ฤทธิ์ยับยั้งเซลล์แมคโครฟาจ J774A.1 ที่ถูกกระตุ้นด้วยแอลพีเอสของน้ำมันหอมระเหย

จากอบเชยจีนและซินนามัลดีไฮด์

นางพรรณี ชินเจริญพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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ปีการศึกษา 2556

INHIBITORY EFFECTS OF ESSENTIAL OIL FROM THE LEAVES OF *CINNAMOMUM CASSIA* AND CINNAMALDEHYDE ON LPS-STIMULATED MACROPHAGE J774A.1

Mrs. Pannee Chinjarernpan

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmacology
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Thesis Title	INHIBITORY EFFECTS OF ESSENTIAL OIL FROM THE LEAVES OF *CINNAMOMUM CASSIA* AND CINNAMALDEHYDE ON LPS-STIMULATED MACROPHAGE J774A.1
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พรรณี ชินเจริญพันธ์ : ฤทธิ์ยับยั้งเซลล์แมคโครฟาจ J774A.1 ที่ถูกกระตุ้นด้วยแอลพีเอสของน้ำมันหอมระเหย จากอบเชยจีนและซินนามัลดีไฮด์. (INHIBITORY EFFECTS OF ESSENTIAL OIL FROM THE LEAVES OF *CINNAMOMUM CASSIA* AND CINNAMALDEHYDE ON LPS-STIMULATED MACROPHAGE J774A.1) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. จันทนี อิทธิพานิชพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.วัชรี ลิมปนสิทธิกุล, , หน้า.

การศึกษานี้เป็นการศึกษาและเปรียบเทียบผลของน้ำมันหอมระเหยจากใบอบเชยจีนและซินนามัลดีไฮด์ต่อเซลล์แมคโครฟาจ J774A.1 ที่ถูกกระตุ้นด้วยไลโพพอลีแซคคาไรด์ จากวิธีการวิเคราะห์โดย GC-MS/MS พบว่าสารประกอบหลักของน้ำมันหอมระเหยจากอบเชยจีนที่ใช้ในการศึกษานี้คือ ซินนามัลดีไฮด์ (78.35 %) โดยน้ำมันหอมระเหยจากอบเชยจีนและซินนามัลดีไฮด์ที่ความเข้มข้น 1-20 μg/ml ยับยั้งการสร้างไนตริกออกไซด์ในเซลล์ J774A.1 ที่ถูกกระตุ้นด้วยแอลพีเอสได้ตามขนาดความเข้มข้นที่ใช้ในการศึกษา โดยมีค่า IC_{50} 6.1±0.25 และ 9.97±0.35 μg/ml ตามลำดับ น้ำมันหอมระเหยทั้งสองชนิดที่ความเข้มข้น 5-20 μg/ml สามารถยับยั้งการเกิดกระบวนการจับกินสิ่งแปลกปลอมได้ โดยการศึกษาด้วย RT-PCR พบว่าน้ำมันหอมระเหยทั้งสองชนิดลดการแสดงออกระดับ mRNA ของสารสื่ออักเสบหลายชนิดในเซลล์ J774A.1 ที่ถูกกระตุ้นด้วยแอลพีเอส เช่น tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) และ macrophage inflammatory protein-1α (MIP-1α) นอกจากนี้สารทั้งสองชนิดยังลดการแสดงออกของเอนไซม์ inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) และ microsomal prostaglandin-E synthase (mPGES-1) ได้อย่างมีนัยสำคัญทางสถิติและยับยั้งปริมาณการสร้าง TNF-α ที่วัดด้วยวิธี ELISA ในทางตรงกันข้ามน้ำมันหอมระเหยทั้งสองชนิดนี้เพิ่มการแสดงออกระดับ mRNA ของไซโตไคน์ที่มีฤทธิ์ยับยั้งการอักเสบคือ interleukin-10 (IL-10) และ transforming growth factor (TGF-β) และเพิ่มการสร้างปริมาณ IL-10 ที่วัดโดยวิธี ELISA ด้วย นอกจากนี้สารทั้งสองยังสามารถเพิ่มการแสดงออกของ ferroportin 1 (Fpn1) ซึ่งเป็นโปรตีนที่ทำหน้าที่ขนส่งเหล็กออกนอกเซลล์ ผลจากการศึกษาครั้งนี้แสดงให้เห็นว่า ผลการยับยั้งการอักเสบของน้ำมันหอมระเหยจากใบอบเชยจีนน่าจะเกิดขึ้นจากสารประกอบหลัก ซินนามัลดีไฮด์ ซึ่งนอกจากสารดังกล่าวมีฤทธิ์ยับยั้งสารสื่อที่ทำให้เกิดการอักเสบแล้ว ยังมีฤทธิ์กระตุ้นสารสื่อยับยั้งการอักเสบรวมทั้งยังมีผลต่อกระบวนการควบคุมการขนส่งเหล็กในเซลล์แมคโครฟาจในภาวะการอักเสบ

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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PANNEE CHINJARERNPAN: INHIBITORY EFFECTS OF ESSENTIAL OIL FROM THE LEAVES OF *CINNAMOMUM CASSIA* AND CINNAMALDEHYDE ON LPS-STIMULATED MACROPHAGE J774A.1. ADVISOR: ASSOC. PROF. CHANDHANEE ITTHIPANICHPONG, CO-ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., pp.

This study investigated and compared the effects of leaf cassia oil from *Cinnamomum cassia* and cinnamaldehyde on lipopolysaccharide (LPS)-activated macrophage J774A.1 cells. By GC-MS/MS, cinnamaldehyde (78.35 %) was the main component of leaf cassia oil in this study. Cassia oil and cinnamaldehyde at 1-20 µg/ml were markedly inhibited nitric oxide (NO) production in LPS-activated J774A.1 cells in concentration-dependent manner with IC50 value of 6.1±0.25 and 9.97±0.35 µg/ml, respectively. They markedly inhibited phagocytic activity at both 10 and 20 µg/ml. Using RT-PCR, they down-regulated mRNA expression of cytokines and chemokines involve in inflammation in the LPS-activated cells. These mediators included tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α). They also significantly decreased mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), microsomal prostaglandin-E synthase (mPGES-1) determined by RT-PCR and inhibited the production of TNF-α determined by ELISA. In the opposite way, they increased mRNA expression and the production of inhibitory cytokines IL-10 and transforming growth factor (TGF-β). They also up-regulated IL-10 production determined by ELISA. In addition, they promoted the expression of the iron exporter protein ferroportin 1 (Fpn1). The results from this study demonstrated that inhibitory effect of leaf cassia oil from *C. cassia* came mainly from cinnamaldehyde. This compound not only inhibited inflammatory mediators but also activated anti-inflammatory mediators in LPS activated J774A.1 cells. It may also have effect on iron regulatory proteins in activated macrophages.

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

Academic Year: 2013 Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percent
/	Per
<	Less than
µg	Microgram
µl	Microliter (s)
µm	Micrometer (s)
µM	Micromolar
°C	Degree Celsius
AA	Arachidonic acid
ACD	Anemia of chronic disease
AI	Anemia of inflammation
C3b	Complement 3b
C3bi	Complement 3bi
CO_2	Carbon dioxide
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CR1	Complement receptor 1
CR2	Complement receptor 2
CTLDs	C-type lectin-like domains
CAT	Catalase
C. cassia	*Cinnamomum cassia*
CIN	Cinnamaldehyde
CO	Cassia oil
DEX	Dexamethasone
dl	Deciliter (s)
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FcR	Fc receptor
Fpn1	Ferroportin1
ft	Feet
g	Gram
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response eliment
h	Hour
Hb	Hemoglobin
HO-1	Heme oxygenase
H_2O_2	Hydrogen peroxide
Hp	Haptoglobin
IC_{50}	Half maximal inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IFN- γ	Interferon-gamma
IgG	Immunoglobulin G
IkB	NF-kappa-B inhibitor alpha
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
JNKs	c-Jun N-terminal kinase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
M	Molarity
mAb	Monoclonal antibody

MCP-1	Monocyte chemoattractant protein-1
min	Minute (s)
MIP-1α	Macrophage inflammatory protein-1 alpha
ml	Milliliter (s)
mPGES-1	Microsomal prostaglandin E synthase-1
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MyD88	Myeloid differentiation protein 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NED	*N*-1-napthylethylenediamine dihydrochloride
ng	Nanogram (s)
NK cell	Natural killer cell
nm	Nanometer (s)
NO	Nitric oxide
NOSs	Nitric oxide synthases
nNOS	Neuronal nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2	Oxygen
O_2^-	Superoxide anion
OD	Optical density
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pg	Picogram (s)
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PHSC	Pluripotent haematopoietic stem cell
PRR	Pattern recognition receptor

PS	Phosphatidylserine
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
S.E.M	Standard error of mean
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
SR-AI, AII	Scavenger receptor-AI, AII
Src	Tyrosine kinase Src
Syk	Spleen tyrosine kinase
Tf	Transferrin
TGF	Transforming growth factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
U	Unit
CAM-1	Vascular cell adhesion molecule-1

CHAPTER I

INTRODUCTION

Background and Rationale

Inflammation is the body's natural immune response to eliminate harmful stimuli including microorganisms, pathogens, damaged cells, toxin and irritants. It is classified into acute and chronic inflammation. Acute inflammation is a short-term process lasting for 24-48 hours and neutrophils are prominent in injured area. Chronic inflammation is a prolong inflammation which can last for several months or even years. The hallmark of chronic inflammation is the increasing of recruited macrophages and lymphocytes at injured tissue. These cells are recruited from the circulation by the static release of chemoattractant mediators. Macrophages are major cells involved in chronic inflammation. They possess phagocytic and bactericidal activity. During inflammation macrophages produce mediators involved in inflammation including chemoattractant mediators e.g. Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1 α), interleukin-8 (IL-8), pro-inflammatory cytokines e.g. interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF-α), prostaglandin E_2 (PGE_2), nitric oxide (NO) and reactive oxygen species (ROS) which promote the killing of microorganisms and removal of harmful stimuli. Additionally, activated macrophages also produce anti-inflmmatory cytokines including interleukin-10 (IL-10) and transforming growth factor-beta1 (TGF-β1) which promote negative feedback mechanism to protect tissue destruction and induce tissue repair. However, when macrophages are allowed to continue activation, chronic inflammation will occur (1, 2). Chronic inflammation has been shown to be involved in the development of a variety of serious diseases, such as rheumatoid arthritis, atherosclerosis, autoimmune disorders, neurodegenerative disease, cancer and often lead to development of anemia, called anemia of inflammation (AI) (3). AI is a mild to moderate anemia, attributed by insufficient erythrocyte production resulting from the limitation of iron supply for erythropoiesis.

Macrophages play a central role in the organism as they recycle iron after phagocytosis of aged erythrocytes from blood circulation. In lysosomes, erythrocytes are degraded, heme is released and iron is extracted from heme. The extracted iron can be stored or exported to blood circulation through iron exporter protein on macrophage membrane, ferroportin1 (Fpn1). Previous studies have shown that acute and chronic inflammatory states account for the restriction of iron efflux from macrophages by down-regulation of Fpn1. This effect is mediated by pro-inflammatory cytokines to induce sequestration of iron into macrophages and reduction of plasma iron (4). Thus, the compounds with the ability to reduce expression of pro-inflammatory cytokines, chemokines and inflammatory mediators or increase expression of anti-inflammatory cytokines and cellular iron transporter protein can be beneficial to chronic inflammatory diseases and anemia of inflammation.

Nowadays, steroidal compounds or non-steroidal anti-inflammatory drugs (NSAIDs) including non-selective inhibitors of cyclooxygenase (COX) enzyme and selective COX-2 inhibitors are often prescribed by physicians for the treatment of acute and chronic inflammation (5). However, GI complications are the most common found side effects or even serious adverse side effect like heart attack encountered regarding their use (6, 7). Thus, a variety of safe and effective anti-inflammatory agents in long-term use is growing needed.

Over the past decade, many researchers are interested in herbal medicine as the potential therapeutic compounds in the treatment of inflammatory diseases. *Cinnamomum cassia* (Lauraceae) or Chinese cinnamon is an evergreen tall tree with aromatic bark, native to southern China, India, Bangladesh, Sri Lanka and Vietnam. It is popularly used in foods, bakery, beverages, and traditional herb medicine (8). It contains several pharmacologically active substances such as essential oil, tannin, and carbohydrate (9). Essential oil or cassia oil is extracted from the leaves or barks of *C. cassia* by steam distillation (10, 11). Cassia oil has been traditionally used in the treatment of cold, influenza, fevers, dyspepsia and other inflammatory diseases such as gastritis, arthritis and rheumatism (12). It has been demonstrated anti-oxidant, antibacterial and antifungal activities (13, 14). The main constituent in cassia oil is

cinnamaldehyde. It has been reported that essential oil from the barks and leaves of C. *cassia* contain cinnamaldehyde in different amount.(15, 16). Previous studies have demonstrated that some herbal components such as genistein and ginsenoside significantly increased cellular iron exporter in mice and U373MG cells (17, 18). However, there is limited evidences for other plant constituents. Additionally, it has not been reported on the effect of cassia leaf oil and cinnamaldehyde on anti-inflammatory mediators and iron exporter in activated macrophages. Therefore, in this study we aim to compare the effects of cassia leaf oil and cinnamaldehyde on pro-inflammatory and also anti-inflammatory cytokine production in LPS-stimulated macrophage J774A.1 cell including the effect of both compounds on the cellular iron transporter (Fpn1).

Research Objectives

1. To investigate and compare the activities of cassia oil from the leaves of *C. cassia* and cinnamaldehyde on mRNA expression in LPS-stimulated macrophage J774A.1cells, including pro-inflammatory cytokines (IL-β, IL-6, TNF-α), anti-inflammatory cytokines (IL-10, TGF-β), chemokines (MCP-1, MIP-1α), inflammatory mediators (NO) and inflammatory enzymes (iNOS, COX-2, mPGES-1).

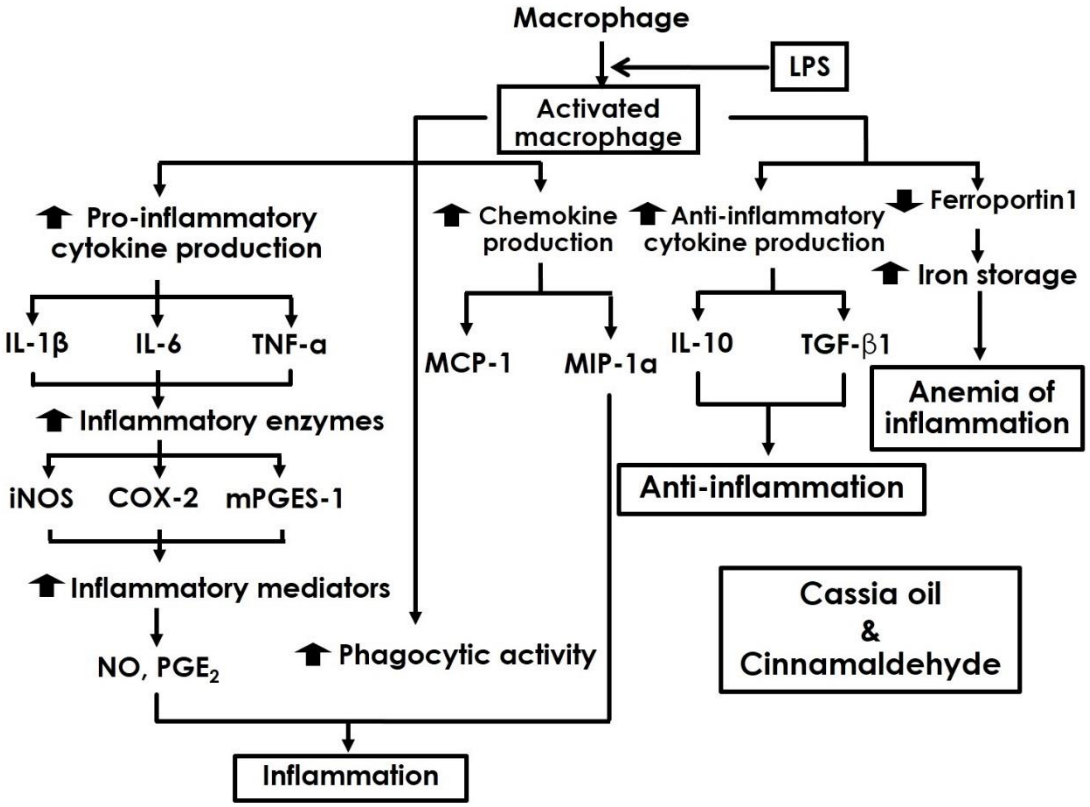
2. To examine and compare the effect of cassia oil from the leaves of *C. cassia* and cinnamaldehyde on phagocytic activity of LPS-activated macrophage J774A.1cells.

3. To investigate and compare the effect of cassia oil from the leaves of *C. cassia* and cinnamaldehyde on ferroportin (Fpn1) mRNA expression in LPS-activated macrophage J774A.1cells.

Hypothesis

Cassia oil from the leaves of *C. cassia* and cinnamaldehyde exert inhibitory effects on LPS-activated macrophages by inhibiting phagocytic activity, decrease expression of inflammatory mediators, increase expression of anti-inflammatory mediators, and up-regulating the expression of Fpn1.

Conceptual framework



Expected benefit and application

The results of its inhibitory effects on the activated-macrophages may support the anti-inflammatory activity of cassia oil from the leaves of *Cinnamomum cassia*. The results also provide the inhibitory activities of cassia leaf oil in comparison to cinnamaldehyde which is the main component of cassia oil in order to prove whether cinnamaldehyde is the key compound in its inhibitory effects on macrophage function. The information from this study would promote further investigations in order to obtain adequate sciencetific information to support the natural health products developement.

Key Words

Cinnamomum cassia, J774A.1 cells, phagocytosis, pro-inflammatory cytokines, cinnamaldehyde, ferroportin

CHAPTER II

LITERATURE REVEIWS

Inflammation is one of the most important and useful of our host defense mechanisms to endogenous and exogenous stimuli, including physical injury, bacteria, immune complexes, neurotoxins, endotoxin, etc. Inflammation has four distinct phases: recognition of foreign particles, recruitment of cells, elimination of the foreign particles, and resolution of inflammation. It can also be classified into acute and chronic inflammation based on duration of the inflammatory lesion (1).

Acute inflammation

Acute inflammation is an immediate and early response to harmful stimuli. It is a short-term response, lasting for minutes to several hours or a few days. It is characterized by exudates, fluid with high protein concentration and neutrophils accumulation in injured area. Acute inflammation can be divided into an early vascular and late cellular response. When tissue is injured, the chemical mediators derived from plasma or cells are complement, histamine, serotonin, bradykinin, prostaglandins, leukotrienes, activated oxygen species, nitric oxide and cytokines. These chemical mediators induce vasodilatation, increased blood flow and vascular permeability resulting in redness, heat and swelling. Bradykinin, prostaglandins, and serotonin induce pain (1). Pain and severe swelling at the inflammed area also cause physically immobilize of the tissue or loss of function. In cellular response, recruited leukocytes from the blood stream and finally from the bone marrow are targeted to the site of inflammation. The cellular response covers the following phenomenons (Fig.1) (19):

1.) Migration, rolling and adhesion of leukocytes

Red blood cells or erythrocytes are normally localized to the central (axial) area in blood vessels. When vascular permeability is increased, more neutrophils and other leukocytes accumulate along the endothelial surface. Thereafter, they

bind to endothelium facilitated by cell adhesion molecules; selectins, immunoglobulins, integrins, etc.

2.) Transmigration of leukocytes

Leukocytes transmigrate from blood vessels by extending pseudopodia through the vascular wall by a process called diapedesis. The most important mechanism of leukocyte transmigration is via widening of epithelial junctions to produce gaps after vasodilatation and increases vascular permeability.

3.) Chemotaxis

The recruitment of leukocytes from blood circulation towards the site of inflammation guided by chemical gradients is called chemotaxis. The components of the complement system (C5a), bacterial and mitochondrial products of arachidonic acid metabolism and IL-8 are the most important chemotactic factors for neutrophils. Chemoattractants react with receptors on cell membrane of leukocytes resulting in activation of phospholipase C that finally leading to cytosolic calcium ions release from endoplasmic reticulum and these ions stimulate cell movement towards the stimulus. Pro-inflammatory cytokines (TNF-α, IL-1β) production and secretion in innate immune response to harmful stimuli induce the expression of adhesion molecules selectins and integrin on endothelial cells. Luekocytes in the circulation bind to these adhesion molecules and respond to the chemokines, leading to recruitment of the leukocytes into the tissues. Leukocytes and other immune cells release some biochemical mediators including cytokines, neuropeptide, growth factors and neurotransmitters to the site of inflammation. The symptoms of inflammation such as pain, erythema, heat, and edema are caused by changes in local blood vessels which resulting from these biochemical mediators.

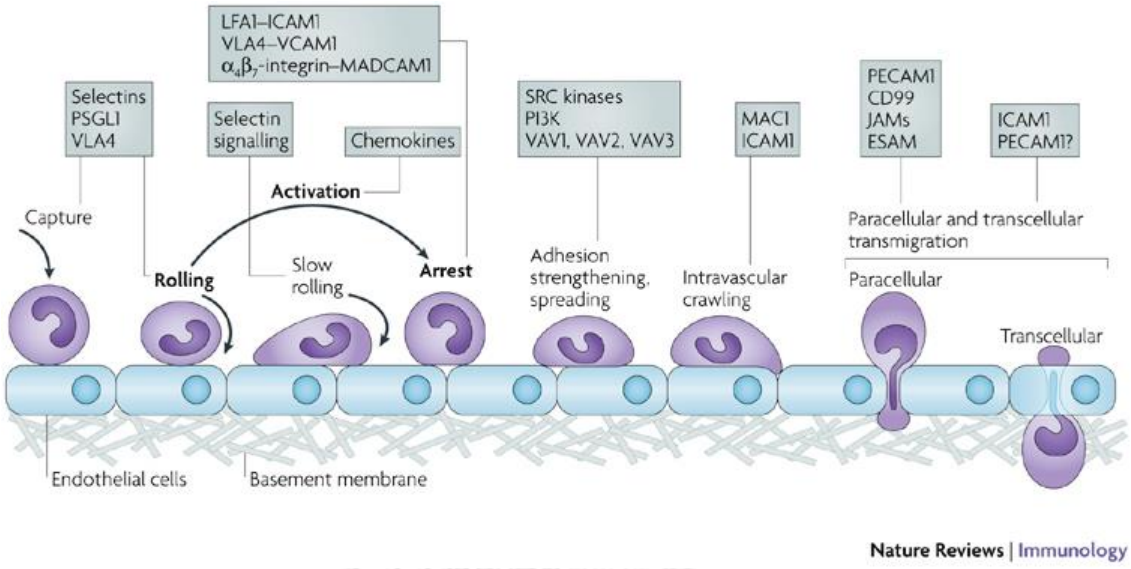


Figure 1: The cellular response of inflammation. Leukocyte migrate from blood circulation to site of tissue injury during the innate immune response (19).

Chronic inflammation

Chronic inflammation is a process proceeding simultaneously along with active inflammation, tissue destruction and tissue repair. Macrophages are the main cells involved in chronic inflammation. They are known as mononuclear cells because of having single nuclei. They live far longer than neutrophils. They are activated by chemical mediators such as lymphokines, lipopolysaccharide (endotoxin from gram negative bacteria cell walls) or interferon-**γ** (IFN-**γ**). After activation, macrophages do an excellent job of engulfing and neutralizing or killing foreign antigens and present the antigenic peptide to activate other immune cell in adaptive immunity system (1). Previous studies have shown that chronic inflammation contributes to the pathogenesis of various diseases such as heart disease, cancer, stroke, Alzheimer's disease, diabetes, nephritis and etc (20-23).

Macrophages

Macrophages are mononuclear phagocytes derived from pluripotent haematopoietic stem cells (PHSC) in bone marrow. They are delivered to peripheral blood as monocytes and mature in various tissues with specific name like microglia (central nervous system), Kupffer cell (liver), histiocytes (connective tissue) and osteoclasts (bone) (Fig.2). They are mainly classified to classically and alternatively activated macrophage (24).

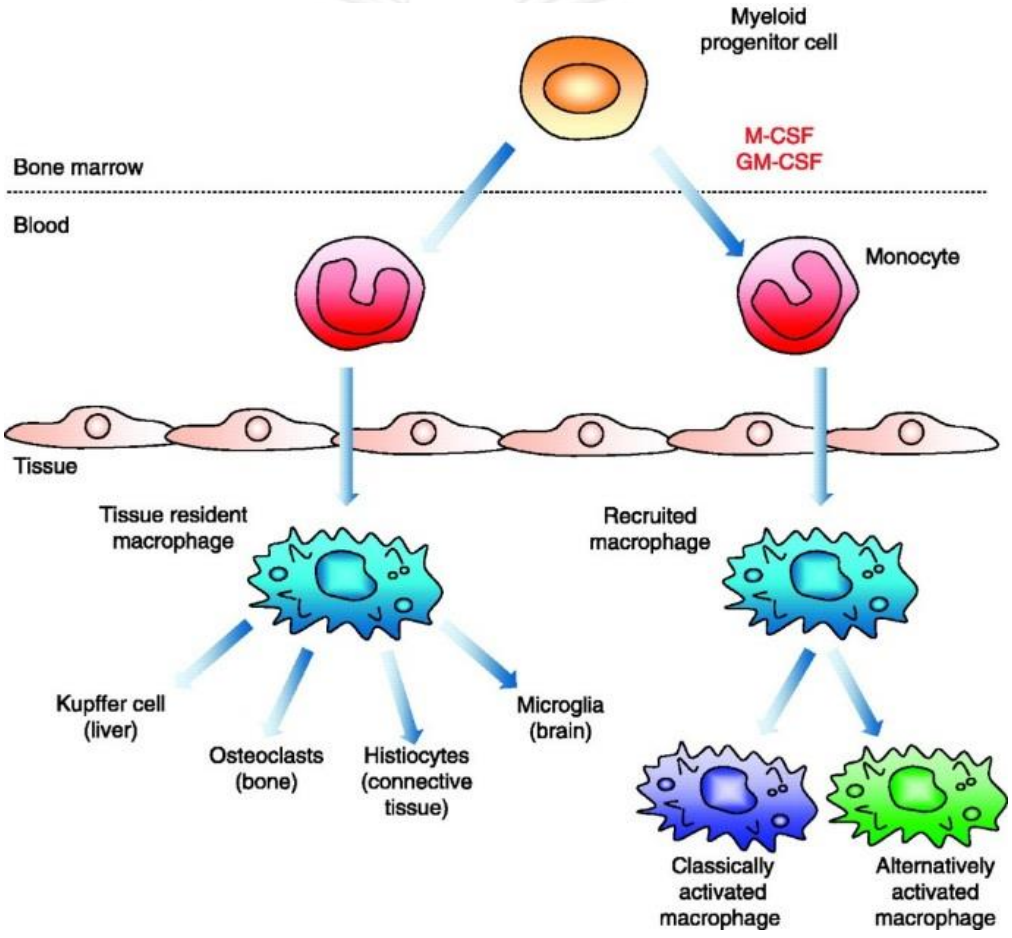


Figure 2: Macrophage maturation. Myeloid progenitor cell in bone marrow is differentiated to monocyte which released to blood circulation and differentiated to macrophage in various tissues (24).

Macrophages play many roles in our body. In normal condition, they perform general functions by clearing aged red blood cells (RBCs) and apoptotic cells by little

or without immune mediator production. There are several types of surface receptors on macrophages membrane which mediate the homeostatic clearance process including scavenger receptors, phosphatidylserine receptors, the thrombospondin receptor, integrins and complement receptors. They are constitutively expressed on their cell surface. During aging of RBCs, auto-oxidative damage occurs to lipid and protein components in their membrane. Macrophages of reticuloendothelial system recognize aged red blood cells which express phosphatidylserine on their outer surface and subsequent phagocytose them. Thus, aged red blood cells are removed. This process is called eryptosis, erythrocyte programmed cell death. Furthermore, macrophages are hallmark of chronic inflammation; they live for months in the body up to a maximum of several months. The type of macrophage resulting from the differentiation of monocyte depends on the types of cytokines which they are encountered. The two major subpopulations of macrophages are M1 and M2 (Fig.3) (25).

In classical (M1) activation, macrophages are activated by pathogen and cytokines particularly IFN-γ, typically involved in potentially harmful inflammation. When they are activated, they secrete a number of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines (MCP-1, MIP-1α and IL-8) that assist in recruitment of other leukocytes to inflammatory area (26). In addition, they also increase the production of toxic oxygen species and induction of the inducible nitric oxide synthase (iNOS) gene to produce NO and synthesis inflammatory mediators such as PGE_2, collagenase and elastase to promote their ability to destroy pathogens (27, 28). However, when macrophages are allowed to continue activation, chronic inflammation will occur (1, 2). These inflammatory mediators released by M1 macrophages are extensive capable to injure normal host tissue which has been implicated in the pathogenesis of many disease processes. Previous studies have shown that chronic inflammation has been shown to be involved in the development of a variety of serious diseases, such as rheumatoid arthritis, atherosclerosis, autoimmune disorders, neurodegenerative disease, cancer (29-31) and often lead to development of anemia, called anemia of inflammation (AI) (3).

In alternative (M2) activation, macrophages are activated by IL-4, IL-10, IL-13 or LPS (32). It can be divided into three subtypes (M2a, M2b, and M2c), which have different functions such as regulation of immunity, maintenance of tolerance and tissue repair/wound healing. After activation, they produce the immunoregulatory cytokines IL-10 and transforming growth factor-beta1 (TGF-β1) involved in negative feedback mechanism to protect tissue destruction, resolution of inflammation and promotion of tissue repair (33). M2-macrophages can also up-regulate enzyme arginase activities, which is involved in proline and polyamine biosynthesis. Proline promotes extracellular matrix (ECM) building while polyamines promote cell proliferation. TGF-β1 also functions indirectly to promote ECM building by inducing fibroblasts to produce ECM components (34). These distinct activation phenotypes contribute different or opposing biological functions to impact on tissue homeostasis and pathological condition such as infectious diseases, cancer and anemia of inflammation.

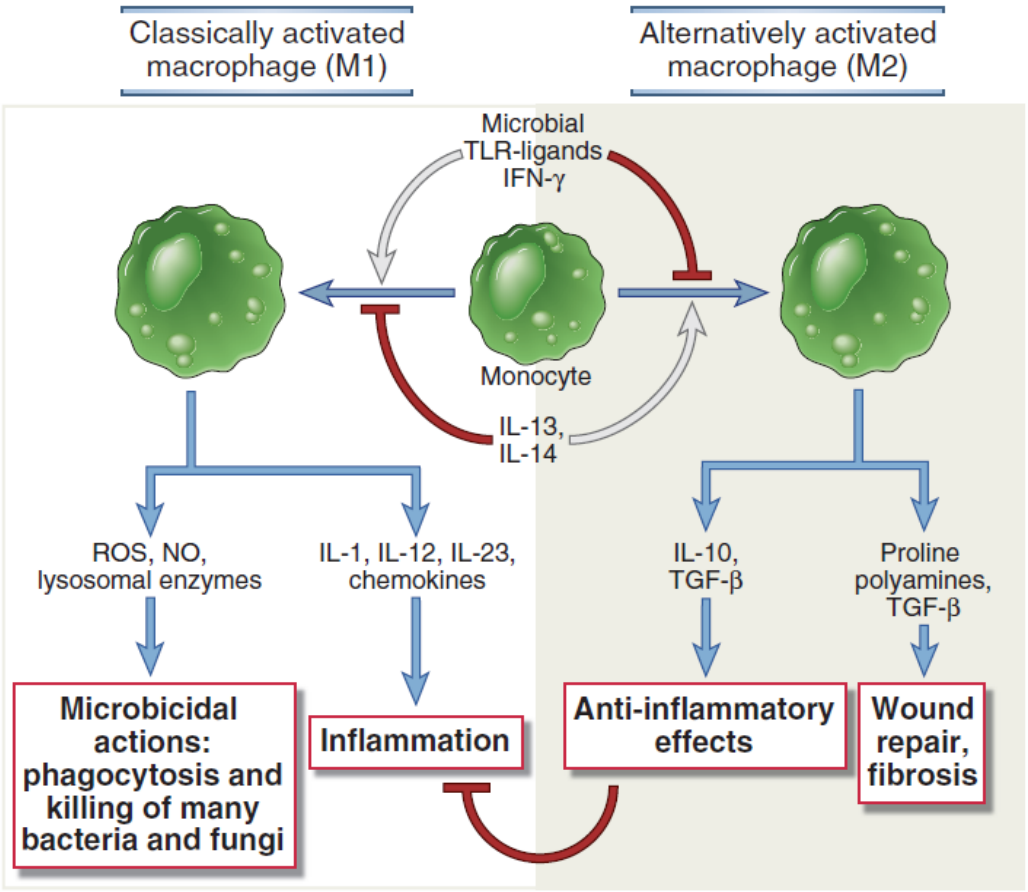


Figure 3: Macrophage activation. The two major subpopulations of macrophages are classically activated macrophage (M1) and alternatively activated macrophage (M2). During inflammation; microbial, TLR-ligands and IFN-γ promote M1 response that produce inflammatory mediators for destroying harmful stimuli. Cytokines IL-4 and IL-13 promote M2 activation that play a critical role in negative feedback mechanism to suppress immune function and promote wound healing (25).

Macrophage functions

During chronic inflammation, macrophages play an important role in host defense mechanism. They have three major functions consisted of the recognition of infective organisms and phagocytose them into phagosomes then followed by destruction of ingested organisms (phagocytosis). After that, they produce several mediators including cytokines and chemokines. Finally, they present the processed particles to T cells for T cells activation in adaptive immune responses (35).

Macrophage act as phagocyte

Phagocytosis is an important process to eliminate foreign particles and cellular debris. It is the process of engulfment and internalization of particulate material size over 0.5μm. They play an important role to promote antigen presentation to stimulate adaptive immune response. The phagocytic cells include neutrophils, monocytes and tissue macrophages.

Phagocytosis involves in three interrelated steps as follow (Fig.4):

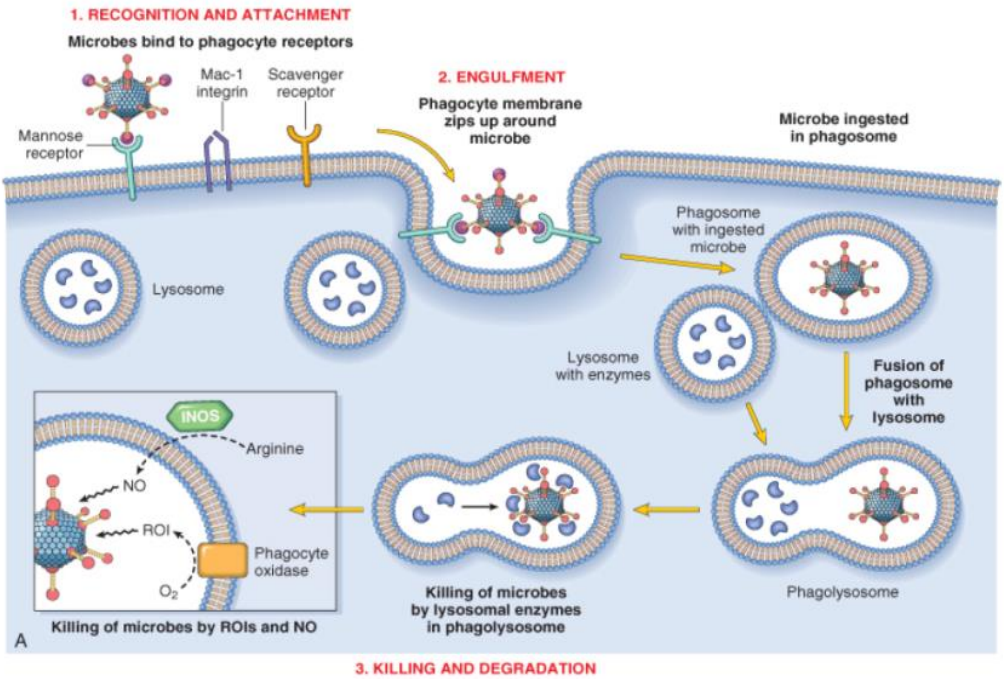


Figure 4: Steps involved in phagocytosis of microbe by macrophage. Macrophage recognizes microbe by their surface receptors. The microbe is engulfed into phagosome which fused with lysosome and then it is destroyed by free radicals and enzymes (40).

1. Recognition and attachment

Phagocytosis is enhanced if the particle is coated with certain plasma proteins called opsonins. They promote the adhesion between the particle and the phagocyte's membrane. There are three major opsonins: the Fc fragment of immunoglobulin bind to the receptor for Fc region of the IgG molecule (FcR), components of the complement system C3b and C3bi bind to complement receptors (CR1 and CR2), and the carbohydrate-binding proteins-lectins. Phagocytes recognize foreign substances by several kinds of pattern recognition receptors (PRRs) such as mannose receptor, dectin-1, scavenger receptor and Mac-1 integrin.

- **Dectin-1 and mannose receptors** are members of C-type lectins which compose of one or more C-type lectin-like domains (CTLDs). C-type lectins can be divided into 17 groups based on their domain composition. They acted 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. Mannose receptor is PRR which recognizes part of mannose of bacteria, virus, protozoa component and fungal cell wall. The main example of PRRs which is expressed on macrophage membrane is the Dectin-1 (Fig.5). It plays a major role in anti-fungal innate immunity that can be up-regulated by many cytokines and microbial factor and down-regulated by IL-10 and dexamethasone. Dectin-1 recognizes β-1, 3-linked glucans, glucose polymers which are fungal cell wall component (36). This receptor was activated by β-glucan to produce a variety of cellular response including endocytosis and phagocytosis, arachidonic acid metabolites production, the respiratory burst, and cytokines and chemokines production such as TNF, CXCL2 (MIP-2), IL-23, IL-6 and IL-10. Zymosan, a yeast cell wall derived from *Saccharomyces cerevisiae* which composed of β-glucan, mannan, mannoprotein and chitin. Zymosan induces immune responses depend on both Dectin-1 and TLR2. After binding to Dectin-1, Dectin-1 is phosphorylated by tyrosinase kinase Src. Then, Syk is activated and induces the CARD9-Bcl10-Malt1 complex leading to activate transcription factor NF-kB and subsequent pro-inflammatory cytokines and chemokines production. Its signaling is promoted and cooperative signaling from MyD88 coupled TLRs. The Syk is a kinase which mediates production of reactive oxygen species (ROS) via respiratory burst induction (37).

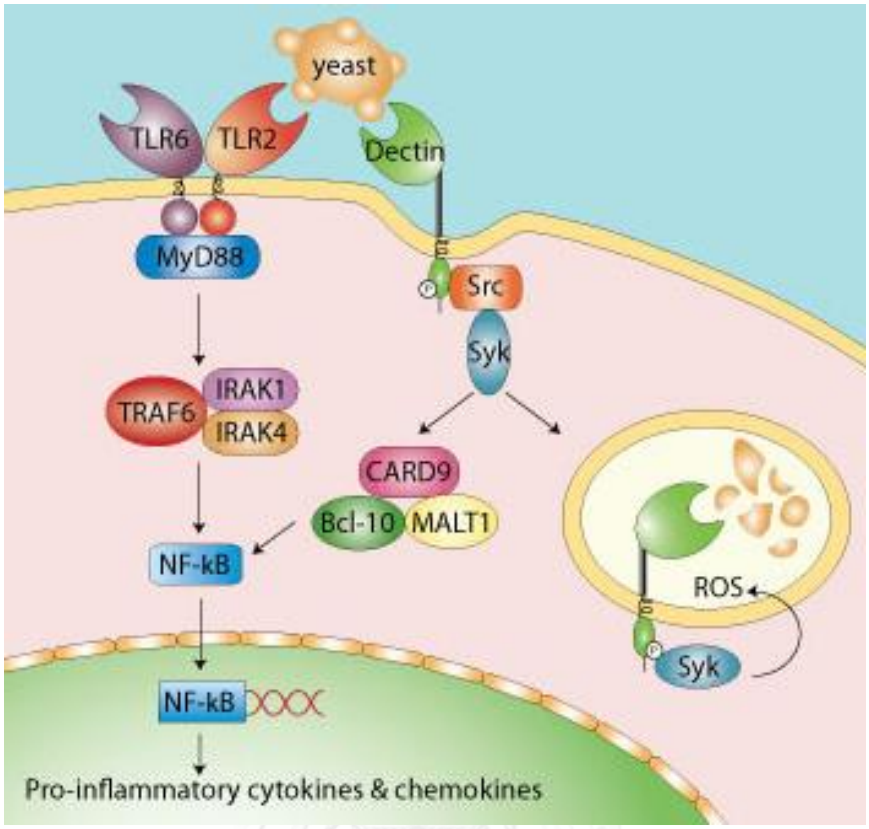


Figure 5: The recognition of yeast and intracellular signaling in macrophage. Yeast is recognized by Dectin-1 and TLR2 to generate signaling cascade by Src/Syk-dependent and MyD88 coupled TLRs pathways to produce several cytokines and chemokines (37).

- **Scavenger receptors (SR)** are trimeric, transmembrane glycoprotein receptors which involve 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). In addition, 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 while integrins and phosphatidylserine (PS) receptor are play role in phagocytosis of various apoptotic cells such as aged red blood cells. At the end of their life span, macrophages remove aged red blood cells by erythrophagocytosis (38, 39).

2. Engulfment

Phagocytes engulf foreign particles by prolongation of the cytoplasm (pseudopods) around the particle to be engulfed, until complete enclosure of the particle within the phagosome. After that, phagosome is fused with lysosome to become a phagolysosome and the engulfed particle is exposed to degradative lysosomal enzymes.

3. Killing or degradation

After engulfment, the foreign particles are killed or degraded by two complex mechanisms.

Oxygen-dependent mechanisms

During the processes of phagocytosis, increased in oxygen uptake by phagocyte which is called the respiratory burst occurs. Oxygen molecules can be converted to reactive oxygen species (ROS) like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme. Furthermore, peroxynitrite (ONOO-) is one of product which generated from the combination of NO which is catalyzed by inducible nitric oxide synthase (iNOS) and O_2^- to induce microbial toxicity. These species have single unpaired electrons in their outer orbits that easily react with other molecules in any biological cell membrane or nucleus to cause damages. These mechanisms use large amounts of O_2 which cause neutrophils die shortly after ingestion and destroying microbes (40).

Oxygen-independent mechanisms

These mechanisms are mediated by the constituents of phagocyte's granules; defensins, lysozymes, lactoferrin, cathepsins and cationic proteins to degrade the engulfed microbes. For example, peptidoglycan wall of bacteria is broken down by lysozyme, thereby lysing the cell. Lysozyms hydrolyse both peptidoglycan of gram-positive bacteria and some of gram-negative bacteria. Furthermore, the acidification

of the phagosome which low pH 4.0-5.0 is an opposed environment that impedes the microbial growth (40, 41).

Macrophage act as secretory cells

For destroy pathogens and tissue repair, activated macrophages act as secretory cell to produce pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines that play an important role in immunological signaling and function.

The major pro-inflammatory cytokines and chemokines secreted from macrophages

Pro-inflammatory cytokines and chemokines play important roles in leukocyte movement and migration from blood circulation to inflammatory tissues. They also increase T and B cell activation (42).

Pro-inflammatory cytokines

Interleukin-1 (IL-1)

IL-1 is produced by fibroblasts, keratinocytes, endothelial cells, T and B lymphocytes and macrophages. It increases expression of adhesion molecules that mediate leukocyte adhesion to other cells, inflammatory processes activation and induction expression of acute-phase proteins. IL-1 and TNF secrete to the blood circulation share synergistic biological effects in sepsis. IL-1 also induces the production of PGE_2 from vascular endothelium of the hypothalamus which causes fever. There are two isoforms of IL-1, IL-1α and IL-β. IL-1α is mainly produced from keratinocytes and endothelial cells and acts locally, while IL-1β is mainly produced by monocytes and macrophages after activated by LPS, interferon-γ and other stimuli (43). IL-1β is the main biologically active secreted form. It is secreted in a processed form and functions as an endocrine signaling molecule. IL-1β production usually requires two distinct signals, one that activates new gene transcription and production of a precursor pro–IL-1β polypeptide. This signal is induced by TLR signaling pathway which activates transcription factor, NF-kB. The second signal activates the NLRP3 inflammasome to proteolytically cleave the precursor to generate the mature IL-1β protein (Fig. 6) (44). IL-1β functions as an endocrine

signaling molecule through type I IL-1 receptor, which is expressed on endothelial cells, epithelial cells, and leukocytes (27, 45).

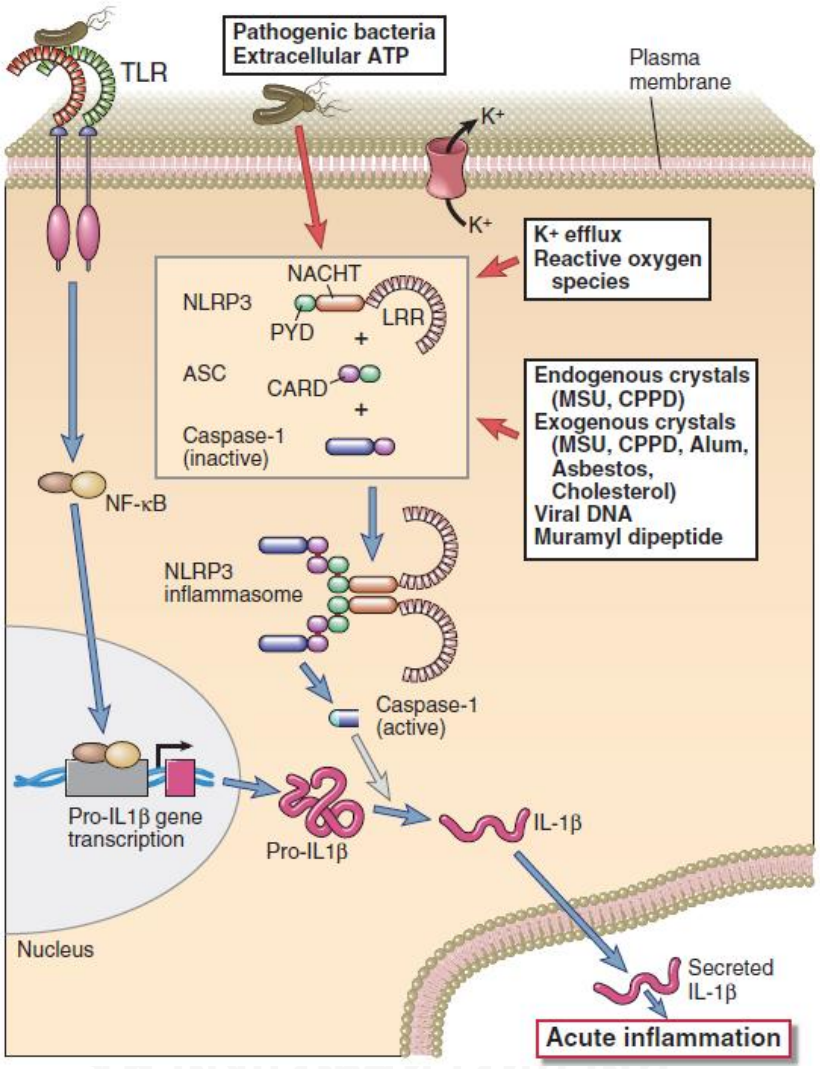


Figure 6: Inflammasome. NLRP3 inflammasome was activated. Activated inflammasome processes pro-IL-1β to active IL-1β. Various PAMPs induce pro-IL-1β expression through pattern recognition receptor signaling, including TLR (44).

Interleukin-6 (IL-6)

Interleukin-6 is an immune protein produced by monocytes, macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. IL-6 is released in response to infection, burns, trauma, and neoplasia. Its functions range from an important role in acute-phase protein production, complement and clotting cascades activation,

inducing B- and T- cell growth and the inducing B-cells differentiation into antibody forming cells or plasma cells. IL-6 has been shown to be an endogenous pyrogen capable of inducing fever in people with infections (46).

Tumor necrosis factor (TNF-α)

TNF-α is produced by macrophages, neutrophils, fibroblast, mast cells, T cells and NK cells, but especially by macrophages in response to inflammation, infection, and other environmental stress. Lipopolysaccharide is an especially potent stimulus for TNF-α synthesis. TNF- α has a wide spectrum of biological activities. It is a pro-inflammatory cytokine like IL-1 and IL-6 and acts as an activator to activate cytokine production in macrophage. TNF-α is also be an acute phase protein which can increase vascular permeability resulting in recruiting macrophages and neutrophils to site of infection. It also induces the proliferation of neutrophils during inflammation and causes necrosis of some types of tumors. Prolonged over production of TNF-α induce sepsis (47).

Chemokines

Chemokines or chemotactic cytokines are family of cytokines which induce directed chemotaxis in adjacent responsive cells by gradient of them. They are divided into four sub-families: C–*x*–C, C–C, C, and C–x_3–C, based on the position of the first two cysteines in N-terminal, as well as the remaining cysteines in the C-terminal of the molecule. Monocytes/macrophages, T lymphocytes, basophils and eosinophils are mainly chemotactic by the C-C subfamily of chemokines like MCP-1 and MIP-1 α.
MCP-1 and MIP-1 α are induced during an immune response to activate and recruit leukocytes to inflamed area by interacting with G protein-linked transmembrane receptors called chemokine receptors that are found on surface of leukocytes. Chemokines and their receptors are considered important contributors in cell migration and inflammation in chronic inflammatory disorders. Therefore, agents that inhibit the action of chemokines affecting monocytes/macrophages function may be a useful drug for controlling chronic inflammation (48).

Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 is a chemokine that attracts monocytes, memory T cells, dendritic cells and natural killer cells to the site of injury but not neutrophils. It can be produced by monocytes, macrophages, vascular endothelial cells, smooth muscle cells and osteoblastic cells upon induction with the activators such as growth factors, cytokines, double-strand RNA and LPS. It regulates the expression of adhesion molecules and the expression of IL-1 and IL-6. In addition, previous studies have shown that MCP-1 and its receptor, CCR2 played a critical role in many inflammatory and autoimmune diseases like atherosclerosis, multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (49-52).

Macrophage Inflammatory Protein-1α (MIP-1α)

MIP-1α is a member of the C-C subfamily of chemokines that has a chemotaxis activity to induce eosinophils and macrophages to the inflammatory site. It is produced by monocytes, macrophages, mast cells, Langerhans cells, fibroblast and T lymphocytes (53). It was found that the production of MIP-1α correlated with increasing acute disease severity and remained elevated throughout chronic diseases.

Inflammatory mediators

Nitric *oxide (NO)*

Nitric oxide is a soluble gas generated by a variety of cell types including monocytes/macrophages, neutrophils, vascular endothelial cells, fibroblasts and platelets. It is synthesized from amino acid L-arginine to L-citrulline by three isoforms of nitric oxide synthase (NOSs) enzymes depending on type of tissue from which they are produced. The three isoforms are endothelial NOS (eNOS or NOS I), inducible NOS (iNOS or NOS II) and neuronal NOS (nNOS or NOS III). Endothelial NOS and neuronal NOS are produced from vascular endothelial cells and neuronal cells, respectively. Another isoform of NOS is inducible NOS. It is found upon inflammatory stimulation in fibroblasts, endothelial cells, keratinocytes, chondrocytes, NK cells and monocytes/macrophages (54). NO activity is strongly depending on its concentration.

eNOS and nNOS in resting cells generate low level of NO which acts in a short period as a vascular-relaxing agent, an inhibitor of platelet aggregation and a neurotransmitter. iNOS in macrophages is induced by pro-inflammatory cytokines, bacterial products or infectious cells. Large amounts of nitric oxide synthesized primarily by iNOS in macrophages acting as a free radical which is toxic to invading pathogens. In chronic inflammation, NO is an important inflammatory mediator is working together with other pro-inflammatory cytokines; TNF- α, IL-1 and IL-6, resulting in up regulation of iNOS in macrophages (55). Over production of NO by iNOS has been shown to be involved in various pathophysiology of human diseases. They are septic shock, rheumatoid arthritis and atherosclerosis (56).

Prostaglandins

Prostaglandins are hormone-like substances derived from arachidonic acid and have several important functions. Arachidonic acid (AA) is released from membrane phospholipid and is converted to prostaglandin H_2 (PGH_2) by cyclooxygenase enzymes (COX). They have two isoforms; COX-1 and COX-2. COX-1 is a constitutively enzyme that generates prostaglandins in physiological amount for normal functions of tissues and organs (57). COX-2 is selectively induced by pro-inflammatory cytokines and generates inflammatory mediators like PGE_2 and $PGF_{2\alpha}$. PGE_2 is a well characterized mediator involved in inflammation and pain (58). It is converted from PGH_2 by microsomal prostaglandin E synthase-1 (mPGES-1). Excessive and chronic production of PGE_2 causes pain and fever. Thus compounds which specifically block COX-2 or mPGES-1 generation may be beneficial for the treatment of chronic inflammatory diseases (59).

Anti-inflammatory cytokines

Interleukin-10

IL-10 is a cytokine produced by macrophages, dendritic cells, regulatory T cells and keratinocytes. The production of various inflammatory cytokines both at mRNA and protein levels by LPS-activated macrophages and dendritic cells, including IL-1β, TNF-α, IFN-γ, IL-8, and IL-12 are inhibited by IL-10 (60). Because IL-10 is produced by

macrophages and it also inhibits macrophage functions, therefore it is found relatively late following activation of macrophages. IL-10 is a negative feedback regulator to control innate immune reactions and cell-mediated immunity (61). IL-10 also inhibits the expression of co-stimulatory molecules and MHC class II molecules on dendritic cells and macrophages. Previous studies has shown that IL-10 suppresses macrophages by down-regulate the pro-inflammatory M1 phenotype (62). Therefore, IL-10 serves to inhibit T cell activation and terminate cell-mediated immune reactions (63).

Transforming Growth Factor-beta1 (TGF-β1)

TGF-β was discovered as a tumor product that promoted the survival of tumor cells *in vitro*. It has three isoforms TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is mainly synthesized and secreted by cells of immune system; CD4+ regulatory T cells and activated macrophages. It is a potent immunosuppressive and 'macrophage-deactivating' agent. Previous study has shown that TGF-β1 suppressed TNF-α and IL-6 production in LPS-activated macrophages (64). It inhibits the proliferation and the effector functions of T cells, and macrophage activation. Furthermore, it stimulates collagen synthesis and matrix-modifying enzyme production by macrophages and fibroblasts. It also promote angiogenesis leading to resolution of the inflammatory response (65, 66).

Metabolism of iron by macrophages

Iron is an essential element for cell survival and proliferation. Iron in the body is regulated to guarantee enough iron availability for the various essential functions including oxygen transport, electron transfer, and DNA synthesis. Most of total body iron in vertebrates comes from recycle iron because dietary iron availability is very low (1-2 mg daily). Erythroid precursors in bone marrow are the major site of iron employment. They need larger amount of iron (about 20 mg daily) for their proliferation and hemoglobin synthesis. Most of the body iron is provided by macrophages that clear senescent red blood cells (RBC) and release iron from heme destruction back to blood circulation. Normally, 60-70% of total body iron is in

hemoglobin of circulating RBC. Iron is transported within the body between sites of absorption and storage. This iron binds reversibly with plasma glycoprotein transferrin and 80% of this iron is transported for hemoglobin synthesis in the bone marrow (Fig.7) (67).

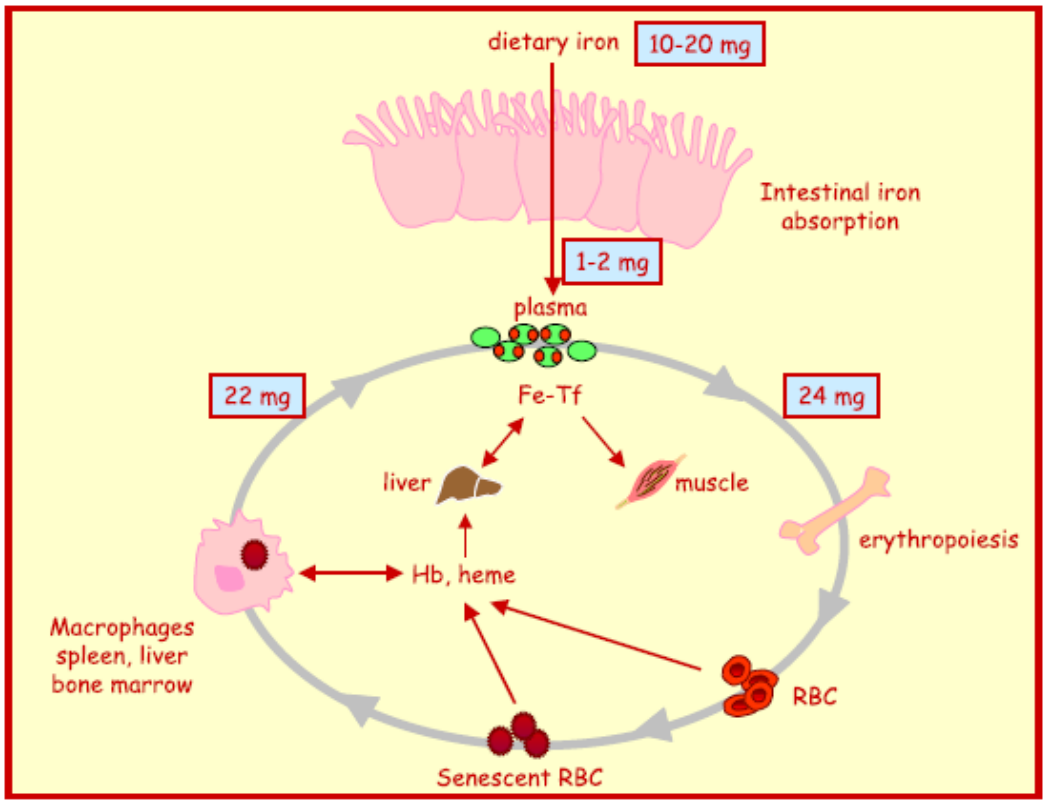


Figure 7: Metabolism of iron by macrophage. Dietary iron is absorbed from intestinal and bound to plasma transferrin (Tf). Fe-Tf is transported for red blood cell production in bone marrow. Macrophages phagocytose aged RBC and release iron from heme destruction back to circulation (67).

There are two pathways of iron acquisition by tissue macrophages; intravascular hemolysis and extravascular hemolysis or erythrophagocytosis.

Intravascular hemolysis, erythrocytes about 10–20% are eliminated through this pathway. Glycoprotein haptoglobin (Hp) which is synthesized from the liver is formed complex to hemoglobin in blood circulation. This complex is then bound to

specific receptor CD163 on macrophages membrane and internalized by macrophages. If Hp saturation, free Hb in plasma can be oxidized to methemoglobin, then heme binds to the plasma protein hemopexin to form complex. After that, this complex is internalized by macrophages through specific receptor CD91.

Extravascular hemolysis is the main pathway of iron acquisition by macrophages which occurs in the spleen and bone marrow and to a lesser scope in the Küpffer cells. During aging, RBC accumulate multiple modifications (cell shrinkage, externalization of phosphatidyl-serine, peroxydation of the membrane lipoproteins, loss of sialic acid residues, appearance of neoantigens of senescence), which have essential signals for the macrophage to eliminate them. Macrophages phagocytose senescent RBC. Senescent RBC are internalized and degraded in an erythrophagolysosome. After that, heme is released and iron is extracted from heme by heme oxygenase 1 enzyme. This extracted iron is then exported back to the plasma through iron exporter, ferroportin or stored in their cytoplasmic ferritin (Fig. 8) (67)

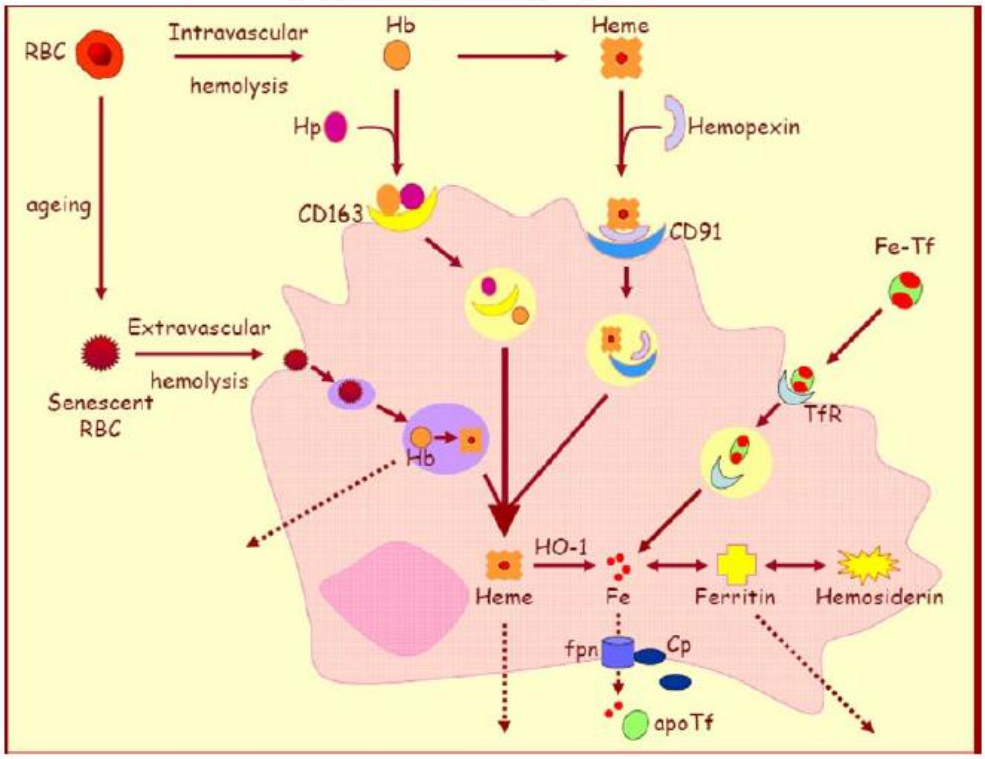


Figure 8: Pathways of iron acquisition and iron release by macrophage. After inntravascular hemolysis, heme and hemoglobin (Hb) are released in blood circulation. Hb is then form complex with haptoglobin (Hp) and is taken up by specific receptor CD163. The complex of free heme and hemopexin is internalized by CD91. Extravascular hemolysis, macrophage phagocytoses senescent RBCs and heme is degraded by heme oxygenase 1 (HO-1). Iron is exported to the plasma by ferroportin (fpn) (67).

Anemia of inflammation (AI)

Anemia of inflammation or anemia of chronic disease is usually a mild to moderate anemia with hemoglobin 7-12 g/dl. AI is characterized by hypoferremia due to iron sequestration, resulting in the limitation of iron supply to erythropoiesis. It develops in many infections and inflammatory diseases, and some malignancies (3, 68, 69). The restriction of iron from microorganisms is one of host defence mechanism for limiting intracellular growth within activated macrophages. The molecular mechanisms of iron sequestration are come from cytokine-stimulated overproduction of iron-regulatory hormone hepcidin, particularly IL-6 (Fig. 9) (67). Hepcidin is a peptide hormone for iron homeostasis produced mainly by hepatocytes. It causes the endocytosis and proteolysis of cellular iron exporter, ferroportin-1 (Fnp1). Fnp-1 is mainly expressed in duodenal enterocytes and macrophages. In AI, hepcidin is up-regulated by pro-inflammatory cytokines, suppresses intestinal iron absorption and sequesters iron by macrophages in reticuloendothelial system by down-regulating Fpn1, causing to iron deficient anemia (3, 70). Inflammation can also affect expression of Fpn1. Previous studies have shown that injection of LPS into mice and rats stimulate hepcidin production and reduce Fpn1 transcription in spleen, liver and intestine (71-73). In addition, LPS or IFN-γ also down-regulate macrophage Fpn1 mRNA expression in hepicidin-independent manner (71, 74).

Recent study has shown that gene expression profiles related to iron metabolism are exactly polarized between different types of activated macrophages. Iron is an essential growth factor for both mammalian cells and microorganisms. During inflammation, M1 macrophages sequester iron as bacteriostatic mechanism to

restrict iron from invading pathogen by decreasing the expression of cellular iron exporter (Fpn1). In addition, pro-inflammatory cytokines also induce liver hepcidin production to inhibit macrophage iron release by targeting Fpn1 degradation leading to iron deficient anemia (75). Conversely, M2 macrophages are express Fpn1 in high levels displaying a phenotype associated with iron release, which is clearly different from the iron sequestration response induced in M1 macrophages (76). Thus, any process increasing the iron export from macrophages during inflammatory state should be regards as a protective mechanism causing to attenuation of anemia.

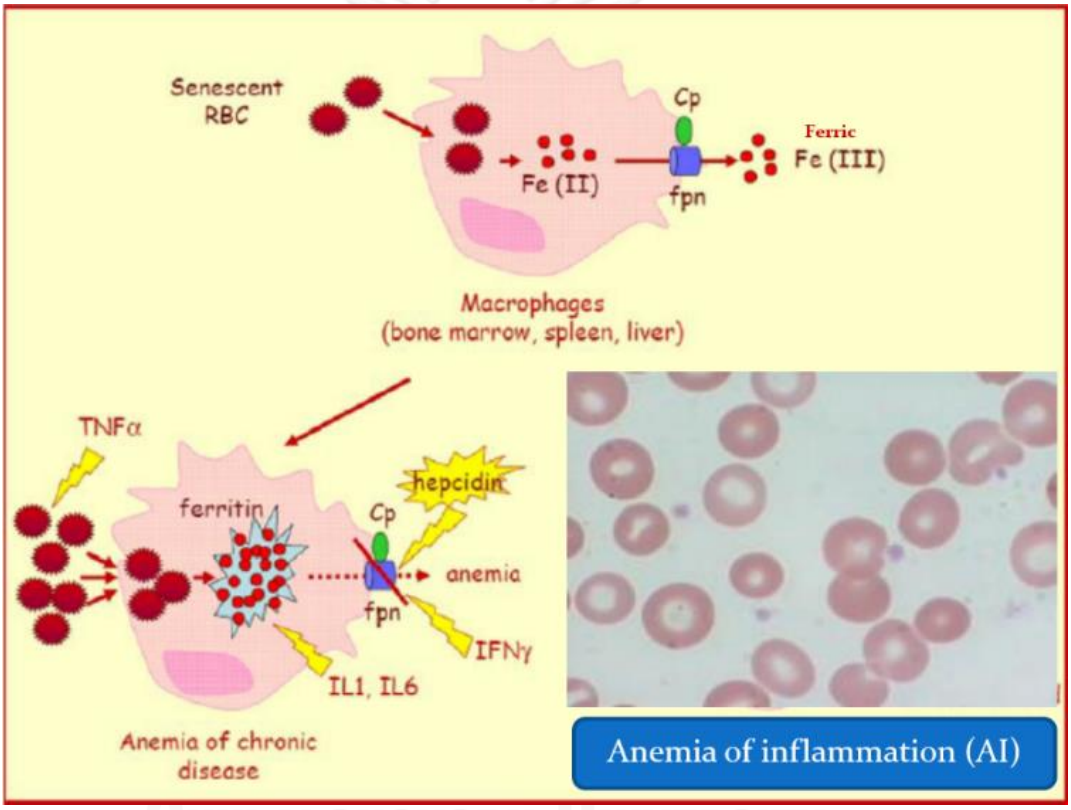


Figure 9: Diseases related to defects in iron metabolism. Alterations of erythropoiesis and iron metabolism cause anemia of chronic disease. Hereditary hemochromatosis is the consequence of increased intestinal iron absorption but also increased iron efflux from macrophages following defective hepcidin expression (67).

Anti-inflammatory drugs

At present anti-inflammatory drugs are classified into 3 classes; steroidal drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and biologic drugs. They are

used for the treatment of inflammation in order to reduce pain, fever, swelling and inflammation.

Steroidal drugs

Dexamethasone, fludrocortisones, triamcinolone and betamethasone are corticosteroids commonly used in inflammatory and autoimmune diseases as ashma, ulcerative colitis and prevention of organ transplant rejection. In moderate to severe inflammatory diseases, steroidal drugs are used to reduce inflammation or swelling irrespective to the inflammation's cause. They bind to glucocorticoid receptors (GR) in cytosol of the cell to form steroid-receptor heterodimer complex (activated GR complex) and translocate to the nucleus. In the nucleus, this complex binds to glucocorticoid response elements (GRE) on glucocorticoid-responsive gene leading to increase the expression of anti-inflammatory proteins, lipocortin-1 (annexin-1). Lipocortin-1 suppresses phospholipase A_2 activity thereby inhibits arachidonic acid releasing from membrane phospholipid and other main products of inflammation. In addition, this activated GR complex suppresses the expression of pro-inflammatory cytokines, iNOS and COX-2 by preventing the translocation of the transcription factor from the cytosol into the nucleus. Steroidal drugs cannot be consumed over long periods of time because they often produce numerous harmful side effects like immune deficiency, glaucoma and steroid-induced osteopororsis (77).

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are commonly used to reduce mild to moderate pain, swelling and inflammation. They can be classified into two groups according to the selectivity to COX enzyme. They are non-selective COX inhibitors and selective COX-2 inhibitors (78).

- Non-selective COX inhibitors or standard NSAIDs like diclofenac, ibuprofen, indometacin, aspirin and naproxen are indicated for management of acute or chronic inflammatory diseases in rheumatoid arthritis, osteoarthritis, acute gout etc. They inhibit both COX-1 and COX-2 enzymes and cause the inhibition of prostaglandins production from arachidonic acid resulting in reduction of pain and inflammation at sites of damage. Most people who take NSAIDs for a long time will experience some common adverse effects including gastrointestinal (GI) and renal effects. The GI

effects are caused by the acidic molecules of NSAIDs directly irritate the gastric mucosa. In addition to the acidic molecules, reduction of protective prostaglandins synthesis by inhibition of COX-1 enzymes can lead to increase in gastric acid secretion, decrease bicarbonate and mucus secretion (79).

- Celecoxib, etoricoxib, and lumiracoxib are selective COX-2 inhibitors which selectively block COX-2 enzyme. These medicines reduce risk of peptic ulceration but they increased risk of heart attack, thrombosis and stroke by a relative increase in thromboxane A_2 production (6, 7, 80).

Biologic drugs

Biologic drugs used for the treatment of chronic inflammatory diseases are anti-TNF-α the IL-1 receptor antagonist (IL-1Ra). They are genetically engineered drugs targeted to specific proteins involved in inflammation. They are usually used in patients who have not responded to the conventional drugs. Large amount of proteins produced by several immune cells can be neutralized by biologic drugs before they cause inflammation. Biologic drugs are a relatively new treatment, so their long-term efficacy and side effects are not fully known, but the common side effects of them are infections, hypersensitivity and immune system disorder (81-84).

Cinnamomum cassia

The genus *Cinnamomum* in family Lauraceae comprises over 250 plant species. *C. cassia* is an evergreen tall tree grows up to 65 ft (20 meters) high with aromatic bark. It is a native plant in Southern China, India, Bangladesh, Sri Lanka and Vietnam. The leaves of this plant are opposite, ovate with three prominent veins. The young leaves are reddish and turn dark green when they get older. The fruit is green fleshy which contains one seed and turn dark purple or black on maturation and similar in size to a small olive. The leaves and barks are popularly used as flavoring agents in dental and pharmaceutical preparations, foods, bakery and beverages in several cultures due to their delicate aroma and sweet taste (85). In traditional medicine, *C. cassia* is used for the treatment of chronic bronchitis, inflammation of eye, vaginitis, toothaches and rheumatism. In Ayurvedic medicine, it

is also used as an antiemetic, antidiarrheal, antispasmodic and antiflatulant remedy (9, 86). It contains several pharmacologically active substances like essential oil, tannin and carbohydrate (85, 87).

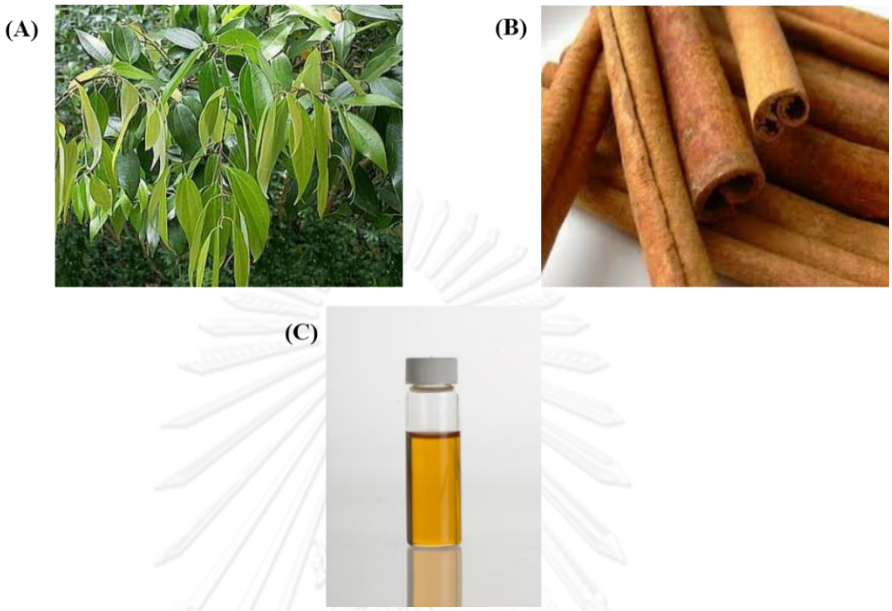


Figure 10: *Cinnamomum cassia* Blume. (A) The leaves of *C. cassia*. (B) The barks of *C. cassia.* (C) Cassia oil from the leaves of *C. cassia*.

Essential oil

Essential oil of *C. cassia* or cassia oil is extracted from the barks or leaves of *Cinnamomum cassia* by steam distillation. It has been traditionally used in the treatment of colds, influenza, fevers, dyspepsia and other inflammatory diseases such as gastritis, arthritis and rheumatism. In addition, it is used medicinally as carminative, antidiarrheal, antimicrobial, and antiemetic remedy (85). Some pharmacological effects of cassia oil are demonstrated including hypouricemia, anti-oxidant, anti-inflammatory, antibacterial and antifungal activities (14, 16). The chemical compositions of cassia oil vary depending on the parts and the species of the plants (10). Previous studies have shown that essential oil from different parts of *C. cassia* contain some different amount and volatile compositions (15). Cassia oil from the barks and leaves are composed of alcohol, aldehyde, alkane, ester, ether and ketone (11). Cassia oil from the leaves of *C. cassia* will be used in this study. Previous study found that cassia oil from the leaf of *C cassia* contained 22 volatile

components. The main components were trans-cinnamaldehyde, 3-methoxy-1, 2-propanediol, o-methoxy-cinnamaldehyde, coumarin and glycerin at 30.36%, 29.30%, 25.39%, 6.36% and 3.01%, respectively. The amount of others volatile components was less than 1% (15).

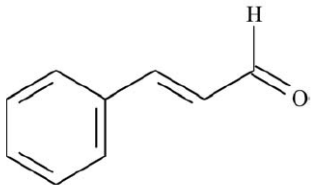


Figure 11: Chemical structure of cinnamaldehyde (88).

Table1: Volatile compounds identified from the essential oil of *C. cassia* leaf and their pharmacological properties.

Compound	Percentage (%)	Pharmacological properties
trans-Cinnamaldehyde (0, 6.25, 12.5, 25, and 50 μM)	30.36	- Inhibit NO, iNOS, COX-2, TNF-α, PGE_2, NF-kB and IkBα in LPS-stimulated RAW-264.7 cells with IC_{50} value 45 μM. - Reduce serum NO, TNF-α, and PGE_2 levels in carrageenan-induced mouse paw edema model (89).
o-Methoxy-cinnamaldehyde (1, 10, 20, 50 μM)	25.39	- Decrease vascular cell adhesion molecule-1 (VCAM-1) expression in TNF-α-activated endothelial cells (90).
Coumarin (1, 5, 10 μM)	6.36	- Inhibit iNOS, COX-2, TNF-α, IL-1β and activity of NF-kB in LPS-activated HT-29 human colon carcinoma cells (91). - Inhibit NF-kB, IkBα in LPS-stimulated RAW-264.7 cells (92).

Pharmacological activities of *Cinnamomum* species

Antimicrobial activities

Cassia oil has been reported to exert inhibitory effects on fungal spore production and bacterial growth on tomatoes (93). Cassia oil, as well as the major components cinnamaldehyde and eugenol had antimicrobial effects on *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes*, and *Salmonella enteric*, and *Morganella morganii in vitro* and *in vivo* studies (94, 95). Methylene chloride extracts 50 μg/ml of cinnamon completely inhibited *Helicobacter pylori* growth (96, 97). Furthermore, the essential oils of several *Cinnamomum* species had a significant inhibitory effect against *Candida* and pathogenic bacteria in *in vitro* studies (98, 99).

Antiviral activities

Cinnamomum cassia bark extract was highly effective against HIV-1 and HIV-2 replication by inhibition of virus induced cytopathogenicity in MT-4 cells infected with HIV (100).

Anti-cancer/tumor activities

The essential oil from the bark of *Cinnamomum zeylanicum* has been reported to possess anticarcinogenic effect on fibroblasts cell lines with the IC_{50} value less than 20 μg/ml (101). Cinnamaldehyde which was the main active compound found in essential oil from the barks and leaves of *Cinnamomum cassia* was a potent inducer of apoptosis in human promyelocytic leukemia HL-60 cells. It induced reactive oxygen species (ROS) generation resulting in mitochondrial permeability transition and cytochrome c release to the cytosol (88, 102). 2'-Hydroxy-cinnamaldehyde and 2'-benzoyloxycinnamaldehyde which were isolated from *Cinnamomum cassia* significantly inhibited the growth of 29 kinds of human cancer cells *in vitro* and the growth of SW-620 human tumor xenograft in nude mice at the cellular level (103).

Cardiovascular activities

Cinnamomum cassia has been shown to affect the blood and cardiovascular system by reducing the water and sodium on the circulatory system resulting in reducing blood pressure in mice after oral administration of *Cinnamomum cassia* bark extract (104, 105). Previous studies have shown that essential oil from *Cinnamomum magio* could reduce systolic and diastolic arterial blood pressure, heart rate, carbon monoxide level in anesthetized open-chest cat after intradermal application (106).

Anti-inflammatory and antioxidant activities

Cinnamomum cassia exerted anti-inflammatory both in *in vivo* and *in vitro* studies. Cinnamic aldehyde from bark and leaf essential oil acted as an anti-inflammatory agent to reduce inflammation in the edema paw after carrageenan injection. It reduced the nitric oxide, tumor necrosis factor and prostaglandin E_2 levels in blood circulation and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in paw tissue of carrageenan-induced mouse paw edema model. Cinnamic aldehyde could also inhibit NO, TNF-α, PGE_2 levels and significantly blocked protein expression of iNOS, COX-2, NF-kB and IkB in LPS-stimulated mouse macrophage (RAW264.7) (107). *In vitro* studies, the adhesion of TNF-α-induced monocytes to endothelial cells and the expression of the cell adhesion molecules, VCAM-1 and ICAM-1 were inhibited by cinnamaldehyde (50-200μM) resulting from suppressing NF-kB activation (89). Cinnamaldehyde (24-80μM) isolated from essential oil produced from leaves of *Cinnamomum osmophloeum* has been reported to inhibit LPS-induced pro-inflammatory cytokines secretion (TNF- α, IL-1β, IL-6), reactive oxygen species in murine J774A.1 macrophages by inhibiting the phosphorylation of extracellular signal-regulated kinase (ERKs) and c-Jun N-terminal kinase (JNKs) (108).

Although essential oil extracted from the bark of *C. cassia* and cinnamaldehyde have been reported to have biological activities such as antioxidant

and

anti-inflammatory activities. However, very limited reports on the anti-inflammatory effect of essential oil extracted from the leaves of *C. cassia*. Depend on these data, the objectives of this study were to investigate and compare the anti-inflammatory activities of cassia oil and cinnamaldehyde in LPS-stimulated macrophage J774A.1cells on phagocytic activity and the expression of pro-inflammatory cytokines, chemokines, inflammatory enzymes and NO production. In addition, effects of cassia oil and cinnamaldehyde on the expression of anti-inflammatory cytokines and Fpn1 were also evaluated in LPS-stimulated macrophage J774A.1cells.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Cassia oil and cinnamaldehyde

Cassia oil was purchased from Thai-China flavors and fragrances industry, Thailand. It was extracted from leaves of *Cinnamomum cassia* by steam distillation. Trans-cinnamaldehyde was purchased from Sigma, USA. Cassia oil and cinnamaldehyde were prepared as stock solution by diluting in 80% ethanol in incomplete Dulbeco's Modified Eagle's Medium (DMEM). The stock solutions were stored in dark glass bottles at 4°C before use. These stock solutions were diluted to desired final concentrations with constant 0.4% ethanol in DMEM to the same desired concentrations.

2. Cell culture

Mouse macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin and incubated in 5%CO_2 at 37°C. They were subcultured three times weekly during use. Their viability was determined by 0.4% trypan blue. The cells with their viability not less than 90% in the exponential growth phase were used in all experiments in this study.

3. Chemicals and reagents

The following chemicals, reagents and reagent kits were used in this study; DMEM medium (Gibco, USA), fetal bovine serum (Gibco, USA), penicillin/streptomycin (Gibco, USA), 0.4% trypan blue dye (Sigma, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), Resazurin (Sigma, USA), nitroblue tetrazolium (NBT) (Sigma, USA), zymosan A from *Saccharomyces cervisiae* (Sigma, USA), lipopolysaccharide (Sigma, USA), nitric

oxide assay kit (Promega, USA), TRIzol Reagent (Invitrogen, USA), dietyl pyrocarbonate (DEPC) (Molekula, UK), absolute ethanol (Merck, Germany), absolute methanol (Merck, Germany), chloroform (Merck, USA), ImProm-II™ reverse transcription system (Promega, USA), primer (Bio Basic, Canada), Taq polymerase (Vivantis, USA), Mouse IL-1β ELISA kit (Thermo scientific, USA), Mouse PGE_2 ELISA kit (Abnova, Taiwan)

4. Equipments and Instruments

The following equipments and instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autopipette (Gilson, USA), biohazard laminar flow hood (Science, Germany and Labconco, USA), centrifuge (Hettich, USA and Eppendrof, Germany), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), scrapper (Greiner, UK), autoclave (Hirayama, Japan), pH meter (Mettler Tuledo, Switzerland), refrigerator 4°C and -20°C (Sanyo, Japan), Incubator (Thermo, USA), Light microscope (Nikon, Japan), vortex mixer (Scientific Industries, USA), PCR thermal cycler (Eppendorf, Germany), T25 tissue culture flasks (Corning, USA), 96- and 24-well plates (Corning, USA)

Methods

All assays were done in duplication or triplication with 3-5 optimal concentrations and in at least three independent experiments. Dexamethasone and 0.4% ethanol were used as the positive and the negative control, respectively. The effects of cassia leaves oil were compared to the effects of cinnamaldehyde.

1. Analysis of essential oil

Essential oil from leaves of *C. cassia* was analyzed by gas chromatography (GC) and mass spectrometry (MS) using Finnigan Trace GC ultra Finnigan DSQ Quadrupole detector system (Allured: Illinois, USA). The components of *C. cassia* leaves oil were identified by matching their mass spectra and retention indices with Adams EO Mass Spectral library and NIST05 Mass Spectral library. The relative concentration of each compound in essential oil was computed from GC peak areas by the analysis program.

2. Determination of effect of cassia oil and cinnamaldehyde on NO production in LPS-activated macrophages by Griess reaction assay

1. J774A.1 cells at a density of $4x10^5$cells/ml were cultured in 96-well plate for 24h.

2. The cells were treated with 5 concentrations of cassia oil or cinnamaldehyde in 0.4% ethanol and 100 ng/ml LPS for 24h at 37°C.

3. Hundred µl supernatant of the treated cells in both conditions were collected for determining amount of NO in nitrite form.

4. The supernatants were reacted with 20 µl of sulfanilamide for 10 min and with 20 µl of NED (N-1-napthylethylenediamine dihydrochloride) for 10 min in the dark at room temperature.

5. The reaction mixture was measured at 540 nm by a microplate reader. The concentrations of NO were determined from a standard curve.

6. The percentage of nitric oxide inhibition of cassia oil or cinnamaldehyde was determined by comparing to the LPS-activated condition using the following formula:

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

3. Determination of effect of cassia oil and cinnamaldehyde on cell viability resazurin reduction assay

Resazurin is a dye which can be used as an indicator of viable cells. In viable cells, this dye (dark-blue, absorption peak at 600 nm) is reduced by mitochondrial reductase enzyme to resorufin (pink, absorption peak at 570 nm). The experiment was performed with the following procedures.

1. The treated cells from NO production determination were incubated in 100 µl fresh DMEM medium containing 50 µg/ml resazurin for 2h at 37°C.

2. The reaction mixture was measured at 570 nm and 600 nm by a microplate reader. The percentage of cell viability was calculated as follows:

$$\% \text{ Cell viability} = [\Delta OD_{(control)} - (\Delta OD_{(sample)} / \Delta OD_{(control)})] \quad X \quad 100$$

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

4. Determination of effect of cassia oil and cinnamaldehyde on phagocytic activity of LPS-activated macrophages

The effect of cassia oil and cinnamaldehyde on phagocytic activity in macrophage J774A.1 cells was determined by zymosan-nitroblue tetrazolium (NBT) reduction assay. Zymosan is prepared from cell wall of yeast (*Saccharomyces cerevisiae*) and consists of protein-carbohydrate complexes. It is used as pathogen in phagocytosis assays. During the processes of phagocytosis by macrophage can lead to increase in oxygen uptake also called respiratory burst. Therefore the activated macrophage is able to make the reactive oxygen species, super oxide free radical that can reduce the yellow NBT dye to the insoluble purple-blue formazan which has absorption peak at 570 nm. The procedure was as below;

1. Macrophage J774A.1 cells at a density of $4x10^5$cells/ml were cultured in 96-well plate for 24h.

2. The cells were treated with cassia oil or cinnamaldehyde and 100 ng/ml LPS at 37°C for 4h.

3. The treated cells were washed with incomplete DMEM and further incubated with 800 µg/ml zymosan and 600µg/ml NBT for 1h.

4. The cells were washed thrice with absolute methanol and the insoluble purple-blue formazan in each well was dissolved by adding 2M KOH solution and 100% DMSO.

5. The reaction mixture will be measured at 570 nm by a microplate reader.

6. The inhibitory effect of cassia oil and cinnamaldehyde were presented as percentage of phagocytosis inhibition comparing to the LPS-stimulated condition by the following formula:

$$\% \text{ Phagocytic inhibition} = [(OD_{(control)} - OD_{(sample)}) / OD_{(control)}] \times 10$$

5. Determination of effect of cassia oil and cinnamaldehyde on mRNA expression of IL-1β, IL-6, TNF-α, IL-10, TGF-β1, Fpn1, iNOS, COX-2, mPGES-1, MCP-1 and MIP-1 α in LPS-activated macrophages

The effects of cassia oil and cinnamaldehyde on the mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF- α), inflammatory enzymes (iNOS, COX-2 and mPGES-1) and chemokines (MCP-1 and MIP-1α), anti-inflammatory cytokines (IL-10, TGF-β1) and iron exporter protein (Fpn1) were determined by reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis as following steps.

1. J774A.1 cells at a density of $4x10^5$ cells/ml in 24-well plate were treated with cassia oil or cinnamaldehyde in the presence or absence of 100 ng/ml LPS at 37°C for 4h in order to determine the mRNA expression of IL-1β, IL-6, TNF- α, MCP-1, MIP-1α, IL-10 and TGF-β1, Fpn1.

2. The cells were treated with the same conditions as before for 24h in order to determine the mRNA expression of iNOS, COX-2, mPGES-1enzyme, IL-10, TGF-β1 and Fpn1.

3. The supernatant of the treated cells at 24h were used to determine the level of IL-10 and TNF-α by ELISA.

Isolation of total RNA from the treated cells

Methods for isolation of total RNA were determined by using the following procedures.

1. After removed the supernatant, the remaining cells were lyses and homogenized in 500 µl/well TRIzol® reagents.

2. The lysate were transfer to 1.5 ml eppendorf tube and incubated at room temperature for 5 min.

3. The total RNA in the lysate were isolated by adding 200 µl chloroform, vigorously shaken for 15 sec, incubated at room temperature for 5 min and finally centrifuged at 12,000g, 4°C for 15 min.

4. The total RNA in aqueous phase was isolated by adding 500 µl of isopropanol and incubating at -20°C for 1h.

5. The RNA pellets were collected by centrifuging at 12,000g, 4°C for 10 min.

6. The pellet were washed twice with 1 ml 75% ethanol in DEPC treated water using centrifugation at 12,000g, 4°C for 5 min.

7. The pellet were air-dried for 5 min and dissolved in RNase-free water.

8. The RNA concentration and its DNA contamination were measured by Nanodrop at 260 and 280 nm. The ratio of absorbance$_{260/280}$ must be more than 1.8. The total RNA solution was stored at -70°C until used.

Synthesis cDNA from total RNA by reverse transcription

mRNA in total RNA were converted to cDNA using Impromt II reverse transcription system by following procedures.

1. One µl oligo dT15 primer was mixed with 1.5 µg total RNA in 0.2 ml PCR tube.

2. The mixture were heated at 70°C for 5 min and immediately chilled on ice for 5 min.

3. The transcription mixture solution containing 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor and reverse transcriptase were prepared and 15 µl added into each tube.

4. cDNA were synthesized in a thermocycler machine by using the following conditions: 25°C for 5 min then 42°C for 1.5 hour, and 70°C for 15 min.

5. The cDNA samples were stored at -20°C until used.

Synthesis PCR products from cDNA samples by polymerase chain reaction

1. PCR product of interested genes will be amplified from cDNA samples.

2. One µl cDNA sample were mixed with 24 µl PCR reaction mixture solution containing suitable primers, dNTP, Taq polymerase in each 200 µl thin layer tube.

3. The PCR products were generated in a thermocycler under the following conditions: denature at 94 °C for 45 sec, annealing at 53-66 °C for 45 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min.

4. The PCR products were identified by running on 1.5% agarose gel electrophoresis and staining with 0.5 µg/ml ethidium bromide in TBE buffer for 15 min and de-staining with TBE buffer for 30 min.

5. Identify and analyze density of PCR product by gel documentation, and normalized density with the density of β-actin PCR product as follow:

% Internal control = (band density of sample /band density of β-actin) x 100

Table 2: Primer sequences of the investigated genes in RT-PCR

Gene		Primer sequences	(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'	
MCP-1	F	5'- ACTGAAGCCAGCTCTCTCTTCTC-3'	274
	R	5'- TTCCTTCTTGGGGTCAGCACAGAC-3'	
MIP-1α	F	5'- GCCCTTGCTGTTCTTCTCTGT-3'	258
	R	5'- GGCAATCAGTTCCAGGTCAGT3'	
iNOS	F	5'- CCCTTCCGAAGTTTCTGGCAGCAGC-3'	496
	R	5'- GGCTGTCAGAGCCTCGTGGCTTTGG-3'	
COX-2	F	5'- CACTACATCCTGACCCACTT-3'	696
	R	5'- ATGCTCCTGCTTGAGTATGT -3'	
mPGES-1	F	5'- CCTTGAGCTGACAGCCTACC-3'	565
	R	5'- CAGCCTAATGTTCAGCGACA-3'	
IL-10	F	5'- GGACTTTAAGGGTTACTTGGGTTGCC-3'	313
	R	5'- CATTTTGATCATCATGTATGCTTCT-3'	
TGF-β	F	5'- TGGACCGCAACAACGCCATCTATGAGAAAACC-3'	525
	R	5'- TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT-3'	
Fpn1	F	5'- TGGCCTTGTTCGGACTGGTCTG-3'	449
	R	5'- TCAGGATTTGGGGCCAAGATGAC-3'	
β-Actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

Table 3: PCR condition

Gene	Denature	Annealing	Extension	Cycles
TNF-α	T 94°C 30 sec.	T 53°C 45 sec.	T 72°C 1 min.	24
IL-1β	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
IL-6	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
MCP-1	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
MIP-1α	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
iNOS	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
COX-2	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
mPGES-1	T 94°C 30 sec.	T 66°C 45 sec.	T 72°C 1 min.	29
IL-10	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24
TGF-β	T 94°C 30 sec.	T 65°C 45 sec.	T 72°C 1 min.	34
Fpn1	T 94°C 30 sec.	T 60°C 45 sec.	T 72°C 1 min.	34
β-Actin	T 94°C 30 sec.	T 57°C 45 sec.	T 72°C 1 min.	24

6. Determination of TNF-α and IL-10 secretion

The effects of cassia oil or cinnamaldehyde on TNF-α and IL-10 production were determined by analyzing TNF-α and IL-10 levels in the supernatants of the treated cells above (5) using an enzyme linked immunosorbent assay (ELISA) kit.

Sandwich ELISA procedures were performed as following steps:

1. ELISA plates were coated with 100 µl capture antibody which specific for mouse TNF-α or IL-10 at room temperature, overnight.

2. The plate was washed 4 times with 300 µl washing buffer solution.

3. Add 300 µl of blocking buffer in each well and incubated at room temperature for 1h.

4. The plate was washed 4 times with 300 µl washing buffer solution.

5. One hundred µl of the supernatants or standard were added in coated plate and incubated at room temperature for 2h.

6. The plate was washed 4 times with 300 µl washing buffer solution.

7. Add 100 µl of detection antibody in coated plate and incubated at room temperature for 2h.

8. The plate was washed 4 times with 300 µl washing buffer solution.

9. The sample in each well was incubated with 100 µl avidin-horseradish peroxidase (HRP) conjugate solution at room temperature for 30 min.

10. The sample was washed 4 times and incubated with 100µl of ABTS substrate at room temperature for 30 min.

11. Measure color development at 450 nm with wavelength correction set at 650 nm by a microplate reader.

12. The concentration of TNF-α and IL-10 in each sample was determined from a standard curve prepared from standard TNF-α and IL-10 solutions.

Statistical analysis

Data from at least three independent experiments was presented as mean with standard error of mean (mean ± S.E.M.). The data of treated group with cassia oil or cinnamaldehyde were compared with control group by using One-way analysis of variance (ANOVA) followed by *Tukey's* post hoc test for multiple comparisons. SPSS program version 17.0 was used to perform all statistical analysis. The p-value less than 0.05 were considered as statistically significant.

CHAPTER IV

RESULTS

1. Chemical composition of Cassia oil from the leaf of C. cassia

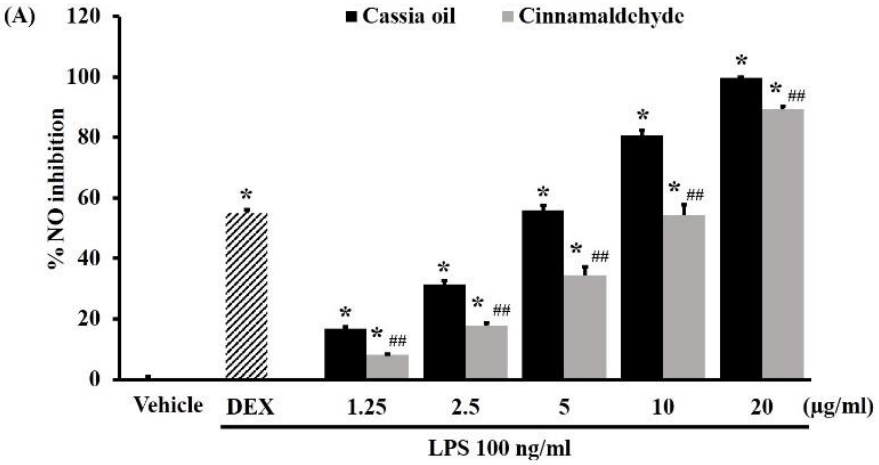
The compositions of cassia leaf oil from *C. cassia* used in this study were evaluated by matching their mass spectra and retention indices with Adams EO Mass Spectral library and NIST05 Mass Spectral library. The percentage of each composition was computed from GC peak areas. The main compound of the oil was cinnamaldehyde. The other compounds found in this oil were cinnamyl acetate, terpeneols, eugenol, and caryophyllene and etc (Table 4).

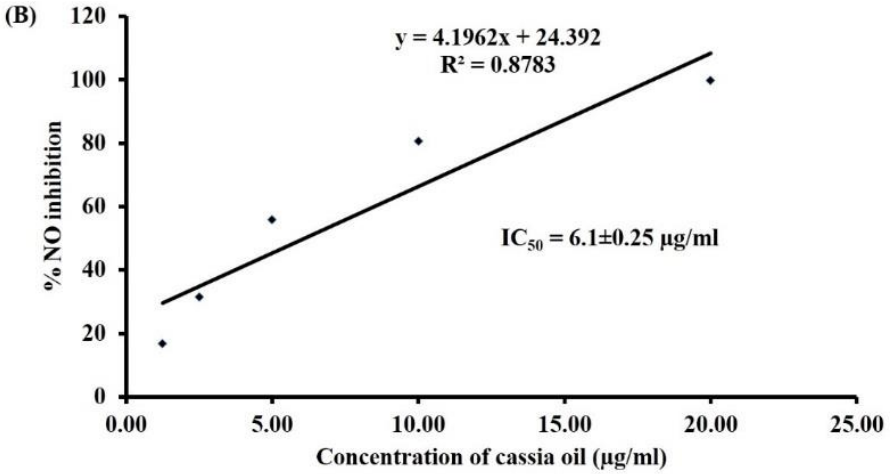
Table 4: Chemical composition of the volatile oils from the leaves of *Cinnamomum cassia*.

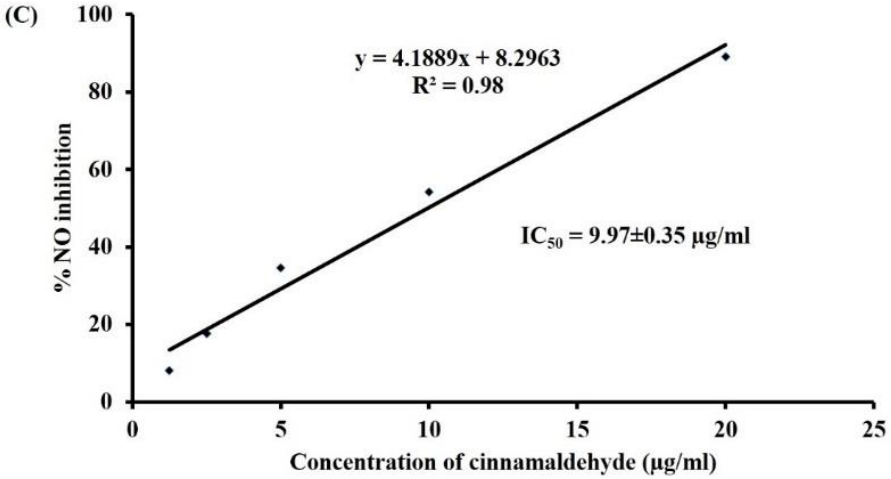
RT (min.)	Chemical composition	Kovat's index	% Area
13.97	Terpineol<1->	1133	0.18
14.4	Terpineol<cis-beta->	1144	0.75
15.21	Terpineol<trans-beta->	1163	0.42
16.35	Terpineol<alpha->	1188	4.23
16.64	Terpineol<gamma->	1199	2.18
19.73	Cinnamaldehyde<E->	1270	78.35
23.43	Eugenol	1359	4.52
26	Caryophyllene(E-)	1419	0.21
26.99	Cinnamyl acetate<E->	1446	9.16

2. Effects of cassia oil and cinnamaldehyde on LPS-induced NO production and iNOS mRNA expression

Activated macrophages up-regulate iNOS expression for producing a large amount of NO. NO plays role as an inflammatory mediator in chronic inflammation. Cassia oil and cinnamaldehyde both at 1.25–20 µg/ml significantly inhibited NO production in a concentration-dependent manner (Fig. 12A) with the IC_{50} value of 6.1 ± 0.25 (Fig. 12B) and 9.97 ± 0.35 µg/ml (Fig. 12C), respectively. Cassia oil at the concentration of 1.25, 2.5, 5, 10 and 20 µg/ml were significantly inhibited NO production by 17 ± 0.36, 31 ± 1.3, 57 ± 1.91, 81 ± 2.47 and 100 ± 0.28%, respectively. In LPS-activated J774A.1 cells treated with cinnamaldehyde with the corresponding concentration, NO production was significantly inhibited by 8 ± 0.27, 18 ± 0.83, 35 ± 2.76, 54 ± 3.47 and 89 ± 1.16%, respectively. The inhibitory effect of cassia oil was significantly higher than its main component cinnamaldehyde ($p < 0.01$). The inhibitory effects of the oil and its component did not affect the viability of J774A.1 cells (Fig. 12D). The inhibitory effect of cassia oil and cinnalmaldehyde on NO production was supported by the reduction of iNOS expression determined by RT-PCR as presented in Fig. 12E. Cassia oil and cinnamaldehyde concentration-dependently attenuated iNOS mRNA expression in LPS-activated cells. Cassia oil at the concentration of 5, 10 and 20 µg/ml were significantly inhibited iNOS expression by 17.4 ± 8.3, 53 ± 3.9, and 76.4 ± 6.8%, respectively. Cinnamaldehyde with the corresponding concentration also inhibited iNOS expression by 3.7 ± 0.3, 26.6 ± 4.4, and 47.1 ± 2.2, respectively. Cinnamaldehyde showed less iNOS inhibitory effect ($p < 0.01$) comparing with cassia oil in the corresponding concentration (Fig. 12E).


(A)
Cassia oil
Cinnamaldehyde
% NO inhibition
120
100
80
60
40
20
0
Vehicle
DEX
1.25
2.5
5
10
20
(µg/ml)
LPS 100 ng/ml
*
*##


(B)
y = 4.1962x + 24.392
R² = 0.8783
IC50 = 6.1±0.25 µg/ml
% NO inhibition
120
100
80
60
40
20
0
0.00
5.00
10.00
15.00
20.00
25.00
Concentration of cassia oil (µg/ml)


(C)
y = 4.1889x + 8.2963
R² = 0.98
IC50 = 9.97±0.35 µg/ml
% NO inhibition
100
80
60
40
20
0
0
5
10
15
20
25
Concentration of cinnamaldehyde (µg/ml)

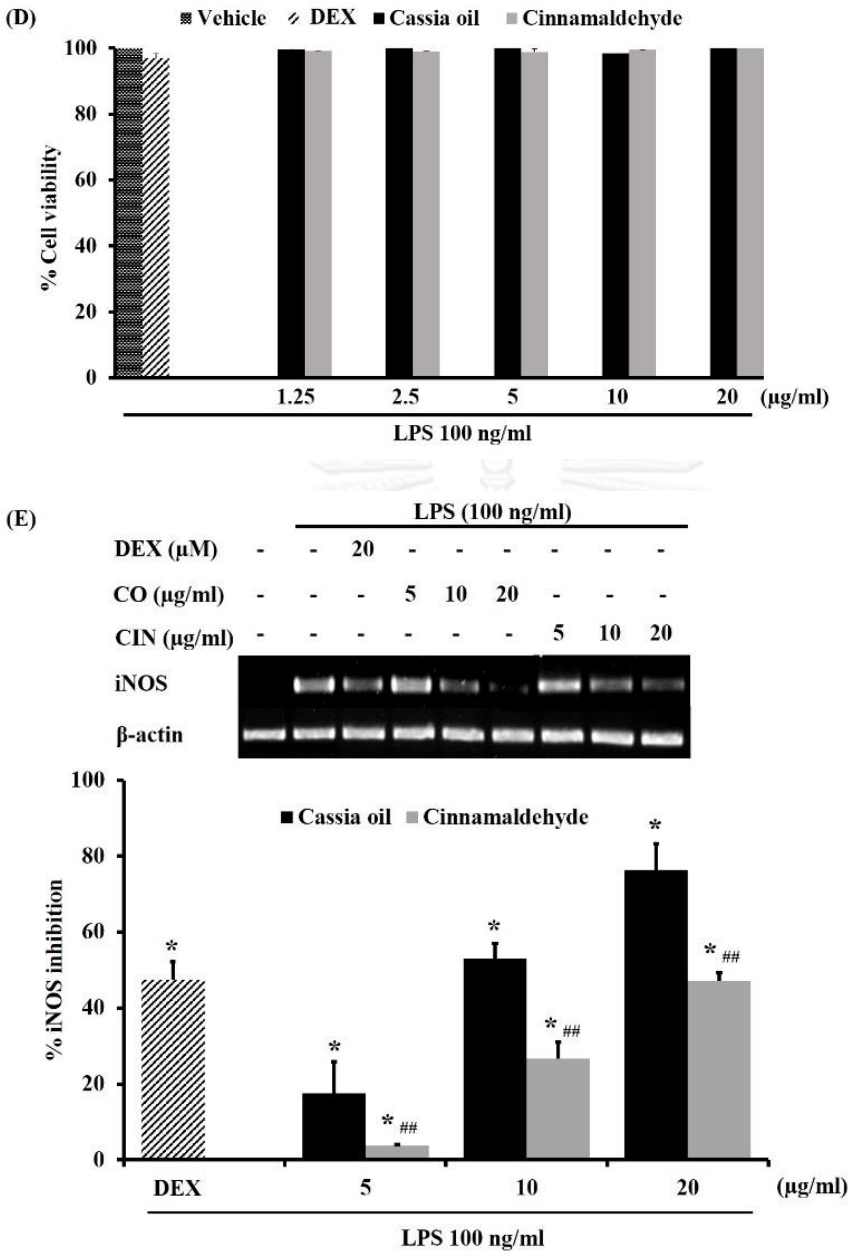


Figure 12: Effects of cassia oil and cinnamaldehyde on NO production and iNOS mRNA expression in LPS-stimulated J774A.1cells. Cells were co-treated with either cassia oil or cinnamaldehyde (1.25, 2.5, 5, 10 and 20 μg/ml) or dexamethasone (20 μM) and LPS (100 ng/ml) for 24 h. NO levels were measured in the cell culture media by Griess reaction assay (A). The IC_{50} of cassia oil and cinnamaldehyde (B, C). Cell viability was determined by resazurin reduction assay (12D). Semi-quantitative RT-PCR was used to determine the expression of iNOS mRNA (E). *$P < 0.05$ compared with LPS-stimulated control; $^{\#\#}P < 0.01$ compared with cassia oil treated cells.

3. Effects of cassia oil and cinnamaldehyde on phagocytic activity in LPS-stimulated macrophages

As phagocytosis is a process enhancing toxic oxygen species production responsible for eliminating pathogens which leading to normal tissue destruction in chronic inflammation. The further investigation is the influence of cassia oil and cinnamaldehyde on phagocytic activity in LPS-stimulated J774A.1 cells using zymosan-nitroblue tetrazolium (NBT) reduction assay. In J774A.1 cells treated with LPS showed significantly increase in phagocytic activity compared with the un-treated negative control (data not show). Co-treatment of cassia oil at the concentration of 5, 10 and 20 µg/ml and LPS were significantly inhibited phagocytic activity in a concentration-dependent manner by 13.92 ± 0.94, 24.59 ± 1.26, and 50.50 ± 5.91%, respectively (Fig. 13). In LPS-activated J774A.1 cells that treated with cinnamaldehyde at the concentration of 5, 10 and 20 µg/ml, the phagocytic activity was significantly inhibited by 11.89 ± 3.95, 22.99 ± 3.78 and 49.27 ± 6.68%, respectively. However, there was no statistical difference in phagocytic inhibition between cassia oil and cinnamaldehyde.

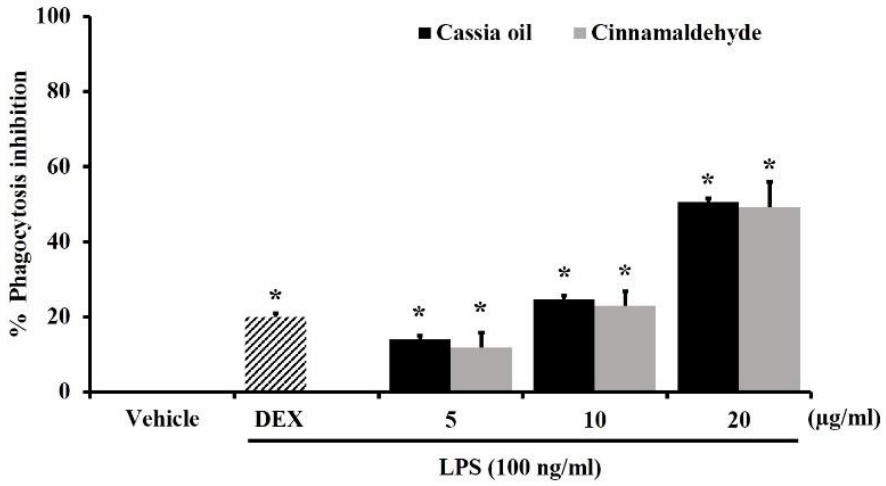


Figure 13: Effect of cassia oil and cinnamaldehyde on phagocytic activity of LPS-stimulated macrophage J774A.1 cells, cells were co-treated with cassia oil or cinnamaldehyde (1.25, 2.5, 5, 10 and 20 µg/ml) or dexamethasone (20 µM) and LPS (100 ng/ml). Phagocytic activity of LPS-stimulated J774A.1 cells was determined by zymosan-NBT reduction assay. Results represent mean ± S.E.M. relative to LPS-stimulated control. *$P < 0.05$ compared with LPS-stimulated control

4. Effects of cassia oil and cinnamaldehyde on LPS-induced COX-2 and mPGES-1 mRNA expression

PGE_2 is the most well-known inflammatory mediator in inflammatory process. It is produced in a large amount by activated macrophages during chronic inflammation by COX-2 and mPGES-1. These enzymes are inducible enzymes expressed in macrophages only when the cells are activated. These enzymes were not expressed in untreated J774A.1 cells but they were highly expressed in the LPS-activated cells (Fig. 14A). Cassia oil and cinnamaldehyde at 5, 10, and 20 µg/ml significantly and similarly inhibited the mRNA expression of COX-2 enzymes as shown in Figure 14B. Cinnamaldehyde at 5 and 10 µg/ml significantly exerted higher inhibitory activity than cassia oil on mPGES-1 mRNA expression (Fig. 14C).

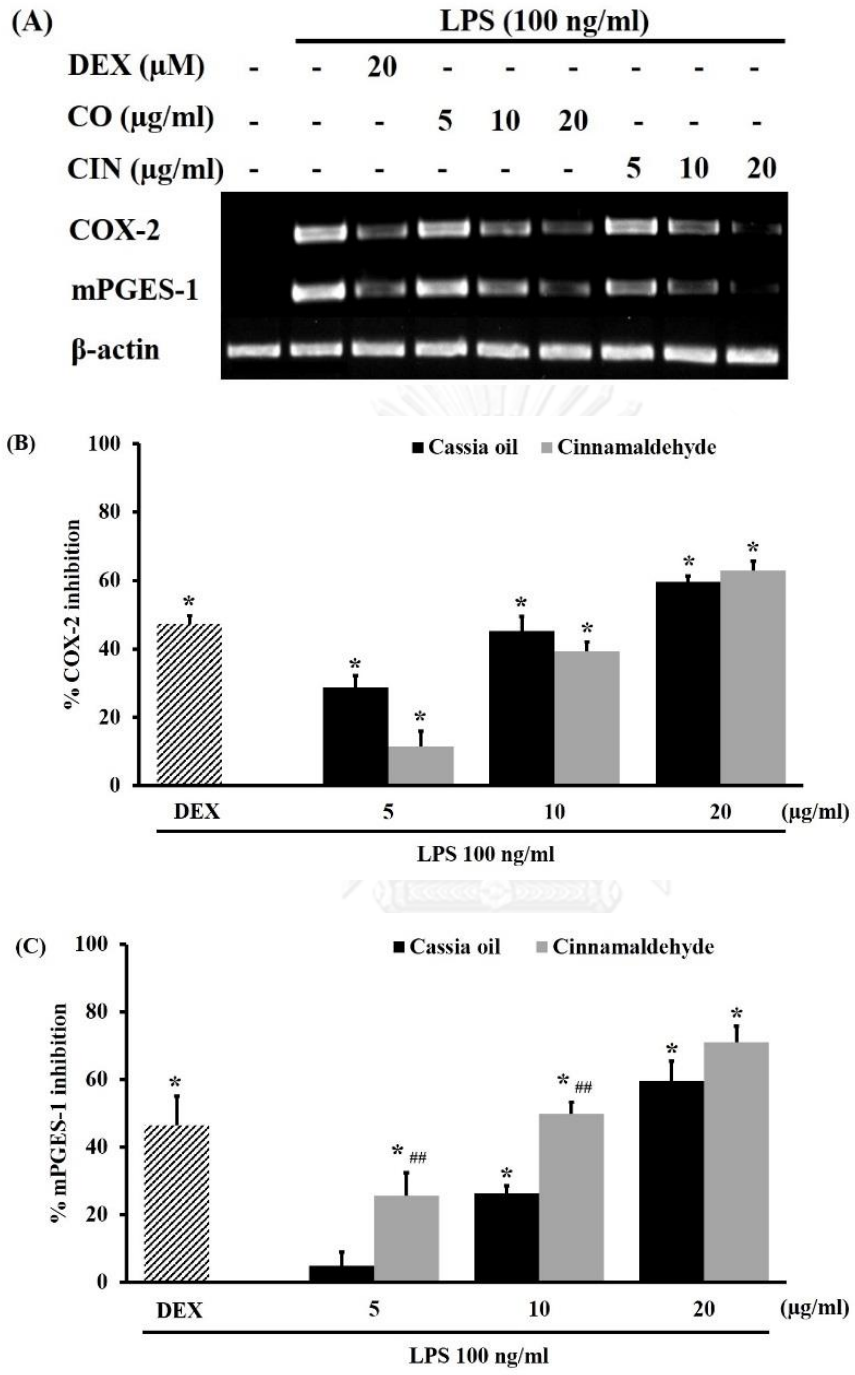
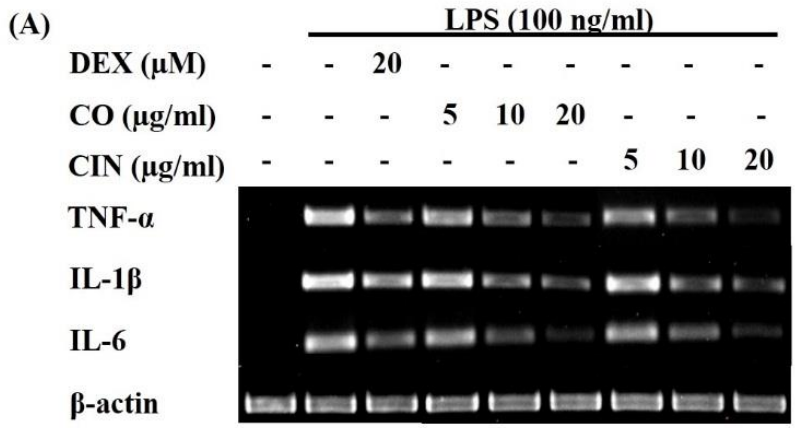


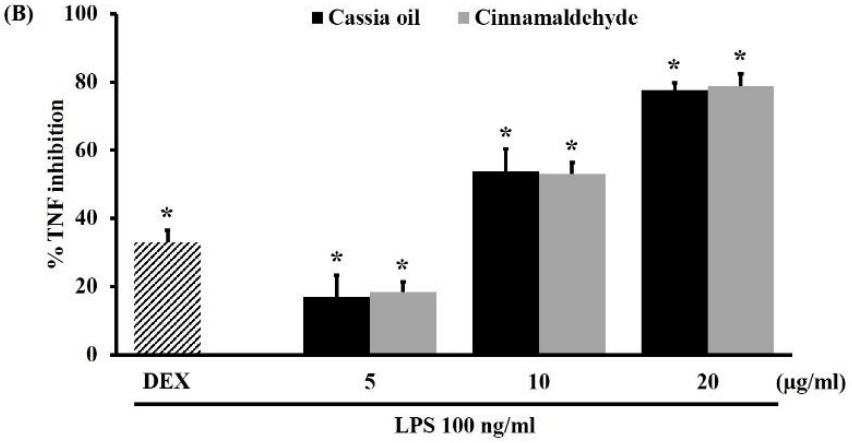
Figure 14: Effects of cassia oil and cinnamaldehyde on COX-2 (B) and mPGES-1 (C) mRNA expression in J774A.1 cells, cells were stimulated with LPS (100 ng/ml) in the presence or absence of cassia oil or cinnamaldehyde (5, 10 and 20 μg/ml) or dexamethasone (20 μM). Inflammatory enzyme mRNA expression were determined after incubation for 24 h by semi-quantitative RT-PCR. Results represent mean ±

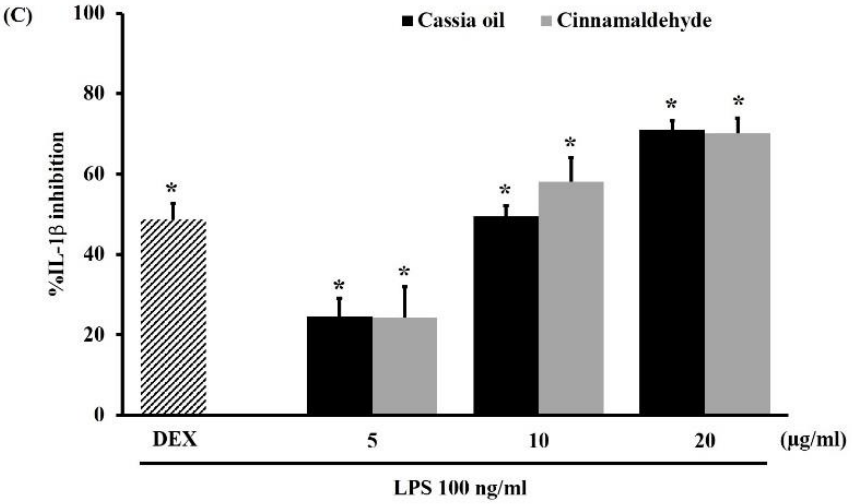
S.E.M. relative to LPS-stimulated control. *P < 0.05 compared with LPS-stimulated control.

5. Effects of cassia oil and cinnamaldehyde on LPS-induced pro-inflammatory cytokines TNF-α, IL-1β and IL-6 expression and TNF-α protein production

Pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in activated macrophages play major roles during chronic inflammation by inducing the production of several inflammatory mediators such as NO and PGE_2. These cytokines did not express in untreated macrophage J774A.1 cells but they were highly expressed in LPS-activated cells (Fig. 15A). Both cassia oil and cinnamaldehyde at 5, 10 and 20 μg/ml similarly and significantly inhibited the expression of TNF-α, IL-1β and IL-6 in activated J774A.1 cells in a concentration-dependent manner (Fig. 15B-15D). Inhibitory effects of these two compounds on pro-inflammatory cytokines were confirmed by determining TNF-α production in supernatants of the treated cells at 24 h. TNF-α production was undetectable in untreated macrophages (Fig. 15E). LPS-activated macrophages produced a large amount of TNF-α in the supernatant (5517±357 pg/ml). Cassia oil and cinnamaldehyde similarly and markedly inhibited TNF-α production in LPS-stimulated J774A.1 cells in a concentration-dependent manner. Cassia oil at 5, 10 and 20 μg/ml decreased TNF-α levels to 4,367±526, 3,513±732 and 243±182 pg/ml, respectively. Cinnamaldehyde at 5, 10 and 20 μg/ml decreased TNF-α levels to 5,093±226, 3,437±547, 220±200 pg/ml, respectively. Thus, cassia oil and cinnamaldehyde inhibited both mRNA expression and production of TNF-α.


(A)
LPS (100 ng/ml)
DEX (μM) - - 20 - - - - - -
CO (μg/ml) - - - 5 10 20 - - -
CIN (μg/ml) - - - - - - 5 10 20
TNF-α
IL-1β
IL-6
β-actin


(B)
Cassia oil
Cinnamaldehyde
% TNF inhibition
100
80
60
40
20
0
DEX
5
10
20
(μg/ml)
LPS 100 ng/ml


(C)
Cassia oil
Cinnamaldehyde
%IL-1β inhibition
100
80
60
40
20
0
DEX
5
10
20
(μg/ml)
LPS 100 ng/ml

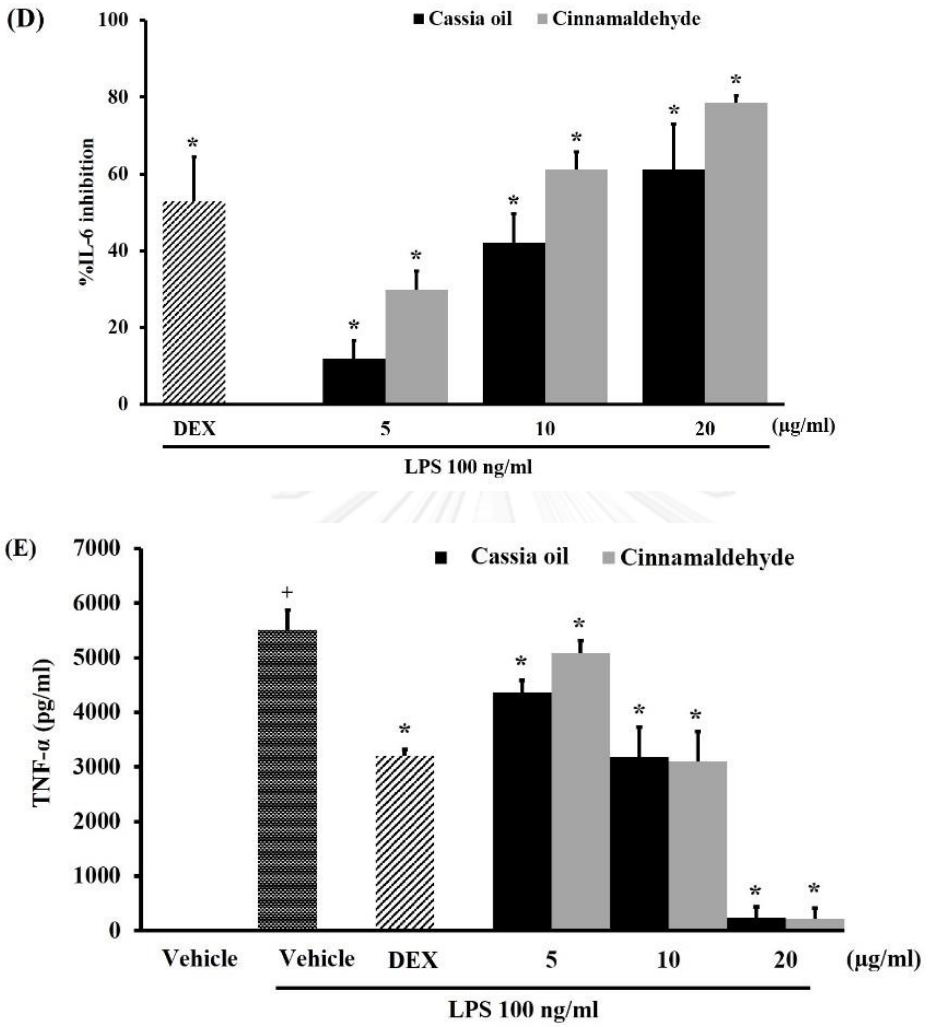


Figure 15: Effects of cassia oil and cinnamaldehyde on mRNA expression of TNF-α (B), IL-1β (C), IL-6 (D) and TNF-α production (E) in LPS-stimulated J774A.1 cells, cells were co-treated with LPS (100 ng/ml) and cassia oil or cinnamaldehyde (5, 10 and 20 μg/ml) or dexamethasone (20 μM) for 4 h (B, C and D) and 24h (15E). The mRNA levels of pro-inflammatory cytokines and TNF-α concentration were examined by semi-quantitative RT-PCR and specific ELISA, respectively. Results represent mean ± S.E.M. relative to LPS-stimulated control. *P < 0.05 compared with LPS-stimulated control.

6. Effects of cassia oil and cinnamaldehyde on LPS-induced MCP-1 and MIP-1α expression

Chemokines from activated macrophages play role to recruit more leukocytes to inflammatory sites during chronic inflammation. Cassia oil and cinnamaldehyde significantly inhibited mRNA expression of chemokines MCP-1 and MIP-1α in LPS-activated J774A.1 cells (Fig. 16A). They have similar inhibitory effect on MIP-1α expression (Fig. 16C). At 5 and 10 μg/ml cassia oil demonstrated higher inhibitory activity than cinnamaldehyde on MCP-1 expression (Fig. 16B).

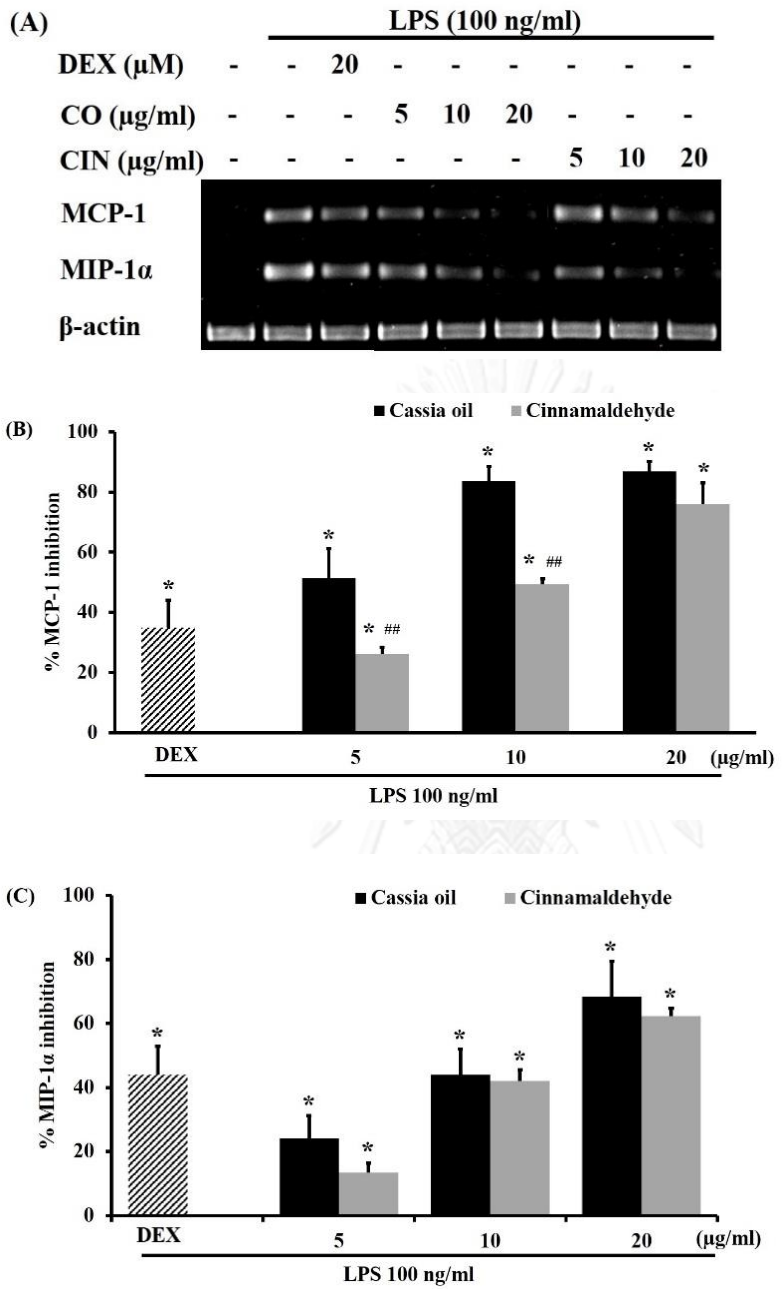
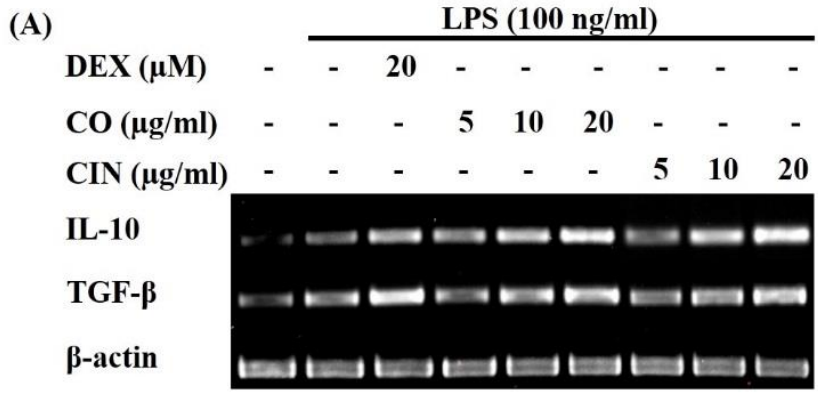
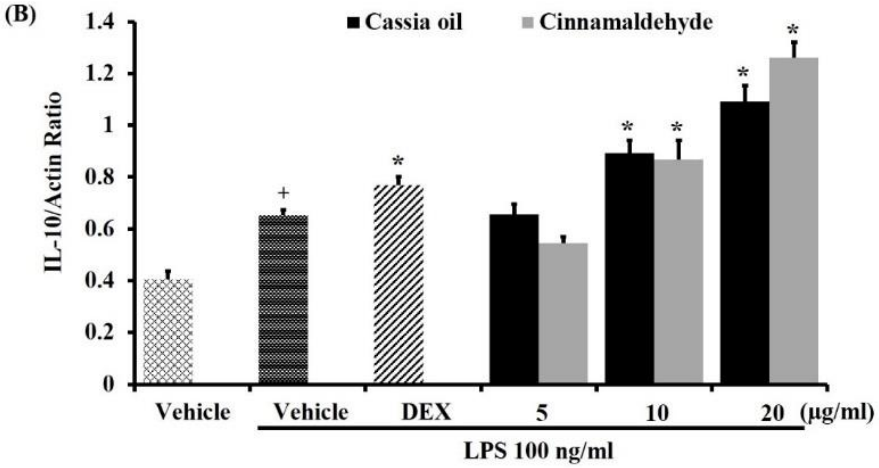


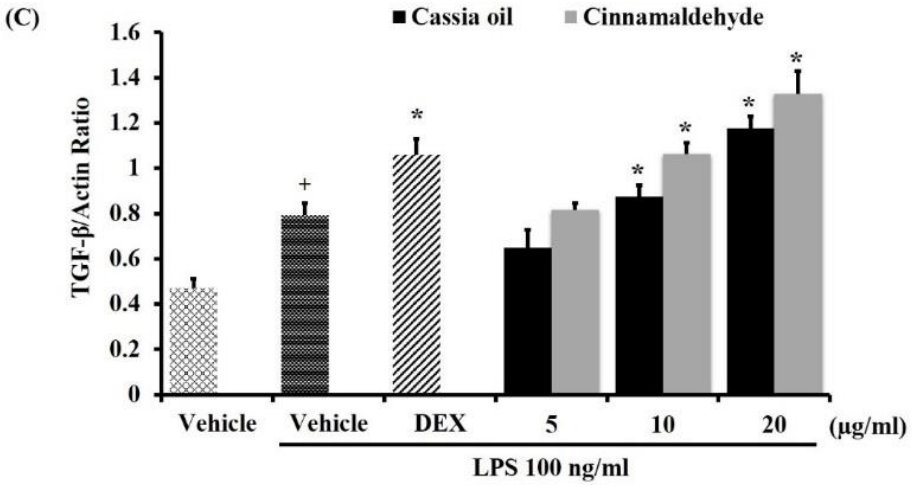
Figure 16: Effects of cassia oil and cinnamaldehyde on chemokines mRNA expression in LPS-stimulated J774A.1 cells (A), cells were co-treated with LPS (100 ng/ml) and cassia oil or cinnamaldehyde (5, 10 and 20 μg/ml) or dexamethasone (20 μM) for 4 h. Semi-quantitative RT-PCT was used to determine the MCP-1 (B) and MIP-1 α (C) mRNA levels. Results represent mean ± S.E.M. relative to LPS-stimulated control. *P < 0.05 compared with LPS-stimulated control; $^{\#\#}P$ < 0.01 compared with cassia oil treated cells.

7. Effects of cassia oil and cinnamaldehyde on LPS-induced anti-inflammatory cytokines IL-10 and TGF-β expression

Induction of anti-inflammatory cytokines should benefit the reduction of inflammatory process during chronic inflammation. Effects of cassia oil and cinnamaldehyde on expression and production of major anti-inflammatory cytokines IL-10 and TGF-β in LPS activated macrophages were evaluated. At 4 h of treatment, 100 ng/ml LPS up-regulated mRNA expression of anti-inflammatory cytokines IL-10 and TGF-β in activated cells when compared to non-stimulated cells (Fig. 17A). Cassia oil and cinnamaldehyde with the concentration at 10 and 20 µg/ml similarly and significantly increased the expression of these anti-inflammatory cytokines in LPS activated J774A.1 cells (Fig. 17B and 17C). These two oils with corresponding concentration also significantly increased IL-10 production in supernatants of LPS-activated J774A.1 cells determined by ELISA (Fig. 17D). Dexamethasone at 20 µM also increased both the expression and production of these anti-inflammatory cytokines in LPS-activated J774A.1 cells.


(A)
LPS (100 ng/ml)
DEX (μM) - - 20 - - - - - -
CO (μg/ml) - - - 5 10 20 - - -
CIN (μg/ml) - - - - - - 5 10 20
IL-10
TGF-β
β-actin


(B)
Cassia oil
Cinnamaldehyde
IL-10/Actin Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
Vehicle
DEX
5
10
20
(μg/ml)
LPS 100 ng/ml


(C)
Cassia oil
Cinnamaldehyde
TGF-β/Actin Ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
Vehicle
DEX
5
10
20
(μg/ml)
LPS 100 ng/ml

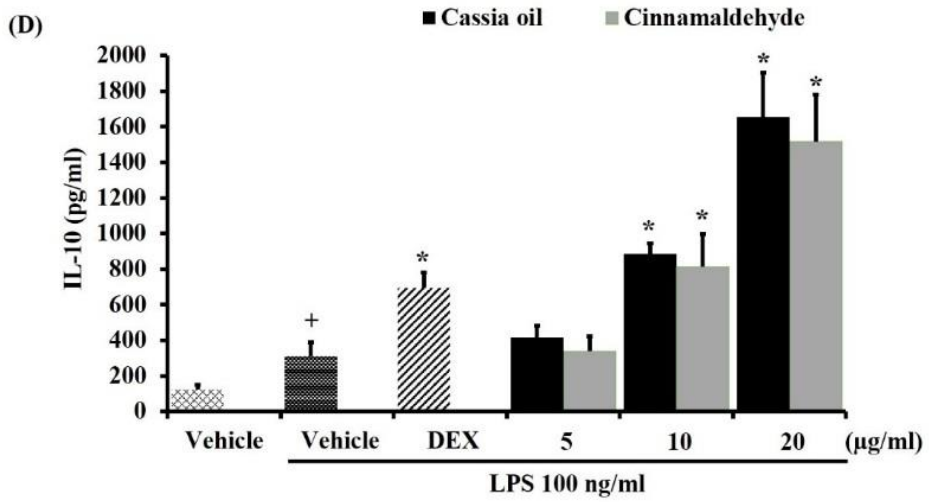


Figure 17: Effects of cassia oil and cinnamaldehyde on mRNA expression of IL-10 (B), TGF- β (C) and the secretion of IL-10 (D) in LPS-stimulated J774A.1 cells, cells were co-treated with LPS (100 ng/ml) and cassia oil or cinnamaldehyde (5, 10 and 20 μg/ml) or dexamethasone (20 μM) for 4 h (A) and 24 h (D).The mRNA levels of anti-inflammatory cytokines and IL-10 production were determined by semi-quantitative RT-PCR and specific ELISA, respectively. All experiments were repeated three times in duplicate. *$P < 0.05$ compared with LPS-stimulated control.

8. Effects of cassia oil and cinnamaldehyde on LPS-suppressed Fpn-1expression

During chronic inflammation, pro-inflammatory cytokines can directly reduce iron exporter Fpn-1 expression. This effect leads in part to increase iron storage in activated macrophages and reduce plasma iron resulting in anemia of chronic inflammation. LPS at 100 ng/ml significantly decreased Fpn1 expression (Fig. 18A). When compared to LPS control, cassia oil and cinnamaldehyde similarly and significantly increased mRNA expression of Fpn1 in LPS-activated cells (Fig. 18B). Dexamethasone at 20 μM also increased expression of the iron exporter protein in activated J774A.1 cells.

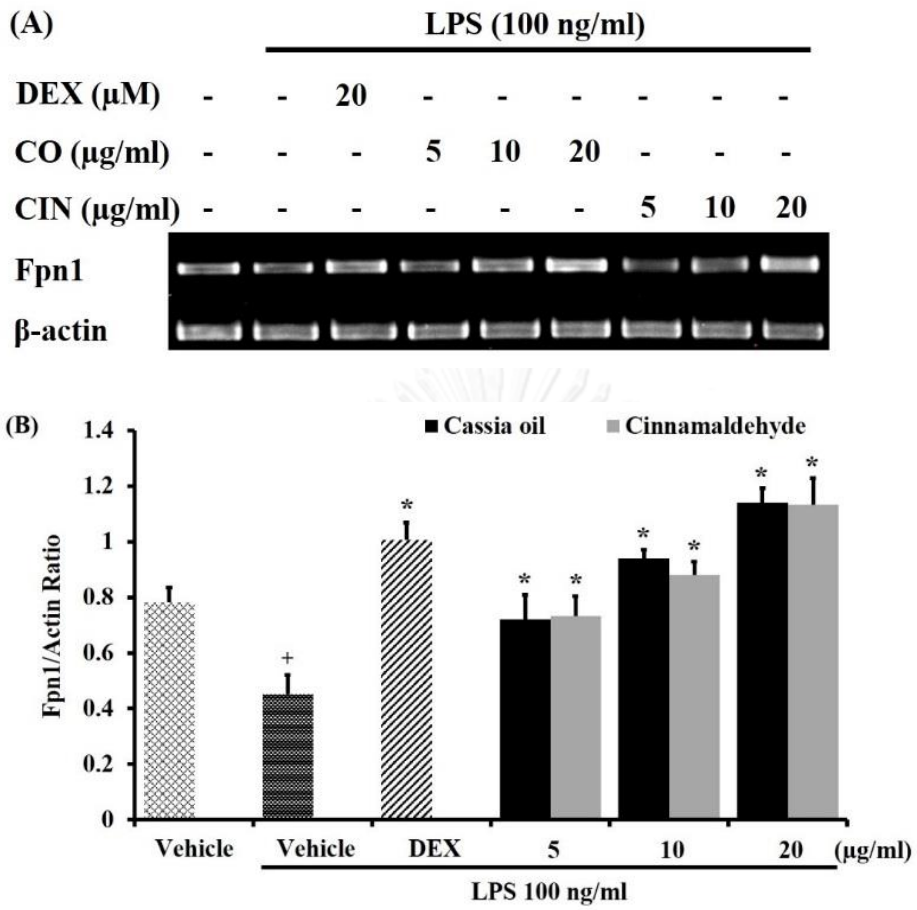


Figure 18: Effects of cassia oil and cinnamaldehyde on mRNA expression of Fpn-1 in LPS-stimulated J774A.1 cells (A and B), cells were co-treated with LPS (100 ng/ml) and cassia oil or cinnamaldehyde (5, 10 and 20 μg/ml) or dexamethasone (20 μM) for 4 h. Levels of mRNA for Fpn-1 were examined by semi-quantitative RT-PCR. Results represent mean ± S.E.M. relative to LPS-stimulated control. *$P < 0.05$ compared with LPS-stimulated control

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Macrophages play major roles in chronic inflammatory diseases. They are sources of several inflammatory mediators such as pro-inflammatory cytokines, chemokines, PGs and NO (109). These mediators in excess amount are capable to injure normal host tissues and involve in several chronic inflammatory diseases (21, 22, 110) . During chronic inflammation, macrophages also sequester iron by decreasing expression of cellular iron exporter Fpn1 and lead to iron deficient anemia or anemia of inflammation (AI) (23). Suppression of macrophage activation is usually the goal of several clinically used anti-inflammatory agents for treating many chronic inflammatory diseases. Several natural compounds have been investigated for their anti-inflammatory activities both *in vitro* and *in vivo* by focusing on macrophage functions (111-114).

The present study investigated anti-inflammatory potential of cassia oil from of *Cinnamomum cassia* leaves compared its activity with the main constituent cinnamaldehyde. Cassia leaf oil in this study contained mainly cinnamaldehyde 78.35% and other constituents including cinnamyl acetate, eugenol, terpineol, and caryophyllene. Previous study reported that cinnamaldehyde was the main volatile component of *C. cassia* bark and leaf essential oil at 91% and 50%, respectively (115). The variation of cinnamadehyde in each studies may be due to difference in parts of the plants, geographical region, ages of the plant, harvest seasons, and method of oil extraction. Other constituents of cassia oil in this study were also different from reported constituents of bark cassia oil. We could not detect coumarin in cassia leaf oil used in the study. Different compounds can modulate unrelated signaling and therefore, can possess synergistic effects in inhibitory effect on activated-macrophages.

Effects of cassia leaf oil and cinnalmaldehyde on expression and production of several inflammatory mediators in LPS-activated macrophage J774A.1 cells were

evaluated. All concentrations of these oils (1.25-20 μg/ml) were proved to be nontoxic to J774A.1 cells by resazurin assay. Both cassia oil and cinnamaldehyde significantly inhibited NO production in a concentration dependent manner. Inhibition of NO production of these oils correlated to their inhibitory effects on iNOS expression. It is known that iNOS only expressed in macrophages when these cells are activated. This enzyme is responsible for a large amount NO production. Elevations of NO and iNOS in activated macrophages are associated with the pathological process of acute and chronic inflammatory conditions (2). Cassia oil demonstrated more potent inhibition than cinnamaldehyde on iNOS expression and NO production. It is possible that other constituents in cassia oil may also have inhibitory effect along with cinnamaldehyde. Eugenol has been reported to inhibit NO and iNOS production in murine macrophage RAW 264.7 cells and human macrophages stimulated by LPS (116, 117). It possibly indicated that eugenol in this oil exerted synergistic effect in inhibiting NO and iNOS expression in murine macrophage J774A.1 cells.

PGE_2 is the most well-known inflammatory mediator involved in chronic inflammation (118, 119). Inducible enzymes COX-2 and mPGES-1 play major roles in biosynthesis of PGE_2 from arachidonic acid and the unstable intermediate, PGH_2 in activated macrophages (120). Expression of these enzymes is known to be induced by bacterial products, pro-inflammatory cytokines TNF-α and IL-1β and up regulation of them was detected in many chronic inflammatory diseases (121-123). mPGES-1 enzyme is recently expected to be a novel therapeutic target for inhibiting inflammatory diseases due to the cardiovascular side effects of the long-term use of selective COX-2 inhibitors (124, 125). Cassia oil and cinnamaldehyde significantly inhibited COX-2 and mPGES-1 mRNA expression in LPS-stimulated J774A.1 cells. These results suggest that they should be able to inhibit PGE_2 synthesis in activated macrophages. It has been reported that cinnamaldehyde is the only constituent in bark cassia oil that could inhibit iNOS and COX-2 expression (92). The effect of cinnalmaldehyde on mPGES-1 was first evaluated in this study.

As phagocytosis process promotes the production of toxic oxygen species and exzymes which responsible for activation of the respiratory burst of phagocytes. This process is a key first line cellular defense against invading microorganisms. Over production of these molecules leading to normal tissue destruction in chronic inflammation. Many evidences have shown that down-regulation of phagocytic activity, pro-inflammatory cytokines and chemokines expression has been found to exert a beneficial effect on various inflammatory diseases (126). This study was the first report to show that the phagocytic activity of LPS-stimulated J774A.1 was inhibited by both cassia leaf oil and cinnamaldehyde.

Several pro-inflammatory cytokines are also important mediators in acute and chronic inflammation. They have several beneficial effects during acute inflammation but overexpression of cytokines and chemokines including TNF-α, IL-1β, IL-6, MCP-1 and MIP-1α from activated macrophages during chronic inflammation can cause several inflammatory diseases (50-52). In this study, cassia oil and cinnamaldehyde significantly inhibited expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6. They also decreased mRNA expression of chemokines MCP-1 and MIP-1α which can activate endothelial cells to express adhesion molecules resulting in enhanced the recruitment of leukocytes to the site of inflammation (127). Cinnamaldehyde has been reported to inhibit LPS-induced pro-inflammatory cytokine production in murine macrophage RAW264.7 cells (92) and porcine alveolar macrophage (128). Our results confirmed the previous studies and extended the information that these oils also had inhibitory effect on chemokines MCP-1 and MIP-1α.

Activation or elevation of anti-inflammatory mediators should benefit the attenuation of chronic inflammatory condition (63). This study also evaluated the effects of cassia leaf oil and cinnamaldehyde on anti-inflammatory cytokines. LPS activated macrophages and induced both pro-inflammatory and anti-inflammatory cytokines expression and production. Cassia leaf oil and cinnamaldehyde up-regulated expression and production of anti-inflammatory cytokines IL-10 and TGF-β while they down-regulated pro-inflammatory cytokine and chemokine expression and production in LPS-activated macrophages. Activities of these compounds are

similar to dexamethasone. This study demonstrated that anti-inflammatory activities of cassia oil and cinnamaldehyde may come from inhibition of inflammatory mediator production and stimulation of anti-inflammatory mediator production.

Anemia of chronic diseases (ACD) is inflammation-related pathology caused by inhibition of intestinal iron absorption and iron sequestration in macrophages by Fpn1 down-regulation. It is reported that inflammatory cytokines or LPS down-regulate macrophage Fpn1 mRNA expression and induce iron sequestration (3). This study also demonstrated that LPS decreased mRNA expression of Fpn1. Cassia oil and cinnamaldehyde similarly and significantly increased mRNA expression of Fpn1 in LPS-activated J774A.1 cells. This result demonstrated that these two compounds can reverse LPS suppressed Fpn1 mRNA expression. Although this study did not determine the intracellular iron level, it is possible that restoration of Fpn1 mRNA expression should increase iron release. This effect of the compounds may be benefit the improvement of ACD. The effect of cassia oil and cinnamaldehyde on an iron regulatory protein was first reported in this study.

In summary, these results demonstrated comparable anti-inflammatory activity of cassia leaf oil and cinnamaldehyde on LPS-activated J774A.1 cells. It suggested that almost but not all activities on macrophage mediators of cassia oil come from cinnamaldehyde. Cassia leaf oil and cinnalmaldehyde exhibited anti-inflammatory activity by decreasing phagocytic activity, pro-inflammatory cytokines, chemokines, inflammatory enzymes and inflammatory mediators. Additionally, both compounds increased anti-inflammatory mediator mRNA expression. All these results supported the alteration of LPS-activated macrophages from M1 phenotype (inflammatory macrophages) to M2 phenotype (tissue-healing macrophages). It may also affect the iron homeostasis in activated macrophages. Nevertheless, *in vivo* studies in animal model of inflammation and toxicity test should be performed to further validate the therapeutic effects of cassia oil extracted from the leaves of *C. cassia*.

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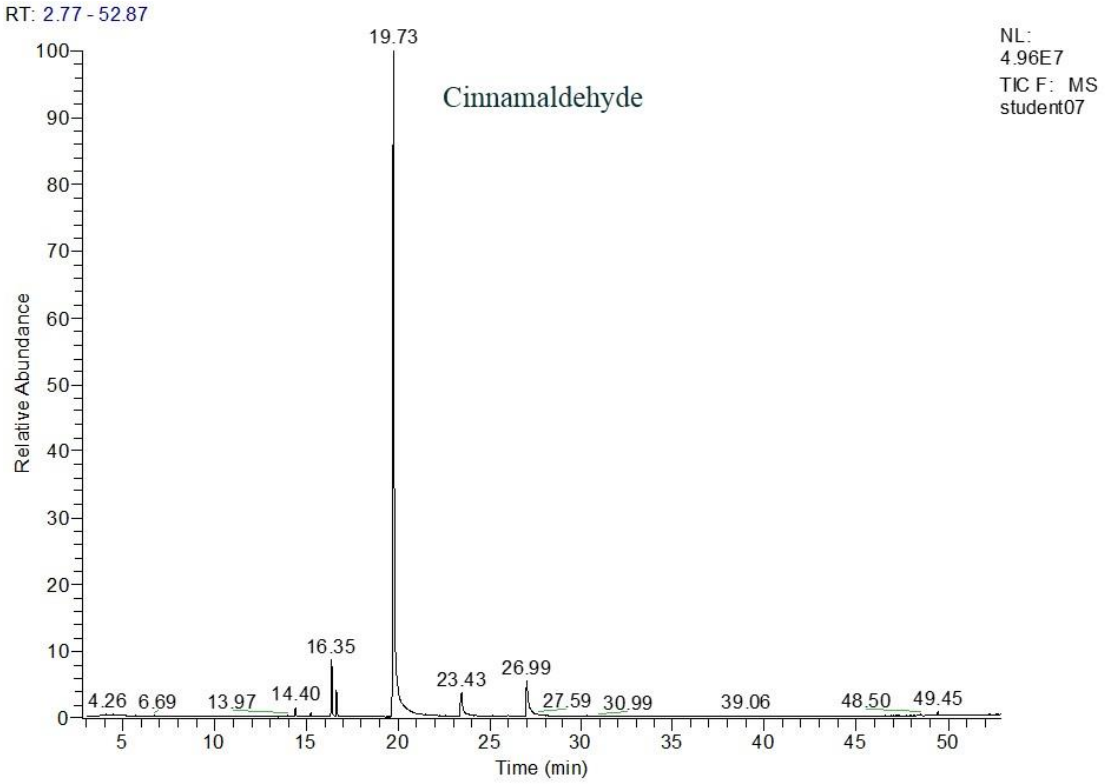
APPENDIX

A: GC-MS/MS fingerprint of cassia leaf oil

B: Experimental results

APPENDIX A



Appendix A: GC-MS/MS fingerprint of cassia leaf oil

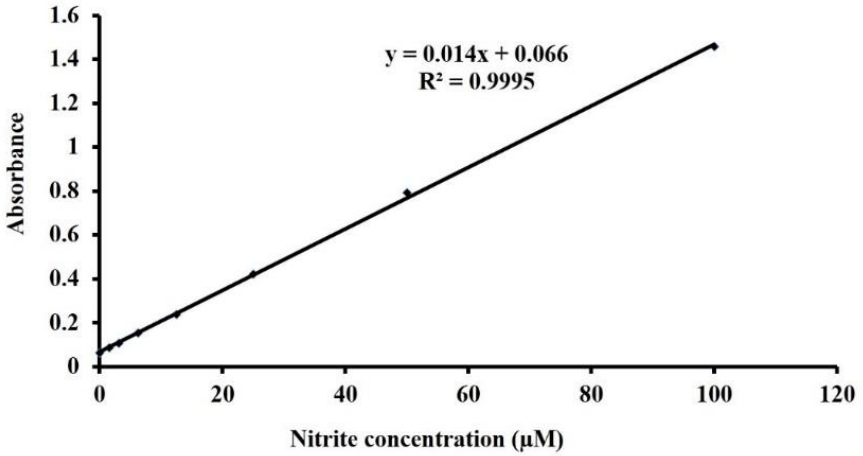
APPENDIX B

EXPERIMENTAL RESULTS

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

Nitrite concentration (µg/ml)	Absorbance at 540 nm.		Mean
	1	2	
0	0.063	0.06	0.062
1.5625	0.087	0.084	0.086
3.125	0.105	0.105	0.105
6.25	0.152	0.154	0.153
12.5	0.24	0.237	0.239
25	0.417	0.424	0.421
50	0.821	0.762	0.792
100	1.408	1.505	1.457

Appendix B-2: Standard calibration curve of nitrite



Appendix B-3: Data of the inhibitory effect of cassia oil and cinnamaldehyde on NO production in LPS- stimulated macrophage J774A.1 cells. The data was represented as mean ± S.E.M.

Tested compounds	% NO inhibition			mean ± SE
	n1	n2	n3	
Media	0	0	0	0
Vehicle	0	3	0	1 ± 0.86
DEX	67	65	69	66 ± 1.09
1.25 µg/ml CO	17	18	17	17 ± 0.36
2.5 µg/ml CO	31	29	33	31 ± 1.30
5 µg/ml CO	58	53	59	57 ± 1.91
10 µg/ml CO	82	76	85	81 ± 2.47
20 µg/ml CO	100	99	100	100 ± 0.28
1.25 µg/ml CIN	8	8	8	8 ± 0.27
2.5 µg/ml CIN	18	19	16	18 ± 0.83
5 µg/ml CIN	40	31	32	35 ± 2.76
10 µg/ml CIN	61	50	52	54 ± 3.47
20 µg/ml CIN	90	87	91	89 ± 1.16

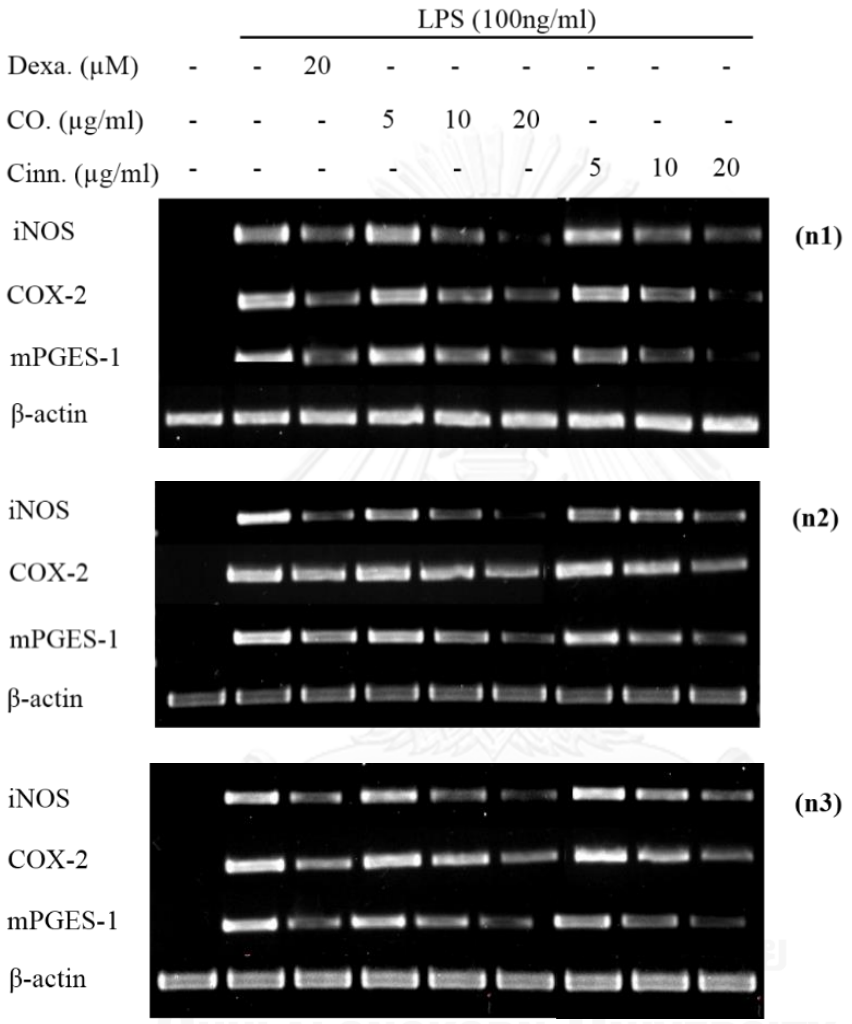
Appendix B-4: Data of cytotoxic effect of cassia oil and cinnamaldehyde on LPS-stimulated macrophage J774A.1 cells.

Tested compounds	% Cell viability			mean ± SE
	n1	n2	n3	
Media	100.00	100.00	100.00	100
Vehicle	100.00	100.00	100.00	100
DEX	96.14	100.00	93.50	96.55 ± 1.88
1.25 µg/ml CO	100.00	100.00	99.17	97.72 ± 0.27
2.5 µg/ml CO	100.00	100.00	100.00	100
5 µg/ml CO	100.00	100.00	100.00	100
10 µg/ml CO	100.00	96.62	98.75	98.46 ± 0.99
20 µg/ml CO	100.00	100.00	100.00	100
1.25 µg/ml CIN	98.45	100.00	98.88	99.11 ± 0.46
2.5 µg/ml CIN	98.27	99.05	99.83	99.05 ± 0.45
5 µg/ml CIN	97.63	99.31	99.78	98.91 ± 0.66
10 µg/ml CIN	100.00	98.41	100.00	99.47 ± 0.53
20 µg/ml CIN	100.00	100.00	100.00	100

Appendix B-5: Data of the inhibitory effect of cassia oil and cinnamaldehyde on phagocytic activity of LPS-stimulated macrophage J774A.1 cells examined by zymosan-NBT assay. The data was expressed as the percentage of phagocytosis inhibition compared to the vehicle control (mean ± S.E.M.).

Tested compounds	% Phagocytic inhibition			Mean ± SE
	n1	n2	n3	
Vehicle	0.00	0.00	0.00	0
DEX	9.32	16.39	21.53	16 ± 2.66
5 µg/ml CO	14.49	14.52	11.18	13.92 ± 0.94
10 µg/ml CO	25.67	20.95	25.05	24.59 ± 1.26
20 µg/ml CO	41.41	40.46	65.22	50.50 ± 5.91
5 µg/ml CIN	19.26	10.67	5.75	11.89 ± 3.95
10 µg/ml CIN	30.37	17.90	20.69	22.99 ± 3.78
20 µg/ml CIN	57.22	35.99	54.60	49.27 ± 6.68

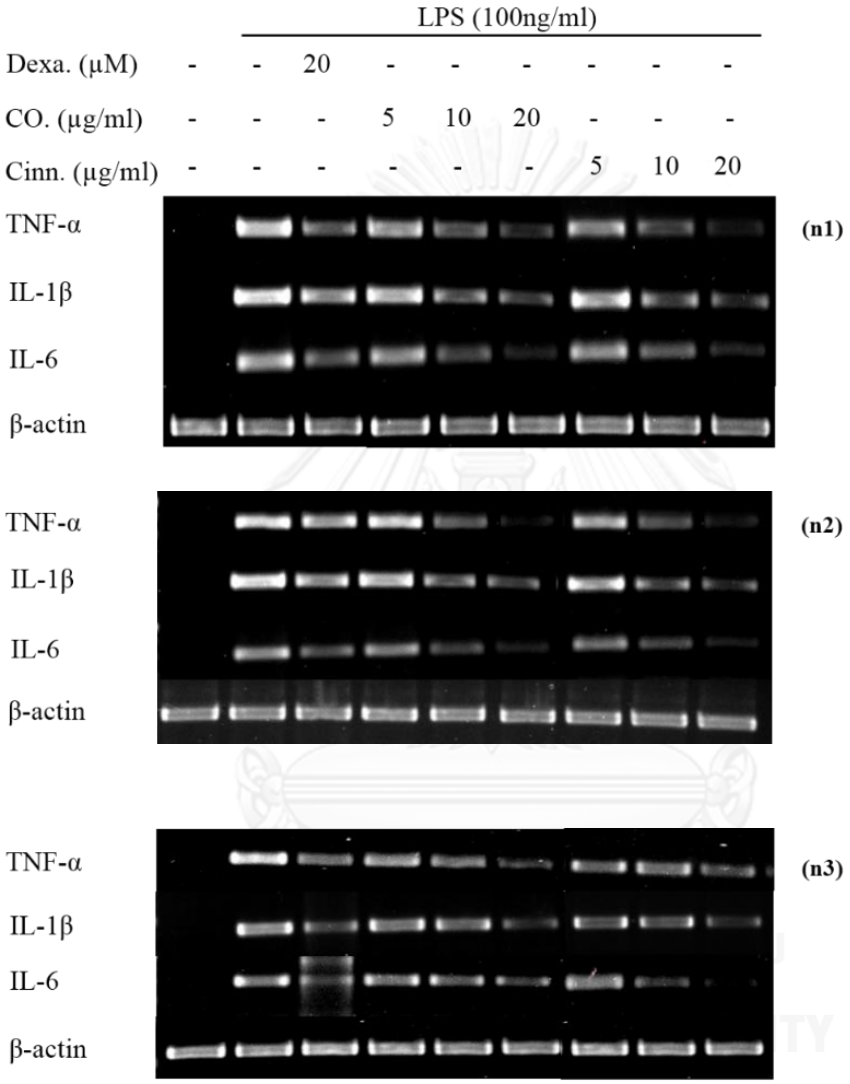
Appendix B-6: The inhibitory effect of cassia oil and cinnamaldehyde on the mRNA expression of iNOS, COX-2 and mPGES-1 enzyme in LPS-stimulated macrophage J774A.1 cells.



Appendex B-7: Data of the inhibitory effect of cassia oil and cinnamaldehyde on mRNA expression of inflammatory enzyme; iNOS, COX-2 and mPGES-1) in LPS-stimulated macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M. compared to untreated control.

Tested compound	% inhibition of iNOS			Mean ± SE	% inhibition of COX-2			Mean ± SE	% inhibition of mPGES-1			Mean ± SE
	n1	n2	n3		n1	n2	n3		n1	n2	n3	
Vehicle	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LPS control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DEX	56.7	57.5	42.5	52.2 ± 4.8	52.6	45.3	45.1	47.6 ± 2.4	47.1	22.7	50.0	39.9 ± 8.6
5 µg/ml CO	25.6	26.1	0.8	17.4 ± 8.3	28.0	35.1	23.2	28.8 ± 3.4	0.0	3.7	12.4	5.3 ± 3.9
10 µg/ml CO	50.8	60.6	47.6	53.0 ± 3.9	53.5	42.8	39.5	45.2 ± 4.2	22.8	25.3	30.8	26.3 ± 2.3
20 µg/ml CO	88.6	65.1	75.6	76.4 ± 6.8	62.3	56.2	60.3	59.5 ± 1.7	47.9	65.2	65.8	59.6 ± 5.8
5 µg/ml CIN	3.9	4.1	3.1	3.7 ± 0.3	20.2	6.5	8.0	11.9 ± 4.3	24.1	38.2	14.7	25.7 ± 6.8
10 µg/ml CIN	19.1	26.3	34.4	26.6 ± 4.4	43.1	33.5	40.9	39.1 ± 2.9	50.3	55.6	43.8	49.9 ± 3.4
20 µg/ml CIN	43.0	47.8	50.6	47.1 ± 2.2	68.0	62.0	58.9	62.9 ± 2.6	73.2	77.9	62.0	71.0 ± 4.7

Appendix B-8: The inhibitory effect of cassia oil and cinnamaldehyde on the mRNA expression of pro-inflammatory cytokine; TNF-α, IL-1β and IL-6 in LPS-stimulated macrophage J774A.1 cells.



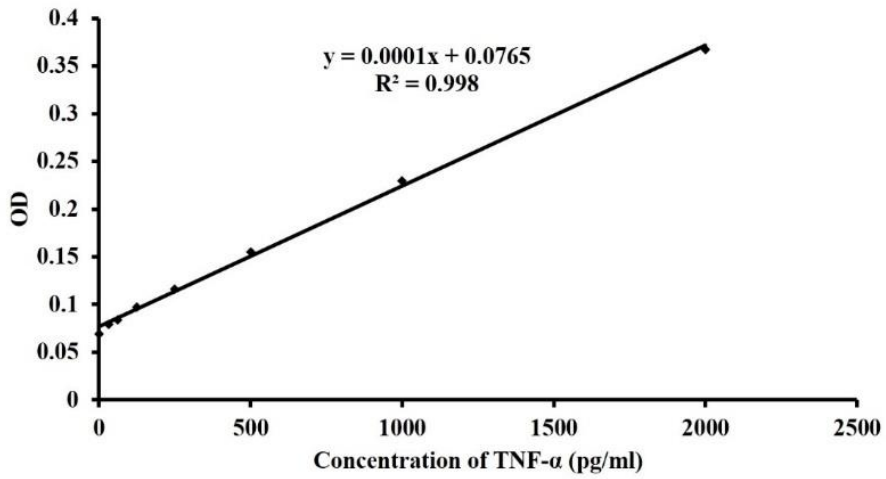
Appendex B-9: Data of the inhibitory effect of cassia oil and cinnamaldehyde on mRNA expression of pro-inflammatory cytokine; TNF-α, IL-1β and IL-6 in LPS-stimulated macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M. compared to untreated control.

Tested compound	% inhibition of TNF-α			Mean ± SE	% inhibition of IL-1β			Mean ± SE	% inhibition of IL-6			Mean ± SE
	n1	n2	n3		n1	n2	n3		n1	n2	n3	
Vehicle	0	0	0	0	0	0	0	0	0	0	0	0
LPS control	0	0	0	0	0	0	0	0	0	0	0	0
DEX	34.3	35.5	24.4	31.4 ± 3.5	59.2	45.9	49.3	51.4 ± 4.0	75.0	52.3	35.3	54.2 ± 11.5
5 µg/ml CO	24.1	22.6	4.6	17.1 ± 6.3	25.7	31.6	16.4	24.6 ± 4.4	9.3	5.1	21.1	11.9 ±4.8
10 µg/ml CO	62.2	58.5	40.6	53.7 ± 6.7	54.2	49.1	45.0	49.4 ± 2.7	35.7	33.2	57.1	42.0 ± 7.6
20 µg/ml CO	74.0	78.1	80.9	77.7 ± 2.0	67.6	70.1	75.3	71.0 ± 2.3	43.5	56.7	83.4	61.2 ± 11.7
5 µg/ml CIN	13.2	23.3	18.9	18.5 ± 2.9	16.8	16.2	39.7	24.2 ± 7.7	36.9	20.8	32.0	29.9 ± 4.8
10 µg/ml CIN	46.5	55.3	57.2	53.0 ± 3.3	50.7	53.3	70.1	58.0 ± 6.1	67.8	52.3	63.4	61.1 ± 4.6
20 µg/ml CIN	78.7	73.0	85.2	79.0 ± 3.5	66.4	66.2	77.7	70.1 ± 3.8	76.0	77.4	82.0	78.5 ± 1.8

Appendix B-10: Data of standard calibration curve for TNF- production by ELISA

Standard TNF-α (pg/ml)	OD			
	n1	n2	n3	Mean
0	0.067	0.07	0.071	0.0693
31.25	0.078	0.079	0.08	0.0790
62.5	0.085	0.084	0.083	0.0840
125	0.101	0.088	0.103	0.0973
250	0.116	0.115	0.118	0.1163
500	0.154	0.157	0.154	0.1550
1000	0.229	0.23	0.23	0.2297
2000	0.317	0.409	0.376	0.3673

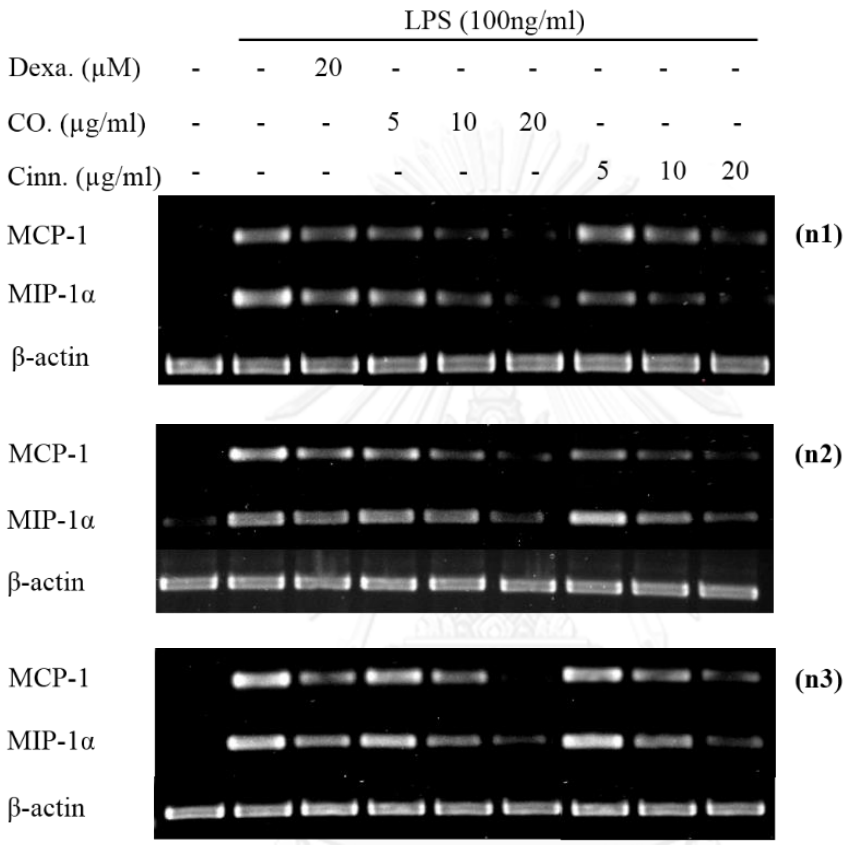
Appendix B-11: Standard calibration curve of TNF-α



Appendex B-12: Data of the inhibitory effect of cassia oil and cinnamaldehyde on TNF-α production in LPS-stimulated macrophage J774A.1 cells. The production of TNF-α was evaluated by ELISA. The data are expressed as mean ± S.E.M. compared to LPS-control.

Tested compound	OD n1			OD n2			OD n3			Mean TNF-α (pg/ml)
	1	2	Mean	1	2	Mean	1	2	Mean	
Vehicle	0.061	0.06	0.0605	0.054	0.066	0.06	0.057	0.054	0.0555	0
LPS control	0.332	0.327	0.3295	0.348	0.332	0.34	0.346	0.429	0.3875	5517 ± 357
DEX	0.229	0.223	0.226	0.24	0.233	0.2365	0.233	0.261	0.247	3200 ± 121
5ug/ml CO	0.238	0.248	0.243	0.319	0.307	0.313	0.319	0.338	0.3285	4367 ± 226
10ug/ml CO	0.187	0.186	0.1865	0.256	0.258	0.257	0.309	0.317	0.313	3513 ± 547
20ug/ml CO	0.055	0.056	0.0555	0.107	0.106	0.1065	0.085	0.081	0.083	243 ± 200
5ug/ml CIN	0.31	0.315	0.3125	0.328	0.331	0.3295	0.352	0.351	0.3515	5093 ± 526
10ug/ml CIN	0.194	0.197	0.1955	0.262	0.263	0.2625	0.269	0.305	0.287	3437 ± 732
20ug/ml CIN	0.055	0.059	0.057	0.108	0.107	0.1075	0.077	0.08	0.0785	220 ± 182

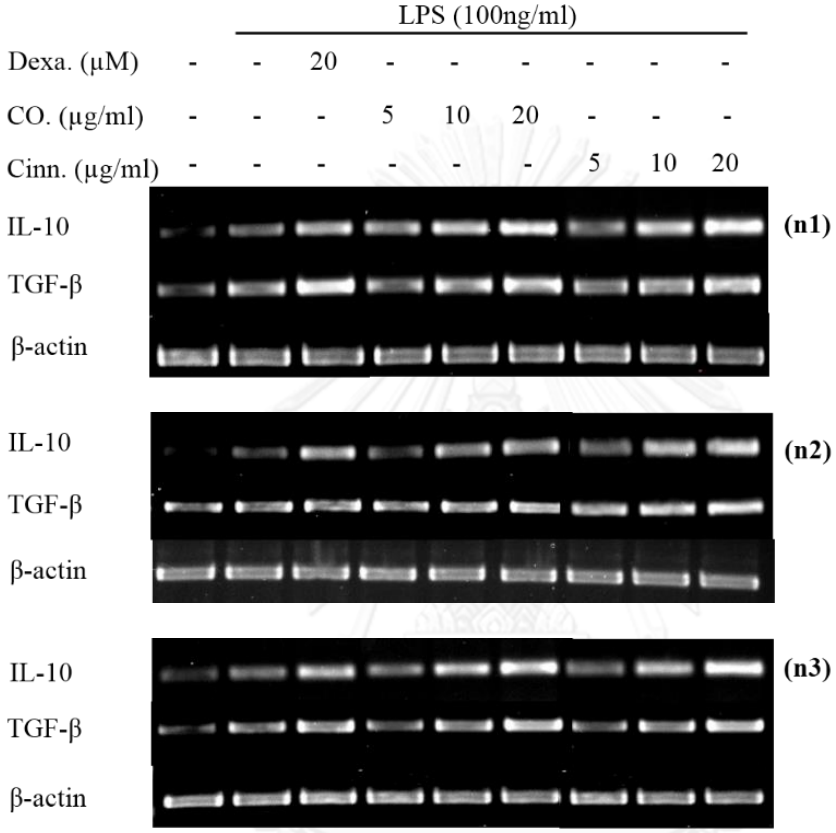
Appendix B-13: The inhibitory effect of cassia oil and cinnamaldehyde on the mRNA expression of chemokine; MCP-1 and MIP-1α in LPS-stimulated macrophage J774A.1 cells



Appendex B-14: Data of the inhibitory effect of cassia oil and cinnamaldehyde on mRNA expression of chemokine; MCP-1 and MIP-1α in LPS-stimulated macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by gel documentation and compared with β-actin PCR products. The data are expressed as mean ± S.E.M. compared to untreated control.

Tested compound	% inhibition of MCP-1			Mean ± SE	% inhibition of MIP-1α			Mean ± SE
	n1	n2	n3		n1	n2	n3	
Vehicle	0	0	0	0	0	0	0	0
LPS control	0	0	0	0	0	0	0	0
DEX	48.3	35.1	17.1	33.5 ± 9.0	65.4	37.4	39.9	47.5 ± 8.9
5 µg/ml CO	34.4	68.3	51.5	51.4 ± 9.8	30.3	9.7	32.2	24.1 ± 7.2
10 µg/ml CO	76.2	92.6	82.1	83.6 ± 4.8	51.5	28.0	52.5	44.0 ± 8.0
20 µg/ml CO	80.9	92.0	87.8	86.9 ± 3.2	78.6	46.2	80.2	68.4 ± 11.1
5 µg/ml CIN	22.1	28.6	28.1	26.2 ± 2.1	8.9	12.7	18.8	13.4 ± 2.9
10 µg/ml CIN	45.5	51.0	51.3	49.3 ± 1.9	41.3	36.2	48.3	42.0 ± 3.5
20 µg/ml CIN	65.1	73.0	89.5	75.9 ± 7.2	58.3	61.5	67.0	62.3 ± 2.5

Appendix B-15: The effect of cassia oil and cinnamaldehyde on the mRNA expression of anti-inflammatory cytokine; IL-10 and TGF-β in LPS-stimulated macrophage J774A.1 cells.



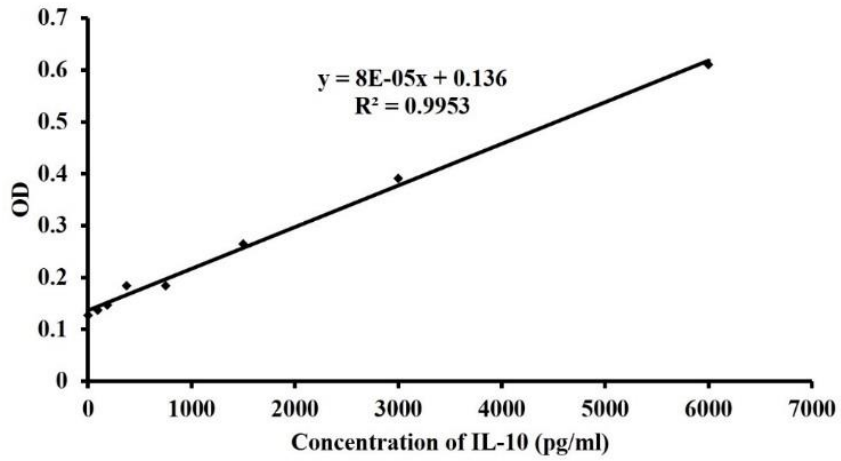
Appendex B-16: Data of the stimulatory effect of cassia oil and cinnamaldehyde on mRNA expression of anti-inflammatory cytokine; IL-10 and TGF-β in LPS-stimulated macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by gel documentation and normalized with β-actin PCR products. The data are expressed as mean ± S.E.M. compared to untreated control.

Test compound	IL-10/Actin ratio			Mean ± SE	TGF-β/Actin ratio			Mean ± SE
	n1	n2	n3		n1	n2	n3	
Vehicle	0.41	0.28	0.35	0.405 ± 0.03	0.61	0.33	0.47	0.470 ± 0.04
LPS control	0.73	0.49	0.53	0.653 ± 0.02	1.09	0.58	0.71	0.794 ± 0.05
DEX	1.02	0.67	0.74	0.770 ± 0.03	1.45	0.78	0.95	1.060 ± 0.07
5 µg/ml CO	0.68	0.52	0.61	0.603 ± 0.04	0.88	0.49	0.58	0.649 ± 0.08
10 µg/ml CO	0.96	0.80	0.77	0.843 ± 0.05	1.12	0.69	0.81	0.874 ± 0.05
20 µg/ml CO	1.25	0.90	0.92	1.022 ± 0.06	1.57	0.92	1.04	1.177 ± 0.05
5 µg/ml CIN	0.64	0.47	0.52	0.544 ± 0.05	0.91	0.66	0.88	0.814 ± 0.03
10 µg/ml CIN	0.98	0.77	0.85	0.866 ± 0.05	1.09	0.86	1.23	1.061 ± 0.05
20 µg/ml CIN	1.52	1.07	1.19	1.262 ± 0.13	1.57	1.14	1.27	1.327 ± 0.1

Appendix B-17: Data of standard calibration curve for IL-10 production by ELISA

Standard IL-10 (pg/ml)	OD			
	n1	n2	n3	Mean
0	0.115	0.144	0.122	0.1271
93.75	0.13	0.15	0.13	0.1366
187.5	0.143	0.147	0.153	0.1476
375	0.181	0.199	0.171	0.1836
750	0.186	0.184	0.183	0.1843
1500	0.28	0.256	0.258	0.2646
3000	0.381	0.405	0.388	0.3913
6000	0.589	0.589	0.653	0.6103

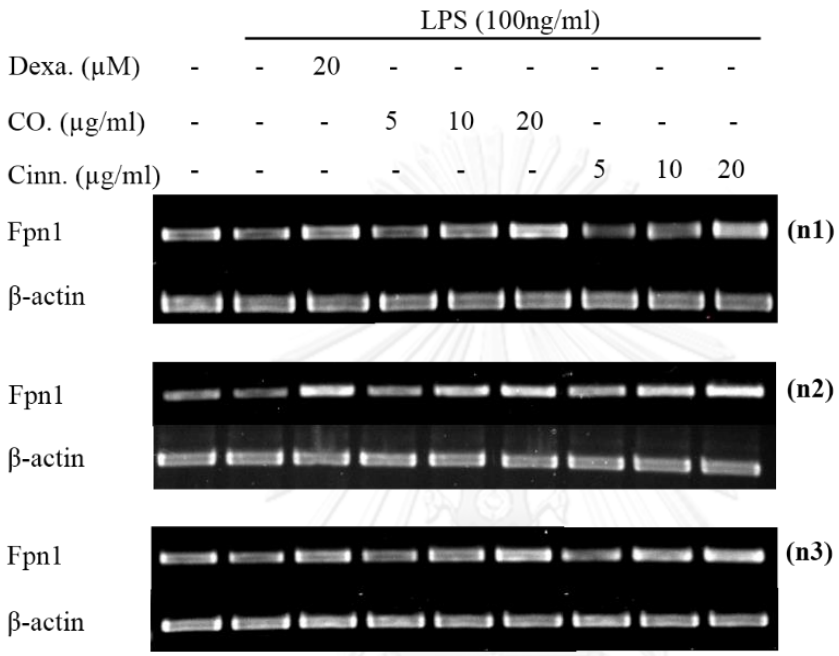
Appendix B-18: Standard calibration curve of IL-10



Appendex B-19: Data of the stimulatory effect of cassia oil and cinnamaldehyde on IL-10 production in LPS-stimulated macrophage J774A.1 cells by ELISA. The data are expressed as mean ± S.E.M. compared to untreated control.

Tested compound	OD n1		OD n2		OD n3		Mean IL-10 (pg/ml)	SE
	1	2	1	2	1	2		
Vehicle	0.121	0.109	0.124	0.115	0.099	0.114	0	0
LPS control	0.152	0.153	0.167	0.165	0.169	0.166	310	95
DEX	0.171	0.197	0.193	0.192	0.167	0.189	697	84
5ug/ml CO	0.145	0.164	0.169	0.168	0.171	0.158	340	83
10ug/ml CO	0.201	0.181	0.189	0.217	0.205	0.138	813	184
20ug/ml CO	0.227	0.227	0.272	0.273	0.26	0.24	1517	263
5ug/ml CIN	0.162	0.161	0.178	0.168	0.168	0.167	417	66
10ug/ml CIN	0.23	0.167	0.177	0.236	0.214	0.18	887	59
20ug/ml CIN	0.243	0.236	0.277	0.284	0.248	0.248	1653	250

Appendix B-20: The effect of cassia oil and cinnamaldehyde on the mRNA expression of iron exporter protein on macrophages membrane ferroportin1 (Fpn1) in LPS-stimulated macrophage J774A.1 cells.



Appendex B-21: Data of the inhibitory effect of cassia oil and cinnamaldehyde on mRNA expression of iron exporter protein on macrophages membrane ferroportin1 (Fpn1) in LPS-stimulated macrophage J774A.1 cells. The expression of mRNA was evaluated by RT-PCR and determined densities of PCR products by gel documentation and normalized with β-actin PCR products. The data are expressed as mean ± S.E.M. compared to untreated control.

Tested compound	Fpn1/Actin ratio			Mean ± SE
	n1	n2	n3	
Vehicle	0.92	0.66	0.76	0.781 ± 0.06
LPS control	0.71	0.62	0.43	0.588 ± 0.08
DEX	1.37	0.66	0.99	1.005 ± 0.05
5 μg/ml CO	0.93	0.52	0.72	0.720 ± 0.11
10 μg/ml CO	1.14	0.75	0.92	0.939 ± 0.11
20 μg/ml CO	1.53	0.81	1.08	1.140 ± 0.2
5 μg/ml CIN	0.76	0.84	0.59	0.731 ± 0.07
10 μg/ml CIN	0.86	1.01	0.78	0.881 ± 0.06
20 μg/ml CIN	1.18	1.06	0.85	1.032 ± 0.09

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