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IMMUNOMODULATORY EFFECTS OF *PIPER NIGRUM* EXTRACT ON MACROPHAGE CELL J774A.1

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ณกันต์วลัย วิศิฎศรี : ฤทธิ์ปรับเปลี่ยนภูมิคุ้มกันร่างกายของสารสกัดพริกไทยดำต่อเซลล์แมคโครฟาจ (IMMUNOMODULATORY EFFECTS OF *PIPER NIGRUM* EXTRACT ON MACROPHAGE CELL J774A.1) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.สพ.ญ.รท.หญิง ดร. เนาวรัตน์ สุธัมนาถพงษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. วัชรี ลิมปนสิทธิกุล, 101 หน้า.

Piper nigrum หรือพริกไทยดำเป็นพืชสมุนไพรของไทยที่ถูกนำมาใช้เป็นทั้งเครื่องเทศ และยาพื้นบ้าน มีสรรพคุณในการขับลม และแก้อาการอาหารไม่ย่อย พริกไทยดำถูกใช้เป็นทั้งยาสมุนไพรเดี่ยวและผสมกับสมุนไพรอื่นๆ เป็นยาตำรับ พริกไทยดำประกอบด้วยพฤกษเคมีหลายกลุ่ม จากการทดสอบฤทธิ์เบื้องต้นพบว่า สารสกัดน้ำของพริกไทยดำมีฤทธิ์กระตุ้นการทำงานของเซลล์แมคโครฟาจในการสร้างไนตริกออกไซด์ ไนตริกออกไซด์จัดเป็นสารสื่อที่สำคัญในการทำให้เกิดกระบวนการอักเสบ ซึ่งเป็นการตอบสนองของร่างกายเพื่อทำลายสิ่งแปลกปลอม การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดน้ำของพริกไทยดำต่อการทำงานของเซลล์แมคโครฟาจ ซึ่งทำหน้าที่หลากหลาย และมีผลต่อการปรับเปลี่ยนภูมิคุ้มกันร่างกาย

ผลการศึกษาพบว่าสารสกัดน้ำของพริกไทยดำ ที่ความเข้มข้น 3.125-100 μ g/ml สามารถกระตุ้นให้เกิดกระบวนการจับกินสิ่งแปลกปลอมของเซลล์แมคโครฟาจได้เพิ่มขึ้น รวมถึงเพิ่มการสร้างไนตริก ออกไซด์ซึ่งเป็นโมเลกุลที่สำคัญในการทำลายเชื้อโรค และสิ่งแปลกปลอม นอกจากนั้นสารสกัดน้ำของพริกไทยดำที่ความเข้มข้น 12.5-50 μg/ml ยังสามารถเพิ่มการแสดงออกของเอนไซม์ inducible nitric oxide synthase (iNOS) ซึ่งเป็นเอนไซม์ที่ใช้สร้างไนตริกออกไซด์อีกด้วย ในส่วนการทำลายสิ่งแปลกปลอมของแมคโครฟาจโดยไม่อาศัยออกซิเจน สารสกัดน้ำของพริกไทยดำสามารถเพิ่มการแสดงออกของเอนไซม์ lysozyme M ซึ่งเป็นเอนไซม์ที่ย่อยทำลายแบคทีเรีย นอกจากนั้นสารสกัดที่ความเข้มข้น 50 μg/ml ยังเพิ่มการแสดงออกของ Dectin-1 ซึ่งเป็นตัวรับที่รับรู้ส่วนประกอบผนังเซลล์ของราอีกด้วย สารสกัดน้ำของพริกไทยดำยังเพิ่มการแสดงออกของ IL-1β, IL-6 และ TNF-α ซึ่งเป็นสารสื่ออักเสบ ทำให้เกิดกระบวนการต่อต้านสิ่งแปลกปลอม และสารสกัดน้ำของพริกไทยดำยังทำให้แมคโครฟาจเพิ่มการแสดงออกของโมเลกุลที่จำเป็นในการกระตุ้นที เซลล์คือ B7-1, B7-2 และ ICAM-1

ผลจากการศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดน้ำของพริกไทยดำ สามารถกระตุ้นการทำงานของเซลล์แมคโครฟาจ ซึ่งมีผลเกี่ยวข้องกับการปรับเปลี่ยนภูมิคุ้มกันร่างกาย การศึกษาครั้งนี้จึงอาจเป็นประโยชน์ในการพัฒนายา และเป็นการทำให้เกิดความมั่นใจในประสิทธิภาพของสมุนไพรไทยอีกด้วย

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

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

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5287149920 : MAJOR PHARMACOLOGY
KEYWORDS : *PIPER NIGRUM*/ MACROPHAGE/ PHAGOCYTOSIS/ PRO-INFLAMMATORY CYTOKINES/ ANTIGEN PRESENTATION

NAKUNTWALAI WISIDSRI: IMMUNOMODULATORY EFFECTS OF *PIPER NIGRUM* EXTRACT ON MACROPHAGE CELL J774A.1. ADVISOR: ASSIST. PROF. NAOWARAT SUTHAMNATPONG, D.M.V., Ph.D., CO-ADVISOR: ASSIST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D. 101 pp.

Piper nigrum (*P. nigrum*) or black pepper is one of Thai herbs which has been used both as seasoning and traditional medicine. This plant has healing properties in relieving flatulence or dyspepsia. *P. nigrum* composed of several phytochemical compounds. Preliminary study showed that the water extract of *P. nigrum* stimulated nitric oxide (NO) production in macrophage cells. NO is one of inflammatory mediators using for host defense mechanism. The present study then aim to investigate the immunomodulatory effects of the water extract of *P. nigrum* on macrophage cells.

The results showed that the water extract of *P. nigrum* at concentration of 3.125-100 µg/ml increased phagocytosis by macrophage cells. Moreover, *P. nigrum* has stimulatory effect on NO production. NO is an important molecule involved in destruction of microbes. Furthermore, this extract at 12.5-50 µg/ml also increased the expression of inducible nitric oxide synthase (iNOS), the enzyme which catalyzes NO production. In part of oxygen-independent pathway, the water extract of *P. nigrum* promoted the production of lysozyme M, the enzyme capable of hydrolyzing bacteria. The extract at 50 µg/ml also enhanced the expression of Dectin-1 receptor, the receptor which recognizes fungal cell wall. The water extract of *P. nigrum* at 12.5-50 µg/ml promoted the expression of the pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α. Furthermore, the water extract also enhanced the expression of costimulatory molecules necessary for T cell activation including B7-1, B7-2 and ICAM-1.

This study suggests that the water extract of *P. nigrum* is ability to stimulate macrophage functions which involve in immunomodulation. It may be benefit in developing new drug and also ensuring the efficiency of Thai herb.

Field of Study: Pharmacology ..... Student's Signature .....
Academic Year: 2011 ..... Advisor's Signature .....
Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percent
/	Per
<	Less than
µl	Microliter (s)
µM	Micromolar
°C	Degree Celsius
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guerin
Ca^{2+}	Calcium
CaM	Calcium and calmodulin
CARD	Caspase-associated recruitment domain
CAT	Catalase
cDNA	Complementary DNA
Con-A	Concanavalin-A
COX-2	Cyclo-oxygenase-2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTLDs	C-type lectin-like domains
DC	Dendritic cell
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
eNOS	endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum

FDA	Food and Drug Administration
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferese
h	hour
HSC	Haematopoietic stem cell
ICAMs	Intercellular adhesion molecules
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
iNOS	inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine-based activation motif
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
M	Molarities
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
min	minute
MIP-1	Macrophage inflammatory protein-1
ml	Milliliter(s)
mlg	Membrane-bound immunoglobulin
MMP	Matrix metalloproteinase
MPL	Monophosphoryl lipid
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NED	*N*-1-napthylethylenediamine dihydrochloride
NFAT	Nuclear factor of activated T-cell

NF-kB	Nuclear factor-kappaB
ng	Nanogram (s)
NK cell	Natural killer cell
nm	Nanometer
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
O_2	Oxygen
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PFC	Plaque forming cell
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptor
PS	Phosphatidyserine
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reverse transcription
S.E.M	Standard error of mean
SOD	Superoxide dismutase
Syk	Spleen tyrosine kinase
TCR	T cell receptor
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
U	Unit

CHAPTER I

INTRODUCTION

Background and Rationale

Defense against harmful matters is an essential necessity for host. Consequently, the strategies for protecting the body are designed to limit this danger. The immune system performs by associated compositions in part of innate and adaptive immunity. These two parts of the immune system interact to fight against hazard attacks which include cancer cells, foreign particles and pathogens. Innate immune responses occur immediately and with non specificity. While it takes several day or weeks for adaptive immunity to become active. Adaptive immunity develops antigen-specific reactions through memory cells, T and B lymphocytes whose actions require help from the innate immune activities [1].

Innate immunity consists of the cells which are able to recognize invading microbes, such as natural killer (NK) cells, dendritic cells (DCs), mast cells, granulocytes cells (neutrophils, eosinophils, basophils) and macrophages. Macrophages play important roles in both innate and adaptive immune responses. They have at least three major functions including phagocytosis, production of mediators and cytokines, and antigen presentation [2]. The foreign particles and microbes are recognized by pattern recognition receptors (PRRs) on the immune cells surface and the responses are developed. Macrophages express a wide variety of PRRs which mediate recognition and uptake both dead cells/debris and microbial pathogens.

Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens, which are recognized by cells of the innate immune system. For instance, scavenger receptors (SR-A) binding to lipopolysacharide (LPS) from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria. Members of Toll-like receptors (TLRs) family have TLR4 binding to

LPS and peptidoglycan, the bacterial cell wall structures. In addition, macrophage's receptors are also involving in fungal recognition. The C-type lectin receptors recognize fungal cell wall structures such as mannose receptor which recognizes mannan and Dectin-1 which recognizes β-glucan [1, 3].

After confrontation with pathogens, macrophages are activated and initiate phagocytosis. The microbes are engulfed into phagosomes which subsequently fuse with lysosomes resulting in phagolysosomes. Microbes are killed by combination of non-oxidative and oxidative mechanisms in lysosome. Non-oxidative mechanisms including acidification of the phagosome, antimicrobial proteins and peptides act as enzyme to hydrolyze microbes such as defensins, cathelicidins, lysozymes and assorted lipases and proteases. Furthermore, oxidative mechanisms, reactive oxygen and nitrogen species (ROS and RNS) including nitric oxide (NO) catalyzed by inducible nitric oxide synthase (iNOS) or NOS-2 are generated and act as toxic molecules to destroy microbes [3-4].

At the same time, the capability of activated macrophages to produce several chemokines such as macrophage inflammatory protein-1 (MIP-1), monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8) and cytokines which act as pro-inflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor-α (TNF-α) is increased. These pro-inflammatory cytokines lead to inflammation which is the host defense mechanism to protect host from hazard attacks [2]. However, phagocytosis of apoptotic cells by macrophages are normal mechanism and without activating the pro-inflammatory cytokine production [5]. Moreover, macrophages present digested antigen by major histocompatibility complex (MHC) to T cell receptors (TCR) and express costimulatory molecules. These costimulatory molecules generate the second signal such as B7-1 (CD80) and B7-2 (CD86) which binding to CD-28 and CD152 on T cell surface and resulting in T cell activation. In addition the intercellular adhesion molecules (ICAMs) including ICAM-1, ICAM-2, and ICAM-3 are expressed and bound to lymphocyte

function-associated antigen 1 (LFA-1) receptor result in tightly grip between antigen presenting cell (APC) and T cell [6].

Macrophages are functional cells of the immune system. Therefore, macrophage activation is crucial in the immunomodulation. Immunomodulation is important for elderly people [7] and cancer patients [8] to improve the body's defense against pathogens or tumor cells. At present, one of strategies to boost the immune system is known as immunotherapy which is used for cancers therapy as well as an adjuvant in vaccines. Adjuvant acts as an immunogenicity enhancer to activate cells in innate immunity such as dendritic cells and macrophages to become activated cells or mature cells. These cells then further activate other immune components to generate specific immune response [9].

It has been known for a long time that drugs derived from medicinal plants make a large contribution to new drug discovery. Plants are the rich sources of several conventional drugs used clinically. Thai ancestors used Thai herbs as food as well as medicines. The herbal medicinal plants are valuable heritages handed down. Many of Thai herbs are still widely used including *P. nigrum* or black pepper. It is a component of Thai medicine remedies and used as monotherapy in traditional medicine. *P. nigrum*'s indication is for treatment of flatulence or dyspepsia. Several phytochemical ingredients of this plant are identified such as lignans, neolignans, terpenes and alkaloid/amides etc [10]. The pharmacological effects of *P. nigrum* are demonstrated including anti-microbial, anti-oxidant, anti-tumor, anthelmintic, anti-inflammation and immunostimulation. However, the immunostimulatory effects are rarely studies.

Preliminary study of *P. nigrum* water extract on macrophage J774A.1 cells showed that this extract could stimulate NO production. NO is an important molecule involved in phagocytosis and inflammation. Therefore, it is of interested to study further on other parameters which involving in immunomodulation by macrophages including (1) pathogen recognition and phagocytosis, (2) production of inflammatory mediators, (3) ability of

macrophages to function as an APC to activate adaptive immunity. The results from this study may provide important scientific information to ensure that this plant contains substances which is useful in traditional medicine, especially in immunological aspects.

Objectives

To study *in vitro* immunomodulatory effects of the water extracted of *P. nigrum* on macrophage J774A.1 cell functions in phagocytic activity and the involving mechanism including NO production, expression of Dectin-1 and expression of lysozyme M. In addition, to evaluate effects of *P. nigrum* extract on the expression of pro-inflammatory cytokines and costimulatory molecules.

Hypothesis

The water extract of *P. nigrum* activates macrophage J774A.1 cells to produce and express involving molecules including NO, iNOS, Dectin-1 receptor, Lysozyme M, IL-1β, IL-6, TNF-α, B7-1, B7-2 and ICAM-1.

Keywords

Piper nigrum, macrophage, phagocytosis, pro-inflammatory cytokine, antigen presentation.

CHAPTER II

LITERATURE REVEIWS

The function of the immune system is to protect host from several foreign antigens including pathogens, cancer cells and foreign particles. It is composed of two parts which divided by the speed and specificity of the responses. The first is the innate immunity which responds rapidly and in a non-specific manner against harmful matters. The second is the adaptive immunity which takes longer duration to develop. The adaptive immunity consists of antigen-specific reactions generated by T lymphocytes and B lymphocytes which have memory and respond vigorously and rapidly to the repeated exposure antigens. However, the innate and adaptive immune systems do not work separately. Instead they work in concert through a highly complicated interaction network [11].

The innate immunity

The innate immunity is the first line of defense against pathogens. It is composed of mechanical, chemical and cellular components. The mechanical component is provided by the skin and mucosa which act as physical barriers to impede the invading pathogens and the physiological components such as mucus secretion, motility and cilia oscillation. The chemical components can be divided in three subcomponents including PRRs, proteins or peptides which hydrolyse microorganism, and several mediators such as cytokines and chemokines which amplify the immune responses. The third subcomponent is the cells such as natural killer (NK) cells, granulocytes cells (neutrophil, basophil and eosinophil), dendritic cells, mast cells and macrophages. These cellular components are also the initiator of chemical components [12].

The adaptive immunity

The adaptive immunity characterizes by antigen-specific receptors on T and B lymphocytes which recognize the antigens in different ways. B cells recognize antigens by membrane-bound immunoglobulin (mIg) on their cell surface and then become activated B cells. The activated cells proliferate and differentiate into plasma cells. The plasma cells are capable of producing antibodies which protect the body by toxin neutralization, complement activation and bacterial opsonization for phagocytosis. The activated plasma cells finally differentiate to become memory B cells, a specialized cell which can remember antigens for a long period of time. In contrast to B cells, T cells recognize antigens in form of short antigenic peptide complex with the major histocompatibility complex (MHC) that presented by APC to T cell receptors (TCR). After recognizing antigen, T cells proliferate and differentiate to become effector cells to eliminate pathogen and finally become memory cells [11].

The innate and adaptive immunity are the complicated interaction network. The innate immunity can bridge to adaptive immunity by information of innate immune cells and their generated mediators which amplify immune responses. Similarly, adaptive immunity can control and work together with innate immunity through cytokines from T cell. Macrophages are an immune cell which plays important roles in the innate immune response and as the bridge to adaptive immune response.

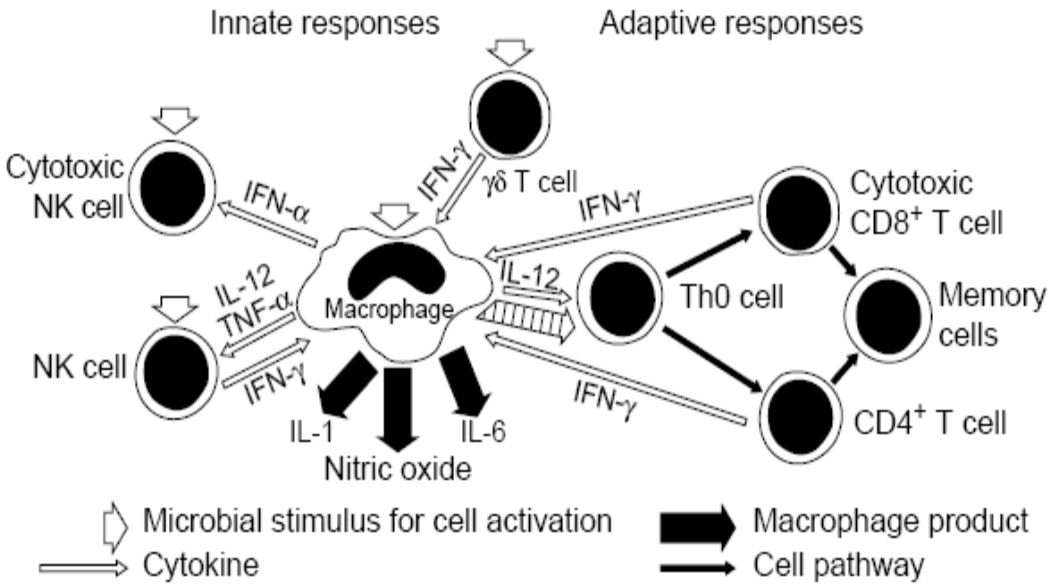


Figure 1: Interaction network between innate and adaptive immune responses. Macrophages are activated by microbial stimulus. Activated macrophages are able to release several cytokines to promote other immune cell activation and lead to T cells response in adaptive immunity. These adaptive immune responses can also promote macrophage activation for microbicidal activities [13].

Macrophages

Macrophages are mononuclear phagocytes derived from pluripotent haematopoietic stem cells (PHSC) in the bone marrow. Their precursor cells are in myeloid lineage. They passage in differentiation steps to monoblasts, pro-monocytes before delivery to monocytes in the peripheral blood. Monocytes migrate from blood circulation to various tissues and become mature cells as tissue-specific macrophages such as osteoclast which are the bone macrophages, microglial cells which are central nervous system macrophages and Kuffer cells which are liver macrophages (Fig.2) [14].

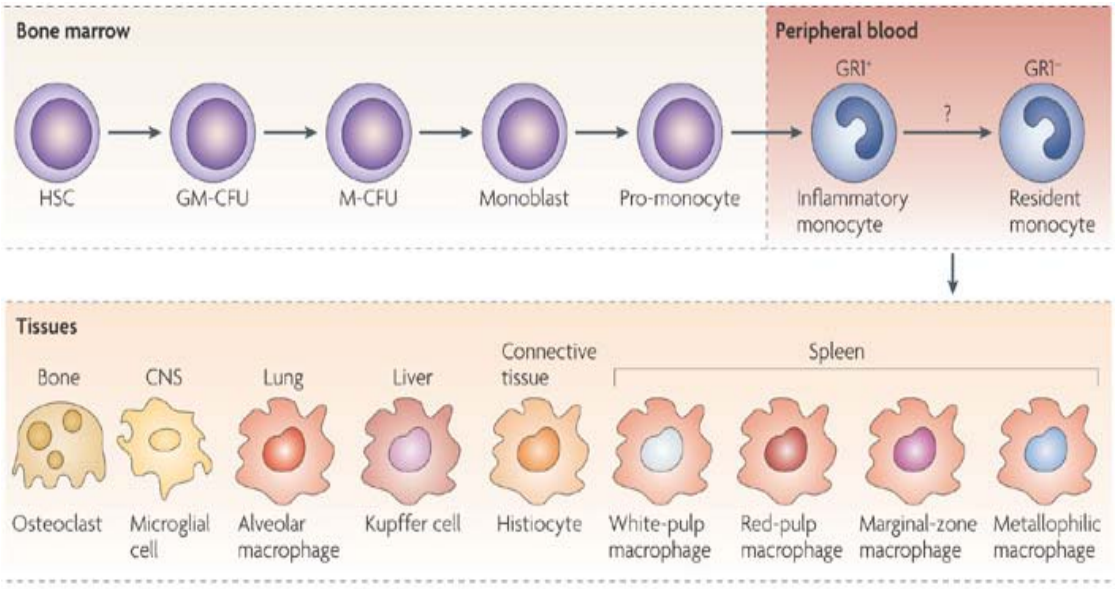


Figure 2: The Macrophage maturation processes. Monocytes derived from bone marrow and differentiated to monoblasts and pro-monocytes before releasing to blood stream. They become mature cells as macrophages in the various tissues [14].

Macrophages are phagocytic cells which maintain homeostasis of tissues as well as function in castrating of pathogens. They serve general functions without special stimulation or ontogeny activation such as clearance of aged red blood cells and apoptotic cells by little or without immune mediators' production. They constitutively express a variety of receptors on their cell surface. The receptors which mediate the homeostatic clearance process include scravenger receptors, phosphatidylserine receptors, the thrombospondin receptor, integrins and complement receptors. When macrophages are activated by several stimuli after tissue injury or during infection, their responses are enhanced in order to eradicate these stimuli. Macrophages express PRRs which recognize pathogen-associated or damage-associated molecular patterns both from microbial patterns such as lipopolysaccharide as well as nuclear and cytosolic proteins resulting from cellular stress. These receptors transmit intracellular signal to activate

macrophages which then produce several mediators which promote inflammation and recruit other immune cells such as neutrophils [14-15].

The macrophage receptors recognize different of signature molecules on the particle cell surface. In the case of pathogens, it is known as PAMPs which differ in vary of pathogens.

The macrophage receptors

Macrophages express diverse receptors which react to natural and altered-self components of the host as well as varieties of microbes (Fig.3). These receptors such as TLRs, scavenger receptors, receptor for opsonin and C-type lectins have been shown to be involved in microbial recognition and uptake [4].

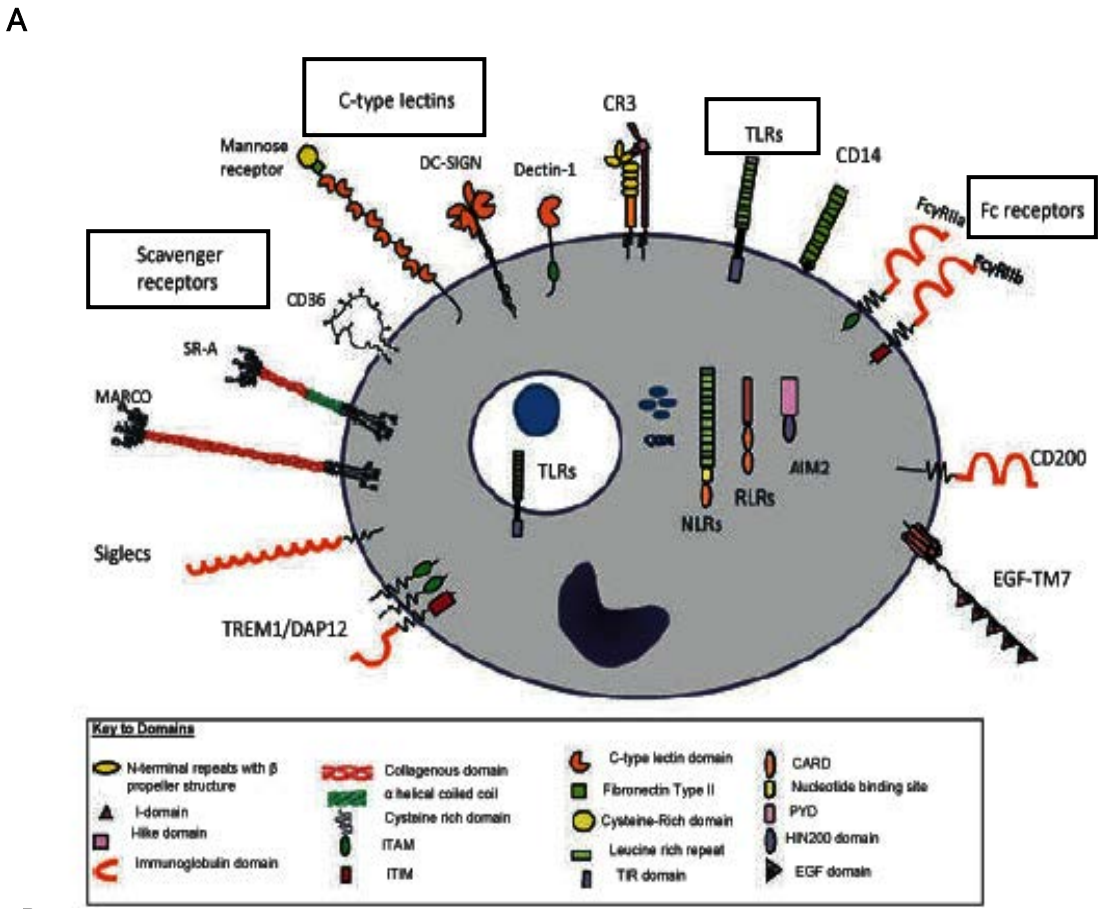
TLRs are type I transmembrane receptor. At present, there are 13 members in mammals. In humans, 10 members (TLR1 to TLR10) have been identified, while in mouse, TLR1 to TLR9 and TLR11 which is absent in humans and TLR12, TLR13 have been identified. The function of the last two TLRs remains unclear. TLR1, 2, 4, 5 and 6 are primarily expressed on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa, whereas TLR3, 7, 8 and 9 are specially express within endocytic parts and recognize nucleic acid PAMPs derived from various viruses and bacteria [16]. TLRs are found in several immune cells including macrophages, dendritic cells (DCs), B cells, specific types of T cells. In addition, TLRs are also found in non immune cells such as fibroblasts and epithelial cells [17].

Scavenger receptors (SR) are trimeric, transmembrane glycoprotein. Several of these receptors are involved in phagocytosis of both microorganisms and apoptotic cells. These receptors contain a variety of molecules involved in receptor-mediated endocytosis of selected polyanionic ligands including modified low density lipoproteins (LDL). Furthermore, they are also involved in cell adhesion process. The SR receptors including SR-AI,

SR-AII and MARCO are SR involved in recognition of PAMPs of microbes. The integrins and phosphatidyserine (PS) receptor have been shown to play a role in phagocytosis of various apoptotic cells [18-19].

Receptor for opsonins sush as Fc-receptors (FcRs) and complement receptors (CRs) that recognize opsonins coated pathogens. These opsonins are the 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 display to promote phagocytic mechanisms and subsequent cellular responses [20-21].

C-Type lectins are the receptors composed of one or more C-type lectin-like domains (CTLDs). These receptors can be divided into 17 groups based on their phylogeny and domain composition. C-type lectins are involved in diverse functions and in difference processes including adhesion of cell, integration of tissue and remodeling, activation of platelet and complement, recognition of pathogen, endocytosis and phagocytosis. The member of C-type lectins such as mannose receptors and Dectin-1 were shown to participate in phagocytosis and host defense. Mannose receptors are PRR which recognize bacteria, virus and protozoa component. Furthermore, they can recognize fungal cell wall in part of mannose while Dectin-1 is PRR which recognizes fungal cell wall in part of β-glucan [22].



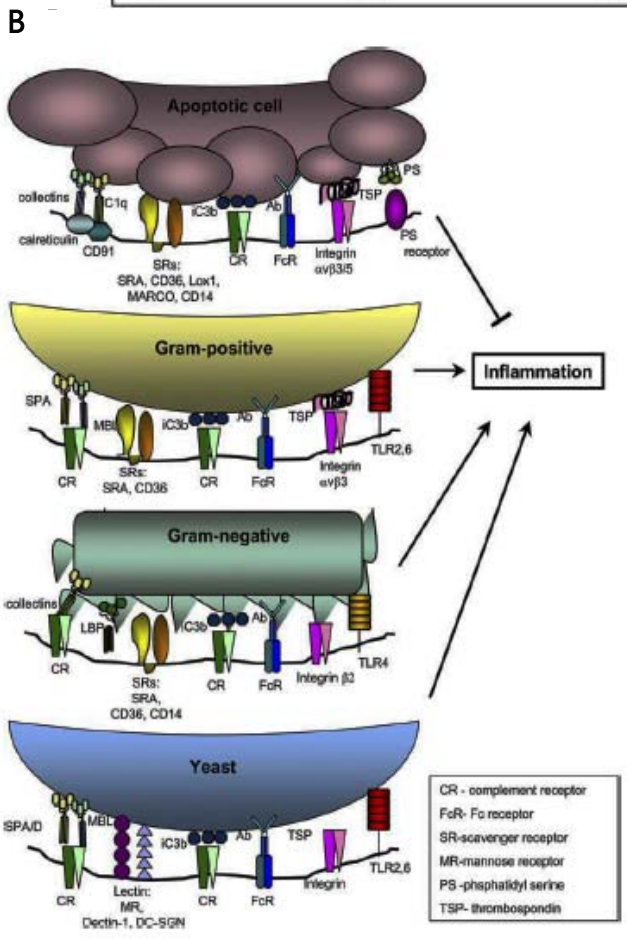


Figure 3: The macrophage receptors. (A) The groups of macrophage receptors which use to recognize pathogens including TLRs, scavenger receptors and C-type lectins [4]. (B) The various receptors which recognize pathogens and apoptotic cell. The receptors which recognize bacterial component both Gram-positive and Gram-negative bacteria are involved in inflammation while the recognition of apoptotic cell is without inflammatory activation [23].

Functions of macrophages

Macrophages play many important roles in both innate and adaptive immunity. 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 then followed by destruction of engulfed foreign bodies and production of several mediators including cytokines and chemokines which serve in innate and adaptive immune responses. Finally, macrophages act as APC to present the digested particles to T cells and express important molecules to activate T cells.

The three major functions of macrophages are

1. Phagocytosis

Macrophages are phagocytic cells which respond when the body is stimulated by pathogens or injury. Phagocytosis is the first step of reaction to these stimulation [24]. Furthermore, it is an initiate step in innate immune response and the destruction of pathogens by macrophages is the important process to promote antigen presentation to stimulate an adaptive immune response. Phagocytosis is the mechanism used to clear foreign particles whose size exceeds 0.5 μm. This process is the clearance of invading microbes as well as apoptotic cells [3, 25].

After macrophage 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 which are vesicles for destroying the engulfed particles by oxygen-dependent and oxygen-independent mechanisms. In addition, cytokines and chemokines are generated as well as molecules involved in presenting an antigen to T cells are also expressed [26].

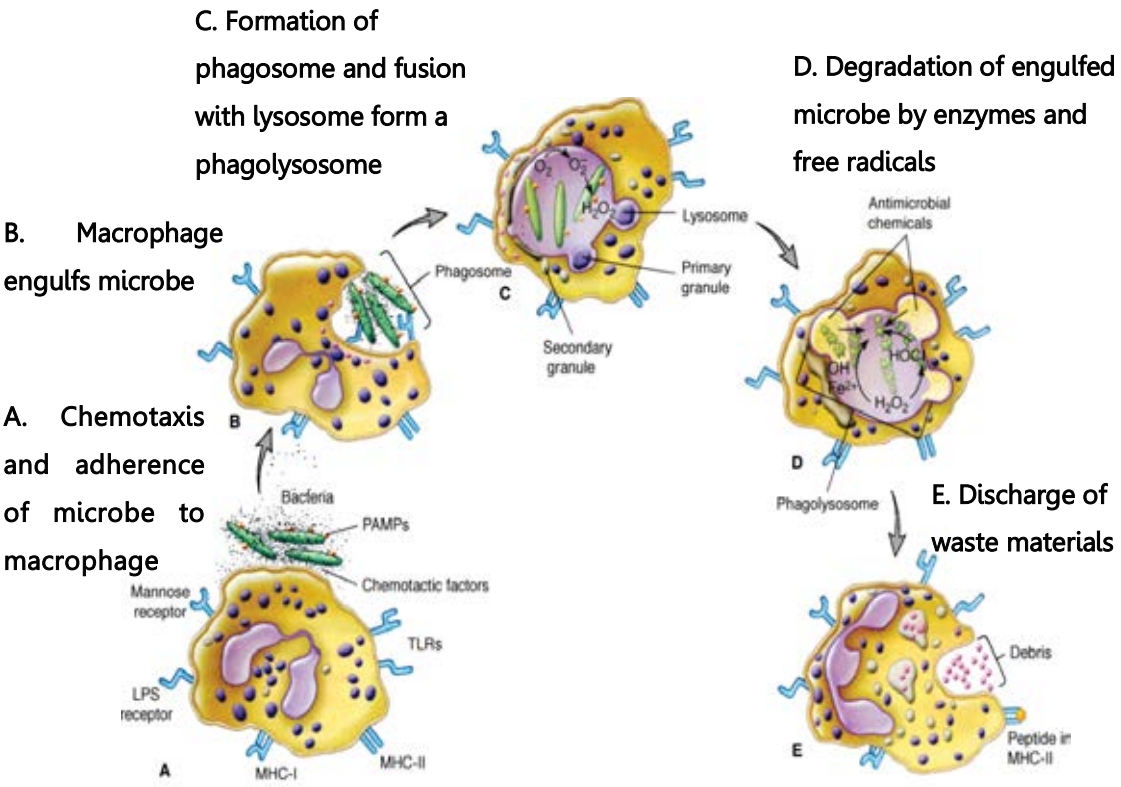


Figure 4: Steps of phagocytosis microbes by macrophage. Macrophages recognize bacteria by their surface receptors then phagocytosis is activated. The bacterium is engulfed into phagosome which fused with lysosome and then it is destroyed by free radicals and enzymes. After digestion of bacteria, the waste materials are discharged and the short peptide is presented by MHC-II [27].

The steps of phagocytosis

Phagocytosis occurs in sequential steps. The process of phagocytosis is consisted of recognition of microbes or foreign particles by varieties of receptors, engulfment and the triggering of different signaling pathway, and killing microbes by several mechanisms in phagosome [26].

I. Recognition of microbes and foreign particles

When pathogens or microorganisms enter the body, macrophages recognize them by PRRs and engulf them into phagosomes. In the case of

fungi, Dectin-1, one of macrophage receptor belongs to C-type lectins receptor recognizes fungal cell wall component.

Dectin-1 is expressed on many cell types in mice and human. In mice, it is expressed on dendritic cells, monocytes, neutrophils, a subset of T cells and macrophages while in human it is also expressed on B cells, eosinophils and mast cells. Many of cytokines and microbial factors can influence the up-regulation of Dectin-1 expression including IL-4, IL-13 and granulocyte macrophage-colony stimulating factor (GM-CSF). In contrast, Dectin-1 are down- regulated by IL-10, LPS and dexamethasone [22].

This receptor recognizes β-1,3-linked glucans, carbohydrate polymers which are fungal cell wall component both in plants and in some of bacteria. Furthermore, Dectin-1 also recognizes zymosan presented in the cell wall of *Saccharomyces cerevisiae.* Zymosan is consisted mainly of β-glucan and also mannans, chitin, protein and lipids. It has been widely used to study immune function both *in vitro* and *in vivo* [28]. Dectin-1 also recognizes PAMPs of mycobacteria. The β-glucans triggers this receptor to generate a variety of cellular response including ligand uptake by endocytosis and phagocytosis, arachidonic acid metabolites production, the respiratory burst, and cytokines and chemokines production such as TNF, CXCL2 (MIP2), IL-23, IL-6 and IL-10. In some cases, its signaling is promoted and cooperative signaling from MyD88 coupled TLRs [29].

The intracellular signaling by Dectin-1 is mediated through the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like motif and involved in multiple pathways both spleen tyrosine kinase (Syk)-dependent and Syk-independent pathway. The Syk is a pivotal kinase which mediates downstream cellular responses including cytokines and chemokines production and respiratory burst induction. Downstream signaling through Syk kinase-dependent pathway involves extracellular signal-regulated kinases (ERK), caspase recruitment domain 9 (CARD9) and nuclear factor of activated T-cells (NFAT) molecules. Signalling through these cascades resulted in

activation of the transcriptional factor such as NF-kB, however, there are still not completely understood. The requirement of Syk for Dectin-1 functions is restricted and specific for certain cell types. In macrophages, Syk is required for induction of respiratory burst and production of cytokines but not required for phagocytosis (Fig. 5) [22, 28-30].

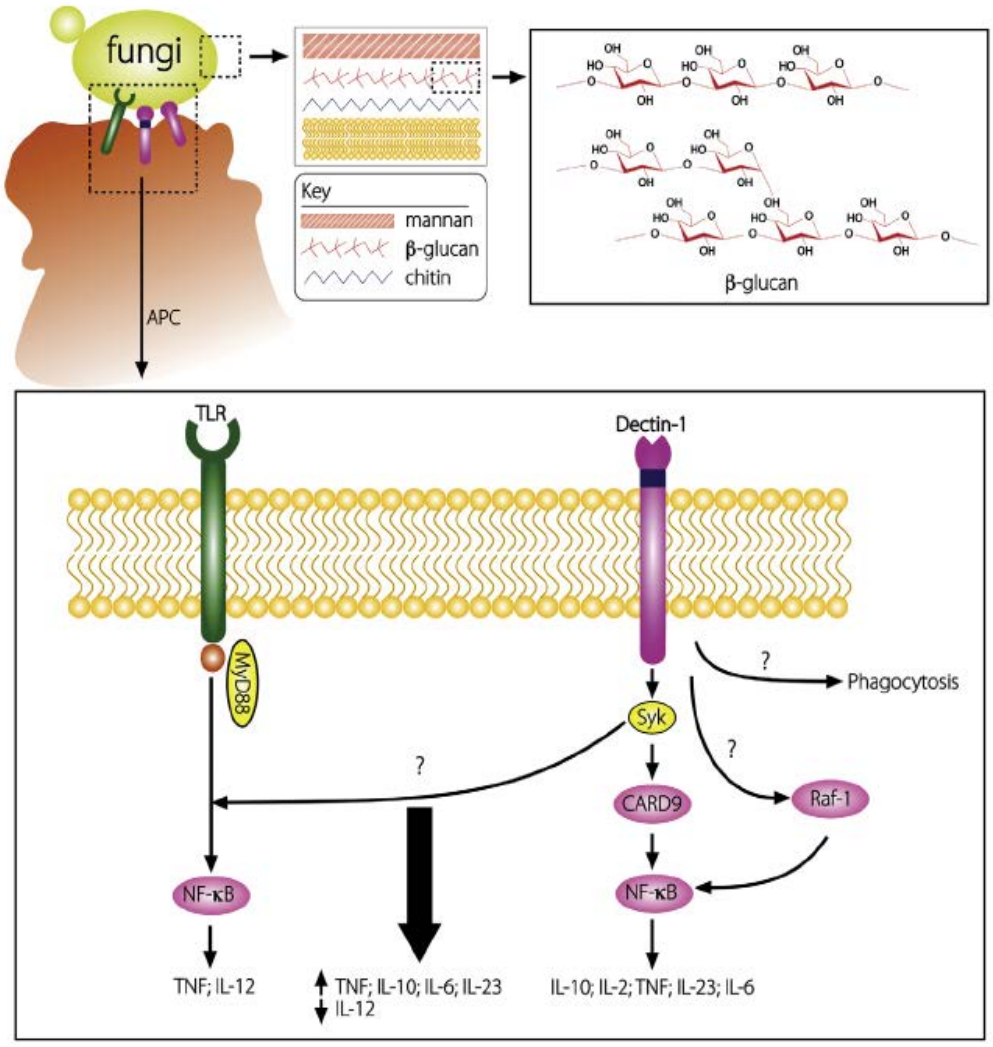


Figure 5: The recognition of fungi by several receptors including Dectin-1 and the generation of intracellular signaling. Dectin-1 recognizes β-glucan component of fungi and generates signaling cascade both by Syk-dependent and Syk-independent pathways to produce several cytokines [29].

II. Engulfment particles and generation of intracellular signal

After recognition of particle, plasma membrane is activated to form pseudopods which are extended to get close to the recognized particle and engulf particle into phagosome. In addition, during particle-receptor binding, several intracellular signals and several molecules required for many pathways are activated to assign the phagocytosis and regulate internalization. The signaling which generated during phagocytosis may subsequently support the activation or inhibition of phagocytosis and microbe-induced responses (Fig.6) [26].

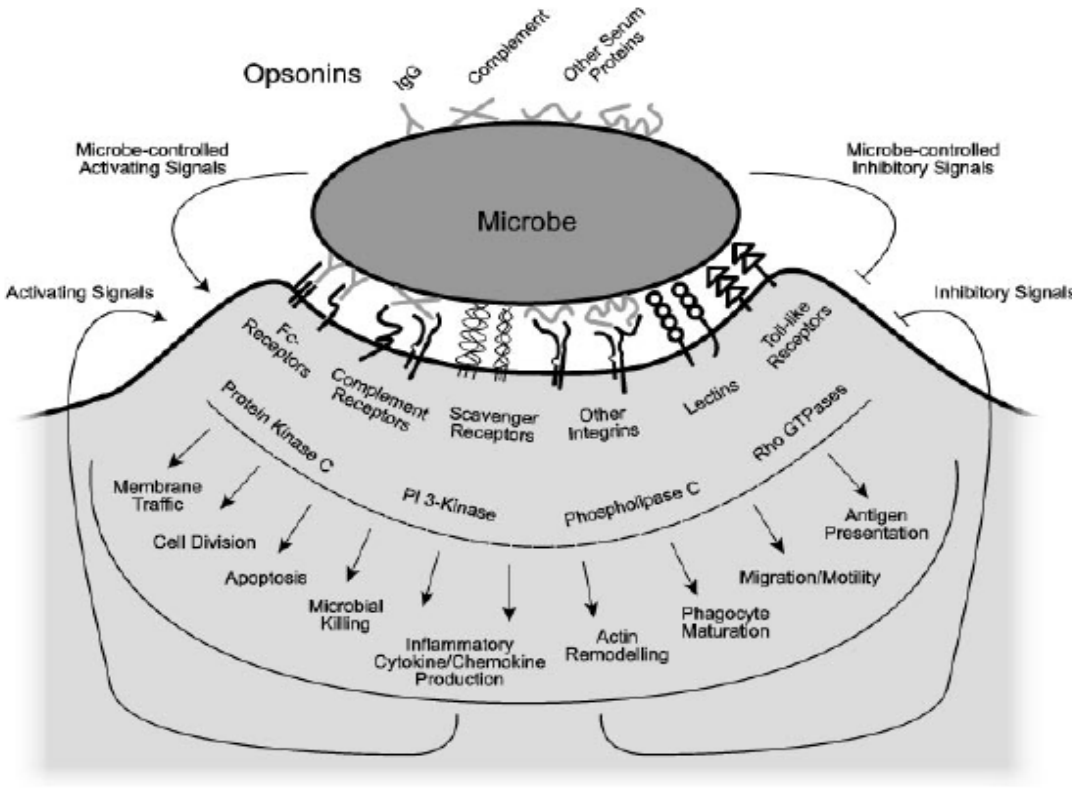


Figure 6: Recognition of microbe by macrophage receptors and the generation of parallel signaling during phagocytosis. During phagocytosis of microbes, many intracellular signals are induced through several intracellular molecules serve in many pathways. Signaling during phagocytosis may subsequently support the activation or inhibition of further phagocytosis and microbe-induced responses [26].

III. Killing microbes by several mechanisms in phagosome

After engulfment, the microbes are trapped in phagosomes which mature to become phagolysosome by fusion with lysosome. The pathogen is degraded by complex mechanisms which are organized into two broad groups including oxygen-dependent and oxygen-independent mechanisms.

Oxygen-dependent mechanisms

In the oxygen-dependent mechanisms, the free radicals are generated to damage microbial DNA. The binding of pathogen lead to increase in oxygen uptake by macrophage which is called the respiratory burst. This influx of oxygen is used in various mechanisms to cause damage to pathogens inside the phagolysosome. In activated macrophages, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase initiates conversion of molecular oxygen (O_2) to reactive oxygen intermediates (ROIs), superoxide (O_2^-) which rapidly transformed to other reactive oxygen species (ROS) such as H_2O_2. Furthermore, peroxynitrite ($ONOO^-$) is one of product which generated from combination of NO which is catalyzed by inducible nitric oxide synthase (iNOS) and O_2^- to induce microbial toxicity [31].

Nitric oxide is generated by many cell types including macrophages. It is a soluble gas whose functions as a vascular-relaxing agent, a neurotransmitter and an inhibitor of platelet aggregation. Furthermore, it is involved in immune response as a toxic agent toward invading pathogens and inflammatory mechanism [32].

NO is synthesized from L-arginine by nitric oxide synthases (NOSs) which is consisted of three isoforms depending on the type of tissue. One is endothelial NOS (eNOS or NOS I) which is produced in vascular endothelial cells. The second is neuronal NOS (nNOS or NOS III) which produced in neuronal cells. These two isoforms of NOS are constitutively present in resting cells and depend on intracellular calcium and calmodulin (CaM) to activate them. The eNOS and nNOS generate NO in low level for normal physiological

functions and action in short period. Another isoform of NOS is inducible NOS (iNOS or NOS II) which is induced by pro-inflammatory cytokines (TNF-α, IL-1 and IL-6), bacterial products or infectious cells and expressed in many activated cell types including fibroblast, endothelial cells, keratinocytes, chondrocytes, NK cells and activated macrophages. NO which is produced by iNOS is high level and action in long period and is not presented in resting cells. The enzyme iNOS generates NO independently of intracellular calcium concentrations but depend on how long it presents in the activated cells [32-33].

NO is not only a toxic agent used to destroy the invaded pathogen but it is also involved in regulation of many immune cells including NK cells, neutrophils and mast cells [32].

Oxygen-independent mechanisms

The important mechanism to destroy pathogen also come from other materials in phagolysosomes. The phagolysosomes contain several mechanisms for microbicidal and microbes degradation. One is the acidification of the phagosome which low pH 4.0-5.0 is an opposed environment that impedes the microbial growth [3]. Furthermore, the oxygen-independent mechanism by several enzymes and antimicrobial peptides such as protease, cathepsins, defensin and lysozyme involved in degradation of the engulfed microbes. These enzymes are produced in many tissue and cell types including lysosomal granule of macrophages [3, 34].

Lysozyme is one of antimicrobial peptide which presents in neutrophils, monocytes and macrophages as well as in many biological fluids such as tear, saliva and airway fluid. It is capable of interrupting the repletion of pathogens by hydrolysing peptidoglycan layer of bacterial cell wall at glycosidic linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid [35]. Lysozyme hydrolyses both peptidoglycan of Gram-positive bacteria and some of Gram-negative bacteria. However, modification of peptidoglycan in Gram-positive

bacteria may lead to resistant to lysozyme. The genes which encode lysozyme are varied among species. There are two lysozyme genes: lysozyme M and lysozyme P. Both genes are expressed in mice while only lysozyme M is expressed in human. Therefore, lysozyme M is the predicted orthologue of human lysozyme [36-37]. Lysozyme is expressed at low level during macrophage differentiation and is constantly expressed in high level in mature macrophages. An increased expression of cytokines and antibacterial proteins including lysozyme was shown in LPS-activated macrophages [38].

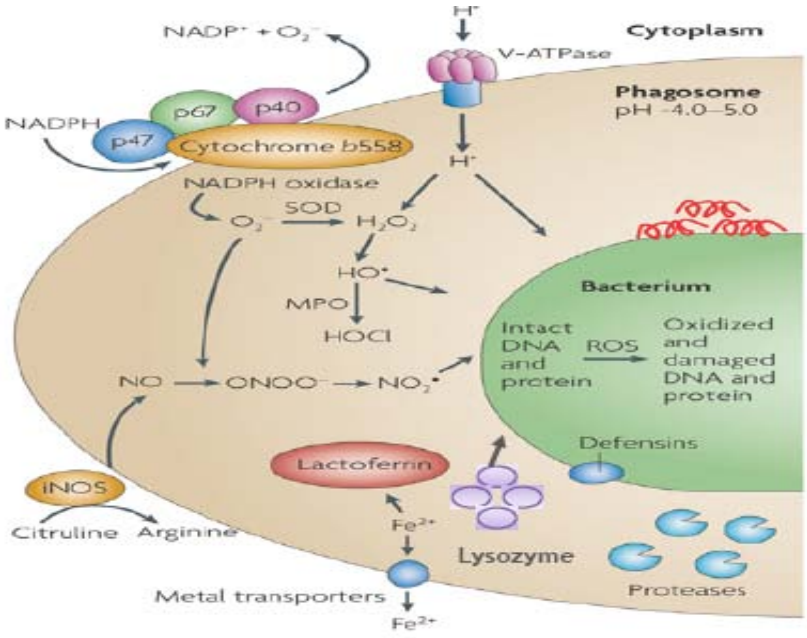


Figure 7: The phagolysosome which contained several mechanisms for destroying engulfed pathogens. Bacteria are destroying by both oxygen dependent pathway including NO, ROS and oxygen independent pathway such as low pH environment and many enzymes including lysozyme [3].

In addition, to destroy pathogens, activated macrophages also capable of producing several cytokines which play an important role in immunological signaling and function.

2. Mediators production

Activated macrophages produce several cytokines and chemokines which participate in the immune responses such as pro-inflammatory cytokines including IL-1, IL-6 and TNF-α and chemokines such as IL-8, MCP-1 and MIP-1.

Pro-inflammatory cytokines

These pro-inflammatory cytokines and chemokines play role in leukocyte movement and migration from the circulation to tissues [2]. In addition, pro-inflammatory cytokines also function as a third signal for T cell activation [39].

Interleukin-1 (IL-1) has two isoforms, IL-1α and IL-1β, which are encoded by different genes and generated by different process. It is produced by many cell types including fibroblasts, keratinocytes, T and B lymphocytes and macrophages. They bind to IL-1 receptor and have similar biological functions. IL-1β is secreted in a processed form and functions as an endocrine signaling molecule while IL-1α is mainly active locally in cell-associated forms and functions as an autocrine signaling molecule [40]. IL-1 is able to stimulate T cells proliferation and control T cell response. IL-1 functions together with antigen as a costimulator to generate third signal for activation of $CD4^+$ T cells [39, 41].

Interleukin 6 (IL-6) is produced by many cell types including monocytes, macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. It is a pro-inflammatory cytokine as well as a growth factor for B cells maturation to plasma cells. Furthermore, IL-6 also stimulates T cells activation and differentiation [42-43]. IL-6 has anti-apoptotic function which controls $CD4^+$ T cells survival and controls the activated $CD4^+$ T cells expansion following immunization [41].

Tumor necrosis factor-α (TNF-α) is produced by fibroblast, mast cells, T cells, NK cells and activated macrophages. It is pro-inflammatory cytokine like IL-1 and IL-6 and acts as an activator to activate cytokine production in macrophage [43]. TNF-α is involved in a wide range of the immune responses including tumoricidal activity either by apoptosis or necrosis, inflammatory effects, proliferative or growth-promoting effects, and hematopoetic effects [44]. It stimulates recruitment of neutrophils and monocytes to infectious sites and these cells are activated and co-response to eradicate microbes. Furthermore, TNF-α induces the expression of adhesion molecules such as selectins and ligands for leukocyte integrins which make the endothelial cell surface adhesive for leukocytes. These are the initial event in the recruitment of leukocytes to infectious sites [45].

As described above, macrophages are an important cell which plays central roles in the innate immune response. Moreover, the responses initiated from macrophages are also linked to adaptive immunity. Adaptive immunity is characterized by antigen specificity and immunologic memory. B and T cells are the chief components which develop responses in different way. B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response. B cells produce antibodies to neutralize and opzonize the pathogens. In addition the antibodies are also capable of activating the complement cascade. On the other hand, T cells response to short antigenic peptide-MHC complexes presented on APCs [46]. Macrophages are effector cells for generating adaptive immune responses by both B and T cells activation. Another important function of macrophages is presentation of antigen to T cell after phagocytosis.

3. Antigen presentation

The presentation of antigen is prepared by antigen-presenting cells which have the special properties in expression of utility molecules. The professional antigen presenting cells are DCs, B cells and macrophages. These

cells are able to process the antigen into small peptide which suitable for presenting to lymphocytes. In addition, they have ability to express the required molecules which deliver additional signal for lymphocyte activation [47]. T lymphocytes are activated when T cell receptor (TCR) recognizes an antigenic peptide with MHC molecule which is located on antigen presenting cell surface. After recognition of presented antigen trough TCR, the intracellular signaling is generated lead to T cell activation and resulted in cell proliferation, production of cytokines, or killing target cells [48].

Effector T cells is divided by CD molecule on their cell surface into two subtypes. The first is $CD4^+$ T lymphocyte or T helper cells which can recognize presented antigen by MHC class II. T helper cells are divided to two types: T helper 1 (Th1) and T helper 2 (Th2) depend on the type of secreted cytokines. Th1 produce IL-2 to activate T cell proliferation and IFN-γ to activate macrophage. Th2 produce IL-4, IL-5, IL-6, IL-10 and IL-13 to activate B cells and promote growth of B cells. Indeed, T helper cell also includes Th17 and regulatory T cell (Treg) [47]. The second type of effector T cells is $CD8^+$ T lymphocytes or cytotoxic T cells (CTL) which can recognize presented antigen by MHC class I. Cytotoxic T cells can produce IL-2, IFN- γ and release perforins and granzymes to destroy the targets cells [11, 50].

T cells activation requires at least two signaling. The first comes from the stimulation by MHC-peptide complex binding to TCR and another comes from costimulatory molecules on APCs surface including B7-1 (CD80) and B7-2 (CD86) binding to CD28 on T cells [51]. Furthermore, binding of accessory molecules such as ICAM-1 on APC surface to LFA-1 on T cells help tightly binding between APCs and T cells. The generation of second signaling leads to complete T cell activation. Triggering of T cells by only one signal is not enough for T cell activation and they become anergic T cells and programmed to apoptosis [51-52].

After digestion of pathogen, macrophages become a professional-presenting cell to present digested pathogen by MHC class II to TCR receptor on $CD4^+$ T cells. Activated macrophages also express costimulatory molecules

on their cell surface to stimulate $CD4^+$ T cell together with TCR activation (Fig 7).

Costimulatory molecules and accessory molecules

B7-1 (CD80) and B7-2 (CD86) are type 1 transmembrane protein with a membrane distal IgV and a membrane proximal IgC domain. Many cell types express these molecules on their cell surface including dentritic cells, B cells and macrophages. Both molecules are able to bind with the same receptors, CD28 and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). The expression of B7-1 is induced slowly however it is expressed steadily for 4-5 day longer than B7-2. B7-2 is constitutively expressed and is rapidly upregulated to maximum expression at 48 h [53]. The expression of both molecules is promoted by IFN-γ and LPS. B7-1 and B7-2 function as potent costimulators to trigger T-cell proliferation and cytokines production. Professional APCs are able to express these molecules to interact with T-cells receptors [54]. Both CD28 and CTLA-4 are TCRs which interact with B7-1 and B7-2. However, B7-1 and B7-2 have high affinity for CTLA-4 receptor found on activated T-cells while they have a lower affinity for CD28 receptor constitutively expressed on resting T-cells [55].

ICAM-1 (CD54) is an adhesion molecule which binds to LFA-1 receptor on T cells. The interaction of ICAM-1 and LFA-1 is important in cell-cell interaction which promotes the grip between T cells and APCs. This molecule is widely expressed on many cell types including dentritic cells and macrophages. In addition, it is expressed on non-hematopoietic cells such as vascular endothelial cells, thymic and mucosal epithelial cells, and dermal fibroblasts [56]. It is a cell surface glycoprotein which is constitutively expressed on some tissues and can be induced by pro-inflammatory cytokines such as IL-1 or IFN-γ to upregurate and promote adhesion both in immunological and inflammatory responses [57].

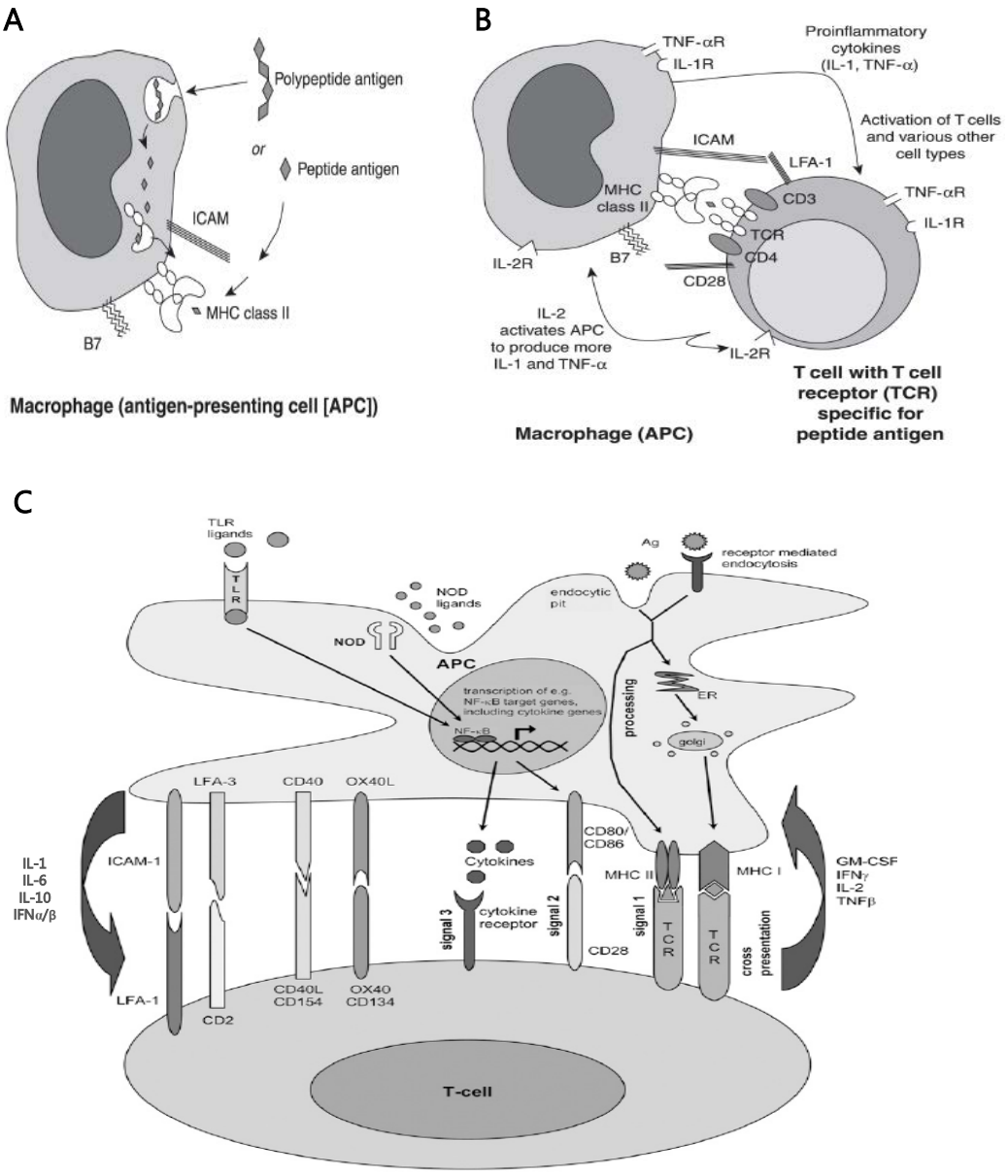


Figure 8: The presentation of antigens by macrophages. (A) Macrophages act as antigen-presenting cells by expression of beneficial molecules on their cell surface and (B) these molecules specifically bind to receptor on T cell [58]. (C) T cells are activated when they are triggered by at least two signals. [59].

Piper nigrum Linn.

Many of Thai herbs are valuable both as food and as traditional medicines. At present, several Thai traditional medicines are approved in the list of herbal medicinal products by National Drug Committee of Thailand. This recognition emphasizes the value of Thai traditional herbs. In addition, several herbs and remedy drug of Thailand are studied to find the novel pharmacological effects and to ensure drug efficiency.

Piper nigrum (*P. nigrum*) or black pepper is well known in Thailand not only as spice food but also as medicinal herbs. *P. nigrum* has pungent property and is widely used as a seasoning in many countries including Thailand. It is a member of genus Piper and family Piperaceae. *P. nigrum* is the most well known species in more than 1000 species of genus piper. It has several groups of chemical components including phenolics, lignans, terpenes, chalcones, flavonoid, alkaloid and steroids [10]. The alkaloid components including Piperine, Piperidine and Piperittine are contained in *P. nigurm* seeds for about 5-9%. In addition, it contains volatile oil for about 1-2.5%. The major volatile oil constituents found in *P. nigrum* are α and β-pinene, limonene and phellandrene [60-61].

Both fresh and mashed dried seeds of *P. nigrum* have been used as a food spice. *P. nigrum* can be classified as black pepper, white pepper and green pepper. The black pepper is green dried seeds included pericarp while white pepper is prepared by removing pericarp. In folk medicine, *P. nigrum* is regarded as an aromatic and carminative. Moreover, white pepper can be used for treatment of cholera, malaria and stomachache while black pepper can be used for treatment of abdominal fullness, adenitis, cancer, cholera, cold, colic, gravel and headache [63].

P. nigrum is monoecious plant and has stout climber and rooting at nodes. Its petiole grooved is about 0.8-1.5 cm. long. The leaf is egg shape and blade fleshy coriaceous, about 4-6 cm. wide and about 9-11 cm. long. The flower of *P. nigrum* is consists of spikes compose of male and female flowers together and it is about 5-13 cm. long. The fruit is also spike which is long

about 7-10 cm. Its fruits are drupe globose and arranged loosely on rachis. The flowering and fruiting are on the year round. It distributed in Thailand and native to South-eastern Asia [63].

It has been shown that *P. nigrum* contains various compounds with several pharmacological effects. *P. nigrum* water extract has antimicrobial effects on both Gram positive and Gram negative bacteria including *Bacillus subtilis, Bacillus sphaericus, Staphylococcus aureus, Klebssiella aerogenes* and *Chromobacterium violaceum violaceum* [64] as well as antimicrobial effects on *Klebsiella pneumonia* and *Pseudomonas aeruginosa* which isolated from oral cavity [65]. Furthermore, *P. nigrum* which extracted by other solvents including ether and ethanol also have antimicrobial effect on *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi* [66]. The fractionations of *P. nigrum* including water, ethanol, methanol, acetone, hexane and butanol have antimicrobial effects on oral candida in candiditis patients [67].

P. nigrum also has antioxidant properties both *in vivo* and *in vitro* studies. The dried powder of *P. nigrum* pods (black pepper) used as supplement with high-fat diet has ability to maintain enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferese (GST) and glutathione (GSH)) levels in various tissue including liver, heart, kidney, intestine and aorta to the level closed to those of normal rats and these enzyme levels were lower than those of rats fed only a high-fat diet [68] as well as antioxidant and anti-atherogenic effects in hamsters which induced with high cholesterol diet [69]. The water and ethanol extracts of *P. nigrum* have antioxidant activity in *in vitro* tests [70] as well as many essential oils from *P. nigrum* such as β-caryophylline, β-pinene and subinene [71].

Both anti-inflammatory and immunostimulatory effects of *P. nigrum* extract have been shown. The DMSO extract of *P. nigrum* inhibited pro-inflammatory cytokines including IL-6 and TNF-α as well as decreased the expression of iNOS and cyclo-oxygenase-2 (COX-2) in LPS-stimulated

macrophages. It also increased IL-10 which is anti-inflammatory cytokine in activated macrophages [72]. In addition, piperine, the important active compound of *P. nigrum* inhibited NO and TNF-α production in LPS-stimulated macrophage and Concanavalin-A (Con-A) stimulated-Balb/c mice [73]. Moreover, piperine also decreased nociception in carrageena-induced paw hyperalgesia and arthritic symptoms in carrageenan-induced arthritis in rat as well as decreased inflammatory area in rat ankle joints. While in *in vitro* model, piperine decreased the level of IL-6, matrix metalloproteinase 13 (MMP 13), COX-2 and prostaglandin E_2 (PGE_2) in IL-1β-stimulated fibroblast-like synoviocytes derived from rheumatoid arthritis patients [74]. Anti-inflammatory effects of piperine both in *in vitro* [72-74] and *in vivo* have also been reported in several studies [74-77].

However, some reports showed immunostimulatory effects of *P. nigrum* extract. In India, *P. nigrum* is the component of ACII remedy. ACII composes of *Syzygium aromaticum* (flower buds), *Mesua ferrea* (flower buds), *Elettaria cardamomum* (seeds), *Piper longum* (seeds), *Zingiber officinale* (rhizome), *Withania somnifera* (rhizome) and *Piper nigrum* (seeds). It increased circulating antibody titer, plaque forming cells (PFC) and WBC count as well as improved bone marrow cellularity in radiation-induced immunosuppressive mice [76]. Furthermore, this remedy also induced antibody-dependent cellular cytotoxicity (ADCC) by NK cells in normal and tumor-bearing mouse. In addition, it also induced T lymphocyte proliferation and elevated level of IL-2, TNF-α and IFN-γ in normal mice serum [79].

Moreover, aqueous extract of *P. nigrum* was served immunostimulatory effects by enhanced splenocyte proliferation and induced IFN-γ production by mouse splenocyte. It also induced pro-inflammatory cytokine production including IL-6, TNF-α as well as NO production in peritoneal mouse macrophages [80].

The immunological effects of *P. nigrum* extract are still under dispute. Although *P. nigrum* have been shown to possess both anti-inflammatory and immunostimulatory effects, its immunostimulatory effect has been shown

albeit that this effect was mainly studied in *in vivo* models. Therefore, the purpose of my study is to further explore the other side of immunomodulatory effect of *P. nigrum*.

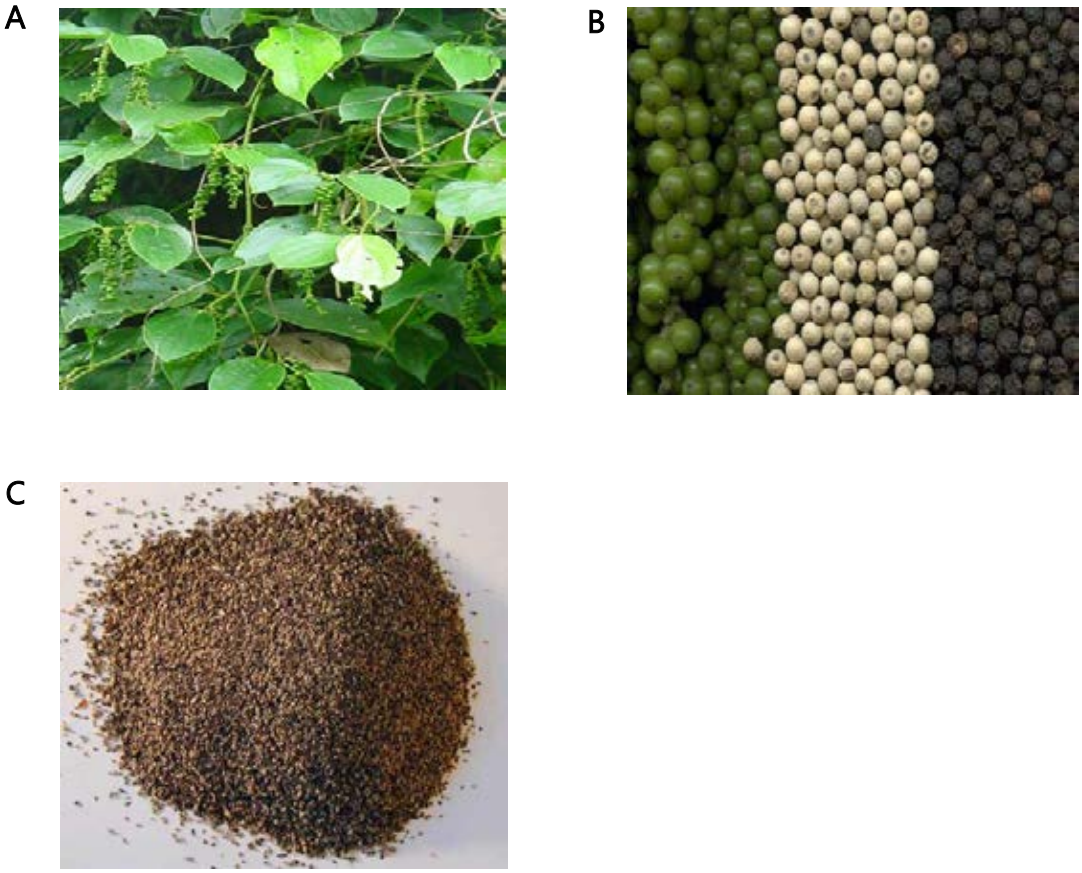


Figure 9: *Piper nigrum* Linn. (A) The appearance of *P. nigrum* Linn. (B) The *P. nigrum* seeds in form of green pepper or pepper pod, white pepper and black pepper. (C) The black pepper powder.

In the present day, immunomodulation is involved in many fields under concept of immunotherapy such as vaccination and cancer therapy. For cancer therapy, its concept is based on induction of cell-mediated immunity against tumor cells which using one's own immune system to specifically target cancerous cells without parallel injury to normal tissue and set up the collection of memory cells which can prevent relapses in the future [79]. The method used in tumor immunotherapy is the combination of tumor cell preparation and the adjuvants to potentiate anti-tumor vaccine effects. The advantage of this method is the ability to restrain tolerance to self, and to induce potent responses and durable of $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells to engage the tumor cells [9].

Adjuvant immunotherapy

These adjuvants can be divided into 4 major groups as following,

1. Toll-like receptor (TLR) agonists

TLR activation generates several inflammatory cytokines, MHC and costimulatory molecules that connect the innate and adaptive immunity [82]. Food and Drug Administration (FDA) approved TLR agonists including;

- TLR-7 agonist (imiquimod) for treatment of superficial basal cell carcinoma, genital warts and actinic keratosis [83].
- TLR-2 agonist [Bacillus Calmette-Guerin (BCG)] prepared from inactive *Mycobacterium bovis.* BCG is used as an immunotherapeutic against bladder cancer [9].
- TLR-4 agonist [monophosphoryl lipid (MPL) A] which is a derivative of the lipopolysacharide (LPS) from *Salmonella Minnesota.* Its toxicity is reduced by removal of specific groups on the sugar moieties or alteration of the amount and length of acyl side chain. The products of MPL® including Fendrix® for HBV and Cervarix® for HPV contain the combination of alum and MPL®. Furthermore, the new generations of TLR-4 agonists are developed [84].

2. Non-specific immunomodulators

- Montanide ISA 51 and 720 are water-in-oil emulsions which consisted of mineral oil mixed with surfactant mannide mono-oleate. Several studies showed that they induced high antibody titers and CTL responses. Furthermore, in phase I and phase II trials, Montanide ISA 51 and 720 are found to be save and well-tolerated for using as vaccines against malaria, HIV and several cancers [9].

- Alum or Aluminium salt (Aluminum phosphate and aluminum hydroxide) is effective in activating macrophages to produce cytokines and chemokines, promoting phagocytosis and enhancing the expression of MHC class II which is necessary for antigen presentation. Alum is used in the composition of FDA-approved vaccines including diphtheria-pertussis-tetanus (DPT), diphtheria-tetanus (DT), DT combined with Hepatitis B virus (HBV), Haemophilus influenza B or inactivated polio virus (IPV), hepatitis A (HAV), Streptococcus pneumonia, meningococcal and human papilloma virus (HPV) [84].

3. Cytokines

- Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) a cytokine that functions as a white blood cell growth factor. It stimulates differentiation and proliferation of hematopoietic progenitor cells and maturation of professional APCs including DC, monocytes and macropahges. Furthermore, it also promotes antigen presentation of APCs to cytotoxic T cells. GM-CSF is now in phase I clinical trials for using as an adjuvant combined with chemotherapy to treat ovarian cancer patients [9]. GM-CSF was approved by FDA to be used as a medication to stimulate the production of white blood cells following chemotherapy. In addition, other cytokines including TNF-α, IFN- α and IL-2 were approved by FDA for treatment of soft tissue sarcoma, renal cell carcinoma and melanoma [83].

4. Immunostimulatory antibodies

- Anti-CD antibodies has been studied in phase I clinical trials by using anti-CD40 monoclonal antibody (mAb) CP-870,893. It is well-tolerated in treatment of patients with solid tumors [9].

This study reveals the screening of new plant which may be used for immunostimulatory agent. In addition this study also showed the other sides of the Thai traditional herb effects. It may support the usage as well as ensure the efficiency of Thai herb especially *P. nigrum*. This study may bring about to benefit in developing of the new immunostimulant drug in the future.

CHAPTER III

MATERIALS AND METHODS

Materials

Plant extract

The powder of *Piper nigrum* was prepared by Puangmalai Clinic, the thai traditional medicine clinic at Phetchaburi province. The seeds of this plant from Chanthaburi province, Thailand were basked in the sunshine before grinding included peels. After that, it was extracted with water by Faculty of Science, Kasetsart University. The method of the extraction is showed as follow.

The dried powder of *P. nigrum* 30 g was heated in distilled water 300 ml at 60°C for 2 h. The solution was filtered through a 5 µ filter and lyophilized. The yield of this extraction was 2.38%. The lyophilized powder was stored in closed container at -20°C until used. The powder was dissolved in sterile deionized water as the stock solution. This stock solution was diluted to various concentrations in incomplete Dulbeco's Modified Eagle's Medium (DMEM) for the experiments.

Cell culture

The mouse macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in 5%CO_2/95% air. The cells were subcultured three times weekly during use.

The viability of cells was determined by 0.4% trypan blue. The viability of cells not less than 85% was used for experiments.

Equipments and Instruments

The following equipments and instruments were used in this study; autopipette (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 machin (Eppendorf, USA) และ vortex mixer (Scientific industries, USA)

Chemicals and reagents

The following reagents were used in this study; dimethyl sulfloxide (DMSO) (Sigma, USA), Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), nitroblue tetrazolium (NBT) (Sigma, USA), resazurin (Sigma, USA), zymosan A from *Saccharomyces cervisiae* (Sigma, USA), lipopolysaccharide (Sigma, USA), nitric oxide assay kit (Promega, USA), penicillin/streptomycin (Gibco, USA) 0.4% trypan blue dye, TRIzol® reagent (Invitrogen, UK), chloroform (Sigma, USA), DEPC (Molekula, UK), ImProm-II™ reverse transcription system (Promega, USA), primer (Bio Basic, Canada), Taq polymerase (Vivantis, USA)

Methods

1. Determination of NO production

The effect of *P. nigrum* water extract on NO production by macrophage J774A.1 cells was determined by Griess reaction assay [85]. The results were obtained from more than three-independent experiments. The experiment details are explained below:

1. Incubate J774A.1 cells at 135 µl/well ($4x10^5$cells/ml) in 96-well plates for 24 h at 37°C, 5% CO_2 / 95% air.

2. Treat cells with *P. nigrum* extract 1.56-100 µg/ml and then incubate for 24 h. Incomplete DMEM and 100 ng/ml of lipopolysaccharide (LPS) were used as the negative and the positive controls, respectively.
3. Collect the supernatant for measuring NO concentration in nitrite form by Griess reaction assay as in the following procedures.
   - Aliquot 100 µl of the supernatant from each well into 96-well plate.
   - Add 20 µl of sulfanilamide to each well and incubate for 10 min in the dark at room temperature.
   - Add 20 µl of NED (*N*-1-napthylethylenediamine dihydrochloride) and further incubate for 10 min in the dark at room temperature.
   - Measure the changed color by microplate reader at the absobance 540 nm.
   - Calculate the NO concentration from sodium nitrite standard curve. The standard nitrite prepare by using 1.56-100 µM of standard nitrite solution in two-fold dilution.

2. Determination of cell viability

The effect of *P. nigrum* water extract on cell viability after treating cells by resazurin reduction assay was determined as the following procedures.

1. Remove supernatant from the treated cells.
2. Add 100 µl of fresh complete DMEM medium containing resazurin 50 µg/ml
3. Incubate for 2 h at 37°C, 5%CO_2/95% air
4. Determine the reduced resazurin to resorufin by viable cells by measuring absorbance at 570 and 600 nm using microplate reader.
5. Calculate the percentage of viable cells by comparing with control using the following formula.

$$\% \text{ viability} = \left(\frac{(OD_{570} - OD_{600})_{sample}}{(OD_{570} - OD_{600})_{DMEM}} \right) \times 100$$

3. Determination of phagocytic activity

The effect of *P. nigrum* water extract on phagocytic activity in macrophage J774A.1 cells was determined by zymosan-nitroblue tetrazolium (NBT) reduction assay. The results were obtained from at least three-independent experiments.

1. Incubate J774A.1 cells at 135 µl/well of $4x10^5$cells/ml in 96-well plates for 24 h at 37°C, 5%CO_2/95% air.
2. Treat cells with 15 µl/well of 1.56-100 µg/ml of *P. nigrum* water extract and then incubate for 24 h. Incomplete DMEM and 100 ng/ml lipopolysaccharide (LPS) were used as the negative and the positive controls, respectively.
3. Carefully remove supernatant and then wash the cells twice with DMEM
4. Add 800 µg/ml of zymosan and 600 µg/ml of NBT in each well and further incubate at 37°C for 1 h.
5. Wash the cells three times with 200 µl of methanol.
6. Vigorously add 120 µl of 2M KOH and 140 µl of DMSO
7. Determine NBT reduction by measuring the absorbance at 570 nm by microplate reader.
8. Calculate the percentage of phagocytosis stimulation using the following formula.

$$\% \text{ Phagocytosis stimulation} = \left(\frac{OD_{570sample} - OD_{570control}}{OD_{570control}} \right) \times 100$$

4. Determination of mRNA expression of dectin-1, lysozyme M, enzyme iNOS, pro-inflammatory cytokines, accessory molecule and co-stimulatory molecules

The effects of *P. nigrum* water extract on the mRNA expression of involving molecules in phagocytic activity (dectin-1 and lysozyme), pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), accessory molecules (ICAM-1) and co-stimulatory molecules (B7-1 and B7-2) were determined using the following procedures.

4.1 Treat J774A.1 cells with the water extract

- Incubate J774A.1 cells 450 µl/well ($4x10^5$cells/ml) in 24-well plates for 24 h at 37°C, 5% CO_2 / 95% air.
- Treat the cells with *P. nigrum* water extract 12.5-50 µg/ml and then incubate for 24 h. Incomplete DMEM and 100 ng/ml of LPS were used as the negative and the positive controls, respectively.

4.2 Isolate total RNA from the treated cells

- Remove supernatant from the treated cells.
- Add 500 µl of TRIzol® reagent in each well then lyse and homogenize the treated cells.
- Incubate at room temperature for 5 min.
- Transfer the homogenized samples to eppendorf tubes.
- Add 200 µl of chloroform to each tube and vigorously shake for 15 seconds.
- Incubate tubes at room temperature for 5 min then centrifuge at 12,000 g for 15 min at 4°C.
- Carefully collect the aqueous phase from each tube to a new eppendorf tube.
- Add 500 µl of isopropanol to each eppendorf tube of aqueous phase.
- Mix and incubate at room temperature for 30 min.
- Separate the RNA pellets by centrifugation at 12,000 g for 10 min at 4°C
- Remove the supernatant and wash the pellets with 1 ml of 75% ethanol by centrifugation at 7,500 g for 5 min at 4°C.
- Air-dry the pellets and dissolve it in DEPC treated water.
- Measure total RNA concentration and contamination of each sample by Nanodrop at the absorbance 260 and 280 nm.
- Store the RNA samples at -70 °C until use.

4.3 Synthesis cDNA by reverse transcription

- Prepare 1.5 µg of total RNA of each sample and mix with 1 µl oligo dT_{15} primer in nuclease-free water in the 200 µl thin layer tubes.
- Heat all tubes at 70°C for 5 min
- Immediately keep the tube on ice for 5 min
- Prepare reverse transcription mixture solution containing 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor and reverse transcriptase.
- Add 15 µl of mixture solution into each tube of samples
- Put the tubes in a thermocycler machine for cDNA synthesis with following condition ; 25°C for 5 min then 42°C for 1 h and 30 min, and follow by 70°C for 15 min at final.
- Store the cDNA samples at -20°C until use.

4.4 Amplify cDNA by polymerase chain reaction (PCR) with specific primers

- Prepare PCR reaction mixture containing primer (Table 1) and mix with dNTP, Taq polymerase in PCR buffer.
- Mix PCR reaction mixture and 1 µl of cDNA in the 200 µl thin layer tubes.
- Put them in the thermocycler machine using the following condition described in table 2.
- Mix 8 µl of PCR products with 2 µl of loading dye and run on 1.5% agarose gel electrophoresis at 100 volt for 60 min in TBE buffer.
- Stain the agarose gel with 0.5 µg/ml of ethidium bromide in TBE buffer for 3 min and de-stain the gel with TBE buffer for 30 min
- Identify and analyze the PCR product densities by gel-documentation.

Table 1: Specific primers for PCR

Gene		Primer sequences	Fragment size (bp)
TNF-α	F	5′- TTGACCTCAGCGCTGAGTTG-3′	364
	R	5′- CCTGTAGCCCACGTCGTAGC-3′	
IL-1β	F	5′- CAGGATGAGGACATGAGCACC-3′	447
	R	5′- CTCTGCAGACTCAAACTCCAC-3′	
IL-6	F	5′- GTACTCCAGAAGACCAGAGG-3′	308
	R	5′- TGCTGGTGACAACCACGGCC-3′	
iNOS	F	5′- CCCTTCCGAAGTTTCTGGCAGCAGC-3′	496
	R	5′- GGCTGTCAGAGCCTCGTGGCTTTGG-3′	
Dectin-1	F	5′- AGGCCCTATGAAGAACTACAGACA-3′	447
	R	5′-TGGCCAGACAGCATAAGGAAAC-3′	
Lysozyme M	F	5′-GAATGGCTGGCTACTA-3′	189
	R	5′-TCCCACAGGCATTCACA-3′	
ICAM-1	F	5'-TCTCGGAAGGGAGCCAAGTAA-3'	272
	R	5'-CTCTTGCCAGGTCCAGTTCC-3'	
B7-1 (CD80)	F	5′-TGGTGCTGTCTGTCATTG-3′	650
	R	5′-GGTAAGGCTGTTGTTTGTT-3′	
B7-2 (CD86)	F	5′-CAGTCAGGATGGGAGTGG TA-3′	435
	R	5′-TTGAGTACTTGGCTGTCTTA-3′	
β-Actin	F	5′-GTGGGCCGCCCTAGGCACCAG-3′	603
	R	5′-GGAGGAAGAGGATGCGGCAGT-3′	

Table 2: PCR condition

Gene	Denature	Annealing	Extension	Cycles
Dectin-1	T 94°C 30 sec	T 65°C 30 sec	T 72°C 30 sec	35
Lysozyme M	T 94°C 10 sec	T 60°C 10 sec	T 72°C 15 sec	35
iNOS	T 94°C 30 sec	T 60°C 45 sec	T 72°C 1 min	25
TNF-α	T 94°C 30 sec	T 53°C 45 sec	T 72°C 1 min	25
IL-1β	T 94°C 30 sec	T 57°C 45 sec	T 72°C 1 min	30
IL-6	T 94°C 30 sec	T 57°C 45 sec	T 72°C 1 min	30
ICAM-1	T 94°C 60 sec	T 55°C 60 sec	T 72°C 1 min	30
B7-1	T 95°C 30 sec	T 57°C 30 sec	T 72°C 1 min	45
B7-2	T 95°C 30 sec	T 57°C 30 sec	T 72°C 1 min	45

Statistical analysis

Data are expressed as means ± standard error of means (S.E.M) from at least three independent experiments. Statistical significance of difference of the data was analyzed by One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test. $p < 0.05$ was regarded as statistically significant.

CHAPTER IV

RESULTS

1. Effect of *P. nigrum* water extract on NO production by macrophage J774A.1 cells

By oxygen-dependent pathway to kill engulfed pathogens after phagocytosis, NO is generated and plays role in destroying pathogens. Moreover, NO is commonly parameter which used as a marker for macrophage activation. Therefore, it is the first parameter that used to assess ability of the *P. nigrum* water extract on macrophage cell activation.

The water extract of *P. nigrum* at concentration of 1.56 µg/ml was able to increase NO production in macrophage J774A.1 cells. This extract at 3.125-100 µg/ml significantly enhanced NO production in a concentration-dependent manner (Fig.10). The water extract of *P. nigrum* exhibited maximum effects at 25 µg/ml.

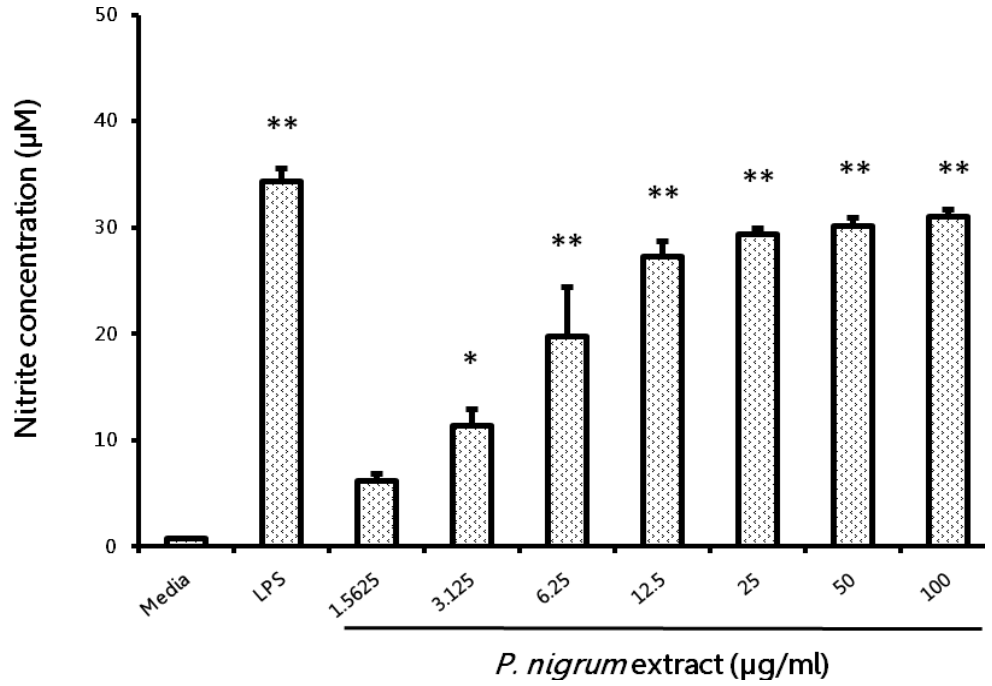


Figure 10: Effect of *P. nigrum* water extract on NO production in macrophage J774A.1 cells. Cells were treated with 1.5625-100 µg/ml of extract or LPS (100 ng/ml) for 24 h. Nitrite levels (µM) in supernatant were determined by using Griess reagent. The concentration of nitrite in each condition was calculated from sodium nitrite standard curve. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). **p<0.05, **p<0.01* compared to untreated control (media).

2. Effect of *P. nigrum* water extract on cell viability

For selecting the suitable concentration of the water extract of *P. nigrum* and testing the possible cytotoxicity of the extract, the effects to the cell viability was assessed by resazurin reduction assay. The viable cells are able to reduce the blue color of resazurin to pink color of resorufin by mitochondrial reductase enzyme. The cell viability was determined after incubating cells with the various concentrations of the water extract of *P. nigrum* or 100 ng/ml of LPS for 24 h.

The results revealed that the *P. nigrum* water extract at all concentration tested had no effects on the viability of J774A.1 cells (Fig.11). Thus, the *P. nigrum* water extract has no cytotoxic effect and all concentrations of the extract were suitable for experiments.

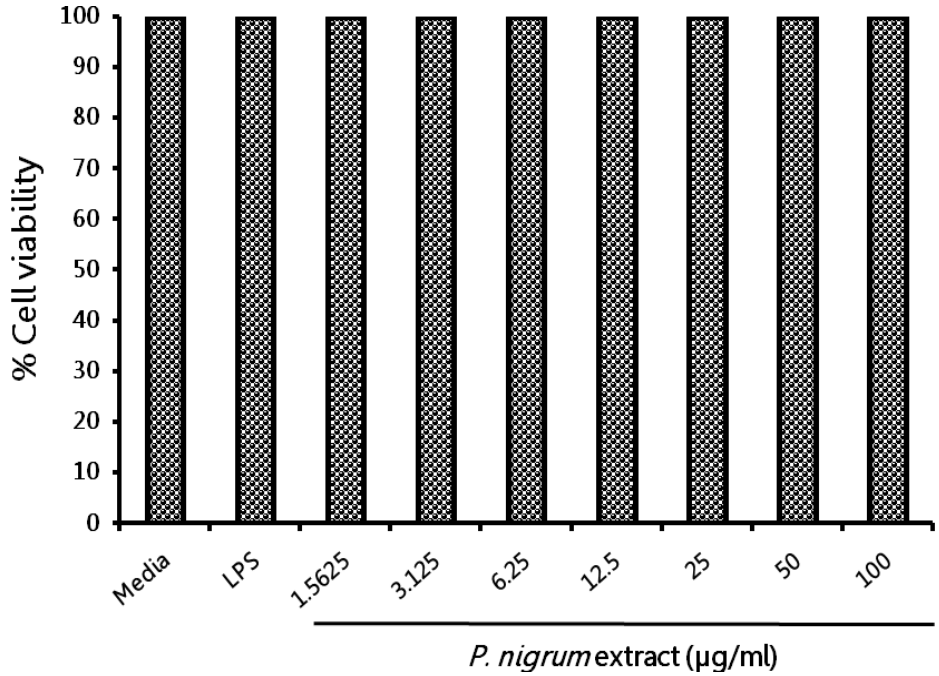


Figure 2: The effect of the *P. nigrum* water extract on macrophage J774A.1 cell viability. The cells were treated with the water extract of *P. nigrum* or 100 ng/ml of LPS for 24 h. The viability of the treated cells was determined by resazurin reduction assay. The data are expressed as the mean ± S.E.M. from 3 independent experiments (n=3).

3. Effect of *P. nigrum* water extract on phagocytic activity of macrophage J774A.1 cells

Macrophages are major tissue phagocytes which have phagocytic ability to engulf and destroy foreign particles or pathogens. In this experiment, the phagocytic activity was determined by using zymosan-nitroblue tetrazolium (NBT) reduction assay. During phagocytosis processes, respiratory burst is occurred and increased the production of hydrogen peroxide (H_2O_2) and superoxide free radical. The free radical is the reducing agent to reduce the yellow color of NBT dye to the blue color of formazan.

The results showed that the water extract of *P. nigrum* at 3.125, 6.25, 12.5, 25, and 50 µg/ml significantly promoted zymosan phagocytosis by macrophage J774A.1 cells in a concentration-dependent manner to 20.49%, 33.95%, 45.87%, 53.04% and 66.48%, respectively. However, its stimulatory effect decreased at 100 µg/ml of the extract to 31.33%. Nevertheless, at the concentration of 100 µg/ml, it significantly promoted macrophage phagocytosed zymosan when compared with untreated control. LPS (100 ng/ml), positive control, also increased macrophage phagocytosis (Fig. 12).

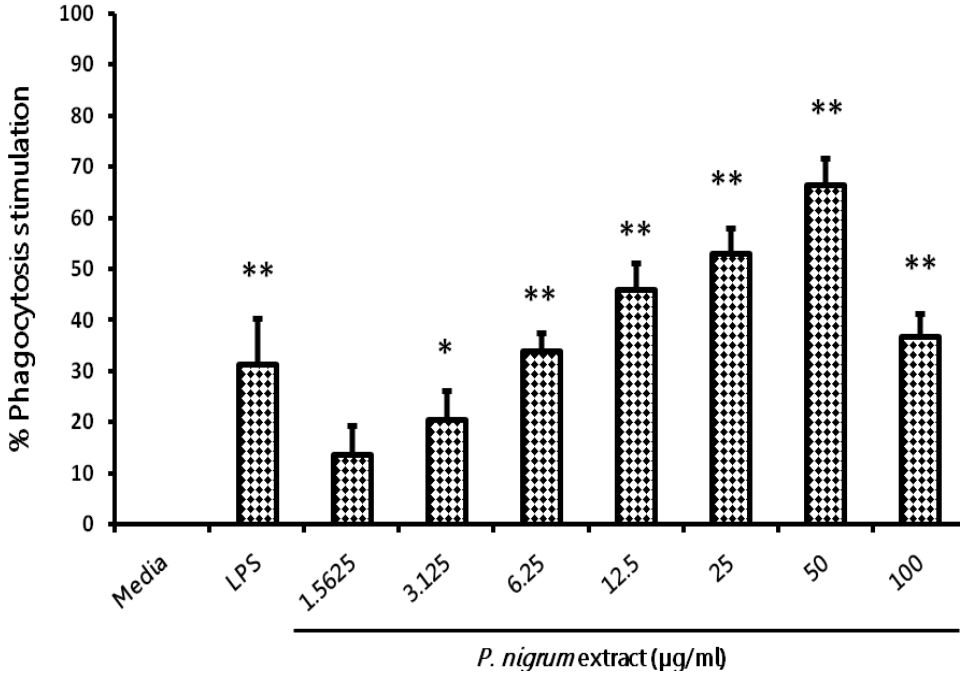


Figure 12: Effect of *P. nigrum* water extract on phagocytic activity in macrophage J774A.1 cells. Cells were treated with 1.56-100 µg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. The phagocytic activity of the treated cells was evaluated by zymosan-NBT reduction assay. The percentage of phagocytosis stimulation of the extract was compared to the solvent control. Data are expressed as mean ± S.E.M from 4 independent experiments (n=4). **p<0.05, **p<0.01* compared to untreated control.

4. Effect of *P. nigrum* water extract on dectin-1, lysozyme M and iNOS mRNA expression by macrophage J774A.1 cells

Macrophages can recognize pathogens by using varieties PRRs located on their cell surface and then engulf the pathogens into phagosome vesicles. These phagosomes fuse with lyzosomes and become phagolysosomes. The phagolysosome are vesicles for destroying the engulfed pathogens by using oxygen-dependent mechanisms such as NO and also oxygen-independent mechanisms such as enzymes which hydrolyse phatogens. Lysozyme is one of macrophage lysosomal enzyme which used for bacteriolysis.

Dectin-1 is one of PRRs located on macrophage cell surface. Dectin-1 recognizes β-glucan structure of yeast cell wall lead to phagocytosis of yeast cells.

NO is an inducible toxic molecule generated by iNOS enzyme after macrophages are activated. NO is one of O_2-dependent mechanism used by macrophages to kill the invaded microorganisms. NO combines with O_2^- to become peroxynitrite ($OONO^-$) which induces microbial toxicity.

By oxygen-independent mechanism, lysozyme M is an enzyme in macrophage lysosomal granules. It is upregurated for hydrolyzing engulfed bacteria.

The water extract of *P. nigrum* at 50 µg/ml significantly enhanced Dectin-1 mRNA expression, while this extract at 12.5 and 25 µg/ml had no effect on macrophages in the expression of Dectin-1 mRNA. In this experiment, LPS (100 ng/ml)-treated cells was not different from untreated control in the expression of Dectin-1 mRNA (Fig. 13).

The water extract of *P. nigrum* at concentration of 12.5, 25 and 50 µg/ml also significantly promoted the expression of lysozyme M mRNA in concentration dependent manner (Fig. 14).

Furthermore, the water extract of *P. nigrum* at 12.5, 25 and 50 µg/ml significantly promoted the expression of iNOS mRNA in concentration dependent manner (Fig. 15).

LPS-treated cells also significantly enhanced both the expression of lysozyme M and iNOS.

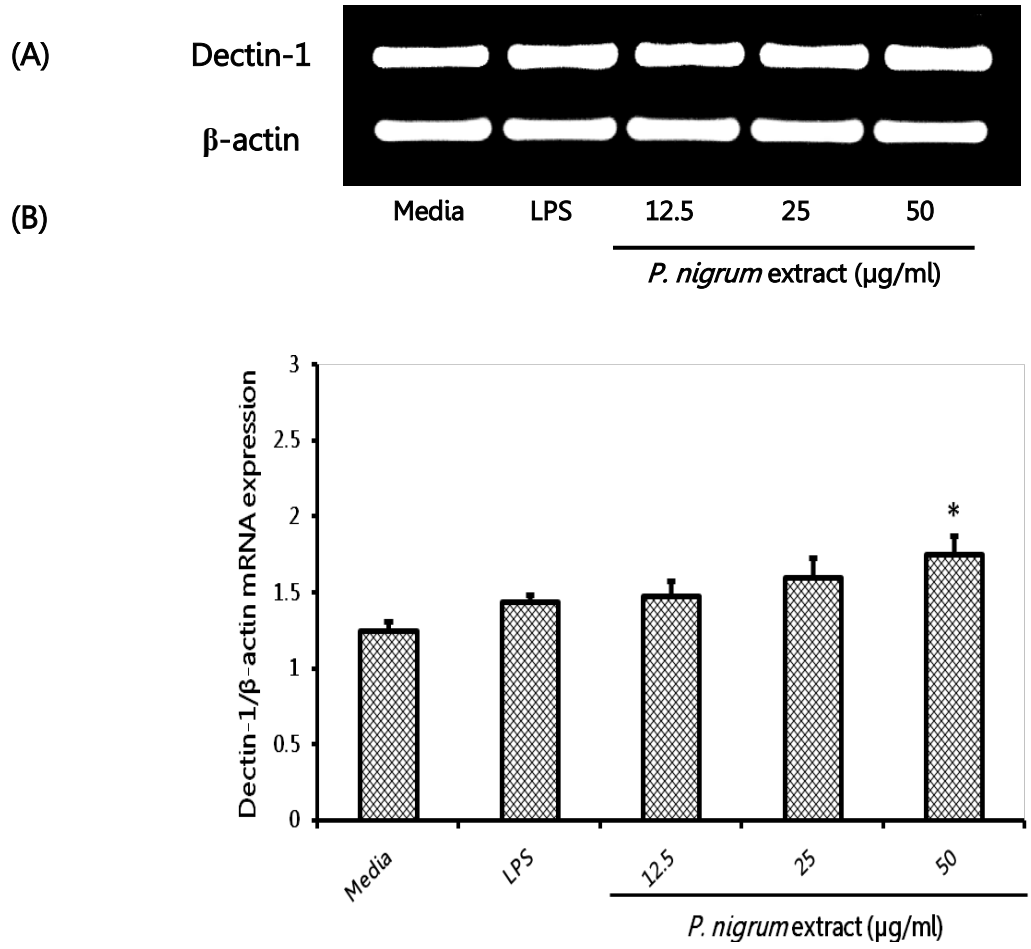


Figure 13: Effect of *P. nigrum* water extract on Dectin-1 mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from the treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of Dectin-1 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$*p<0.05$* compared to untreated control.

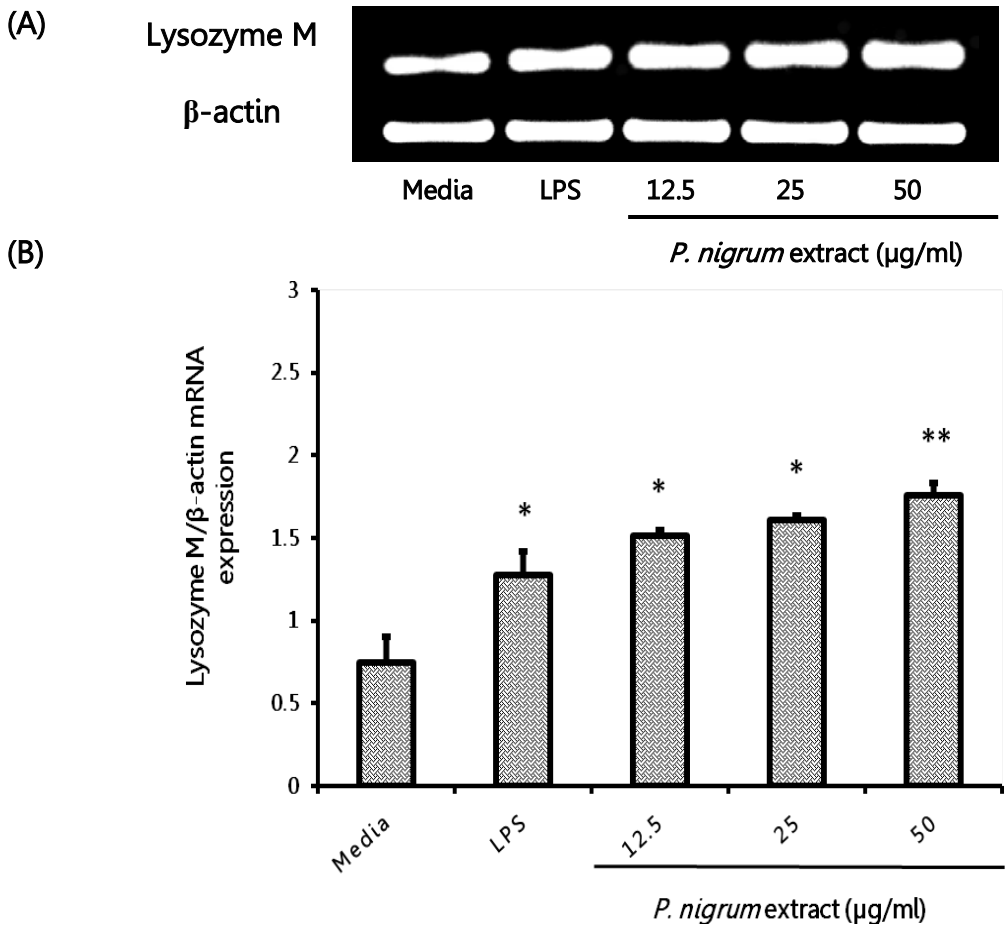


Figure 14: Effect of *P. nigrum* water extract on lysozyme M mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of the water extract of *P. nigrum* or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of lysozyme M with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). **p<0.05, *p<0.01* compared to untreated control.

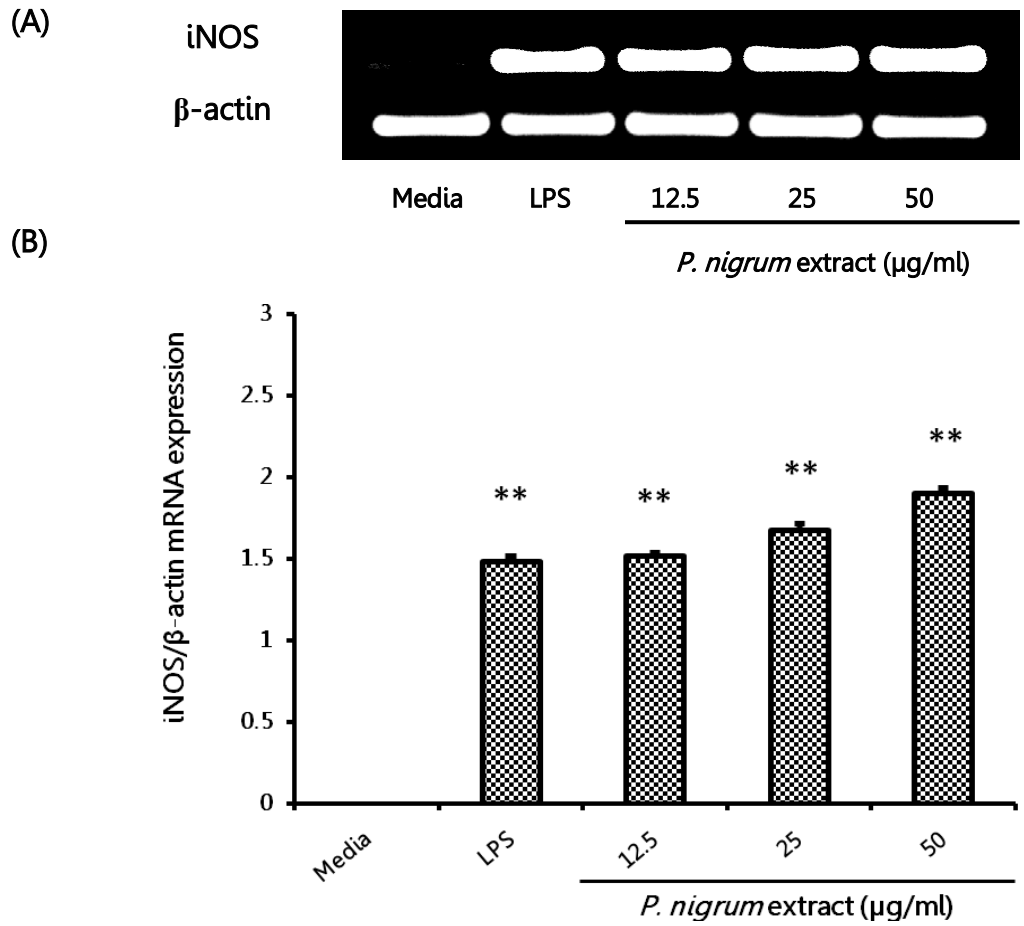


Figure 15: Effect of *P. nigrum* water extract on iNOS mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of the water extract of *P. nigrum* or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of iNOS with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). ***p<0.01* compared to untreated control.

5. Effect of *P. nigrum* water extract on pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α) mRNA expression in macrophage J774A.1 cells

After recognizing pathogens by pattern recognition receptors, macrophages to become activated cells with high ability to generate several cytokines and other immune mediators including pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α.

J774A.1 cells were activated by LPS, the positive control, to significantly express pro-inflammatory cytokines mRNA including IL-1β, IL-6 and TNF-α. The water extract of *P. nigrum* at concentration of 12.5, 25 and 50 µg/ml also greatly induced IL-1β, IL-6 and TNF-α mRNA expression in macrophage J774A.1 cells in a concentration dependent manner (Fig. 16, 17, 18).

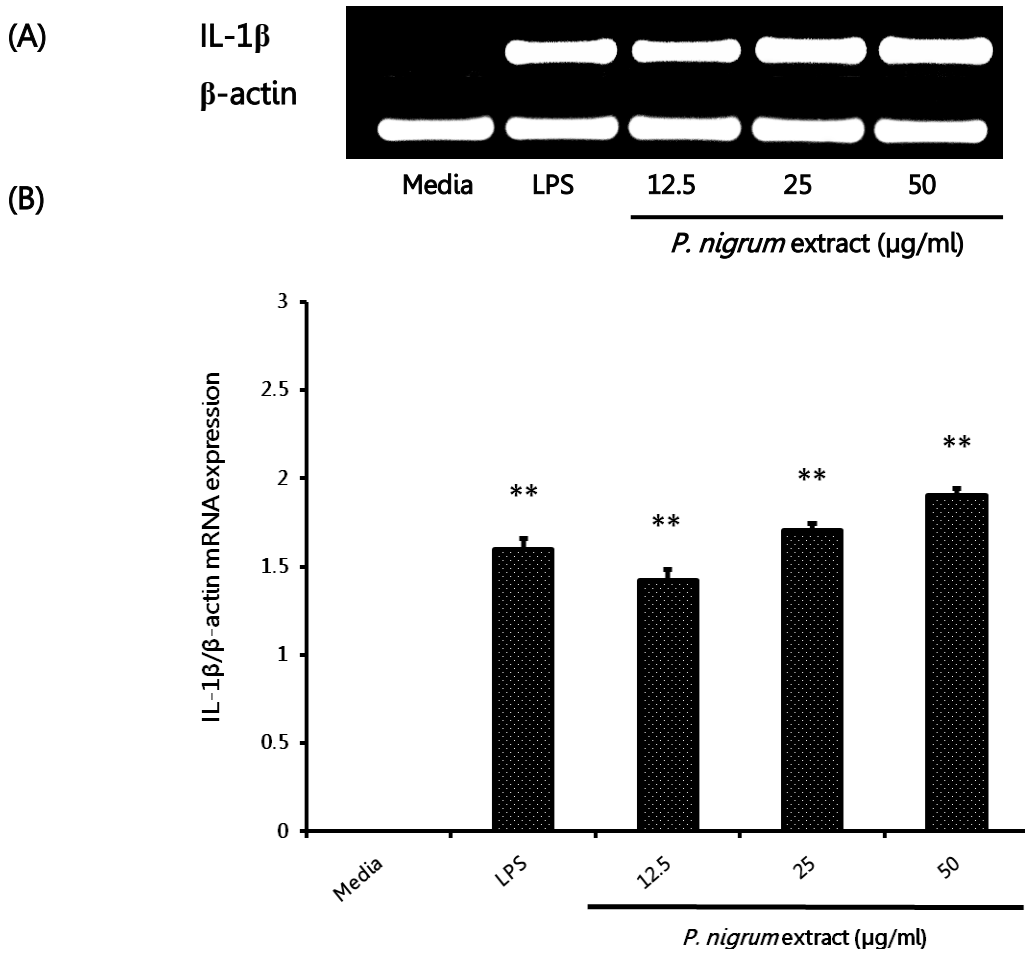


Figure 16: Effect of *P. nigrum* water extract on IL-1β mRNA expression of macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of IL-1β with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). ***p<0.01* compared to untreated control.

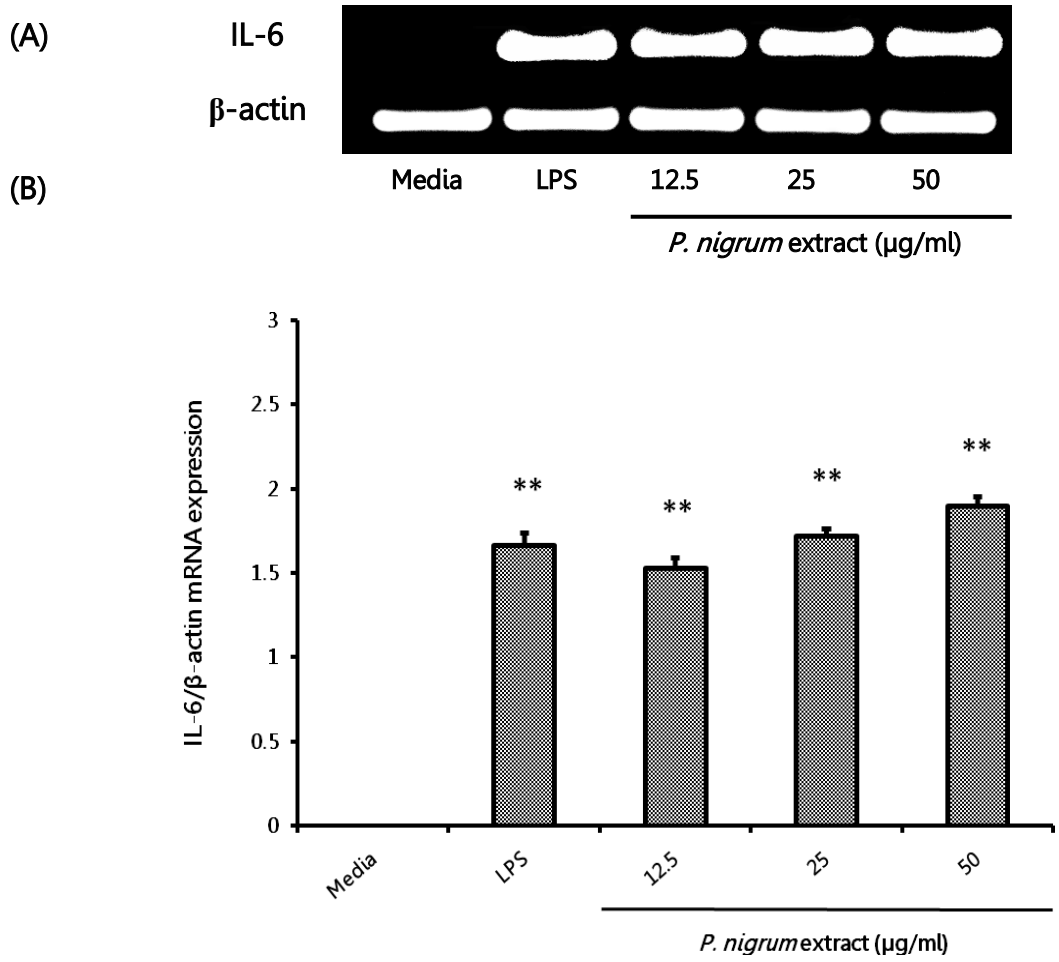


Figure 17: Effect of *P. nigrum* water extract on IL-6 mRNA expression on macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 µg/ml of extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of IL-6 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). **p<0.01* compared to untreated control.

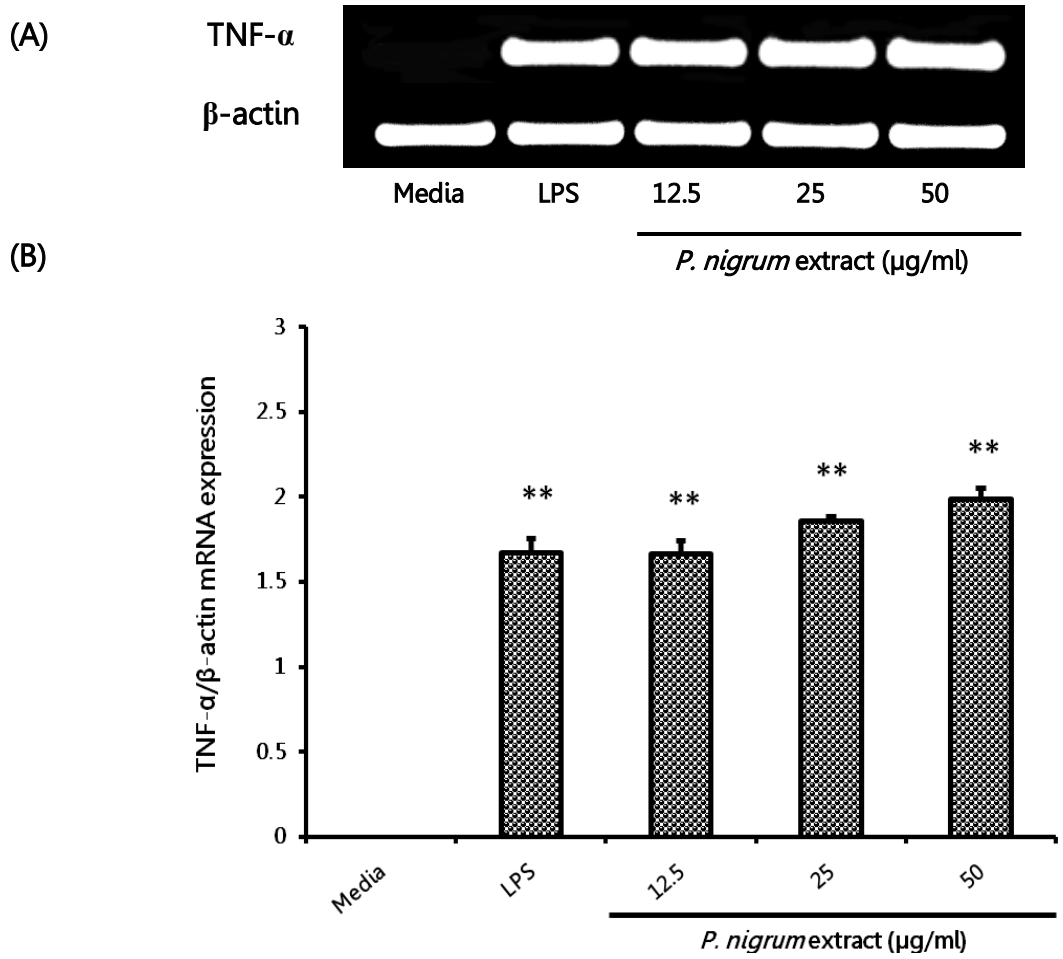


Figure 18: Effect of *P. nigrum* water extract on TNF-α mRNA expression on macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 µg/ml of extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of TNF-α 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 products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). **p<0.01* compared to untreated control.

6. Effect of *P. nigrum* water extract on accessory molecule (ICAM-1) and co-stimulatory molecules (B7-1 and B7-2) mRNA expression by macrophage J774A.1 cells

Activated macrophages are not only phagocytes but also function as antigen presenting cells (APCs) to present digested antigen to T cells. They express several molecules which participate in T cells activation including accessory molecule ICAM-1. ICAM-1 is adhesion molecule important for signaling between APC and T cells. Furthermore, B7-1 and B7-2 which are costimulatory molecules are important second signaling for T cell activation.

Both LPS and the extract significantly enhanced the expression of ICAM-1, B7-1 and B7-2 mRNA of J774A.1 cells. The *P. nigrum* water extract at 12.5, 25 and 50 µg/ml activated ICAM-1, B7-1 and B7-2 mRNA expression in concentration dependent manner (Fig. 19, 20, 21).

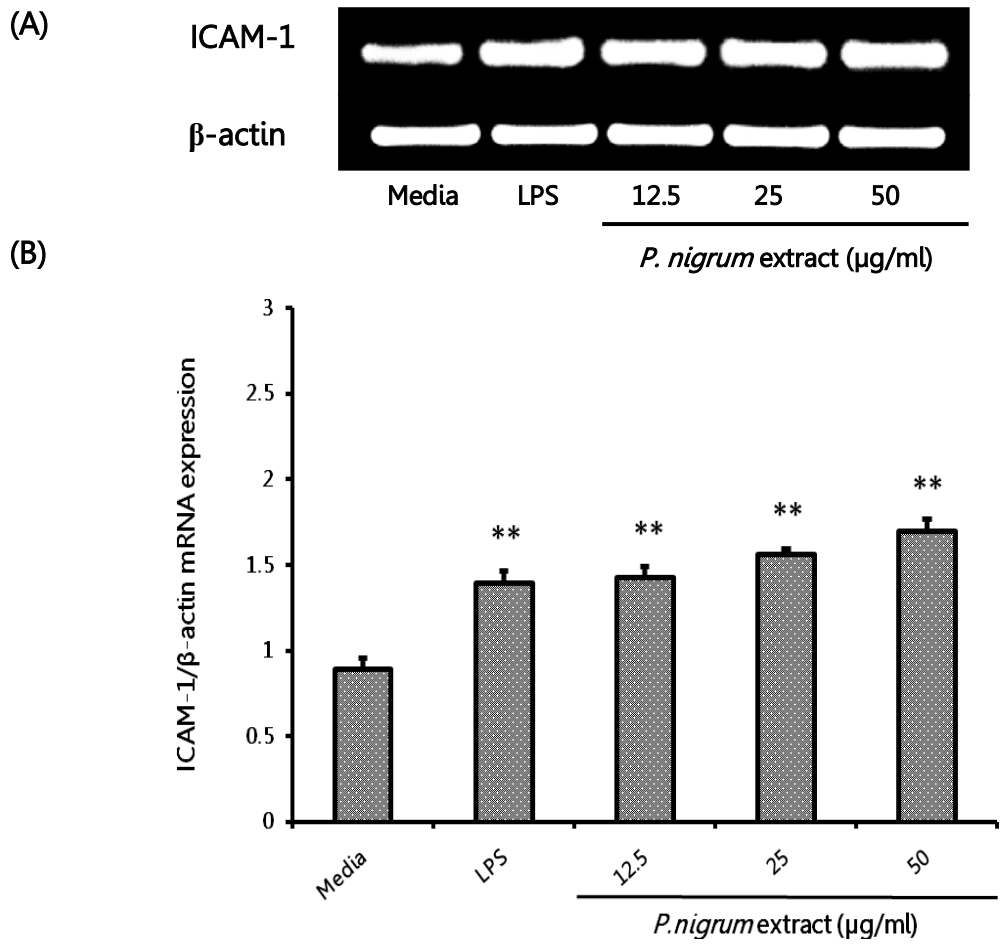


Figure 19: Effect of *P. nigrum* water extract on ICAM-1 mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of ICAM-1 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). ***p<0.01* compared to untreated control.

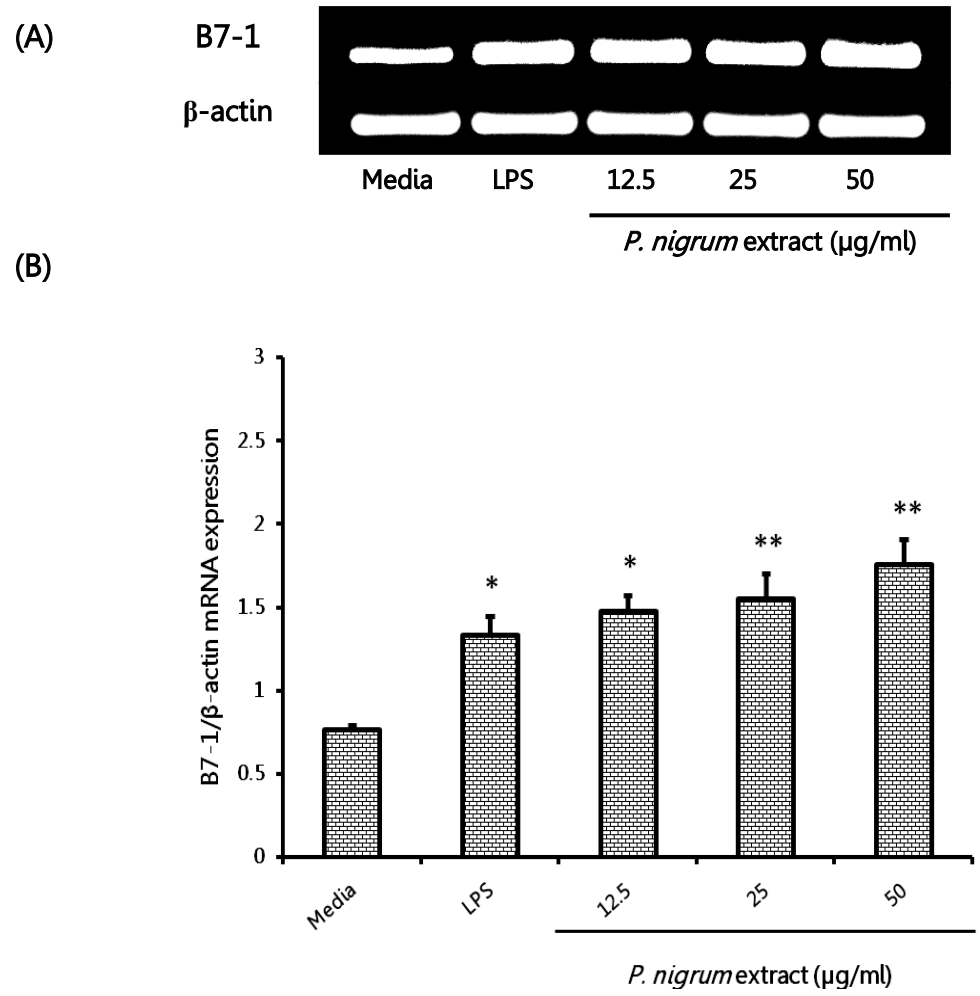


Figure 20: Effect of *P. nigrum* water extraction on B7-1 mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of B7-1 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). **p<0.05, **p<0.01* compared to untreated control.

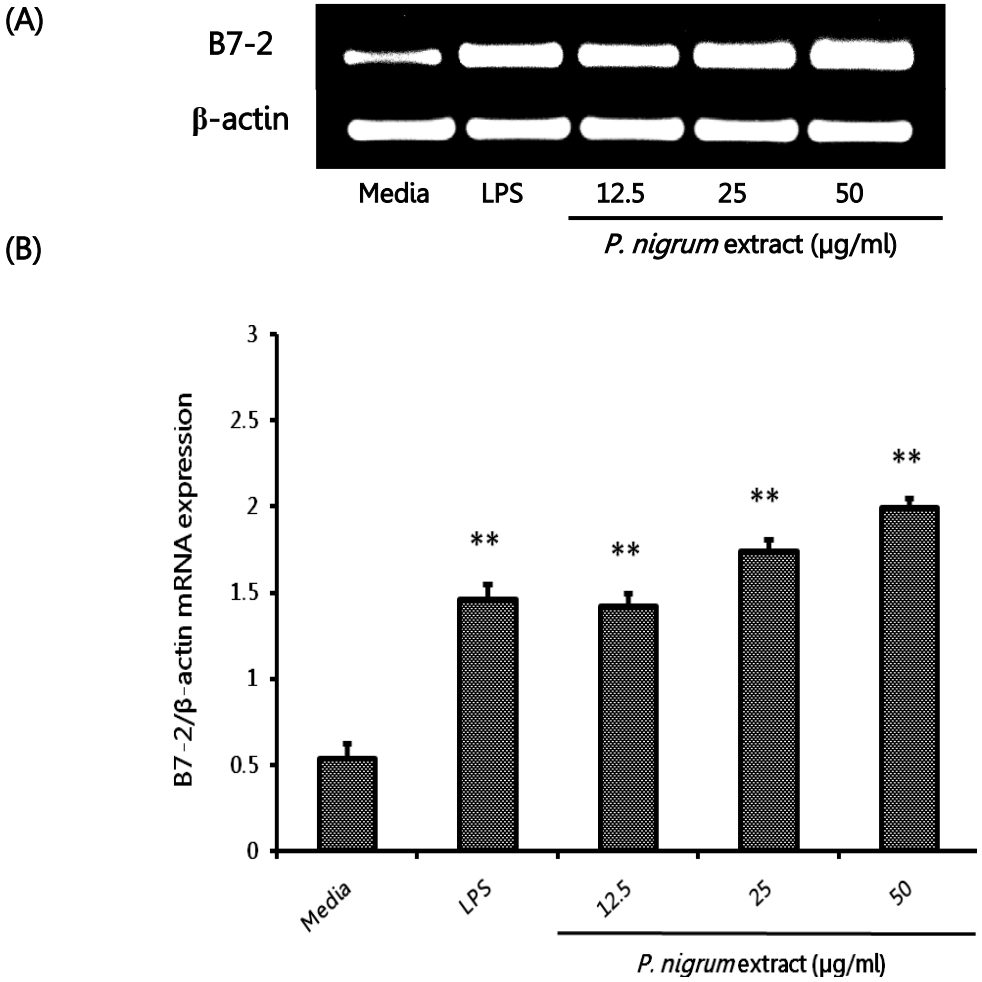


Figure 21: Effect of *P. nigrum* water extract on B7-2 mRNA expression in macrophage J774A.1 cells. Cells were treated with 12.5, 25 and 50 μg/ml of *P. nigrum* water extract or LPS (100 ng/ml) for 24 h. Total RNA was isolated from treated cells and reverse transcribed to cDNA. The cDNA was used as the template to amplify mRNA of B7-2 with specific primer by PCR. (A) The PCR products were detected by agarose gel electrophoresis and (B) the densities of PCR products were determined by using gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). ***p<0.01* compared to untreated control.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion and Conclusion

Immunomodulation involves in expressing, amplifying or inhibiting any part or phase of the immune responses by chemicals or biological which are called immunomodulators. There are two types of immunomodulators based on their effects on the immune system including immunosuppressants and immunostimulators. Immunosuppressants are used for suppressing unwanted or excessive immune reactions such as graft rejection from transplantation or autoimmune diseases. Immunostimulators are used for wanted immune responses such as reconstitution of immune deficiencies in patients with primary immunodeficiencies, radiation treated patients and chemotherapy treated patients. Immunostimulators are also used as adjuvants in cancer therapy and as constituents of vaccines for several cancer treatments [87]. Many mediators, immune cells, as well as macrophages are targets of immunomodulators. Macrophages are good target for immune enhancers because these cells have several functions in both innate and adaptive immune responses.

P. nigrum is one of many plants which have been shown to have immunomodulatory effects. It has been reported to have both inhibitory as well as stimulatory effects on macrophage functions. Inhibitory effects of this plant mostly came from studies of the major alkaloid component of the plant piperine. This study focused on studying the *in vitro* immunomodulatory effects of the water extract of *P. nigrum* seeds on mouse macrophage J774A.1 cells.

The water extract of *P. nigrum* at 3.125-50 µg/ml significantly promoted phagocytic activity of J774A.1 cells by increasing NBT dye reduction after engulfment of zymosan. Phagocytosis is the main host defense

mechanism of macrophages for destroying pathogens, debris cells, as well as apoptotic cells [88]. The effects of the water extract on some sequential steps of phagocytosis process were also evaluated in this study. The extract at 50 µg/ml was able to promote Dectin-1 mRNA expression in macrophage. Dectin-1 is a PRR that recognized β-1-3-glucans of several fungi, and including yeast cell wall. Dectin-1 highly expressed on myeloid cells as well as macrophages [89]. Increase in Dectin-1 expression may be a part of stimulatory activity of the water extract on phagocytic activity of J774A.1 cells.

After engulfing pathogens, macrophages destroy pathogens in phagolysomes by both oxygen-dependent and oxygen independent mechanisms. The water extract demonstrated the stimulatory effects on both pathways. It significantly promoted NO production as well as increased the mRNA expression of iNOS of J774A.1 cells. NO is reactive nitrogen intermediate generated in very high concentration by iNOS in activated macrophages. It interacts with superoxide to become a potent cytotoxic agent peroxynitrite free radical ($ONOO^-$) against pathogens as well as cancer cells [31, 90]. It also functions as one of inflammatory mediators in inflammatory process and plays role as mediator for activating other immune cells [91]. These results suggest that the *P. nigrum* water extract may stimulate oxygen-dependent pathway of phagocytosis.

The water extract also increased the mRNA expression of lysozyme M which is an orthologue of human lysozyme. This enzyme is constantly expressed in high level in mature macrophages but it can be induced to increase expression in LPS-activated macrophages [36, 38]. It plays important roles in protecting host form infection. Many previous studies suggested that lysozyme M was important in protection host form several bacterial infections. Lysozyme M was showed to protect middle ear from *Streptococcus pneumonia* induced otitis media [92] and showed to defense against *Klebsiella pneumonia* in protecting pulmonary infection [93]. In the present study, the water extract of *P. nigrum* also significantly enhanced the expression of lysozyme M by macrophages in concentration dependent

manner. The stimulatory effect of the water extract on lysozyme M expression suggests that this extract may be able to promote oxygen-independent pathway of phagocytosis against bacterial infection.

Phagocytosis process not only destroys engulfed pathogens but also mediates other processes of immune responses by activating signaling cascades of intracellular molecules involve in both innate and adaptive immune responses [94]. This process can transform resting macrophages into activated macrophages which can produce or express several cytokines and their receptors, enzymes, reactive oxygen/nitrogen species, other mediators, and cell surface molecules for both types of immune responses. This study chose the expression of pro-inflammatory cytokines as ones of the marker of activated macrophages for studying the immunostimulatory effect of the water extract of *P. nigrum*. The extract increased the mRNA expression of IL-1β, IL-6 and TNF-α. These pro-inflammatory cytokines and NO not only generate inflammation for host defense but also as mediators directly induce tumoricidal activity by activated macrophages [95-96]. These cytokines can also stimulate T cells [59].

Activated macrophages can also act as APCs to activate CD4+ T cells for generating adaptive immune response. They present the processed antigen by MHC class II to interact with TCR of CD4+ T cells. They also express B7-1, B7-2 and ICAM-1 costimulatory molecules for T cell activation [47]. The stimulatory effects of the water extract on molecules involve in the APC function of activated macrophages were also determined. The water extract of *P. nigrum* may be able to increase antigen presentation ability of macrophages.

Several previous studies have been reported that DMSO extract of *P. nigrum* and its main alkaloid piperine had inhibitory effects on macrophage function [97]. Piperine has been widely studied and known to have diversity of pharmacological effects as well as anti-inflammation by inhibiting macrophage activation. It inhibited NO and pro-inflammatory cytokines production by macrophages [76, 98]. It is less likely that the stimulatory effects

of the water extract of *P. nigrum* on macrophages come from piperine. It is possible that these stimulatory effects might come from polysaccharides components of *P. nigrum* seeds. It has been reported that the chemical of *P. nigrum* in form of ground black pepper was composed of starch about 30% [99]. Several polysaccharides from various plants also have been demonstrated to have immunostimulatory effects on macrophage functions. Polysaccharides isolated from fruiting body of *Inonotus obiquus* and *Ganoderma atrum* activated NO production, increased phagocytic activity, and promoted the expression of pro-inflammatory cytokines and costimulatory molecules in macrophages [24, 100].

The immunostimulatory effects of the water extract of *P. nigrum* in this study are supported by a previous study. The water extract of *P. nigrum* activated stimulated-mouse peritoneal macrophages to produce NO, IL-6 and TNF-α. It also activated splenocytes to proliferate and to produce IFN-γ. It also inhibited splenocytes to produce inhibitory cytokines IL-4 and IL-10 [80].

In summary, the finding in this study demonstrates the immunostimulatory activities of the water extract of *P. nigrum* on macrophage functions. The extract promoted zymosan might induce phagocytosis of macrophage J774A.1 cells in part by up-regulation of Dectin-1 receptor. It may enhance both oxygen-dependent and oxygen-independent pathways of pathogen degradation in phagolysosomes. It activates macrophage cells to express pro-inflammatory cytokines and costimulatory molecules for T cell activation. These immunostimulatory effects of the water extract need to be further confirmed in *in vivo* studies.

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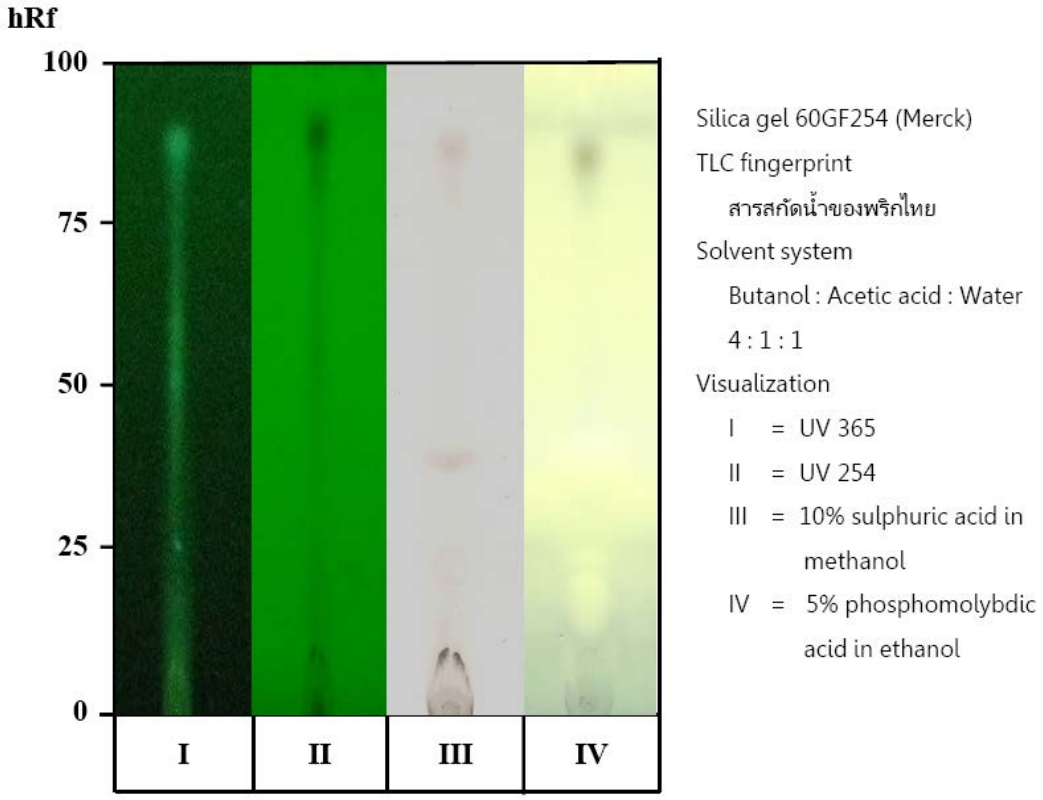
APPENDICES

A : TLC fingerprint from *Piper nigrum* water extract

B : Experiment results

APPENDIX A



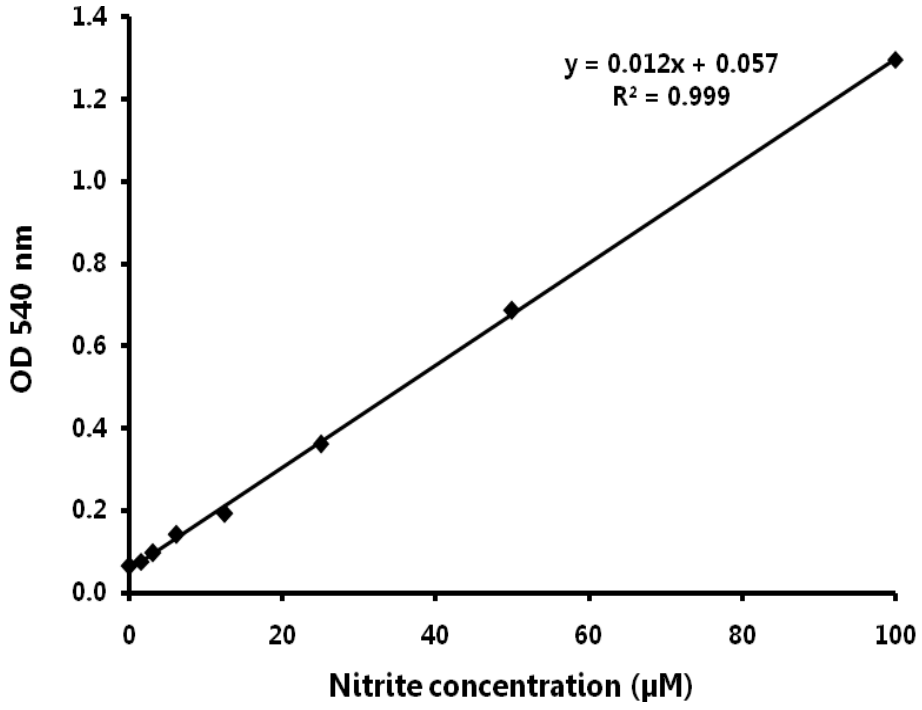
Appendix A: TLC fingerprint from *P. nigrum* water extract

APPENDIX B

EXPERIMENT RESULTS

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

Concentration (µM)	Absorbance 540 nm.		Mean
	1	2	
0	0.065	0.066	0.066
1.531	0.075	0.078	0.077
3.063	0.097	0.099	0.098
6.125	0.140	0.142	0.141
12.5	0.191	0.194	0.193
25	0.361	0.361	0.361
50	0.687	0.688	0.688
100	1.296	1.299	1.298



Appendix B-2: Standard nitrite calibration curve by Greiss reaction

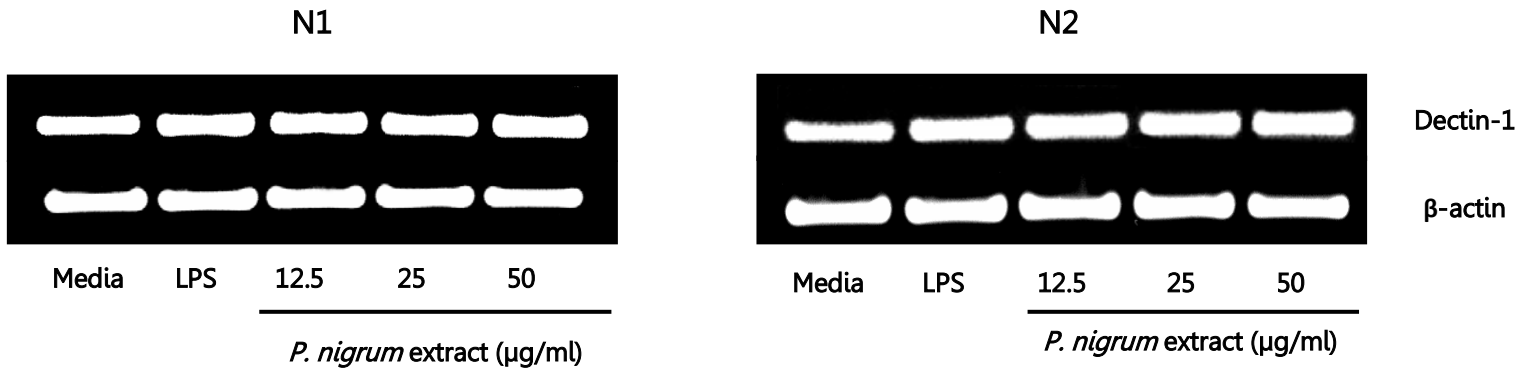
Appendix B-3: Data of the effect of *P. nigrum* water extract on NO production released from macrophage J774A.1 cells by Griess reaction (n=3). The data are expressed as mean ± S.E.M. **p<0.05, **p<0.01* compared to untreated control.

Test compounds		NO concentration			Mean± S.E.
		1	2	3	
Media		0.83	0.92	0.67	0.81±0.07
LPS		33.71	32.71	36.67	34.36±1.19**
P. nigrum (µg/ml)	1.5625	5.33	5.83	7.46	6.20±0.64
	3.125	10.67	9.33	14.38	11.45±1.51*
	6.25	15.83	14.46	29.08	19.79±4.66**
	12.5	26.46	25.33	30.13	27.31±1.45**
	25	29.00	28.75	30.54	29.43±0.56**
	50	30.38	28.71	31.46	30.18±0.80**
	100	31.54	29.63	31.92	31.03±0.71**

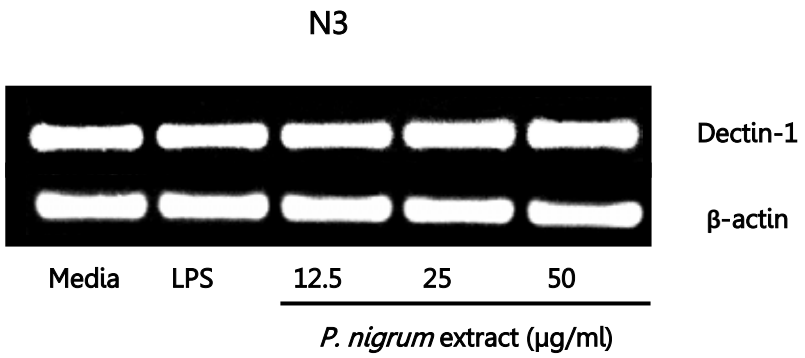
Appendix B-4: Data of the effect of *P. nigrum* water extract on phagocytic activity by macrophage J774A.1 cells evaluated by zymosan-NBT assay (n=4).

The results were expressed as the percentage of phagocytosis stimulation compared to the solvent control (mean ± S.E.M). **p<0.05, **p<0.01* compared to untreated control.

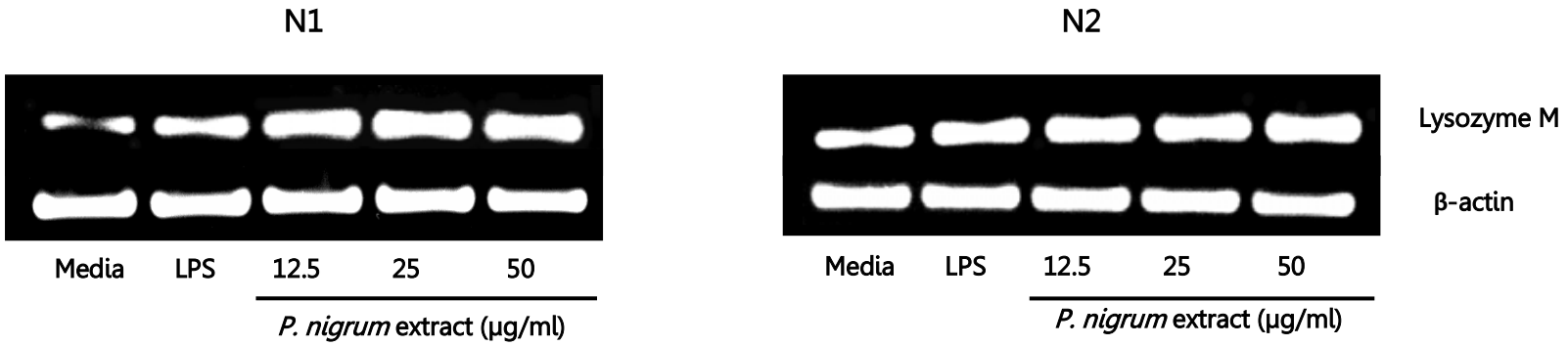
Test compound		% Phagocytosis				Mean ± S.E.M
		1	2	3	4	
Media		0.00	0.00	0.00	0.00	0.00 ± 0.00
LPS		39.95	18.65	52.39	14.33	31.33 ± 8.98**
P. nigrum (µg/ml)	1.5625	24.73	2.70	22.34	5.34	13.78 ± 5.68
	3.125	27.99	10.27	32.45	11.24	20.49 ± 5.70*
	6.25	38.32	26.22	41.76	29.49	33.95 ± 3.65**
	12.5	36.41	36.76	56.65	53.65	45.88 ± 5.39**
	25	39.67	52.97	63.03	56.46	53.04 ± 4.92**
	50	51.36	67.84	73.67	73.03	66.48 ± 5.21**
	100	42.94	28.92	46.28	29.21	36.84 ± 4.54**



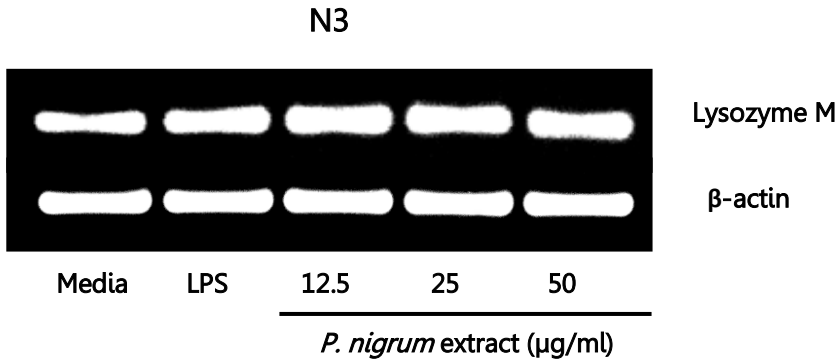
Appendix B-5: Effect of *P. nigrum* water extract on dectin-1 mRNA expression in macrophage J774A.1 cells.



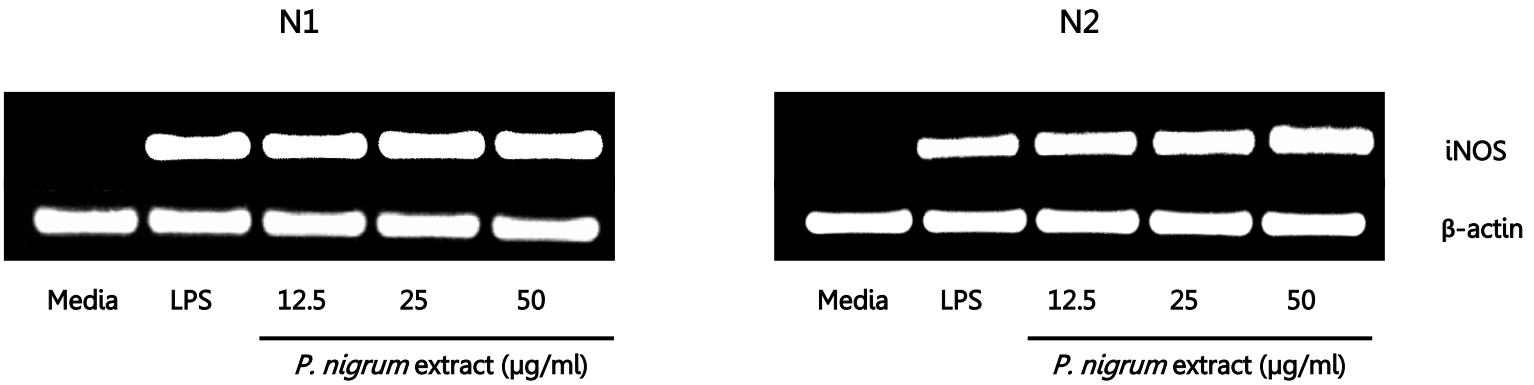
Appendix B-6: Effect of *P. nigrum* water extract on dectin-1 mRNA expression in macrophage J774A.1 cells.



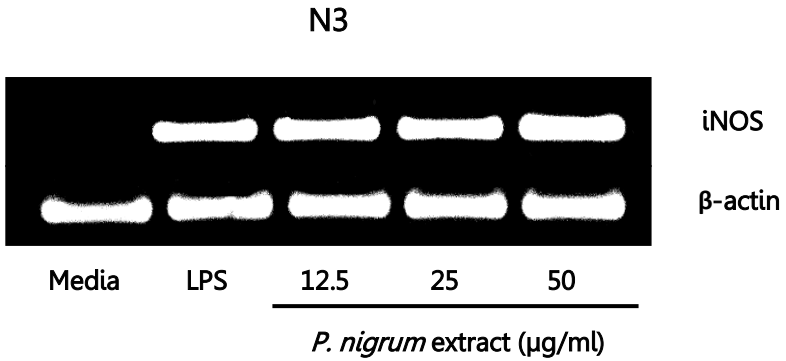
Appendix B-7: Effect of *P. nigrum* water extract on lysozyme M mRNA expression in macrophage J774A.1 cells.



Appendix B-8: Effect of *P. nigrum* water extract on lysozyme M mRNA expression in macrophage J774A.1 cells.



Appendix B-9: Effect of *P. nigrum* water extract on iNOS mRNA expression in macrophage J774A.1 cells.



Appendix B-10: Effect of *P. nigrum* water extract on iNOS mRNA expression in macrophage J774A.1 cells.

Appendix B-11: Data of the effect of *P. nigrum* water extract on mRNA expression of molecules which involving in phagocytosis (dectin-1, lysozyme M and iNOS) by macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR products (n=3). The data are expressed as mean ± S.E.M. **p<0.05, **p<0.01* compared to untreated control.

Test compound		Dectin-1/β-actin mRNA expression			Mean± S.E.	Lys M/β-actin mRNA expression			Mean± S.E.	iNOS/β-actin mRNA expression			Mean± S.E.
		1	2	3		1	2	3		1	2	3	
Media		1.20	1.18	1.37	1.25 ± 0.06	0.43	0.89	0.92	0.75 ±0.16	0.00	0.00	0.00	0.00 ± 0.00
LPS		1.38	1.52	1.42	1.44± 0.04	1.07	1.23	1.53	1.28 ± 0.14*	1.53	1.51	1.42	1.49 ± 0.03**
P. nigrum (µg/ml)	12.5	1.29	1.61	1.53	1.48 ± 0.09	1.54	1.45	1.56	1.52 ± 0.03*	1.51	1.56	1.49	1.52 ± 0.02**
	25	1.35	1.68	1.77	1.60 ± 0.13	1.62	1.55	1.65	1.61 ± 0.03*	1.76	1.63	1.64	1.68 ± 0.04**
	50	1.51	1.89	1.86	1.75 ± 0.12*	1.90	1.73	1.65	1.76 ±0.07**	1.85	1.92	1.95	1.90 ± 0.18**

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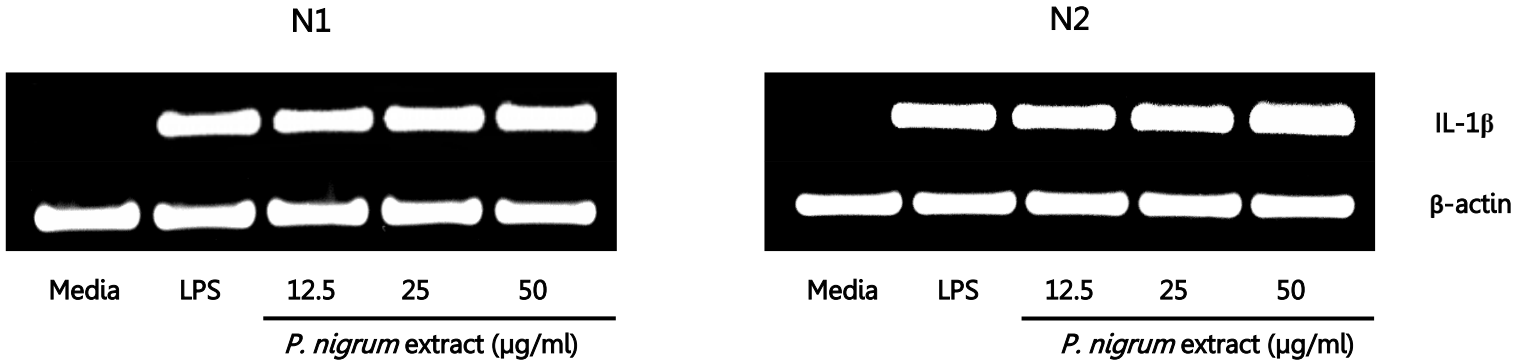
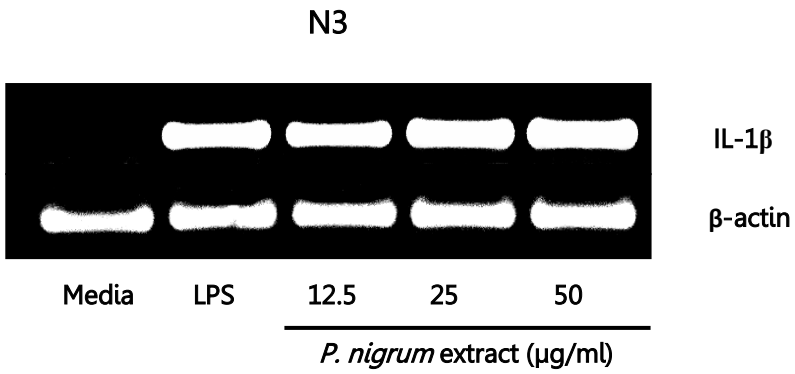
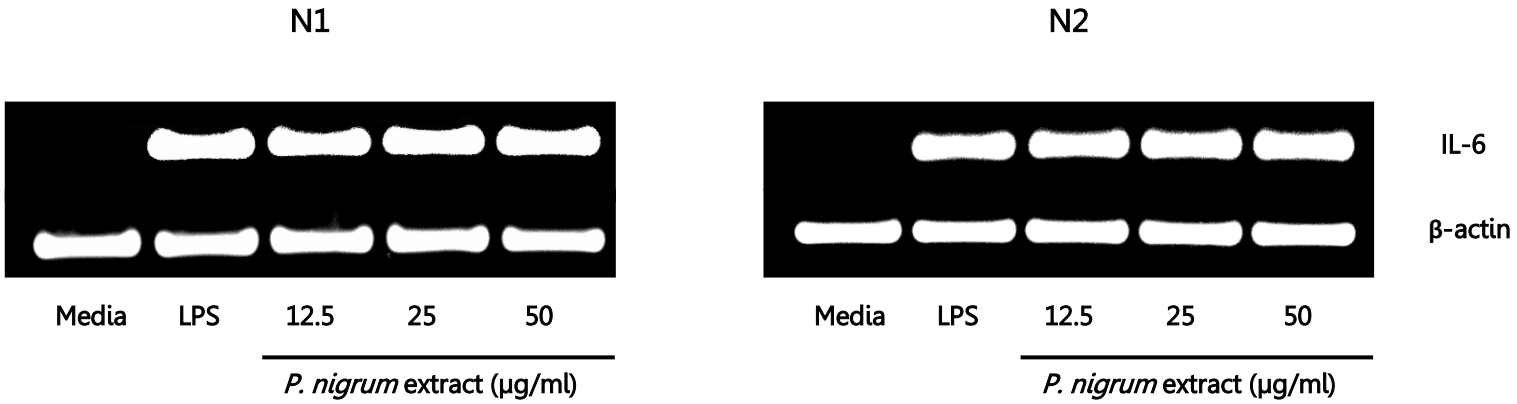


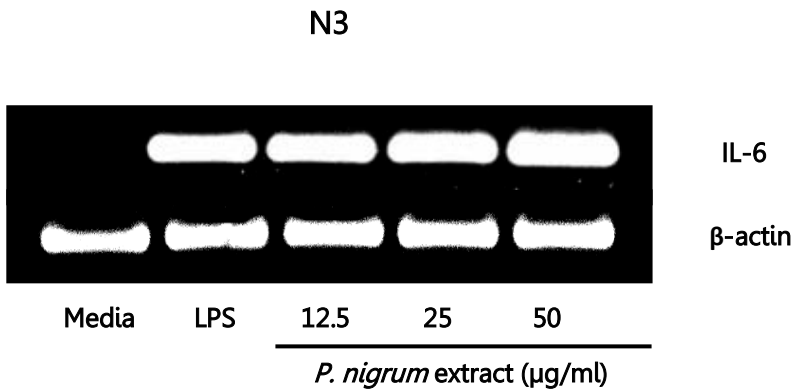
Appendix B-12: Effect of *P. nigrum* water extract on IL-1β mRNA expression in macrophage J774A.1 cells.



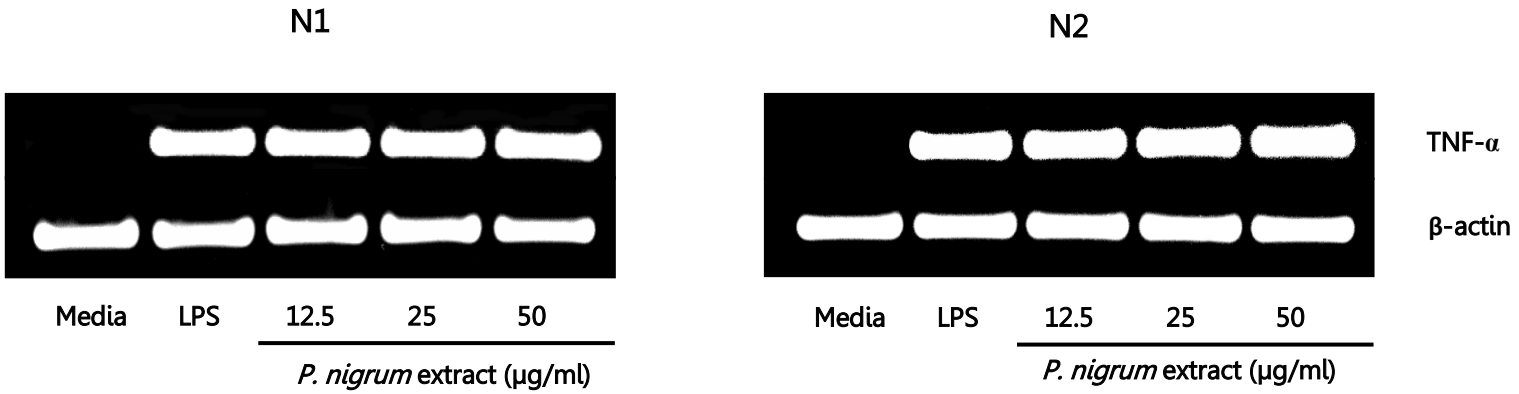
Appendix B-13: Effect of *P. nigrum* water extract on IL-1β mRNA expression in macrophage J774A.1 cells.



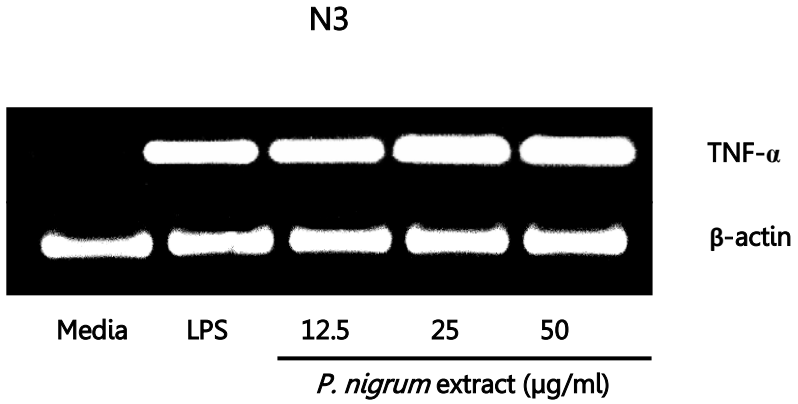
Appendix B-14: Effect of *P. nigrum* water extract on IL-6 mRNA expression in macrophage J774A.1 cells.



Appendix B-15: Effect of *P. nigrum* water extract on IL-6 mRNA expression in macrophage J774A.1 cells.



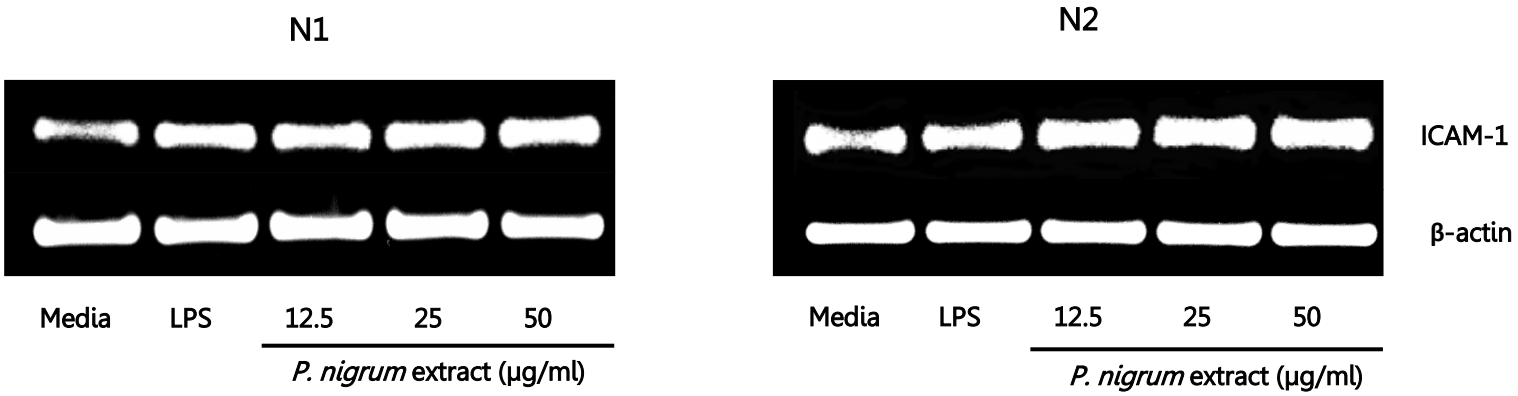
Appendix B-16: Effect of *P. nigrum* water extract on TNF-α mRNA expression in macrophage J774A.1 cells.



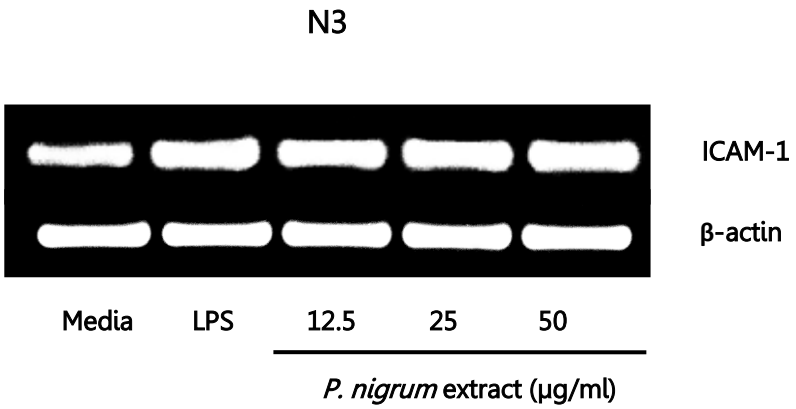
Appendix B-17: Effect of *P. nigrum* water extract on TNF-α mRNA expression in macrophage J774A.1 cells.

Appendix B-18: Data of the effect of *P. nigrum* water extract on mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) by macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR products (n=3). The data are expressed as mean ± S.E.M. **p<0.05, **p<0.01* compared to untreated control.

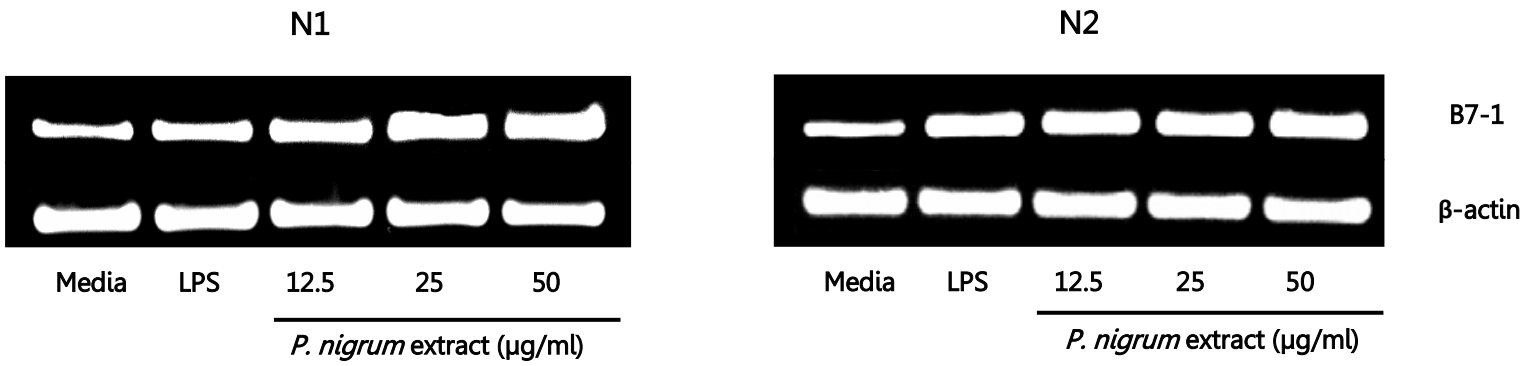
Test compound		IL-1β/β-actin mRNA expression			Mean± S.E.	IL-6/β-actin mRNA expression			Mean± S.E.	TNF-α/β-actin mRNA expression			Mean± S.E.
		1	2	3		1	2	3		1	2	3	
Media		0.00	0.00	0.00	0.00 ± 0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00	0.00	0.00	0.00 ± 0.00
LPS		1.68	1.63	1.48	1.60 ± 0.06**	1.78	1.70	1.52	1.67 ± 0.08**	1.81	1.70	1.51	1.67 ± 0.09**
P. nigrum (µg/ml)	12.5	1.47	1.50	1.29	1.42 ± 0.06**	1.45	1.65	1.50	1.53 ± 0.06**	1.78	1.71	1.51	1.67 ± 0.08**
	25	1.69	1.65	1.79	1.71 ± 0.04**	1.64	1.78	1.75	1.72 ± 0.04**	1.89	1.81	1.89	1.86 ± 0.03**
	50	1.91	1.97	1.84	1.91 ± 0.04**	1.83	1.87	2.01	1.90 ± 0.05**	2.11	1.92	1.94	1.99 ± 0.06**



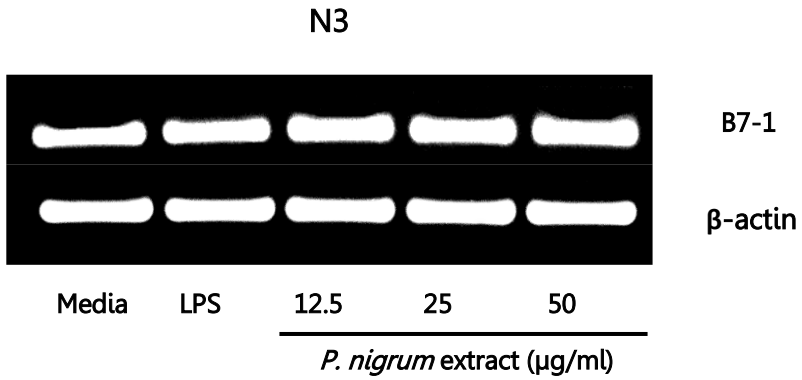
Appendix B-19: Effect of *P. nigrum* water extract on ICAM-1 mRNA expression in macrophage J774A.1 cells.



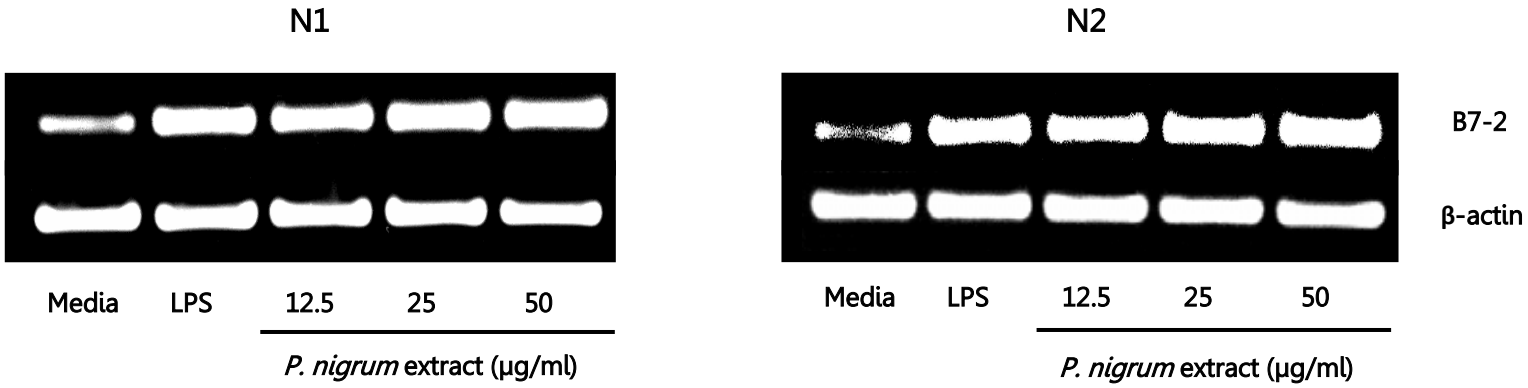
Appendix B-20: Effect of *P. nigrum* water extract on ICAM-1 mRNA expression in macrophage J774A.1 cells.



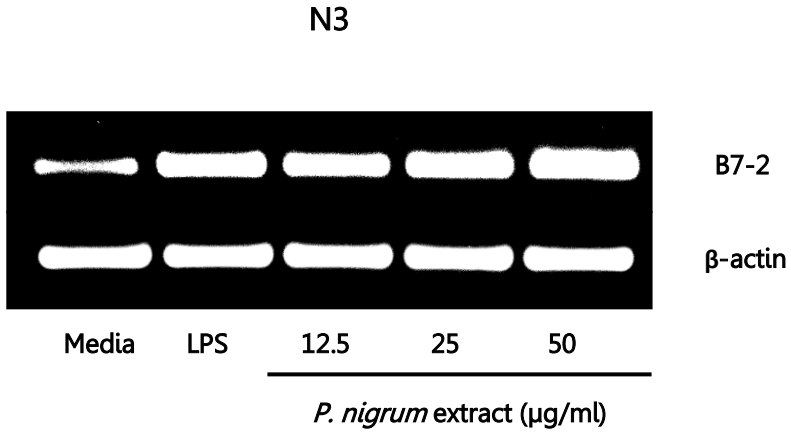
Appendix B-21: Effect of *P. nigrum* water extract on B7-1 mRNA expression in macrophage J774A.1 cells.



Appendix B-22: Effect of *P. nigrum* water extract on B7-1 mRNA expression in macrophage J774A.1 cells.



Appendix B-23: Effect of *P. nigrum* water extract on B7-2 mRNA expression in macrophage J774A.1 cells.



Appendix B-24: Effect of *P. nigrum* water extract on B7-2 mRNA expression in macrophage J774A.1 cells.

Appendix B-25: Data of the effect of *P. nigrum* water extract on mRNA expression of accessory molecule (ICAM-1) and co-stimulatory molecules (B7-1 and B7-2) by macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by using gel documentation and compared with β-actin PCR products (n=3). The data are expressed as mean ± S.E.M. **p<0.05, **p<0.01* compared to untreated control.

Test compounds		ICAM-1/β-actin mRNA expression			Mean± S.E.	B7-1/β-actin mRNA expression			Mean± S.E.	B7-2/β-actin mRNA expression			Mean± S.E.
		1	2	3		1	2	3		1	2	3	
Media		1.01	0.88	0.78	0.89 ± 0.07	0.74	0.76	0.81	0.77 ± 0.02	0.71	0.47	0.43	0.54 ± 0.09
LPS		1.49	1.44	1.26	1.40 ± 0.07**	1.56	1.24	1.19	1.33 ± 0.12*	1.64	1.35	1.41	1.47 ± 0.09**
P. nigrum (μg/ml)	12.5	1.48	1.50	1.30	1.43 ± 0.06**	1.53	1.28	1.62	1.48 ± 0.10*	1.55	1.42	1.30	1.42 ± 0.07**
	25	1.63	1.54	1.52	1.56 ± 0.03**	1.44	1.36	1.85	1.55 ± 0.15**	1.85	1.76	1.62	1.74 ± 0.07**
	50	1.84	1.60	1.66	1.70 ± 0.07**	1.71	1.52	2.04	1.76 ± 0.15**	2.08	2.00	1.90	1.99 ± 0.05**

BIOGRAPHY

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