ฤทธิ์ของสิ่งสกัดเอทานอลจากตำรับยาวิสัมพยาใหญ่ต่อเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์

นางสาวสุณิษา ประสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ACTIVITIES OF THE ETHANOLIC EXTRACT OF WISUMPAYAYAI REMEDY ON LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES

Miss Sunisa Prasit

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	ACTIVITIES OF THE ETHANOLIC EXTRACT OF WISUMPAYAYAI REMEDY ON LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES
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สุณิษา ประสิทธิ์ : ฤทธิ์ของสิ่งสกัดเอทานอลจากตำรับยาวิสัมพยาใหญ่ต่อเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ (ACTIVITIES OF THE ETHANOLIC EXTRACT OF WISUMPAYAYAI REMEDY ON LIPOPOLYSACCHARIDE-STIMULATED MCROPHAGES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.จันทนี อิทธิพานิชพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี และ ผศ. ดร.วัชรี ลิมปนสิทธิกุล; 95 หน้า.

วิสัมพยาใหญ่เป็นตำรับยาสมุนไพรในบัญชียาหลักแห่งชาติที่ประกอบด้วยสมุนไพร 20 ชนิด ได้แก่ ลูกผักชีลา ลูกจันทน์ ดอกจันทน์ กระวาน กานพลู โกศสอ โกฐเขมา โกฐหัวบัว โกฐเชียง โกศจุฬาลัมพา อบเชย สมุลแว้ง สมอเทศ สมอไทย รากไคร้เครือ ว่านน้ำ บอระเพ็ด ขิงแห้ง พญารากขาว และดีปลี มีข้อบ่งใช้เพื่อแก้ท้องขึ้น อืดเฟ้อ จุกเสียด มีรายงานการศึกษาพบว่าสมุนไพรที่เป็นองค์ประกอบหลายชนิดมีฤทธิ์ต้านการอักเสบ แต่ยังไม่มีรายงานการศึกษาฤทธิ์ต้านการอักเสบของตำรับยาวิสัมพยาใหญ่ การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสิ่งสกัดเอทานอลของตำรับยาวิสัมพยาใหญ่ต่อเซลล์แมคโครฟาจ J774A.1 ที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์และศึกษาฤทธิ์ต้านอนุมูลอิสระโดย FRAP assay ผลการทดสอบพบว่าเมื่อให้สิ่งสกัดเอทานอลของตำรับยาวิสัมพยาใหญ่ที่ความเข้มข้น 12.5-100 µg/ml ก่อนและหลังการกระตุ้นเซลล์แมคโครฟาจ สามารถลดการเกิดกระบวนการจับกินสิ่งแปลกปลอมของเซลล์แมคโครฟาจเมื่อทดสอบด้วยวิธี zymosan-NBT reduction assay ได้ อีกทั้งมีฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ โดยมีค่า IC_{50} เป็น 40.77 และ 37.39 µg/ml ตามลำดับและไม่เป็นพิษต่อเซลล์แมคโครฟาจ และสิ่งสกัดที่ความเข้มข้น 25-100 µg/ml สามารถลดการแสดงออกในระดับ mRNA ของเอนไซม์ cyclooxygenase 2 (COX-2) และ inducible nitric oxide synthase (iNOS) ซึ่งเป็นเอนไซม์ที่ใช้ผลิตพลอสตาแกลนดินและไนตริกออกไซด์ตามลำดับ อีกทั้งสิ่งสกัดยังสามารถลดการแสดงออกของไซโตไคน์ IL-1β, IL-6, TNF-α และคีโมไคน์ MCP-1, MIP-1α, IL-8 ซึ่งเอนไซม์และไซโตไคน์เหล่านี้เป็นสารสื่อกลางการอักเสบที่ถูกกระตุ้นเมื่อเกิดการอักเสบ นอกจากนั้นสิ่งสกัดยังมีฤทธิ์ต้านอนุมูลอิสระได้สูงที่ความเข้มข้น 100 µg/ml ผลจากการศึกษานี้แสดงให้เห็นว่าสิ่งสกัดเอทานอลของตำรับยาวิสัมพยาใหญ่มีฤทธิ์ยับยั้งการทำงานของเซลล์แมคโครฟาจ ดังนั้นสิ่งสกัดเอทานอลของตำรับยาวิสัมพยาใหญ่น่าจะมีศักยภาพต้านการอักเสบได้

สาขาวิชา……เภสัชวิทยา……ลายมือชื่อนิสิต……

ปีการศึกษา…2554…….. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……..

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……

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SUNISA PRASIT: ACTIVITIES OF THE ETHANOLIC EXTRACT OF WISUMPAYAYAI REMEDY ON LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES. ADVISOR: ASSOC. PROF. CHANDHANEE ITTHIPANICHPONG, CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., ASSIST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D. 95 pp.

Wisumpayayai is one of the household traditional remedy approved by the Ministry of Public Health of Thailand in The National Drug List as anti-flatulence and anti-dyspepsia medication. It is composed of 20 herbal plants including; *Coriandrum sativum, Diospyros decandra, Myristica fragrans, Amomum krervanh, Syzygium aromaticum, Angelica dahurica, Atractylodes lancea, Conioselinum univitatum, Angelica sinensis, Artemisia pallens, Cinnamomum verum, Cinnamomum bejolghota, Terminalia arjuna, Terminalia chebula, Acorus calamus, Aristolochia* sp., *Tinospora crispa, Zingiber kerrii, Melastoma saugnineum, Piper retrofractum.* Some of the plants in this remedy have been reported to exert anti-inflammatory activities and a variety of the pharmacological properties. No evidences have been found in the anti-inflammatory effect of Wisumpayayai remedy. This study aimed to investigate the effect of the ethanol extract of Wisumpayayai remedy on lipopolysaccharide-stimulated macrophages and to determine its total anti-oxidant activities by FRAP assay. The results demonstrated that the ethanol extracts of Wisumpayayai remedy significantly inhibited phagocytic activity of the activated macrophages by zymosan-NBT reduction assay in a concentration-dependent manner (12.5-100 µg/ml). It also significantly inhibited nitric oxide (NO) production either by pretreatment and cotreatment of the extract and without cytotoxic effect to the cells. Their IC_{50} values for NO inhibition were 40.77 and 37.39 µg/ml for pre-treatment and co-treatment respectively. The extract 50 and 100µg/m significantly decreased mRNA expression of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS). These enzymes were responsible for PGE2 and NO production in the activated macrophages. Moreover, the extract 50 and 100 µg/ml were able to inhibit mRNA expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α as well as chemokines MCP-1, MIP-1α and IL-8. These enzymes and cytokines are inflammatory mediators produced during inflammatory process. The antioxidant assay showed high antioxidant activity of the extracts at 100 µg/ml. The results obtained from this study indicated that Wisumpayayai ethanol extract were able to inhibit activated macrophages functions. Therefore, this remedy may possess anti-inflammatory activity.

Field of Study:……Pharmacology……Student's Signature……...

Academic Year:……2011……... Advisor's Signature……...

Co-advisor's Signature……

Co-advisor's Signature……

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LIST OF ABBREVIATIONS

%	Percent
/	Per
<	Less than
=	equal
µ	Micro
µg	Microgram (s)
µl	Microliter (s)
µM	Micromolar
°C	Degree Celsius
5-LOX	5-lipooxygenase
AA	Arachidonic acid
APC	antigen-presenting cells
ATCC	American Type Culture Collection
Ca^{2+}	Calcium
CAMs	Cellular adhesion molecules
CAMs	Cell-adhesion molecules
$CD4^{+}$	Cluster of differentiation 4
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CO_2	Carbon dioxide
COX	Cyclooxygenases
CRP	Creactive protein
DEX	Dexamethasone
DMEM	Dulbeco's Modified Eagle"s Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate

eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
FRAP	Ferric Reducing Antioxidant Power
GI	Gastrointestinal
Glu	Glutamate
GM-CFU	Granulocyte/macrophage colony-forming unit
GPCRs	G protein-coupled receptor
GRE	Glucocorticoid response elements
H_2O_2	Hydrogen peroxide
HSC	Haematopoietic stem cell
HPA	Hypothalamic-pituitary –adrenal
IC_{50}	Inhibition concentration 50%
IFN- γ	Interferon-gamma
IgG	Immunoglobulin G
IkB-α	IkappaB-alpha
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
M	Molarities (mole per liter)
MCP-1	Monocyte chemotactic protein-1
M-CFU	Macrophage colony-forming unit
mg	Milligram (s)
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentrations
Min	Minute
MIP-1α	Macrophage inflammatory protein-1
Ml	Milliliter

MPO	Myeloperoxidase
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NED	N-1-napthylethylenediamine dihydrochloride
NF-kB	Nuclear Factor-KappaB
ng	Nanogram
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
O_2	Oxygen
OD	Optical density
OH^-	Hydroxyl radical
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGs	Prostaglandins
PLA	Phospholipase A
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
S.D.	Standard deviation
SAA	Serum amyloid A
Sec	Second
SRs	Scavenger receptors
TBE	Tris-borate-EDTA
TLRs	Toll-like receptors
TPTZ	Tripyridyltriazine

CHAPTER I
INTRODUCTION

Macrophages are important cells in the immune system. They play several essential roles both in innate and adaptive immune responses against pathogens. Macrophages are known to be key players in the inflammatory process, especially in chronic inflammation [1]. They are involved in initiation, maintenance and resolution of inflammation in tissue. They have at least three major functions including phagocytosis, antigen presentation, and immunomodulations [2]. Activated macrophages generate various pro-inflammatory cytokines and inflammatory mediators lead to acute inflammation and chronic inflammation.

Macrophages are major tissue phagocyte in innate immunity. Phagocytosis is the first step in the macrophages response to pathogens. They recognize pathogens by varieties of pattern recognition receptors (PRRs), such as toll like receptor (TLRs) family. TLR4 binds to lipopolysacharide (LPS) from Gram-negative bacteria and peptidoglycan from Gram-positive bacteria. In addition, macrophage receptors are also involved in fungal recognition. The C-type lectin receptors recognize fungal cell wall structures (manan and β-glucan) [3].

After recognizing pathogen, macrophages are activated and initiate phagocytosis. They engulf pathogens into phagosome which subsequently fuse with lyzosomes resulting in phagolysosomes for destroying the engulfed pathogens. These pathogen are degraded by oxygen-dependent and oxygen-independent mechanisms. Oxygen-dependent mechanisms require reactive oxygen (ROS) and nitrogen species (RNS). Nitric oxide (NO) catalyzed by inducible nitric oxide synthase (iNOS) is generated and act as toxic molecules to destroy microbes. Furthermore, oxygen-independent mechanisms require antimicrobial proteins and peptide (lysozymes, defensins) to hydrolyze microbes. Antigenic peptides are produced following enzyme degradation and they activate adaptive immunity [4, 5].

Macrophages become a professional antigen presenting cells (APCs). They present antigenic peptide by major histocompatability (MHC) to T cells receptor and express co-stimulatory molecules for generation of second signal to trigger T cells proliferation and production of cytokines. These cytokines stimulate cells in the immune system, or killing target cells such as infection and cancer [6].

Macrophages activation is a crucial in the immunomodulation. After pathogens recognition, macrophages become activated macrophages and produce several mediators, such as pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (IL-8, MCP-1, and MIP-1α) and inflammatory mediators (nitric oxide and prostaglandins). These mediators not only play roles in both innate and adaptive immune response but also induce neighbor tissue injury and bring on cardinal signs of inflammation (pain, edema, red and fever). The secretion of inflammatory cytokines and inflammatory mediators by activated macrophages are associated with some chronic inflammatory disease, such as rheumatoid arthritis (RA) and osteoarthritis [7].

There are two main classes anti-inflammatory agents, corticosteroids and non-steroidal anti-inflammatory drug (NSIADs). They inhibit production of pro-inflammatory cytokine and mediator production resulting in impaired inflammatory response. Gastrointestinal, liver and kidney disorder are associated with drug utilization [8]. Thai traditional medicines have been used for a long time as alternative treatment for relieving inflammatory symptoms since Thai people believe in the efficacy and safety of the compound drugs. Wisumpayayai is one of the household traditional remedy approved by the Ministry of Public Health of Thailand in National Drug List as anti-flatulence and anti-dyspeptic medication. It is composed of twenty herbal plants. There are several studies demonstrate that fourteen of the plants composition have anti-inflammatory activities. It is of interest whether this remedy possesses anti-inflammatory activity. The purpose of the present study is to investigate the *in vitro* anti-inflammatory activity of the ethanol extract of Wisumpayayai remedy on activated macrophages.

Objectives of the study

1. This study aimed to investigate the activity of the ethanol extract of Wisumpayayai remedy on LPS stimulated-macrophages J774A.1.

1.1 To examine the *in vitro* effect of the ethanol extract of Wisumpayayai remedy on NO production and phagocytic activity.

1.2 To investigate the effect of the ethanol extract of Wisumpayayai remedy on mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (MCP-1, MIP-1α and IL-8) and inflammatory enzymes (iNOS and COX-2)

2. To investigate the antioxidant activity of the ethanol extract of Wisumpayayai remedy

Hypothesis

1. The ethanol extract of Wisumpayayai remedy can inhibit LPS-stimulated macrophages activities by modulation of the mRNA expression of pro-inflammatory cytokines and inflammatory mediators.

2. The ethanol extract of Wisumpayayai remedy possesses antioxidant activity.

Research design

Experimental research

Keywords

Wisumpayayai remedy, Macrophages, Phagocytosis, Cytokines, Inflammatory mediator

CHAPTER II

LITERATURE REVIEWS

Macrophages

Macrophages are white blood cells of the mononuclear phagocytic system in tissue. They are generated from pluripotent hematopoietic stem cells (PHSC) in the bone marrow. Their precursor cells are in myeloid lineage. The growing and differentiation of macrophages depend on lineage-determining cytokines, macrophages colony-stimulating factor (M-CSF) and granulocyte colony stimulating factor (GM-CSF). They divide and differentiate into monoblasts and pro-monocytes before becoming monocytes and release into the peripheral blood, where they circulate with an estimated half-life of 8-9 hours. Monocytes constitutively enter and develop into macrophages in all tissue of the body. The differentiation of resident macrophages populations are based on their anatomical location, especially tissue-resident macrophages, such as alveolar macrophages (lung), osteoclasts (bone), histiocytes (interstitial connective tissue), Kupffer cells (liver) and microglia (central nervous system, CNS) adapted to their local microenvironment (Fig.1). [9].

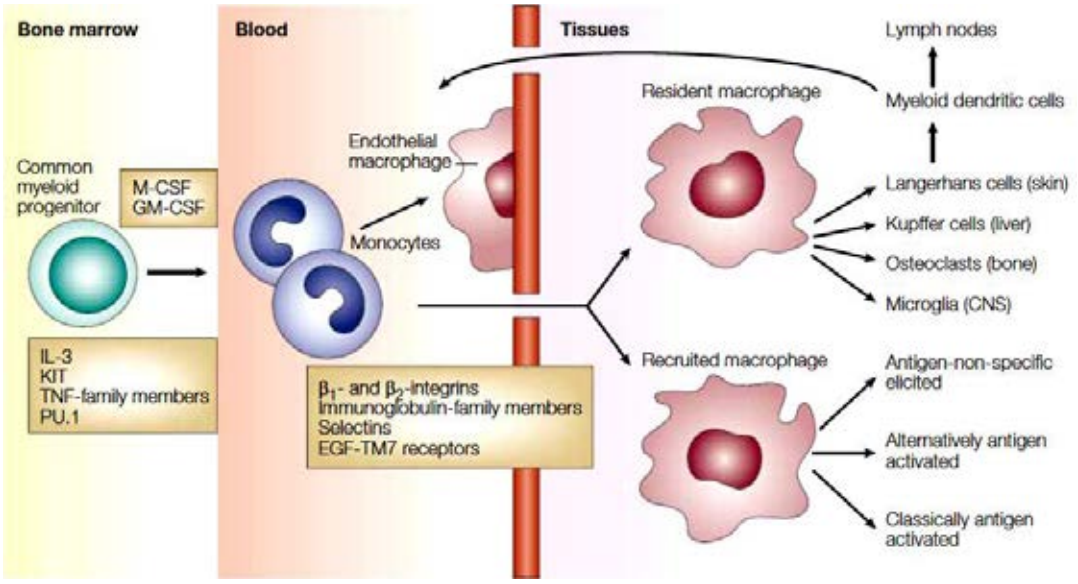


Figure 1: The Macrophages maturation processes [9].

Macrophages activation

Macrophages are important cells in both innate and adaptive immune responses. They play several essential roles such as foreign body eradication, degradation of pathogens or inflammatory response [10]. Normally tissue-resident macrophages are resting cells. Their normal functions are clearance of aged red blood cells and apoptotic cells without immune mediator productions and inflammation (Fig.2) [11]. Resting macrophages cells are wide-spread tissue distribution and respond to many endogenous stimuli (necrotic cells, tissue damaged and cytokines) and exogenous stimuli (microbial, allergens, irritants, foreign bodies and toxic compounds). They are activated and recognized LPS or cytokines (interferon-γ (IFN-γ)) by PRRs. TLR-4 are PRRs in response to LPS activation and exert intracellular signaling. After recognition of pathogen macrophages produce a variety of pro-inflammatory mediators for killing of microbes (Fig 3) [12-13].

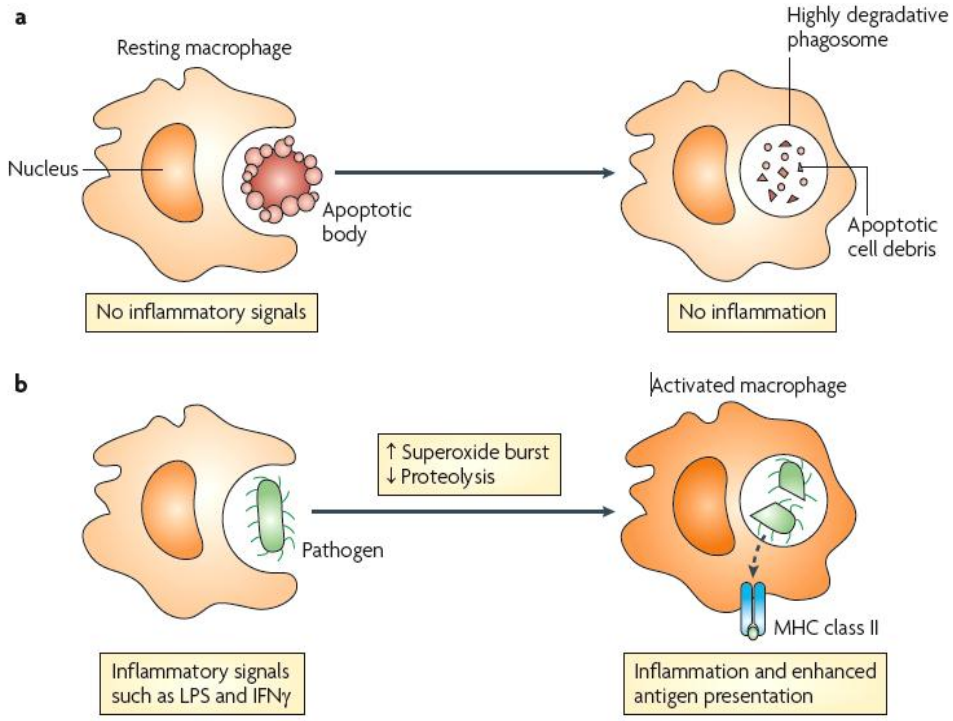


Figure 2: The phenotypic differences between resting and activated macrophages [11]

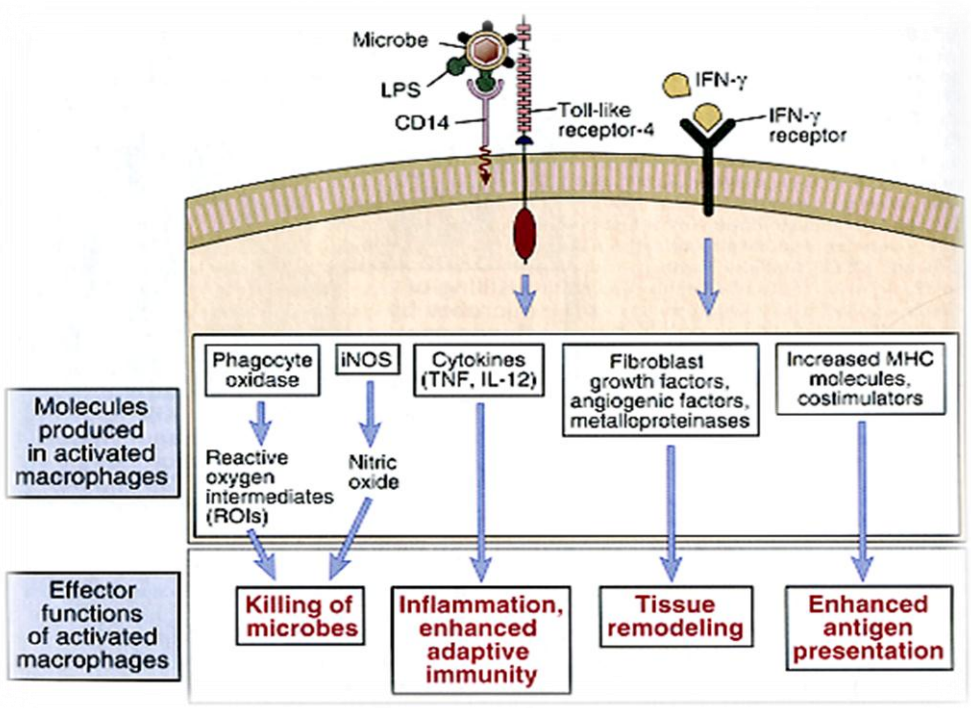


Figure 3: Macrophage activation mechanism [12].

Functions of macrophages

It is known that macrophage is key players in the inflammatory process, especially in chronic inflammation. They have at least three major functions which occur in sequential steps consisted of the recognition of pathogens or foreign particles and phagocytose them into phagosomes followed by destruction of engulfed foreign particles or microbial pathogens. Macrophages also act as APC to present the digested particles to T cells and express important molecules to activate T cells. After confrontation with pathogens, macrophages are activated and produce pro-inflammatory cytokines, chemokines and inflammatory mediators which serve in innate and adaptive immune responses and leading to acute and chronic inflammation [2].

The main function of activated macrophages

1. Phagocytosis

Macrophages are major tissue phagocytic cell in innate immunity responds to microbial pathogen or tissue injury. Phagocytosis is the first step confrontation with pathogen [14]. The destruction of pathogens by macrophages is a process to promote antigen for the adaptive immune response. In addition, phagocytosis causes release of the enzymes responsible for eliminating pathogens from neutrophils and macrophages [15].

The macrophages cells are activated by directly recognizing foreign particles or pathogen-associated microbial patterns (PAMPs) by varieties of PRRs. LPS is one of bacterial PAMPs to interact with PRRs. Furthermore, a fungal PAMP, zymosan presented in the cells wall of *Saccharomyces cerevisiae* is also interact with PRRs. LPS and zymosan are widely used for study immune function both *in vitro* and *in vivo* [16]. After macrophages receptors bind to their particulate ligands which are constituent of cells, intracellular signals are generated to trigger phagocytosis of the particles. The particles are engulfed into phagosome vesicles. These phagosomes can fuse with lyzosomes to become phagolysosomes where the engulfed particles are destroyed by oxygen-dependent and oxygen-independent mechanisms [17-18].

The macrophages receptors

Macrophages express diverse receptors react to natural and altered-self components of microbes. Example of these receptors are; (Fig.4) [19].

(1) The mannose receptors (MRs) and Scavenger receptors (SRs)

Mannose receptors recognize a wide range of gram-negative and gram-positive bacteria, yeasts, parasites and mycobacteria. MRs are PRRs present on the surface of macrophages and dendritic cells. They recognize mannose, N-acetylglucosamine that are located on the surfaces of tissue. These receptors are associated with a signal transduction pathway and exhibit phagocytosis and cytokine production. Scavenger

receptors (SRs) are trimeric transmembrane glycoprotein expressed by macrophages. Some of these receptors are involved in phagocytosis of both microorganism and apoptotic cells. SR-AI, SR-AII and MARCO are SRs invole in recognition of PAMPs of microbes. [20].

(2) Receptor for opsonins (Fc-receptors (FcRs) and complement receptors (CRs)) recognize opsonins coated pathogens. These opsonins are wide range of proteins including antibodies, complement proteins and lectin. They provide the opsonization for allowing the more visible coated pathogens to phagocytic cells. These receptors promote phagocytic mechanisms and subsequent cellular responses [21].

(3) Toll-like receptors (TLRs) are found in macrophages, dendritic cells (DCs), B cells, specific types of T cells. They activate leukocytes in response to different types and components of microbes. At present, there are 13 members of TLRs in mammals. In humans, 10 members (TLR1 to TLR10) have been identified. Different TLRs play essential roles in cellular response to bacterial product such as LPS, endotoxin, proteoglycans, and unmethylated CpG nucleotides. TLRs' functions are mediated through receptor-associated kinases to stimulate the production of anti-microbial substances and cytokines in the leukocytes. [22]

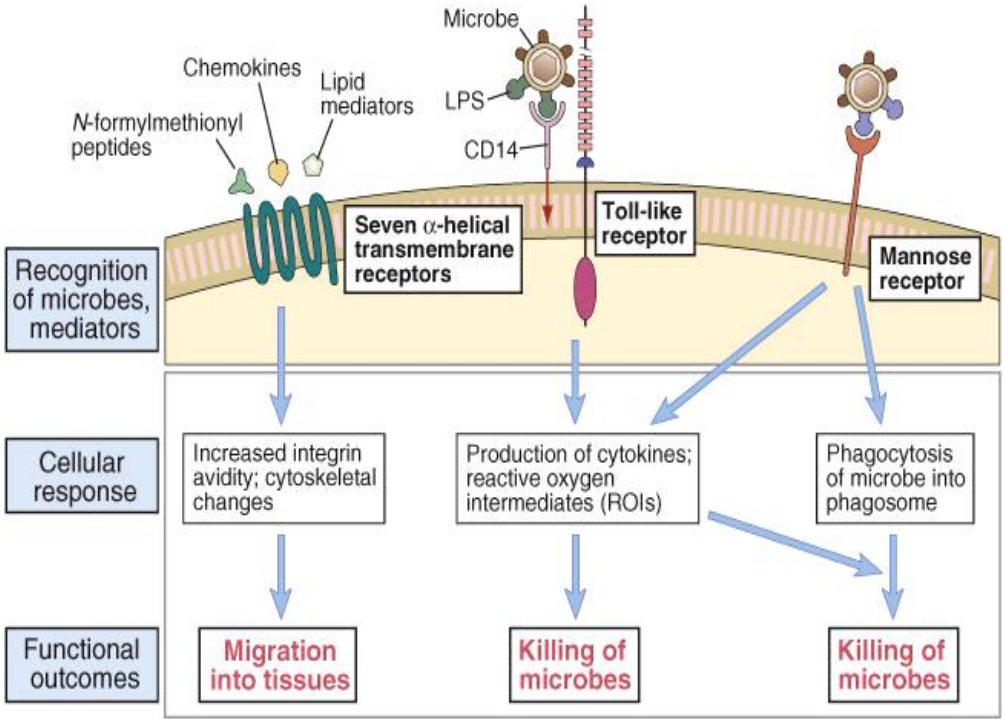


Figure 4: Cells surface receptors of macrophages. [19].

The steps of phagocytosis

Phagocytosis occurs in sequential steps. The process of phagocytosis is consisted of recognition of microbial pathogens or foreign particles by varieties of receptors, engulfment through triggering of different signaling pathway, and killing microbes by several mechanisms in phagolysosome (Fig.5) [23].

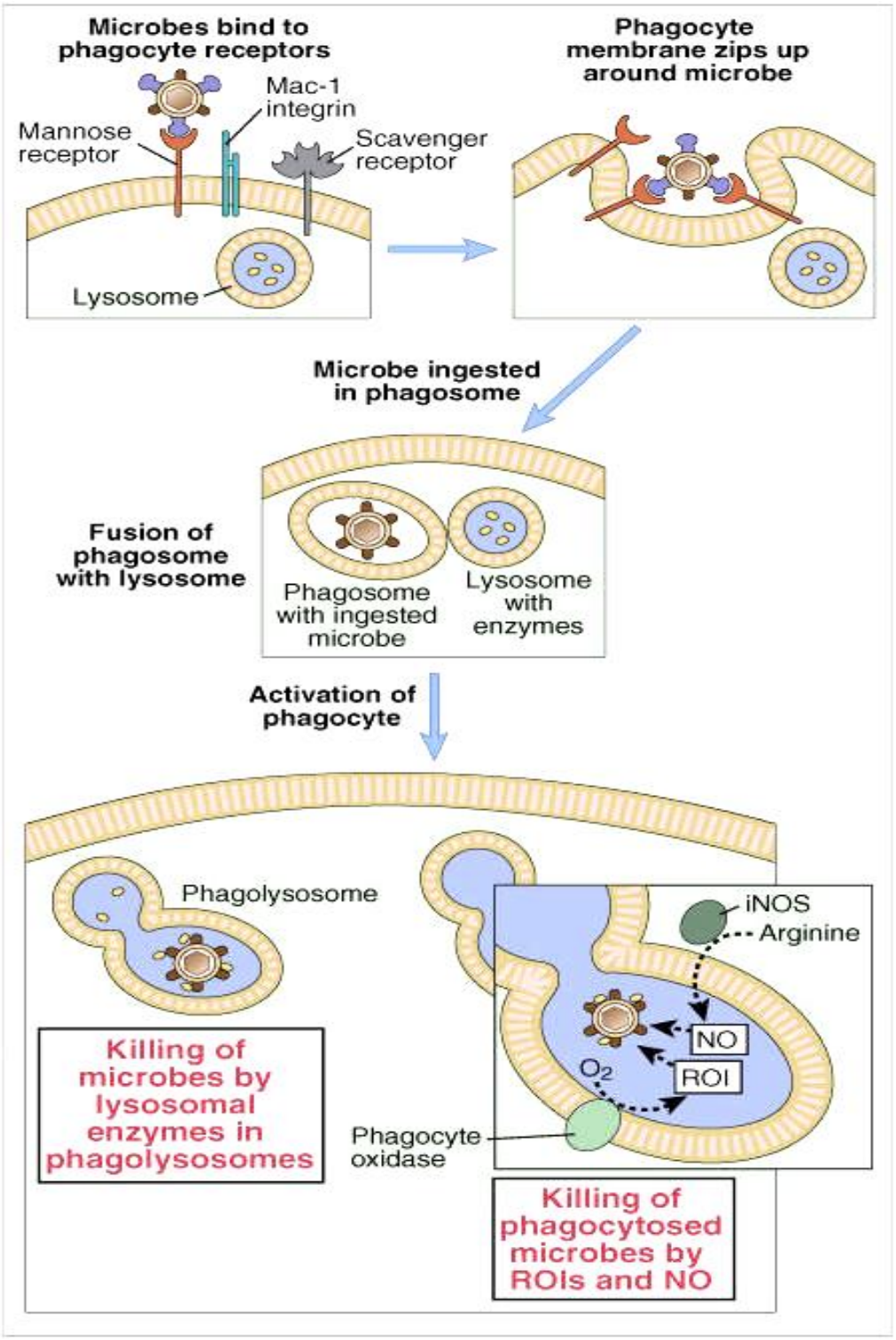


Figure 5: Steps of microbe phagocytosis by macrophages [23].

1. Recognition of microbial pathogens and foreign particles. When pathogens or microorganisms enter the body, macrophages recognize them by PRRs and engulf into phagosomes.

2. After phagocytes engulf the recognized pathogens into phagosomes, they fuse with lyzosomes to become phagolysosomes with the pH of 4.5–5.0. Lysosomes are in golgi apparatus of leukocytes that contain various digestive enzymes and toxic oxygen radicals to destroy the pathogen. In addition, during particle-receptor binding, intracellular signals are activated to assign phagocytosis and regulate internalization. The signal generated during phagocytosis may support the activation or inhibition of phagocytosis and microbe-induced responses (Fig.6) [24].

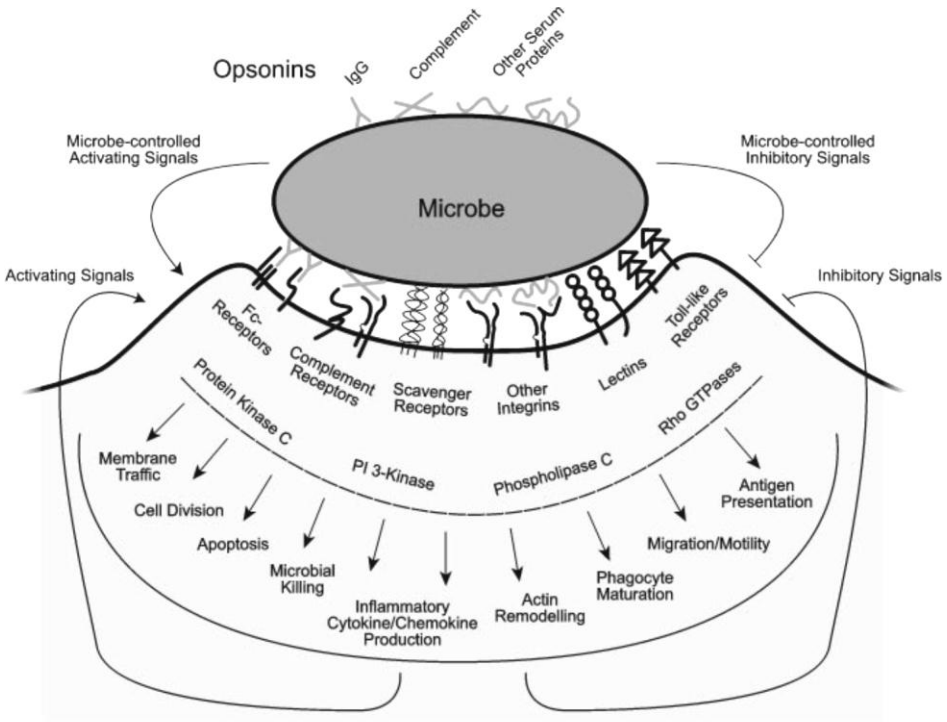


Figure 6: Recognition of microbe by macrophages receptors and the generation of parallel signaling during phagocytosis [24].

3. Killing microbes in phagolysosome are classified into two groups including oxygen-dependent and oxygen-independent mechanisms.

(1) Oxygen-dependent mechanism: The binding of pathogen lead to increase in oxygen uptake by macrophages which are called the respiratory burst. This influx of oxygen causes damage to pathogens inside phagolysosome. The cytoplasmic membrane of activated macrophages contains nicotinamide adenine dinucleotide phosphate (NADPH) oxidase acting as oxygen converter to reactive oxygen intermediates (ROIs), superoxide anion (O^{2-}). Hydrogen peroxide (H_2O_2) and hydroxyl (OH) radicals are formed by interaction of ROIs and water which catalyse by enzyme dismutase. In case of neutrophils, the interaction between hydrogen peroxide and chloride (Cl^{2-}) ions by enzyme myeloperoxidase (MPO) produce hypochlorous acid (HOCL), and singlet oxygen. While in macrophages, NO combine with O_2^- to form peroxynitrite radicals ($ONOO^-$) by inducible nitric oxide synthase (iNOS). (Fig.7). These compounds are potent anti-microbial agent because they oxidize most of the essential groups found in proteins, enzymes, carbohydrates, DNA, and lipids [25].

Nitric oxide (NO)

Nitric oxide (NO) is one of the important free radical. It is also a soluble gas released from the endothelial cells and macrophages. It is synthesized from L-arginine by nitric oxide synthases (NOSs) which is consisted of three isoforms depending on the type of tissue. They are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The nNOS and eNOS are constitutive enzyme primarily expressed in neuronal and endothelial cells types, respectively, and are highly dependent on intracellular Ca^{2+}. On the other hand, inducible NOS (iNOS) is primarily present in the inflammatory cells (e.g. macrophages), where it is induced during inflammatory conditions. High level NO is produced by iNOS and it exerts long action. iNOS generates NO independently of intracellular Ca^{2+}.

NO plays important roles in regulation of diverse physiological and pathophysiological mechanisms. Normally, NO produced at physiological function exerts host defense in the immune system, smooth muscle cells relaxation, neurotransmission in the neuronal system and inhibitor of platelet aggregation (Fig.7). However, overproduction of NO and NO_2 by iNOS associate with various pathophysiology of human diseases such as multiple sclerosis, septic shock, tumor development, asthma and neurodegenerative diseases [26-28].

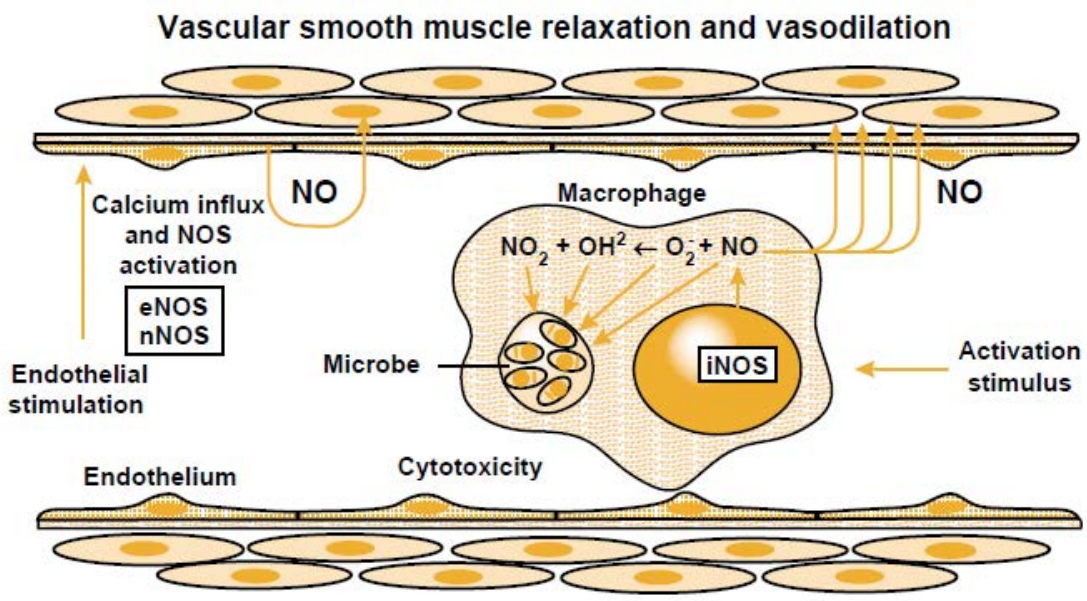


Figure 7: Functions of nitric oxide (NO) in blood vessels and macrophages [27].

(2) Oxygen-independent mechanism: Microorganism or foreign particles are destroyed by using enzyme and protein in phagolysosome. Enzymes and antimicrobial peptide in phagolysosome are defensins, cationic peptides, lysozyme, lactoferrin, cathepsin G, elastase, collagenase and digestive enzymes. They exhibit antimicrobial activity by breaking down proteins, RNA, lipids, and carbohydrates [29].

2. Antigen presentation

Another important function of macrophages is presentation of antigen to T cells after phagocytosis. After digestion of microbial pathogen, antigenic peptides are formed and macrophages become a professional antigen presenting cells (APCs) to present antigenic peptide by MHC to interact with T cells receptor (TCR). Accessory molecules, intracellular adhesion molecule-1 (ICAM-1; CD54)) and co-stimulatory molecules (B7-1 and B7-2), are express on cell surface of macrophage and interact with co-stimulatory molecules on T cells to enhanced lymphocyte function [30]. Intracellular signaling is generated in response to T cells activation and causes T cell proliferation, cytokine production. T helper 1 (Th1) produce IL-2 to activate T cells proliferation and IFN-γ to activate macrophages. Th2 produce IL-4, IL-5, IL-6, IL-10 and IL-13 to activate B cells and promote B cells proliferation and differentiation [Fig.8) [31]. In addition, the cytokines generated by activated T cells are able to kill target cells from infective tissue and cancer cells.

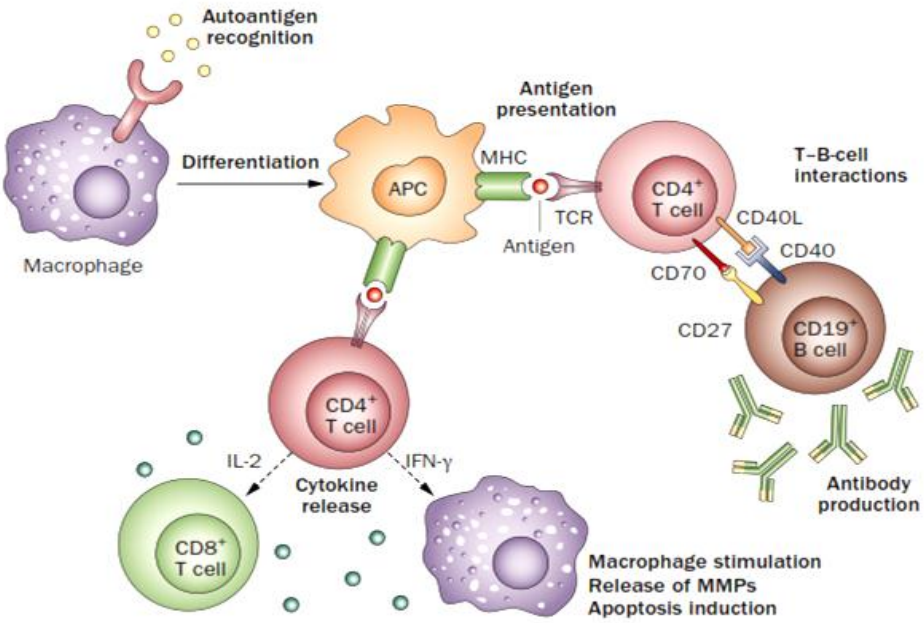


Figure 8: The presentation of antigens by macrophages [31].

3. Inflammatory function and immunomodulations.

Inflammatory function

Macrophages are phagocytic cells which maintain homeostasis of tissue as well as function in inflammations. Inflammation is an adaptive response that is stimulated by exogenous and endogenous stimuli. Considerable progress has been made in understanding cellular and molecular events involved in acute inflammatory response to infection and injury. It is generally known that a controlled inflammatory response is benefit to protect against pathogens and it become harmful in uncontrolled inflammatory response [32].

A typical inflammatory response consists of four components including inflammatory inducers, the sensors that detect signal, the inflammatory mediators induced by the sensors, and target tissues that are affected by the inflammatory mediators. Inflammatory response stimulated by microbial infection or tissue injury involved the coordinated delivery of inflammatory cells. Monocytes, dendritic, macrophages migrate to the site of infection or tissue injury and the macrophages are activated. The activated macrophages release a variety of inflammatory mediators, for example pro-inflammatory cytokines (TNF, IL-1 and IL-6), chemokines (MCP-1, MIP-1α and IL-8), vasoactive amines (histamine, bradykinin), nitric oxide (NO) and eicosanoids (prostaglandins (PGs)) to initiate inflammation (Fig.9) [2]. These inflammatory cytokines and mediators then act on target tissues, including local blood vessels causing vasodilatation and resulting in an increase in blood volume. Higher blood volume causes tissue heating and redness. Increases vascular permeability, leakage of fluid from blood vessels, cause accumulation of fluid (edema) and leading to tissue swollen. Pain, edema, red and fever are cardinal signs appearing in response to this phenomenon (Table 1) [33].

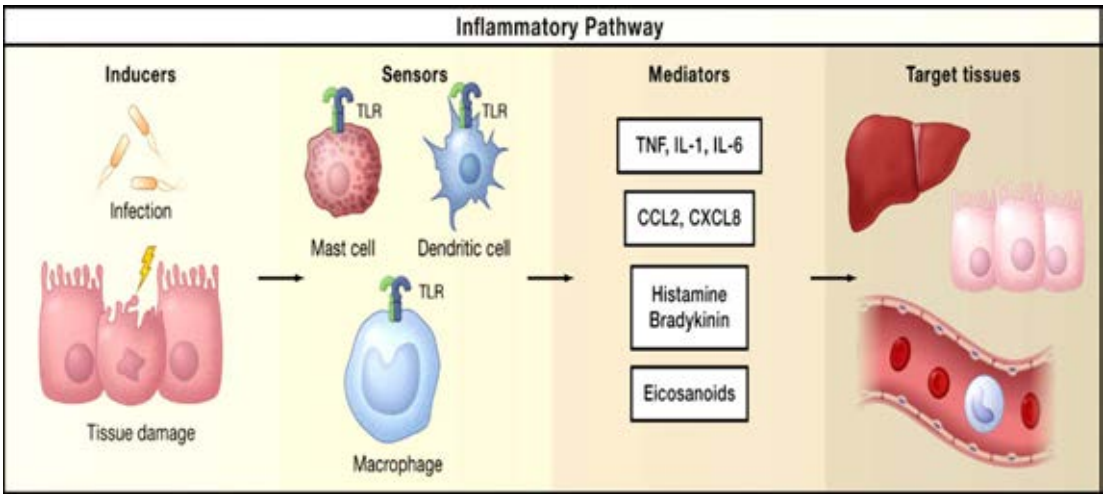


Figure 9: Inflammatory pathway [32].

Table1: Relationships between inflammatory mediators and symptoms of inflammation. [33].

Symptom	Mediators
Vascular permeability	Bradykinin, Leukotrienes, Vasoactive amines, Complement (C3a and C5a), C4, D4, E4 , PAF, Substance P, NO
Vasodilatation	Prostaglandins (PGI2, PGE1, PGE2, PGD2), Hydrogen peroxide, NO
Vasoconstriction	Tromboxane A2, Leukotrienes C4, D4, E4, Superoxide
Chemotaxis and leukocyte adhesion	Chemokines, Complement (C5a)
Pain	Prostaglandins, Bradykinin, Superoxide
Fever	Prostaglandins, Proinflammatory cytokines (TNF-α, IL-1β, IL-6)
Tissue and endothelial damage	Lyzosomal enzyme, ROS, NO

Immunomodulations

Macrophages are functional cells in immune system. Therefore, the macrophages activation is crucial in immunomodulation. In innate immunity, macrophages respond to microbes by secreting cytokines that activate phagocytes and stimulate cellular reaction and promoting acute and chronic inflammation [34-35].

Cytokines

Cytokines usually regulate innate immunity; they are primarily produced by mononuclear phagocytes such as monocytes, macrophages and dendritic cells. Moreover, they are also produced by T-lymphocytes, NK cells, and endothelial cells in inflammatory response [36-37].

Pro-inflammatory cytokines

Activated macrophages produce pro-inflammatory cytokines participated in the immune responses. They are TNF-α, IL-1, and IL-6. These pro-inflammatory cytokines play role in leukocyte movement and migration from circulation to tissues [38-39].

Tumor necrosis factor-α (TNF-α)

TNF-α is a potent pro-inflammatory cytokines that exerts diverse effects by stimulating a variety of cells. It is a soluble 17-kd protein and composed of three identical subunits. It is produced mainly by monocytes and macrophages and alternatively by B cells, T cells, and fibroblasts. The secretion of TNF-α can be stimulated by endotoxin, immune complexes, physical injury and inflammatory stimuli. TNF-α is autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines, chemokines, eicosanoids, and NO. TNF-α promotes the immune responses and enhances tumoricidal activity, inflammatory effects and hematopoietic effects. TNF-α in low concentration ($<10^{-9}$ M) promotes inflammation by stimulating fibroblasts and endothelial cells to express adhesion molecules. These adhesion molecules interact with their ligands on the surface of leukocytes and increase the recruitment of neutrophils and monocytes to site of infection. In addition, TNF-α in moderate concentration ($>10^{-9}$-$<10^{-7}$ M) is able to induced PGE2 synthesis in hypothalamus and

increase body temperature. When serum concentration of TNF-α reachs 10^{-7} M or more, it results in a marked fall in blood pressure, or shock caused by decrease myocardial contractility and vascular smooth muscle tone. Furthermore, TNF-α causes intravascular thrombosis, mainly as a result of abnormal coagulation function of the endothelium. It causes severe metabolic disturbances as well (Fig.10) [39-41].

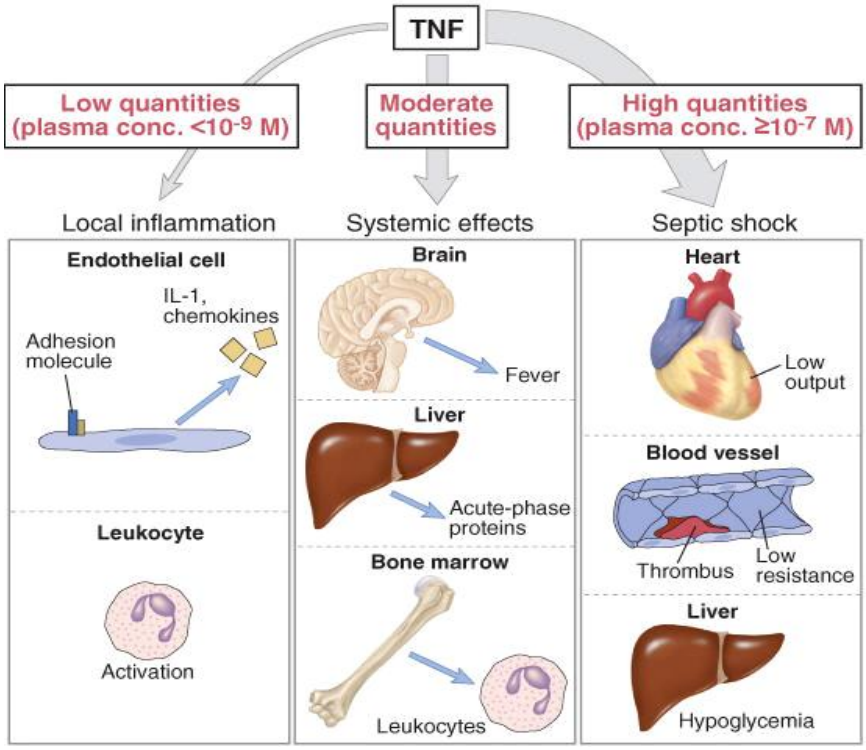


Figure 10: Biological actions of TNF-α [40].

Interleukin-1 (IL-1)

Interleukin-1 (IL-1) is a potent pro-inflammatory cytokine in the inflammatory response and autoimmune diseases rheumatoid arthritis. IL-1 has two isoforms, IL-1α and IL-1β, which are encoded by different genes and generated by different process. They bind to IL-1 receptor and have similar biological functions. IL-1β is a soluble 17.5 kDa protein and function as an endocrine signaling molecule while IL-1α is a soluble 22-31 kDa protein and function as an autocrine signaling molecule [47]. They are mainly

produced by monocytes and macrophages. Both IL-1α and IL-1β trigger fever by increase PGE2 production. In addition, IL-1 share important inflammatory property with TNF-α in increases synthesis of inflammatory mediators promoting inflammation (Fig.11). The pro-inflammatory effects of IL-1 can be inhibited by IL-1 receptor antagonist (IL-1Ra), originally referred to as IL-1 inhibitor [42-44].

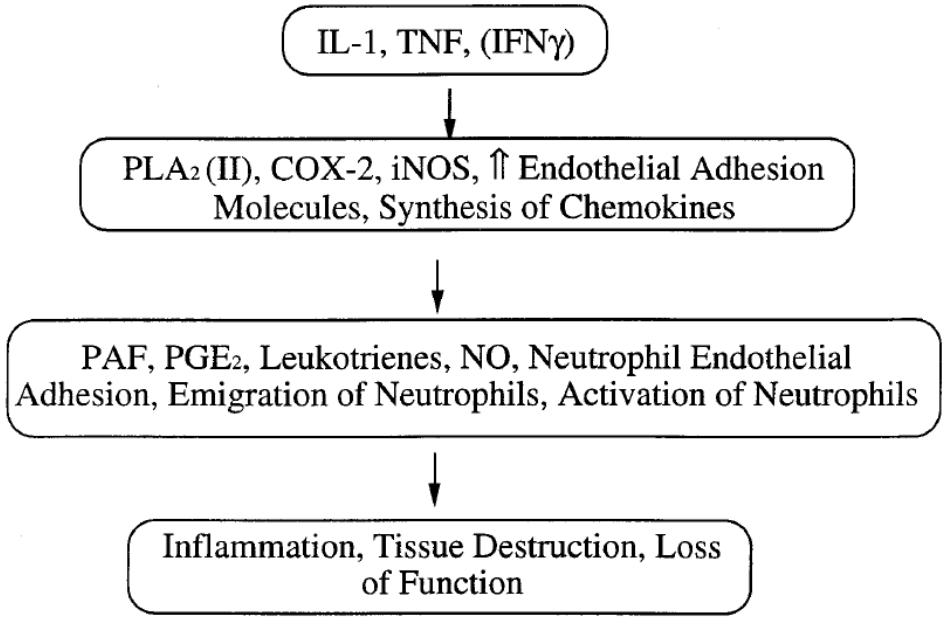


Figure 11: The inflammatory cascade triggered by IL-1 and TNF-α [44].

Interleukin-6 (IL-6)

IL-6 is a glycoprotein with the molecular weight ranging from 21 to 28 kDa. IL-6 is a pleiotropic inflammatory cytokine produced by monocytes, macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. IL-6 involves in a series of biologic processes dealing with the T cells activation and differentiation, stimulation of the growth factor for B cells maturation to plasma cells, and proliferation of synovial fibroblasts. Liver synthesis of acute phase protein is also stimulated by IL-6. In addition, IL-6 induces changes in the levels of complement proteins, fibrinogen, C-reactive protein, and serum amyloid A (SAA). [45]

Chemokines

Chemokines (chemotactic proteins) are members of cytokines that control leukocytes recruitment to site of inflammatory tissues. Chemotactic proteins consist of a conserved secondary structure with a flexible N-terminal segment followed by three antiparallel β-sheets and a C-terminal α-helix. According to the relative position of cysteine residues, members of these large families have been classified into four subfamilies (CXC, CC, C and CX3C). Chemokines induce leukocyte migration and activation by binding with specific G protein–coupled seven transmembrane receptors. The interaction of different chemokines with their receptors on leukocytes allows selective activation and chemotaxis of neutrophils, lymphocytes, or monocytes necessary for migration to the inflammatory sites. The influx of cellular components into inflammatory tissue is activated by gradients of chemokines that support the adhesion of leukocytes to endothelium, transendothelial migration, and movement through the extracellular matrix. Activated monocytes and macrophages are the major chemokine-secreting cells. Several chemokines are produced by monocytes and macrophages with different pathogenic microorganism [46].

Monocyte chemoattractant protein-1(MCP-1)

Monocyte chemoattractant protein-1 (MCP-1; CC chemokine ligand-2 [CCL-2]) is a CC chemokine and a potent chemoattractant or activator for monocytes. The expression of MCP-1 occurs in several cells types upon induction with activators such as growth factors, cytokines, double-stranded RNA and LPS. Resident macrophages in inflamed tissues release chemokines (MCP-1) and cytokines, which activate endothelial cells. The activated endothelial cells express adhesion molecules (P-selectin, CD-34, glyCAM-1) that interacts with their ligands on the surface of leukocytes, resulting in increased the recruitment of monocyte to site of infection [47].

Macrophages-inflammatory protein-1α (MIP-1α*)*

Macrophages-inflammatory protein-1α (MIP-1α) is an 8-kDa peptide that is characterized by its ability of chemotaxis induction in eosinophils and macrophages to inflammatory site. The release of chemokines like MIP-1α is a crucial step in cells recruitment necessary during initiation and maintenance of inflammatory responses. In addition, MIP-1α induces secretion of TNF-α, IL-1β, and IL-6 from murine macrophages. They are key players in the pathogenesis of many inflammatory diseases (Fig. 12) [48].

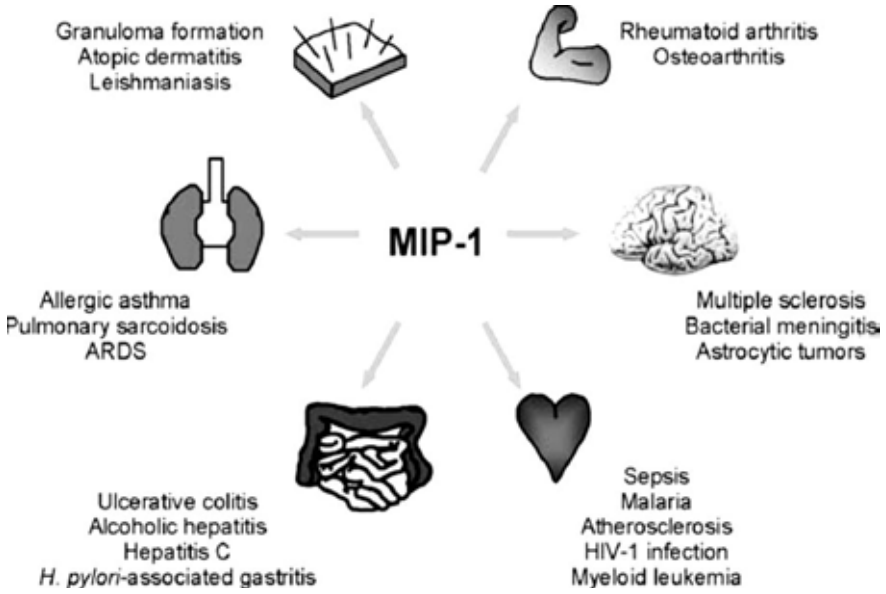


Figure 12: Pathological roles of MIP-1 proteins in the inflammatory diseases [48].

Prostaglandins

Prostaglandins (PGs) are potent small lipid molecules which regulate several processes in the body. They modulate kidney function, platelet aggregation, neurotransmitter release, immune function, gastrointestinal and reproductive functions. The productions of prostaglandins are initiated with the release of arachidonic acid from membrane phospholipids by phospholipase A2. Arachidonic acid (AA) will be converted to PGH2 by the cyclooxygenase enzymes COX-1 and COX-2. COX-1 is normally

expressed in most tissues of the body to maintain homeostatic processes. COX-2, by contrast, is inducible enzyme associated with inflammation and diseases.

Cells-specific prostaglandin synthase will convert PGH2 into a series of prostaglandins, including PGD, PGE, PGF, PGI and tromboxane A2. The important prostaglandins responses to inflammation are PGI2, PGF2α, PGD2 and PGE2 (Fig.13). Prostaglandins are also involved in the pathogenesis of pain and fever. PGE2 and PGF2α cause vasodilation and increase permeability of postcapillary venules, potentiating edema formation. Fever is induced by pyrogens which subsequently stimulate the production of PGE2 and increase body temperature through heat regulating center in hypothalamus [49].

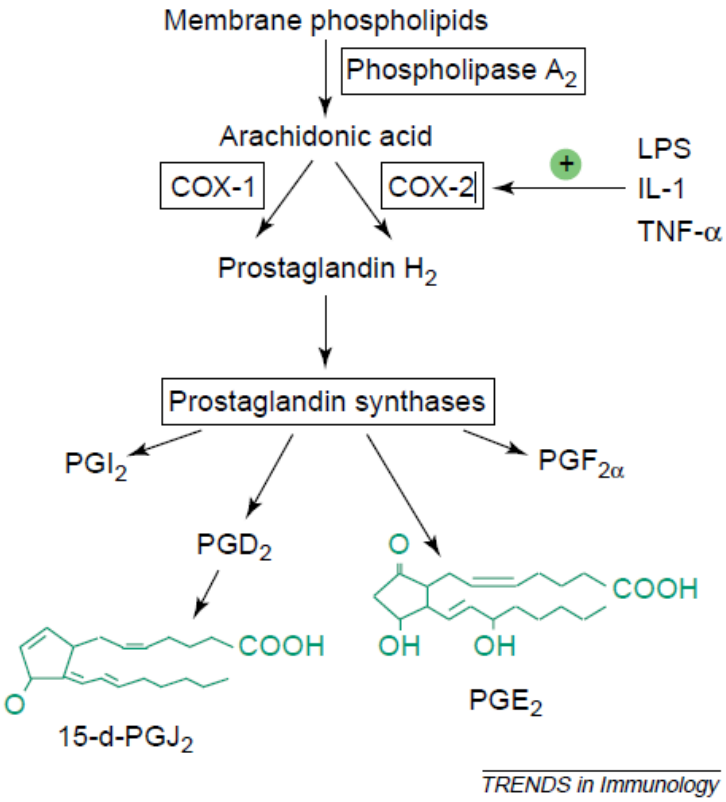


Figure 13: Pathway of prostaglandin production [49].

Anti-inflammatory drugs

Nonsteroidal anti-inflammation drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed classes of medications worldwide. Two major groups of NSAIDs are classified on the basic of their selectivity on COX enzyme. There are non-selective COX inhibitors (e.g. indometacin, ibuprofen, naproxen, piroxicam) and the selective COX-2 inhibitors (celecoxib, etoricoxib). All NSAIDs inhibit cyclooxygenase enzyme (COX-1 and COX-2) which catalyze arachidonic acid to form prostaglandins and thromboxanes. Prostaglandins act as messenger molecules in the process of inflammation especially PGE2, PGI2 and PGF2α. Suppression of these inflammatory prostaglandins result in alleviation of pain, fever, and inflammation [50-51].

Mechanism of action of NSAIDs

1. **Non-selective COX inhibitors:** inhibit both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes. COX-1, a constitutive enzyme synthesizes PGs that regulate physiologic processes. COX-2 is an inducible enzyme expressed throughout the body, primarily during inflammatory responses and in association with pain or fever.

The following adverse effects of NSAIDs related to inhibition of COX-1;

- **Gastrointestinal system:** Abdominal pain, nausea, diarrhea, anorexia, gastric erosions/ulcers, anemia, GI hemorrhage and perforation.

- **Central nervous system:** Headaches, tinnitus, dizziness, confusion, depression, lowering of seizure threshold, hyperventilation (salicylates).

- **Renal system:** Salt and water retention, edema, worsening of renal function in renal/cardiac and cirrhotic patients, decreased effectiveness of antihypertensive medications, decreased effectiveness of diuretic medications, decreased urate excretion (especially with aspirin), hyperkalemia.

- **Platelets:** Inhibited platelet activation.

- **Uterus:** Prolongation of gestation.

- **Hematologic system:** Rare thrombocytopenia, neutropenia, aplastic anemia [56-57].

2. Selective COX-2 inhibitors: Selective COX- 2 inhibitors (coxibs) are developed with better therapeutic activity and safety profile. They selectively inhibit COX-2 enzyme and prostaglandins production. This inhibition is responsible for the analgesic, antipyretic and anti-inflammation.

Adverse effects of selective COX-2 inhibitors

Selective COX-2 inhibitors have been associated with an increase in cardiovascular (fluid retention, hypertension, congestive heart failure, risk of myocardial infarction) as well as cerebrovascular events (stroke) particularly in patients with an elevated risk of thrombosis. This increase risk may be due to COX-2-mediated reduction in synthesis of prostacyclin.

Steroidal compounds

Steroidal compound structure and efficacy are similar to glucocorticoid hormone from adrenal cortex. Their effects are carbohydrate, protein and lipid metabolism, electrolyte and water balance, cardiovascular system, skeletal muscle, central nervous system, forming elements of blood, anti-inflammatory and immunosuppressive action. Glucocorticoids are potent immune suppressive drugs and used widely on a chronic basis to treat most autoimmune diseases. Examples of drugs in this group are dexamethasone, betamethasone, fludrocortisone, triamcinolone and prednisolone.

Mechanism of action of steroid for anti-inflammatory effect

Although there are several mechanisms by which glucocorticoids reduce inflammation, one major mechanism may concern with reduce expression of cytokines-induced genes. Glucocorticoids enter all cells and bind to the cytoplasmic steroid receptor, and then this complex translocates to the nucleus where it is recognized by specific DNA sequences. The major effects of binding to DNA is the suppression of transcription by opposing the activation of the transcription factors AP-1 and NF-kB in the nucleus of cells and reduce expression of pro-inflammatory cytokines and chemokines (Fig.14). Glucocorticoids also suppress expression of inflammatory genes encoding T cells growth factors such as IL-2, IL-4, IL-15, and IL-17 as well as interferon-gamma IFN-γ). In addition, glucocorticoids reduce expression of genes encoding COX-2, inducible nitric oxide synthase (iNOS), and intracellular adhesion molecule-1 (ICAM-1) which are normally induced by the cytokines IL-1β and TNF-α. Glucocorticoids increase expression of genes encoding anti-inflammatory molecules (IL-10 and IL-1 type 2 decoy receptor) [8, 52].

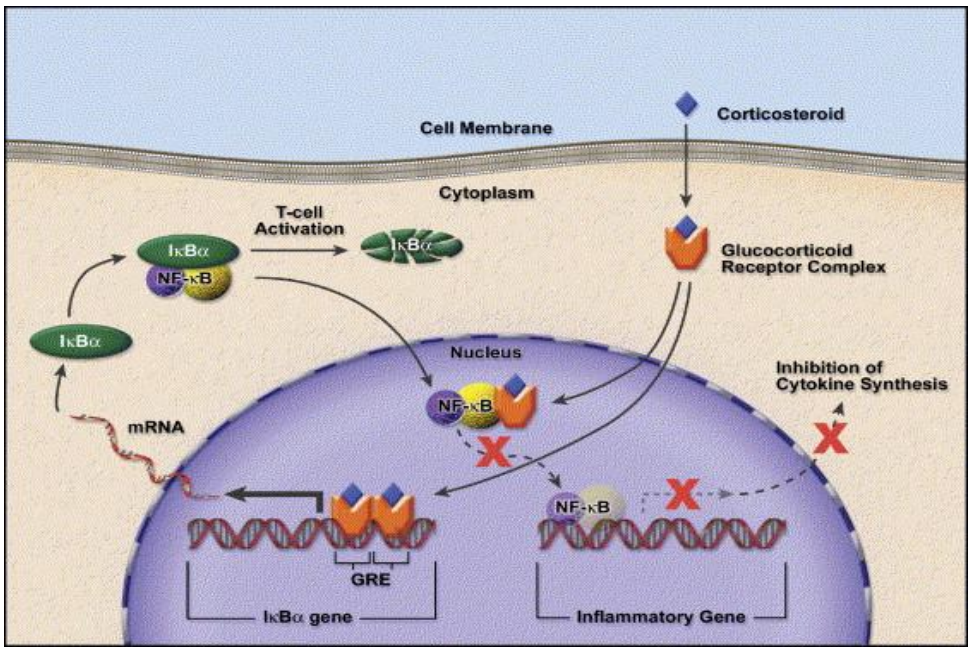


Figure 14: Mechanism of action of steroidal drugs [52].

Adverse effects of steroidal drugs

Steroidal drugs are valued for their therapeutic application but they also cause many systemic side effects in long term administration. Since they suppress the hypothalamic-pituitary–adrenal (HPA) axis and bring about iatrogenic Cushing's syndrome. Fat tends to be redistributed from the extremities to the trunk and the back of the neck. There is an increase growth of fine hair over the face, thighs and trunk. Steroid-induced punctuate acne may appear. Furthermore they cause insomnia, increased appetite, bone loss, peptic ulcers, myopathy, psychoses and glaucoma, increased susceptibility to infection and a risk for reactivation of latent tuberculosis.

The increasing incidence of problems associated with morbidity and mortality of inflammatory diseases have been the subjects of much research with regards to etiology, diagnosis and treatment. Recently major attention has been focused on herbal remedies that inhibit inflammatory processes since they offer much promise in health benefits and disease treatments without excessive side effects. Plants serve as an extensive source of potentially clinically usable natural anti-inflammatory drugs and indicate lead structures for the development of semisynthetic agents.

Thai people commonly use not only modern anti-inflammatory medications but also traditional medicines to relieve inflammation. Wisumpayayai is a household traditional remedy approved by the Ministry of Public Health of Thailand in National drug list as anti-flatulence and anti-dyspeptic medication. It is composed of 20 herbal plants including; *Coriandrum sativum, Diospyros decandra, Myristica fragrans, Amomum krervanh, Syzygium aromaticum, Angelica dahurica, Atractylodes lancea, Conioselinum univitatum, Angelica sinensis, Artemisia pallens, Cinnamomum verum, Cinnamomum bejolghota, Terminalia arjuna, Terminalia chebula, Acorus calamus, Aristolochia* sp., *Tinospora crispa, Zingiber kerrii, Melastoma saugnineum, Piper retrofractum* (Table 2). Fourteen of these plants have been reported to have anti-inflammatory activities (Table 3) and a variety of the pharmacological properties. However, there was no report of anti-inflammatory effect of Wisumpayayai remedy. Therefore, it is interesting to investigate whether this remedy possesses anti-inflammatory activity.

Table 2: The compositions of herbal plants in Wisumpayayai remedy and their pharmacological actions.

Scientific name	Family	Parts in the remedy	Pharmacological activities
1.Coriandrum sativum	Umbelliferae	8	Anti-oxidant activity, anti-bacterial, anti-mutagenicity, anti-convulsant and anti-inflammation [54-56].
2. Diospyros decandra	Myristicaceae	8	Anti-tumor, anti-hepatotoxicity, anti-oxidant and anti-inflammation [57].
3. Myristica fragrans	Myristicaceae	8	Anti-inflammation, anti-cancer, inhibits melanin synthesis, Improvement of mouse memory and hepatoprotective [68-63].
4. Amomum krervanh	Zingiberaceae	2	Anti-flatulence and decrease the motility of the gastrointestinal tract.
5. Syzygium aromaticum	Myrtaceae	2	Anti-fungal, anti-cancer, anti-inflammation and decrease edema [64-66].
6. Angelica dahurica	Umbelliferae	2	Anti-flatulence, anti-inflammation, anti-oxidant, anti-bacterial, anti-cancer [67-70].

Scientific name	Family	Parts in the remedy	Pharmacological activities
7. Atractylodes lancea	Umbelliferae	2	Anti-fungal, anti-neuromuscular, anti-oxidant, anti-inflammatory effect and macrophage activation [71-72].
8. Conioselinum univitatum	Umbelliferae	2	Anti-oxidant and anti-inflammation [73].
9. Angelica sinensis	Umbelliferae	2	Anti-inflammation, anti-cancer, Inhibition of rat vascular smooth muscle cell and activation of macrophages [74-78].
10. Artemisia pallens	Compositae	2	Anti-inflammatory activity in the carrageenan model, anti-oxidant, anti-diabetic effect [79-81].
11. Cinnamomum verum	Lauraceae	2	Anti-flatulence, anti-bacterial, anti-cancer [82-83].
12. Cinnamomum bejolghota	Lauraceae	2	Anti-bacterial, anti-oxidant, anti-inflammation [84-85].
13. Terminalia arjuna	Combretaceae	2	Anti-bacterial, anti-inflammation and anti-oxidant [86-87].
14. Terminalia chebula	Combretaceae	2	Anti-bacterial, anti-diabetic, anti-oxidant, anti-inflammation, anti-cancer [88-92].
15. Aristolochia sp.	Aristolochiaceae	2	Anti-oxidant, anti-cancer [93].

Scientific name	Family	Parts in the remedy	Pharmacological activities
16. Acorus calamus	Araceae	2	Anti-inflammation, wound healing, anti-fungal, Anti-cellular and immunosuppressive properties [94-97].
17. Tinospora crispa	Menispermaceae	2	Anti-diabetic, anti-parasite [98-99].
18. Zingiber kerrii	Zingiberaceae	2	Anti-inflammation, anti-cancer, anti-hapatotoxicity, anti-renaltoxicity [100-103].
19. Melastoma saugnineum	Melastomataceae	2	Anti-oxidant, treatment diarrhea [104].
20. Piper retrofractum	Piperaceae	56	anti-flatulence and anti-dyspepsia, lipid lowering, anti-inflammation, anti-bacterial [105-106].

Table 3: Anti-inflammatory activities of the herbal plants of Wisumpayayai remedy.

Scientific name	Evidence of Anti-inflammatory activity
1. Coriandrum sativum	- The main chemical constituents including; limpnene, terpinene, p-cymene, borneol, citronellol, camphor, geraniol, pyrazine, pyridine, thiazole, furan, tetrahudrofuran derivatives, isocoumarins, coriandrin, dihydrocoriandrin, coriandrons A-E, flavonoids, pthlides, neochidilide, digustilide phenolic acids and sterols. -The ethanol extracts from both stem and leaf of *C. sativum* (CSEE) at 25-150 µg/ml significantly decreased LPS-induced NO and PGE2 production as well as iNOS, COX-2, and inhibit mRNA expression of pro-inflammatory cytokines [56].
2. Diospyros decandra	- The main compound including; lignans, flavonoids, phenylpropanoids, sesquiterpenes, monoterpenes. - The hexane extract at the concentration of 25-100 µg/ml significantly inhibited pro-inflammatory cytokines (TNF- α, IL-1β) and COX-2 in LPS-activated RAW.264.7 macrophages [57].

Scientific name	Evidence of Anti-inflammatory activity
3. Myristica fragrans	- Macelignan at the concentration of 2.5 and 10 μM inhibited neuroinflammation in cultured rat brain microglial cells. It potently suppressed the mRNA expression of COX-2 and iNOS, which consequently resulted in the reduction of PGE2 and NO in LPS-treated microglial cells. It also signficantly suppressed the production of pro-inflammatory cytokines TNF-α and IL-6 [61].
4. Angelica dahurica	- Chemicals structure of five furanocoumarins isolate from the root of *Angelica dahurica*; phellopterin, isoimperatorin, imperatorin, oxypeucedanin, byakagelicol, and methanolate [107]. - Imperatorins is active constituents of *A. dahurica* at the concentration 3-30 μM significantly inhibited activity PGE2 productions. It also inhibited the expression of COX-2 in peritoneal cells macrophages [108]. - The ethanol extract of *A. dahurica* exerted anti-oxidative effect on the production of reactive oxygen species (ROS) and NO in microglial cells [109]. - The pretreatment cells with the ethanol extract of *Angelica dahurica* (ADEE) at concentration 50-200 μg/ml inhibited COX-2 and iNOS expression in LPS-stimulated RAW264.7 cells. It also inhibited production of TNF-α and IL-6 in a concentration-dependent manner. ADEE also inhibited NF-kB translocation to the nucleus by interrupting IkBα degradation [110].

Scientific name	Evidence of Anti-inflammatory activity
5. Atractylodes lancea	- The main constituents including; hinesol and β-eudesmol. β-eudesmol is a major component of the essential oil. - β-eudesmol has been shown inhibit the production and expression of IL-6 in A23187-stimulated human mast cells (HMC). In activated HMC-1 cells, β-eudesmol suppressed activation of p38 mitogen-activated protein kinase (MAPKs) and NF-kB. It also suppressed the activation of caspase-1 and expression of receptor-interacting protein-2 [111].
6. Conioselinum univitatum	- The main chemical constituents including; diligustilide, levistolide, tetramethylpyrazine - The ethyl acetate extract from the branches of this plants inhibited LPS-stimulated macrophages J774A.1 cells. It inhibited NO production and the expression of iNOS, COX-2, and pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) [73].
7. Angelica sinensis	- The main chemical constituents including; ferulic acid, Z-ligustilide, butylidenephthalide and various polysaccharides. Among these compounds, ferulic acid inhibits MIP-2 production and inflammatory mediators (e.g., NO and PGE2) via suppression of transcriptions factor NF-kB in LPS-stimulated-RAW 264.7 macrophage cells. In addition, Z-ligustilide also shows inhibition of the TNF-α and NF-kB activity [112].

	AAP, an acidic polysaccharide fraction isolated from the roots of *Angelica sinensis*. AAP at concentrations of 10-500 μg/ml significantly inhibited NO production and cellular lysosomal enzyme activity in murine peritoneal macrophages [113].
8. Artemisia pallens	- The active compound of *A. pallens* including; saponins, flavonoids, sesquiterpenoids, phenols and tannins. - The methanol extract of *A. pallens* in doses of 100, 200 and 500 mg/ml showed 68.85, 74.53 and 81.13% inhibition of paw edema respectively at the end of three hour. In the hot plate and tail flick model in carrageenin-induced rat paw edema [79].
9. Cinnamomum bejolghota	- The main chemical composition of *C. bejolghota* were extracted in 3 essential oils are trans-anethole, pentadecane and cinnamaldehyde. - The MeOH crude extract of *C. bejolghota* and its fractions obtained from hexane and ethyl acetate (EtOAc) extract significantly inhibited the production of IL-1β, IL-6 and the TNF-α in LPS-stimulated RAW-264.7 cells. It also the MeOH and EtOAc extract (100 μg/ml) strongly suppressed the NO and PGE2 production in LPS/IFN-γ-activated macrophages [85-86].
10. Terminalia arjuna	- Principal components of *T. arjuna* include triterpnoid, saponins, anjantic acid, arjunone, arjunolone. - The methanol extract of *T. arjuna* at the concentrations of 100 and 200 mg/kg body weight inhibit paw edema in carrageenan induced rat paw edema [87].

Scientific name	Evidence of Anti-inflammatory activity
11. Terminalia chebula	- Tannins and flavonoids present in *T. chebula*, flavonoids like rutin and quercetin from methanolic extract of *T. chebula*. Rutin and quercetin exhibits anti-inflammatory activity, inhibited NO, PGE2 production and the expression of iNOS, COX-2, and pro-inflammatory cytokines (IL-1, IL-6 and TNF-α). - The alcohol extract of the leaves of *T. chebula* on the healing of rat dermal wounds, *in vivo* showed decreased period of epithelialization and increase in total protein, DNA and collagen contents in the granulation tissues of treated wounds. It also reduced lipid peroxide levels in treated wounds, as well as measurement of antioxidant activity by DPPH radical quenching, suggested that *T. chebula* possessed antioxidant activities [114]. -The ethanol extract of the fruits of *T. chebula* potent dual inhibition against COX-2 and 5-LOX, Chebulagic acid isolated from fruits of *T. chebula* showed inhibitory activity of COX-2 and 5-LOX [115].
12. Acorus calamus	- Active ingredient: asarone, β-asarone and volatile oil [19]. - The pretreatment cells with the water extracts of ACL concentration at 1-100 μg/ml reduced expression of IL-6 and IL-8 at the RNA and protein levels in PGN and polyI:C.-stimulated Human HaCaT keratinocytes cells [94-95].

Scientific name	Evidence of Anti-inflammatory activity
13. Zingiber kerrii	- The major active ingredients in ginger oil are the sesquiterpenes; bisapolene, zingiberene. - Gingerol has been shown to inhibit the production of NO production and expression of COX-2. - The analgesic and anti-inflammatory effects of gingerol. gingerol inhibition of acetic acid-induced writhing response and formalin-induced licking time in the late phase and inhibition of paw edema induced by carrageenin [100].
14. Piper retrofractum	- Several active groups of chemical components of *P. retrofractum* including phenolics, lignans, terpenes, chalcones, flavonoid, and alkaloid. The components including Piperine, Piperidine piperlonguminine, sylvatine, guineensine, piperlongumine, filfiline, sitosterol, methyl piperate [117]. - The ethanol extraction of *P. retrofractum* inhibited pro-inflammatory cytokines including IL-6 and TNF-α as well as decreased the expression of iNOS and COX-2 in LPS-stimulated macrophages. It also increased IL-10 which is anti-inflammatory cytokines in activated macrophages [118]. - Piperine, the important active compound of *P. retrofractum* inhibited NO and TNF-α production in LPS-stimulated macrophages and concanavalin-A (Con-A) stimulated-Balb/c mice. It also decreased nociceptive in carrageenan-induced paw hyperalgesia and arthritic symptoms in carrageenan induced arthritis [119]. - Piperine decreased the level of IL-6, matrix metalloproteinase 13 (MMP-13), COX2 and PGE2 in IL-1β-stimulated fibroblast-like synoviocytes derived from rheumatoid arthritis patients [120].

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Ethanol extract of Wisumpayayai remedy

Wisumpayayai remedy was purchased from a traditional drug store and was authenticated by Associate Professor Dr. Nijsiri Ruangrungsi, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The extract of Wisumpayayai remedy was also prepared by Dr. Nijsiri Ruangrungsi. The power drug was extracted with 95% ethanol in Soxhlet extractor. After extraction the solvent was removed by rotary evaporator until dry. We obtained the extract with 15.6% yield.

1.2 Cells cultures

The murine macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a CO_2 incubator at 37°C in 5% CO_2/95% air. They were subcultured by scraping three timed weekly during use. The cell viability was determined by staining with 0.4% trypan blue which stains only dead cells. The cells with their viability not less than 85% in the exponential growth phase were used in this study at the density of 2×10^5 cells/ml in all experiments.

1.3 Chemicals and reagents

The following reagents were used in this study: chloroform (Sigma, USA), DEPC (Molekula, UK), disposable cells scrapers (Greiner bio-one, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), hydrochloric acid (Merck,Germany), ImProm-IITM Reverse Transcription system (Promega, USA), lipopolysaccharide (Sigma, USA), nitric

assay kit (Promega, USA), penicillin/streptomycin (Gibco, USA), sodium chloride (Sigma, USA), sodium bicarbonate (Baker, USA), sodium hypochlorite (Clorox, USA), potassium hydroxide (Sigma, USA), primer (Bio Basic, Canada), Taq polymerase (Invitrogen, UK) , trypan blue dye (Sigma, USA), and TRiZol reagent (Invitrogen, UK).

1.4 Equipments and Instruments

The followings equipments and instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hiclave^{TM}, HVE-50, Hirayama, Japan), autopipette (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge machine (Hettich, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), 96 and 24 muti-well plate (Corning, USA), T-25 Tissue Culture flasks (Corning, USA), spectrophotometer (Shimadzu, Japan), scrapper (Greiner, UK), thermocycle machine (Eppendorf, USA), vortex mixer (Scientific industries, USA).

2. Methods

2.1 Preparation of the stock solutions of the ethanol extract of Wisumpayayai remedy

The extract was dissolved in dimethylsulfoxide (DMSO) as the stock solution at 50 mg/ml. This solution was stored at -20°C until use. It was diluted with sterile double-distilled water to working solutions at various concentrations with constant 2% DMSO. There working solutions were used to treat cells at the required final concentrations with constant 0.2% DMSO.

In all experiments, 3.92 µg/ml (10 µM) dexamethasone and 0.2% DMSO solution were used as the positive and the negative controls, respectively. All assays were performed at least three independent experiments in triplication.

2.2 Effect of The ethanol extract of Wisumpayayai remedy on NO production in LPS-stimulated J774A.1 cells

The effect of the extract on NO production was determined by Griess reaction assay. The tests were performed in triplication of three independent experiments.

J774A.1 cells at a density of 2 x 10^5 cells/ml were grown in a 96 well plate at 37° C for 24 h. The cells were treated with 6.25-100 µg/ml the ethanol extract for 24 h and then stimulated with 100 ng/ml LPS for 24 h. the other set of experiments, the cells were simultaneously treated with 6.25-100 µg/ml the ethanol extract and 100 ng/ml LPS at 37° C for 24 h. The supernatant of the treated cells was collected for determining nitric oxide content in nitrite from by Griess reagent. The remaining treated cells were assessed for their viability by rezasurin staining assay.

The assay of nitric oxide content by Griess reagent was performing in the dark at room temperature as in the following procedures;

Hundred µl supernatant was mixed with 20 µl of sulfanilamide reagent for 10 min in a 96-well plate, and then 20 µl of N-1-napthylenediamine dihydrochloride (NED) reagent was added and mixed for further 10 min. Optical density (OD) of all reaction mixtures were measured by microplate reader at 540 nm. The nitric oxide content in each well was determined as nitrite content (µM) by using nitrite standard curve generated by using 5 concentrations of standard nitrite solution. The percentage of nitric oxide inhibition of the ethanol extract was compared to the LPS-activated condition by the following formula;

$$\% \text{ NO inhibition} = \{[NO]_{control} - [NO]_{extract} / [NO\}_{control}\} \times 100$$

The 50% inhibitory concentrations (IC_{50}) on NO production of the extracts was calculated and used for choosing suitable 3 concentrations of the extracts in the following experiments.

Determination of the effect of the ethanol extract of Wisumpayayai remedy on cell viability.

After removing the supernatant for determining amount of NO, the remaining treated cells were assessed their viability by resazurin reduction assay. Hundred µl fresh DMEM containing 50 µg/ml resazurin solution was added in each well. The cells were incubated at 37°C for 2 h. Viability of the cells was determined from the ability of viable cells that can metabolically reduce resazurin in blue color to resorufin by measuring the optical density of each well at 570 from the OD at 600 nm using microplate reader. The percentage of cells viability of was calculated by the following formula;

$$\% \text{ Cells viability} = [\Delta OD_{control} - \Delta_{extract} / \Delta OD_{control}] \times 100$$

$$\Delta OD = OD570\text{-}OD600$$

2.3 Effect of The ethanol extract of Wisumpayayai remedy on phagocytic activity of LPS-stimulated J774A.1 cells.

Effect of the ethanol extract on phagocytic activity of LPS-stimulated J774A.1 cells was determined by using zymosan-nitroblue tetrazolium (NBT) reduction assay. The tests were performed at least 3 independent experiment as in the following procedures;

J774A.1 cells at a density of 2×10^5 cells/ml were grown in a 96 well plate at 37° C for 24 h. The cells were treated with 12.5-100 µg/ml the ethanol extract for 24 h and then stimulated with 100 ng/ml LPS for 24 h. the other set of experiments, the cells were simultaneously treated with 6.25-100 µg/ml the ethanol extract and 100 ng/ml LPS at 37° C for 24 h. the treated cells were washed in DMEM media and incubated with 800 µg/ml zymosan and 600 µg/ml NBT for 60 min at 37°C. The cells were washed thrice with absolute methanol. The formazan precipitates in the cells were dissolved by adding 2M KOH solution and 100% DMSO. The amount of reduced NBT was determined by a microplate reader at 570 nm. The percentage of phagocytosis inhibition of the ethanol extract was compared to the LPS-activated condition as in the following formula;

% Phagocytic activity = [$OD_{control} - OD_{extract} / OD_{control}$] ×100

2.4 Effect of the ethanol extract of Wisumpayayai remedy on mRNA expression of cytokines, chemokines, iNOS and COX-2 in LPS-stimulated J774A.1 cells

The effect of the ethanol extract on the mRNA expression of protein involving in inflammatory process including pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) chemokine genes (MCP-1, MIP-1α and IL-8) and enzyme (iNOS and COX-2) was evaluated by reverse transcription polymerase chain reaction (RT-PCR).

J774A.1 cells at the density of 2 x 10^5 cells/ml were grown in a 24 well plate at 37°C for 24 h. The cells were treated with 25-100 µg/ml the extract and 100 ng/ml LPS for 4 and 24 h. The treated cells were collected for total RNA preparation, cDNA production, and determination of the mRNA expressions of protein in inflammatory process as in the following procedures;

2.4.1 Preparation of total RNA

The treated cells were lyses with 1 ml of TRIzol® reagent in 1.5 ml eppendorf tube for 5 min at room temperature. 0.2 ml chloroform was added into each sample. The samples were vigorously shake for 15 seconds, incubated at room temperature for 2-3 min, separated for collecting aqueous phase by centrifugation 12,000g for 15 min at 4 °C. The aqueous phase was transferred into a fresh eppendorf tubes. 0.5 ml of isopropyl alcohol was added into each tube. The samples were incubated at room temperature for 10 min. The RNA pellets were separated and collected by centrifugation 12,000g for 10 min at 4 °C. The pellet was washed twice with 1 ml of 75% ethanol. In each wash the pellet was separated by centrifugation at 7,500g for 5 min at 4°C. The washed pellets was air-dried for 5-10 min, dissolved in RNase free-water, incubate at 55-60 °C for 10 min, and stored at -70°C until use. The protein contamination in the RNA pellets was determined from the ratio of optical densities of the sample at 260 nm and 280 nm which must be > 1.8. The concentrations of total RNA samples were calculated by the following formula; RNA (µg) = Absorbance at 260 nm X 40 X dilution factor

2.4.2 Preparation of complementary DNA (cDNA) by reverse transcription-polymerase chian reaction (RT-PCR)

The total RNA samples were reversely transcribed to complementary DNA (cDNA) by using Improm II™ reverse transcription system kit as in the following procedures;

For each sample tube, 1.5 µg total RNA and Oligo $(dT)_{15}$ primer in 5 µl final volume of nuclease-free water was heated at 70 °C for 5 min. The tube was immediately chilled on ice for 5 min. Fifteen µl reverse transcription mixture solution containing; 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor and reverse transcriptase was added into each tube. cDNA was generated from the reaction mixture in a thermocycler machine by using the following condition; 25 °C for 5 min, then 42 °C for 1 hour 30 min. and finally at 70 °C for 15 min. The cDNA samples were stored at -20 °C until use.

2.4.3 Determination mRNA expression of interested cytokines, iNOS and COX-2 cDNA by PCR

PCR was performed using gene-specific primer (Table 1) for pro-inflammatory cytokines gene (TNF-α, IL-1β, IL-6), chemokine genes (MCP-1, MIP-1 and IL-8) and enzyme gene (iNOS, COX-II) and the β-actin gene as the internal control. PCR was carried out in a 25 µl reaction mixture containing 1 µl of cDNA sample, 50 mM $MgCl_2$, 10mM dNTP, 0.4 µM of primer, 1 unit of Taq DNA polymerase and PCR buffer. The PCR was performed in a thermocycler machine by the following condition; denaturation for 30 sec at 94 °C, annealing for 45 sec at 55 °C, extension for 1 min at 72 °C and final extension for 7 min at 72 °C at the end of 25th cycles.

The PCR products were separated by 1.5 % agarose gel electrophoresis at 100 volt for 45 min in TBE buffer. The gel was stained with ethidium bromide in 1xTBE buffer for 4 min and distained with TBE buffer for 30 min. The PCR products were imaged and determined their densities by gel documentation. The densities of the PCR products were expressed as % of internal control (ratio of the band density divided by that of the housekeeping gene (β-actin) × 10

Table 4: The gene-specific primers used for RT-PCR.

Gene	Primer sequences	Tm °C	Production length(bp)
TNF-α	Forward: 5'- TTGACCTCAGCGCTGAGTTG-3' Reverse: 5'- CCTGTAGCCCACGTCGTAGC-3'	53	364
IL-1β	Forward: 5'- CAGGATGAGGACATGAGCACC-3' Reverse: 5'- CTCTGCAGACTCAAACTCCAC-3'	57	447
IL-6	Forward: 5'- GTACTCCAGAAGACCAGAGG-3' Reverse: 5'- TGCTGGTGACAACCACGGCC-3'	57	308
IL-8	Forward: 5'- CAATGGACTGGTGAGCCC A -3' Reverse: 5'- AGTTCAAACTCGTCGCCTG -3'	57	486
MCP-1	Forward: 5'- ACTGAAGCCAGCTCTCTCTTCCTC -3' Reverse: 5'- TTCCTTCTTGGGGTCAGCACAGAC -3'	60	618
MIP-1α	Forward: 5'- GCCCTTGCTGTTCTTCTCTGT -3' Reverse: 5'- GGCAATCAGTTCCAGGTCAGT -3'	60	488
iNOS	Forward: 5'- CCCTTCCGAAGTTTCTGGCAGCAGC-3' Reverse: 5'- GGCTGTCAGAGCCTCGTGGCTTTGG-3'	60	496
COX-2	Forward: 5'-CACTACATCCTGACCCACTT-3' Reverse: 5'-ATGCTCCTGCTTGAGTATGT-3'	55	696
β-Actin	Forward: 5'-GTGGGCCGCCCTAGGCACCAG-3' Reverse: 5'-GGAGGAAGAGGATGCGGCAGT-3'	57	603

2.5 Total anti-oxidant activities of the ethanol extract of Wisumpayayai remedy.

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay which uses antioxidants as reductants in a redox-linked colorimetric method. Antioxidant activity based on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) which has intensive blue

color in the presence of antioxidants. Antioxidants can be classified into 4 categories on the basis of theirs antioxidant activities (Table 3).

The working FRAP reagent is the solutions mixture containing 300 mM sodium acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl- s- triazine) in 40 mM HCl; and 20 mM $FeCl_{3.6}$ H_2O in the ratio of 10:1:1 by volume. This solution was heated to 37 ^{0}C in a water bath. Hundred µl the ethanol extract at 6.25-100 µg/ml is mixed and incubated with 180 µl of the working FRAP reagent and 10 µl deionized water in 96-well plate at room temperature for 5 min. Ten µg/ml ascorbic acid and 0.2% DMSO were used as the positive and the negative controls, respectively. The ferrous content was determined by measuring the plate by a microplate reader at 600 nm and calculated the amount of ferrous from a standard curve prepared from standard $FeSO_4.7H_2O$ solutions. The anti-oxidant activity of the ethanol extract was categories based on the classification in Table 5.

Table 5: Antioxidant activity classification according to FRAP assay [121].

anti-oxidant activity	Concentration of $FeSO_4.7H_2O$ (µmol Fe (II)/g)
extremely high	> 500
High anti- oxidant activity	100-500
Medium anti- oxidant activity	10-100
Low anti- oxidant activity	< 10

7. Statistical analysis

All data were presented as mean with standard deviation (mean ± S.D.). Statistical comparisons were determined by one-way ANOVA followed by Tukey's post hoc test. All statistical analyses were performed according to the statistic program, SPSS version 17. Any *p*-value $\leq$ 0.05 will be considered statistically significant.

CHAPTER IV
RESULTS

1. Effect of the ethanol extract of Wisumpayayai remedy on NO production and cell viability in LPS stimulated J774A.1 macrophages cells.

NO is one of the key mediators used to evaluate the role of macrophages in inflammatory process. In this study, NO was determined using Griess reagent and it was showed that 100 ng/ml LPS potently stimulated NO production in J774A.1 cells. It was inhibited by pre-treatment and co-treatment with the ethanol extract of Wisumpayayai remedy 6.25-100 µg/ml in a concentration-dependent manner. Pretreatment with 6.25, 12.5, 25, 50 and 100 µg/ml significantly inhibited NO production in LPS-stimulated J774A.1 cells by 26.55, 29.70, 34.45, 56.26 and 95.47%, respectively (Fig.15A), with the half maximal inhibitory concentration (IC_{50}) values of 40.77 µg/ml (Fig.15B). Cotreatment of the extract at the concentration of 6.25, 12.5, 25, 50 and 100 µg/ml significantly inhibited NO productions in LPS-stimulated J774A.1 cells by 14.02, 30.93, 44.67, 77.04 and 88.24%, respectively (Fig.17A), with IC_{50} values of 37.39 µg/ml (Fig.17B). The reference control, 3.92 µg/ml (10 µM) dexamethasone inhibited NO production by 61.58 and 65.58% in pretreatment and cotreatment, respectively. Neither pretreatment nor cotreatment of the extract affect the viability of J774A.1 cells in all concentrations used (Fig.16, 18). The concentrations obtained from this study were subsequently selected for the further investigation (25-100 ug/ml).

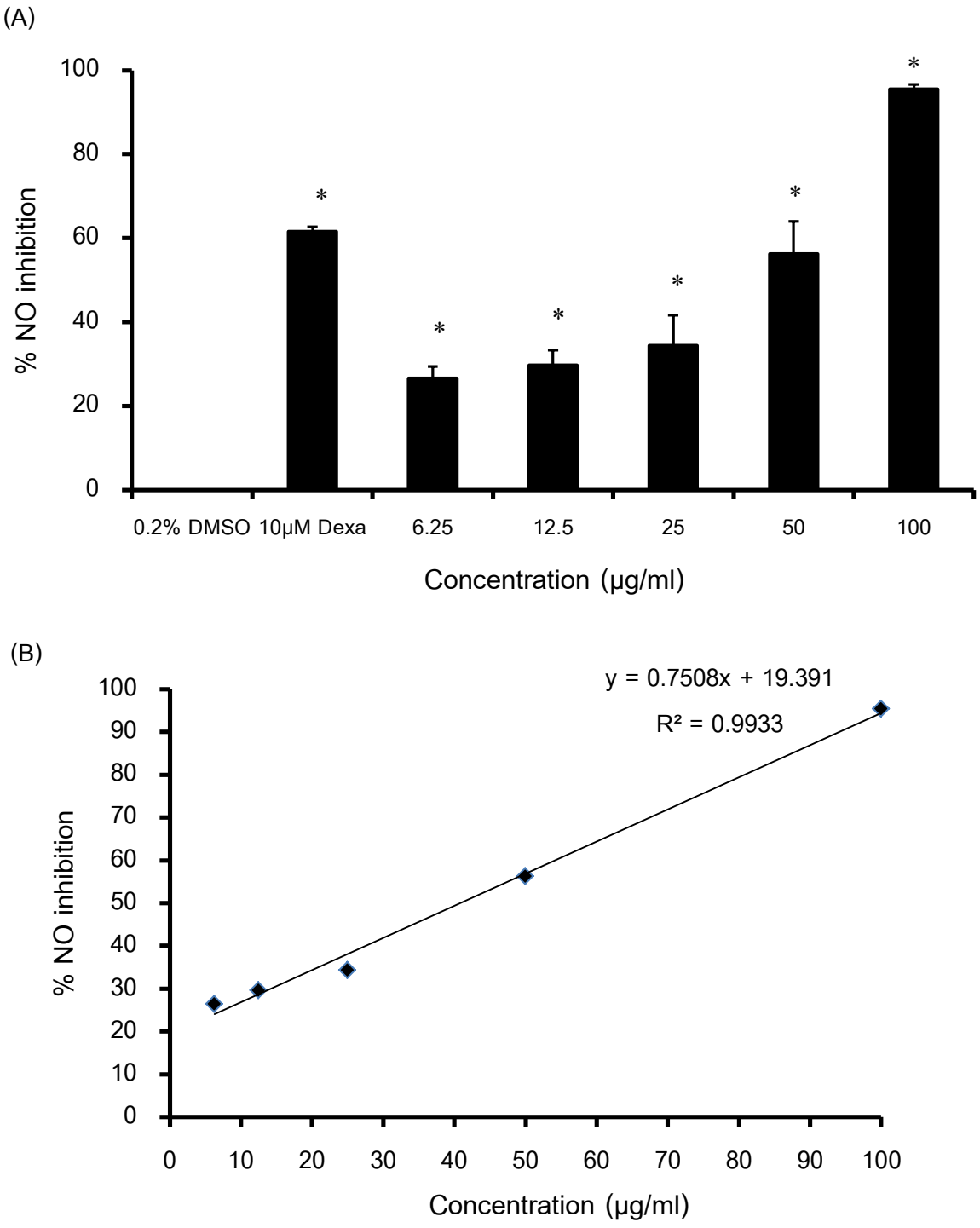


Figure 15: (A) Effect of pretreatment of the ethanol extract of Wisumpayayai remedy 6.25-100 μg/ml on NO production in LPS-stimulated J774A.1 cells. (B) The IC_{50} of pretreatment of the extract (40.77μg/ml). The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *$P < 0.05$ compared with the solvent control.

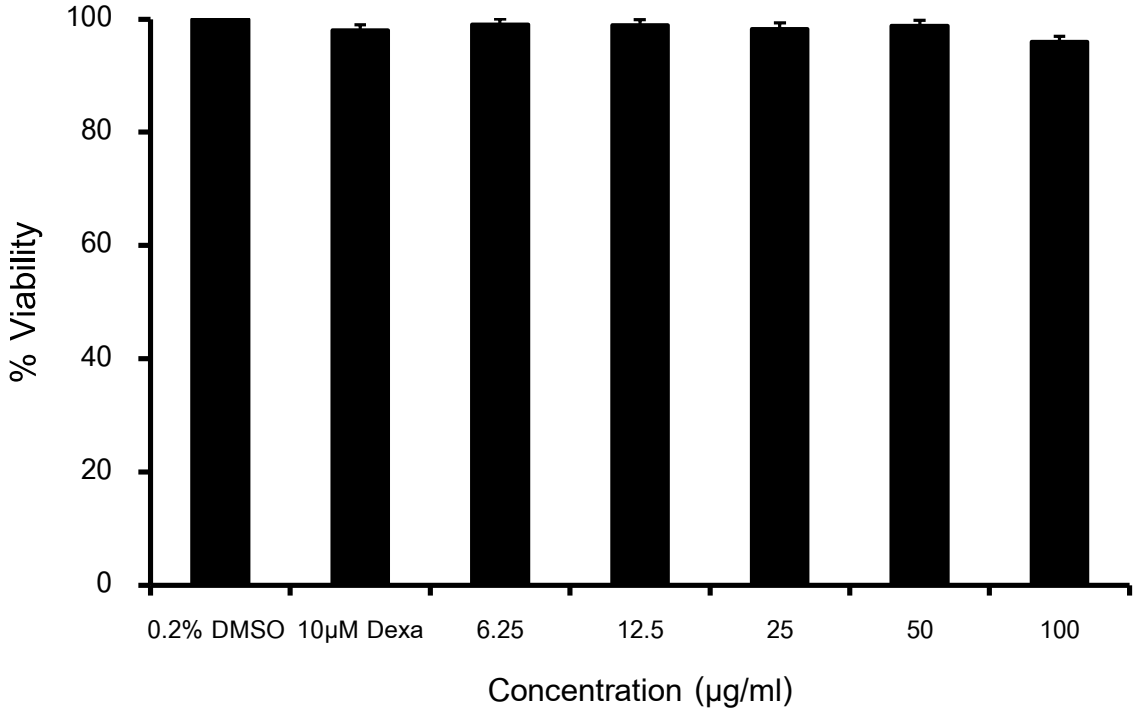


Figure 16: Effect of pretreatment of the ethanol extract of Wisumpayayai remedy at 6.25-100 μg/ml on cell viability in LPS-stimulated J774A.1 cells. The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *$P < 0.05$ compared with the solvent control.

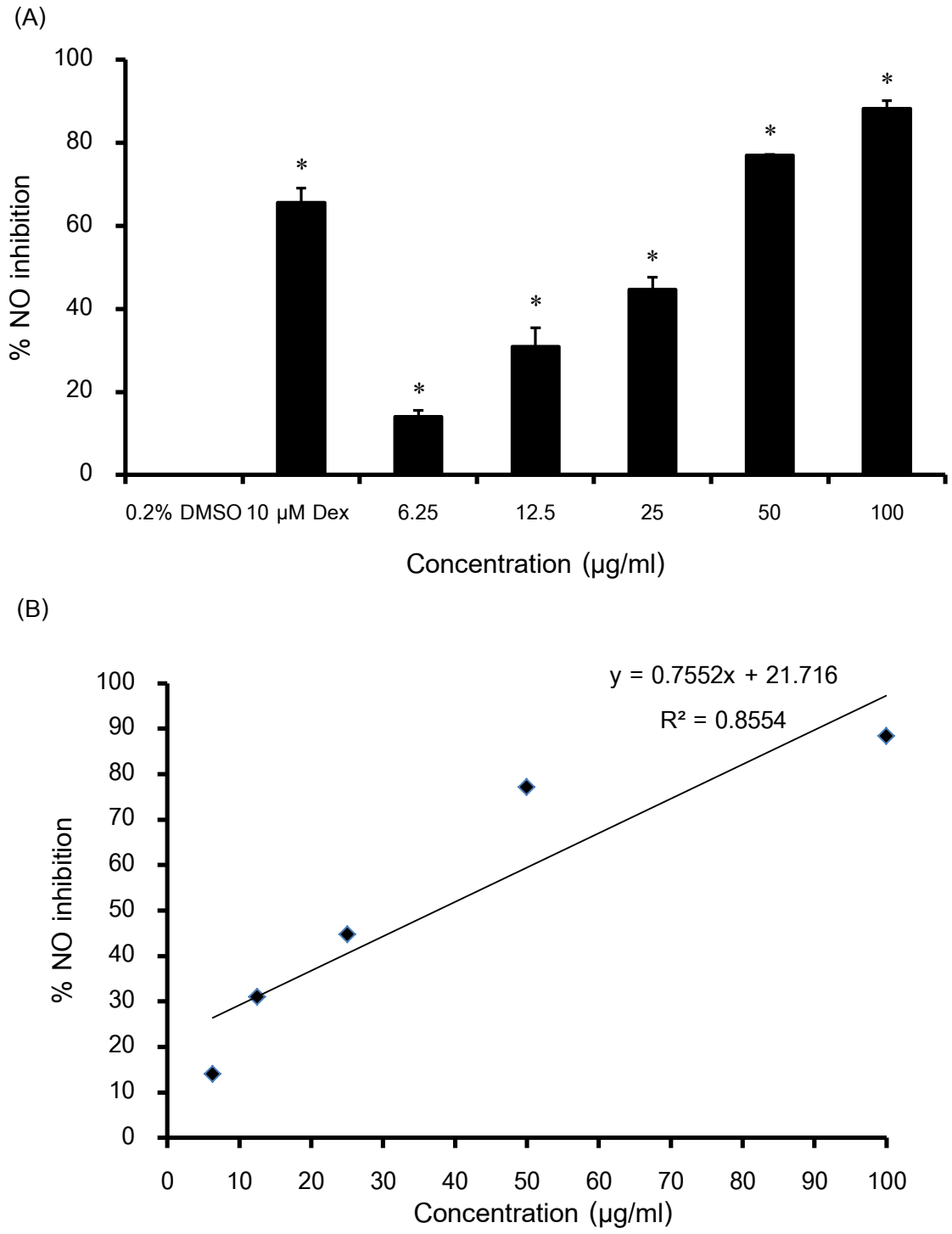


Figure 17: (A) Effect of cotreatment of the ethanol extract of Wisumpayayai remedy 6.25-100 µg/ml on NO production in LPS-stimulated J774A.1 macrophages cells. (B) The IC_{50} of pretreatment of the extract (37.39 µg/ml). The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *$P < 0.05$ compared with the solvent control.

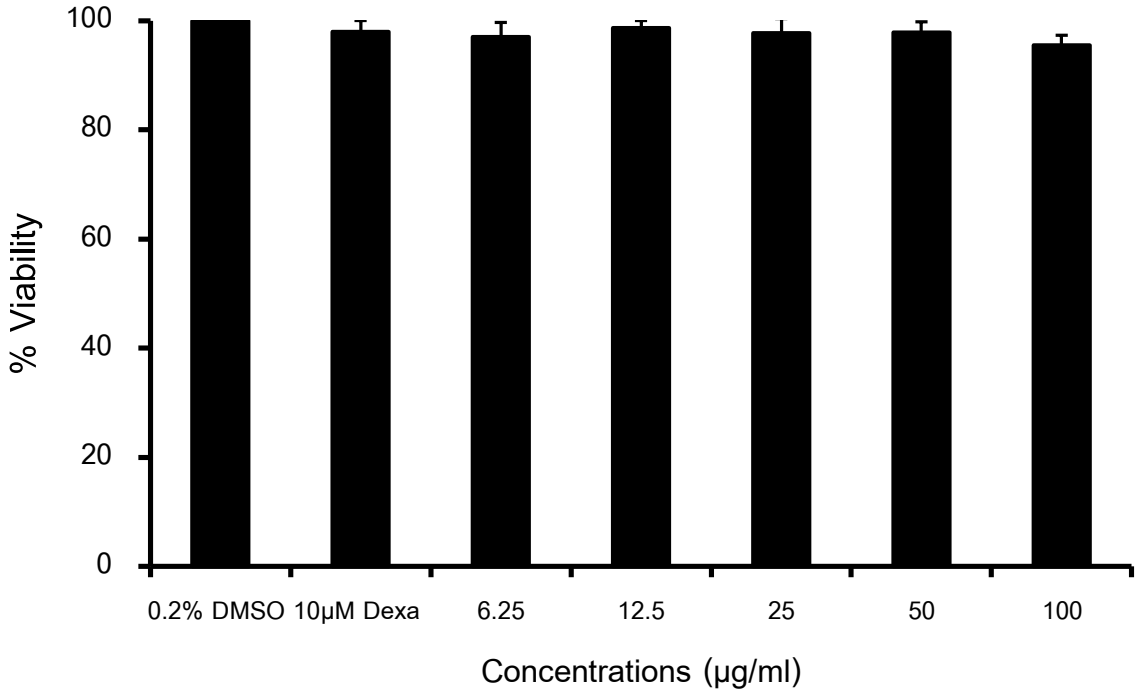


Figure 18: Effect of cotreatment of the ethanol extract of Wisumpayayai remedy 6.25-100 μg/ml on cell viability in LPS-stimulated J774A.1 cells. The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *$P < 0.05$ compared with the solvent control.

2. Effect of the ethanol extract of Wisumpayayai remedy on phagocytic activity in LPS-stimulated J774A.1 macrophages cells.

Macrophages are major tissue phagocytes in innate immunity. They have phagocytic ability to engulf and destroy pathogens. Phagocytic activity is a crucial aspect of macrophage functional assessment. The effects of the ethanol extract of Wisumpayayai remedy on phagocytic activity in LPS-stimulated J774A.1 macrophages cells was further investigated by using zymosan-nitroblue tetrazolium (NBT) reduction assay. The results showed that pre-treatment of the extracts at the concentration of 12.5, 25, 50 and 100 µg/ml were significantly inhibited phagocytic activity in the stimulated cells in a concentration-dependent manner by 5.85, 22.63, 33.34 and 69.48%, respectively (Fig.19A), with IC_{50} value of 71.77 µg/ml (Fig.19B). The similar results were obtained after co-treatment of the extract at the same concentration (12.5-100 µg/ml). The inhibition was found to be 12.65, 25.33, 37.64 and 59.26%, respectively (Fig.20A), with IC_{50} value of 78.99 µg/ml (Fig.20B). However, there was no statistical difference in phagocytic activity between pre-treatment and co-treatment. The reference control, 3.92 µg/ml (10 µM) dexamethasone inhibited phagocytic activity by 16.2, 13.82% in pre-treatment and co-treatment, respectively.

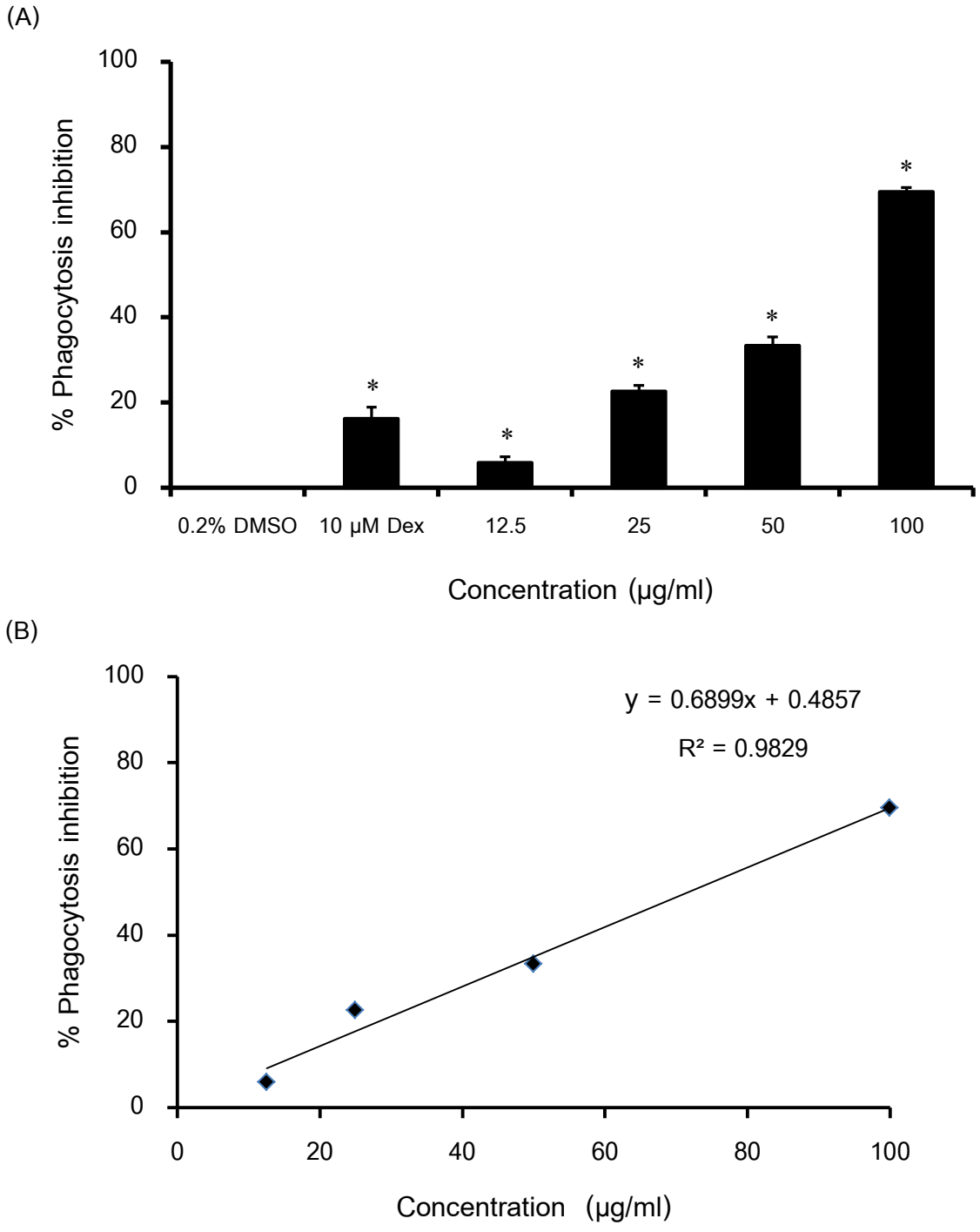


Figure 19: Effect of the ethanol extract of Wisumpayayai remedy on phagocytic activity in LPS-stimulated J774A.1 cells. (A) Inhibitory effect on phagocytic activity of pretreatment with 12.5-100 µg/ml of the extracts. (B) The IC_{50} of pretreatment of the extract (71.77 µg/ml). The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *P < 0.05 compared with the solvent control.

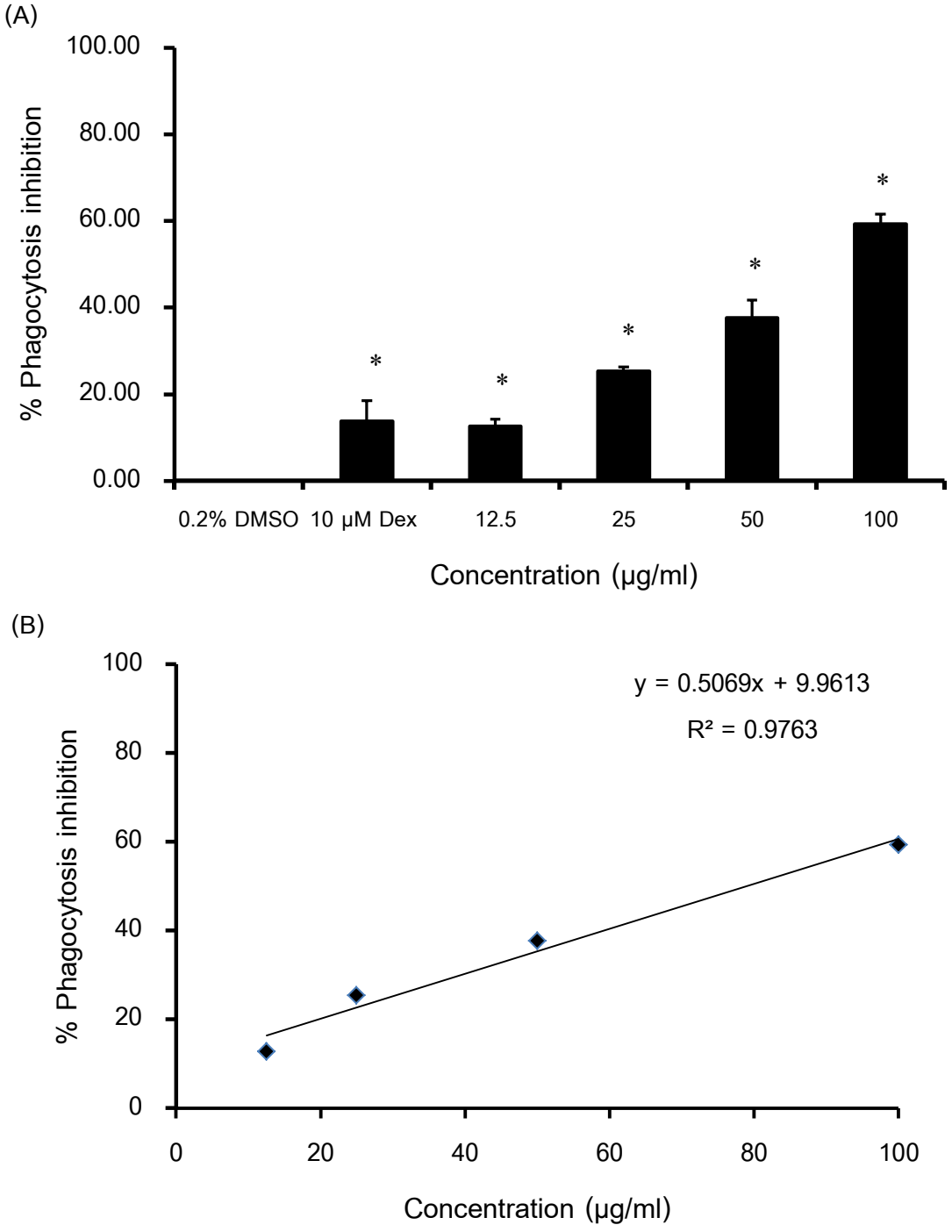
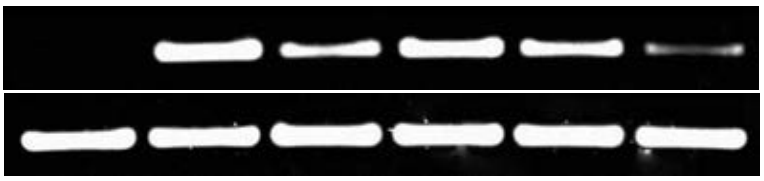


Figure 20: Effect of the ethanol extract of Wisumpayayai remedy on phagocytic activity in LPS-stimulated J774A.1 cells. (A) Inhibitory effect on phagocytic activity of cotreatment with 12.5-100 µg/ml of the extracts. (B) The IC_{50} of cotreatment of the extract (78.99 µg/ml). The data were expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *P < 0.05 compared with the solvent control.

3. Effect of The ethanol extract of Wisumpayayai remedy on the mRNA expression of iNOS in LPS stimulated J774A.1 macrophages cells.

The results obtained from experiment 1 and 2 showed no statistically difference in NO inhibition and phagocytic activity neither pretreatment nor cotreatmnet of the extract, thus, the further subsequent studies were performed under cotreatment condition. To elucidate the underlying mechanisms of the inhibition of NO production by the extracts, the effect of cotreatment with the ethanol extract of Wisumpayayai remedy on the activity of iNOS enzyme in LPS-stimulated J774A.1 macrophages cells were examined with 25, 50 and 100 µg/ml the extract for 24 h. mRNA expression of iNOS were determine by RT-PCR. The results showed that the extracts at the concentration of 50 and 100 µg/ml significantly inhibited mRNA expression of iNOS by 52.20 and 86.87%, respectively (Fig.21, Appendix 7). The reference control, 3.92 µg/ml (10 µM) dexamethasone also significantly inhibited mRNA expression by 75.46%.

(A)



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100

(B)

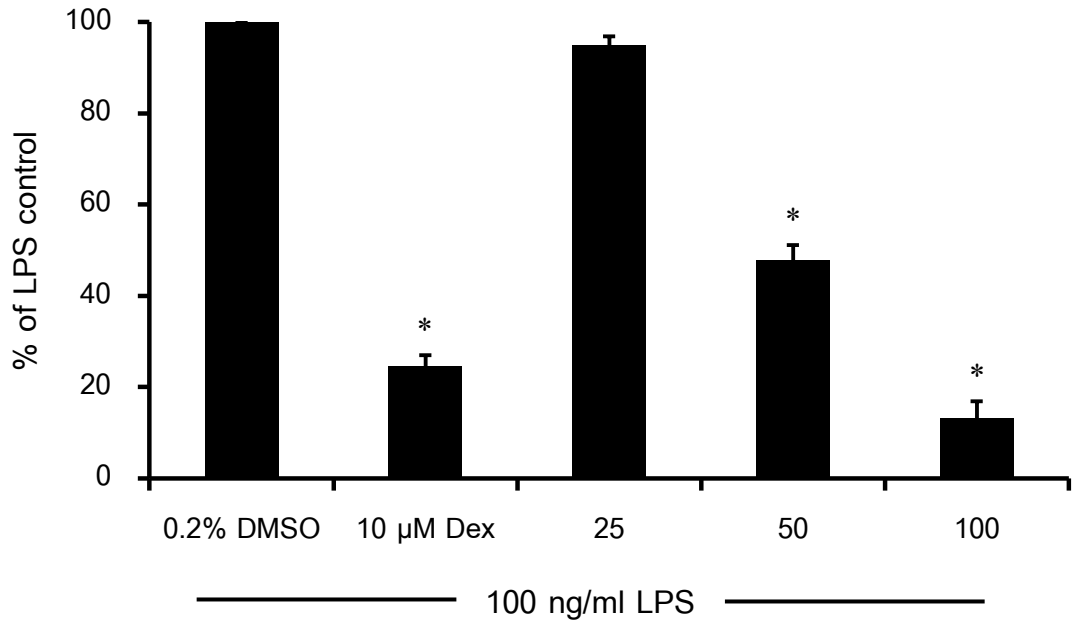
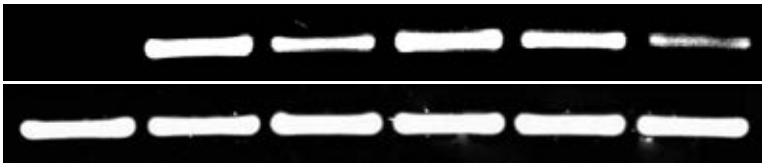


Figure 21: Effect of cotreatment of the ethanol extract of Wisumpayayai remedy 25-100 μg/ml on the mRNA expression of iNOS in LPS-stimulated J774A.1 cells. (A) Representatives of the PCR product of iNOS enzyme from the extract-treated cells were detected by agarose gel electrophoresis. (B) Densitometry analysis the PCR products relative to β-actin represented as % of control. The data is expressed as mean ± S.D. of three independent experiments (n=3). *$P < 0.05$ compared with the untreated control

4. Effect of the ethanol extract of Wisumpayayai remedy on the mRNA expression of COX-2 in stimulated J774A.1 macrophages cells.

COX-2 an inducible enzyme associated with inflammation and inflammatory diseases. In this study, mRNA expression of COX-2 in LPS-stimulated J774A.1 cells was determined by RT-PCR. After co-treatment with 50 and 100 µg/ml of the extract, mRNA expression of COX-2 were significantly inhibited by 32.93 and 70.88%, respectively (Fig.22, Appendix 7). While reference control, 3.92 µg/ml (10 µM) dexamethasone showed 56.77% inhibition of mRNA expression of COX-2.

(A)



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100

(B)

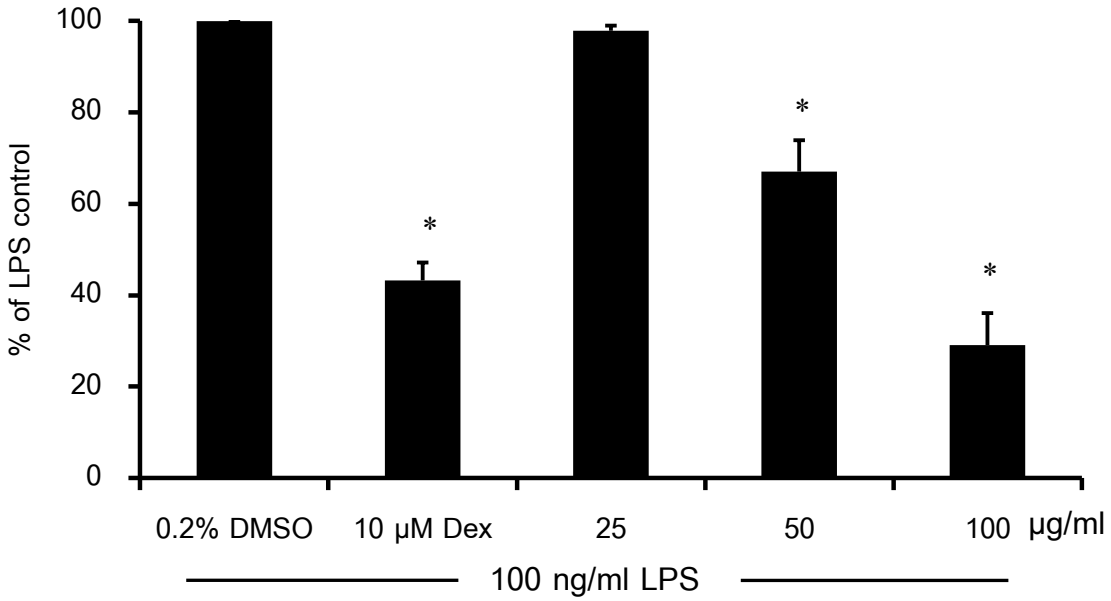
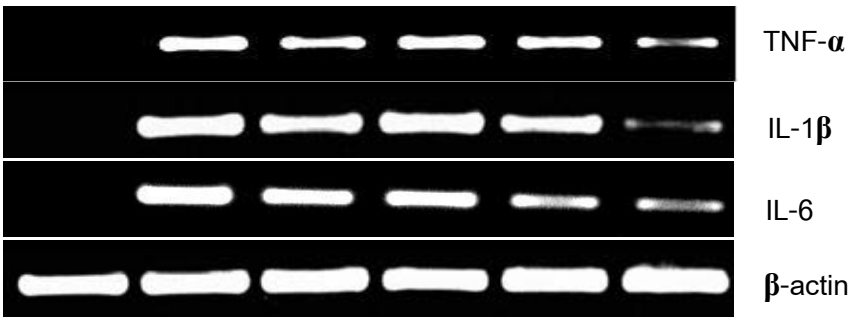


Figure 22: Effect of cotreatment the ethanol extract of Wisumpayayai remedy 25-100 μg/ml on the mRNA expression of COX-2 in LPS-activated J774A.1 cells. (A) Representatives of the PCR product of COX-2 enzyme from the extract-treated cells were detected by agarose gel electrophoresis. (B) Densitometry analysis the PCR products relative to β-actin represented as % of control. The data is expressed as mean ± S.D. of three independent experiments (n=3). *$P < 0.05$ compared with the untreated control.

5. Effect of The ethanol extract of Wisumpayayai remedy on the mRNA expression of inflammatory cytokines.

After pathogens recognition, macrophages become activated macrophages to produce several cytokines, such as pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). After cotreatment with the ethanol extract of Wisumpayayai remedy, it was showed that 50 and 100 µg/ml of the extract were able to inhibit mRNA expression of pro-inflammatory cytokines, TNF-α, by 50.39% and 83.15%, IL-1β by 55.43% and 79.20%, respectively. For mRNA expression of IL-6, the extract 25, 50 and 100 ug/ml were able to inhibit cytokine expression by 46.60, 64.87% and 84.21%, respectively (Fig.23, Appendix 12). The reference control, 3.92 µg/ml (10 µM) dexamethasone also significantly inhibited mRNA expression of TNF-α, IL-1β, and IL-6 by 66.83, 52.82, 49.65%, respectively.

(A)



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100

(B)

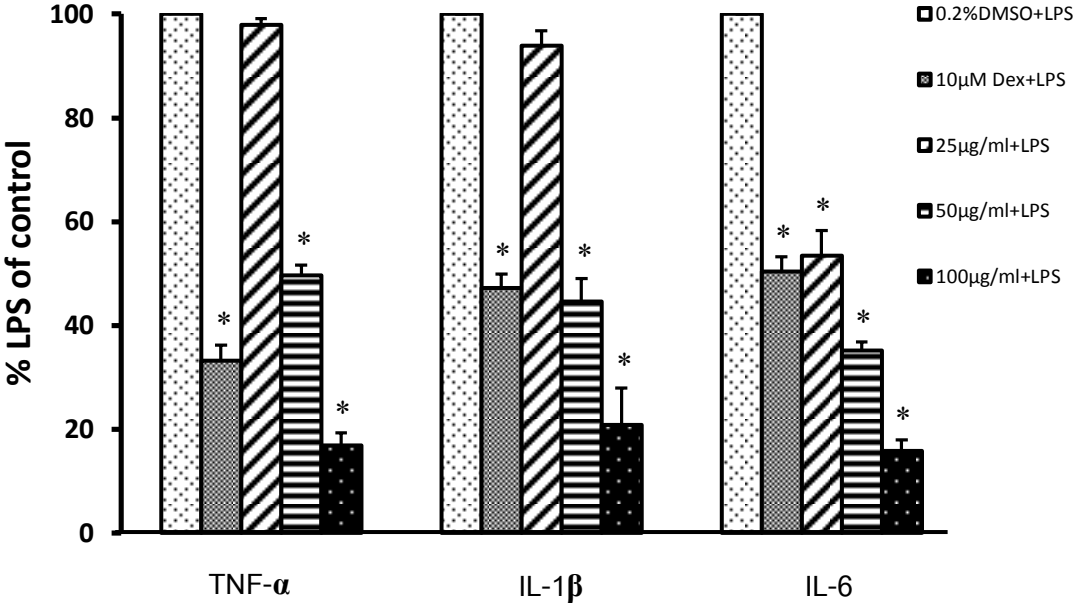
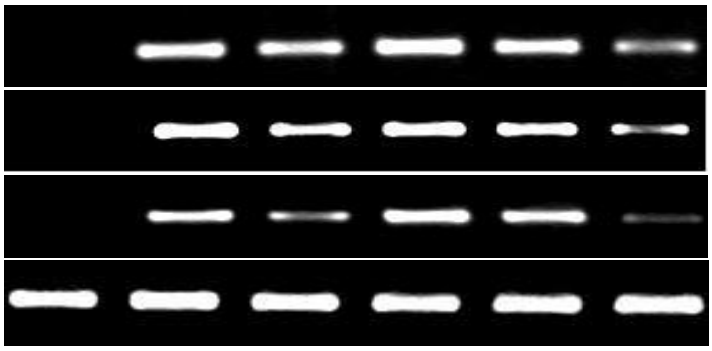


Figure 23: Effect of cotreatment of the ethanol extract of Wisumpayayai remedy 25-100 μg/ml on the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in LPS-stimulated J774A.1. (A) Representatives of the PCR product of pro-inflammatory cytokines from the extract-treated cells were detected by agarose gel electrophoresis. (B) Densitometry analysis the PCR products relative to β-actin represented as % of control. The data is expressed as mean ± S.D. of three independent experiments (n=3) performed in triplicate *$P < 0.05$* compared with the untreated control.

6. Effect of The ethanol extract of Wisumpayayai remedy on the mRNA expression of chemokines in LPS stimulated J774A.1 macrophages cells

The effects of the ethanol extract of Wisumpayayai remedy on the mRNA expression of chemokines (MCP-1, MIP-1α and IL-8) in LPS-stimulated J774A.1 were performed. After co-treatment with 50 and 100 μg/ml, mRNA expression of MCP-1, MIP-1α and IL-8 were significantly inhibited. For MCP-1, percent inhibition was 33.39% and 82.41%, respectively. Percent inhibitions were found to be 45.09% and 80.01%, for MIP-1α, 36.81% and 85.66% for IL-8 respectively (Fig.24, Appendix 12). While reference control, 3.92 μg/ml (10 μM) dexamethasone showed 19.61, 49.85 and 64.19% inhibition of mRNA expression of MCP-1, MIP-1α, IL-8, respectively.

(A)



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100

(B)

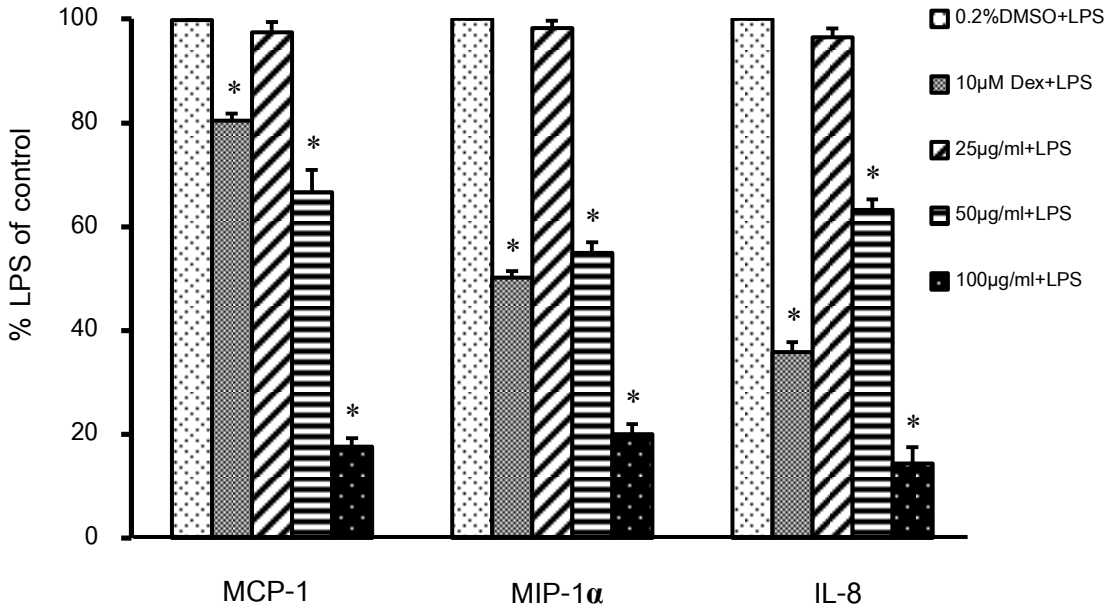


Figure 24: Effect of the ethanol extract of Wisumpayayai remedy 25-100 μg/ml on the mRNA expression of chemokines (MCP-1, MIP-1α and IL-8) in LPS-stimulated J774A.1 cells. (A) Representatives of the PCR product of chemokine from the extract-treated cells were detected by agarose gel electrophoresis. (B) Densitometry analysis the PCR products relative to β-actin represented as % of control. The data is expressed as mean ± S.D. of three independent experiments (n=3). *$P < 0.05$* compared with the untreated control.

7. Total anti-oxidant activities of the ethanol extract of Wisumpayayai remedy.

The desirable preventive or therapeutic properties of the Wisumpayayai remedy has also been considered to be associated with its antioxidant and anti-inflammatory properties. Therefore, we investigated the anti-oxidant activities of the extracts by Ferric reducing antioxidant power (FRAP) assay. In this study, antioxidant activity based on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to ferrous tripyridyltriazine (Fe (II)-TPTZ), resulting in blue color in the presence of antioxidants. It was demonstrated that 6.25, 12.5, 25, 50, 100 µg/ml were able to reduce Fe (III)-TPTZ and 0.25, 36.68, 67.23, 98.67 and 417.67 µmol/g of (Fe (II)-TPTZ) were formed (Fig.25). According to anti-oxidant classification (Table 5). The result showed that the ethanol extract of Wisumpayayai remedy exerted high antioxidant activity at the concentrations of 100 µg/ml and medium antioxidant activity at the concentrations of 12.5-50 µg/ml. While the reference agent, vitamin C 100 µM showed extremely high activity.

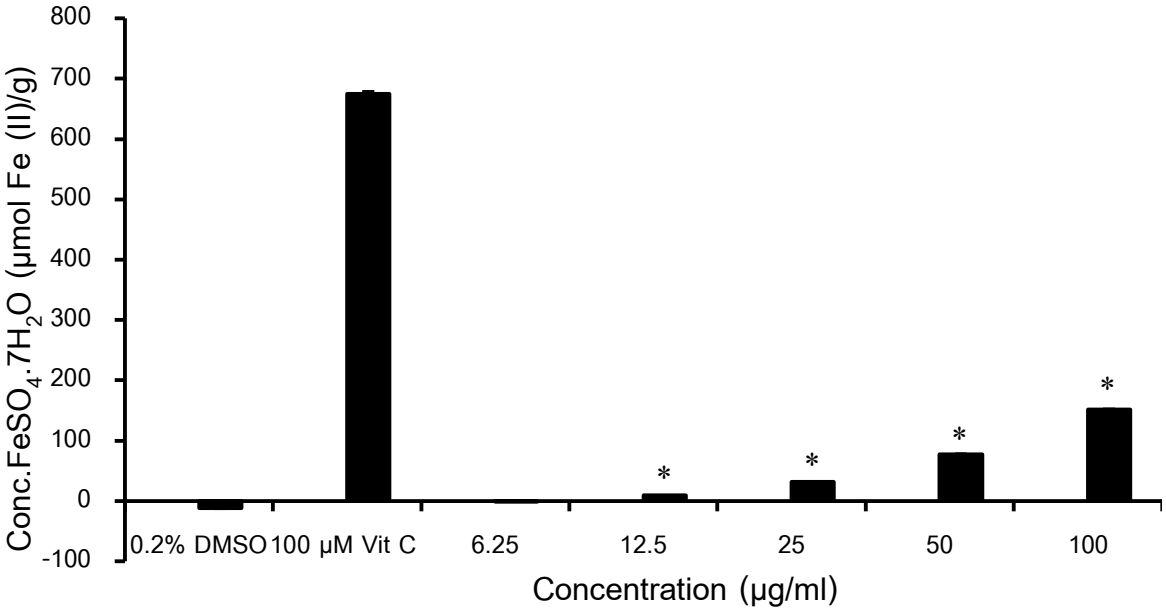


Figure 25: Total anti-oxidant activities of the ethanol extract of Wisumpayayai remedy determined by FRAP assay. The data are expressed as means ± S.D. of three independent experiments (n=3) performed in triplicate. *$P < 0.05$ compared with the solvent control.

CHAPTER V
DISCUSSION AND CONCLUSION

Macrophages play important roles in immune responses, both in innate and in adaptive immunity. It is known that macrophages are key players in the inflammatory process. In terms of innate immunity, microbial components like lipopolysaccharide (LPS), a lipoglycan of the outer membrane of gram negative bacteria, is commonly used to activate macrophages for evaluating anti-inflammatory substances. LPS potently stimulates the production of pro-inflammatory cytokines, chemokines, nitric oxide (NO), prostaglandins (PGs) and other inflammatory mediators. Furthermore, these mediators not only play roles in innate immune response but also initiate and induce neighbor tissue injury and cause many inflammatory diseases, for examples osteoarthritis, rheumatoid arthritis etc [1-2]. Inhibition of these mediators is current or trend therapeutic strategy for the treatment of inflammation.

Wisumpayayai remedy is approved by the Ministry of Public Health of Thailand in National Drug List as the folk remedy for anti-flatulence and anti-dyspepsia. Several plants from this remedy exert anti-inflammatory activity *in vivo* and *in vitro* (Table 3). In this present study, it was showed that the ethanol extract of Wisumpayayai remedy 6.25-100 µg/ml inhibited NO production in LPS-stimulated J774A.1 cells in a concentration dependent manner either by pretreatment and cotreatment of the extract. Their IC_{50} values for NO inhibition were 40.77 and 37.39 µg/ml for pre-treatment and co-treatment respectively. It also inhibited phagocytic activity of the activate cells in a concentration dependent manner at 12.5-100 µg/ml with IC_{50} values 71.77 and 78.99 µg/ml for pre-treatment and co-treatment respectively. However, there were no statistical difference in percent inhibition of NO production and phagocytic activity between pre-treatment and co-treatment of the extract. Moreover, NO inhibitory concentrations of the extract on the LPS-stimulated J774A.1 revealed no cytotoxicity (6.25-100 µg/ml), as assessed by resazurin assays. Thus, we selected the model of cotreatement and the extract

concentrations for the subsequent investigation. The underlying mechanism in NO inhibitory effect was first evaluated through mRNA iNOS expression by RT-PCR. iNOS inhibitory expression effect was found at concentrations 50 and 100 µg/ml. This activity was correlated to the inhibition on NO production of the extract.

The pathogen elimination in the initial stage immediately after phagocytosis is related to the upregulated enzymatic activities of NADPH oxidase and nitric oxide synthase in phagolysosomes of activated macrophages. NADPH oxidase regulates productions of toxic free radicals in eliminating pathogen during phagocytosis such as reactive oxygen species (ROS) and NO. NO is one of the key mediators commonly used as a parameter to determine macrophages activation. Nitrite production is a stable end product of the rapidly occurring oxidation of NO and it is used for Griess reaction assay. NO plays role in destroying pathogen in phagolysosomes by oxygen-dependent pathway. Large amount of NO generation is stimulated by inducible nitric oxide synthase (iNOS) in pathological condition such as infection, inflammation. NO from activated macrophages interact with superoxide anion to generate powerful free radical peroxynitrite ($ONOO^-$). Free radicals also cause tissue injury during inflammation [25]. The desirable preventive or therapeutic properties of Wisumpayayai remedy has also been considered to be associated with its antioxidant and anti-inflammatory properties. It was revealed that the extract showed high anti-oxidant activities at concentration 100 µg/ml by FRAP assay. Thus, the antioxidant activity and NO inhibitory activity found in this extract were the underlying mechanisms subsequent leading to the anti-inflammatory ability.

In addition, prostaglandins are also involved in the pathogenesis of pain and fever in inflammation process. PGs are known to be important mediators in acute and chronic inflammation. They are produced by cyclooxygenase-2 enzyme (COX-2). COX-2 is a key enzyme responsible for the conversion of arachidonic acid (AA) to PGs [49]. Previous studies have been found that some of the herbal plants in Wisumpayayai remedy such as *C. sativum*, *A. sinensis*, *P. retrofractum* etc. inhibit PGE2 production in

LPS-activated RAW 264.7 cells, while they down-regulate COX-2 expression by inhibiting NF-kB activation [56, 112, 118]. The inhibitory effects of the extracts on inflammatory mediators, prostaglandins, were also indirectly investigated by determining mRNA expression of COX-2. From this present result, the extract significantly inhibited mRNA expression of COX-2 in the LPS-stimulated J774A.1 cells at 50 and 100 ug/ml. Decrease COX-2 mRNA expression may result in inhibition of prostaglandins production and improving inflammatory response.

We further determined the effect of Wisumpayayai remedy extract on cytokines and chemokines expression because these mediators are functional outcome of macrophages activation and known to have direct, immediate and protracted impact on inflammation [36]. The maximum production of pro-inflammatory cytokines and chemokines occurs at approximately 4 h after induction with LPS. The results showed that the extract suppressed mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokines (MCP-1, MIP-1α and IL-8) at 4 h after LPS stimulation of the macrophages. TNF-α and IL-1β are rapidly released cytokines after LPS stimulation. They are key pro-inflammatory cytokines that regulate inflammatory processes such as inflammatory cells activation and recruitment. Moreover, they induce the expression of inflammatory cytokines, chemokines and cell adhesion molecules. They together play roles during inflammation. Inhibition of pro-inflammatory cytokine production was subsequent reduce other inflammatory molecules resulting in inflammation alleviation [40].

Although molecular signaling mechanism associated with the inhibition of LPS-stimulated macrophages J774A.1 expression of pro-inflammatory-cytokines (TNF-α, IL-1β and IL-6), chemokine (MCP-1, MIP-1α and IL-8) and inflammatory enzyme (COX-2 and iNOS) by the ethanol extract of Wisumpayayai remedy in this study were not investigated, the evidences from the previous reports showed that some of the herbal plants in Wisumpayayai remedy inhibited pro-inflammatory cytokine and inflammatory mediator production by blocking the activation of NF-kB in activated macrophage cells

(table 3). It is well established that transcriptions factor, NF-kB controls the expression of inflammatory genes. The association of NF-kB and inhibitory effect of the extract are needed to be further elucidated.

Wisumpayayai remedy, a traditional Thai remedy, has been used as anti-flatulence and anti-dyspeptic medication. It is composed of 20 herbal plants including; *Coriandrum sativum, Diospyros decandra, Myristica fragrans, Amomum krervanh, Syzygium aromaticum, Angelica dahurica, Atractylodes lancea, Conioselinum univitatum, Angelica sinensis, Artemisia pallens, Cinnamomum verum, Cinnamomum bejolghota, Terminalia arjuna, Terminalia chebula, Acorus calamus, Aristolochia sp., Tinospora crispa, Zingiber kerrii, Melastoma saugnineum, Piper retrofractum* (Table 2). Fourteen of these plants have been reported to have anti-inflammatory activities including *Coriandrum sativum L., Diospyros decandra, Myristica fragrans, Angelica dahurica, Atractylodes lancea, Conioselinum univitatum, Angelica sinensis, Artemisia pallens, Cinnamomum bejolghota, Terminalia arjuna, Terminalia chebula, Acorus calamus L., Zingiber kerrii, Piper retrofractum* (Table 3). The ancient people believe that combination of phytomedicines lead to provoke synergism effect and reduce interaction among the components presenting in it and also decrease its side effect. At present, some of the herbal plants from Wisumpayayai components have been widely studied in the diversity of pharmacological effects. Several of them demonstrated anti-inflammatory activities by inhibiting macrophages activation, anti-oxidant activity and immunomodulation (table 2). The preliminary result obtained from this study demonstrated the evidences supporting anti-inflammatory activities of Wisumpayayai remedy extract.

In summary, this study primarily evaluated the inhibitory effect of the ethanol extract of Wisumpayayai remedy on the activated macrophages. It exerted NO, phagocytic inhibitory effect. It also decreased mRNA expression of proinflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (MCP-1, MIP-1α and IL-8) enzyme iNOS and COX-2. Moreover, it exhibited antioxidant activity by FRAP assay. Further

investigations involve its molecular mechanism and *in vivo* anti-inflammatory effects are needed to confirm its anti-inflammatory properties.

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Appendices

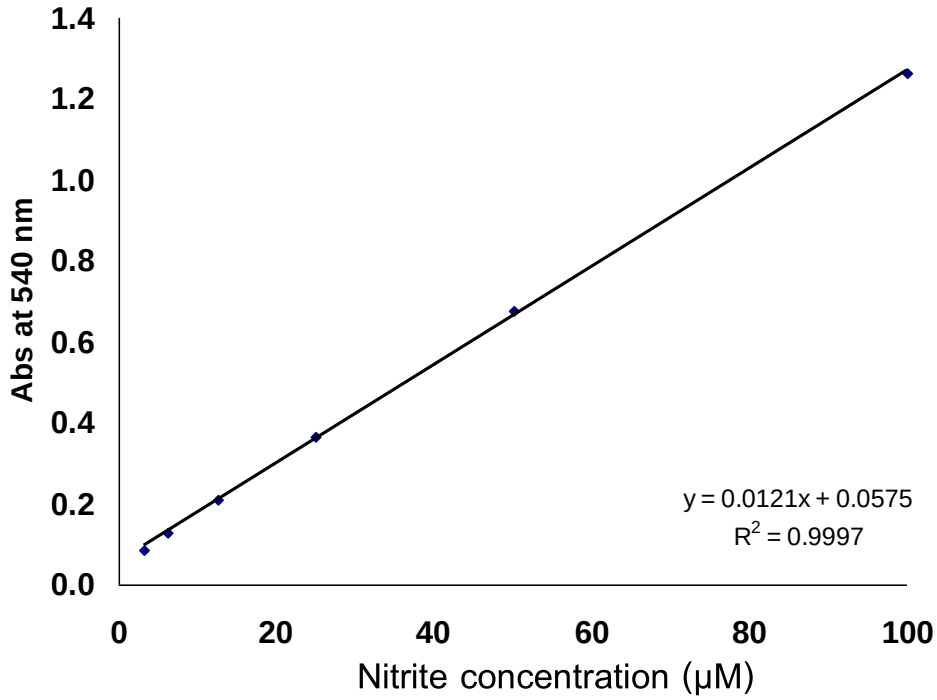
APPENDIX

EXPERIMENT RESULTS

Appendix 1: Data of standard calibration curve of nitrite by Griess reaction

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

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



Appendix 3: Data of pretreatment and cotreatment the effect of the ethanol extract of Wisumpayayai remedy on nitric oxide production in LPS-stimulated J774A.1 cells by Griess reaction (n=3).

Test Compounds		Concentration of nitrite (μM) of pre-treatment			Mean ± S.D.	Concentration of nitrite (μM) of co-treatment			Mean ± S.D.
		1	2	3		1	2	3	
0.2% DMSO		36.19	27.36	26.81	30.12 ± 2.67	30.97	32.39	33.44	32.27 ± 2.67
0.2% DMSO+LPS		14.72	22.19	20.81	19.24 ± 1.09	10.36	12.69	13.56	12.20 ± 1.09
Dexa (10μM)+LPS		27.63	19.33	19.67	22.21 ± 2.83	27.19	27.64	28.36	27.73 ± 2.83
Wisumpayayai (μg/ml)	6.25	27.63	19.33	19.67	26.55 ± 2.83	100.00	100.00	100.00	100.00
	12.5	24.50	19.22	19.56	29.70 ± 3.63	39.44	46.54	43.72	43.23 ± 3.87
	25	20.86	18.47	19.17	19.50 ± 7.13	22.83	22.28	21.64	21.25 ± 3.63
	50	13.67	11.22	14.06	12.98 ± 7.77	17.97	16.92	18.64	17.84 ± 7.13
	100	1.17	1.42	1.39	1.32 ± 1.32	7.14	7.39	7.69	7.41 ± 7.70

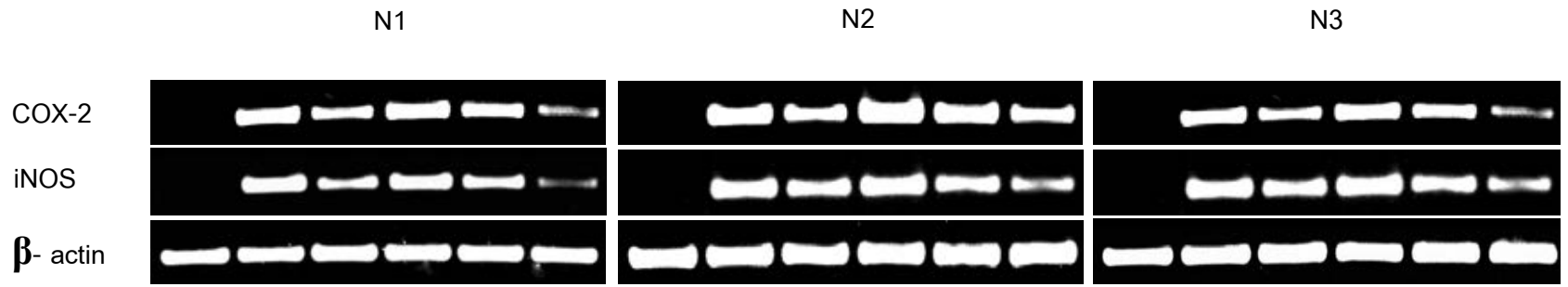
Appendix 4: Data of pretreatment and cotreatment cytotoxicity of the ethanol extract of Wisumpayayai remedy (μg/ml) in LPS stimulated J774A.1 macrophages cells after 2 h exposure by using resazurin assay.

Test Compounds (μg/ml)		Cytotoxicity of pre-treatment			Mean ± S.D.	Cytotoxicity of co-treatment			Mean ± S.D.
		1	2	3		1	2	3	
0.2% DMSO		0.000	0.000	0.000	0.00 ± 0.00	0.000	0.000	0.000	0.00 ± 0.00
0.2% DMSO+LPS		1.805	0.000	0.000	0.602 ± 0.09	0.097	0.065	0.000	0.054 ± 0.09
Dexa (10μM)+LPS		0.342	0.109	0.736	0.396 ± 1.83	0.595	0.021	0.389	0.335 ± 1.83
Wisumpayayai (μg/ml)	6.25	0.342	0.109	0.736	0.396 ± 0.89	0.595	0.021	0.389	0.335 ± 1.32
	12.5	0.952	0.087	0.898	0.646 ± 1.23	0.986	0.545	0.643	0.725 ± 2.12
	25	3.459	0	1.002	1.487 ± 1.67	2.085	1.944	1.745	1.925 ± 2.45
	50	1.462	1.989	2.043	1.831 ± 098	2.374	1.034	1.034	1.481 ± 1.56
	100	4.098	2.094	5.081	3.758 ± 1.12	3.76	2.65	2.034	2.815 ± 2.21

Appendix 5: Data of pretreatment and cotreatment the effect of the ethanol extract of Wisumpayayai remedy on phagocytic activity on LPS-stimulated J774A.1 cells evaluated by zymosan-nitroblue tetrazolium (NBT) reduction assay (n=3). The results were expressed as the percentage of inhibition of phagocytosis compared to the solvent control (mean ± S.D.).

Test Compounds (µg/ml)		% phagocytosis inhibition of pre-treatment			Mean ± S.D.	% phagocytosis inhibition of co-treatment			Mean ± S.D.
		1	2	3		1	2	3	
0.2% DMSO		0.00	0.00	0.00	0	0	0	0	0
0.2% DMSO+LPS		0.00	0.00	0.00	0	0	0	0	0
Dexa (10µM)+LPS		15.70	11.82	21.08	16.2 ± 2.68	5.44	14.44	21.57	13.82 ± 4.66
Wisumpayayai (µg/ml)	12.5	8.67	4.43	4.46	5.85 ± 1.40	12.63	9.80	15.52	12.65 ± 1.65
	25	23.72	19.95	24.21	22.63 ± 1.35	26.49	23.35	26.14	25.33 ± 0.99
	50	37.24	30.54	32.23	33.34 ± 2.01	35.96	31.55	45.42	37.64 ± 4.09
	100	67.69	70.94	69.80	69.48 ± 0.95	59.82	54.90	63.07	59.26 ± 2.37

Appendix 6: Effect of cotreatment the ethanol extract of Wisumpayayai remedy on the mRNA expression of iNOS and COX-2 in LPS stimulated J774A.1 cells.



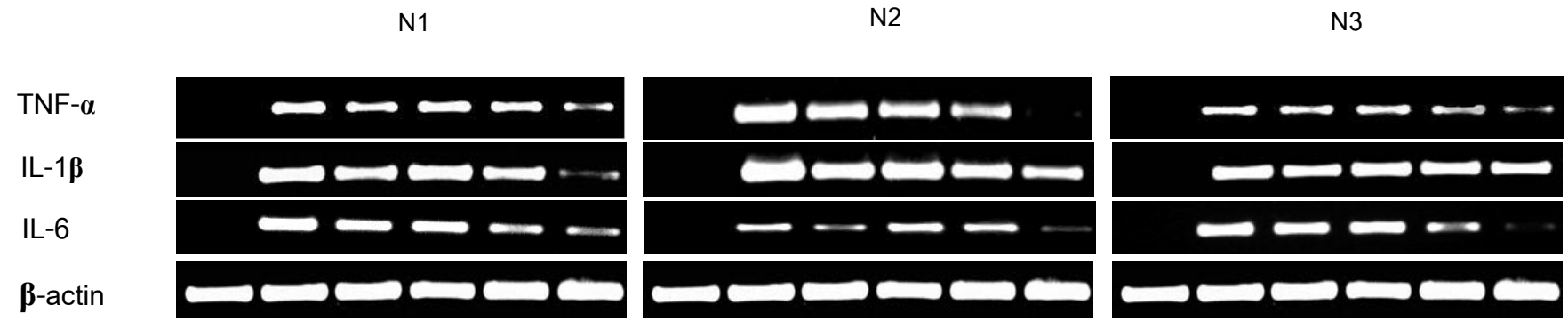
LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100	-	-	-	25	50	100	-	-	-	25	50	100

Cells were treated with 25, 50 and 100 μg/ml of the extract and 100 ng/ml LPS for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplified mRNA of iNOS and COX-2 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR product were determined by using gel documentation and compared with β-actin PCR product. The data are expressed as mean ± S.D. from 3 independent experiments (n=3). *$p<0.05$ compared to untreated control.

Test Compounds		% Of control iNOS			Mean ± S.D.	% inhibition	% Of control COX-2			Mean ± S.D.	% inhibition
		1	2	3			1	2	3		
0.2% DMSO		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.2% DMSO+LPS		100.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00	100.00	0.00
Dexa (10μM)+LPS		29.26	20.91	23.45	24.54 ± 2.47	75.46	39.44	46.54	43.72	43.23 ± 3.87	56.77
Wisumpayayai (μg/ml)	25	92.11	98.52	94.06	94.90 ± 1.90	5.10	95.72	99.72	98.01	97.82 ± 1.16	2.18
	50	47.06	53.95	42.39	47.8 ± 3.36	52.20	54.38	69.06	77.76	67.07 ± 6.82	32.93
	100	10.31	20.69	8.38	13.13 ± 3.82	86.87	16.41	30.81	40.14	29.12 ± 6.90	70.88

Appendix 7: Data of cotreatment the effect of The ethanol extract Wisumpayayai remedy on the mRNA expression of iNOS and COX-2 in LPS-stimulated J774A.1 cells evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR product (n=3). The data are expressed as mean ± S.D.

Appendix 8: Effect of cotreatment the ethanol extracts of Wisumpayayai remedy on the mRNA expression of pro-inflammatory-cytokines (TNF-α, IL-1β, IL-6) in LPS stimulated J774A.1 macrophages cells.



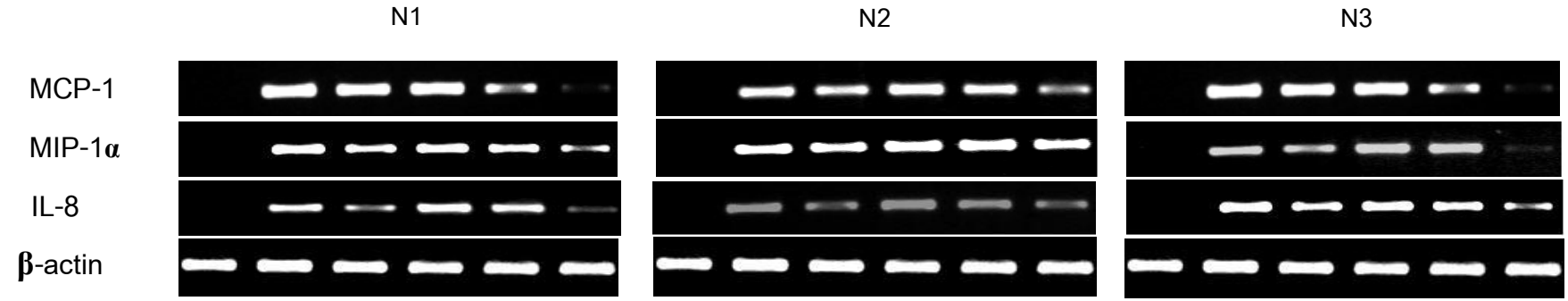
LPS (100 ng/ml)	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-
10 μM Dexa	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Wisumpayayai Remedy (μg/ml)	-	-	-	25	50	100	-	-	-	25	50	100	-	-	-	25	50	100

Cells were treated with 25, 50 and 100 μg/ml of the extract and 100 ng/ml LPS for 4 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplified mRNA of pro-inflammatory cytokines with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR product were determined by using gel documentation and compared with β-actin PCR product. The data are expressed as mean ± S.D. from 3 independent experiments (n=3). *$p<0.05$ compared to untreated control.

Test Compounds	% Of control TNF-α			Mean ± S.D.	% Of control IL-1β			Mean±S.D.	% Of control IL-6			Mean ±S.D.
	1	2	3		1	2	3		1	2	3	
0.2% DMSO	0	0	0	0	0	0	0	0	0	0	0	0
0.2% DMSO+LPS	100.33	100.04	100.43	100.25 ± 0.23	100.04	100.00	100.01	100 ± 0.02	100.0	100.0	105.3	100 ± 1.23
Dexa (10 µM)+LPS	35.89	29.98	33.65	32.27 ± 2.10	41.71	50.43	49.40	47.18 ± 2.75	55.76	45.87	49.42	50.35 ± 2.89
Wisumpayayai 25	96.72	98.54	98.34	98.25 ± 1.23	95.38	97.99	88.20	93.86 ± 2.92	54.26	61.36	44.59	53.40 ± 4.85
Wisumpayayai 50	48.19	51.40	49.23	50.08 ± 2.00	41.74	53.42	38.55	44.57 ± 4.51	37.13	36.53	31.73	35.13 ± 1.70
Wisumpayayai (µg/ml) 100	19.89	12.09	18.56	15.83 ± 3.12	11.30	34.84	16.26	20.80 ± 7.16	19.84	14.92	12.61	15.79 ± 2.13

Appendix 9: Data of cotreatment the ethanol extracts of Wisumpayayai remedy on the mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) by macrophages J774A.1 cells evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR product (n=3). The data are expressed as mean ± S.D.

Appendix 10: Effect of cotreatment the ethanol extracts of Wisumpayayai remedy on the mRNA expression of chemokine (MCP-1, MIP-1α, and IL-8) in LPS-stimulated J774A.1 macrophages cells.



Cells were treated with 25, 50 and 100 μg/ml of the extract and 100 ng/ml LPS for 4 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplified mRNA of chemokine with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR product were determined by using gel documentation and compared with β-actin PCR product. The data are expressed as mean ± S.D. from 3 independent experiments (n=3). *$p<0.05$ compared to untreated control.

Test Compounds	% Of control MCP-1			Mean ± S.D.	% Of control MIP-1α			Mean ± S.D.	% Of control IL-8			Mean ± S.D.
	1	2	3		1	2	3		1	2	3	
0.2% DMSO	0	0	0	0	0	0	0	0	0	0	0	0
0.2% DMSO+LPS	99.28	100.01	100.00	99.76 ± 0.21	100.00	100.01	100.00	100.00 ± 0.0	99.95	100.1	100.1	100.01 ± 0.02
Dexa (10μM)+LPS	78.45	81.11	81.61	80.39 ± 1.45	47.70	49.97	52.78	50.15 ± 1.34	36.26	39.61	31.55	35.81 ± 2.00
25	94.00	98.45	99.78	97.41 ± 2.00	98.67	99.78	96.23	98.23 ± 1.45	95.99	94.71	98.66	96.45 ± 1.77
Wisumpayayai 50	57.85	61.21	80.75	66.61 ± 4.34	55.82	50.25	58.67	54.91 ± 2.13	63.23	58.46	67.87	63.19 ± 2.14
(μg/ml) 100	15.78	19.12	17.86	17.59 ± 1.67	19.48	18.48	22.01	19.99 ± 2.02	17.81	18.01	7.21	14.34 ± 3.15

Appendix 11: Data of cotreatment of the ethanol extracts of Wisumpayayai remedy on the mRNA expression of chemokine (MCP-1, MIP-1α and IL-8) by macrophages J774A.1 cells evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR product (n=3). The data are expressed as mean ± S.D.

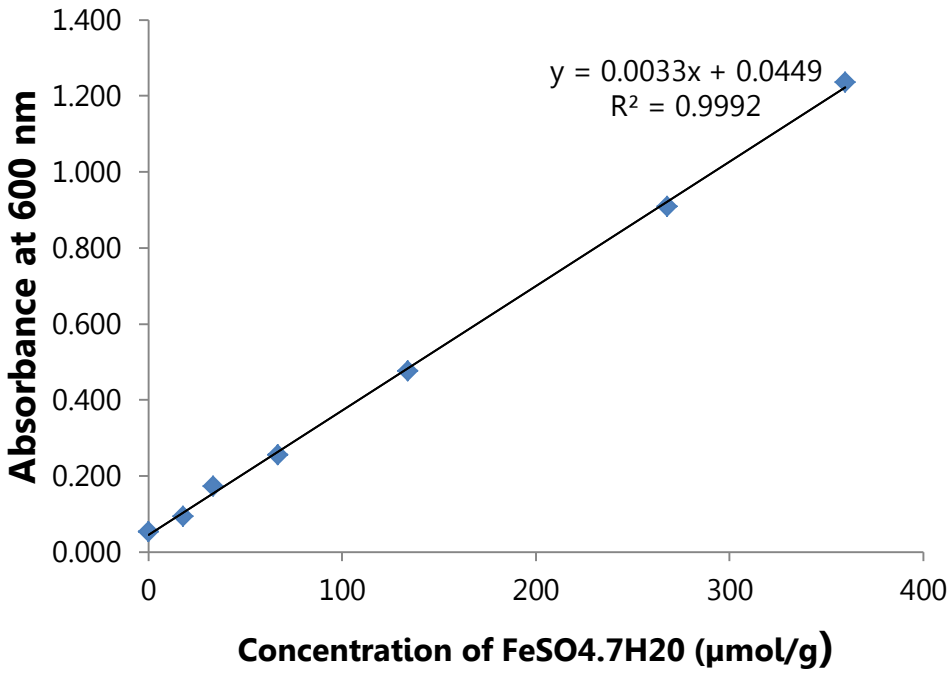
Test Compounds		% Inhibition			% Inhibition		
		TNF-α	IL-1β	IL-6	MCP-1	MIP-1α	IL-8
0.2% DMSO		0	0	0	0	0	0
0.2% DMSO+LPS		0	0	0	0.24	-0.01	-0.01
Dexa (10μM)+LPS		66.83	52.82	49.65	19.61	49.85	64.19
Wisumpayayai (μg/ml)	25	2.13	6.14	46.60	2.59	1.77	3.55
	50	50.39	55.43	64.87	33.39	45.09	36.81
	100	83.15	79.20	84.21	82.41	80.01	85.66

Appendix 12: Data of %inhibition of cotreatment the ethanol extracts of Wisumpayayai remedy on the mRNA expression of cytokine ((IL-1β, IL-6 and TNF-α) and chemokine (MCP-1, MIP-1α and IL-8) by macrophages J774A.1 cells evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR product (n=3). The data are expressed as mean ± S.D.

Appendix 13: Data of standard calibration curve of concentration of FeSO4.7H2O (µmol/g)

concentration of FeSO4.7H2O(µmol/g)	Absorbance at 600 nm		
	1	2	mean
0	0.051	0.056	0.054
17.94	0.093	0.095	0.094
33.48	0.17	0.175	0.173
66.96	0.25	0.26	0.255
133.92	0.453	0.498	0.476
267.85	0.89	0.93	0.910
359.71	1.2	1.271	1.236

Appendix 14: A representation of FeSO4.7H2O (µmol/g) calibration line by FRAP assay.



Appendix 15: Data of the ethanol extracts of Wisumpayayai remedy on antioxidant activity by FRAP assay and compared with β-actin PCR product (n=3). The data are expressed as mean ± S.D.

Test compound		Conc. FeSO4.7H2O (µmol/g)			mean± SE	Antioxidant activities
		1	2	3		
water		-5.83	-6.17	-6.17	-7.42 ±0.113	-
vitamin c 100µM		824.67	875.00	867.50	871.25 ±15.67	extremely high
Wisumpayayai (µg/ml)	6.25	0.83	0.25	0.67	0.25 ± 0.38	low
	12.5	38.98	35.65	37.97	36.98 ± 0.30	medium
	25	64.55	68.38	68.65	67.23 ± 1.70	medium
	50	95.86	98.34	99.54	98.67 ±2.90	medium
	100	154.83	154.00	155.33	417.67 ±0.64	high

BIOGRAPHY

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