ANTI-INFLAMMATORY ACTIVITY OF TREEHOM REMEDY

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ตำรับยาตรีหอมเป็นยาสามัญประจำบ้านแผนโบราณ วัตถุส่วนประกอบมีดังนี้ คือ เนื้อลูกสมอเทศ เนื้อลูกสมอพิเภก เนื้อลูกมะขามป้อม ลูกผักชีลา รากไคร้เครือ โกฐสอ ชะเอมเทศ น้ำประสานทองสะตุ ลูกซัดคั่ว เนื้อลูกสมอไทย โกฐน้ำเต้าใหญ่นึ่งสุก ตำรับยาตรีหอมเป็นตารับยาที่ใช้แก้เด็กท้องผูก ระบายพิษไข้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ ต้านการอักเสบของสิ่งสกัดด้วยน้ำและเอทานอลจากตำรับยาตรีหอมต่อเซลล์แมคโครฟาจ J774A.1ที่ถูกกระตุ้นด้วยไลโพพอลีแซคคาไรด์ และศึกษาฤทธิ์ต้านอนุมูลอิสระโดย FRAP assay ผลการศึกษาพบว่า สิ่งสกัดด้วยเอทานอลและน้ำจากตารับยาตรีหอมสามารถยับยั้งการสร้างไนตริกออกไซด์ได้ตามความเข้มข้นของสิ่งสกัด 6.25-100 µg/mโดยมีค่า IC_{50} เป็น และ 40.05และ 60.05 µg/ml ตามลำดับ ความเข้มข้นของสิ่งสกัดทั้งสองชนิดที่ใช้ในการศึกษา (25-100µg/ml) สามารถลดการแสดงออกในระดับ mRNA ของเอนไซม์ inducible nitric oxide synthase (iNOS) โดยมีเปอร์เซ็นต์การแสดงออก 71.28%- 29.58%และ 73.97%-39.64% สำหรับสิ่งสกัดด้วยเอทานอลและน้ำตามลำดับ ซึ่งสอดคล้องกับผลการยับยั้งการสร้างไนตริกออกไซด์ และยังสามารถลดการแสดงออกของเอนไซม์ cyclooxygenase 2 (COX-2) โดยมีการแสดงออกของเอนไซม์เป็น 75.67%-38.32%และ 86.81%-48.95% สำหรับสิ่งสกัดด้วยเอทานอลและน้ำ ตามลำดับ นอกจากนี้สิ่งสกัดสามารถลดการแสดงออกในระดับ mRNA ของ TNF-α, IL-1β, IL-6 ซึ่งไซโตไคน์เหล่านี้และเอนไซม์ COX-2 เป็นสารสื่อที่ถูกกระตุ้นเมื่อเกิดการอักเสบ อีกทั้งสิ่งสกัดทั้งสองยังมีฤทธิ์ต้านอนุมูลอิสระได้สูงที่ความเข้มข้น 25-100 µg/ml จากผลการศึกษาดังกล่าวแสดงให้เห็นว่าสิ่งสกัดด้วยน้ำและเอทานอลจากตรีหอมน่าจะเป็นสารที่มีศักยภาพต้านการอักเสบได้

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

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JAREE TREEKEAW: ANTI-INFLAMMATORY ACTIVITY OF TREEHOM REMEDY.

ADVISOR: ASSOC.PROF. CHANDHANEE ITTHIPANICHPONG, COADVISOR:

ASSOC.PROF. NIJSIRI RUANGRUNGSI, Ph.D., 112 pp.

Treehom remedy is a Thai traditional medicine used for relieving of pyrexia and constipation in children. Its composition are *Terminalia* sp., *Terminalia bellirica* Roxb., *Phyllanthus emblica* L., *Coriandrum sativum* Linn., *Aristolochia* sp., *Angelica dahurica* Benth., *Glycyrrhiza glabra* L., Sodium borate, *Trigonella foenum-graecum* L., *Terminalia chebula* Retz., *Rheum officinale* Baill. The aim of this study is to investigate the anti-inflammatory activity of the water and ethanol extracts from this remedy on LPS-activated J774A.1 macrophages. Our results demonstrated that the ethanol and water extracts of Treehom remedy significantly inhibited NO production from LPS-stimulated macrophages in concentration-dependent manner (6.25-100 µg/ml). IC_{50} values were 40.05 and 60.05 µg/ml for the ethanol and water extracts respectively. Both extracts 25-100 µg/ml inhibited mRNA expression of inducible nitric oxide synthase (iNOS). The expressions were 71.28%- 29.58% and 73.97%-39.64% for the ethanol and water extracts, respectively and were correlated with the reduction in NO production. These extracts also reduced mRNA expression of cyclooxygenase 2 (COX-2) with the expression of 75.67%-38.32% and 86.81%-48.95% for the ethanol and water extracts. Moreover both extracts decreased mRNA expression of TNF-α, IL-1β, IL-6. These cytokines and COX-2 enzyme are inflammatory mediators produced during inflammatory process. The antioxidant assay also showed high antioxidant activity of both extracts at concentration 25-100 µg/ml. All of these result demonstrated anti-inflammatory potential of the ethanol and water extracts from Treehom remedy.

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

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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
$^{\circ}C$	Degree Celsius
5-LOX	5-lipooxygenase
AA	Arachidonic acid
ACPA	Anti-citrullinated peptide antibodies
AGEs	Advanced glycation end products
AOX	Antioxidant
ATCC	American Type Culture Collection
Ca^{2+}	Calcium
CAMs	Cellular 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
Dexa	Dexamethazone
DMEM	Dulbeco's Modified Eagle"s Medium

DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DVT	DVT deep vein thrombosis
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
F_c	Fragment crystallizable
FRAP	Ferric Reducing Antioxidant Power
GI	Gastrointestinal
Glu	Glutamate
GPCRs	G protein-coupled receptor
GRE	Glucocorticoid response elements
h	Hour
H_2O_2	Hydrogen peroxide
IC_{50}	Inhibition concentration 50%
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IkB-α	IkappaB-alpha
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LF	Lactoferrin
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
M	Molarities (mole per liter)
Mac-1	Macrophage 1 antigen
MCP-1	Monocyte chemotactic protein-1
mg	Milligram (s)
MHC	Major histocompatibility complex

MIC	minimum inhibitory concentrations
min	Minute
MIP-1α	Macrophage inflammatory protein-2
ml	Milliliter(s)
mLDL	Modified forms Low-density lipoprotein
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 (s)
NK	Natural killer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NSAIDs	Non steroidal anti-inflammatory drugs
O_2	Oxygen
OD	Optical density
OH^-	Hydroxyl radical
$OONO^-$	Peroxynitrite
OVA	Ovalbumin-induced allergic
PAF	Platelet activating factor
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGs	Prostaglandins
PLA	Phospholipase A
PRRs	Pattern recognition receptors

RANTES	Regulated on activation, normal T expressed and secreted
RF	Rheumatoid factor
ROS	Reactive oxygen species
S.E.M	Standard error of mean
SAA	Serum amyloid A
sec	Second
SLPI	Secretory leukocyte protease inhibitor
SRs	Scavenger receptors
TBE	Tris-borate-EDTA
TCHE	*Terminalia chebula* hydroalcoholic extract
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TPTZ	Tripyridyltriazine
TXB_2	Thromboxane B_2
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4

CHAPTER I

INTRODUCTION

Background and Rationale

Inflammation is a physiologic response of innate immunity in response tissue and cells to protect against foreign substances. It can be presented as acute or chronic inflammation. Acute inflammation occurs immediately in response to trauma within two hours and lasts for a week. Chronic inflammation is a continuous response to inflammatory stimuli and leading to pathogenic condition. The signs of inflammation are pain, swelling, redness, hotness. Many inflammatory mediators are secreted from leukocytes during inflammation.

Macrophages are white blood cells that play a key role in inflammatory process. Their functions are phagocytosis, antigen presentation, and immunomodulation. Many stimuli such as foreign antigen particle, lipopolysaccharide, cytokines etc. are able to activate macrophages leading to generation of immune response. Activated macrophages produce and secrete many inflammatory mediators and pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 (IL-1) and the enzymes involved in inflammation as well. These enzymes are inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs). They are responsible for nitric oxide and prostaglandins generation. COXs are at least divided into two isoforms; a constitutive form (COX-1) and an inducible isoforms (COX-2). During inflammation, COX-2 is selectively induced by proinflammatory cytokines at the site of inflammation. Inflammatory reactions also induce the production of reactive oxygen species (ROS) by activated macrophages. The inflammatory reaction is beneficial when its effects is

limited to the pathogens while prolonged inflammation is able to causes various kinds of diseases, such as Alzheimer's disease, cancer, rheumatoid arthritis, psoriasis, etc.

At present, anti-inflammatory agents are used to reduce signs of inflammation. These agents are nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs) are drugs commonly used for management of inflammation. They inhibit cyclooxygenase (COX) enzyme resulting in inhibition of prostaglandins production at the site of inflammation. Inhibition of gastrointestinal prostaglandins is associated with mechanism-based toxicities and limits their usefulness. Steroidal drugs inhibit phospholipase A_2 as well as suppress immune cells and reduce inflammatory mediators. Many side effects are related to steroidal drugs utilization such as immunosuppression, peptic ulcer and reduced bone density.

Recently, natural medicines from plants have been wildly researched as anti-inflammatory agents since they are considered to be efficacious and safe. Treehom remedy is a Thai traditional medicine composed of 10 herbal plants including *Terminalia* sp., *Terminalia bellirica* Roxb., *Phyllanthus emblica* L., *Coriandrum sativum* Linn., *Aristolochia* sp., *Angelica dahurica* Benth., *Glycyrrhiza glabra* L., Sodium borate, *Trigonella foenum-graecum* L., *Terminalia chebula* Retz., *Rheum officinale* Baill. It is approved by the Ministry of Public Health of Thailand in National Drug List as the folk remedy for relieving of pyrexia. As far as we know, several composition plants of this compound showed the anti-inflammatory and antioxidant effect in the existing reports. However, there hasn't been reported about the anti-inflammatory activity of this remedy. Therefore, the aim of this study is to investigate the anti-inflammatory activity of the water and ethanol extracts from Treehom remedy on LPS-activated J774A.1 macrophages as well as the antioxidant activity of this extracts by FRAP assay.

Objectives

- To study the inhibitory activities of the water and ethanol extracts from Treehom remedy on pro-inflammatory cytokines and mediators in LPS – stimulated J774A.1 macrophages.
- To study the antioxidant effect of the water and ethanol extracts from Treehom remedy.

Hypothesis

1. The water and ethanol extracts from Treehom remedy can inhibit pro-inflammatory cytokines and mediators in LPS - stimulated J774A.1 macrophages.

2. The water and ethanol extracts from Treehom remedy exhibit antioxidant effect.

Research design

Experimental research

Keyword

Treehom remedy, inflammation, NO, iNOS, TNF-α, IL-1β, IL-6, COX-2, and ROS

CHAPTER II

LITERATURE REVIEWS

Inflammation is an important mechanism of tissues and cells in response to foreign substances. When tissue is injured, it induces redness by increase vasodilation and blood flow. Increase permeability of the blood vessels and leakage of plasma protein or fluid to the site of inflammation cause swelling. In addition, bradykinin, histamine, prostaglandins are released to the site of injury and they increase sensitivity to pain. Furthermore, leukocytes are migrated into a site of inflammation and release a variety of cytokines and the enzymes involve in inflammation as well. These enzymes are inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX). COX-1 is constitutive and COX-2 is inducible isoforms. During inflammation, COX-2 is selectively induced by proinflammatory cytokines. These mediators and cytokines induce increased expression of many cellular adhesion molecules (CAMs) and immunoglobulin. In addition, during inflammation, phagocytosis of bacteria or foreign substances produce high amount of reactive oxygen species (ROS) such as hydroxyl radical (OH^-), superoxide (O_2), and hydrogen peroxide (H_2O_2) etc. ROS induce expression of phospholipase A_2, 5-lipooxygenase (5-LOX), COX-2, iNOS and cause cellular damage by disturbing cellular structure including lipids, proteins and nucleic acids leading to physiological dysfunction and cells death (Kehrer *et al*., 1993; Linton *et al*., 2003; Lee *et al*., 2009).

Inflammatory response

Inflammatory responses are classified into four distinct phases: recognition of foreign particles, recruitment of cells, elimination of the foreign particles, and resolution of inflammation.

Recognition of foreign particles

Inflammation is a physiologic response of innate immunity in host defense against pathogenic microbe or foreign particle. Inflammatory cells recognize foreign substances by the pattern recognition receptors (PRRs) that function as enhanced attachments, induction of complements and coagulation cascades, phagocytosis, induction of proinflammatory signaling pathways. There are several kinds of PRRs involved in the process of inflammation. They are mannose receptor, scavenger receptor, seven α-helical-transmembrane/G protein-coupled receptor (GPCRs), receptors for opsonin, and Toll like receptor (TLRs) (Fig 1) (Charles *et al*., 2002; Riordan *et al*., 2002).

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

The mannose receptors recognize a wide range of Gram-negative and Gram-positive bacteria, yeasts, parasites and mycobacteria. MRs are PRRs presenting on the surface of macrophages and dendritic cells. The functions of these receptors are to recognize mannose, N-acetylglucosamine that located on the surfaces of inflammatory stimuli. These receptors are associated with a signal transduction pathway leading to cytokines production. Scavenger receptors (SRs) are widely expressed on macrophages. These receptors recognize modified forms of low-density lipoprotein (mLDL) (Peiser *et al*., 2001; Stah *et al*., 1998).

- The Seven α-helical transmembrane/G protein-coupled receptor (GPCRs)

These receptors are found on leukocytes. The functions of them are regulation of the inflammatory response via activation of adhesion and migration of the leukocytes to the site of inflammation. GPCRs are characterized by seven membrane-spanning domains with an extracellular N terminus and a cytoplasmic C terminus. GPCRs transduce extracellular stimuli into intracellular signals. These receptors recognize short

peptide (e.g. *N*-formylmethionyl), chemokines, chemotactic breakdown products of complement and lipid mediators of inflammation. Binding of *N*-formylmethionyl to these receptors on phagocyte up-regulate cell motility, chemotaxis, and phagocytosis *(*Jason *et al.,* 1998*;* Abul *et al.,* 2005*;* Kumar *et al*., 2004).

-Receptors for opsonin

Receptors for opsonin such as F_c receptors and complement receptors, induce phagocytosis of foreign particle coated with antibodies, complement, proteins, and lectins. The process is called opsonization.

-Toll like receptors (TLRs)

TLRs are expressed on macrophages, neutrophils, and DCs. These receptors recognize conserved components of bacteria, virus, fungi, protozoa, foreign particle and cause cell activation. At present, there are 13 different TLRs identified in mammal. The TLR ligands include LPS (TLR4); peptidoglycan, lipoteichoic acid, and zyomosan (TLR2); double-stranded RNA (TLR3); flagellin (TLR5); single-stranded RNA. Over expression of TLR4 causes induction of inflammatory cytokine genes and co-stimulatory molecules (Barton *et al.,* 2009*;* Takeda *et al.,* 2003).

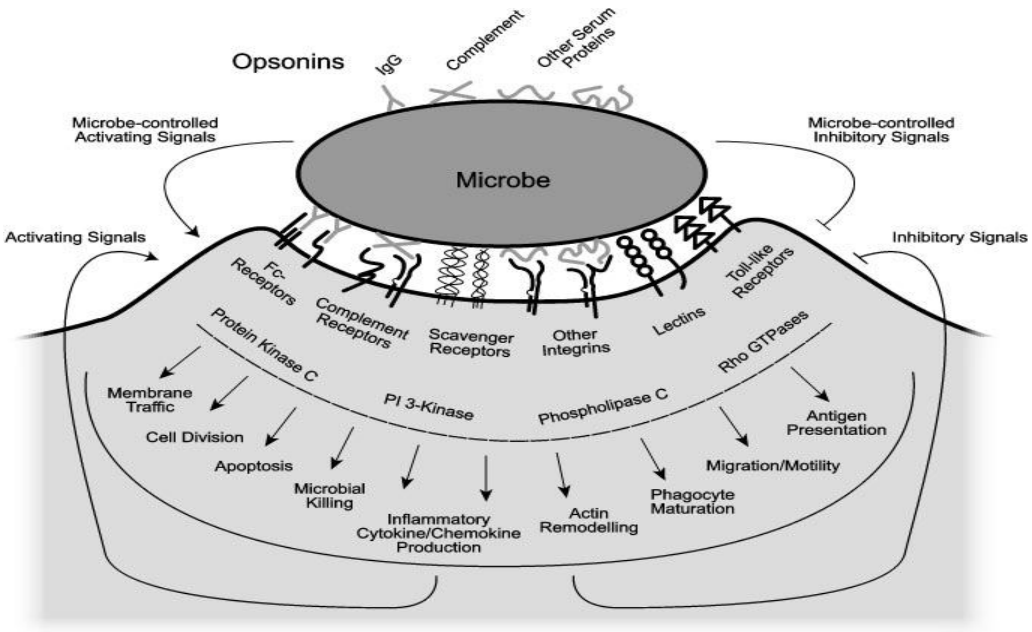


Figure 1: Receptors and signaling interactions during phagocytosis of microbes (Underhill *et al.,* 2002).

Recruitment of the cells to the sites of inflammation and elimination of the foreign particles

Inflammatory response induces changes in local blood vessel and endothelial cells by proinflammatory cytokines. TNF-α, IL-1, and lipid mediators increase migration of leukocytes and flow of plasma to the site of inflammation. The recruitment of leukocytes to the sites of inflammation activate endothelial cells of nearby venules to produce selectins (P, E-selectin), ligand for integrins (ICAM-1, VCAM-1), and chemokines. Selectin causes tethering and rolling of blood leukocytes, Integrin firm adhesion of cells chemokines activate the migration of leukocytes through the endothelium to the site of inflammation (Fig 2). The accumulation of leukocytes occur at the site of the foreign particles. In addition to clearance of apoptotic neutrophils, macrophages also contribute to eliminating of foreign particles organisms. Macrophages engulf and degrade foreign particles using proteases, ROS, and RNS (Fig 1) (Kumar *et al.,* 2007; Barton *et al.,* 2009).

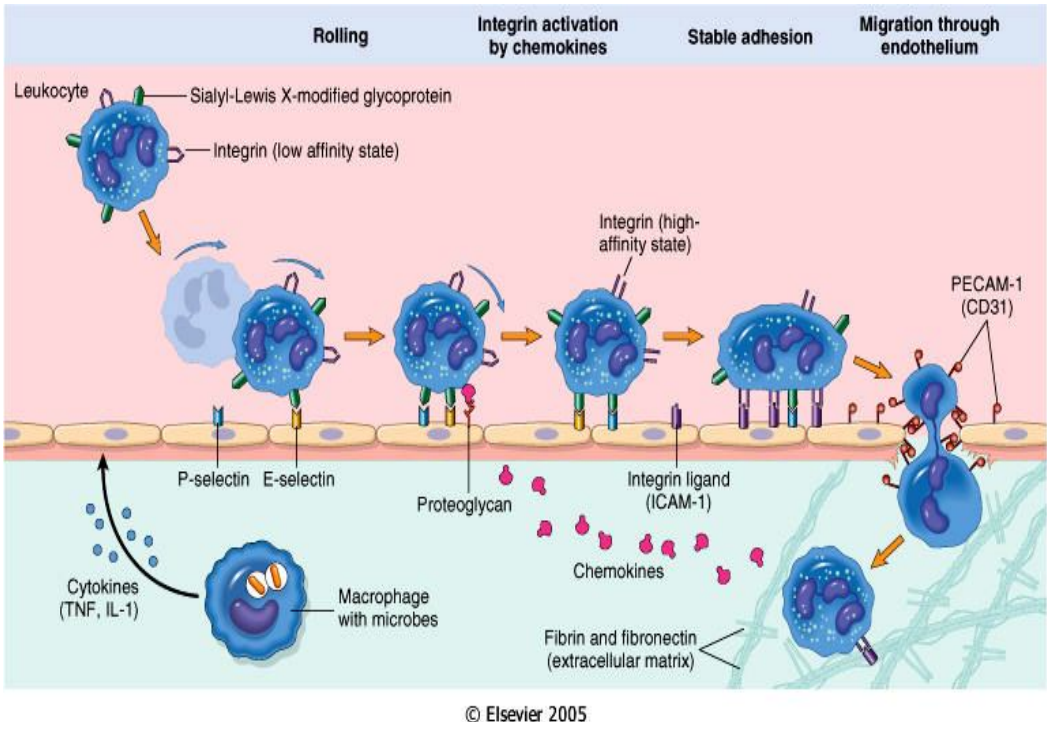


Figure 2: Recruitment of leukocytes to the site of inflammation (Barton *et al.,* 2009).

Resolution of inflammation and return to homeostasis

Many families of lipid-derived mediators play a clear role in resolution of inflammation and tissue repair. The lipid-derived mediators associated with resolution of inflammation are lipoxins, resolvins and protectins. Lipoxins stop the influx of neutrophils, induce the uptake of apoptotic neutrophils, and recruit additional monocytes to help clear away dead cells and tissue debris. Resolvins and protectins enhance neutrophils apoptosis leading to release of anti-inflammatory and reparative cytokines such as transforming growth factor beta 1 (TGFβ-1) to promote repair of damage tissue (Serhan *et al.,* 2005).

Macrophages

Macrophages are white blood cell derived from pluripotent haematopoietic stem cells in the bone marrow. Their precursor cells are in myeloid lineage. Macrophages are generating from circulating monocytes. Monocytes change to become macrophages in tissues. Each type of macrophage has a specific name determined by its location such as in pulmonary airways, it is called alveolar macrophage, in granuloma is epitheloid cell, in bone is osteclast, in connective tissue is histiocytes, in neural tissue is microglia, in liver is Kupffer cells (Fig 3)(Gordon., 2003).

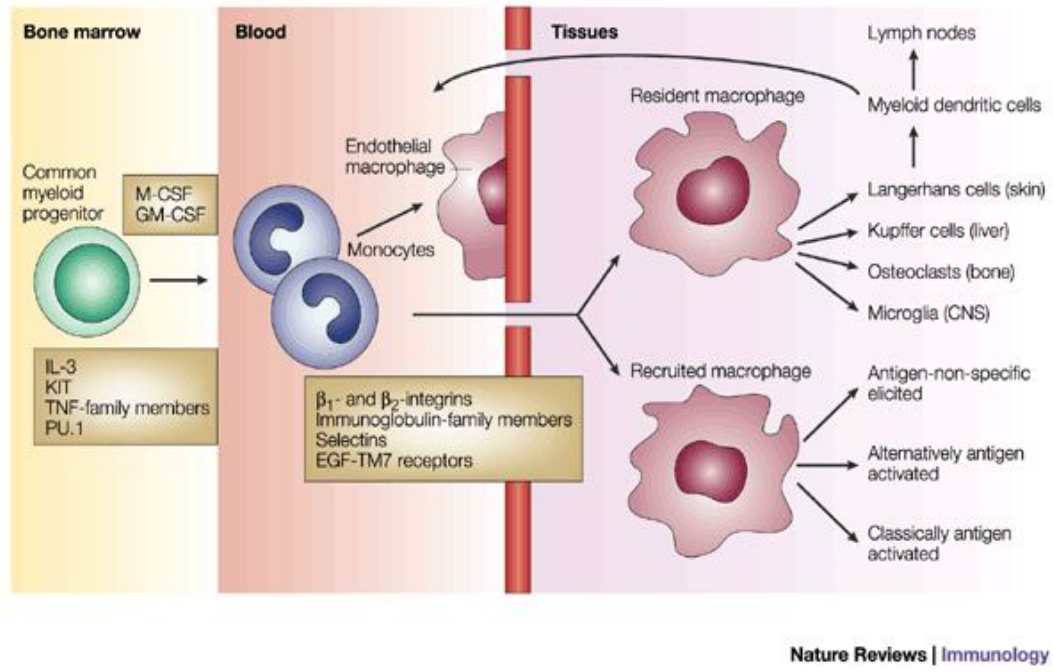


Figure 3: Differentiation, distribution of macrophages in vivo (Gordon., 2003).

Macrophages are involved in the immune response as the antigen presentation, co-activation of T and B lymphocytes, anti-tumor activity, and anti-infectious action. Macrophages exhibit important role in inflammatory process. They are activated by cytokines/endotoxin such as interferon-γ (IFN- γ), lipopolysaccharide (LPS). After activation, they secrete cytokines (TNF-α, IL-1, IL-6) and inflammatory mediators (NO, PGE_2) in relation to the pathogen associated molecular patterns (PAMPs). PAMPs bind to pattern recognition receptors (PRRs). These receptors are on the cell surface of macrophages and induce intracellular signaling cascades leading to increase in inflammatory cytokine production (Fig 4) (Kvietys *et al.,* 2012).

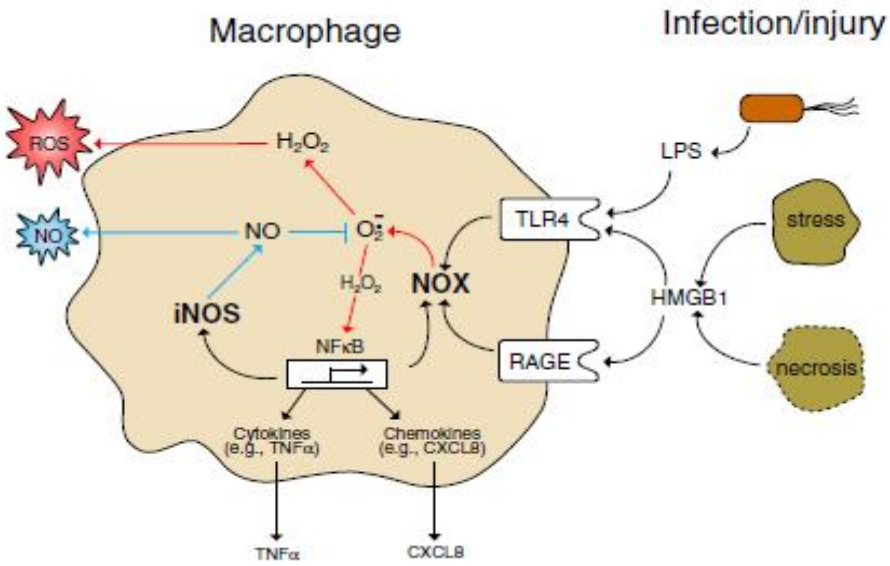


Figure 4: Activated macrophages secrete inflammatory mediators to protect against foreign substances (Kvietys *et al.,* 2012).

Phagocytosis in macrophages is a nonspecific defense mechanism. Many phagocytes engulf and destroy microorganisms of disease. Phagocytosis is necessary for clearance of foreign particles and cellular debris. There are three phases in phagocytic process: attachment phase, ingestion phase, killing and degradation phase. In the first phase, the attachment of particles to phagocytic cell surfaces involves receptors, two of these receptors have been recognized: the receptor for F_c region of the IgG molecule, and the C3b receptor for the activated third component of complement. Next step, phagocytes engulf foreign particles into phagosome. The phagosome fuse with lysosome to become a phagolysosome. The last step, the pathogen is degraded in phagolysosome. Degradation can be oxygen-dependent or oxygen-independent. Oxygen-dependent degradation depends on nicotinamide adenine dinucleotide phosphate (NADPH) and the production of reactive oxygen species (ROS). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase converts molecular oxygen (O_2) to reactive oxygen intermidiates (ROIs), superoxide anion (O_2^-) and free radicals, which are toxic to pathogens. Oxygen-independent degradation depends on the release of granules, containing proteolytic enzymes including defensins, lysozyme, and cationic proteins.

The degradation of foreign proteins generates antigenenic peptide in phagolysosome. The macrophages then become an antigen presenting cells (APC) to present antigenic peptide by major histocompatability (MHC) to T-cell receptors and initiate immune response (Fig 5) (Underhill *et al.,* 2002; Aderem*.,* 2003; Jutras *et al.,* 2005; Stuart *et al.,* 2005).

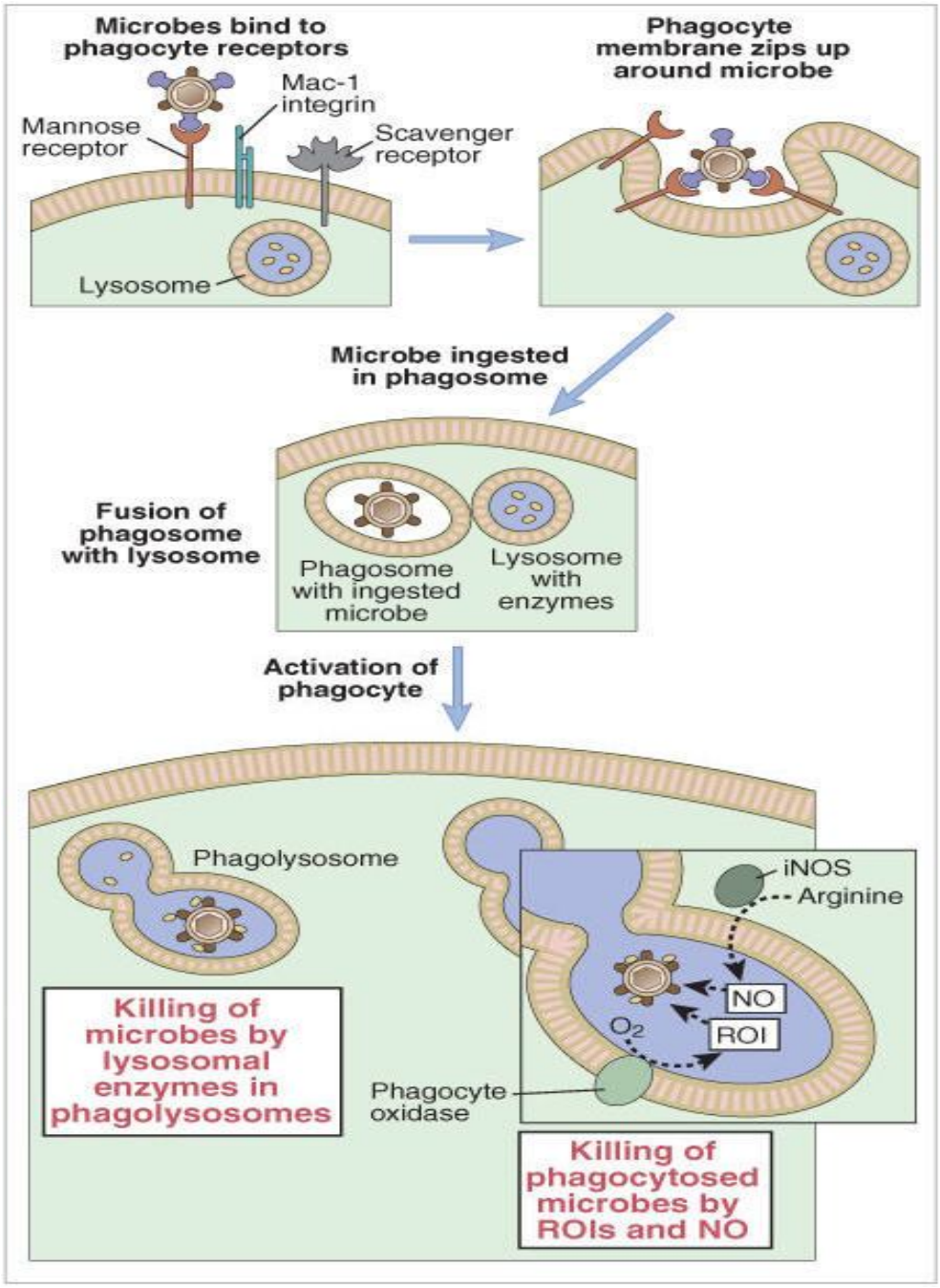


Figure 5: Steps involved in phagocytosis of a particle by macrophages (Abul *et al*., 2005).

Pro-inflammatory cytokines and mediators

-Tumor necrosis factor-α (TNF- α)

TNF- α is a type II transmembrane glycoprotein. It is produced by macrophages, monocytes, neutrophils, NK-cells and T-cells following bacterial lipopolysaccharide (LPS) activation (Table 1). TNF- α induces the recruitments of leukocytes the expression of adhesion molecules which cause the endothelial cell surface adhesive for leukocytes. TNF- α also stimulates endothelial cells and macrophages to produce and secrete chemokines, cytokines and genotoxic molecules. Genotoxic molecules enhance DNA damage and mutations. TNF- α also induces increase synthesis of prostaglandins E_2 (PGE_2) which acts on the hypothalamus and cause fever. Furthermore, TNF-α and IL-1 share several pro-inflammatory properties, they induce adhesion molecules and interleukin 6 (Lin *et al.,* 2007*;* Goetz *et al.,* 2004).

Table 1: The biochemistry of TNF (Tracey *et al.,* 1994).

Structure	Membrane-associated form is 26 kD Secreted form is 17 kD Biologically active form is a bell-shaped structure composed of three noncovalently bound 17 kD molecules
Stimuli to TNF Release	Bacterial toxins: (lipopolysaccharide, enterotoxin, toxic shock syndrome toxin) Viruses: HIV, influenza Mycobacteria Fungi Parasites Products of complement activation Antigen-antibody complexes Cytokines
Cellular sources	Macrophages Lymphocytes Polymorphonuclear leukocytes Eosinophils Astrocytes Langerhans cells Kupffer cells
Cellular biological activities	Cytotoxic to some tumor cells Growth factor for some tumor cells Suppresses LPL in adipocytes Decreases Em in myocytes
Serum half-life	6–20 minutes in mammals following intravenous bolus injection

Interleukin 1(IL-1)

IL-1 is the prototypic pro-inflammatory cytokine. There are two isoforms of IL-1, IL-1α and IL-β. IL-1α is mainly produced by keratinocytes and endothelial cells, while IL-1β is largely produced by monocytes and macrophages following bacterial lipopolysaccharide (LPS) activation. IL-1α from plasma membrane of the producing cell acts locally, whereas IL-1β secreted by macrophages circulates systemically. IL-1 increases the expression of adhesion molecules that mediate leukocyte adhesion (e.g. ligand for integrins). IL-1 secreted to blood circulation leads to acute phase response. It induces increase production of PGE_2 from vascular endothelium of the hypothalamus and causes fever (Fig 6) (Dinarellot *et al.,* 1998*;* Netea *et al.,* 2010).

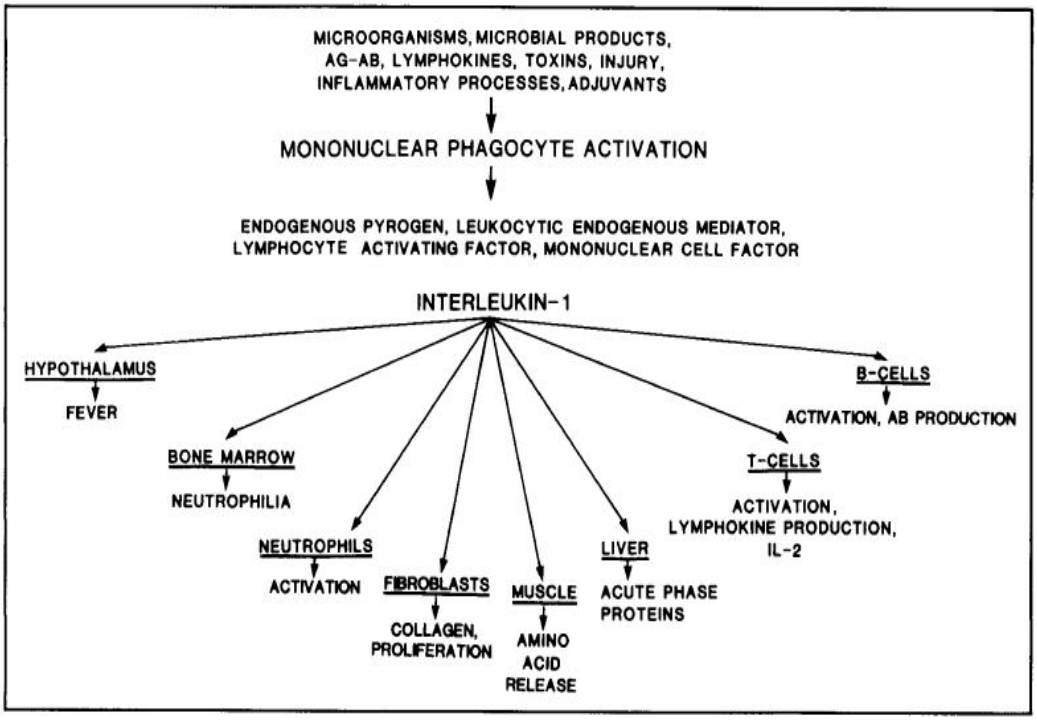


Figure 6: The multiple biological activities of Interleukin – 1 (Dinarello *et al.,* 1984).

Table 2: Immunological effects of IL-1 on various cells (Dinarello *et al.,* 1988).

Cells	Effects
T lymphocytes	Costimulator activity; IL 2 production; increased IL 2 receptor number or binding; growth factor for T cells; induction of interferon-7, IL 3 and other lymphokine synthesis
B lymphocytes	Growth factor for transformed B cells; synergism with B cell growth and differentiation factors (IL 4 and IL 6)
Natural killer cells	Synergism with IL 2 and interferons for tumor lysis; increased binding to tumor cells; induction of cytokine synthesis
Macrophages	Synthesis of PGE_2, induction of cytotoxicity, increased migration; synthesis of IL 1, colony-stimulating factors, and other cytokines
Bone marrow cells	Increased synthesis of colony-stimulating factors; synergism with colony-stimulating factors (hemopoietin 1 activity) on immature precursors

Interleukin 6(IL-6)

IL-6 is produced by mononuclear phagocyte cells, vascular endothelium cells and fibroblast cells following the viral infections or lipopolysaccharide activation. It regulates immune responses, hematopoiesis, and acute phase reactions. IL-6 is one the important mediators that causes fever and acute phase response. IL-6 induces synthesis of PGE_2 in hypothalamus leading to increased body temperature as well as activation of neutrophils to site of infection. IL-6 has biologic effects similar to TNF and IL-1 (Fig 7) (Spooren *et al.,* 2011*;* Kishimoto *et al.,* 1989).

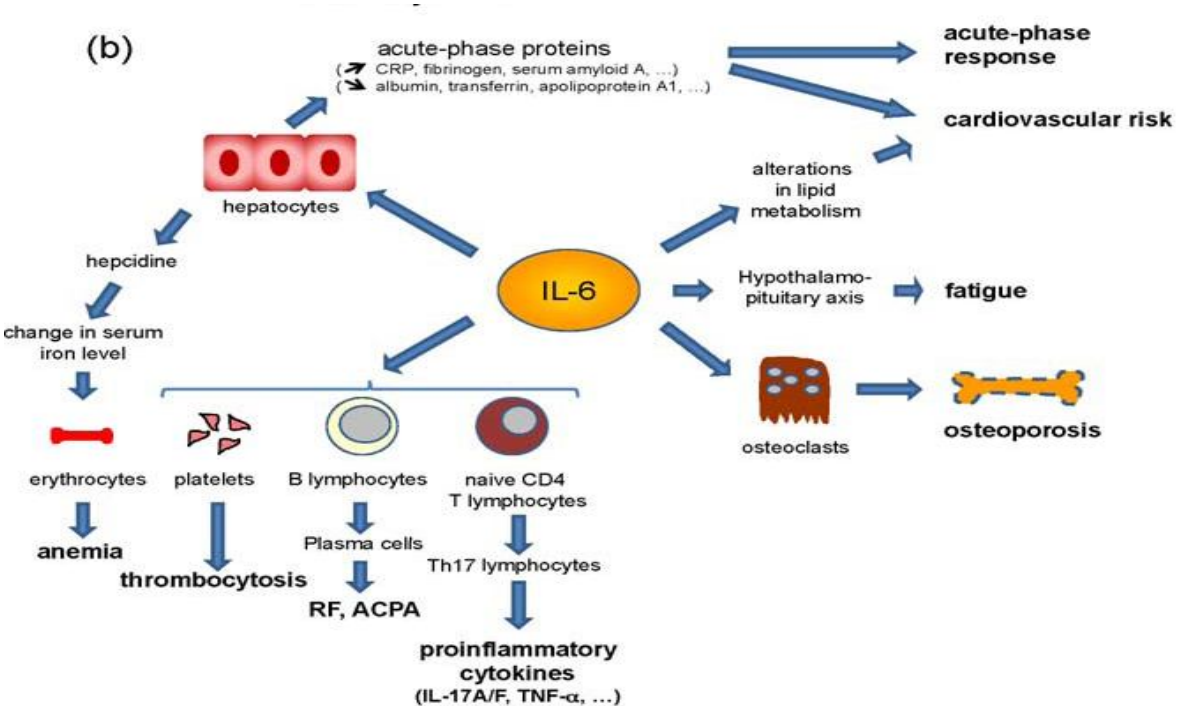


Figure 7: Systemic effects of IL-6 (Assier *et al.,* 2010).

Nitric oxide (NO)

NO, a short-lived gaseous radical, is generated by the enzyme NO synthase (NOS) in mammalian systems. NOS catalyze conversion of L-arginine to L-citrulline resulting in production of NO. NOS exist in three isoforms: the endothelial constitutive NOS (eNOS), the neuronal constitutive NOS (nNOS) and the inducible NOS (iNOS) (Fig 8).

eNOS is constitutively expressed in endothelial platelets cells. They produce NO leading to vasodilatation and maintaining blood pressure homeostasis. NO diffuses to the near vascular smooth muscle and generates soluble guanylate cyclase and increase cyclic guanosine monophosphate (cGMP) levels. Therefore its function is regulation of blood vessel tone. Furthermore, eNOS inhibits the aggregation and adhesion of platelets.

nNOS is found in central and peripheral neurons. It is presented in skeletal muscle, neutrophils, pancreatic islets, endometrium, respiratory, and gastrointestinal epithelia. The function of NO produced from nNOS are neurotransmitter in controlling memory function, depression, gastrointestinal motility and penile erection.

iNOS is expressed by chondrocytes, hepatocytes, macrophages, oteoblasts, lung epithelium, mesangial cells and vascular smooth muscle in response to inflammatory stimuli. When the murine macrophages were induced by cytokines or LPS, they express high levels of iNOS mRNA and protein.

NO is known as a mediator of inflammatory responses. It possesses cytotoxic properties against pathogenic microbe and damaging effects on host tissues in inflammatory response. NO is an important mediator working together with pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and subsequently increases iNOS expression in monocytes, macrophages, neutrophils. NO also upregulates endothelial adhesion molecules (Macmicking *et al.,* 1997; Huang *et al.,* 2001; Bellows *et al.,* 2006; Kim *et al.,* 2011).

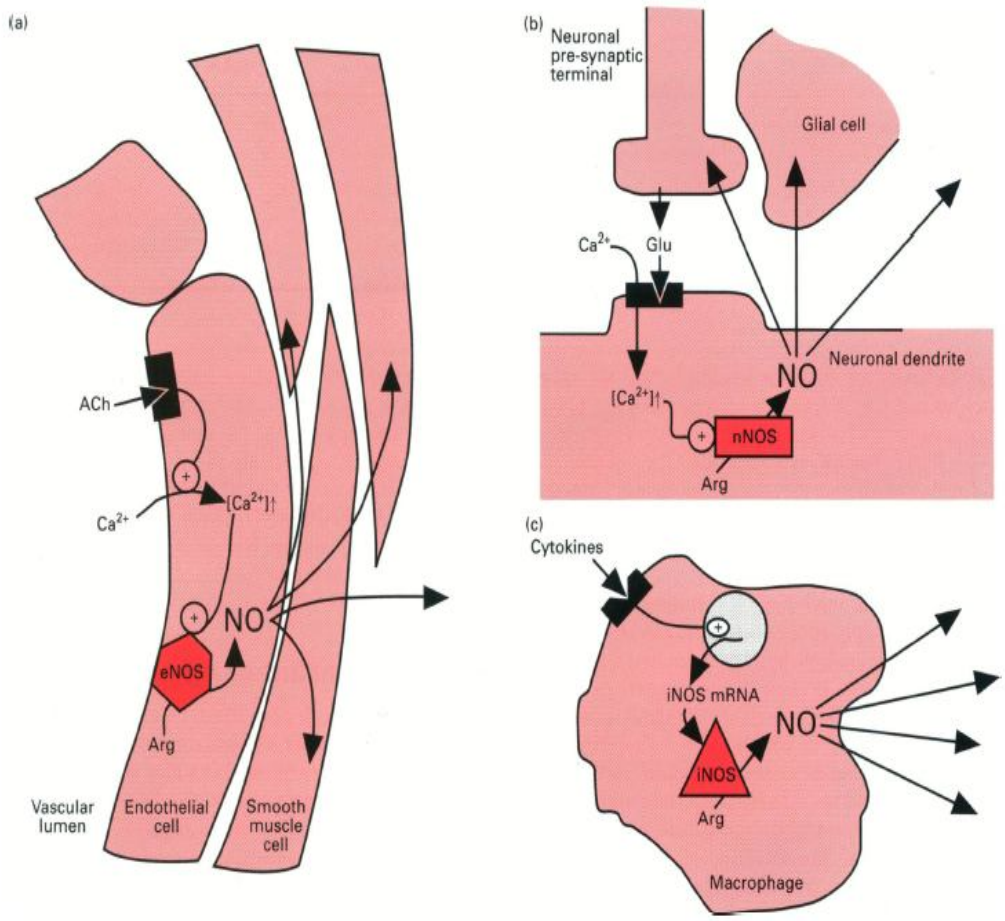


Figure 8: Types and sources of nitric oxide: (a) synthesis of NO by eNOS in a vascular endothelial cell stimulated by acetylcholine (ACh); (b) NO synthesis by nNOS in a neuronal dendrite stimulated by glutamate (Glu); and (c) NO synthesis by iNOS in a macrophage following induction of iNOS mRNA and enzyme by cytokines (Knowles *et al.,* 1994).

Prostaglandins (PGs)

Prostaglandins (PGs) are lipid-derived autacoids, produced following the sequential oxidation of arachidonic acid (AA) by cyclooxygenase (COX) and prostaglandin synthase (Fig 9). COXs exist at least in two isoforms; a constitutive form (COX-1) and an inducible isoforms (COX-2). COX-1 is a constitutively enzyme that generates prostaglandins in physiological amount for normal functions of tissues and

organ. During inflammation, COX-2 is selectively induced by proinflammatory cytokines and generates inflammatory mediators such as PGE_2 and $PGF_{2\alpha}$ at the site of inflammation. They cause vasodilation, increases vascular permeability, edema and fever (Fig 9) (Hata *et al.,* 2004; Botting *et al.,* 2006 ;Khan *et al.,* 2007).

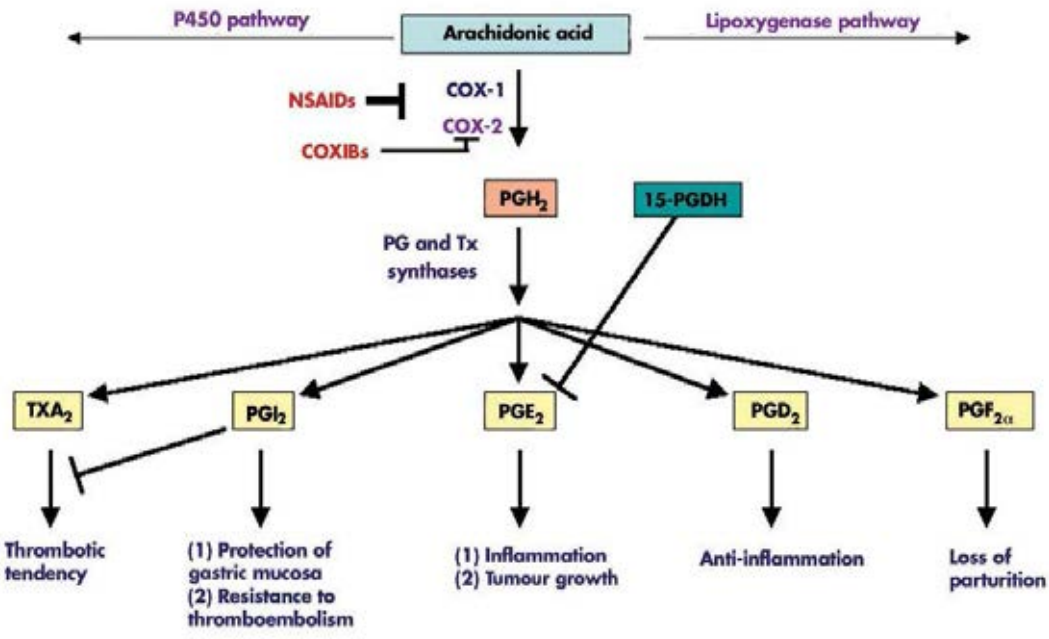


Figure 9: Pathway of prostaglandin production and theirs function (Wang *et al.,* 2006).

Reactive oxygen species (ROS)

Superoxide radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide, are important mediators of cell and tissue injury during inflammation. They are produced by macrophages, neutrophils, mast cell etc. Normal cells are able to balance the production of oxidants and antioxidants to maintain redox equilibrium. Foreign substances cause oxidative stress and imbalance between the level of antioxidant (AOX) and ROS. High level of ROS induce cellular damage by disturbing cellular structure including lipids, proteins and nucleic acids and cause physiological dysfunction or cell death. Cancer, neurodegenerative disorders, cardiovascular diseases, diabetes and autoimmune disorders are consequence imbalance of these mediators (Table 3) (Barnes *et al.,* 1990; Chang *et al.,* 2001; Wong *et al.,* 2006).

Table 3: The role of free radicals in pathophysiology (Chang *et al.,* 2001).

Diseases	Role of free radicals in pathophysiology
Atherosclerosis	Superoxide-mediated endothelial dysfunction, activation of macrophages
Myocardial infarction	ROS driven ischemic reperfusion injury and myocyte necrosis and/or apoptosis
Hypertension	ROS-mediated vascular smooth muscle cell proliferation, oxidant production via NADH/NADPH oxidase and endothelial dysfunction
Diabetes	ROS accelerated formation of advanced glycation end products (AGEs)
Aging	Cell damage and metabolic abnormalities
Cancer	ROS-mediated gene mutations (modification of pyridine and purine bases) and post-translational modifications leading disruption of cellular processes
Parkinson's disease	ROS-mediated mitochondrial dysfunction
Alzheimer's disease	Amyloid peptide and advanced glycation end products ROS-mediated neurotoxicity to hippocampal cells and the synaptosomal membranes

Diseases	Role of free radicals inpathophysiology
Huntington's disease	ROS-mediated transcriptional dysregulation and mitochondrial impairment
Autoimmune disorders	ROS-mediated inflammation and tissue destruction
Age-related macular degeneration	Photochemical reactions in the oxygen-rich environment of the outer retina lead to the liberation of cytotoxic (ROS)
Acute lung injury, acute respiratory distress syndrome, inflammation and hyperoxia	ROS-mediated inflammation and endothelial dysfunction

Anti-inflammatory drugs

Anti-inflammatory agents, nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs, are used for reduction of signs of inflammation (pain, swelling and fever).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are drugs commonly used for management of pain, fever, and inflammation. These agents are classified into two groups, nonselective COX inhibitors and selective COX-2 inhibitors.

-Nonselective COX inhibitors drugs (ibuprofen, indomethacin, sulindac, and aspirin etc.) are indicated for management of acute or chronic inflammatory diseases in rheumatoid arthritis, osteoarthritis, acute gout etc. Their mechanism of action involve inhibition of both cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) enzymes resulting in inhibition of prostaglandins and thromboxane production from arachidonic

acid. The main side effects from use of these drugs are related to gastrointestinal (GI) effects.

-Selective COX-2 inhibitors drugs, celecoxib, etoricoxib, and lumiracoxib etc. are as effective as nonselective COX inhibitors. They reduce risk of peptic ulceration but they increase risk for heart attack, thrombosis and stroke by a relative increase in thromboxane A_2 (Laine *et al.,* 2002; Zell *et al.,* 2009; Ashok *et al.,* 2011).

Steroidal drugs

Steroidal drugs, dexamethasone, fludrocortisones, triamcinolone and betamethasone, contain structure and efficacy similar to glucocorticoid hormone. They are widely used for management of inflammation in chronic inflammatory diseases. Prolong utilization of steroidal drugs cause many systemic and local side effects (Table 4).

Mechanism of action of steroidal drugs

Steroidal drugs infiltrate plasma membrane to their cytosolic receptors in the cytosol and form steroid-receptor heterodimer complex and translocate to the nucleus. They bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes leading to either inhibit or stimulate these gene transcriptions. They also stimulate the expression of lipocortin-1 that blocks phospholipase A_2 and inhibits the secretion of arachidonic acid from phospholipids. These drugs inhibit various aspects of the inflammatory process through increase the transcription of anti-inflammatory genes and decrease the transcription of inflammatory genes (Fig 10) (Table 5) (Baqai *et al.,* 2009; Barnes., 1998).

Table 4: Systemic and local side effects of steroidal drugs (Baqai *et al.,* 2009).

Endocrine	Adrenal suppression, hypercortisolism, cushingoid syndrome, hyperglycemia, precipitation of diabetes mellitus, immunosuppression, hypokalemia, amenorrhea, menstrual disturbances, growth retardation
Metabolic	Hyperglycemia, glucosuria, redistribution of fat, negative nitrogen balance, sodium and water retention
Cardiac	Hypertension, fluid retention, CHF, DVT
Musculoskeletal	Osteopenia/osteoporosis, avascular necrosis of bone, pathologic fracture, muscle wasting and atrophy, muscle and joint pain
Psychological	Mood swings, insomnia, psychosis, anxiety, euphoria, depression
Gastrointestinal	Ulcerative esophagitis, hyperacidity, peptic ulceration, gastric hemorrhage, diarrhea, constipation
Ocular	Retinal hemorrhage, posterior subscapular cataracts, increased intraocular pressure, exophthalmos, glaucoma, damage to optic nerve, secondary fungal and viral infection
Dermatologic	Facial flushing, impaired wound healing, hirsutism, petechiae, ecchymosis, hives, dermatitis, hyperpigmentation, hypopigmentation, cutaneous atrophy, sterile abscess
Nervous System	Headache, vertigo, insomnia, restlessness, increased motor activity, ischemic neuropathy, seizures
Other	Epidural lipomatosis, fever

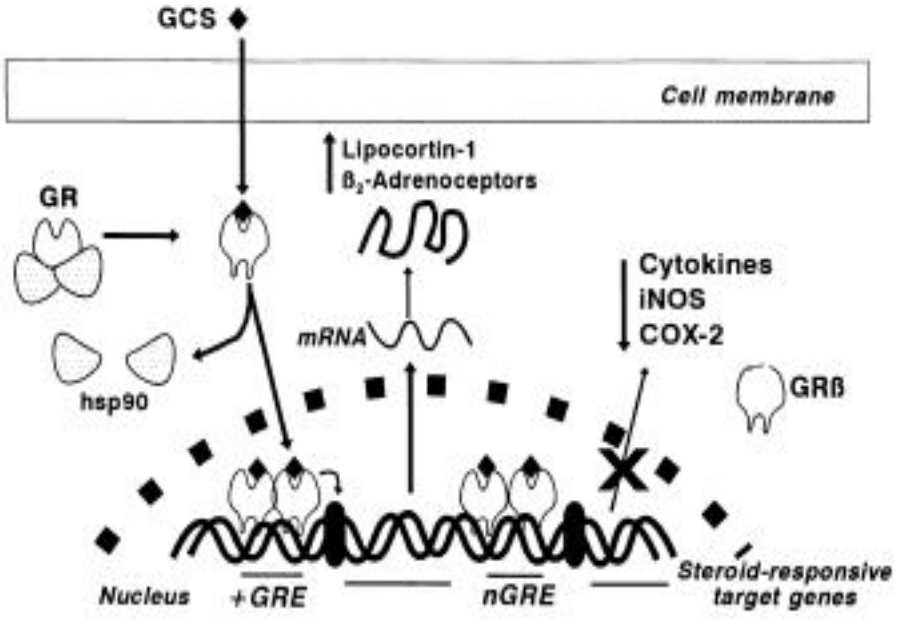


Figure 10: Mechanism of action of steroidal drugs (Barnes., 1998).

Table 5: Effect of glucocorticoids on gene transcription (Barnes., 1998).

Increase transcription	Decrease transcription
Lipocortin-1 β2-Adrenoceptor Secretory leukocyte inhibitory protein Clara cell protein (CC10) IL-1 receptor antagonist IL-1R2 (decoy receptor) IkB-α	Cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12, IL-13, TNF-α, GM-CSF, stem cell factor) Chemokines (IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin) iNOS COX-2 Cytoplasmic PLA_2 Endothelin-1 NK1-receptors, NK2-receptors Adhesion molecules (ICAM-1, E-selectin)

Biological drugs

Apart from nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs, biological drugs are specific drugs used in the treatment of specific chronic inflammatory diseases such as rheumatoid arthritis, SLE ect. They are inhibitors of proinflammatory cytokine production and function. Anti TNF-α drugs, adalimumab, etanercept, infliximab, bind to TNF-α and interfere their cellular function. The recombinant human IL-1 receptor antagonist (IL-1ra), anakinra, acts at a decoy receptor to neutralize IL-1. Side effects of these drugs associated with immune system disorder, infection and hypersensitivity. Parental routes of drug administration and high cost are limitation of their use (Mccoll *et al.,* 2004; So *et al.,* 2007).

Treatment of inflammatory diseases depends on pathological state, drug use and patient response. However, side effects of anti-inflammatory drugs cause a major problem in their clinical use. Current therapies of inflammation focus on inhibition of synthesis or functions of inflammatory mediators. At present, natural medicines from plants have been researched for their efficacy and safety in order to provide the evidences support their use in the treatment of inflammation.

Treehom remedy is composed of ten herbal plant including *Terminalia* sp., *Terminalia bellirica* Roxb., *Phyllanthus emblica* L., *Coriandrum sativum* Linn., *Aristolochia* sp., *Angelica dahurica* Benth., *Glycyrrhiza glabra* L., Sodium borate, *Trigonella foenum-graecum* L., *Terminalia chebula* Retz., *Rheum officinale* Baill (Table 6). It is approved by the Ministry of Public Health of Thailand in National Drug List as the folk remedy for relieving of pyrexia and constipation in children. As far as we known, several composition plants of this remedy showed anti-inflammatory and antioxidant effects (Table 6). However, there hasn't been reported in the anti-inflammatory activity of this remedy. Therefore, the aim of this study is to investigate the anti-inflammatory activity of the water and ethanol extracts from Treehom remedy on LPS-activated J774A.1 macrophages as well as the antioxidant activity of this extracts by FRAP assay.

Table 6: Compositions and pharmacological properties of Treehom remedy.

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Terminalia sp.	Combretaceae	4	- *Terminalia* sp. root showed antimicrobial activity in vitro. (Silva *et al.* 1997) - *Terminalia* sp. showed to prevents glucose-induced hypertension in rats. (Lemba *et al*. 2010)
Terminalia bellirica Roxb.	Combretaceae	4	- *Terminalia belerica* Roxb. showed protective effect against carbon tetrachloride induced damage in albino rats. (Jadon et al., 2007) - *Terminalia belerica* Roxb. showed the anti-Salmonella activity *in vitro* and in vivo studies. (Mandani and Jain., 2008) -*Terminalia belerica* Roxb. was an antioxidant and showed reactive oxygen species scavenging properties *in vitro*. (Hazra et al., 2010)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Phyllanthus emblica L.	Euphorbiaceae	4	- Progallin A isolated from the acetic ether part of the leaves of *Phyllanthus emblica* L. has shown anticancer activities *in vitro* by induced apoptosis of human hepatocellular carcinoma BEL-7404 cells. It up-regulated Bax expression and down-regulated Bcl-2 expression (Chatterjee *et al*., 2010) - *Phyllanthus emblica* Linn. (PE) extract has shown the protective effects on ethanol induced rat hepatic injury. (Zhong *et al*., 2011.) - Phenolics purified from *Phyllanthus emblica* L. has shown antioxidant activities and antiproliferative capacities *in vitro.* (Luo *et al*., 2011)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Coriandrum sativum Linn.	Umbelliferae	4	- Polyphenolic compounds from *Coriandrum sativum* Linn. decreased the activities of antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and caused decreased glutathione content. (Hashim *et.al., 2005*) - *Coriandrum sativum* Linn. has shown inhibition of carrageenan induced rat paw edema. (Sonika *et.al., 2010*)
Aristolochia sp.	Asclepiadaceae	1	*Aristolochia* sp. extracts exhibited antioxidant properties *in vitro.* (Thirugnanasampandan *et.al., 2008*)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Angelica dahurica Benth.	Umbelliferae	1	- *Angelica dahurica* Benth reduced airway inflammation and suppressed oxidative stress in the OVA-induced asthma model. (Lee *et al., 2010*) - *Angelica dahurica* Benth decreased the levels of proinflammatory factors such as tumor necrosis factor-a (TNF-α), interleukin-1β (IL-1 β), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a lipopolysaccharide (LPS)-activated microglial cell line, BV2 cells and alleviated the level of reactive oxygen species in LPS-activated BV2 cells. (Moon *et al., 2012*)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Glycyrrhiza glabra L.	Papilionaceae	1	- *Glycyrrhiza glabra* roots exhibited anti-inflammatory property via the inhibition of PGE_2,TXB_2 and LTB_4 in mammalian cell assay system. (Chandrasekaran *et.al 2010)* - *Glycyrrhiza glabra* roots exhibited antimicrobial activity against both Gram-positive.(Gupta *et al., 2008*) - Aerial Parts and Roots of *Glycyrrhiza glabra* L. was antioxidant and exhibited radical scavenging activity *in vitro*. (Dhingra *et al., 2006*)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Trigonella foenum-graecum L.	Leguminosae	1	- *Trigonella foenum-graecum* L. leaves extract showed anti-inflammatory and antipyretic properties in both i.p. and p.o. administration. (Haouala *et al*., 2008) - The aqueous and organic extracts of *Trigonella foenum-graecum* L. inhibited the mycelia growth of fungi. (Ahmadiani *et al., 2001*) - The extract of fenugreek *(Trigonella foenum graecum) seeds* exhibited scavenging of hydroxyl radicals and inhibition of hydrogen peroxide-induced lipid peroxidation in rat liver mitochondria. (Kaviarasan *et al*., 2007)
Sodium borate	-	1	-

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Terminalia chebula Retz.	Combretaceae	22	-*Terminalia chebula* hydroalcoholic extract (TCHE) showed the anti-arthritic effect in experimental models (Nair *et al*., 2010) -Chebulagic acid isolated from the fruits of *Terminalia chebula* Retz inhibited COX and 5-LOX, the key enzymes involved in and induces apoptosis in COLO-205 cell line. (Reddy *et al*., 2009) -The chloroform extract of *Terminalia* chebula Retz. seeds showed antidiabetic and renoprotective effects in streptozotocin-induced diabetic rats. (Rao *et al*., 2006) -The aqueous extracts of Terminalia chebula showed potent antioxidant in vitro. (Naik *et al*., 2004)

Sciencetific name	Family name	Parts in the remedy	Pharmacological properties
Rheum officinale Baill	Polygonaceae	22	-Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone) isolated from the roots of *Rheum officinale* Baill enhanced cutaneous wound healing in vitro and in vivo. (Tang *et al*., 2007) -The water extract of *Rheum officinale* Baill. showed anticance activity in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines. (Li *et al*., 2009)

CHAPTER III

MATERIALS AND METHODS

Materials

Treehom remedy extracts

- Treehom remedy was purchased from a Thai traditional drug store and authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D. Faculty of Pharmaceutical Sciences, Chulalongkorn University.
- The ethanol extract of Treehom remedy was also prepared by Associate Professor Nijsiri Ruangrungsi, Ph.D. The remedy was extracted using 95% ethanol until exhausted and evaporated until dryness. The marc was further extracted with warm water and lyophilyze until dryness.
- %Yield obtains from the ethanol and water extracts were 35.5% and 14.6%, respectively.
- The ethanol extract was prepared as the stock solution by dissolving in dimethylsulfoxide (DMSO). The final concentrations of this extract were prepared with the constant concentration 0.2% DMSO. The water extract of dissolved in complete media as the stock solution.

Cells

The murine macrophages J774A.1 cells were obtained from the 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 at 37 ^{0}C , 97% humidity ,and 5% CO_2.

They were subcultured when the cells were 80 % confluence. The cells were used in all experiments with their viability more than 85.

Chemicals and reagents

The following chemicals and reagents were used in this study, dimethyl sulfloxide (DMSO) (Sigma, USA), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (Hyclone, USA), hydrochloric acid (Merck,Germany), lipopolysaccharide (Sigma), nitric oxide assay kit (Promega, USA), penicillin/streptomycin (Hyclone, USA), sodium chloride (Sigma, USA), sodium bicarbonate (Baker, USA), sodium hypochlorite (Clorox, USA), 0.4% Trypan blue dye, TRiZol reagent (Invitrogen), Chloroform(Sigma, USA), DEPC(molekula, UK), ImProm-II™ Reverse Transcription system(Promega, USA), primer(Bio Basic,Canada), Taq polymerase(vivantis, USA), 20 mM Iron(II) sulfate heptahydrate, FRAP reagent, and 40mM HCL.

Equipments and Instruments

The following equipments and instruments were used in this study, auto pipette (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge (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), scrapper (Greiner, UK), spectrophotometer (Shimadzu, Japan), T-25 Tissue Culture flasks (Corning, USA), thermocycler machine (Eppendrof, USA), and vortex mixer (Scientific Industries, USA).

Methods

Effects of the ethanol and water extracts from Treehom remedy on nitric oxide production in LPS-stimulated J774A.1 cells.

1. Incubate 2× 10^5 cells/ml J774A.1 cells in a 96-well at 37 ^{0}C, 5% CO_2 for 24 h.
2. Add 6.25-100 µg/ml the ethanol and water extracts and incubated at 37 ^{0}C for 24h. Ten µM dexamethasone and 0.2%DMSO were used as the positive control and negative control, respectively.
3. Stimulated with 100 ng/ml LPS and further incubated at 37 ^{0}C for 24 h.
4. Determine nitric oxide production in the supernatant by Griess reaction assay and cell viability of treated cell by resazurin reduction assay.

4.1 perform Griess reaction assay by the following procedures;

- Pipette 100 µl of the supernatant into 96 well plates.
- Add 20 µl of sulfanilamide solution and incubate for 10 min at room temperature in the dark.
- Add 20 µl of NED solution and further incubate for 10 min at room temperature in the dark.
- Determine NO production by measuring the plate at 540 nm by a microplate reader, calculating the percentages of nitric oxide inhibition of the extracts compared to LPS-stimulated condition.

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

- Determine the 50 % inhibitory concentration (IC_{50}) on NO production of the extracts for using in all experiments after this assay.

4.2 Perform resazurin assay by the following

- Remove all supernatant from the remaining treated cell
- Add 100 µl of 50 µg/ml resazurin and incubate at 37 ^{0}C, 97% huminity,5% CO_2 for 2 h.
- Determine viable cell which can reduce resazurin (blue) to resorufin (red) by measuring the plate at 570 and 600 nm using microplate reader and calculate the percentage of cell viability compared to LPS-stimulated condition by following formular;

$$\% \text{ cell viability} = \left[\Delta OD_{control} - (\Delta OD_{extract} / \Delta OD_{control})\right] x100$$

$$\Delta OD = OD_{570} - OD_{600}$$

Effect of the extracts on antioxidant activities

The antioxidant activity of the extracts was determined by Ferric reducing antioxidant power (FRAP) assay by the following procedure.

- Prepare 180 µl FRAP reagent mixture containing 300 mM sodium acetate buffer pH 3.6, 10 mM 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.
- Warm and mixture at 37 ^{0}C.
- Mix10 µl of extracts, 180 µl FRAP reagent mixture and 10 µl deionized water in 96 well plate and incubate at room temperature for 5 min. Ten µM ascorbic acid, double distilled water and 0.2% DMSO were used as the positive control and the negative control, respectively.

- Measure the reaction mixture at 600 nm by microplate reader.
- Determine the concentration of ferrous tripyridyltriazine by using ferrous standard curve prepared from standard $FeSO_4.7H_2O$ solution.
- Classify the anti-oxidant activities of the ethanol and water extracts according to the classification in table 7.

Table 7: Classification of antioxidant activities (Wong *et al*., 2006)

[$FeSO_4.7H_2O$] (µmol Fe (II)/g)	Antioxidant activities
> 500	extremely high
100-500	high
10-100	medium
< 10	low

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

- Incubate 2×10^5 cell/ml J774A.1cells in 24 well plates at 37 ^{0}C for 24 h.
- Add 25, 50, and 100 µg/ml the water and ethanol extracts and incubate for 24 h. Ten µM dexamethasone and 0.2%DMSO solution were used as the positive control and negative control, respectively.
- Stimulate the cells with 100 ng /ml LPS at 37 ^{0}C for 4 or 24 h in order to determine pro-inflammatory cytokine expression or iNOS and COX-2 expression, respectively.
- Prepare total RNA from the treated cells by the following procedure;
    - Remove the supernatant from treated cells
    - Lyse in 1 ml TRizol reagent at room temperature for 5 min.

- Transfer the homogenized samples to eppendrof tubes.
- Add 0.2 ml chloroform into each tube, vigorously shake for 15 sec and incubate at room temperature for 2-3 min.
- Separate the aqueous phase by centrifugation at 12,000 g at 4^0C for 15 min.
- Carefully collect the aqueous phase from each tube into fresh eppendrof tube.
- Precipitate total RNA by adding 0.5 of isopropyl alcohol and incubating at room temperature for 10 min.
- Separate the aqueous phase by centrifugation at 12000 g at 4^0C for 10 min.
- Wash the pellet with 75% ethanol, precipitate it by centrifugation at 7500 g at 4^0C for 5 min, air-dry and then dissolve it in RNase free-water.
- Determine the RNA concentration and contamination by measuring the absorbance at 260 nm and 280 nm by Nanodrop.
- Store the total RNA sample at -70 ^{0}C.

- Prepare complementary DNA (cDNA) from the total RNA samples by reverse transcription reaction as in the following procedures.

  - Heat 1.5 µg total RNA, Oligo dT_{15} primer and nuclease-free water in 5 µl total volume at 70 ^{0}C for 5 min and then immediately chill on ice for 5 min.

  - Prepare reverse transcription mixture containing 25 nm $MgCl_2$, mixed dNTP, ribonuclease inhibitor, and reverse transcriptase.

  - Add 15 µl the reverse transcription mixture into each total RNA tube.

  - Perform PCR in a themocycler as in the followings; 25 ^{0}C for 5 min, then 42 ^{0}C for 1 hour 30 min, and finally 70 ^{0}C for 15 min.

- Store the cDNA samples at - 20 ^{0}C until used.

- Generate all PCR products from cDNA samples by PCR as in the following procedure;

  - Mix 1 µl of cDNA sample with 24 µl PCR reaction mixture containing 50 $MgCl_2$, 10mM dNTP, 0.4µM of primers, 1 unit of Taq DNA polymerase and PCR buffer primer.

  - Perform PCR using the following conditions; denaturation for 30 sec at 94 ^{0}C, annealing for 45 sec at 55^{0}C, extension for 1 min at 72 ^{0}C, and final extension for 7 min at 72 ^{0}C at the end of 25th cycles .

  - Run 8 µl of PCR product plus 2 µl of loading dye 1.5 % agarose gel electrophoresis in TBE buffer at 100 volt for 45 min.

  - Stain the gel with ethidium bromide for 2 min and destain with TBE buffer for 30 min

  - Identify and analyze the density of PCR products by gel documentation.

  - Determine the amount of the PCR product as

$$\% \text{ Internal control} = \frac{\text{Band density of the PCR}_{\text{product}} \times 100}{\text{Band density of the PCR } \beta\text{-actin product}}$$

Statistical analysis

All data were expressed as means ± standard error *(*mean ± SE). One-way ANOVA by Turkey's post hoc test was used to determine the statistical significance of differences between the values for the various experimental and control groups. All statistical analysis was performed according to the statistic program, SPSS version 17. The p-value of less than 0.05 was considered statistically significant difference.

CHAPTER IV

RESULTS

1. Effects of the ethanol and water extract from Treehom remedy on nitric oxide production in LPS-stimulated J774A.1 cells and cell viability.

Nitric Oxide (NO) is one of the important mediators of inflammatory responses when macrophages are activated by lipopolysaccharide (LPS). Inhibitory effect on nitric oxide production were shown in LPS-stimulated J774A.1 after pretreatment with the ethanol and water extracts from Treehom remedy 6.25-100 μg/ml in concentration-dependent manner. The ethanol extract from Treehom remedy inhibited NO production by 3.23%, 23.63%, 49.11%, 65.83% and 83.09% at the concentrations 6.25, 12.5, 25, 50 and 100μg/ml, respectively (Fig 11A). The reference control, 10μM (3.92 μg/ml) dexamethasone inhibited NO production by 92.63%. For the water extract, it inhibited NO production by 6.77%, 19.6%, 27.45%, 42.53% and 77.85% at the concentrations 6.25, 12.5, 25, 50 and 100μg/ml, respectively (Fig 13A). Dexamethasone at concentration of 10μM (3.92 μg/ml) inhibited NO production by 85.54%. The concentration required for 50% inhibition (IC_{50} value) of NO for the ethanol and water extracts were determined for selecting the concentrations of the extract used in subsequent studies. IC_{50} value of the ethanol and water extracts were 40.05 (Fig 11B) and 60.05 μg/ml (Fig 13B), respectively. The effects of the ethanol and water extracts on J774A.1 cells viability were also determined. Both extracts did not affect J774A.1 cells viability at all concentration treated (Fig 12, 14).

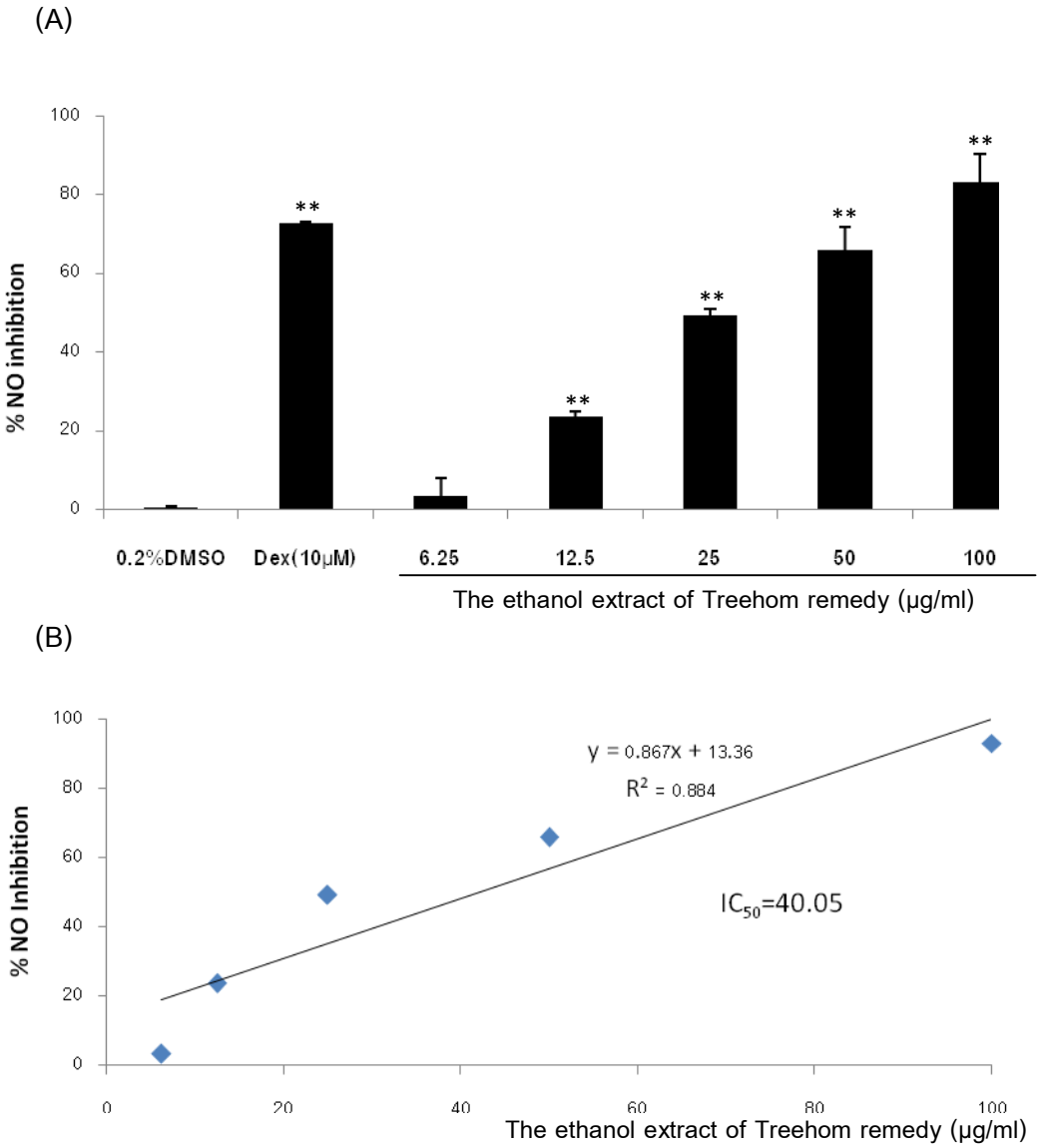


Figure 11: (A) Pretreatment with the ethanol extract of Treehom remedy 6.25-100 μg/ml significantly inhibited LPS-stimulated NO production in concentration-dependent manner. The data are expressed as the mean ± S.E. from three independent experiments (N=3). (B) IC_{50} value the ethanol extract from Treehom remedy was 40.05 μg/ml. **P<0.05 indicates significant difference from 0.2% DMSO-treated LPS activated cells.

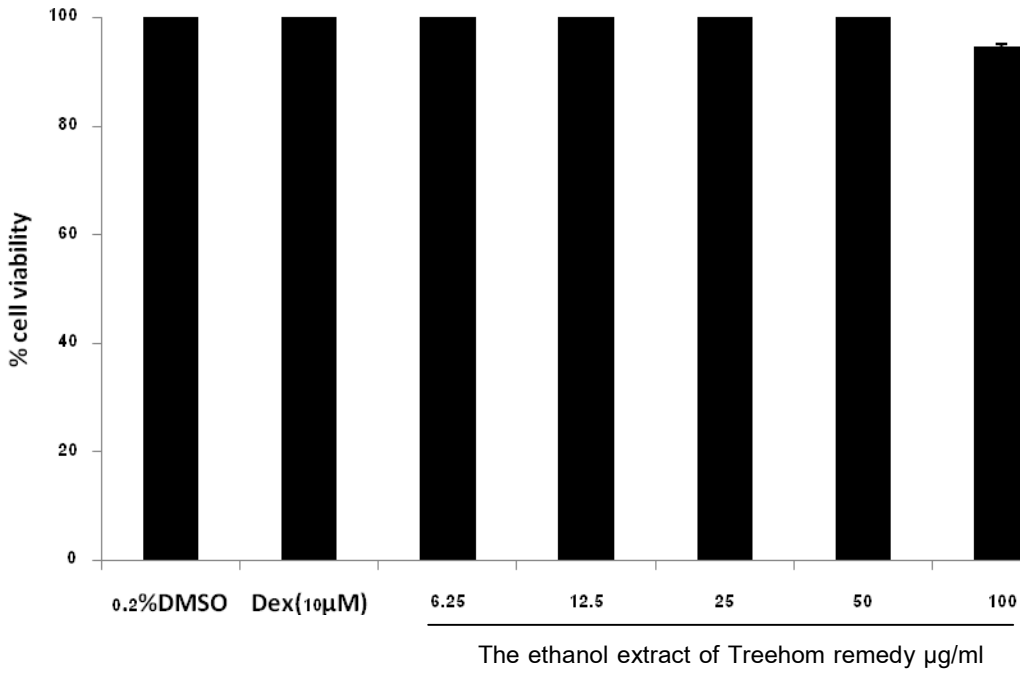


Figure 12: The effect of ethanol extracts from Treehom remedy at the concentrations 6.25-100 μg/ml on cell viability in LPS-stimulated J774A.1 cells. The data are expressed as the mean ± S.E. from three independent experiments (N=3).

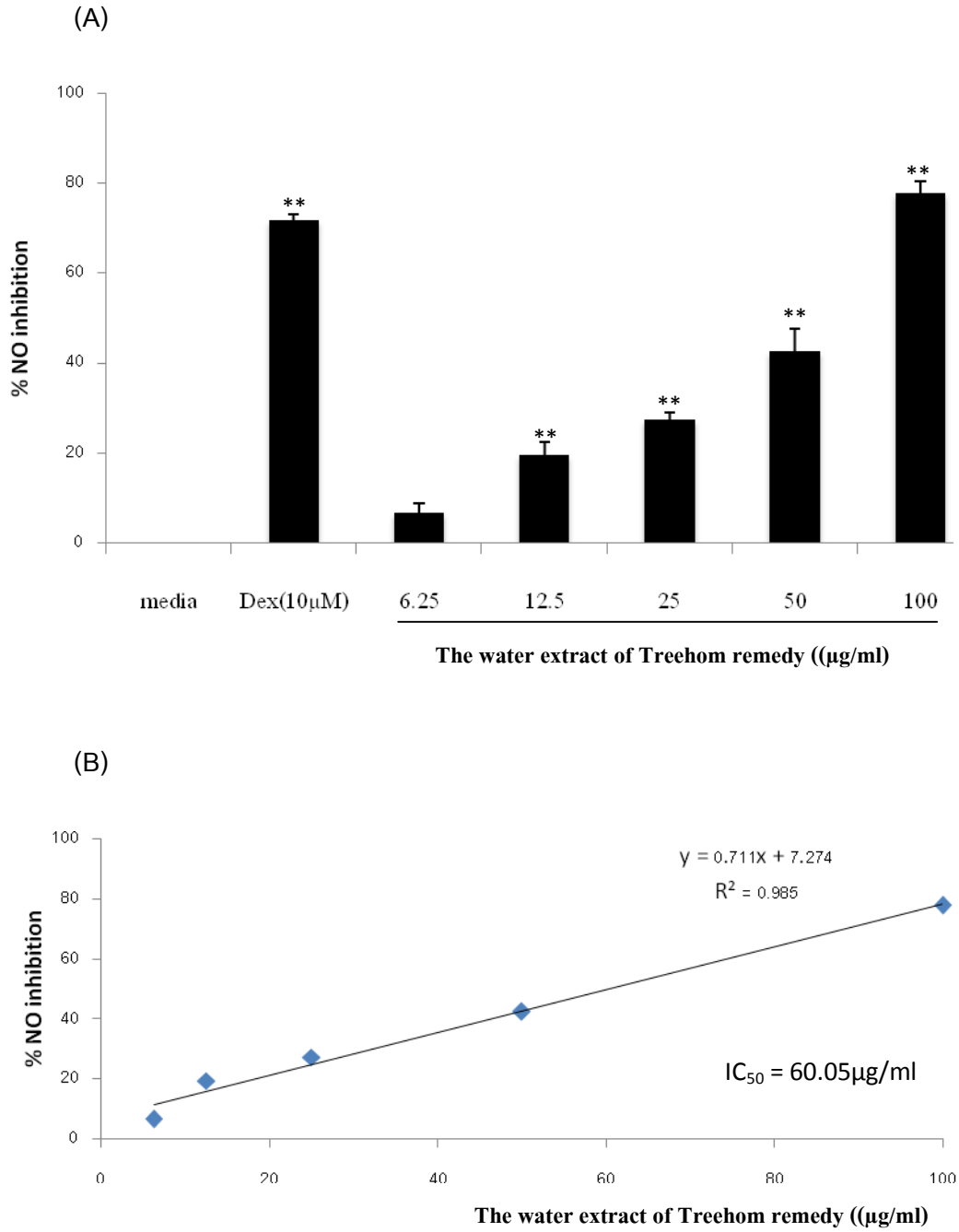


Figure 13: (A) Pretreatment with the water extract of Treehom remedy 6.25-100 μ g/ml significantly inhibited LPS-stimulated NO production in concentration-dependent manner. The data are expressed as the mean ± S.E. from three independent experiments (N=3). (B) IC_{50} value of ethanol extract from Treehom remedy was 60.05 µg/ml. **P<0.05 indicates significant difference from the 0.2% DMSO-treated LPS activated cells.

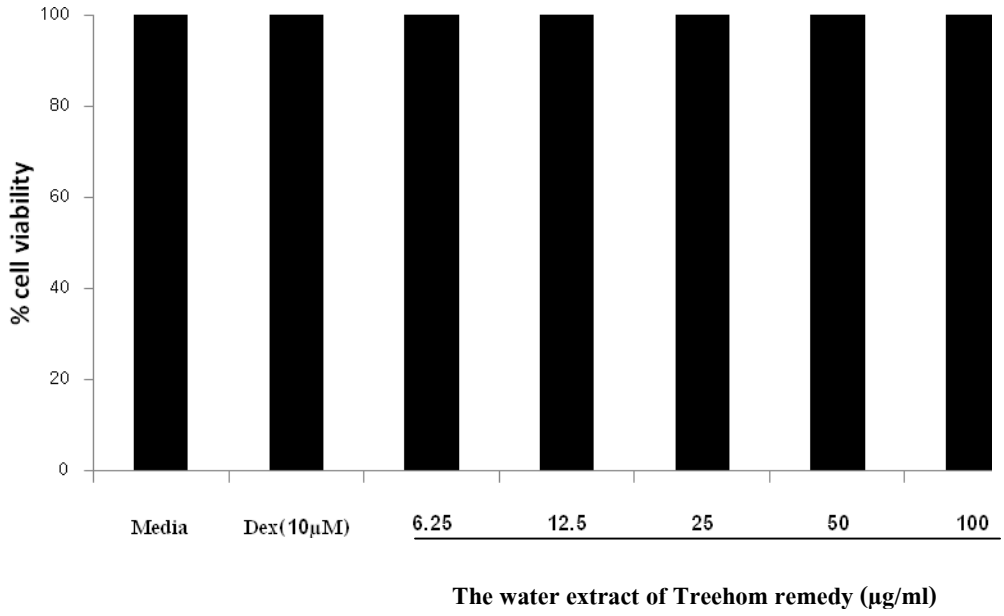


Figure 14: The effect of water extracts from Treehom remedy at the concentrations 6.25-100 μg/ml on cell viability in LPS-stimulated J774A.1 cells. The data are expressed as the mean ± S.E. from three independent experiments (N=3).

2. The effect of the extract on anti-oxidant activity

Effect of Treehom remedy extracts on anti-oxidant activity was determined by ferric reducing antioxidant power (FRAP) assay. In this assay, antioxidant activity base on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ), leading to an intensive blue color. According to table 7, the samples were classified into four categories on the basis of their antioxidant activities. Under this classification, the ethanol extract of Treehom remedy at 25, 50 and 100 µg/ml showed high antioxidant activity because they produced 123.42, 238.67 and 439.92 µmol/g of ferrous tripyridyltriazine (Fe (II)-TPTZ) complex, respectively. Medium antioxidant activity of the extract were found at 6.25 and 12.5µg/ml since 34.25 and 67.4 µmol/g of (Fe (II)-TPTZ complex were formed respectively (Fig 15A). For the water extract, high antioxidant activity were obtained at concentration 25, 50 and 100 µg/ml (Fe (II)-TPTZ complex = 130.83, 283.00 and 417.67 µmol/g, respectively). Medium antioxidant activity were demonstrated at 6.25 and 12.5, µg/ml (Fe (II)-TPTZ complex = 32.25 and 68.42 µmol/g respectively) (Fig 15B).

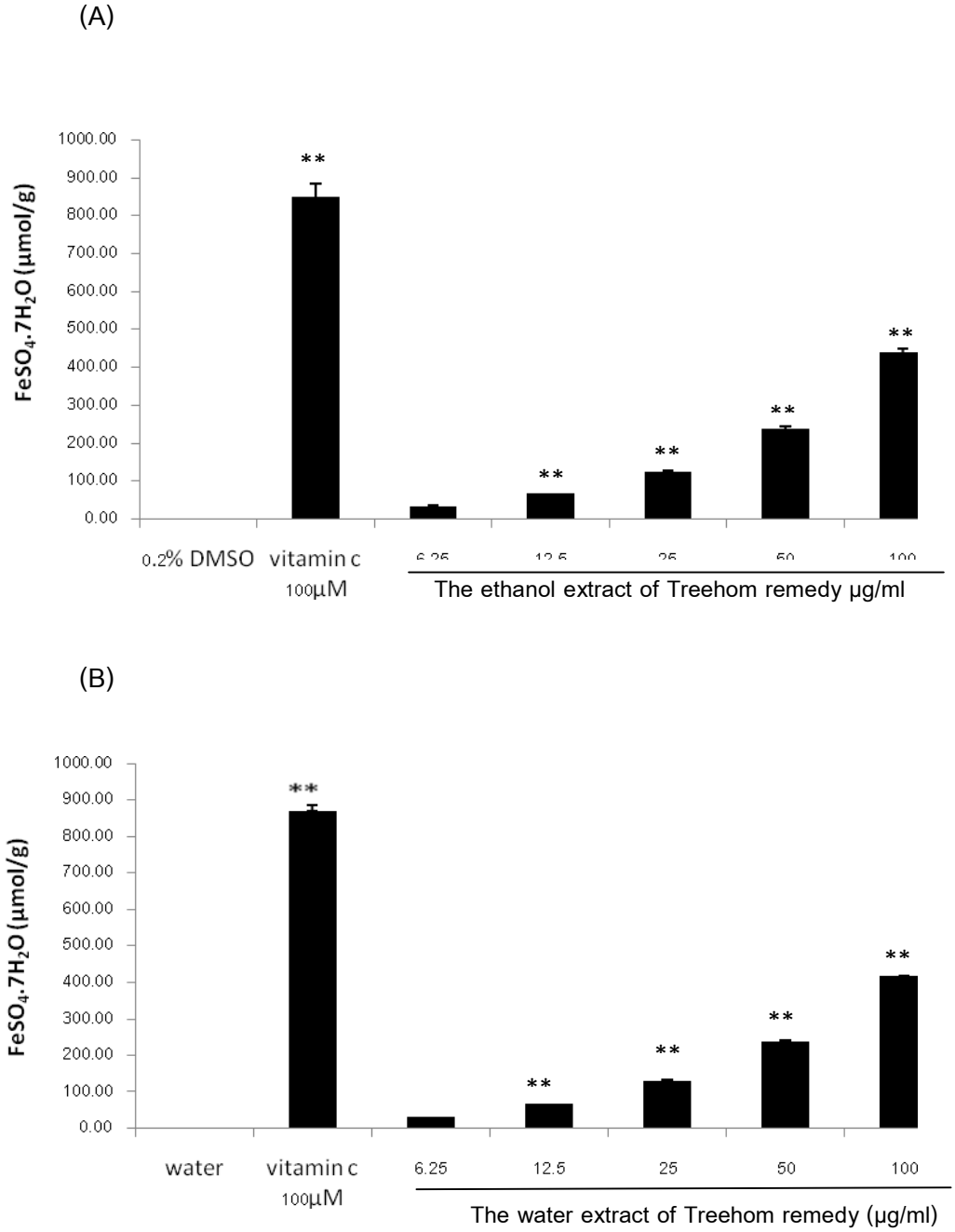


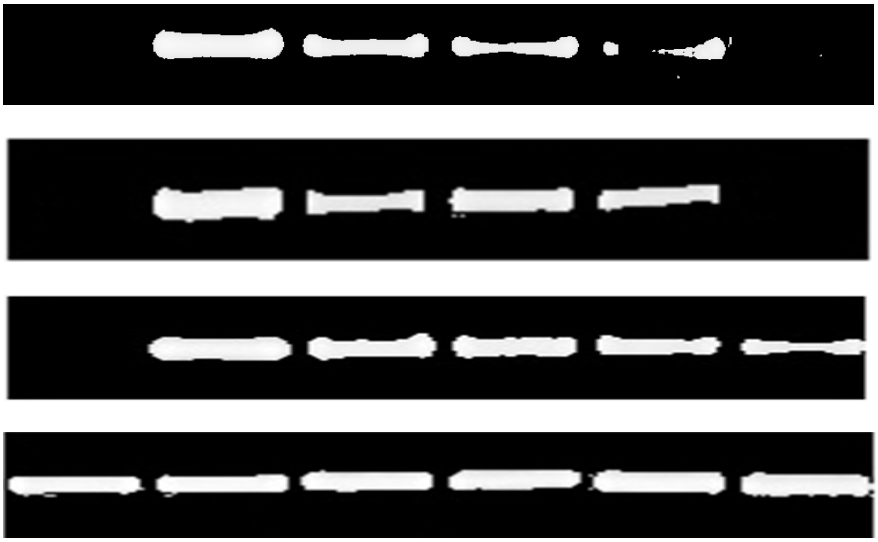
Figure 15: Effects of the ethanol (A) and water extracts (B) from Treehom remedy at the concentrations 25-100 µg/ml on antioxidant activity by FRAP assay. The data are express mean ± S.E. from 3 independent experiments (n=3). *P<0.05.

3. The effects of the extracts on mRNA expression of pro-inflammatory cytokines in LPS-stimulated J774A.1 cells

TNF-α, IL-1β and IL-6 are key proinflammatory cytokines in inflammatory process. From this experiment, inhibitory effect on the expression of TNF-α, IL-1β and IL-6 were shown in stimulated J774A.1 after pretreatment with the ethanol and water extract from Treehom remedy 6.25-100 μg/ml.

The ethanol extract of Treehom remedy 25-100 μg/ml was significantly decreased mRNA expression of TNF-α, IL-1β and IL-6 of LPS-stimulated J774A.1 cells (Fig 16). TNF-α mRNA expression were 75.21%, 57.90%, 37.40% for the ethanol extract 25, 50 and 100 μg/ml respectively while IL-1β mRNA expression were 55.09%, 44.89%, 5.61% at 25, 50 and 100μg/ml. It also decreased mRNA expression of IL-6, the expressions were found to be 42.13%, 19.53%, 4.28% at 25, 50 and 100μg/ml. Dexamethasone 10 μM (3.92μg/ml) also reduced cytokines release by inhibition of mRNA expression. The expression were 59.45%, 30.70% and 54.52% for TNF-α, IL-1β and IL-6, respectively.

The water extracts of Treehom remedy exhibited similar patterns in cytokines expression (Fig 17). It significantly decreased mRNA expression of TNF-α, IL-1β and IL-6 of LPS-stimulated J774A.1. TNF- α mRNA expression were 65.24%, 60.82%, 55.27% at 25, 50 and 100 μg/ml respectively. For IL-1β mRNA, the expression were 71.04%, 42.51%, 6.80% at 25, 50 and 100μg/ml. It was found that IL-6 mRNA expression were 51.96%, 30.62%, 14.87% at 25, 50 and 100 μg/ml of the water extract respectively. Dexamethasone 10 μM (3.92μg/ml), a reference drug, exhibited 59.51%, 41.33 and 55.09% on mRNA expression of TNF-α, IL-1β and IL-6, respectively.



LPS 100 ng/ml	-	+	+	+	+	+
media	+	+	-	-	-	-
Dex (10μM)	-	-	+	-	-	-
Treehom (μg/ml)	-	-	-	25	50	100

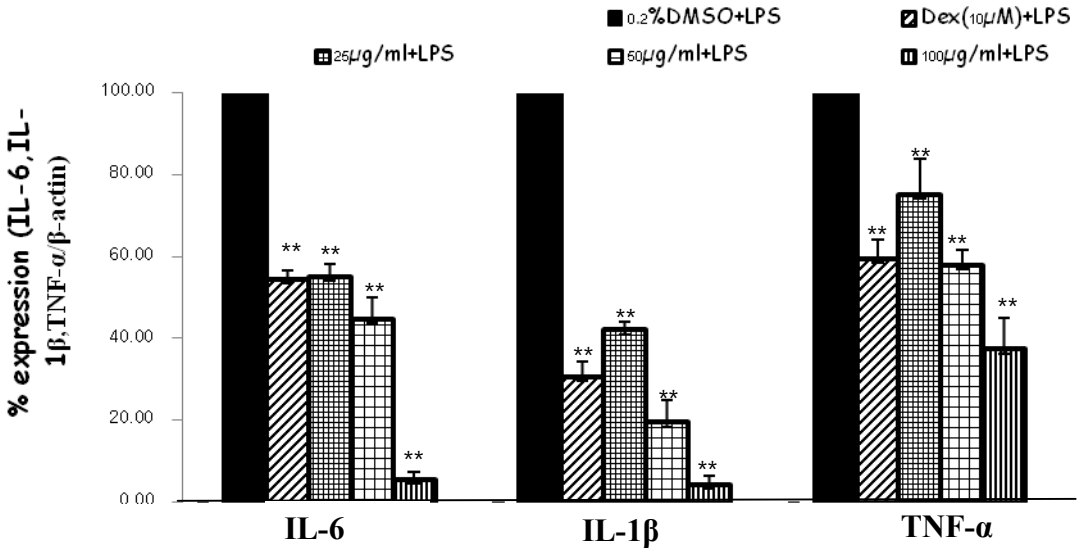
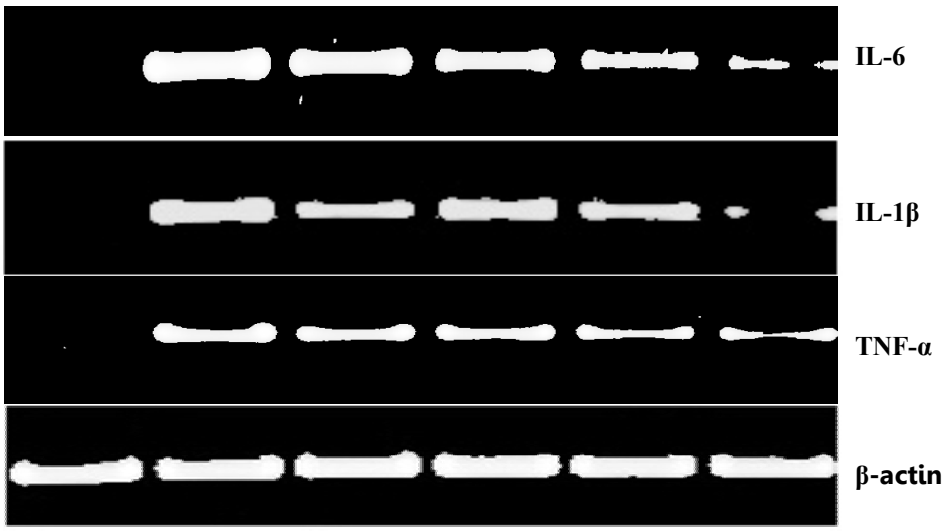


Figure 16: Effects of the ethanol extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of TNF-α, IL-1β, IL-6. The data are express mean ± S.E. from 3 independent experiments (n=3). **P<0.05 compared to untreated LPS activation.



LPS 100 ng/ml	-	+	+	+	+	+
media	+	+	-	-	-	-
Dex (10μM)	-	-	+	-	-	-
Treehom (μg/ml)	-	-	-	25	50	100

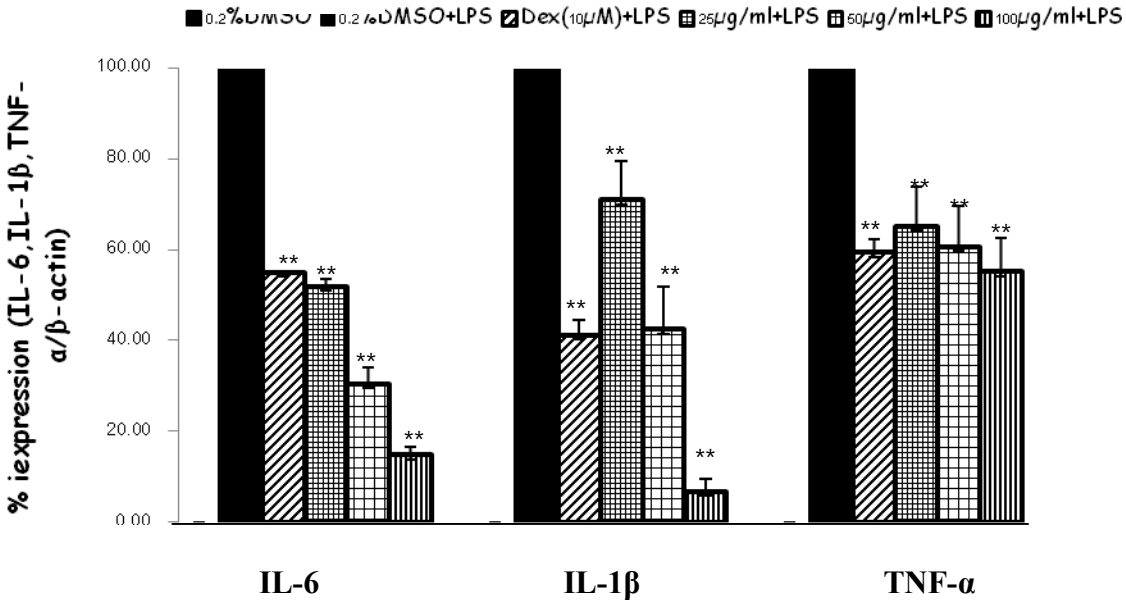


Figure 17: Effects of the water extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of TNF-α, IL-1β, IL-6 .The data are express mean ± S.E. from 3 independent experiments (n=3). **P<0.05 compared to untreated LPS activation.

4. The effect of the extracts on mRNA expression of iNOS in LPS stimulated J774A.1 cells.

Inhibitory effect on expression of iNOS were shown in stimulated J774A.1 cells after pretreatment with the ethanol and water extract from Treehom remedy 6.25-100 µg/ml. The ethanol extract of Treehom remedy 25-100 µg/ml were significantly decreased mRNA expression of iNOS, the expression were 71.28%, 60.60%, and 29.58%at 25,50,100µg/ml respectively)(Fig 18) while dexamethasone 10 µM (3.92µg/ml) exhibited 46.42% mRNA expression of iNOS (Fig 18). The water extract of Treehom remedy also decreased mRNA expression of iNOS, the expression obtained were 73.97%, 60.83%, and 39.64% at concentration 25, 50,100µg/ml respectively (Fig 19). Dexamethasone 10 µM (3.92µg/ml) exhibited 49.68% mRNA expression of iNOS (Fig 19).

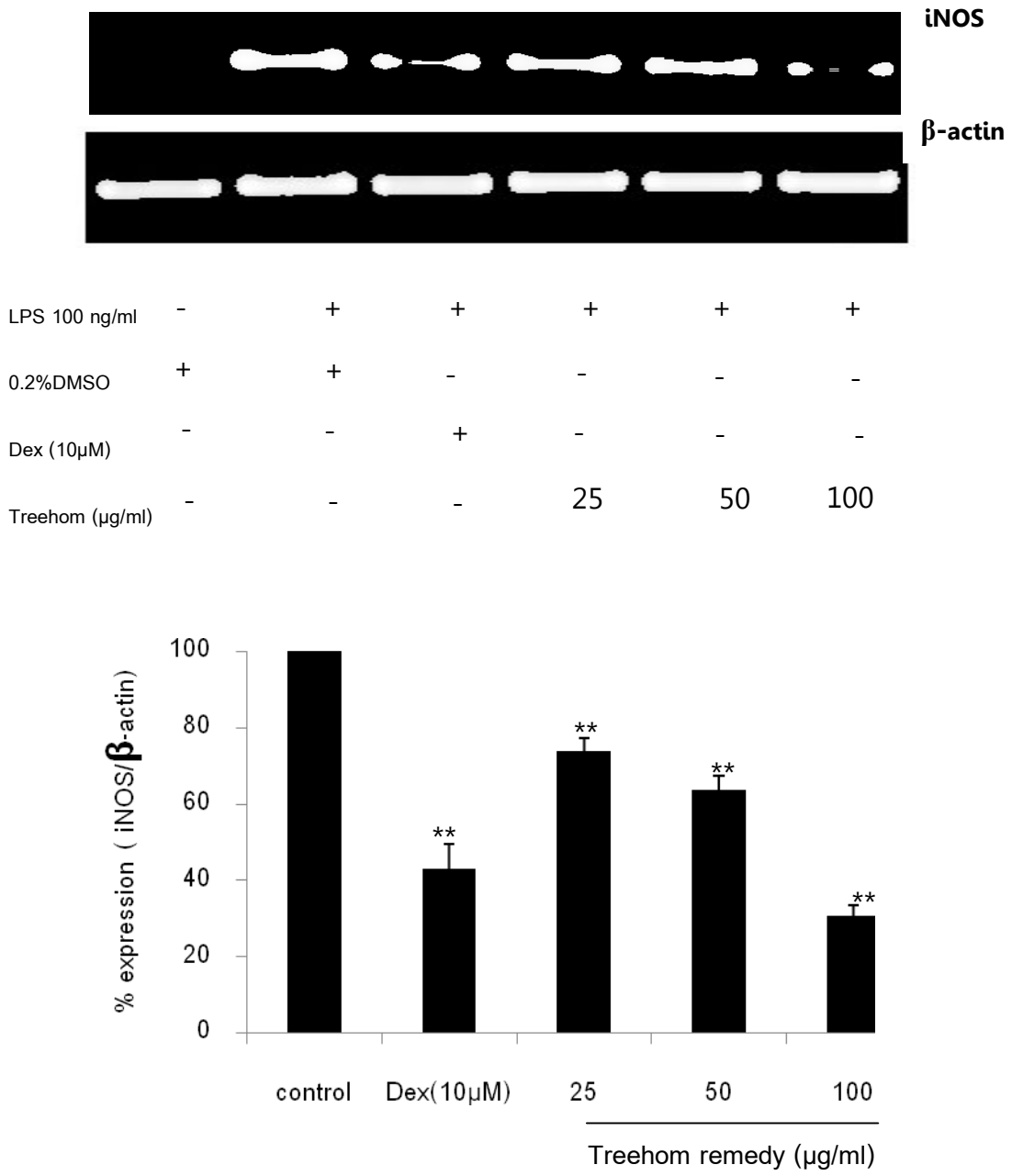


Figure 18: Effects of the ethanol extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of iNOS .The data are express mean ± S.E. from 5 independent experiments (n=5). **P<0.05 compared to untreated LPS activation.

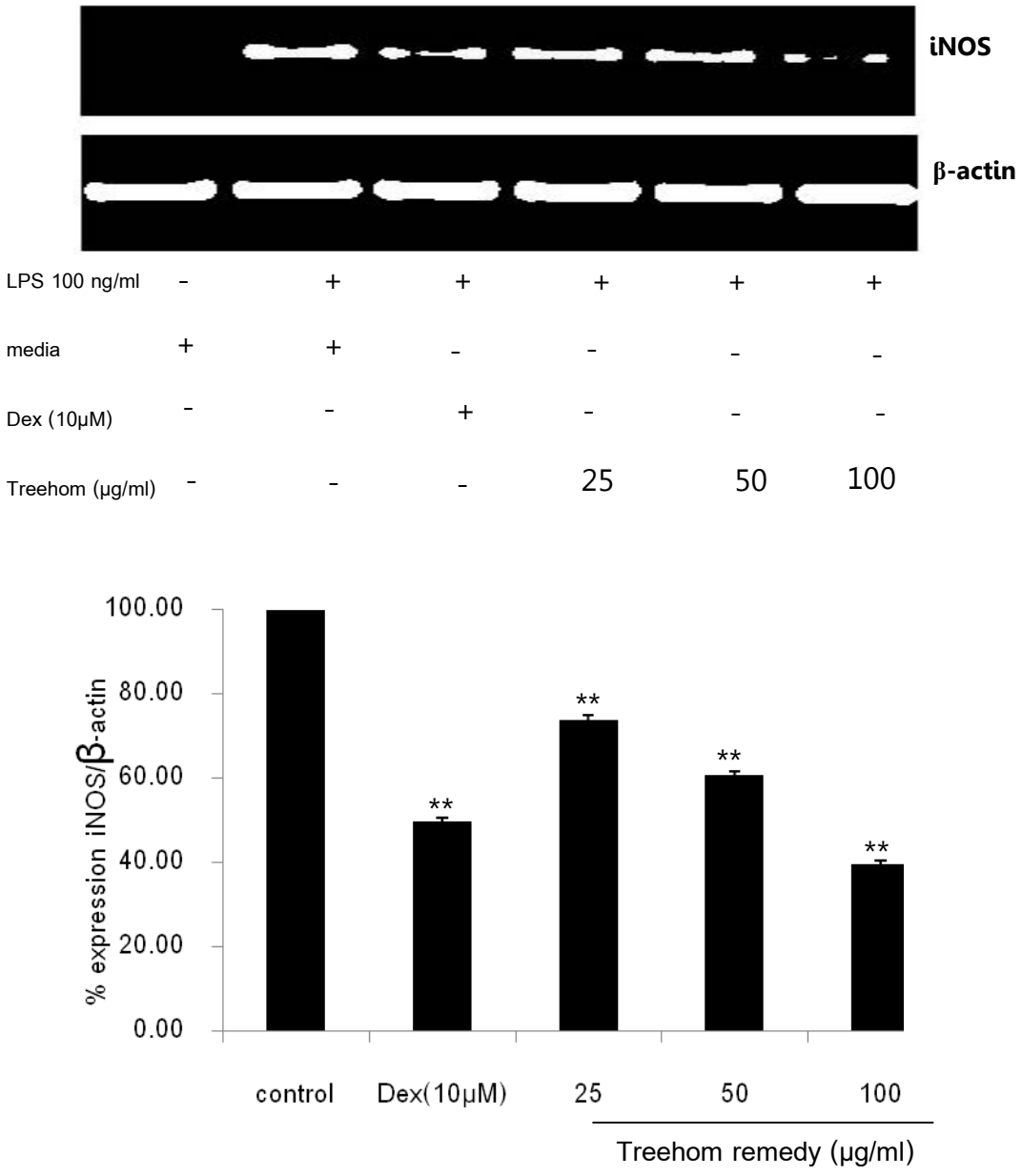


Figure 19: Effects of the water extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of iNOS .The data are express mean ± S.E. from 5 independent experiments (n=5). **P<0.05 compared to untreated LPS activation.

5. The effect of the extracts on mRNA expression of COX-2 in LPS stimulated J774A.1 cells.

COX-2 is an inducible enzyme found in pathological conditions. After pretreatment with the ethanol and water extract from Treehom remedy 6.25-100 µg/ml, inhibitory effect on expression of COX-2 were found in stimulated J774A.1cells. The ethanol extract of Treehom remedy was significantly decreased mRNA expression of COX-2, the expression were 75.67%, 63.68%, and 38.32% at 25,50,100µg/ml respectively (Fig 20).It was found that dexamethasone 10 µM (3.92µg/ml) exhibited 49.84% mRNA expression of COX-2 (Fig 20). The same result were obtained from the water extract since it was significantly decreased mRNA expression of COX-2, the expression were 86.81%, 72.66% and 48.95% at 25,50,100µg/ml respectively(Fig 21). Dexamethasone 10 µM (3.92µg/ml) exhibited 49.84% mRNA expression of COX-2 (Fig 21).

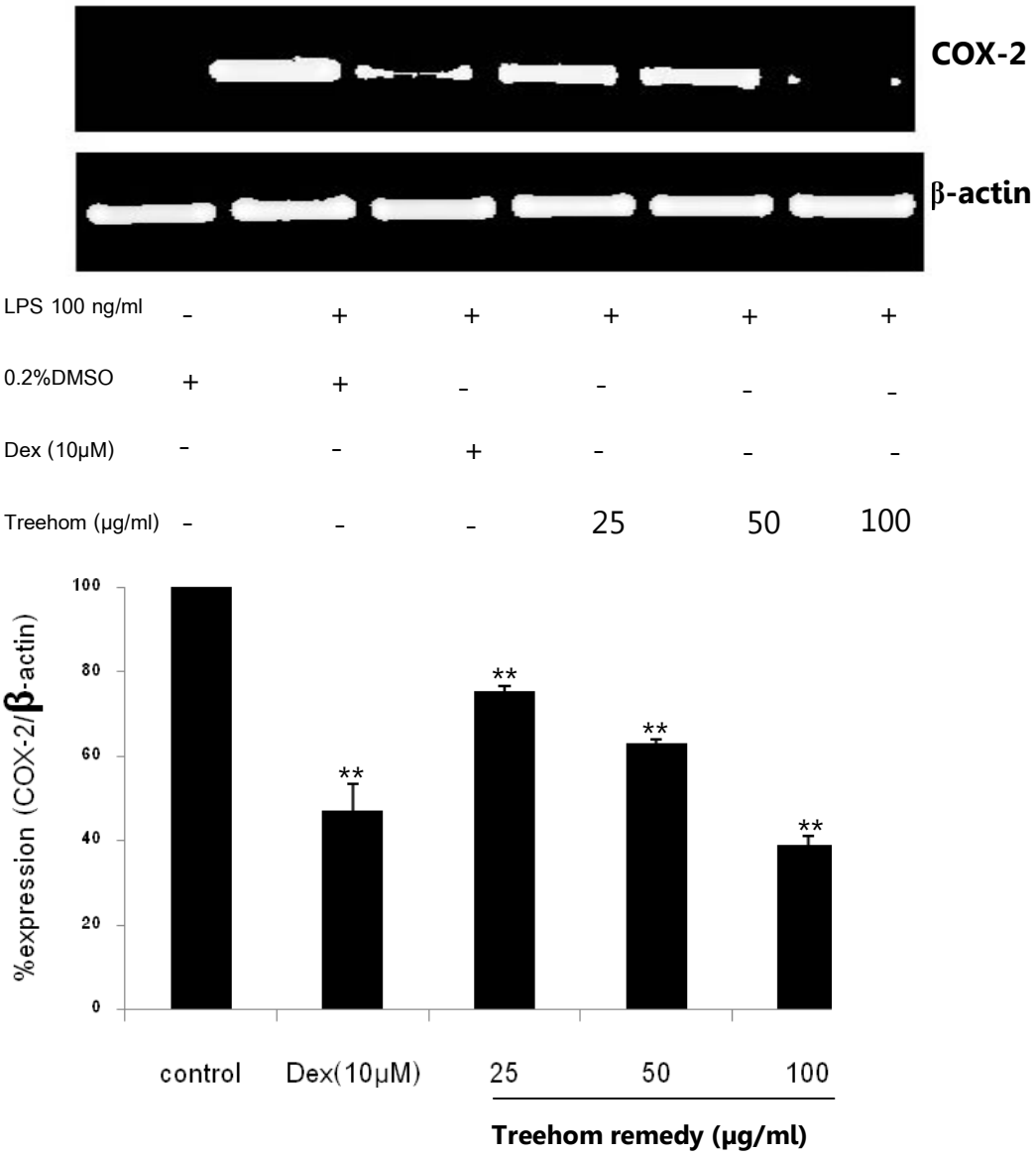
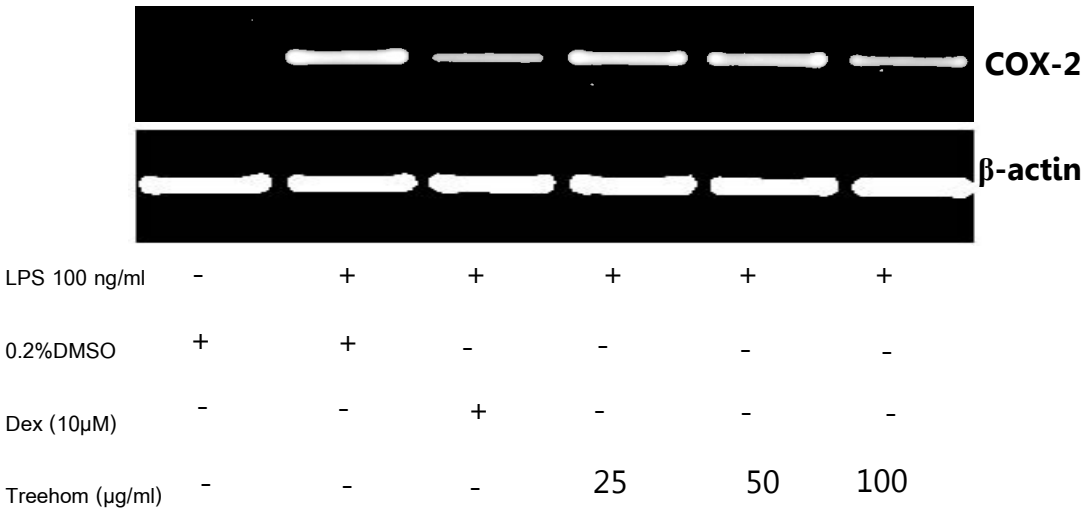


Figure 20: Effect of the ethanol extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of COX-2 .The data are express mean ± S.E. from 3 independent experiments (n=3). **P<0.05 compared to untreated LPS activation.



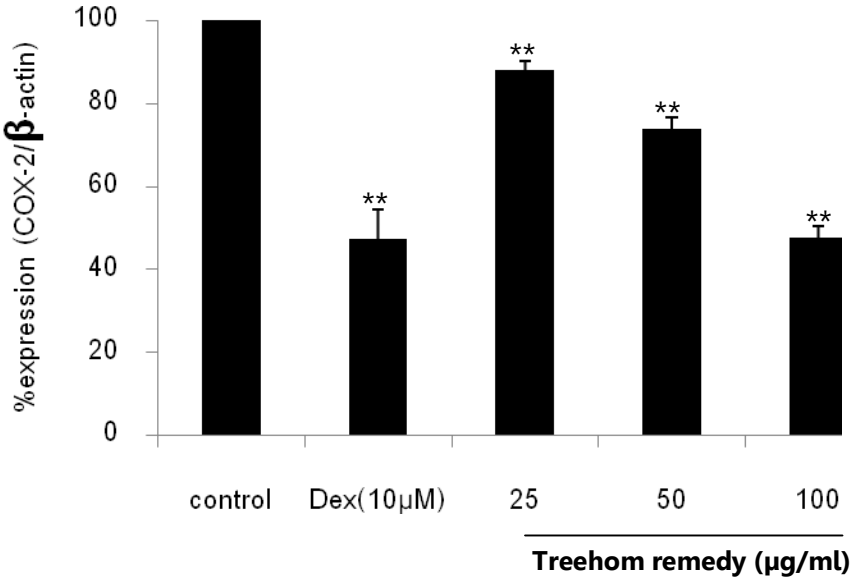


Figure21: Effect of the water extract from Treehom remedy at the concentrations 25-100 μg/ml on mRNA expression of COX-2 .The data are express mean ± S.E. from 3 independent experiments (n=3). **P<0.05 compared to untreated LPS activation.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion and conclusion

Inflammation is a physiologic response of innate immunity in response tissue and cells to protect against foreign substances. The signs of inflammation are pain, swelling, redness, hotness. It is well established that macrophages exhibit important role in inflammatory process. Activated macrophages secrete many inflammatory mediators and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 (IL-1) and the enzymes involved in inflammation as well. These enzymes are inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX). They are responsible for nitric oxide and prostaglandins generation. COXs exist in two isoforms; a constitutive form (COX-1) and an inducible isoforms (COX-2). During inflammation, COX-2 is selectively induced by proinflammatory cytokines at the site of inflammation. These mediators induce increased expression of many cellular adhesion molecules (CAMs) and immunoglobulin. Inflammatory reactions also induce the production of reactive oxygen species (ROS) by activated macrophages (Kehrer *et al*., 1993; Linton *et al*., 2003; Lee *et al*., 2009).

Treehom remedy is approved by the Ministry of Public Health of Thailand in National Drug List as the folk remedy for relieving of pyrexia and constipation in children. Several plants from this remedy exerted anti-inflammatory activity *in vivo* and *in vitro* (Table 6). From this present study, it was shown that the ethanol and water extracts (25-100µg/ml) were able to inhibit NO production from LPS-stimulated J774A.1 cells in dependent manner (Fig 11A,13A).This result was correlated with a decrease in iNOS mRNA expression from both extracts (Fig 18,19). During inflammatory process, leukocytes are migrated to site of inflammation and release a variety of cytokines and the enzymes involved in inflammation. NO is known as an important mediator in

inflammatory responses. NO works together with pro-inflammatory cytokines (TNF-α, IL-1, IL-6) resulting in increase iNOS expression in monocytes, macrophages, neutrophils. NO also upregulates leukocytes and endothelial adhesion molecules. In optimum concentration, NO possesses cytotoxic properties against pathogenic microbe in host defence mechanism and it also possesses damaging effects on host tissues in inflammatory response in high concentration. Decrease in iNOS mRNA expression is subsequent leading to inhibition of nitric oxide production. Decrease in NO production is capable of reduction of inflammation (Huang *et al.,* 2001; Bellows *et al.,* 2006; Kim *et al.,* 2011).

The anti-inflammation's molecular mechanism on NO inhibition of these extracts were further investigated by determination of mRNA expression of TNF -α, IL-1β, IL-6 ,COX-2 on LPS-activated macrophages using RT-PCR. Inhibition of mRNA expression of TNF -α, IL-1β, IL-6, COX-2 in difference of efficacy were found in both extracts at every concentration used (25-100μg/ml) (Fig 16, 17, 20, 21). In inflammatory response, TNF-α induces the recruitments of neutrophils and macrophages as well as the expression of adhesion molecules which cause the endothelial cell surface adhesive for leukocytes to migrate to the site inflammation. TNF-α stimulates endothelial cells and macrophages to produce chemokines, cytokines, as well as genotoxic molecules. NO and ROS cause DNA damage and mutations. TNF- α also induces increase synthesis of prostaglandins (PGE_2) and it acts on the hypothalamus resulting in increase body temperature. Furthermore, TNF-α and IL-1 share several pro-inflammatory properties, they induce adhesion molecules and interleukin 6. IL-6 is secreted into the blood circulation leading to acute phase response. IL-6 also induces increase production of PGE_2 from vascular endothelium of the hypothalamus and cause fever. Together TNF-α, IL-1β and IL-6 play important roles in the pathophysiology of inflammation and they subsequent lead to pain, fever, redness and swelling. Cyclooxygenase-2 (COX-2) enzyme is responsible for prostaglandins generation in response to inflammation, tumor,

shock etc. PGE_2 and $PGF_{2\alpha}$ facilitate inflammatory process as they greatly contribute to vasodilation, increases vascular permeability and edema. Thus decrease in proinflammatory cytokines mRNA and COX-2 expression facilitates anti-inflammation. The inhibitory action on iNOS, COX-2, proinflammatory cytokines mRNA expression in macrophage by LPS is under the control of many transcription factors including NF-kB, AP-1. (Hong *et al.,* 2008)

In the final step of phagocytosis, the pathogen is degraded in phagolysosome through oxygen-dependent or oxygen-independent mechanism. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase converts molecular oxygen (O_2) to reactive oxygen intermidiates (ROIs), superoxide anion (O_2^-) and free radicals, which are toxic to the ingested microbe in oxygen-dependent mechanism.

Antioxidant activity of the extracts from Treehom remedy were evaluated by FRAP assay. The ethanol and water extracts at 25-100 µg/ml showed high antioxidant activity and medium antioxidant activity were found at 12.5,6.25 µg/ml (Fig 16A). High level of ROS induce cellular damage by disturbing cellular structure including lipids, proteins and nucleic acids leading to physiological dysfunction and cell death. The antioxidant activity of both extracts may alleviate inflammation.

The result from the present study also demonstrated that there was no statistically significant difference between the ethanol and water extracts of Treehom remedy in the inhibition of NO production (IC_{50} of the ethanol and water extracts were 40.05 and 60.05 µg/ml respectively), iNOS mRNA expression (IC_{50} 68.65 and 77.44 µg/ml), COX-2 mRNA expression (IC_{50} 80.87 and 95.23 µg/ml), TNF-α mRNA expression (IC_{50} 89.58 and 111.78 µg/ml), IL-1β mRNA expression (IC_{50} 21.60 and 36.53 µg/ml), IL-6 mRNA expression (IC_{50} 36.52 and 21.02 µg/ml) (Appendix B-5, 18-20, 29, 38). The explanation is based on phytochemical constituents relating to their polarity. Both extracts may contain the same category of phytochemical constituents with difference polarity so they exert similar anti-inflammatory action in LPS-stimulated macrophages.

The ancient people believed that combination of phytomedicines causes synergism effect among the components present in it and also decrease its side effect. Treehom remedy is employed for relieving of pyrexia and constipation in children. It composes of *Terminalia* sp., *Terminalia bellirica* Roxb., *Phyllanthus emblica* L., *Coriandrum sativum* Linn., *Aristolochia* sp., *Angelica dahurica* Benth., *Glycyrrhiza glabra* L., Sodium borate, *Trigonella foenum-graecum* L., *Terminalia chebula* Retz., *Rheum officinale* Baill. As far as we known, several plants of this remedy showed the anti-inflammatory and antioxidant effect (Table 6). Fever is one of the inflammatory signs. The present result supports the anti-inflammatory potential of this remedy.

In conclusion, we reported the anti-inflammatory potential of the water and ethanol extract of Treehom remedy since they decreased NO production, iNOS, COX-2, IL-6, IL-1β, TNF-α mRNA enzyme expression on LPS-activated J774A.1 macrophages. It also possessed antioxidant activity by FRAP assay. Further investigations are needed to clarify the mechanism underlying all of these activities for provision of more evidences supporting their anti-inflammatory activity.

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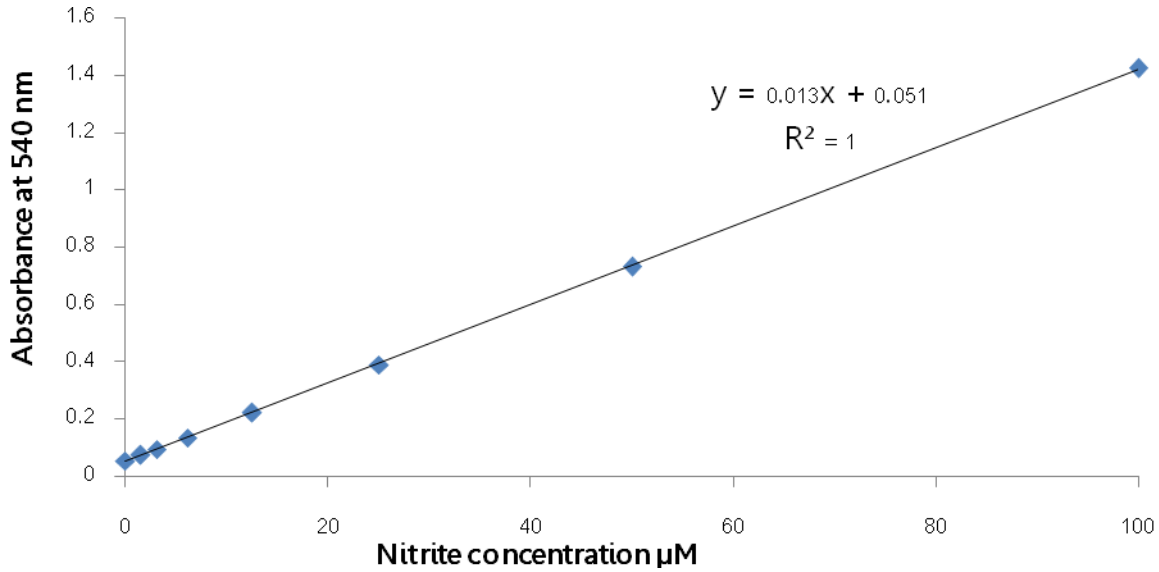
Appendix

Appendix

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

Concentration (μM)	Absorbance at 540 nm		
	1	2	Mean
100	1.484	1.368	1.426
50	0.699	0.765	0.732
25	0.372	0.41	0.391
12.5	0.211	0.233	0.222
6.125	0.133	0.14	0.1365
3.063	0.091	0.098	0.0945
1.531	0.073	0.075	0.074
0	0.052	0.057	0.0545



Appendix -2: Linearity of nitrite standard calibration line.

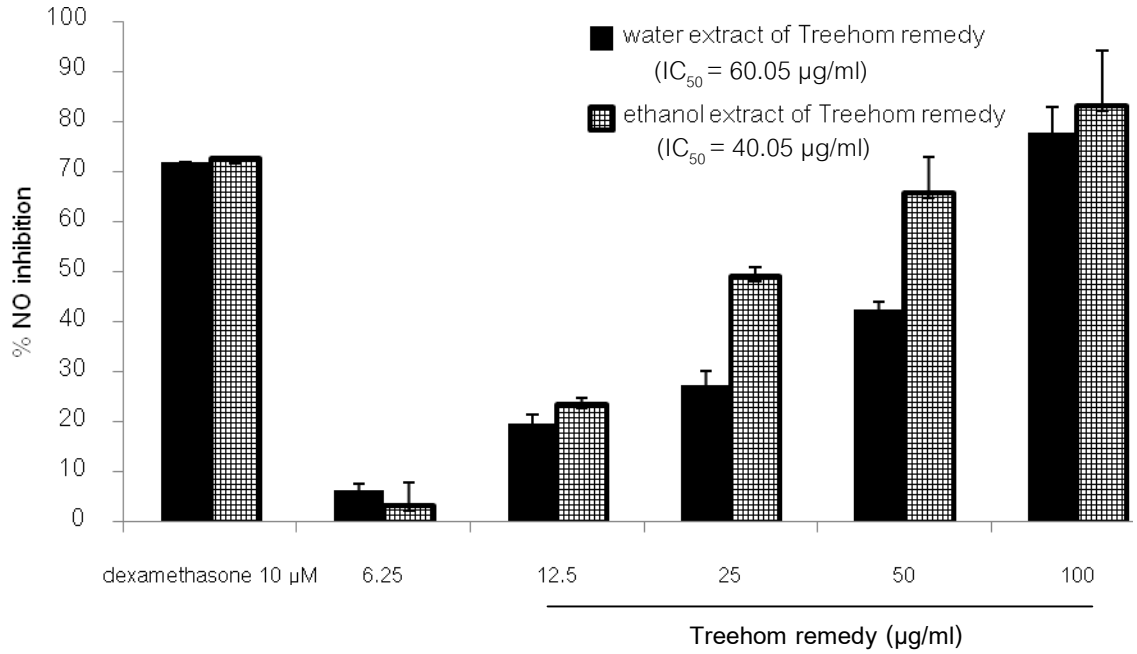
75

Appendix 3: The effect of ethanol extract from Treehom remedy on nitric oxide production in LPS stimulated- J774A.1 macrophages.

Test compounds	% Inhibition NO				mean ± SE
	1	2	3	mean	
0.2%DMSO	1.02	-0.04	-0.02	0.32	0.32 ± 0.35
Dexamethasone(10μM)	83.29	97.82	96.79	92.63	92.63 ± 4.68
Treehom 6.25	5.01	3.59	1.1	3.23	3.23 ± 1.14
Treehom 12.5	21.83	27.12	21.96	23.64	23.64 ± 1.74
Treehom 25	35.4	58.58	53.35	49.11	49.11 ± 7.01
Treehom 50	43.66	80.08	73.76	65.83	65.83 ± 11.23
Treehom 100	82.09	83.02	84.17	83.09	83.09 ± 5.11

Appendix 4: The effect of water extract from Treehom remedy on nitric oxide production in LPS stimulated- J774A.1 macrophages.

Test compounds	% Inhibition NO				mean ± SE
	1	2	3	mean	
Media	0.01	0.01	0	0.0033	0.0033 ± 0.0033
Dexamethasone(10μM)	85.74	83.57	81.32	85.54	85.54 ± 1.27
Treehom 6.25	2.88	8.43	9	6.77	6.77 ± 1.95
Treehom 12.5	19.15	24.55	15.1	19.6	19.6 ± 2.73
Treehom 25	30.17	25.28	26.91	27.45	27.45 ± 1.43
Treehom 50	43.17	33.24	50.68	42.53	42.53 ± 5.06
Treehom 100	81.73	73.02	78.8	77.85	77.85 ± 2.55



Appendix -5: Effect of the water extract compare with ethanol extracts on NO production in LPS-stimulated J774A.1 cells.

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Appendix -6: Data of cytotoxicity of the ethanol extract from Treehom remedy.

Test compounds	% Cytotoxicity				% Viability	% Viability ± SE
	1	2	3	mean		
2%DMSO	0	-0.325	-0.02	-0.29	100	100±0.15
Dexamethasone(10μM)	0.621	-0.7	-1.225	-0.43	100	100±0.54
Treehom 6.25	-0.944	-5.975	-6.3	-4.4	100	100±1.73
Treehom 12.5	-0.87	-1.325	-0.875	-1.02	100	100±0.15
Treehom 25	-1.913	-3.575	-5.5	-3.66	100	100±1.03
Treehom 50	-3.528	-5.5	-4.95	-4.65	100	100±0.58
Treehom 100	4.522	5.5	5.8	5.27	94.73	94.73±0.38

Appendix -7: Data of cytotoxicity of the water extract from Treehom remedy.

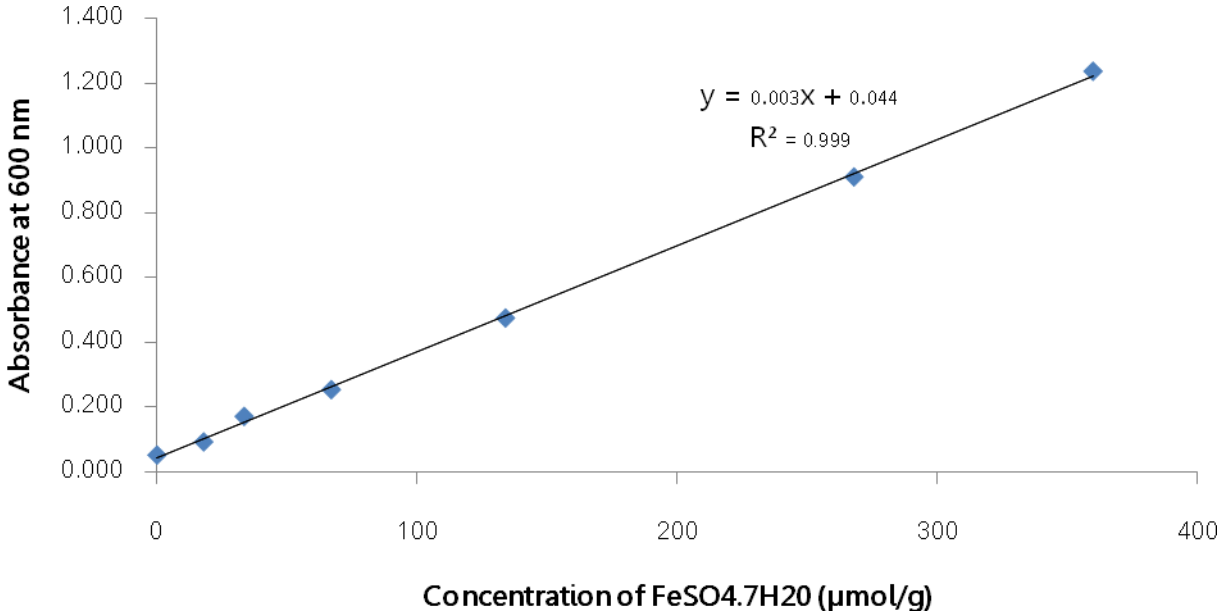
Test compounds	% Cytotoxicity				% Viability	% Viability ± SE
	1	2	3	mean		
Media	0.01	0.01	0	-0.15	100	100 ± 0.73
Dexamethasone(10μM)	85.74	83.57	81.32	-4.84	100	100 ± 4.04
Treehom 6.25	2.88	8.43	9	-4.56	100	100 ± 2.99
Treehom 12.5	19.15	24.55	15.1	-3.58	100	100 ± 3.25
Treehom 25	30.17	25.28	26.91	-3.73	100	100 ± 3.98
Treehom 50	43.17	33.24	50.68	-2.17	100	100 ± 4.45
Treehom 100	81.73	73.02	78.8	-3.03	100	100 ± 2.68

80

Appendix -8: Data for linearity of ferrous standard calibration line.

[$FeSO4.7H_2O$(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

81



Appendix -9: Linearity of ferrous standard calibration line.

82

Appendix -10: Effects of the ethanol extract from Treehom remedy on antioxidant activity by FRAP assay.

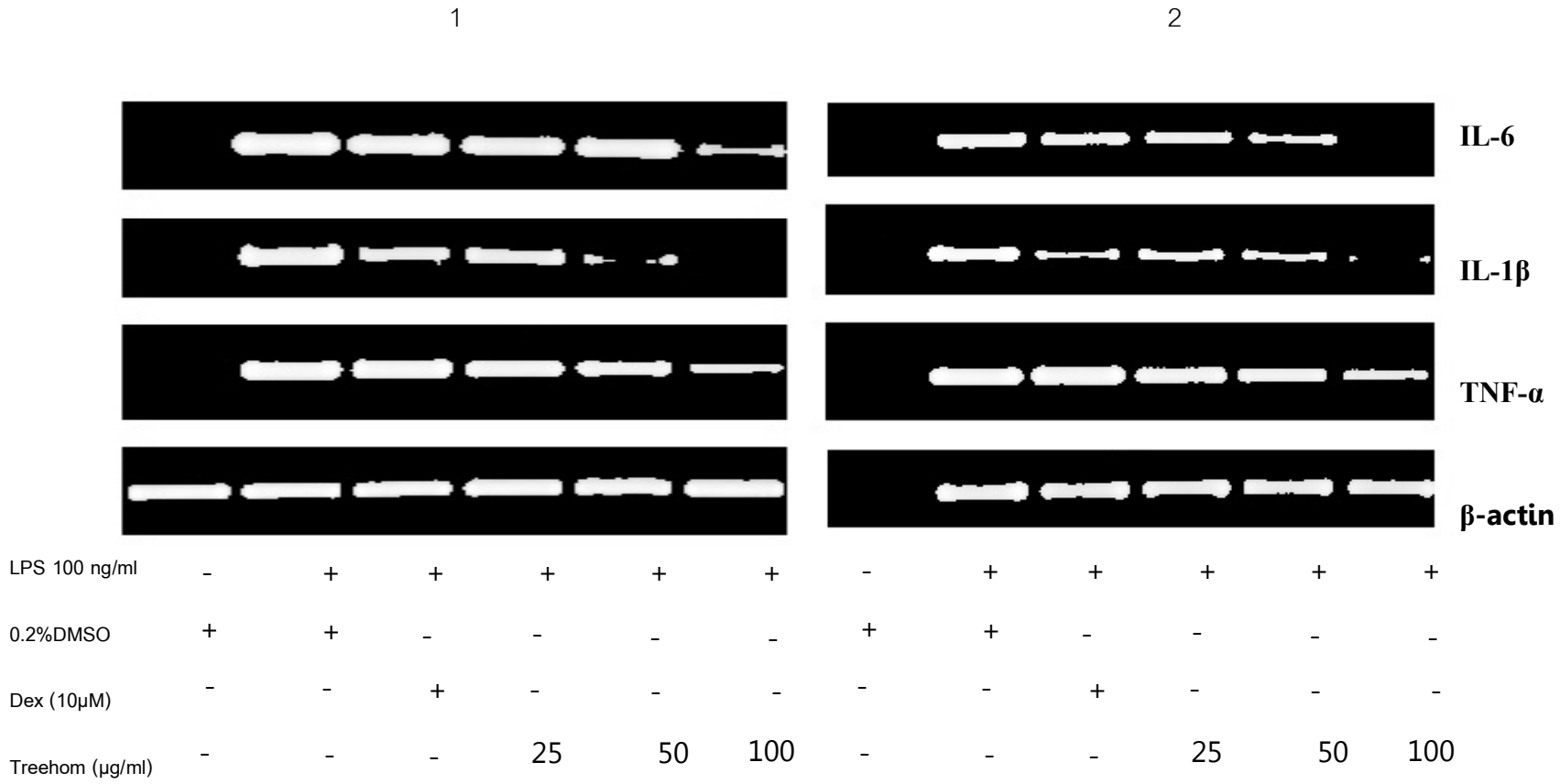
Test compound	[$FeSO_4.7H_2O$ (µmol/g)]			mean ± SE	Antioxidant activity
	1	2	3		
0.2% DMSO	-5.83	-7.50	-7.33	-7.42 ±0.53	
vitamin c 100µM	780.50	806.83	894.67	850.75 ±34.51	extremely high
Treehom 6.25	35.00	33.33	35.17	34.25 ± 0.58	medium
Treehom 12.5	73.50	66.50	67.58	67.04 ± 2.17	medium
Treehom 25	137.83	124.67	122.17	123.42 ± 5.76	high
Treehom 50	251.83	247.67	229.67	238.67 ±6.8	high
Treehom 100	447.33	456.17	423.67	439.92 ±9.7	high

83

Appendix -11: Effects of the water extract from Treehom remedy on antioxidant activity by FRAP assay.

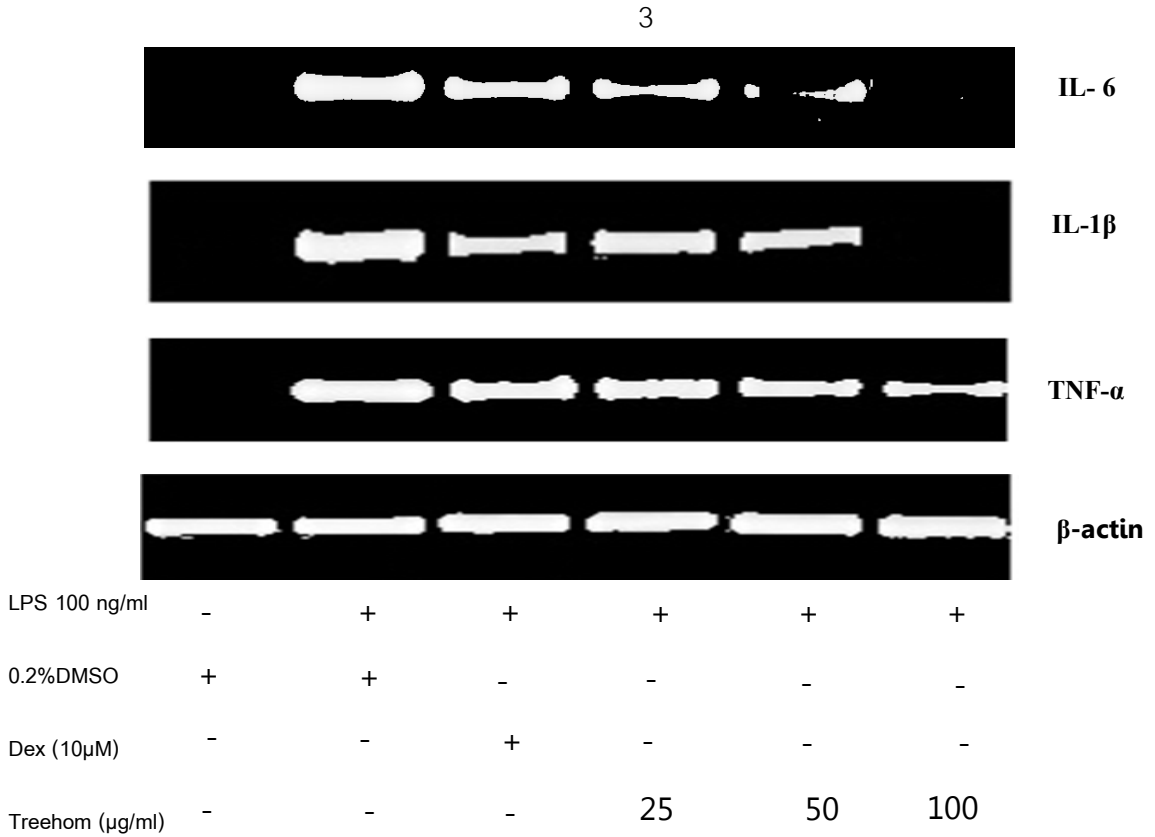
Test compound	[$FeSO_4.7H_2O$ (µmol/g)]			mean± SE	Antioxidant activities
	1	2	3		
water	-5.83	-6.17	-6.17	-7.42 ±0.113	
vitamin c 100µM	824.67	875.00	867.50	871.25 ±15.67	extremely high
Treehom 6.25	32.83	32.83	31.67	32.25 ± 0.38	medium
Treehom 12.5	69.00	68.83	68.00	68.42 ± 0.30	medium
Treehom 25	134.33	133.00	128.67	130.83 ± 1.70	high
Treehom 50	243.67	241.83	234.17	283.00 ±2.90	high
Treehom 100	415.83	418.00	417.33	417.67 ±0.64	high

84



Appendix -12: Effects of the ethanol extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

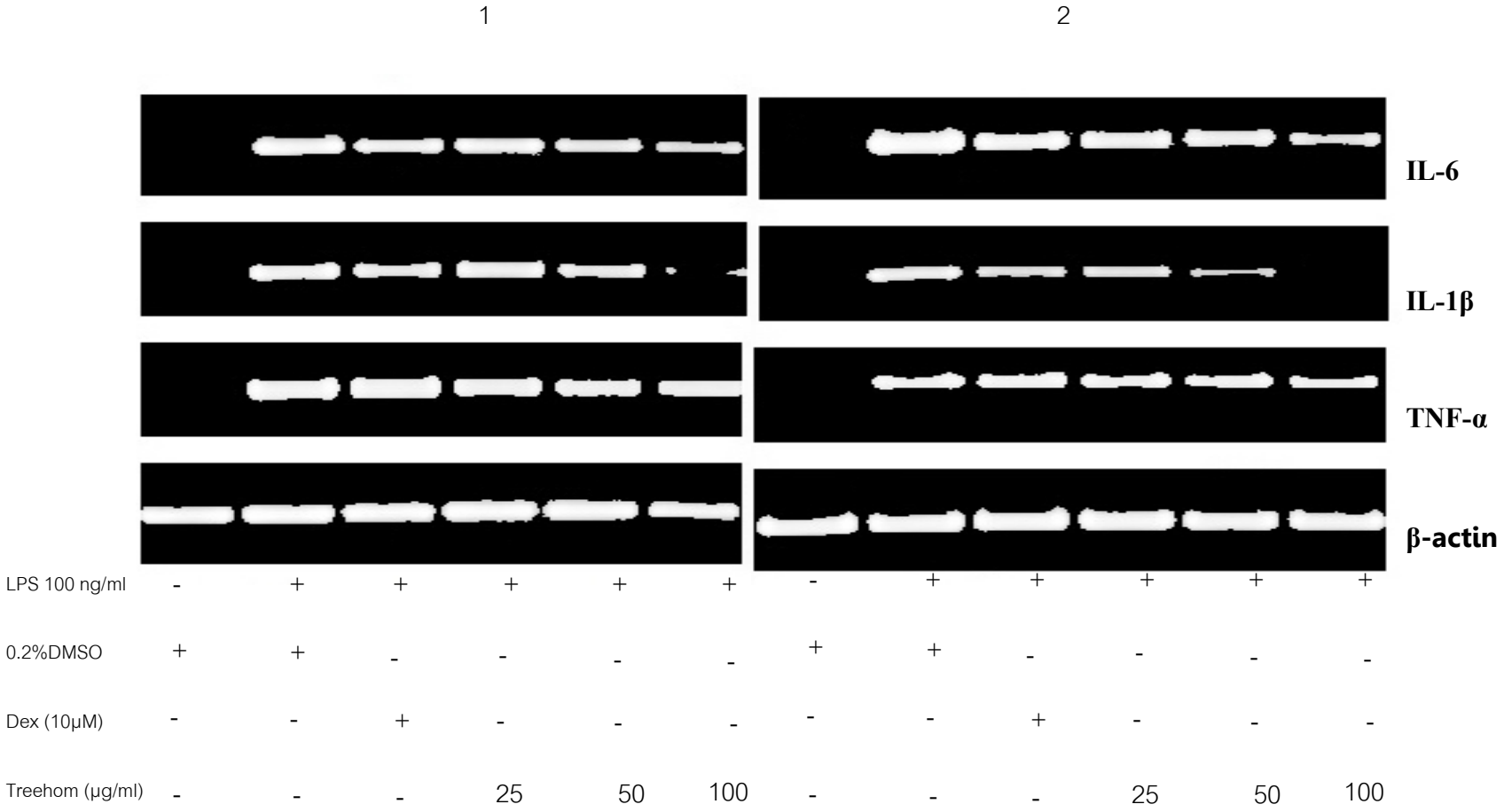
85



Appendix -13: Effects of the ethanol extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

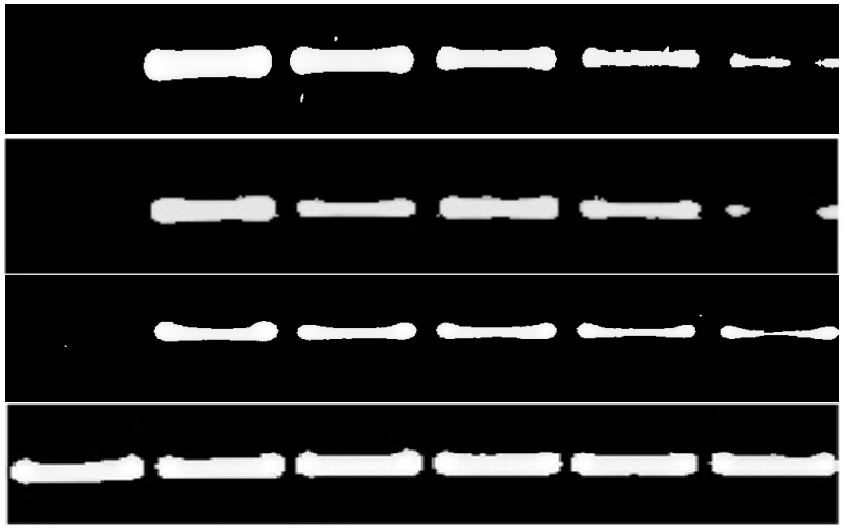
Appendix -14: Effects of the ethanol extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

Test compounds	% of control IL-6			mean±SE	% of control IL-1β			mean±SE	% of control TNF-α			mean±SE
	1	2	3		1	2	3		1	2	3	
0.2%DMSO	0.00	0.00	0.00	0±0.00	0.00	0.00	0.00	0±0.00	0.00	0.00	0.00	0±0.00
0.2%DMSO+LPS	100.00	100.00	100.00	100±0.00	100.00	100.00	100.00	100±0.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	63.51	44.50	55.54	54.52±2.26	36.18	23.50	32.43	30.7±3.76	58.50	59.40	60.45	59.45±4.73
25μg/ml+LPS	58.50	48.52	58.25	55.09±3.28	45.11	38.33	42.95	42.13±1.99	61.00	90.75	73.89	75.21±8.61
50μg/ml+LPS	54.37	35.96	44.36	44.89±5.32	9.90	20.32	28.39	19.53±5.35	53.30	65.21	55.17	57.9±3.69
100μg/ml+LPS	5.17	8.82	2.85	5.61±1.7	2.08	2.28	8.47	4.28±2.09	21.90	44.73	45.57	37.4±7.75



Appendix 15: Effects of the water extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

3



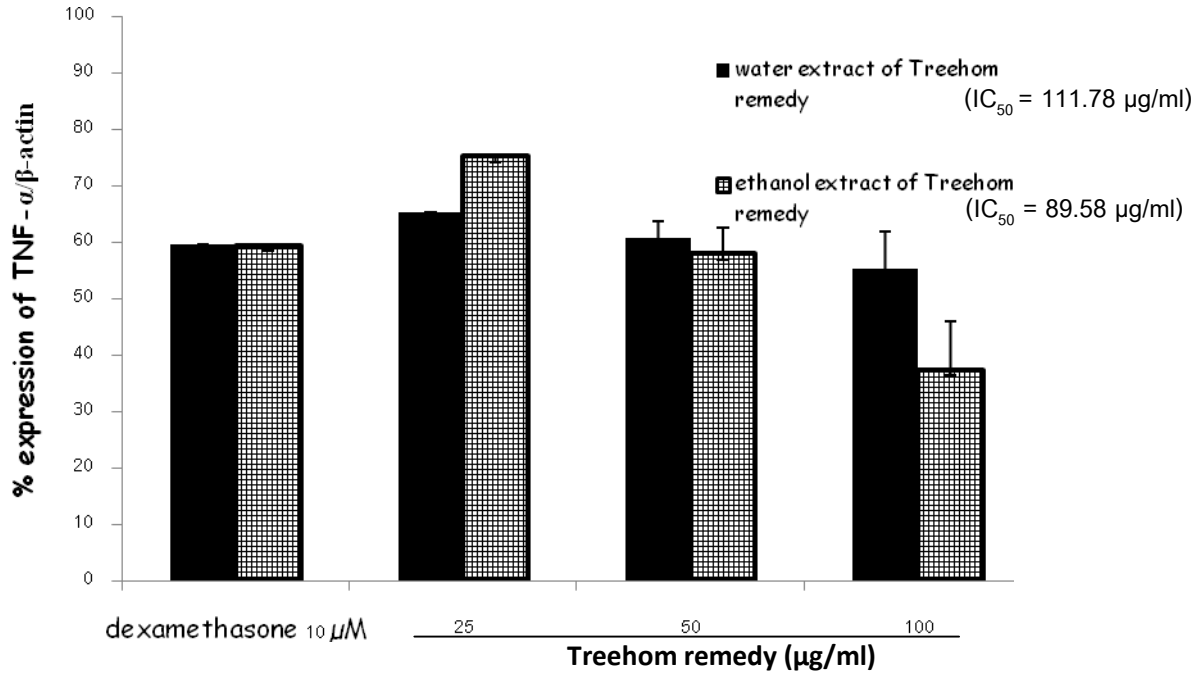
LPS 100 ng/ml	-	+	+	+	+	+
0.2%DMSO	+	+	-	-	-	-
Dex (10μM)	-	-	+	-	-	-
Treehom (μg/ml)	-	-	-	25	50	100

Appendix 16: Effects of the water extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

Appendix 17: Effects of the water extract from Treehom remedy on mRNA expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated J774A.1 cells.

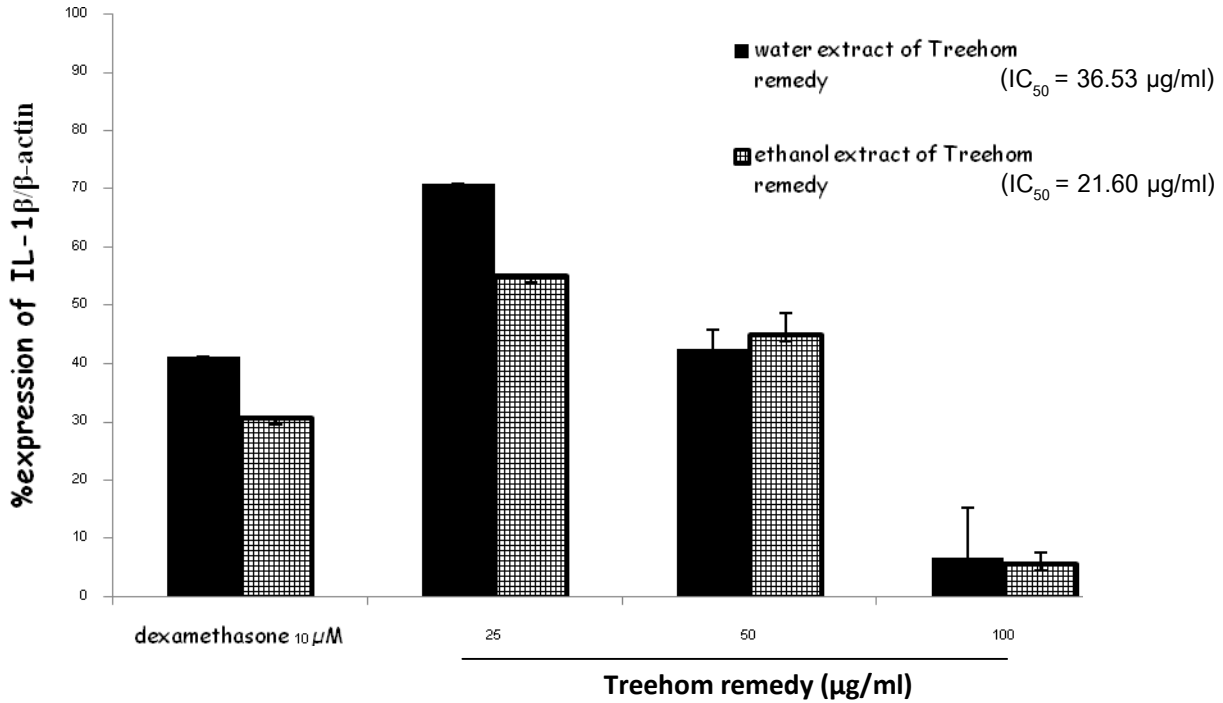
Test compounds	% of control IL-6			mean±SE	% of control IL-1β			mean±SE	% of control TNF-α			mean±SE
	1	2	3		1	2	3		1	2	3	
Media	0.00	0.00	0.00	0±0.00	0.00	0.00	0.00	0±0.00	0.00	0.00	0.00	0±0.00
Media+LPS	100.00	100.00	100.00	100±0.00	100.00	100.00	100.00	100±0.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	54.76	55.55	54.96	55.09±0.23	40.36	31.54	52.08	41.32±3.31	57.08	57.27	64.17	59.50±2.79
25μg/ml+LPS	51.23	54.25	50.42	51.96±0.63	82.59	54.26	76.28	71.04±8.58	60.92	81.89	52.92	65.24±6.63
50μg/ml+LPS	35.19	32.97	23.70	30.62±3.51	48.50	23.88	55.16	42.51±9.51	49.99	81.77	50.70	60.81±7.97
100μg/ml+LPS	16.21	11.11	17.27	14.87±1.9	6.10	2.51	11.79	6.80±2.7	47.91	70.36	47.54	55.27±6.54

90



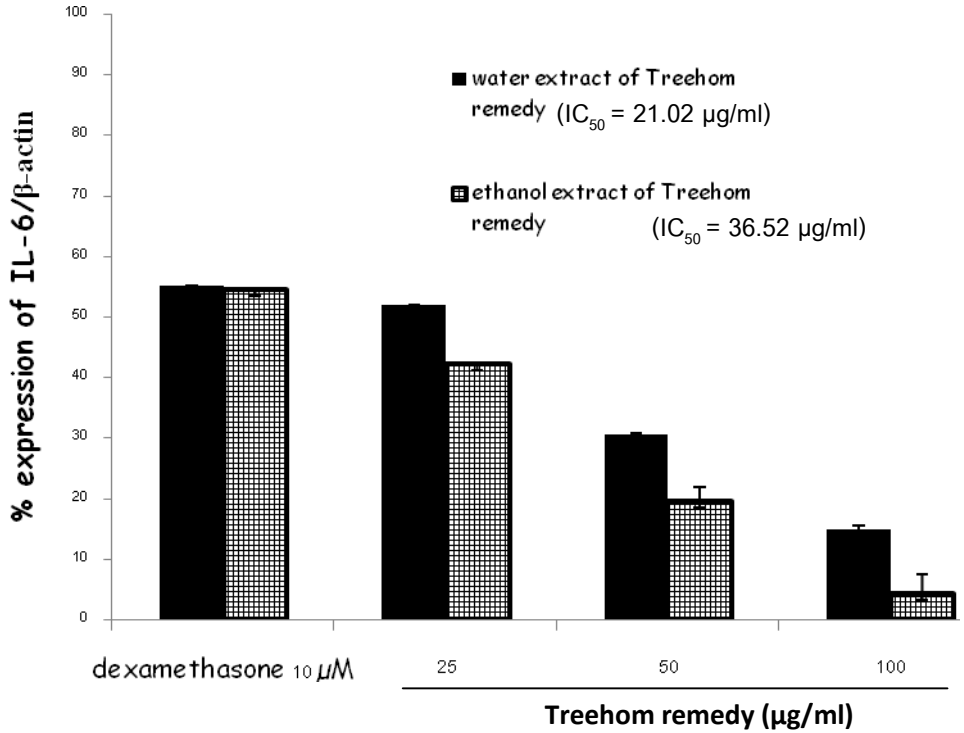
Appendix 18: Effect of the water extract compare with ethanol extracts on mRNA expression of TNF-α in LPS-stimulated J774A.1 cells.

91



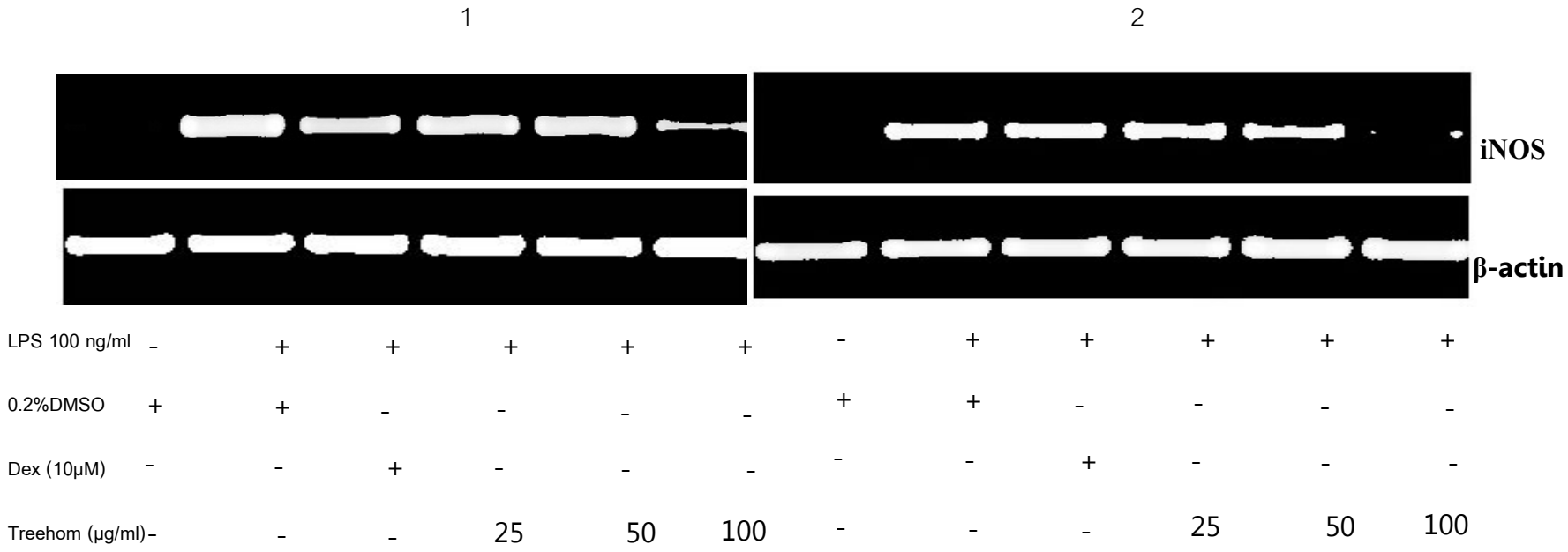
Appendix 19: Effect of the water extract compare with ethanol extracts on mRNA expression of IL-1β in LPS-stimulated J774A.1 cells.

92



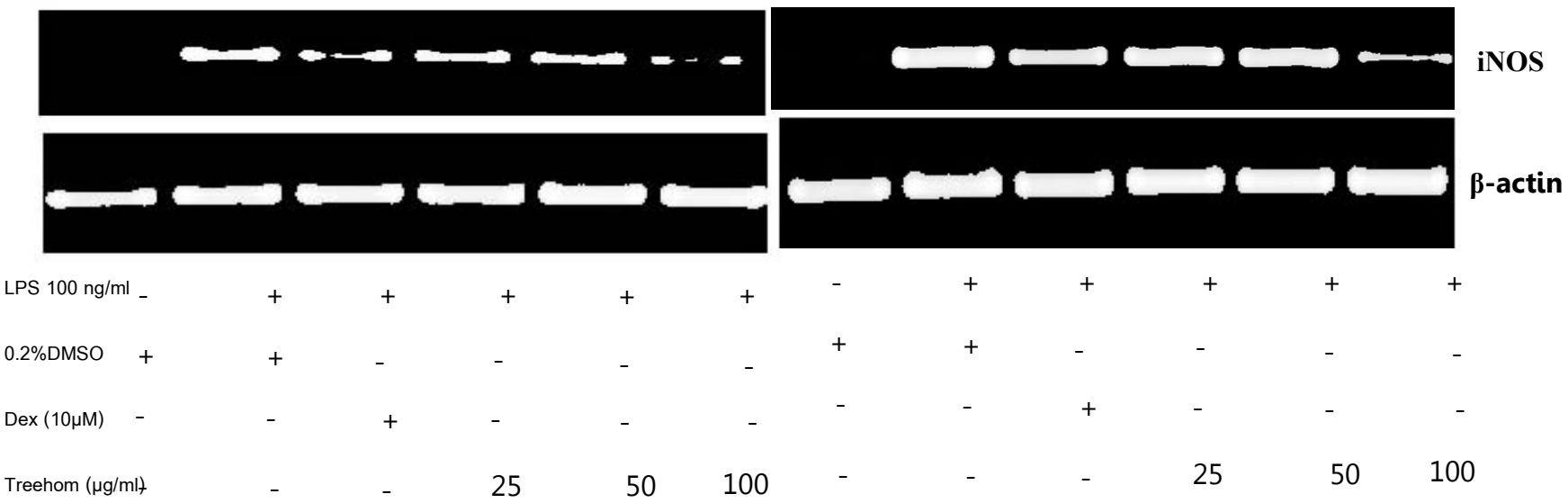
Appendix 20: Effect of the water extract compare with ethanol extracts on mRNA expression of IL-6 in LPS-stimulated J774A.1 cells.

93

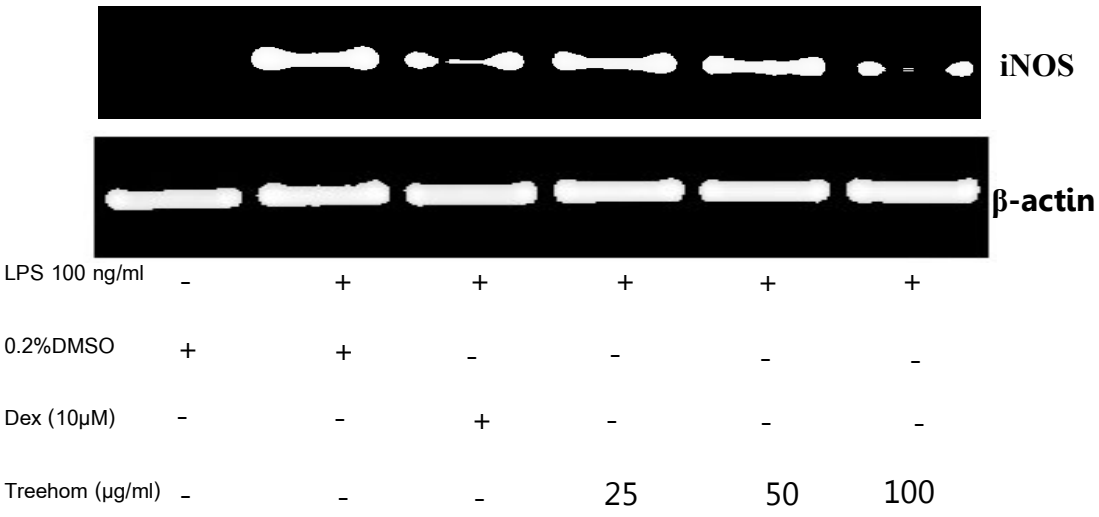


Appendix 21: Effects of the ethanol extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

94



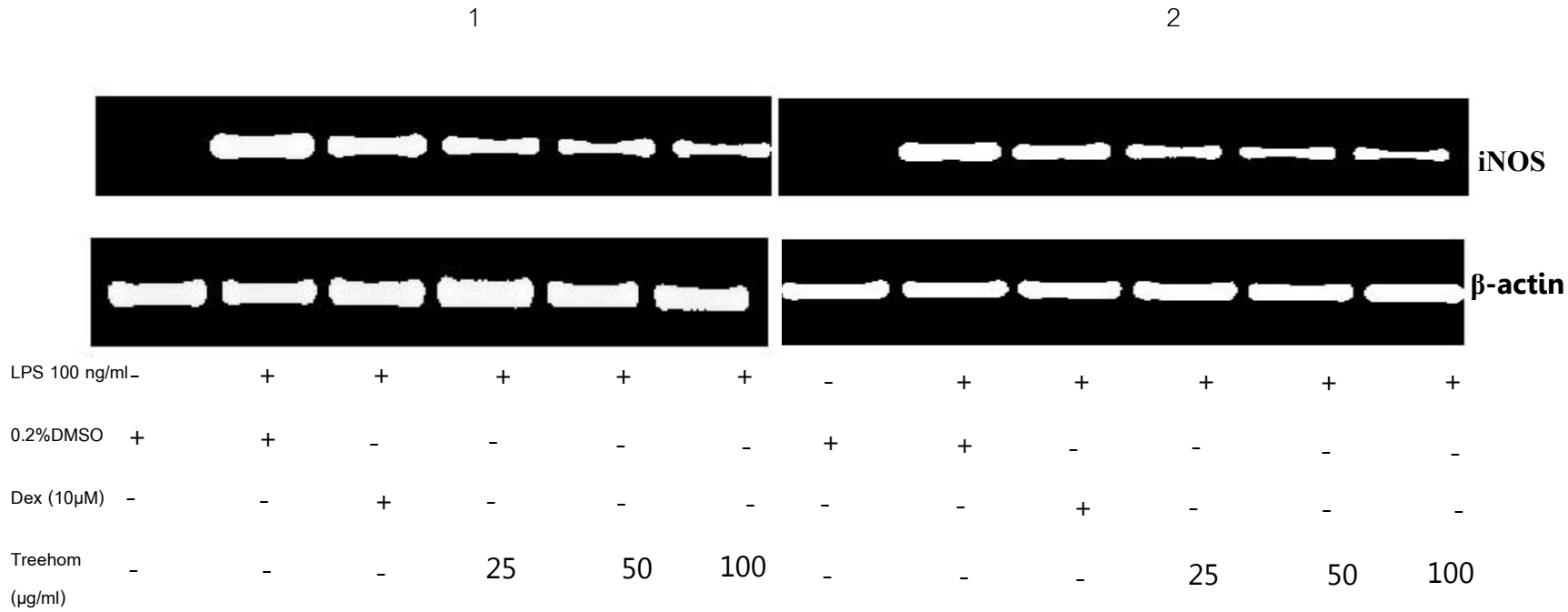
Appendix 22: Effects of the ethanol extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.



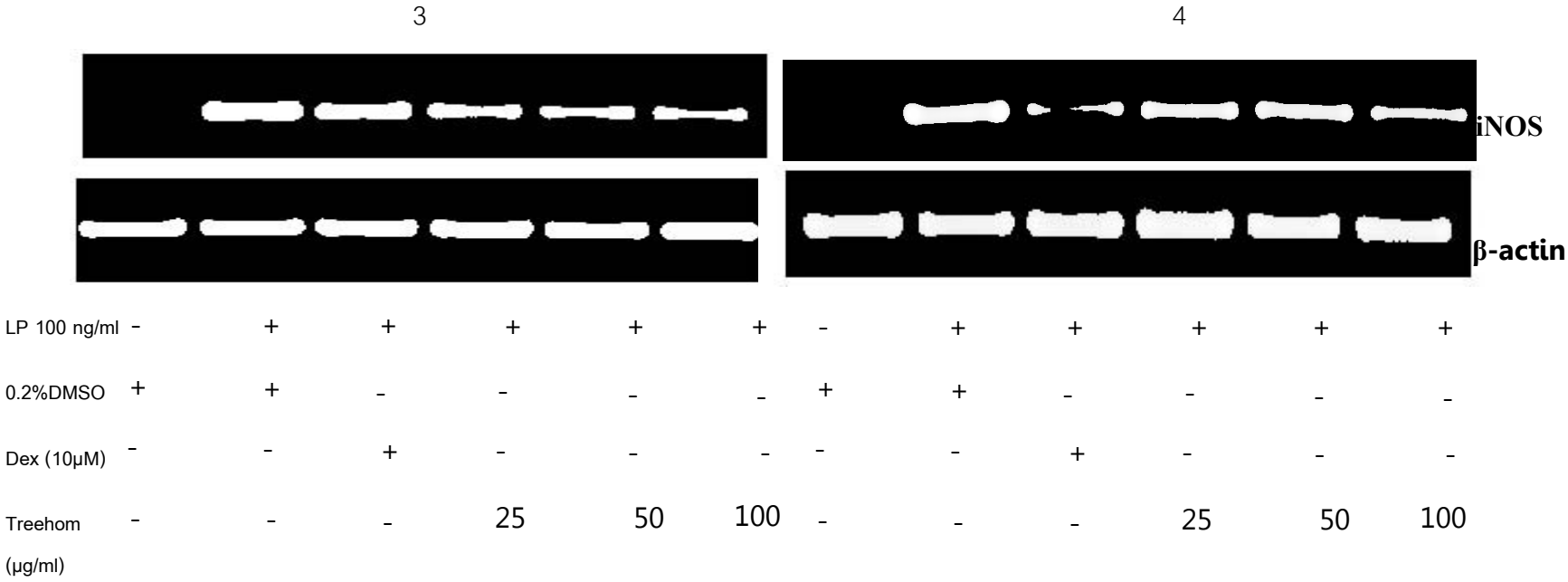
Appendix 23: Effects of the ethanol extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

Appendix 24: Effects of the ethanol extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

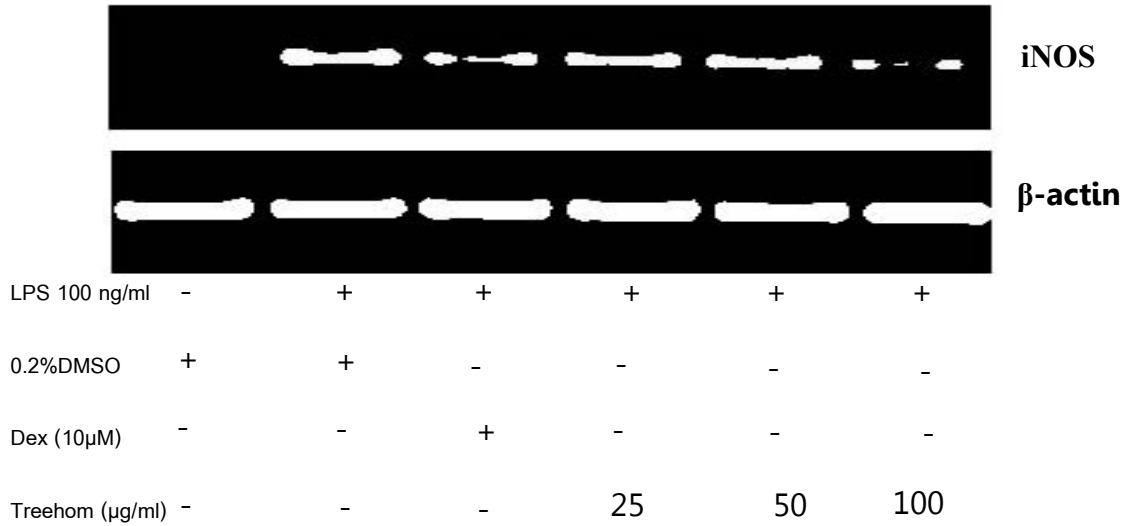
Test compounds	% of control iNOS					mean±SE
	1	2	3	4	5	
0.2%DMSO	0.00	0.00	0.00	0.00	0.00	0±0.00
0.2%DMSO+LPS	100.00	100.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	59.59	62.42	43.84	30.33	35.93	43.13±6.34
25μg/ml+LPS	61.23	65.35	77.86	74.80	77.18	73.8±3.36
50μg/ml+LPS	48.72	55.77	69.23	66.62	62.68	63.58±3.73
100μg/ml+LPS	25.04	34.90	37.07	25.84	25.06	30.72±2.64



Appendix 25: Effects of the water extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.



Appendix 26: Effects of the water extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.



Appendix 27: Effects of the water extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

Appendix 28: Effects of the water extract from Treehom remedy on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

Test compounds	% of control iNOS					mean±SE
	1	2	3	4	5	
Media	0.00	0.00	0.00	0.00	0.00	0±0.00
Media+LPS	100.00	100.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	59.86	63.08	68.28	27.12	30.04	47.13±8.72
25μg/ml+LPS	59.48	74.17	79.26	78.94	78.01	77.6±3.73
50μg/ml+LPS	50.25	68.38	53.53	65.08	66.89	63.47±3.72
100μg/ml+LPS	40.98	36.43	43.59	37.51	39.67	39.3±1.26

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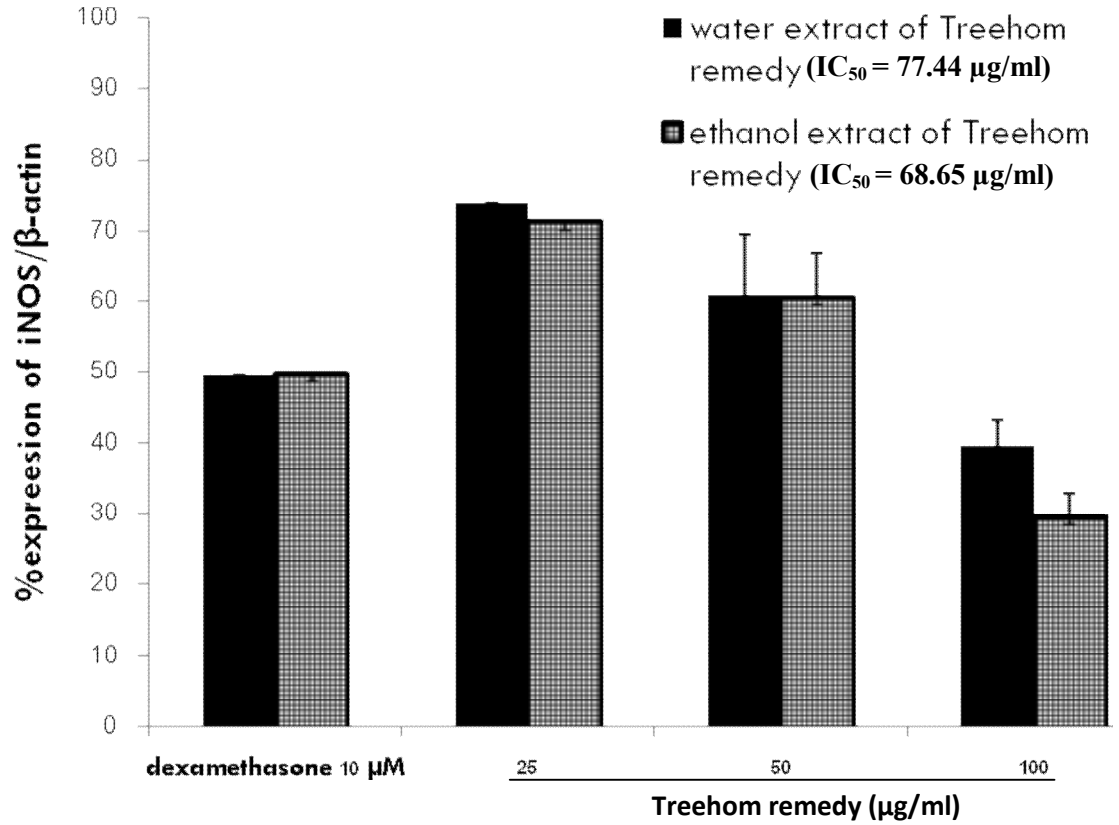

Appendix 29: Effect of the water extract compare with ethanol extracts on mRNA expression of iNOS in LPS-stimulated J774A.1 cells.

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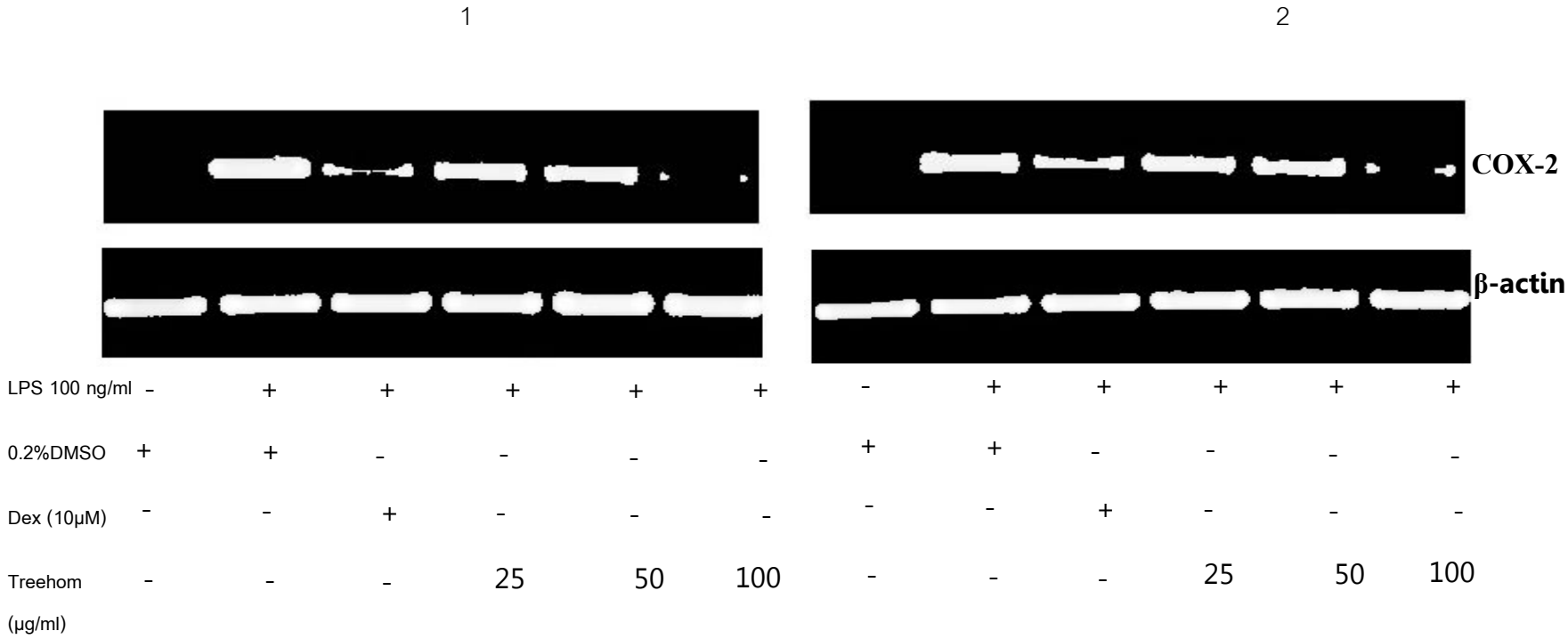

Appendix 30: Effects of the ethanol extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

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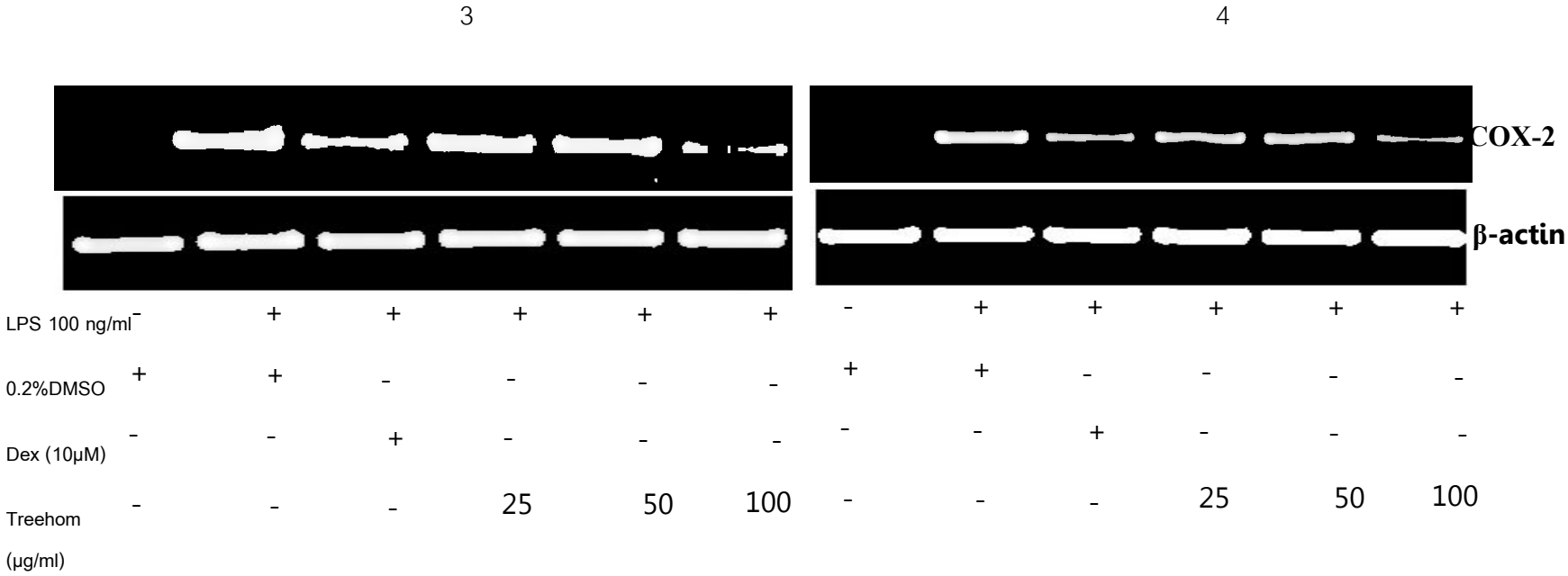
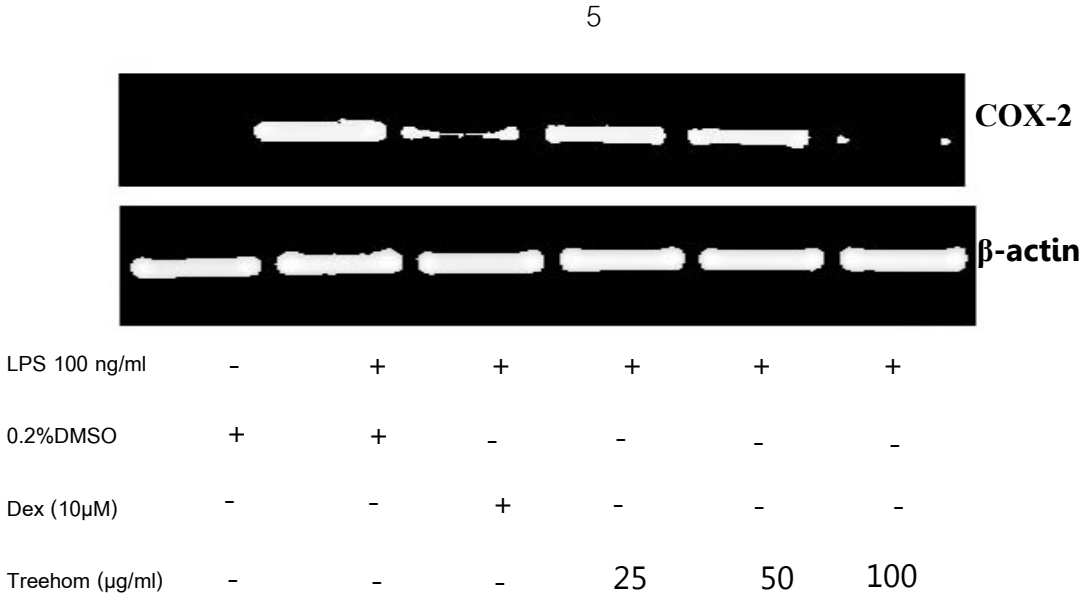


Appendix 31: Effects of the ethanol extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.



Appendix 32: Effects of the ethanol extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

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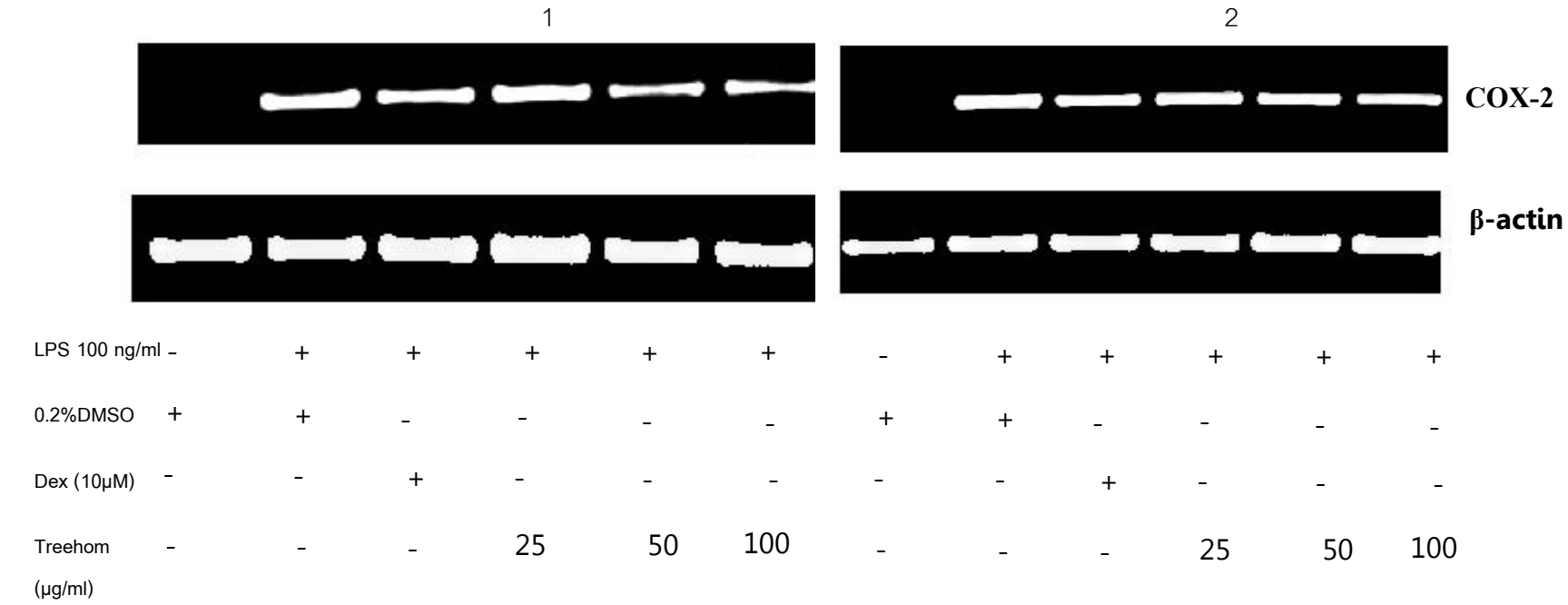
Appendix 33: Effects of the ethanol extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

Test compounds	% of control COX-2					mean±SE
	1	2	3	4	5	
0.2%DMSO	0.00	0.00	0.00	0.00	0.00	0±0.00
0.2%DMSO+LPS	100.00	100.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	60.26	56.92	62.39	35.54	34.08	47.23±6.2
25μg/ml+LPS	76.10	76.40	79.24	74.39	72.20	75.56±1.16
50μg/ml+LPS	66.17	63.43	64.38	62.95	61.49	63.06±0.77
100μg/ml+LPS	35.32	37.91	45.59	36.80	35.99	39.07±1.86



Appendix 34: Effects of the water extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

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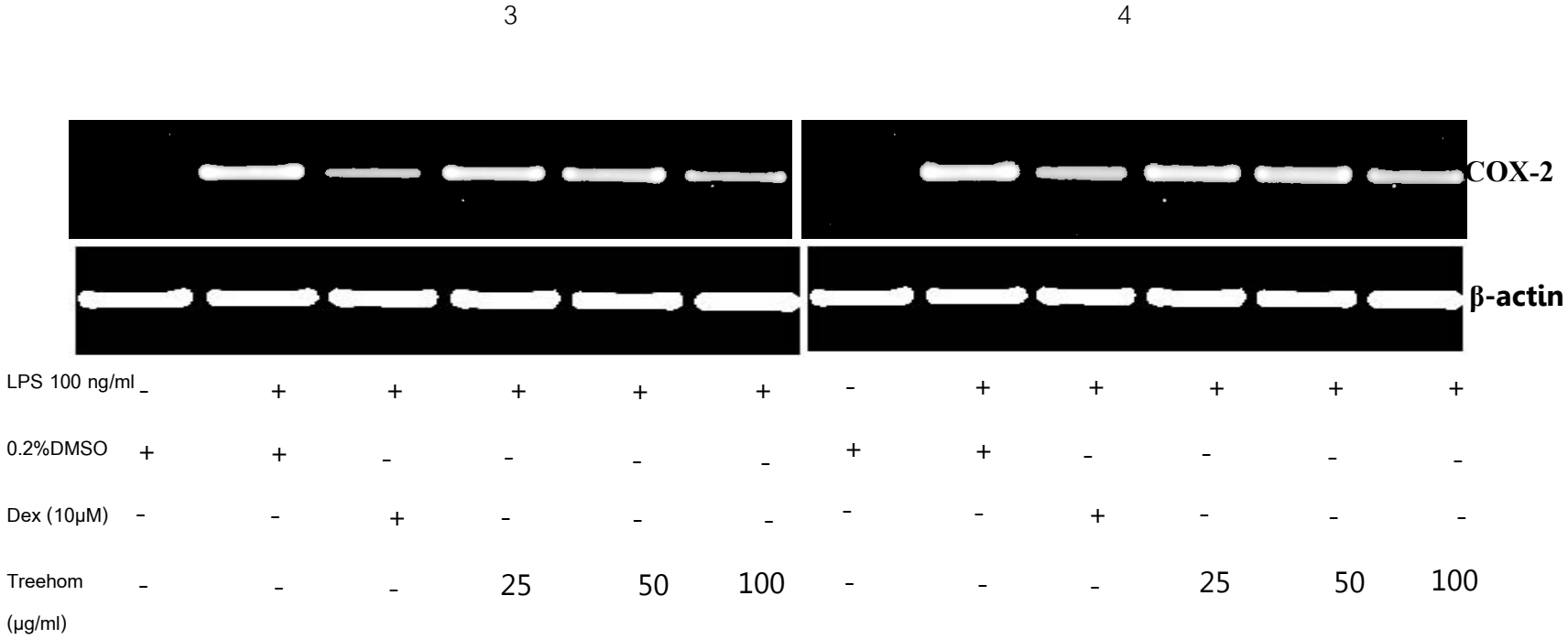

Appendix 35: Effects of the water extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

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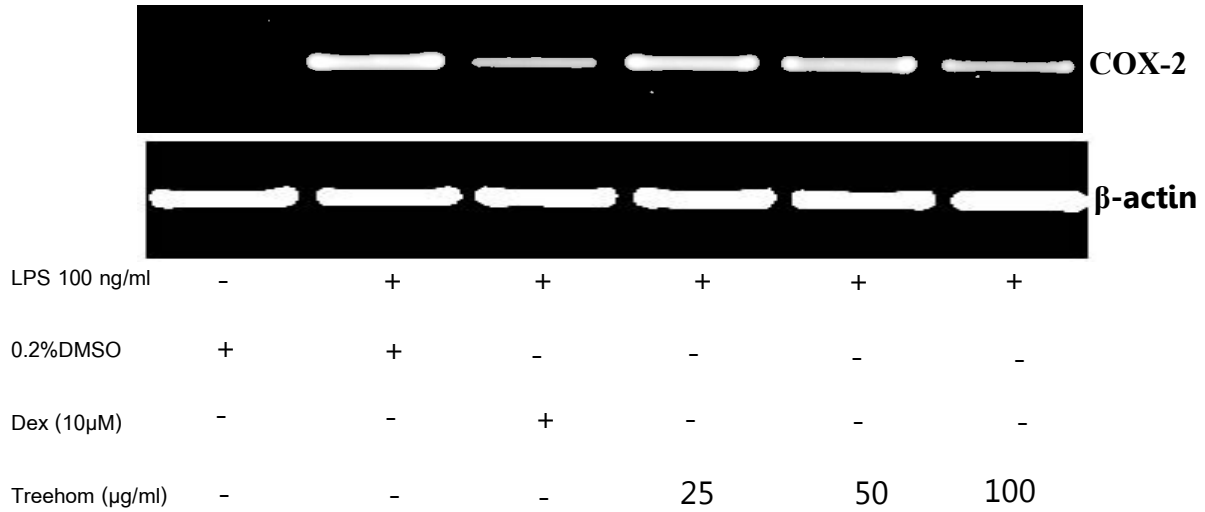

Appendix 36: Effects of the water extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

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Appendix 37: Effects of the water extract from Treehom remedy on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

Test compounds	% of control COX-2					mean±SE
	1	2	3	4	5	
Media	0.00	0.00	0.00	0.00	0.00	0±0.00
Media+LPS	100.00	100.00	100.00	100.00	100.00	100±0.00
Dex(10μM)+LPS	59.52	53.50	68.85	31.25	36.07	47.41±7.08
25μg/ml+LPS	82.33	90.21	91.98	88.74	80.79	87.93±2.21
50μg/ml+LPS	67.60	79.92	78.57	69.66	67.56	73.93±2.72
100μg/ml+LPS	54.71	53.48	53.01	40.65	42.89	47.51±2.96

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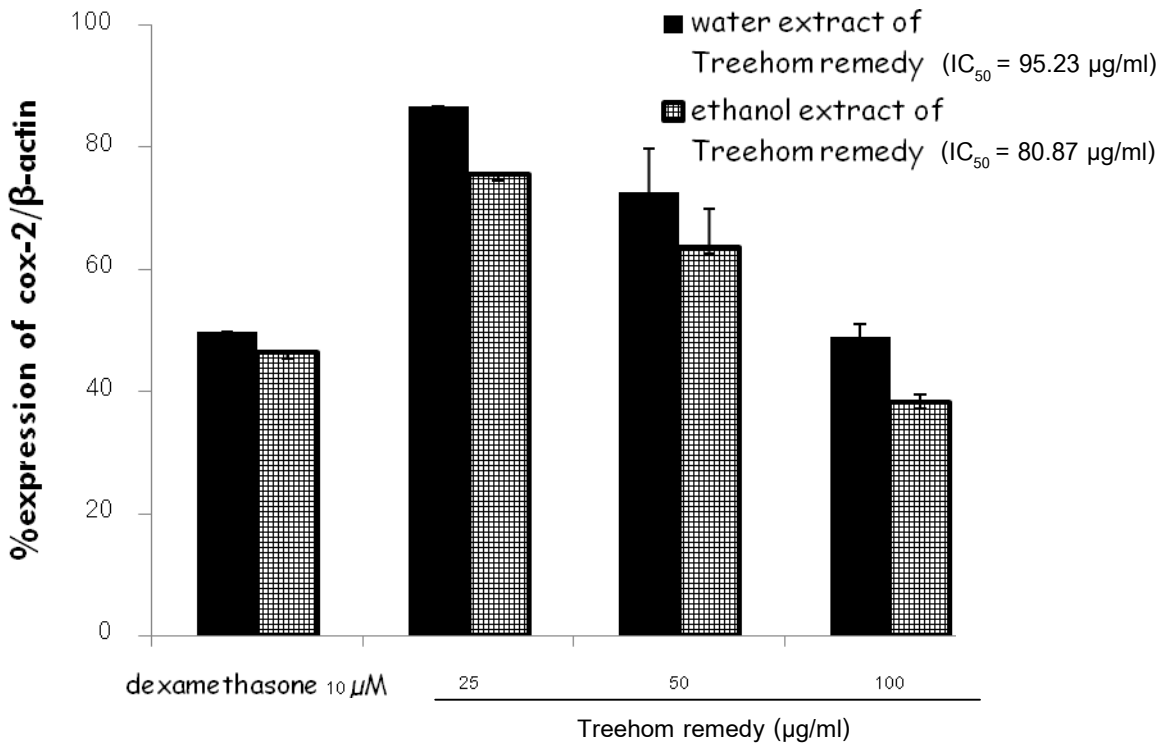

Appendix 38: Effect of the water extract compare with ethanol extracts on mRNA expression of COX-2 in LPS-stimulated J774A.1 cells.

BIOGRAPHY

Name	Miss Jaree Treekeaw
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