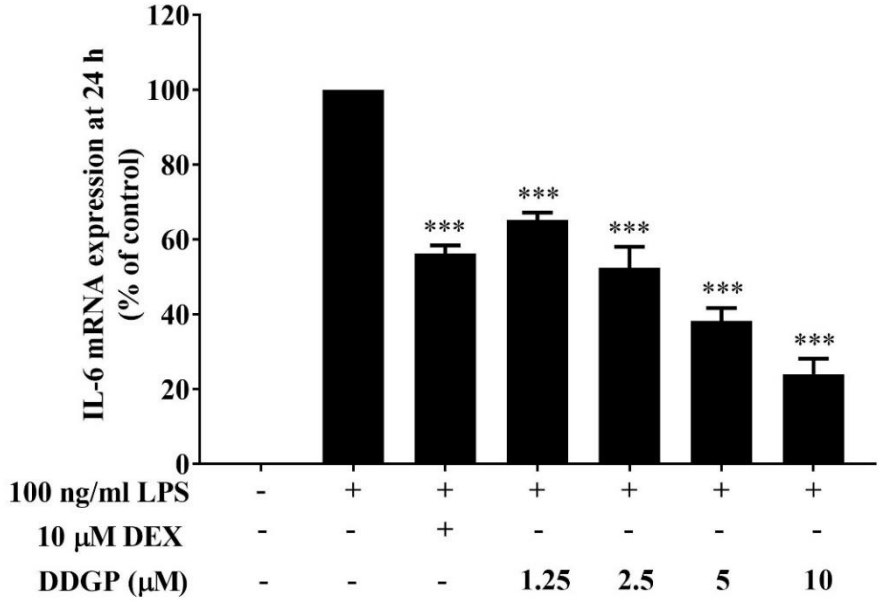


Figure 20 Effects of DDGP on IL-6 expression. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP. The expression of IL-6 was determined after 4h (A) and 24 h (B) of treatment by qPCR. The data are presented as means ± S.E.M. (n = 3). ***$p < 0.001$ indicates statistical difference compared to the LPS control.

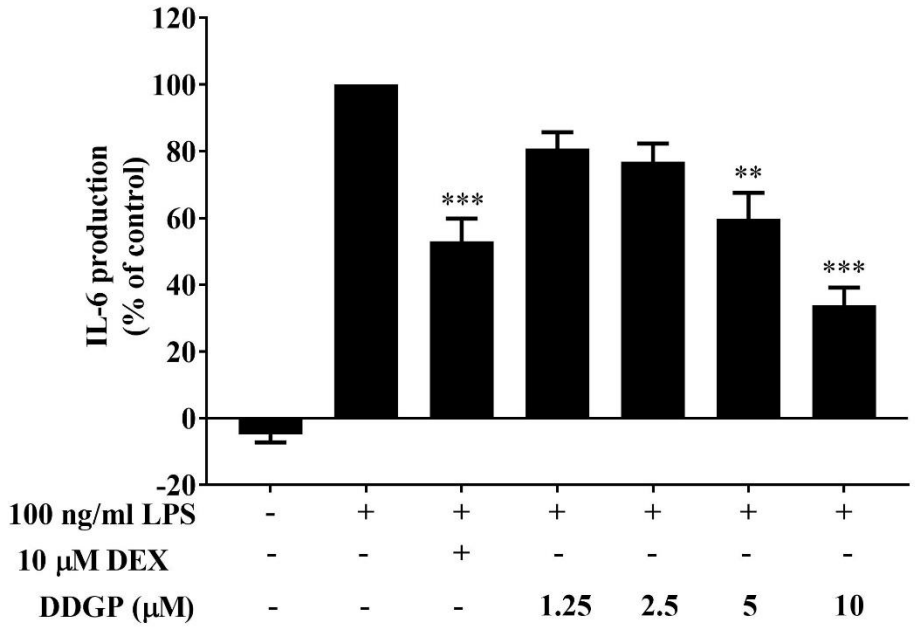


Figure 21 Effects of DDGP on IL-6 levels. LPS-activated RAW 264.7 cells were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. Level of IL-6 in cultured media was determined by ELISA. The data are presented as means ± S.E.M. (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

4.4. Effect of DDGP on the expression of chemokines in LPS-activated RAW264.7 macrophages

The inhibitory effect of DDGP on other inflammatory markers was also evaluated. Effect of the compound on the expression of macrophage chemokines MCP-1 and MIP-1α which play roles in leukocyte migration and recruitment was determined. LPS-activated RAW264.7 cells were treated with 1.25, 2.5, 5, and10 μM DDGP for 24 h for evaluating MCP-1 and MIP-1α mRNA expression by qPCR. DDGP at 5 and 10 μM significantly suppressed the mRNA expression of MCP-1 to 65.14±3.09% and 25.51± 8.96%, respectively (Figure 22). It also decreased the expression of MIP-1α to 64.20± 3.46% and 50.82± 8.81% at 5 and 10 μM, respectively (Figure 23).

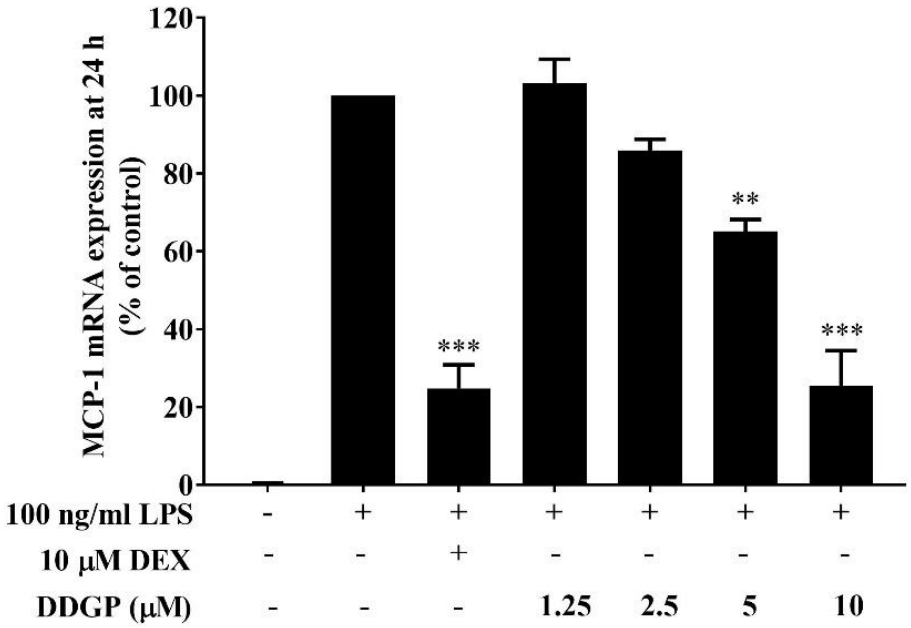


Figure 22 Effects of DDGP on the expression of MCP-1. LPS-activated RAW 264.7 macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The expression of MCP-1 was determined by qPCR. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

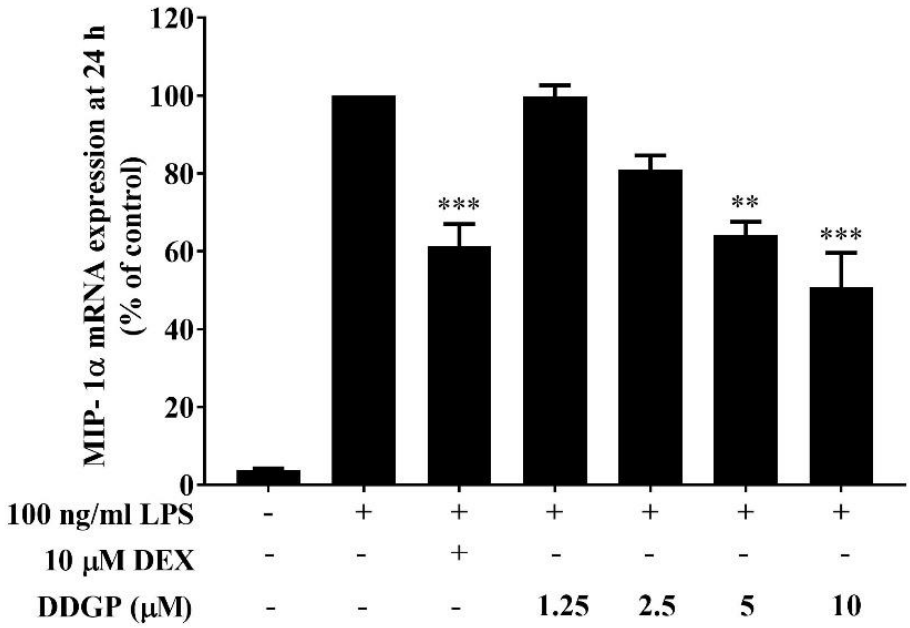


Figure 23 Effects of DDGP on the expression of MIP-1α. LPS-activated RAW 264.7 macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The expression of MIP-1α was determined by qPCR. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

4.5. Effect of DDGP on the expression of COX-2 and mPEGS-1, and the production of PGE2 in LPS-activated RAW264.7 macrophages

PGE2 is the most well-known inflammatory mediator and marker in inflammatory process. In activated macrophages, PGE2 is produced by metabolism of arachidonic acid using inducible enzymes COX-2 and mPGES-1. LPS and pro-inflammatory cytokines can activate macrophages and induce COX-2 and mPGES2 production from the activated cells. In this study, LPS-activated RAW264.7 cells were treated with 1.25, 2.5, 5, and10 µM DDGP for 24 h. Expression of COX-2 and mPGES-1 in the treated cells was determined by qPCR. PGE2 production in the supernatant of the treated cells was determined by ELISA.

DDGP at 5 and 10 µM significantly inhibited PGE2 production in LPS-activated RAW264.7 cells to 78.51±2.92% and 48.89±2.53%, respectively (Figure 24). This inhibitory effect of DDGP on PGE2 may come from the inhibition of COX-2 and/or mPGES-1 expression. These enzymes did not express in the solvent control without LPS stimulation (Figure 25-26). DDGP at 2.5, 5, and 10 µM suppressed COX-2 expression to 51.64±3.28%, 33.69±3.24%, and 10.49±0.50%, respectively, when compared to the LPS-control (Figure 24). The compound at 10 µM significantly decreased the expression of mPGES-1 to 45.65±3.21% (Figure 26).

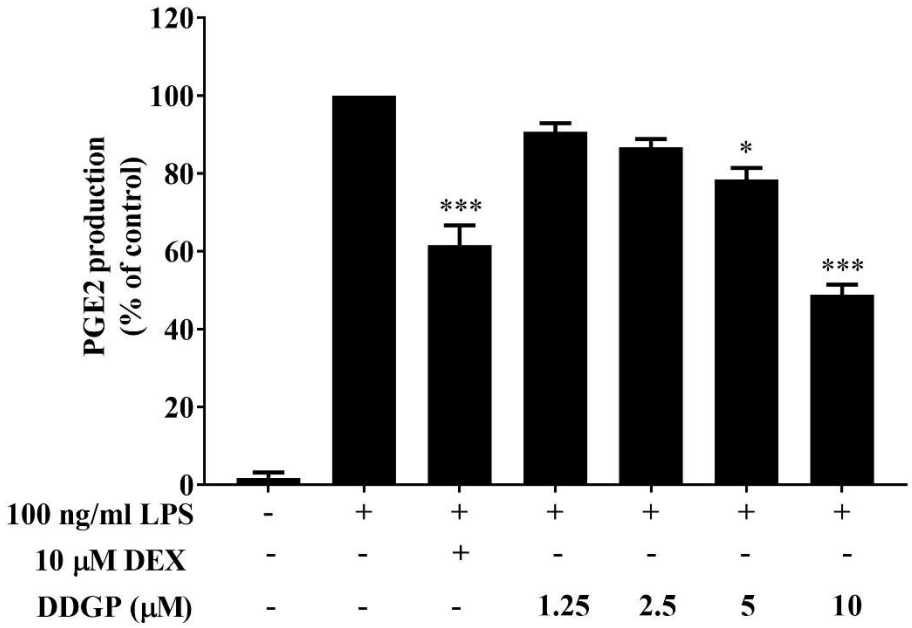


Figure 24 Effects of DDGP on the production of PGE2. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The level of PGE2 in the cultured media was determined by ELISA. The data are presented means ± S.E.M. of three independent experiments (n = 3). $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control.

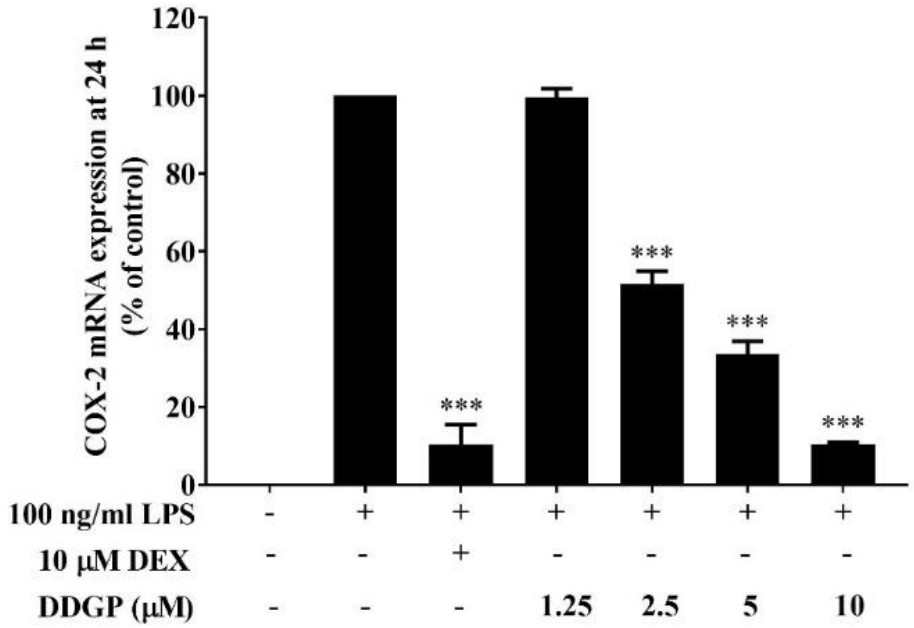


Figure 25 Effects of DDGP on the expression of COX-2. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The mRNA level of COX-2 was determined by qPCR. The data are presented means ± S.E.M. of three independent experiments (n = 3). ***$p < 0.001$ indicates statistical difference compared to the LPS control.

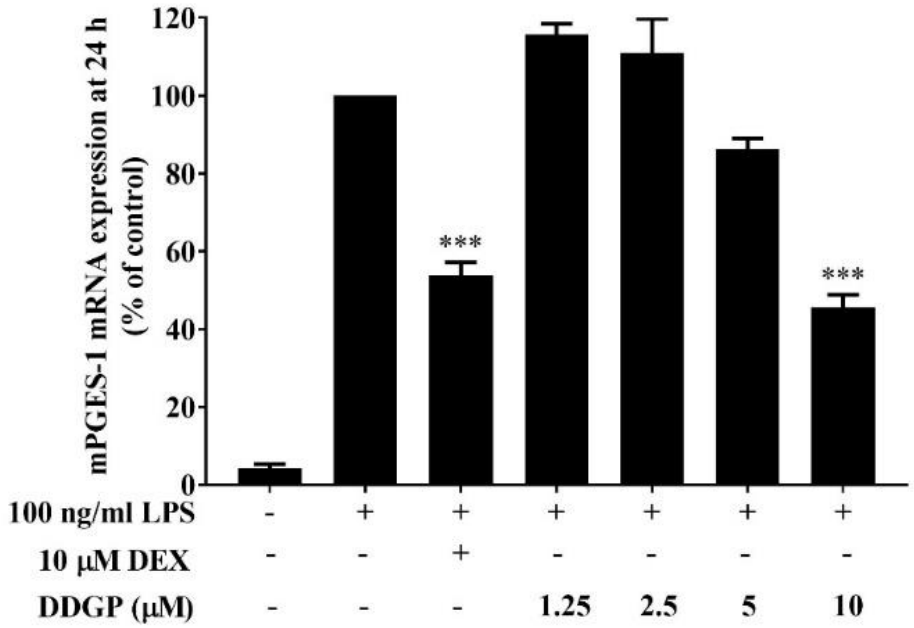


Figure 26 Effects of DDGP on the expression of mPGES-1. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The mRNA level of mPGES-1 was determined by qPCR. The data are presented means ± S.E.M. of three independent experiments (n = 3). ***$p < 0.001$ indicates statistical difference compared to the LPS control.

4.6. Effects of DDGP on anti-inflammatory cytokine and enzyme expression in LPS-activated RAW macrophages

LPS not only induces many inflammatory mediator productions but also activates the production of anti-inflammatory mediators such as IL-10 and TGF-β. During inflammation, arginase-1 is implicated to decrease NO production by competing for the same substrate L-arginine. To investigate effects of DDGP on IL-10, TGF-β, and arginase-1, LPS-activated RAW264.7 cells were treated with 1.25, 2.5, 5, and10 µM DDGP for 4 and/or 24 h. Expression of IL-10, TGF-β, and arginase-1 was determined by qPCR.

DDGP seemed to have little or no effects on anti-inflammatory mediators. Only 10 µM DDGP significantly decreased the expression of IL-10 after 4 and 24 h exposure (Figure 27). There was a trend of increase in TGF-β expression by DDGP but it was significantly increase only at 5 µM DDGP (Figure 28). The compound did not have effect on arginase-1 expression (Figure 29).

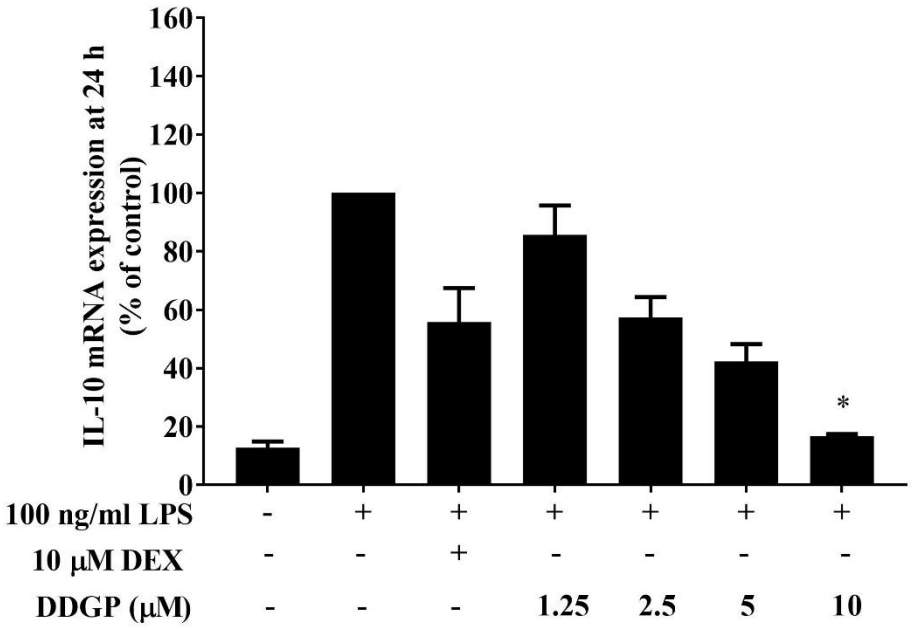


Figure 27 Effects of DDGP on the expression of IL-10. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The mRNA level of IL-10 was determined by qPCR. The data are presented means ± S.E.M. of three independent experiments (n = 3). *$p < 0.05$ indicates statistical difference compared to the LPS control.

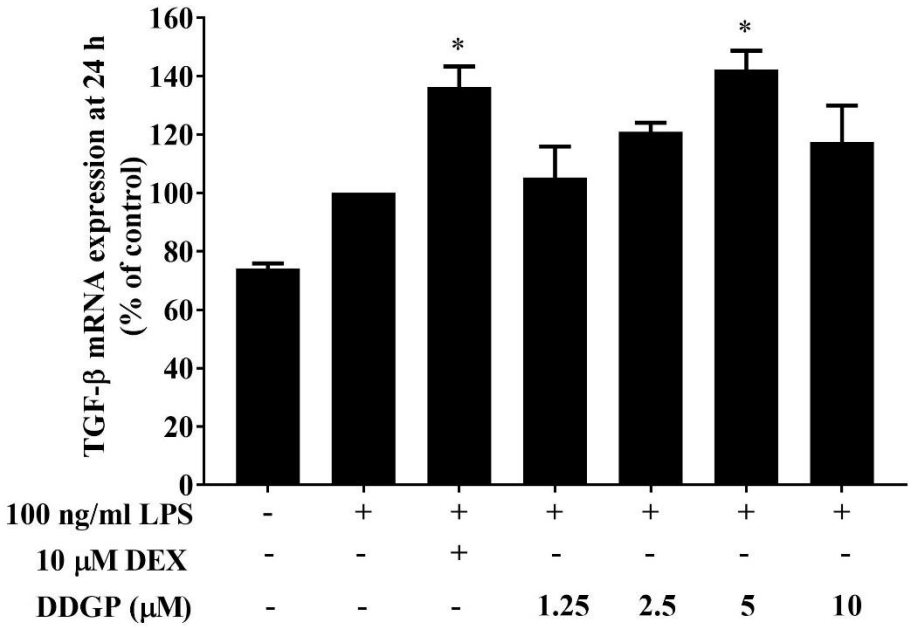


Figure 28 Effects of DDGP on the expression of TGF-β. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The mRNA level TGF-β was determined by qPCR. The data are presented means ± S.E.M. of three independent experiments (n = 3). *$p < 0.05$ indicates statistical difference compared to the LPS control.

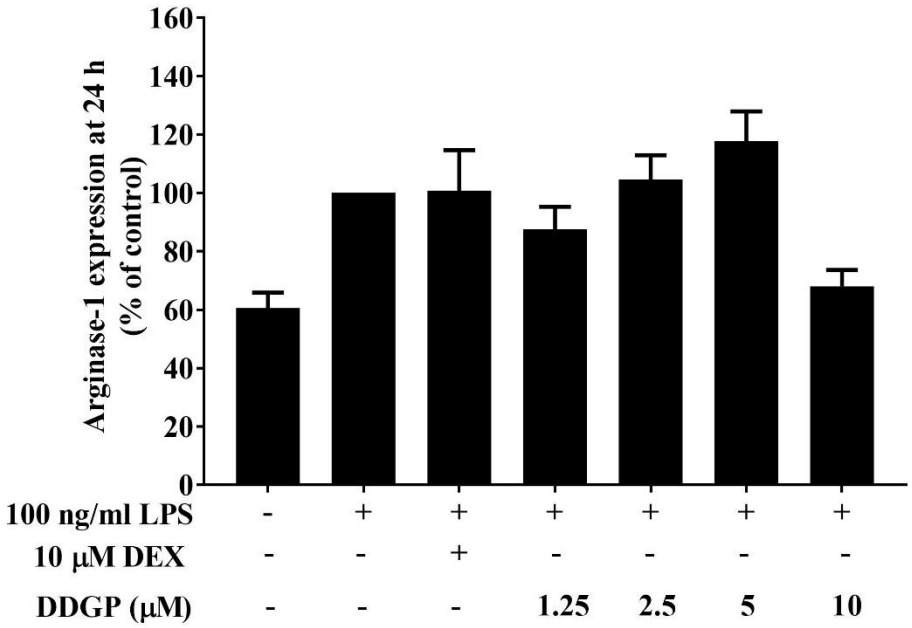


Figure 29 Effects of DDGP on the expression of arginase-1. LPS-activated macrophages were treated with 1.25, 2.5, 5, and 10 μM DDGP for 24 h. The mRNA level of arginase-1 was determined by qPCR. The data are presented mean ± S.E.M. of three independent experiments (n = 3).

4.7. Effect of DDGP on signaling molecules involve in LPS-induced macrophage activation.

The effect of DDGP on two main signaling pathways of the inflammatory response, MAPK pathways and NF- κB activation, was also investigated. LPS-activated RAW cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Cellular, cytoplasmic and nuclear proteins were used to investigate phosphorylation of MAPKs (ERK, JNK, and p38) and NF- κB activation by western blot analysis.

DDGP at 2.5, 5, and 10 μM almost completely inhibited the phosphorylation of p38. It decreased phosphorylated p-38 to 0.7±0.3, 0, and 0 fold, respectively, compared to the LPS control (Figure 30). It had little effect on ERK phosphorylation. Only 10 μM DDGP significantly decreased phosphorylated ERK to 0.39±0.07 fold (Figure 31). DDGP did not have effect on JNK phosphorylation (Figure 32) and NF-κB activation, determined by levels of cytosolic iκB and nuclear NF-κB (Figure 33 and Figure 34), in LPS-activated RAW264.7 cells.

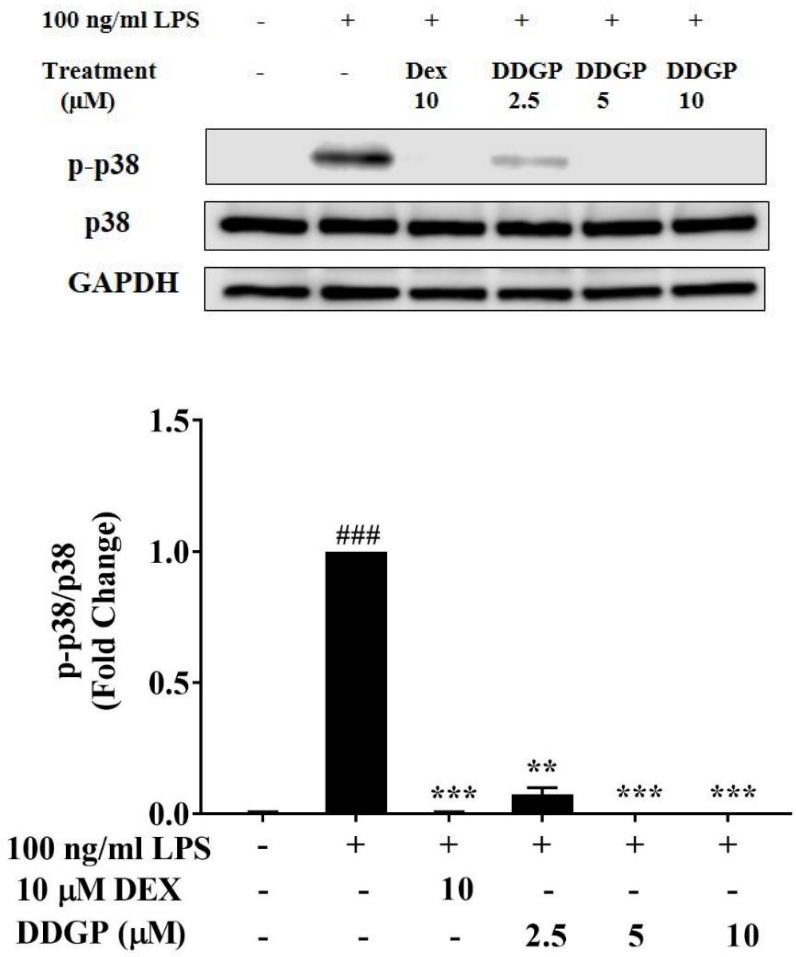


Figure 30 Effects of DDGP on LPS-induced activation of p38 in RAW264.7 cells. LPS-activated cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Cellular proteins were used to determined phosphorylation of p38 by western blot analysis. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference compared to the LPS control, and $^{###}p < 0.001$ indicates statistical difference compared to the solvent control.

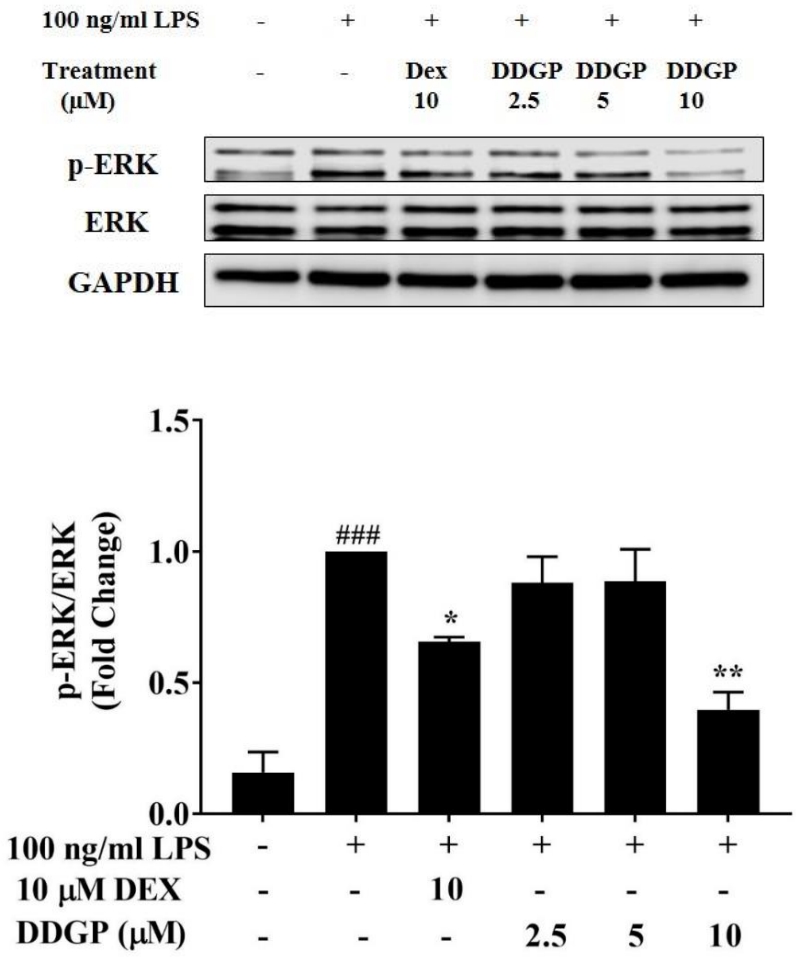


Figure 31 Effects of DDGP on LPS-induced activation of ERK in RAW264.7 cells. LPS-activated cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Cellular proteins were used to determined phosphorylation of ERK by western blot analysis. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference compared to the LPS control, and $^{###}p < 0.001$ indicates statistical difference compared to the solvent control.

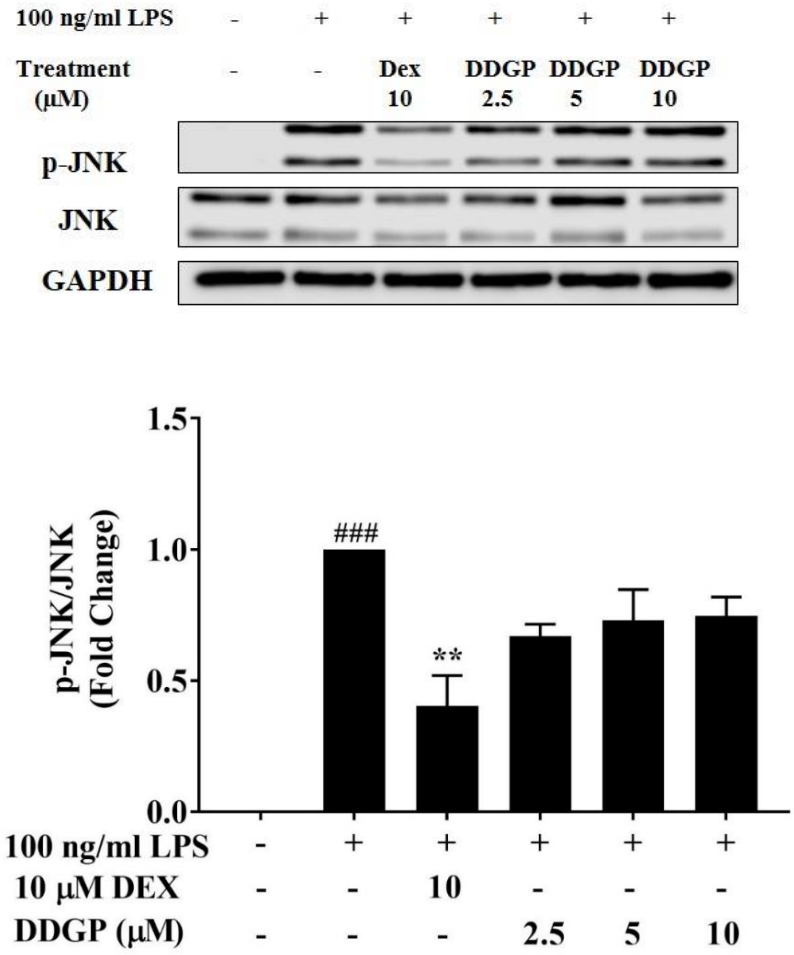


Figure 32 Effects of DDGP on LPS-induced activation of JNK in RAW264.7 cells. LPS-activated cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Cellular proteins were used to determined phosphorylation of JNK by western blot analysis. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{**}p < 0.01$ indicates statistical difference compared to the LPS control and $^{###}p < 0.001$ indicates statistical difference compared to the solvent control.

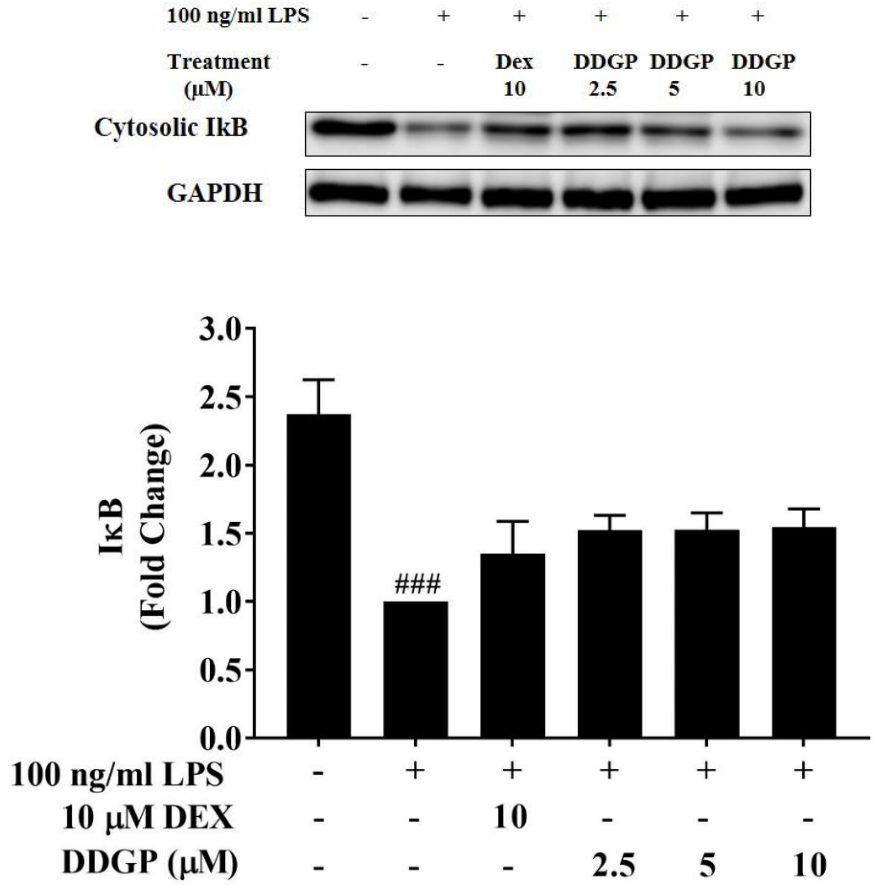


Figure 33 Effects of DDGP on LPS-induced IκB in RAW264.7 cells. LPS-activated cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Cytosolic proteins were fractionated and used to determine cytosolic IκB by western blotting. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{###}p < 0.001$ indicates statistical difference compared to the solvent control.

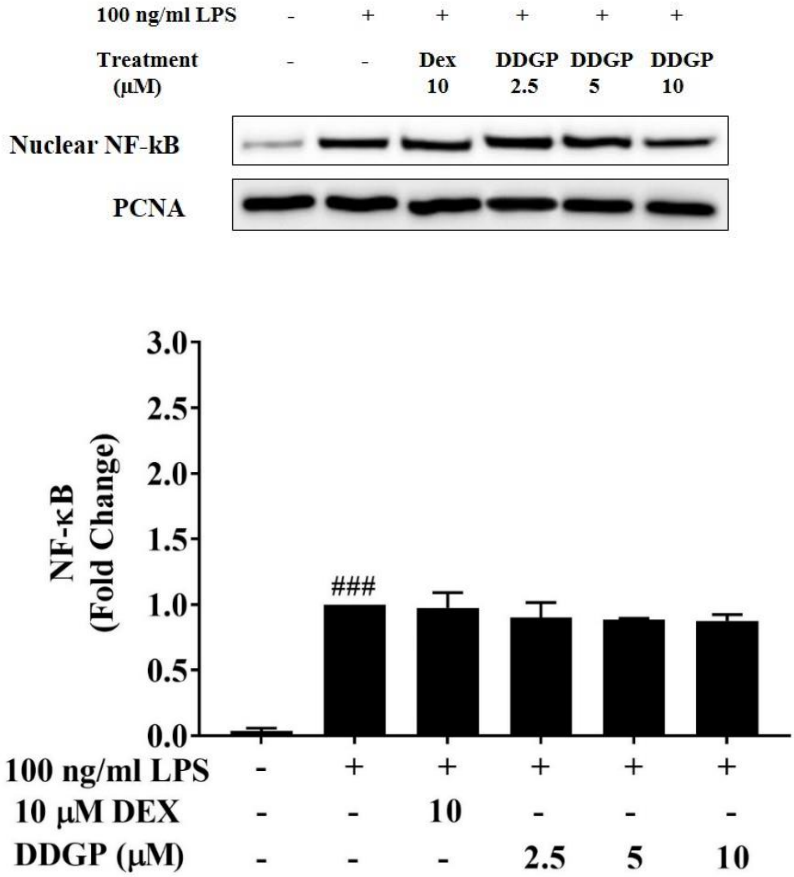


Figure 34 Effects of DDGP on LPS-induced NF-κB activation in RAW264.7 cells. LPS-activated cells were treated with 2.5, 5, and 10 μM DDGP for 30 min. Nuclear proteins were fractionated and used to determine NF-κB p65 in the nucleus by western blotting. PCNA were used as loading controls of nuclear protein. The data are presented as means ± S.E.M. of three independent experiments (n = 3). $^{###}p < 0.001$ indicates statistical difference compared to the solvent control.

CHAPTER V

DISCUSSION AND CONCLUSION

Activated macrophages and their products are key targets for developing novel anti-inflammatory agents. LPS-induced macrophage activation is a classical model used for evaluating anti-inflammatory activities of investigating compounds. LPS activates macrophages via TLR4 to generate pro-inflammatory cytokines which induces the production of chemokines, adhesion molecules, inducible enzymes iNOS and COX-2 for NO and PGE2 production, respectively. LPS-activated RAW264.7 macrophages were used in this study to evaluate anti-inflammatory effects and molecular mechanisms of actions of main compounds, ABN, MPL and DDGP, isolated from the ethyl acetate extracts from branches and leaves of *G. parva* (10). These extracts were preliminary evaluated to have potent inhibitory effects on the mRNA expression of pro-inflammatory cytokines, iNOS, and COX-2 (9). RAW264.7 macrophages were simultaneously activated with LPS and treated with the test compounds. Dexamethasone, a steroidal anti-inflammatory drug, was used as the positive control because it can inhibit the production of all inflammatory markers, pro-inflammatory cytokines, chemokines, iNOS and NO, COX-2 and PGE2 in this study.

Inhibition of NO production in LPS-activated macrophages was used to identify anti-inflammatory potential of ABN, MPL and DDGP. At non-toxic concentrations, only DDGP from the leaves of *G. parva* demonstrated potent inhibitory effect on NO production. DDGP at 10µM suppressed NO production to 19% when compared to the

LPS-activated control. ABN and MPL decreased the production to more than 50% at the same concentration. DDGP was chosen to investigate its inflammatory effect in detail.

DDGP potently suppressed NO production in LPS-activated RAW264.7 macrophages with IC50 3.47 ± 0.1 μM. It also inhibited the expression of iNOS. During inflammatory process, activated macrophages up-regulate iNOS expression for catalyzing L-arginine to NO (34). These results suggest that DDGP suppressed NO production via downregulating iNOS expression.

It has been reported that LPS as well as pro-inflammatory cytokines, TNFα and IL-1β, can induce iNOS expression via MAPK signaling pathways and NF-κB activation (24, 69). Excessive production of pro-inflammatory cytokines has been associated in many inflammatory diseases such as rheumatoid arthritis (70), inflammatory bowel diseases (71) and atherosclerosis (72). Thus, blocking the effect of pro-inflammatory cytokines could be an effective therapeutic strategy. In a previous study, the expression of pro-inflammatory cytokines predominantly was found to increase within 2-6 h after treatment by LPS and continues to decrease until 24-h (73). We evaluated the effect of DDGP on pro-inflammatory cytokines. DDGP decreased the production TNFα, IL-1β, and IL-6 by downregulating their gene expression. Production of these inflammatory cytokines is the early step of LPS induced macrophage activation. These cytokines amplify the activity of LPS by upregulating

the expression and production of chemokines, iNOS/ NO production, COX-2/ PGE2 production in activated cells (24). These results suggest that DDGP inhibits in the early step of LPS-induced macrophage activation.

DDGP downregulated the expression of chemokines MCP-1 and MIP-1α. These chemokines are potent chemokines from activated macrophages for recruiting monocytes and macrophages to inflammation areas leading to the continuation of the inflammatory process (30). DDGP also significantly downregulated the expression of COX-2 and mPGES-1, two inducible enzymes for PGE2 synthesis. This downregulation leads to the decrease of PGE2 production. In activated macrophage, COX-2 converts arachidonic acid to PGH2 and inducible mPGES-1 converts PGH2 to PGE2 (36). LPS-activated macrophages produce high levels of PGE2 at the sites of inflammation (74). PGE2 is a well-known inflammatory mediator which causes vasodilatation and has chemotactic activity at inflammation sites (36). These results support the possibility that DDGP may inhibit in the early step of LPS activation. It had inhibitory effects on many inflammatory markers. Elevation of anti-inflammatory mediators should have benefit for attenuating inflammatory process during chronic inflammation. This study also investigated the effects of DDGP on anti-inflammatory molecules, IL-10, TGF-β, and arginase 1 in activated macrophages. DDGP had little or no effect on modulating the expression of these molecules.

LPS acts through its TLR4 receptor to activate two main signaling pathways, the MAPK signaling pathways and AKT/NF-κB activation, to induce inflammation (41, 47-49, 69). Pro-inflammatory cytokines, TNF-α and IL-1, also act via these two signaling pathways to amplify the inflammatory process (24). MAPKs are protein serine/threonine kinases. There are 3 subtypes of these kinases; JNK, ERK, and p38 kinase. Many evidences revealed that activation of these kinases and NF-κB upregulated inflammatory cytokines and inflammatory enzymes (24, 47, 75). Several compounds demonstrated their anti-inflammatory effects via inactivating signaling molecules in these pathways (76-79). Inhibitory effects of DDGP on LPS-induced JNK, ERK, p38 MAPK and NF-κB activation were examined. DDGP profoundly suppressed phosphorylated p38 at all concentrations (1.25-10 μM). It had little effect on ERK phosphorylation and no effect on JNK phosphorylation. It did not significantly change levels of cytosolic iκB and nuclear NF-κB.

Several reports suggested that p38 activation is involved in inflammation by increasing the expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-12 , and COX-2 (80-82). A strong link between the p38 MAPK activation and many inflammatory diseases such as rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease was also reported (83-86) Several natural compounds exhibited anti-inflammatory activities in part by inactivating p38 phosphorylation (87-92). Moreover, some p38 inhibitory compounds are being under clinical studies for

treating chronic inflammatory diseases such as PH797804, Losmapimod, Pamapimod and BMS-582949 (93-96).

The results in this study suggest that DDGP inhibits LPS-induced macrophages activation mainly via inactivating p38 MAPK. Furthermore, DDGP may inhibit the early step of LPS-induced macrophages via inactivating p38 MAPK leading to the suppression of pro-inflammatory cytokines, chemokines, iNOS and COX-2 expression and production.

In summary, this study revealed for the first time that *S*-deoxydihydroglyparvin from *G. parva* inhibited LPS-induced macrophage activation by suppressing p38 activation. DDGP could be considered a potential anti-inflammatory agent.

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APPENDIX A

PREPARATION OF REAGENTS

1. DMEM stock solution (1L)

DMEM powder	10.4 g
$NaHCO_3$	3.7 g
ddH_2O	900 ml

Adjust pH to 7.4 with 1N HCl or 1N NaOH

Add ddH_2O to 1 L and sterilized by filtering through a 0.2 membrane filter

Store at 4°C

2. 1X Phosphase Buffered Saline (PBS) (1L)

NaCl	8.065 g
KCl	0.2 g
KH_2PO_4	1.15 g
ddH_2O	900 ml

Adjust pH to 7.4 with 1N HCl or 1N NaOH

Add ddH_2O to 1 L and sterilized by autoclaving

Store at room temperature

3. 1X 0.5 M Tris HCl pH 6.8 (Stacking buffer) 500 ml

Tris base	30.285 g
ddH_2O	350 ml

Adjust pH to 6.8 with 1N HCl or 1N NaOH

Add ddH_2O to 500 ml

Store at 4°C

4. 1X 1.5 M Tris HCl pH 8.8 (Separating buffer) 500 ml

Tris base	90.855 g
ddH2O	350 ml

Adjust pH to 8.8 with 1N HCl or 1N NaOH

Add ddH_2O to 500 ml

Store at 4°C

5. 2X Sample Diluting Buffer (200 ml)

10% SDS	80 ml
Glycerol	40 ml
0.05 % bromophenol blue	16 ml
Stacking buffer	50 ml

Add ddH_2O to 200 ml

Store at room temperature

6. 10X Running buffer (1L)

Tris base	30.28 g
Glycine	142.63 g
SDS	10 g

Add ddH_2O to 1 L

Store at 4°C

7. 1X Running buffer (1L)

10X Running buffer	100 ml
Add ddH_2O to 1 L	
Store at 4°C	

8. 10X Transfer buffer (1 L)

Tris base	30.28 g
Glycine	142.63 g
Add ddH_2O to 1 L	
Store at 4°C	

9. 1X Transfer buffer (1L)

10X Transfer buffer	100 ml
Add ddH_2O to 1 L	
Store at 4°C	

10. 10X Tris buffer Saline (TBS) 1 L

Tris base	24.2 g
Nacl	87.7 g
ddH_2O	700 ml
Adjust pH to 7.4 with 1N HCl or 1N NaOH	
Add ddH_2O to 500 ml	
Store at 4°C	

11. 1X TBS (1L)

10X TBS	100 ml

Add ddH_2O to 1 L

Store at 4°C

12. 0.1 % Tween-20 in Tris buffer Saline (TBST) 1 L

1X TBS	100 ml
Tween-20	1 ml

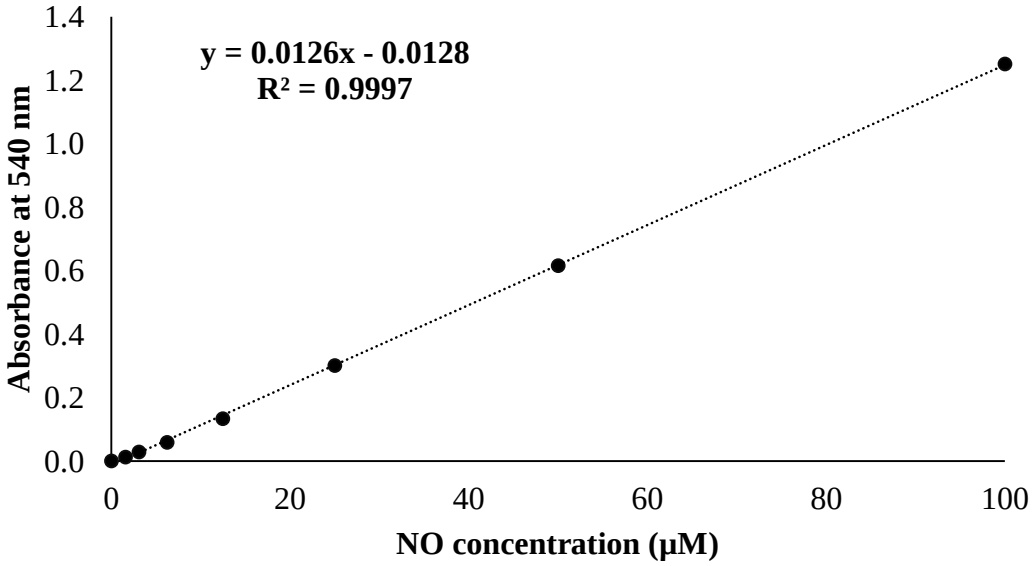
Add ddH_2O to 1 L

Store at 4°C

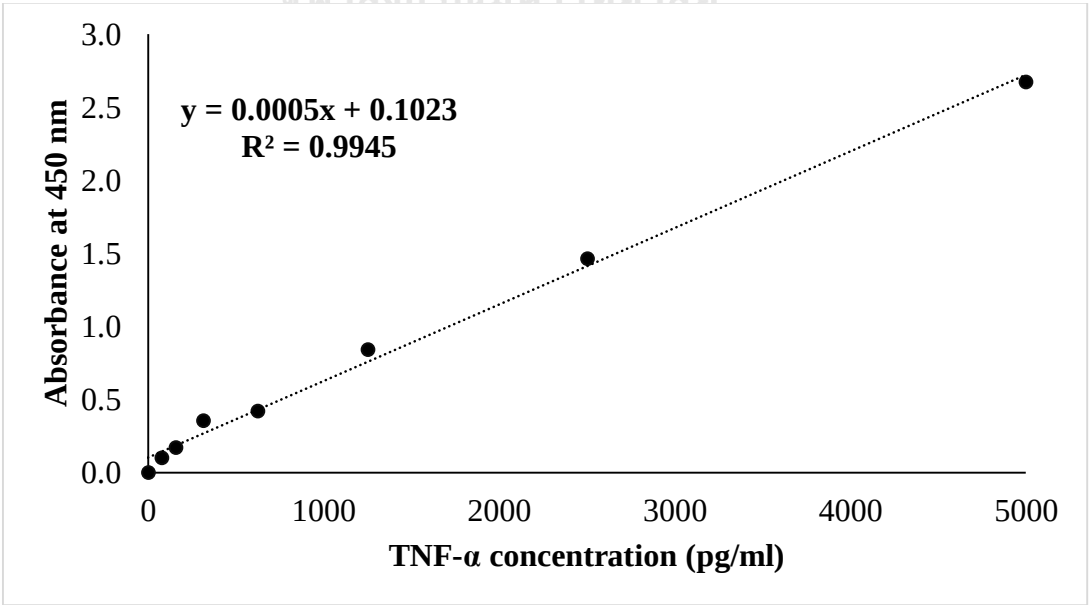
APPENDIX B

STANDARD CALIBRATION CURVES

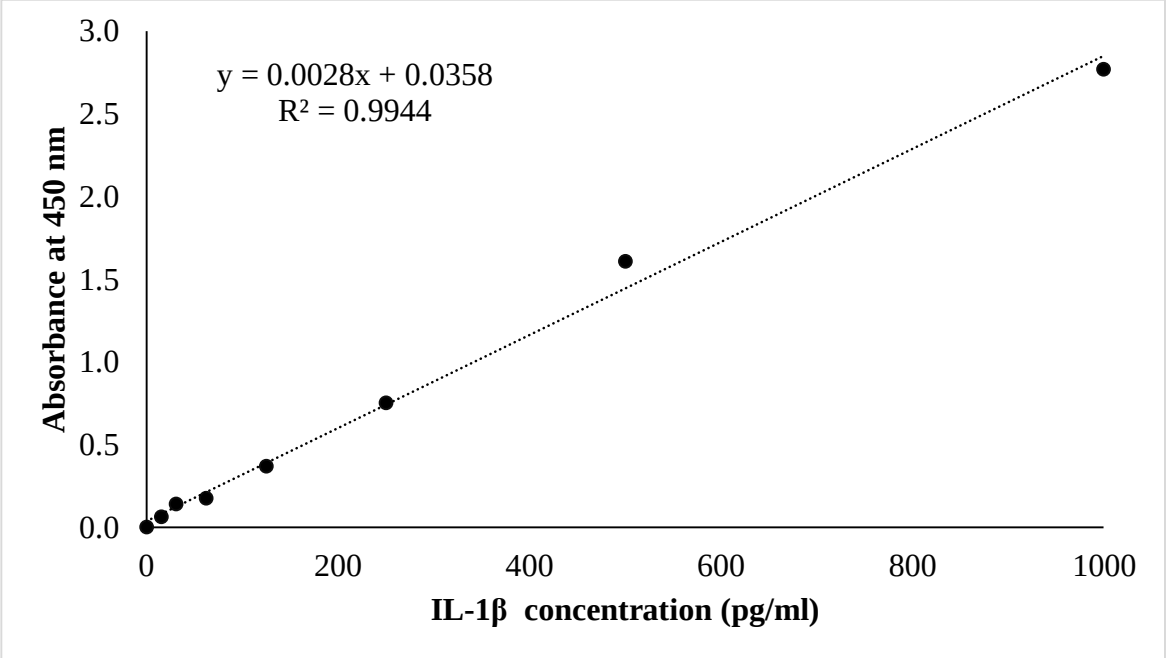
Appendix B1: Standard calibration curve for nitrite



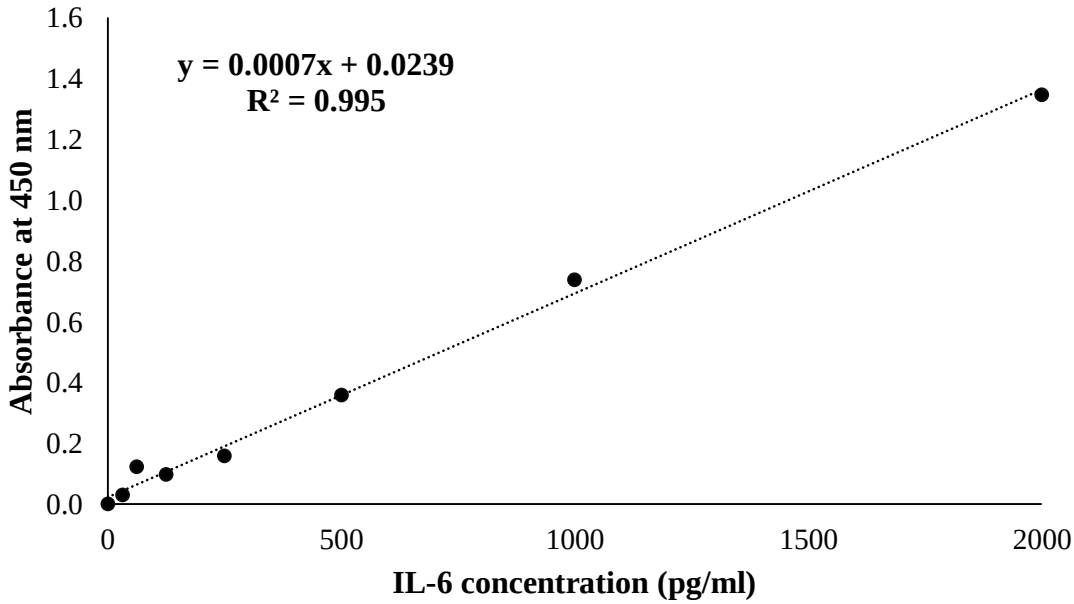
Appendix B2: Standard calibration curve for TNF-α



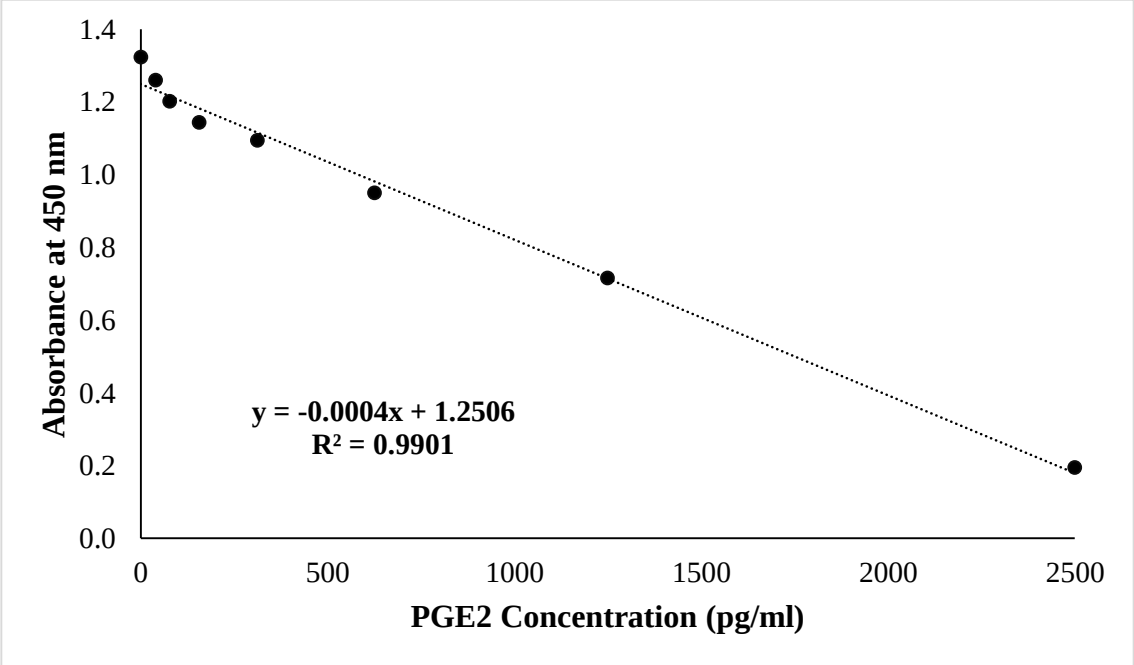
Appendix B3: Standard calibration curve for IL-1β



Appendix B4: Standard calibration curve for IL-6



Appendix B5: Standard calibration curve for PGE2



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