ANTI-INFLAMMATORY EFFECT OF (+)-GONIOFUFURONE FROM *POLYALTHIA CRASSA* IN THE LIPOPOLYSACHARIDE-STIMULATED RAW264.7 MACROPHAGES

WANICHAYA KHAKHUNMALEE

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Miss Rungkaew Phumpho
Candidate

Asst. Prof. Singhanat Nomnian,
Ed.D. (TESOL & Applied Linguistics)
Major advisor

Lect. Sumittra Suraratdecha,
Ph.D. (Linguistics)
Co-advisor

Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

Lect. Kanopporn Wonggarasin,
Ph.D. (Creativity Psychology)
Program Director
Master of Arts Program in
Language and Culture for
Communication and Development
Research Institute for Languages and
Cultures of Asia
Mahidol University

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was submitted to the Faculty of Graduate Studies, Mahidol University
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(Language and Culture for Communication and Development)
on
December 16, 2015

.....
Miss Rungkaew Phumpho
Candidate

.....
Asst. Prof. Sita Yiemkuntitavorn,
Ph.D. (Linguistics)
Chair

.....
Asst. Prof. Singhanat Nomnian,
Ed.D. (TESOL & Applied Linguistics)
Member

.....
Lect. Sumittra Suraratdecha,
Ph.D. (Linguistics)
Member

.....
Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

.....
Assoc. Prof. Sophana Srichampa,
Ph.D. (Linguistics)
Director
Research Institute for Languages and
Cultures of Asia
Mahidol University

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Wanichaya Khakhunmalee

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WANICHAYA KHAKHUNMALEE 5537343 SCBT/M

M.Sc. (BIOTECHNOLOGY)

THESIS ADVISORY COMMITTEE : IDSADA MUNGSANTISUK, Ph.D., PONGSAK UTAISINCHAROEN, Ph.D., PATOOMRATANA TUCHINDA, Ph.D.

ABSTRACT

(+)-Goniofufurone, a styryl lactone, isolated from leaves and twigs of *Polyalthia crassa* was reported to have the antiproliferative activity against various cancer cells. In present study, the cytotoxic effect of (+)-goniofufurone was investigated in RAW264.7 macrophages by MTT assay. The result showed low cytotoxicity of (+)-goniofufurone to RAW264.7 cells with the IC_{50} of 2.08 mM. The anti-inflammatory effect of this compound was also studied. RAW264.7 cells stimulated with lipopolysaccharide (LPS) of *Escherichia coli* exhibited the expression of inflammatory-related genes in this study including TNF-α, IFN-β, IL-1β, IL-6, IL-12p40, COX-2 and iNOS, and increase the production of mediators including NO and PGE_2. Treated RAW264.7 cells with (+)-goniofufurone could suppress LPS-induced inflammatory responses suggesting the effect of (+)-goniofufurone activity on inflammatory genes and cytokines through both MyD88-dependent and-independent pathways.

KEY WORDS : ANTI-INFLAMMATORY/ *POLYALTHIA CRASSA* / (+)-GONIOFUFURONE / MyD88-DEPENDENT / MyD88-INDEPENDENT

72 pages

ผลการต้านการอักเสบของสารสกัดโกนิโอฟุฟูโรนจากพืชโพลีอาวเทียคราสซ่าในเซลล์แมคโคร-ฟาจของหนูที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์

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วนิชญา ข่าขันมะลี 5537343 SCBT/M

วท.ม. (เทคโนโลยีชีวภาพ)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : อิษฎา มุ่งสันติสุข, Ph.D., พงศ์ศักดิ์ อุทัยสินธุเจริญ, Ph.D., ปทุมรัตน์ ตู้จินดา, Ph.D.

บทคัดย่อ

(+)-โกนิโอฟุฟูโรนเป็นสารในกลุ่มสไตริลแลคโตน สกัดจากใบและกิ่งของพืชโพลีอาวเทีย คราสซ่า ซึ่งมีรายงานถึงฤทธิ์ในการต้านเซลล์มะเร็ง สำหรับการศึกษานี้ได้ทดสอบความเป็นพิษต่อเซลล์ของสารกัด (+)-โกนิโอฟุฟูโรนในเซลล์แมคโครฟาจของหนู โดยการทดสอบด้วยวิธี MTT ผลการทดลองพบว่าสารสกัด (+)-โกนิโอฟุฟูโรนมีความเป็นพิษต่อเซลล์แมคโครฟาจของหนูต่ำ โดยมีค่าความเข้มข้นที่สามารถยับยั้งเซลล์ได้ 50% ที่ 2.08 มิลลิโมลาร์ นอกจากนี้ ได้ทำการศึกษาฤทธิ์ในการต้านการอักเสบของสารสกัด (+)-โกนิโอฟุฟูโรน พบว่าเซลล์แมคโคร-ฟาจของหนูที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรค์ของแบคทีเรียอีโคไล จะมีการแสดงออกของยีนที่เกี่ยวข้องกับการอักเสบ ได้แก่ TNF-alfa, IFN-beta, IL-1beta, IL-6, IL-12p40, COX-2 และ iNOS อีกทั้งเพิ่มการผลิตของสารสื่อกลางการอักเสบ ได้แก่ NO และ PGE_2 เพิ่มขึ้นด้วย การตอบสนองเหล่านี้สามารถยับยั้งได้โดย (+)-โกนิโอฟุฟูโรน ซึ่งชี้ให้เห็นว่า สารสกัด (+)-โกนิโอฟุฟูโรนมีผลต่อยีนที่เกี่ยวข้องกับการอักเสบและไซโตไคน์ผ่านทั้งวิถี MyD88-dependent และ MyD88-indepndent

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LIST OF ABBREVIATIONS

/	per
%	percent
*	significance level
AP-1	activating protein-1
APS	ammonium persulfate
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et. al	et. All (Latin), and others
FBS	fetal bovine serum
g	gram
h	hour (s)
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
IC_{50}	Inhibitory concentration of 50%
IFN-β	interferon-beta
IkB	ikappaB
IKKα	ikappaB kinase alpha
IKKβ	ikappaB kinase beta
IKKγ	ikappaB kinase gamma
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-12p40	interleukin-12p40

LIST OF ABBREVIATIONS (cont.)

iNOS	inducible nitric oxide synthase
IP-10	IFN-γ-inducible protein 10
IRAK	IL-1 receptor-associated kinase
IRF3	interferon regulatory factor3
JAK	janus kinase
kDA	kilodalton
LPS	lipopolysaccharide
Mal	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
min	minute
ml	milliliter
mRNA	messenger ribosomal nucleic acid
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-kappa B
NK	natural killer cell
nm	nanometer
ng	nanogram
NO	nitic oxide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
Refs	references
RNA	ribosomal nucleic acid
rpm	round per minute
RT	room temperature
RT-PCR	reverse transcripse-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error

LIST OF ABBREVIATIONS (cont.)

STAT	signal transducer and activator of transcription
TAK	TGF-β-activated kinase
Taq	*Thermus aquaticus*
TBE	tris-borate EDTA buffer
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
TIRAP	TIR-domain-containing adaptor protein
TIR	Toll/IL-1 receptor homologous
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β
vs	versus
μg	microgram

CHAPTER I
INTRODUCTION

Inflammation is a natural response of the mammalian body to a variety of harmful stimuli including parasite, pathogenic microorganism, toxic chemical substances and physical damage to tissue. Once an inflammation occurs, the macrophages release pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and various cytokines, in response to activation signals, including lipopolysaccharide (LPS) of bacteria, cytokines and chemical mediators. Majority of human population worldwide is getting affected by the inflammation related disorders. It is believed that current analgesia inducing drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) is not useful in all cases, because of their side effects like gastrointestinal irritation, liver dysfunction and many others [1-5]. Accordingly, therapeutic agents suitable for the treatment of chronic inflammatory diseases are highly desirable, which has resulted in an increased interest in complementary and alternative medicines.

Large number of herbal species has been used traditionally or as folk medicines against inflammatory. Many of them have been studied scientifically and proved to be beneficial anti-inflammatory agents. The success has been attained to isolate various single chemical responsible for anti-inflammatory activity. However, still many herbal folk medicines for inflammation have not undergone through scientific investigations and careful assessment of their toxic effects. So, it is a need of time to consider herbal medicines for determining their pharmacological activities that responsible for anti-inflammatory effect and developing suitable formulation, beneficial against inflammatory disorders. The plant compounds from genus *Polyalthia* have long been used in traditional medicine in Asia. Study in (+)-goniofufurone extracted from *Polyalthia crassa* reviewed antitumor activity in various cancer cells [107]. However, the anti-inflammatory activity of this compound has not been reported previously. To study anti-inflammatory property, macrophage

cell lines (RAW264.7) were used as a model of study because they play an important anti-inflammatory role and can decrease immune reaction through the release of cytokines. The LPS of *Escherichia coli* was used to stimulate RAW264.7 cells to express inflammatory genes studied in this research. Bacterial LPS can trigger innate immune responses through Toll-like receptor (TLR) 4 which is a member of the TLR family. Upon pathogen recognition, MyD88 (a cytoplasmic protein) is recruited by TLRs. There are two major pathways downstream of TLR4 in response to LPS : the MyD88-dependent and-independent pathways as shown in Figure 1.1. In this study the effects of (+)-goniofufurone activity on gene expression downstream of these two pathways were investigated.

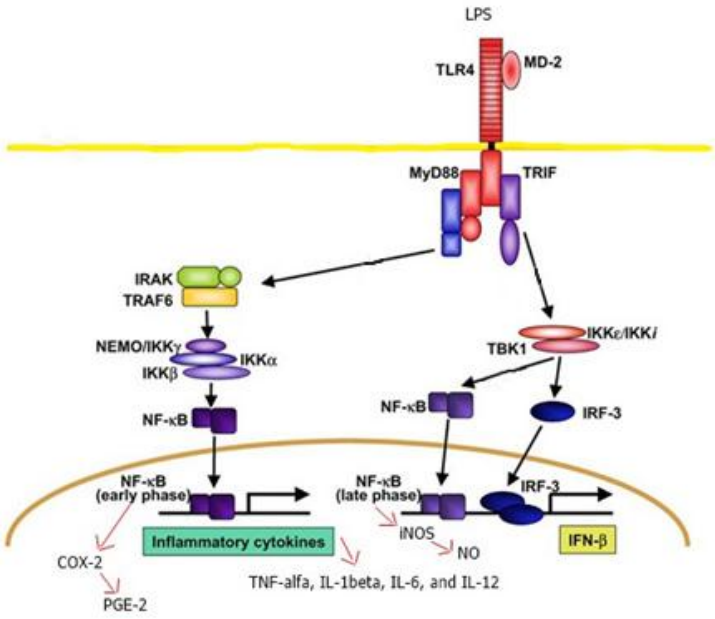


Figure 1.1 Innate immune responses triggered by bacterial LPS through TLR4. There are two major pathways downstream of TLR4 in response to LPS : the MyD88-dependent and-independent pathways

Objective :

1. To study the cytotoxicity of (+)-goniofufurone to RAW264.7 cells.

2. To study the effects of (+)-goniofufurone activity on gene expression downstream of MyD88-dependent and-independent pathways in RAW264.7 cells.

CHAPTER II
LITERATURE REVIEW

2.1 Medicinal Plant

Several plants can produce substances from their parts espectially seeds, flowers, roots, twigs and leaves protecting them to survive. For example, some plants release bad smelling substances to protect themselves against herbivore. In the past, people had gathered these substances to create herbal medicines for disease treatment and later, ethnobotany (the study of using plants in the past of human) became the way to discover future medicines. Fourteen years ago, researchers identified more than a hundred compounds that were used in modern medicine by developing from ethnomedical plant sources. Eighty percentage of the compounds from ethnomedical were identical or related to current use of the active constituents of the plant [6]. Many of current drugs originated in plants.

2.2 Phytochemistry

Chemical compounds are produced in plants as their normal metabolic activities which known as phytochemicals. These phytochemicals are classifided into 2 groups including primary metabolites such as sugars and fats which are found in all plants, and secondary metabolites which are produced in some of plants. The secondary metabolites have specific function more than primary metabolites. For examples, toxins for protecting itself from herbivore and pheromones for attracting insects for pollination. Interestingly, the secondary metabolites and pigments from plant contain therapeutic activity to humans which can be purified to produce and developed for disease treatment. Examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove [7]. In addition, toxic plants also have been used in development of

pharmaceuticals [8]. The examples of phytochemicals that are synthesized from plants include alkaloids, polyphenols, glycosides, terpenes and styryl lactones [9].

2.2.1 Alkaloids

Alkaloids are chemical compounds that contain a nitrogen ring in the structure. These compound can be produced from many organisms including bacteria, fungi, plants, and animals. They are the natural products and secondary metabolites. Many of them can be purified from crude extracts by acid-base extraction which most alkaloids are toxic to organisms. In addition, alkaloids also have pharmacological activities and are used as medicines. Examples of alkaloids are cocaine, caffeine and nicotine used for stimulant, psilocin used for psychedelic, morphine used for analgesic, berberine used for antibacterial, vincristine compound used for anticancer, reserpine agent used for anti-hypertension, galatamine used as cholinomimeric, atropine agent used for spasmolysis, vincamine used for vasodilator, quinidine compound used for anti-arhythmia, ephedrine used for anti-asthma, and quinine drug used for antimalarial.

2.2.2 Polyphenols

Polyphenols or phenolics are compounds containing phenol rings. Example of polyphenols include the anthocyanins from purple grapes, the isoflavones and the phytoestrogens from soy, and the tannin from tea.

2.2.3 Glycosides

Glycosides are molecules that contain a sugar part bound to a non-carbohydrate, usually a small organic molecule. Glycosides play important function in living organisms. Normally the plants store chemical compounds in the inactive form and can be activated by breaking off the sugar part using enzyme hydrolysis, making the chemical compounds are activated. Many plant glycosides are used as medicine to treat disease. Examples of glycosides are the cyanoglycosides produced from cherry pits which there toxins will be released only when bitten by herbivore.

2.2.4 Terpenes

Terpenes are organic compounds produced from plants such as conifers which have strong smelling and may have function to protect plant. Terpenes are the major components of resin, turpentine (terpene was derived from turpentine), and are the major biosynthetic within living creature. Steroids are the example of triterpene derivatives. The modification of terpenes by the oxidation or rearrangement at the carbon skeleton leads to the terpenoids. Terpenes and terpenoids are used in the primary constituents of the essential oils in many types of plants and flower. Essential oils are extracted from plant to use as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines, mainly aroma therapy. Synthetic derivatives of terpenes and terpenoids also expand distribute the variety of aromas used in perfumery and flavors used in food additives. An example of a terpene is vitamin A. Monoterpenes cause odor of rose and lavender. The carotenoids are component of the red, yellow and orange colors of pumpkin, corn and tomatoes, respectively.

2.2.5 Styryl lactones

Styryl lactones are secondary metabolites purified from plant in the genus *Goniothalamus* and *Polyalthia* [16]. They have important biological properties in the antiproliferative activity against cancer cells. Nomally, the styryl lactones have toxicity specific to cancer cells. Many reports suggests that antiproliferative activity of styryl lactones is involved with the induction of apoptosis in cancer cells [75]. Some research reported styryl lactone with activity against several human tumor cell line and inhibition of inflammatory related genes such as NF-κB, iNOS and COX-2 in LPS-activated RAW264.7 macrophage cells [107].

2.3 *Polyalthia crassa*

Polyalthia spp. are classified in *Annonaceae* family which consists of about 150 genera and 2,500 species. *Polyalthia spp.* are found in tropical Africa and Asia [109]. Chemical analysis of *Polyalthia spp.* has revealed the compounds including alkaloids, diterpenes, triterpenes, clerodane, benzopyran derivatives,

polyacetylenic compounds and styryl lactones. Several genus of *Polyalthia* have been widely used in traditional medicine in Asia. In Thailand, extracts from the roots of *P. evecta* and *P. debilis* are used as drug for stimulate secretion of milk in mother with her infant (galactagogue) and for abdominal pain relieve [10], respectively. In Malaysia, *Polyalthia bullata* is used as drug for stimulating sex requirement (aphrodisiac) [11]. Purified compounds from *Polyalthia* species show bioactivities, including the purified compounds from *P. sclerophylla* with anti-HIV activities [12], from *P. suberosa* with anti-HIV activity [14] , anti-oxidative stress and cell death activities [13], and from *P.longifolia* with antifungal activity [15]. Some compounds from the extract of *P. crassa* (or Nom maeo noi as the local name) show cytotoxicity against human cancer cells [75]. There are 11 compounds isolated from an ethyl acetate-soluble extract of the leaves and twigs of *P. crassa* including (1-4) crassalactones A–D, (5) (+)-howiinol A, (6) (+)-goniofufurone, (7) (+)-altholactone, (8) (+)-3-acetylaltholactone, (9) (+)-goniopypyrone, (10) aristolactam AII, and (11) cinnamic acid as shown in Figure 2.1 [16].

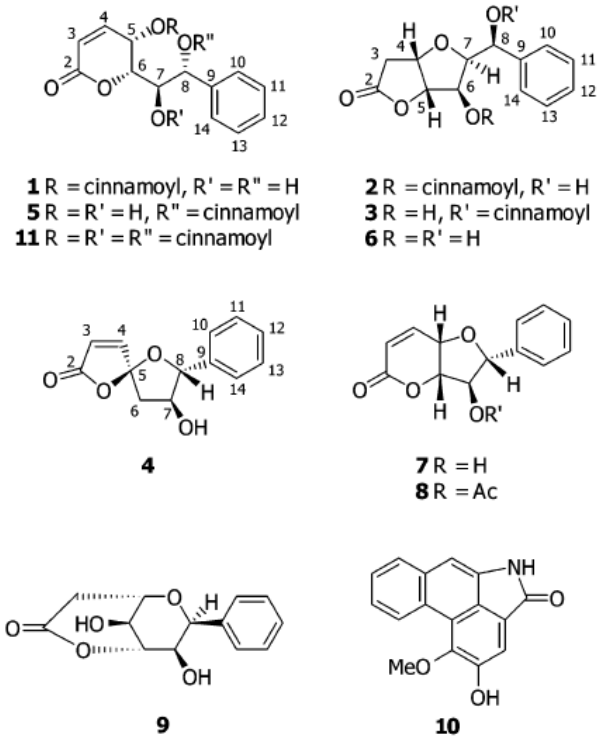


Figure 2.1 Eleven compounds from the leaves and twigs of *P. crassa* [16].

(+)-Goniofufurone is one of an interesting natural compound because it possesses significant cytotoxic activity to several human tumor cell lines [16]. This compound is a styryl lactone (Figure 2.2). Previously, the (+)-goniofufurone was isolated from stem bark of *Goniothalamus giganteus* which is classified in *Annonaceae* family by McLaughlin and co-workers [76, 77]. Plants of the genus *Goniothalamus* have been known for a long time in South East Asia for their properties in folk medicine. This plant genus provides active molecules known as styryl lactones which associated with a biological activity in anti-tumor, anti-parasitic, abortifacient, and preventing plant from insect. Structure of styryl lactones can be classified into six main groups as shown in Figure 2.3 [110]. The extract of the seeds of *Goniothalamus amuyon* from Taiwan was reported to be used for the treatment of edema and rheumatism [78]. The leaves of *Goniothalamus sesquipedalis* have been used as drug to relieve labor pain in women, and the burning leaves have been used to dispel the mosquitoes [79]. The leaves of *Goniothalamus macrophyllus* have been used for abortion in rural areas of North Malaysia [80].

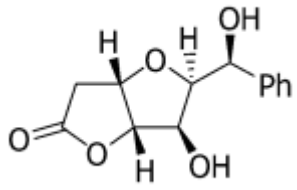


Figure 2.2. Chemical structure of (+)-goniofufurone. Molecular formula : $C_{13}H_{14}O_5$; molecular weight : 250.247 dalton.

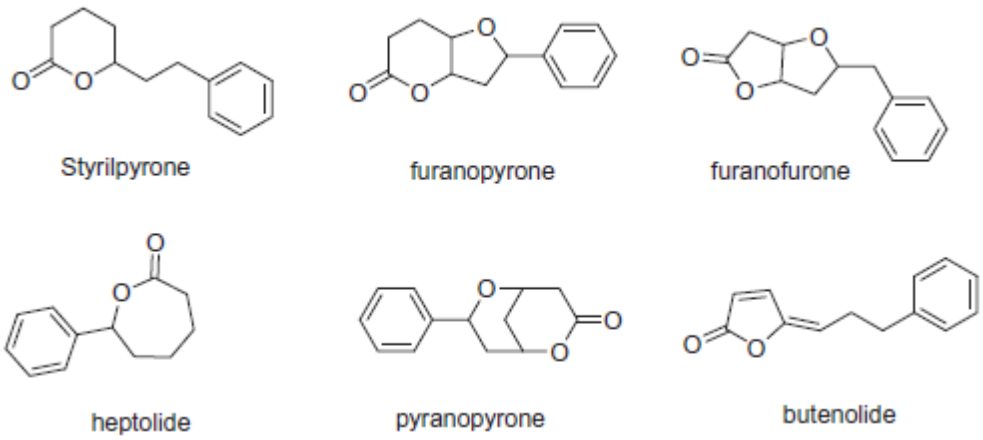


Figure 2.3. Main styryl lactones skeletons found in plants of *Goniothalamus* genus [110].

2.4 Inflammation

Inflammation is a response of physiological body to harmful stimuli such as heat shock protein, cellular stress, physical injury, chemical or toxin, and microorganisms. This is a process of body protection to destroy invading organisms or to repair tissues after injury [17,18]. This process works together with a regulation of immunological and physiological processes under the controling of cytokines.

When the body is affected by inflammation, signs and symtoms of inflammation include pain, heat, redness, swelling, and loss of function [115,130] which are defined from mechanism of the body response to tissue injury involving vascular, humoral and cellular factors. Redness and heat occur from increasing of blood flow to the inflamed tissues. Accumulation of the fluid in the inflamed tissues results to swelling and releasing of chemicals to stimulate nerve endings leads to pain, Finally, loss of function occurs from a combination of many factors from various causes [115]. Inflammation is divided into 2 types including acute and chronic inflammation which occurs over seconds or days, and over longer times, respectively.

2.4.1 Acute inflammation

Acute inflammation is the first process of the body responding to inflammatory harmful stimuli by increasing the movement of plasma and leukocytes from the blood vessels into the injured tissues [112]. Process of inflammatory response involves with vascular system, immune system and various immune cells within the injured tissue to remove pathogens or stimuli. The first step of the inflammation is recognition of infection or damage. Initiation occurs by the detection of pathogen-associated molecular patterns (PAMPs) which are specific to general molecules expressed by pathogens and essential for pathogen survival. During infection, the levels of heat shock proteins (HSPs) are increased [49], which the HSPs function as intracellular molecular chaperones of newly synthesized polypeptide chains, preventing protein denaturation and loss of function. HSPs are classified into 6 families, but there are only 2 families that have potential to trigger immunoregulatory pathways (including the HSP60 and HSP70). During bacterial infections, the bacterial members of the HSP60 and HSP70 families are common targets of humoral and cell-mediated immune responses [57, 58]. For the response of damage cells or foreign particles, damage-associated molecular patterns (DAMPs) are molecules that signal damage and are recognized by the innate immune system. This response has been characterized by receptors, mainly Toll-like receptors (TLRs) and nucleotide binding domain and leucine-rich-repeat-containing receptors (NLRs) [59, 60, 113, 122]. This initial recognition is mediated by macrophages, leading to the production of variety of inflammatory mediators including chemokines, cytokines, vasoactive amines and eicosanoids. A successful response of acute inflammatory results in elimination of the infectious agents or harmful stimuli followed by the resolution and repairing [114].

2.4.1.1 Process of acute inflammation

There are 2 phases involving in the process of acute inflammation including a vascular phase that occurs first, followed by a cellular phase involving immune cells. The process of acute inflammation is initiated by immune cells, mainly macrophages, dendritic cells, histiocytes, Kupffer cells and mastocytes. These cells contain receptors on their surfaces which known as pattern recognition receptors (PRRs). PRRs recognize generic molecules such as PAMPs and DAMPs. Upon harmful stimuli, contacting with PAMPs or DAMPs at the origin of an infection,

or physical injuries, immune cells are activated and release inflammatory mediators such as NO and PGE_2 responsible for the signs of inflammation. These mediators stimulate the vasodilation, leading to the increase of blood flow which can cause redness and heat at the inflammatory areas. Increase of the permeability of blood vessels results in an exudation of plasma proteins including fibrin and immunoglobulins (antibodies) and fluid into the tissue, which exhibits swelling tissue. This exuded tissue fluid contains several antimicrobial mediators, mainly complement, lysozyme and antibodies to destroy the microbes, and prepare the microbes for the cellular phase by opsonization. Some of the exuded tissue fluid also works like funnel to the region of lymph nodes to flushing bacteria together with initiating the recognition and preparing to adaptive immune system. Some of the released mediators such as bradykinin increase the sensitivity to pain. The mediator molecules also control the blood vessels to permit the migration of leukocytes such as neutrophils from blood vessels into the injury tissue to attract with harmful stimuli which the leukocytes migrate to reach the site of injury by chemotactic. The process of leukocyte movement from the blood to the tissues is known as extravasation. Some leukocytes act as phagocytic cells to ingest bacteria, viruses and cellular debris. Most PAMPs that bind to PRRs and initiate phagocytosis are cell wall components of leukocytes including complex carbohydrates, lipopolysaccharides (LPS), peptidoglycans, and surface proteins. Some leukocytes such as mast cells, neutrophils, eosinophils, macrophages, lymphocytes, endothelial cells and platelets can release inflammatory mediators that develop and maintain the inflammatory response which several mediators have been demonstrated to play an essential role in inflammation as shown in Table 2.1.

Table 2.1 Cell derived mediators

Name	Type	Source	Description
Lysosome granules	Enzymes	Granulocytes	These cells contain enzymes that perform a functions to break down a number of substances.
Histamine	Vasoactive amine	Mast cells, basophils, platelets	Histamine is stored in granules and released to response harmful stimuli of inflammatory. It causes arteriole dilation and increased venous permeability.
IFN-γ	Cytokine	T-cells, NK cells	IFN-γ has antiviral activities, immunoregulatory and anti-tumour properties. This interferon is macrophage-activating factor, and is important in the maintenance of chronic inflammation.
IL-8	Chemokine	Macrophages	IL-8 activates and induces chemotaxis in target cells, mainly neutrophils.
Leukotriene B4	Eicosanoid	Leukocytes	Leukotriene is able to mediate leukocyte adhesion and activation to bind with endothelium of vessels and migrate across it..
Nitric-oxide	Soluble gas	Macrophag, endothelial cells, some neurons	NO has potential to be a vasodilator. NO relaxes smooth muscle, reduces platelet aggregation and has antimicrobial activity in high concentrations.
Prostaglan-dins	Eicosanoid	Mast cells	Prostaglandins are cause of vasodilation, fever and pain in the response of inflammatory.

Table 2.1 Cell derived mediators (Cont.)

Name	Type	Source	Description
TNF-α and IL-1	Cytokines	Macrophages	Both affect induce inflammatory reactions: fever, production of cytokines, gene regulation, chemotaxis and leukocyte adherence.

2.4.2. Chronic inflammation

Prolonged inflammation is known as chronic inflammation which occurs over several months and even year. It results from failure to pathogen elimination or stimuli agent, self antigen response, and irritant from chronic disease. Chronic inflammation can lead to a severity in the cells that present in the site of inflammation and is characterized by having the destruction of tissue and reparing the tissue from the inflammatory process.

Chronic inflammation results in the up-regulation of enzymes and signaling proteins in affected tissues and cells. These proinflammatory enzymes include the inducible forms of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for the increasing of NO and prostaglandins (PGs), respectively. They are involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's diseases, and colon cancer [124]. The proinflammatory activity of NO lead to the excessive production of NO by iNOS and is stimulated by factor such as interaction of NO with other oxidants and during of NO production. Previous study showed that the chronic inflammation correlates with the increasing of iNOS activity [125]. The evidence that NO is a mediator of tissue injury is obtained from studies on an animal arthritis model, human osteoarthritis, and rheumatoid arthritis [126]. In contrast, iNOS is constitutively expressed in epithelial and neuronal cell which forms of NOS are known to involve with inflammation and carcinogenesis slightly. COX is the enzyme that converts arachidonic acid to PGs. COX-2 is an inducible form responsible for the production of PGs at the inflammatory site [127]. Furthermore, tumor necrosis factor (TNF)-α is proinflammatory cytokine that plays an important role in immunity and inflammation. It activates vascular endothelium and increases permeability leading to the increasing

entry of IgG, complement, and cells to tissue at the site of inflammatory and increases fluid drainage to lymph nodes. It also has systemic effects such as fever, systemic edema and shock [128].

2.5 Toll like receptor (TLR)

Toll-like receptors (TLRs) are Type I transmembrane receptors in recognition site of conserved patterns characteristic of bacterial, viral or fungal invasions, but distinguishable from host molecules, the so-called Pathogen-Associated Molecular Patterns (PAMPs) [120]. Their activation induces the secretion of pro-inflammatory mediators such as cytokines, chemokines, type I interferon (IFN), reactive oxygen species, and other mediators.

TLR signaling pathway is mediated through the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with a TIR domain-containing adaptor protein. Five TIR-domain-containing adapter molecules are known to activate TLRs signaling including (1) myeloid differentiation factor 88 (MyD88), (2) MyD88 adapter-like (Mal), also called TIR-domain-containing adapter protein (TIRAP), (3) TIR-domain-containing adapter including interferon-β (TRIF), (4) TRIF-related adapter molecule (TRAM), and (5) sterile alpha and HEAT/armadillo motif protein (SARM) [23]. The first four adaptor proteins have activating function but SARM functions as a negative regulator of TRIF-dependent signaling [24].

2.5.1 Signaling pathways via TLRs

The signalling cascades induced by TLR ligands can be divided into two main cascades depending on the intracellular adaptor molecules they recruit. The MyD88 is the main adaptor of the first signaling cascade leading to the activation of Nuclear Factor-κB (NF-κB) and Activated Protein-1 (AP-1) transcription factors. The second cascade is dependent on the activation of interferon (IFN)-regulatory factors (IRFs) by TRIF or MyD88-independent.

2.5.1.1 MyD88-dependent pathway

MyD88 is the immediate adapter protein which is common to all mammalian TLRs except for TLR3. Upon LPS stimulation, MyD88 induces

signalling cascades downstream of IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways such as JNK, and induces the activation of the transcription factors AP-1 [29-32]. A complex of IKK phosphorylates IκB (inhibitor of NF-κB transcription factor) proteins. The phosphorylation of IκB leads to degradation the IκB from transcription factor NF-κB and NF-κB is translocated into the nucleus (Figure 2.4) to control the expression of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-12, and IL-1β, and also inducible enzymes including COX-2 [33]. Interestingly, LPS-stimulated MyD88 knockout macrophages can not induce proinflammatory cytokines.

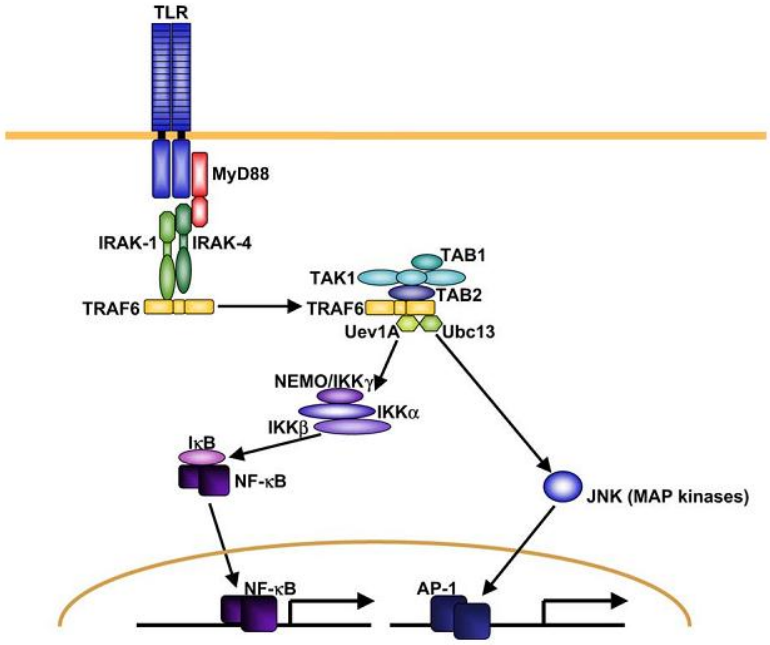


Figure 2.4 TLR-mediated MyD88-dependent signaling pathway [143].

2.5.1.2 MyD88-independent pathway

TRIF is an important adaptor protein that mediates MyD88-independent signaling by activating the signalling through TLR3 and TLR4. Initiation of signaling induces activation of TRIF adaptor. TRIF activates the downstream kinases leading to the activation of IFN refulatory factor 3 (IRF3) [34] and also induces delayed activation of NF-kB [35] (Figure 2.5). Target genes that are regulated

through TRIF signaling pathways include IFNβ and IFN-inducible genes such as inducible nitric oxide synthase (iNOS) and inducible protein-10 (IP-10) [36,37].

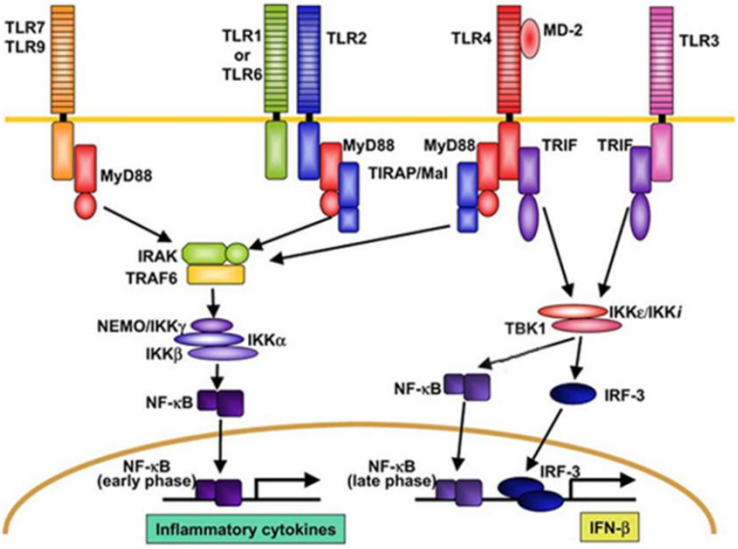


Figure 2.5 TLR-mediated MyD88-independent signaling pathway [143].

2.6 Cytokines

Cytokine is a protein secreted largely by activated cells. The responding cells bear matching transmembrane receptor. Cytokines play a major role in the communication between cells especially in immune system. They regulate the survival, proliferation, differentiation and function of immune cells. Cytokines can be classified into proinflammatory cytokine and interferon-β. Examples of proinflammatory cytokines are IL-1β, IL-6, IL-12 and TNF-α.

2.6.1 Interleukin-1 (IL-1)β

IL-1β is proinflammatory cytokine that plays roles in acute and chronic inflammatory. This cytokine is produced by activated macrophages and important for the response of inflammatory. It is involved in activity of immune cells, mainly cell proliferation, differentiation and apoptosis. In the central nervous system (CNS), IL-1β can induce COX-2 to contribute inflammatory pain hypersensitivity [38].

2.6.2 Interleukin-6 (IL-6)

IL-6 is a cytokine that functions in both innate and adaptive immunity [39]. It is synthesized by a number of cell types including fibroblast, macrophages, dendritic cells, T and B lymphocytes, endothelial cells, glia cells and keratinocytes [40]. IL-6 has several diverse functions. In innate immunity, it stimulates the synthesis of acute phase response proteins by hepatocytes and thus contributes to the systemic effects of inflammation [39]. In adaptive immunity, IL-6 stimulates terminal differentiation of B cells to antibody producing plasma cells, differentiation of monocytes to macrophages, and growth of hematopoetic stem cells [40]. IL-6 uses the common receptor subunit gp130 for signal transduction [41]. After IL-6 binds to the surface, IL-6 receptor (IL-6R)α (gp130) is dimeried and forms the receptor complex before activating two intracellular signaling pathways including JAK-STAT pathway and the CCAAT/enhancer binding protein (C/EBP) pathway [42,43].

2.6.3 Interleukin-12 (IL-12)

IL-12 is expressed in macrophages, dendritic cells and B cells [20-22,25]. IL-12 is believed to be a central mediator in the development of T cell responding to exogenous microbes or noninfectious by the induction of IFN-γ production [28]. IL-12 is produced mainly by activated monocytes, macrophages, neutrophils, B cells and dendritic cells. From the study of Wang SZ, et. al. 2015, it was suggested that IL-12p40 deficiency increased Respiratory Syncytial Virus (RSV)-induced lung inflammation. IL-12 is composed of two subunits, IL-12p35 and IL-p40. However, p35 shares homology with other cytokines such as IL-6 [117], whereas p40 is homologous to the receptor of IL-12. The major functions of IL-12 include induction IFN-γ production from natural killer (NK) cells and T cells, which in turn activates monocytes/macrophages for production of IL-12 [118], enhancement of cytotoxity of NK and cytotoxic T cells, differentiation of naïve T cells into Th1 effectors [44,116]. Although IL-12 is important for host defence, over expression of IL-12 can cause persistent inflammation leading to autoimmune disorders.

2.6.4 Tumor necrosis factor-alpha (TNF-α)

TNF-α is the principal mediator of the acute inflammatory response to Gram negative bacteria and other infectious microbes. It is not usually detectable in healthy persons, but elevated levels in inflammatory and infectious conditions [39,46]. TNF-α is mainly expressed by activating macrophages, NK-cells, T-cells, fibroblasts, astrocytes, Kupffer cells, smooth-muscle cells, keratinocytes and tumor cells [45]. Cytokine binding to TNF receptor leads to recruitment and signaling cascades are initiated resulting in the activation of effector proteins and protein kinases (MAPK, IKK) followed by the activation of transcription factors (AP-1, NF-kB) [45]. TNF-α also induces expression of COX-2 resulting in vasodilation [47], causing redness and heat through increased local blood flow. In addition, TNF-α has been implicated in many inflammatory lung diseases [119]. For example, TNF-α is increasingly released in lung of asthma patients [121].

2.6.5 Interferons-beta (IFN-β)

IFN-β is produced by several cell types including macrophages, fibroblasts, NK cells, T cells, dendritic cells and plasmacytoid monocytes [50-54]. However, macrophages are the major cellular source of IFN-β in the immune system [55]. Several reports demonstrated a low-level constitutive expression of IFN-β in resting macrophages [50, 56]. IFN-β is expressed in rheumatoid arthritis (RA) and can enhance the production of anti-inflammmatory mediators [48]. Futhermore, IFN-β is essential in the induction of iNOS by LPS stimulation [61]. Expression of IFN-β gene is rapidly induced by TLR4 activation in an MyD88-independent manner [62]. Binding of these cytokines to cell surface receptors generates an intracellular signal through the JAKs. Activated JAKs phosphorylates STAT-1 which then translocates to nucleus and regulates cytokine-dependent transcription [63].

2.7 Mediators

Mediators are the chemical released during inflammation and propagate the inflammatory response. These mediators are soluble and diffusible molecules that

can act locally and systemically. The mediators that involved in inflammation are PGE_2 and NO.

2.7.1 Prostaglandins (PGs)

Prostaglandins (PGs) are small lipid messengers and have a wide variety of biological activities. They belong to the class of prostanoid fatty acid derivatives of arachidonic acid and participate in pain and inflammation responses. Arachidonic acid is liberated from membrane phospholipids by the action of phospholipases, metabolized into PGG_2 and PGH_2 by the cyclooxygenases. Both prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) are unstable and rapidly converted into PGE_2 by prostaglandin E synthetase (PGES) [132], which PGE_2 is the main form of prostaglandins as shown in Figure 2.6 [142]. They have potency to dilate arteriol and enhance the effects of other mediators by increasing small vein permeability. Cyclooxygenases (COX) is a key enzyme in the biosynthetic pathway of prostaglandin (PG) synthesis. COX-1 and COX-2 are the two isoforms of COX [136]. COX-1 is constitutively expressed in all tissues and is involved in many physiological functions [133], while COX-2 is an enzyme to generate prostaglandins from arachidonic acid and is associated with inflammation and tumorigenesis [129,134]. Over-expression of COX-2 catalyzes the synthesis of PGE_2 through the transcription factor NF-κB, contributing to physiological processes including transformation, survival, proliferation, metastasis and angiogenesis by up-regulating several signaling pathways and down-regulating apoptotic protein [137]. COX-2 can be induced by several intracellular and extracellular factors including lipopolysaccharide (LPS), epidermal growth factor (EGF), and tumor necrosis factor [138].

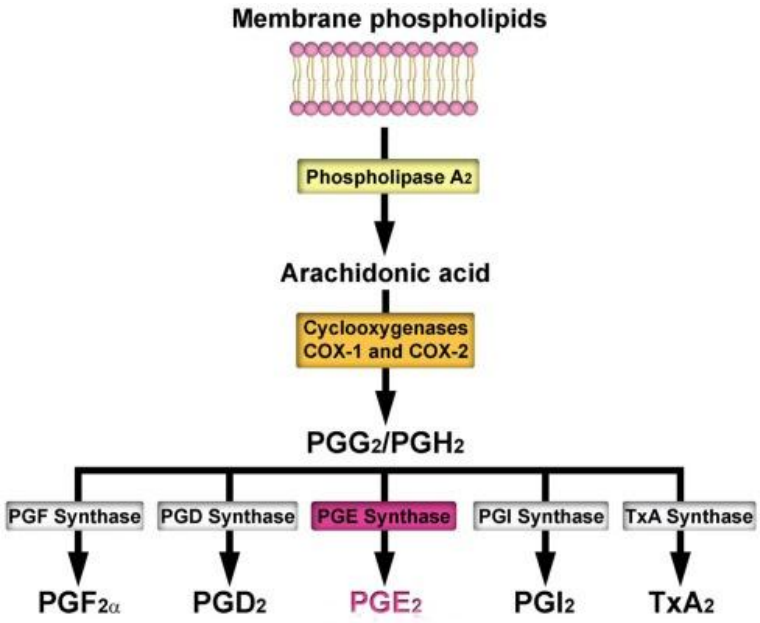


Figure 2.6 Prostaglandin E_2 biosynthesis. Upon phospholipids release from plasma membrane by phospholipase A_2, arachidonic acid is converted to prostaglandin G_2 (PGG2) and prostaglandin H_2 (PGH2) by the endocyclooxygenases (COX-1 and COX-2). Both COX isoforms catalyze the same reaction. While COX-1 is constitutively expressed, COX-2 is upregulated by cytokines and growth factors. Terminal synthases convert both PGG2 and PGH2 into prostaglandins, which PGE_2 is the main form of prostaglandins [142].

2.7.2 Nitric oxide (NO)

One of the most studied molecules secreted by macrophage is NO. NO is an important cell-signaling molecule that directly regulates the activity of intracellular proteins promoting inflammation, tumor angiogenesis [135] and mediates the oxidative burst leading to cell death in the hypersensitive response. Small amounts of NO play a role in maintaining resting vascular tone, vasodilation, and antiagregation of platelets. In response to cytokines (TNF-α, IL-1) and other inflammatory mediators, the production of large quantities of NO is stimulated. In larger quantities, NO is a potent vasodilator, facilitates macrophage-induced cytotoxicity, and may contribute to joint destruction in some type of arthritis. NO is produced by the deamination of L-arginine, catalyzed by NO synthases as shown in Figure 2.7 [141]. At least two NO synthases exist, one is constitutive NOS (cNOS) and the other is cytokine-inducble

NOS (iNOS), have been reported. NO is increased in inflammation and has pro-inflammatory and regulatory effects [131]. iNOS, induced by bacterial products and inflammatory cytokines through the transcription factor NF-κB in macrophages and responsible for prolonged production of larger amounts of NO.

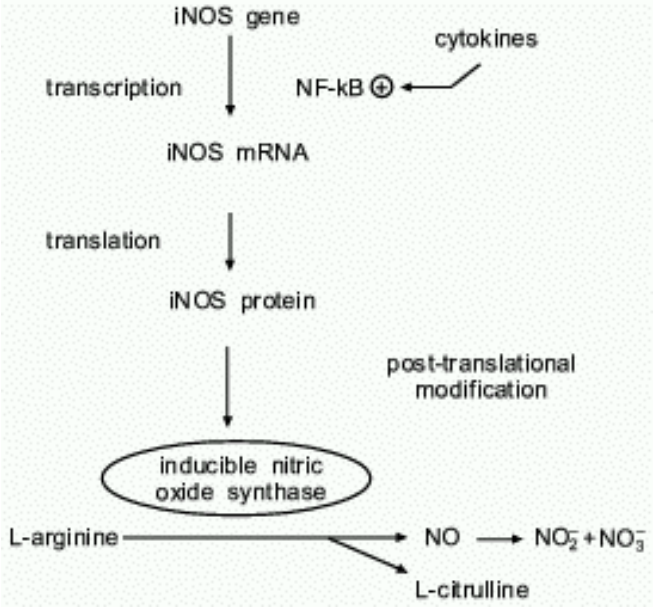


Figure 2.7 NO biosynthetic pathway. Inducible nitric oxide synthase catalyzes the oxidation-reduction reaction of L-arginine to form the free radical NO. iNOS expression is upregulated by cytokines through the transcription factor NF-κB [141].

CHAPTER III
MATERIALS AND METHODS

3.1 Cell culture

Mouse macrophage cell line (RAW264.7), obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS) (Hyclone). The cells were incubated at 37°C under 5% CO_2 atmosphere. The cells were subcultured at 80-90% confluency before detaching from plastic surface using cell scraper. The cell suspension was centrifuged at 1000 rpm, 4 °C, for 5 min and packed cells were suspended in 1 ml of supplemented DMEM medium before transferring to fresh medium and cultured as decribed.

3.2 Purified compound

Purified compound from leaves and twigs of *Polyalthia crassa* was obtained from Prof. Patoomratana Tuchinda. The air-dried and finely powdered mixed leaves and twigs of *P. crassa* were sequentially percolated with hexane, EtOAc, and MeOH, at room temperature. The solvents of the hexane, EtOAc, and MeOH extracts were removed, respectively. The EtOAc extract was subjected to column chromatography, eluting with EtOAc-hexane, followed by MeOH-EtOAc to give fractions A1-A11. The fraction A9 (eluted with 40-50% EtOAc-hexane) was rechromatographed on a Sephadex LH-20 column (MeOH as eluent) to give fractions C1-C4. Further separation of fraction C4 on a silica gel column (MeOH-CH_2Cl_2-hexane, 1:12:7, as eluent) yielded fractions D1-D5. Fraction D4 was further separated by passage on a silica gel column (10% EtOH-CH_2Cl_2 as eluent) to yield fractions G1-G6. Fraction G3 provided (+)-goniofufurone after column chromatography (50%EtOAc-hexane as eluent) and recrystallization with EtOAc-hexane [16].

3.3 Cell viability by trypan blue dye exclusion

The cells were detached from plastic surface with cell scraper. The cell suspension was centrifuged at 1000 rpm, 4 °C, for 5 min and packed cells were suspended in 1 ml of supplemented DMEM medium. A 10 µl of cell suspension was mixed with 590 µl of 1X trypan blue dye solution (Sigma-Aldrich, USA) and stained cells were counted using hemacytometer under light microscope. The amount of viable cell was counted and % cell viability was calculated from below equation.

$$\text{Viable cells/ml} = \text{viable cells counted} \times \frac{\text{dilution factor}}{\text{\# of squares}} \times 10{,}000 \text{ cells/ml}$$

$$\%\ \text{Viability} = \frac{\text{viable cell count}}{\text{total cell count}} \times 100$$

3.4 Determination of cytotoxicity using MTT assay

RAW264.7 cells were seeded at 5.0×10^4 cells/well (150 µl of medium) in 96-well plates and incubated for overnight. The cells were treated with tested samples by various concentrations at 0-800 µg/ml (total volume was 100 µl per well) for 24 h and 20 µl of MTT solution (Sigma-Aldrich,USA) (stock concentration 5 mg/ml) was added. The cells were incubated at 37 °C for 3.5 h. The MTT solution will be reduced to purple formazan in the mitochondria of living cells. Then, the solution was discarded and 150 µl of DMSO was added. The absorbance at 590 nm was read and calculated % cell viabllity from below equation.

$$\%\ \text{Cell viability} = \frac{\text{OD treat} - \text{OD blank}}{\text{OD untreat} - \text{OD blank}} \times 100$$

3.5 Treatment of mouse macrophage cell line (RAW 264.7)

Mouse macrophage cells at initial density of 5×10^5 cells were cultured overnight in 6-well plate. When the cell density reached 1×10^6 cells/ml, the medium was replaced with fresh medium. The cells were pretreated with compound at various

concentrations for 2 h and co-treated with or without 100 ng/ml of LPS from *Escherichia coli* strain 0111:B4 (Sigma, St Louis, MO) for 22 h.

3.6 Determination of NO production using Griess reagent system

After the RAW264.7 cells were pre-treated with tested sample for 2 h and co-treated with or without 100 ng/ml of LPS for 22 h. The supernatant was collected to analyze NO production using Griess reagent kit (Invitrogen^{TM}, USA) according to the manufacturer's instructions. In brief, solutions A and solution B were mixed in the ratio 1:1 which was called Griess reagent. For the analytical, 150 µl of cultured medium samples, 20 µl of griess reagent and 130 µl of distilled water were added respectively and mixed in 96 well assay plate. NO production was determined by measuring absorption at 548 nm and absorbance reading was converted to nitrite concentrations by comparing with standard curve (see appendix).

3.7 Enzyme-linked immunosorbent assay (ELISA)

The level of prostaglandin E_2 production was determined using ELISA assays. The culture supernatant obtained from the stimulated or unstimulated cell cultures were kept at 80 °C until used.

3.7.1 ELISA for mouse prostaglandin E_2

The production of PGE_2 in culture supernatant was analyzed using commercially available ELISA kit (R&D system, Inc., Minneapolis, MN). The 96-well plate was coated with goat anti-mouse. The 150 µl of sample or PGE_2 standard (dilute 1:3 with calibrator diluent) (see appendix) was added, then 50 µl of primary antibody solution was added to each well and incubated at RT for 1 h on a shaker set at 500 rpm. Then 50 µl of PGE_2 conjugate was added to each well and incubated at RT for 2 h on a shaker set at 500 rpm. Plate was then washed for 4 times with washing buffer and 200 µl of substrate solution (color reagent A and color reagent B mixed 1:1) was added to each well and incubated at RT for 30 min in dark. The reaction was stopped by adding 100 µl of stop solution (2N H_2SO_4). The intensity of color was

determined immediately by measurement with microplate reader (Softmax) at absorbance 450 nm. A standard curve was prepared for each microtiter plate and used to quantify the amount of PGE_2 present in test samples (see appendix).

3.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

3.8.1 Preparation of separating and stacking gels

Electrophoresis was performed on 8x10x0.1 cm gel. A 5 ml of an 8% acrylamide solution in 3.0 M gel buffer pH 8.8 containing 1% sodium dodecyl sulfate (SDS) (Plusone, Uppsala, Sweden), 0.05% (v/v) *N*,*N*,*N*',*N*'-tetrametyhlethylene diamine (TEMED) (Plusone) and 0.05% ammonium persulfate (APS) (Plusone) was carefully added and allowed to polymerize at RT for at least 1 h. The gel prepared as described could be kept at 4 °C with distilled water covering the top surface if the electrophoresis was not performed immediately. Distilled water was removed and the comb was gently inserted on the top of the gel to make wells for sample application before adding stacking gel solution. Five millilitre of 30% acrylamide in 0.5 M gel buffer pH 6.8 (see appendix) used as stacking gel was layered over the separating gel and left for 30 min before used. After polymerization, the comb was carefully removed, samples then electrophoresed immediately.

3.8.2 Preparation of samples

Cell samples were centrifugated at 10,000 rpm. Supernatant was discarded and the pellet was solubilized in lysis buffer (see appendix) containing 0.0625 M Tris-HCl (USB) pH 6.8, 1% SDS, 10% glycerol (Sigma), 5% 2-mercaptoethanol (2-ME) (BDH) and bromophenol blue. The solution was sonicated on ice for 1 min before heated for 5 min in the heat box. The samples were centrifuged at 10,000 rpm for 5 min at 4 °C and the supernatant was subjected to electrophoresis. Protein marker (Infinigen Biotechnology, USA) was used to indicate the size of aspected protein.

3.8.3 Electrophoresis

The electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, CA) was prepared with cathode at the top and the anode at the bottom chambers. Electrophoresis was carried out at the room temperature with a constant current of 20 mA per gel in descending direction until the blue dye marker reached the bottom of the gel, approximately 1 h to complete this process.

3.9 Immunobloting

After electrophoresis, the gels were electrotransferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England) at a constant voltage (80 volts) for 3 h. The membrane was blocked with 5% blocking solution (Roche Diagnostics, Mannheim, Germany) in 1X PBS (see appendix) for 1 h before incubating at 4 °C for overnight with rabbit anti-mouse iNOS antibody (Santa Cruz, Santa Cruz, CA) in 5% blocking solution in 1X PBS. The concentration of each antibody was used as manufacturer's recommendation. The membranes were washed for 3 times with 0.1% Tween20 in PBS for 10 min. After that, the blots were then allowed to react with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Pierce, Rockford, IL) or HRP-conjugated goat anti-rabbit IgG (Pierce). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics) which nitrocellulose membrane was exposed to hyperfilm (Amersham Biosciences). The intensity of each bands measured by using Gel Documentation (GeneSystem).

3.10 Reverse transcription-polymerase chain reaction (RT-PCR) assay

3.10.1 Total cellular RNA extraction

Total RNA was extractd from the treated cells according to the manufacture's instruction (GE healthcare). In brief, after the culture medium was

removed, the cells were washed three time with 1 ml of PBS. The cells were lyzed with 350 µl of lysis buffer (RA1 buffer with 3.5 µl of β-mercaptoethanol) before transferred to a sterile 1.5 ml-microcentrifuge tube and centrifuged at 14,000 rpm for 5 min. The supernatant was added with 350 µl of 70% alcohol and gently mixed before transferred to an RNA binding column. The homogenized lysate was added with 700 µl of low stringency wash solution and centrifuged at 14,000 rpm for 30 sec. The supernatant was discarded before adding diluted DNase I and incubated at room temperature for 15 min. The samples were subjected to centrifugation at 14,000 rpm for 30 sec. This step was repeated twice. The RNA binding column was transferred to a new tube. Forty microliters of RNA elution (RNase-free H_2O) was added to the sample and incubated at room temperature for 1 min. The sample was then centrifuged at 14,000 rpm for 2 min. Total RNA concentration in solution was determined by spectrophotometer at 260/280 nm (Multiskan Spectrum, ThermoLabsystems, Helsinki, Finland). The mRNA samples were used for cDNA synthesis.

3.10.2 First-strand cDNA synthesis

First-strand cDNA was synthesized from the mRNA. Total RNA (0.1 µg) was used to synthesize cDNA in 20 µl of reaction. One reaction contained 4 µl of 5X AMV reverse transcriptase reaction buffer, 1 µl of 100 mM dTT, 2 µl of 100 mM dNTP, 1 µl of 500 µg/ml oligo (dT), 100 units of RNase Ribonuclease inhibitors, 30 units of AMV reverse transcriptase and nuclease-free water to make final volume of 20 µl. After incubation at 42 °C for 1 h, the first-strand cDNA was synthesized. The reaction mixture was kept at -20 °C until used.

3.10.3 Polymerase-chain reaction (PCR)

The PCR reaction was performed in a 25 µl of total volume using 10X PCR buffer (Invitrogen, Carlsbad, CA), 20 µM of specific primer, 5U/µl of *Thermus aquaticus* (*Taq*) DNA polymerase (Invitrogen, Carlsbad, CA) and DNase-RNase free water (Gibco Labs). The nucleotide primers specific for all genes of interested and their expected size used in the PCR amplification were shown in Table 3.1. The TGradiant thermalcycler 96 (Biometra, Goettingen) was used for PCR amplification. The conditions for all PCR amplification were shown in Table 3.2.

3.10.4 Detection of PCR products

All amplified products were mixed with loading dye in the ratio of 5:1. The mixture was electrophoresed in 1.5% agarose gel (see appendix). After finished, the gel was stained with 0.01% ethidium bromide (Plusone, Amersham Pharmacia Biotech). The gel was electrophoresed in 1X TBE buffer (see appendix) at a constant voltage (90 volts) for 40 min. Quantitative analysis of PCR band was done by using Gel Documentation (GeneSystem).

Table 3.1: Primer specifications for RT-PCR

Gene	Primer	Sequence (5' → 3')	Size (bp)	(Refs.)
TNF-α	Forward	gta gcc cac gtc gta gca aa	350	65
	Reverse	ccc ttc tcc agc tgg gag ac		
IL-1β	Forward	tca tgg gat gat gat gat aac ctg ct	502	-
	Reverse	ccc ata ctt tag gaa gac acg gga tt		
IL-6	Forward	aac gat gat gca ctt gca ga	452	-
	Reverse	gag cat tgg aaa ttg ggg ta		
IL-12p40	Forward	agg tgc gtt cct cgt aga ga	241	-
	Reverse	aaa gcc aac caa gca gaa ga		
COX-2	Forward	aga agg aaa tgg ctg cag aa	291	-
	Reverse	gct cgg ctt cca gta ttg ag		
IFN-β	Forward	tcc aag aaa gga cga aca ttc g	312	64
	Reverse	tga gga cat ctc cca cgt caa		
iNOS	Forward	gca gaa tgt gac cat cat gg	414	65
	Reverse	aca acc ttg gtg ttg aag gc		
Actin	Forward	cca gag caa gag agg tat cc	436	66
	Reverse	ctg tgg tgg tga agc tgt ag		

Table 3.2 The conditions for all PCR amplification

A.

Gene	Parameter			
COX-2	First denature	94°C	5 min	1 cycle
	Denature	94°C	45 sec	30 cycles
	Annealing	54.5°C	1 min	
	Extention	72°C	1 min	
	Final extension	72°C	7 min	1 cycle

B.

Gene	Parameter			
IL-1β, IFN-β, and iNOS	First denature	94°C	5 min	1 cycle
	Denature	94°C	45 sec	30 cycles
	Annealing	55°C	1 min	
	Extention	72°C	1 min	
	Final extension	72°C	7 min	1 cycle

C.

Gene	Parameter			
TNF-α, IL-12p40, and Actin	First denature	94°C	5 min	1 cycle
	Denature	94°C	45 sec	30 cycles
	Annealing	58°C	1 min	
	Extention	72°C	1 min	
	Final extension	72°C	7 min	1 cycle

Table 3.2 The conditions for all PCR amplification (Cont.)

D.

Gene	Parameter			
IL-6	First denature	94°C	5 min	1 cycle
	Denature	94°C	45 sec	30 cycles
	Annealing	60°C	1 min	
	Extention	72°C	1 min	
	Final extension	72°C	7 min	1 cycle

3.11 Statistical analysis

All statistical analyzes reported were performed using SigmaPlot 18.0 (SPSS). The results were expressed as mean ± standard error (SE) from three separated experiments. Significance of differences between stimulated groups and treatment groups were analyzed by the Student's *t-test*. $P<0.05$ was considered statistically significance.

CHAPTER IV
RESULTS

4.1 Cytotoxicity of (+)-goniofufurone to RAW264.7 cells

Cytotoxicity of the plant compound to mouse macrophage cells (RAW264.7) was determined to obtain the IC_{50} (inhibitory concentration 50) and the optimum concentration of compound was used to assess the potential anti-inflammatory activity for further study. The compound used in this study was (+)-goniofufurone (C-865-1-LF+TW-1056-E002-F4) or MUC760 compound extracted from the leaves and twigs of *Polyalthia crassa*.

RAW264.7 cells were treated with the compound at various concentrations (0, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μg/ml) for 24 h. MTT assay was performed to determine cell viability. Exposure of cells to the compound decreased cell viability in a dose-dependent manner (Table 4.1). The IC_{50} of (+)-goniofufurone was 520 μg/ml or 2.08 mM as shown in Figure 4.1. Exposure of cells to (+)-goniofufurone at concentration less than 200 μg/ml could maintain the cell viability more than 80% and most cells exhibited round shape phagocytic-like phenotype as found with untreated control cells (Figure 4.2). However, treated cells with (+)-goniofufurone at the concentration between 200-800 μg/ml showed a mixture of round and spindle-shaped cells in which actin might be diffusely distributed throughout the cell and partially concentrated in the cell cortex. Concentration of the compound higher than 800 μg/ml displayed a large amount of cellular debris. Therefore, from this study the concentration of compound at 200 μg/ml or 0.8 mM was chosen for further experiments.

Table 4.1 Cell viability of RAW264.7 cells treated with (+)-goniofufurone by MTT assay

Concentrations of compound (µg/ml)	% Cell viability (mean ± SE)
0	100.00 ± 0.00
6.25	97.84 ± 1.74
12.5	96.98 ± 2.25
25	95.78 ± 2.51
50	94.44 ± 2.90
100	92.30 ± 4.11
200	81.98 ± 1.34
400	56.72 ± 4.83
800	27.59 ± 4.40

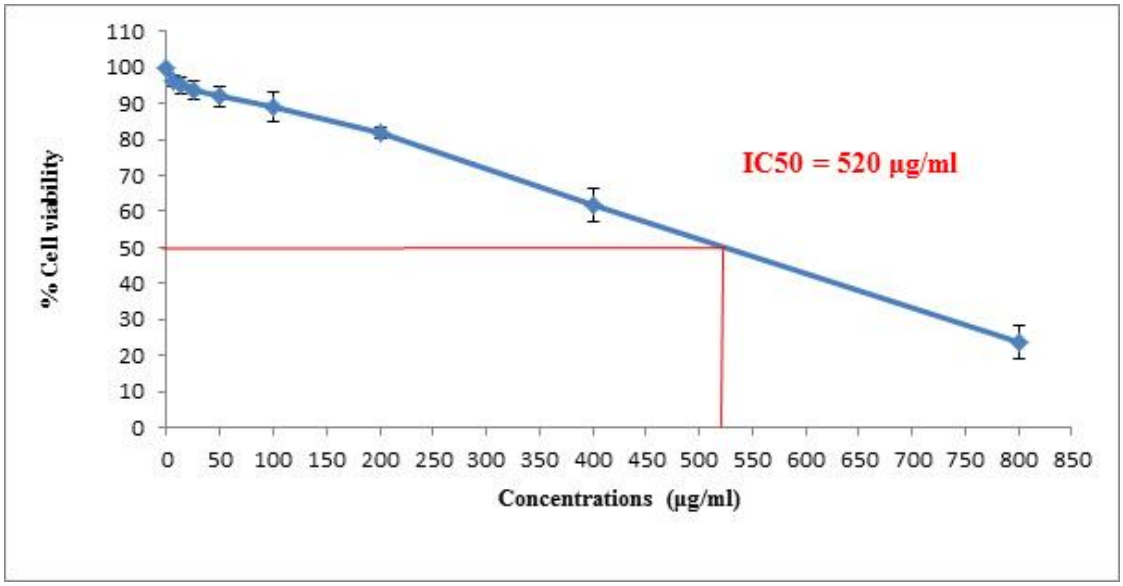


Figure 4.1 Percentage of cell viability from MTT assay and IC_{50} of (+)-goniofufurone from the leaves and twigs of *Polyalthia crassa*.

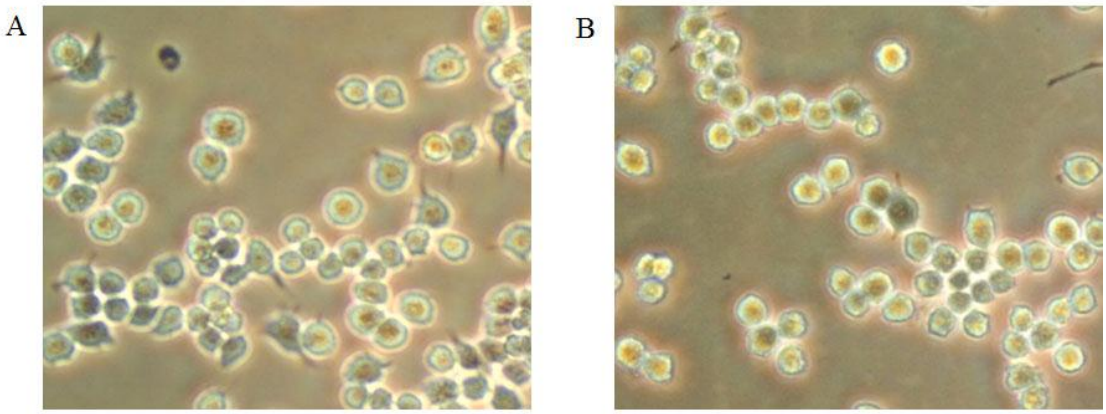
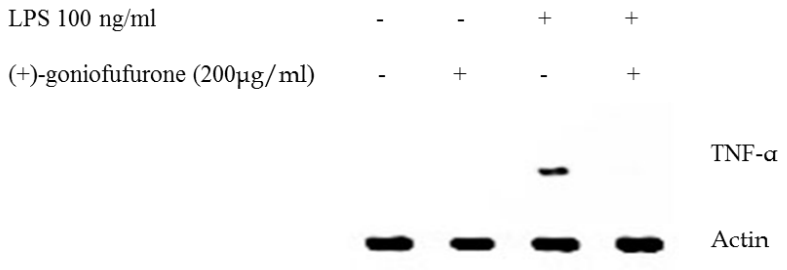


Figure 4.2 Cell morphology of macrophages. RAW264.7 cell lines were cultured without (+)-goniofufurone (A) and with 200 μg/ml of (+)-goniofufurone (B) for 24 h. The cells were visualized under the light microscope (20X).

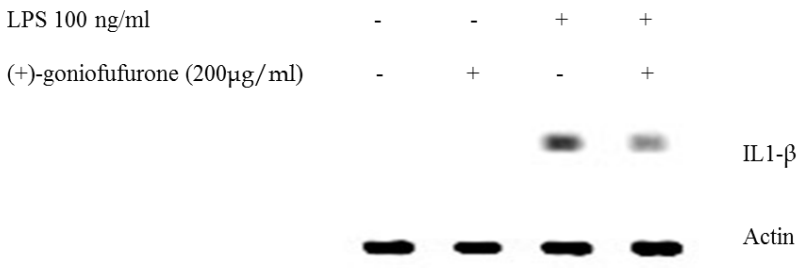
4.2 Suppression of gene expression downstream of MyD88-dependent pathway by (+)-goniofufurone in LPS activated RAW264.7 cells

The RAW264.7 cells were pretreated with (+)-goniofufurone at concentration of 200 μg/ml for 2 h before co-treated with 100 ng/ml of *E.coli* LPS for 22 h. After 24 h of treatment, gene expression downstream of MyD88-dependent pathway was determined by RT-PCR. The results showed that LPS from *E.coli* could stimulate the gene expression studied in this experiment including TNF-α, IL-1β, IL-6, IL-12p40 and COX-2 (Figure 4.3A-4.3E). However, the expression of these genes could be reduced by (+)-goniofufurone with 100%, 42.47%, 35.96%, 100% and 41.33% suppression, respectively (Figure 4.3F). It is suggested that this suppression is dependent on MyD88-downstream molecules.

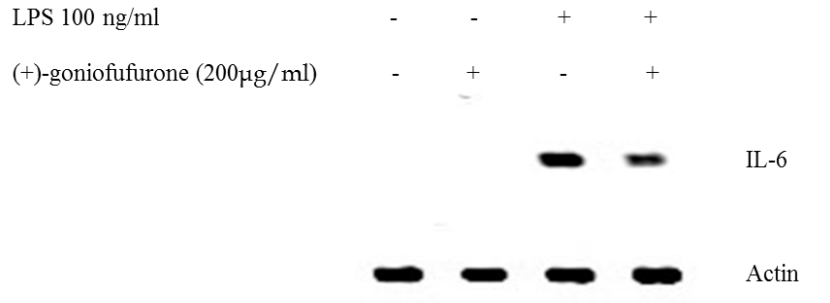
4.3A



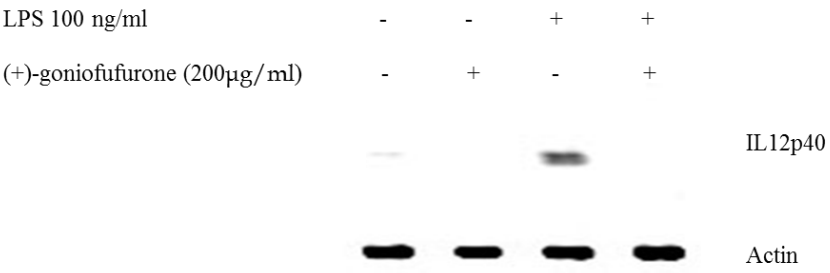
4.3B



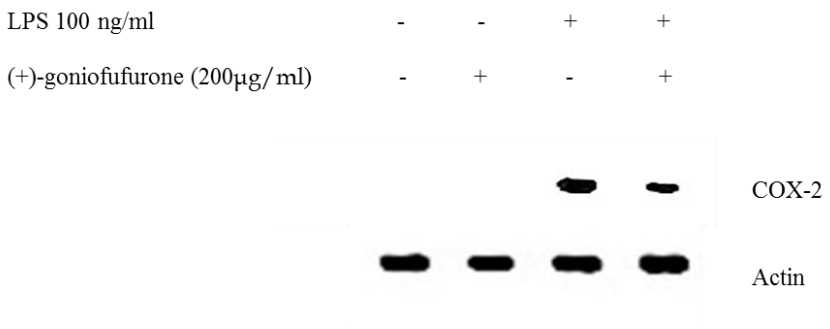
4.3C


LPS 100 ng/ml - - + +
(+)-goniofufurone (200µg/ml) - + - +
IL-6
Actin

4.3D


LPS 100 ng/ml - - + +
(+)-goniofufurone (200µg/ml) - + - +
IL12p40
Actin

4.3E


LPS 100 ng/ml - - + +
(+)-goniofufurone (200µg/ml) - + - +
COX-2
Actin

4.3F

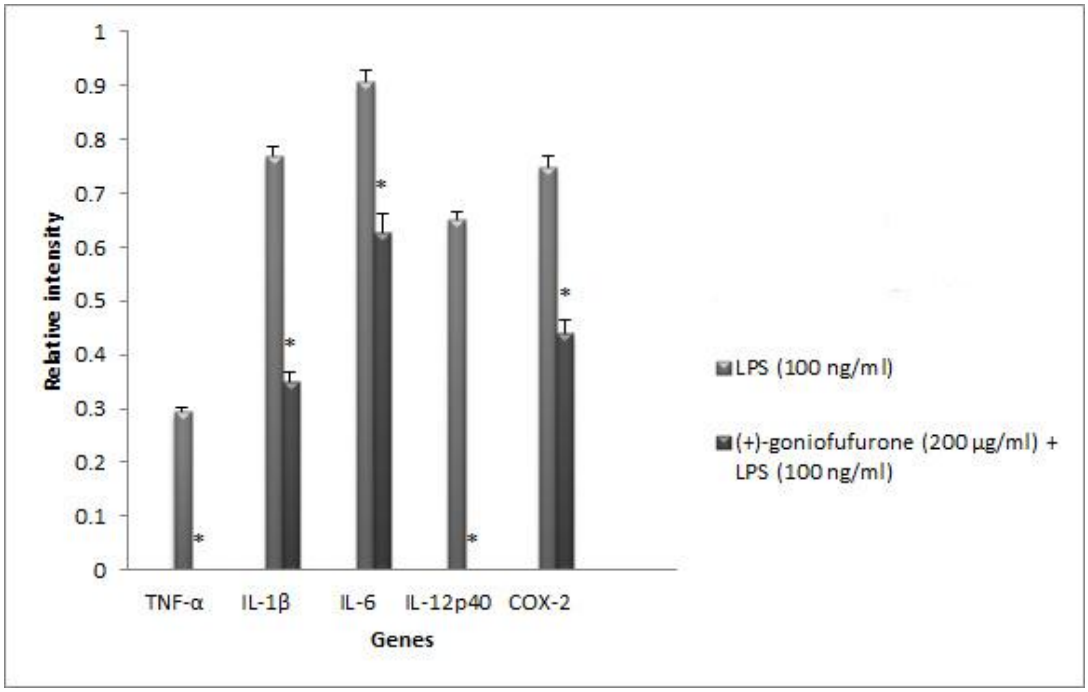
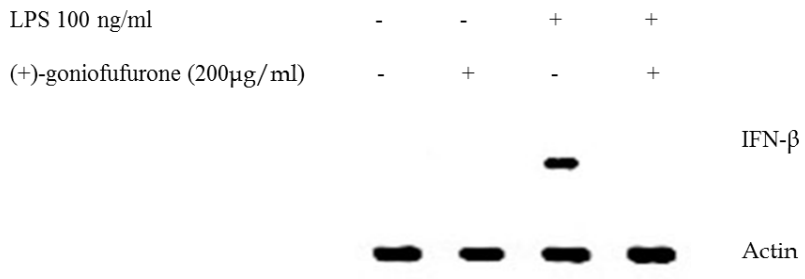


Figure 4.3 Effects of (+)-goniofufurone on MyD88-dependent gene expression in LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with 200 μg/ml of (+)-goniofufurone for 2 h and stimulated with 100 ng/ml of LPS for 22 h. Total cellular RNA was subjected to RT-PCR and PCR product was run on agarose gel electrophoresis. Figure 4.3A-4.3E showed PCR product of TNF-α, IL-1β, IL-6, IL-12p40 and COX-2, respectively. Figure 4.3F showed the rrelative intensity of TNF-α, IL-1β, IL-6, IL-12p40 and COX-2 mRNA which were normalized with β-actin mRNA. The data are mean ± SE of three independent experiments. * $p < 0.05$ vs. LPS group.

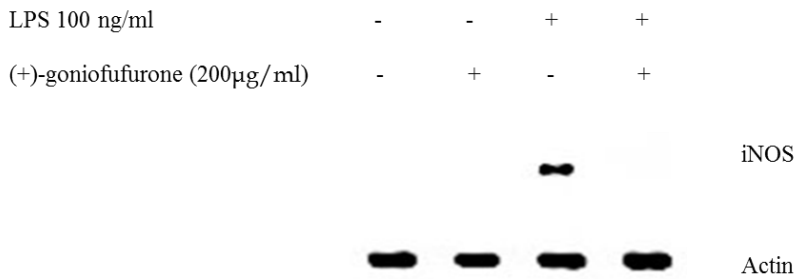
4.3 Suppression of gene expression downstream of MyD88-independent pathway by (+)-goniofufurone in LPS activated RAW264.7 cells

Since previous results showed that (+)-goniofufurone could suppress the expression of genes in MyD88-dependent pathway, the next experiment was carried out to investigate whether the compound (+)-goniofufurone suppressed genes in MyD88-independent pathway. The RAW264.7 cells were pretreated with (+)-goniofufurone at concentration of 200 μg/ml for 2 h before co-treated with 100 ng/ml of *E.coli* LPS for 22 h. After 24 h of treatment, gene expression downstream of MyD88-independent pathway including IFN-βwas determined by RT-PCR. The results showed that LPS from *E.coli* could induce both IFN-β and iNOS gene expression (Figure 4.4A-4.4B). Treatment of LPS-stimulated cells with (+)-goniofufurone could suppress the expression of these genes. It is suggested that this suppression is dependent on TRIF-downstream molecules.

4.4A



4.4B



4.4C

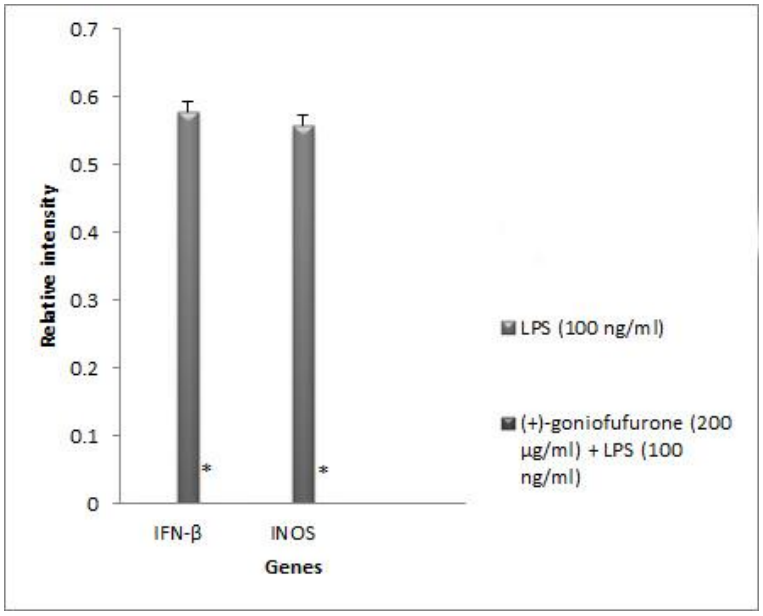


Figure 4.4 Effects of (+)-goniofufurone on MyD88-independent gene expression in LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with 200 µg/ml of (+)-goniofufurone for 2 h and stimulated with 100 ng/ml of LPS for 22 h. Total cellular RNA was subjected to RT-PCR and PCR product was run on agarose gel electrophoresis. Figure 4.4A and 4.4B showed PCR product of IFN-β and iNOS, respectively. Figure 4.4C showed the relative intensity of IFN-β and iNOS mRNA which were normalized with β-actin mRNA. The data are mean ± SE of three independent experiments. * $p < 0.05$ vs. LPS group.

4.4 Inhibition of nitric oxide production by (+)-goniofufurone in LPS activated RAW264.7 cells

iNOS is involved in immune response by producing NO as an immune defense mechanism. iNOS produces large quantities of NO upon stimulation, such as by proinflammatory cytokines (e.g. IL-1 and TNF-α) [67, 68]. Therefore, NO is a critical mediator of macrophage function and its expression is associated with the cytotoxic activity of macrophages against transformed cell, invader and/or any substance. High levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity. It is the proximate cause of septic shock and may function in autoimmune disease. Therefore, inhibitors of NO production may have indications in many immune diseases.

To measure the ability of (+)-goniofufurone to inhibit NO production, various concentrations of compound were pre-treated on RAW264.7 cells for 2 h and then, co-treated with LPS of *E.coli* (100 ng/ml) for 22 h. The cultured supernatant was collected to analyze NO productions using Griess reagent. NO production was determined by measuring absorption at 548 nm and converted absorbance reading to nitric concentrations by comparing with standard curve (see appendix).

The results showed that (+)-goniofufurone alone did not activate NO production. However, RAW264.7 cells activated with LPS could increase NO unit to 19.7 ± 3.2 μM. Furthermore, in the present of (+)-goniofufurone, the reduction of NO production level in LPS-treated cells was decreased as concentration-dependent (% reduction at 50, 100 and 200 μg/ml were 29.24, 44.16 and 66.50%, respectively, as shown in Figure 4.5). From this result, the inhibition concentration 50 (IC_{50}) of (+)-goniofufurone on LPS induced NO production in RAW264.7 macrophage cells was obtained at 135 μg/ml (0.54 mM) as shown in Figure 4.6.

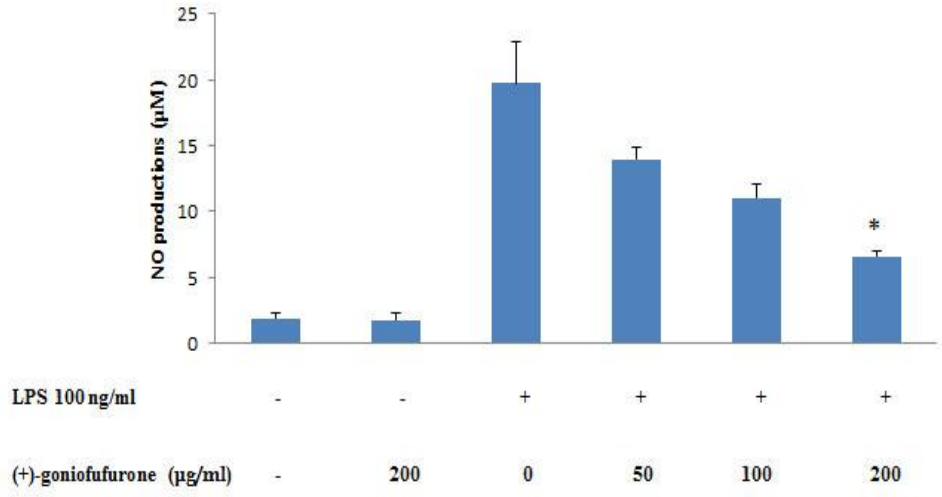


Figure 4.5 Effect of (+)-goniofufurone on LPS-activated NO production in RAW264.7 macrophage cells. NO production was expressed as NO concentration. * indicated $p<0.05$ as compared to LPS-alone group.

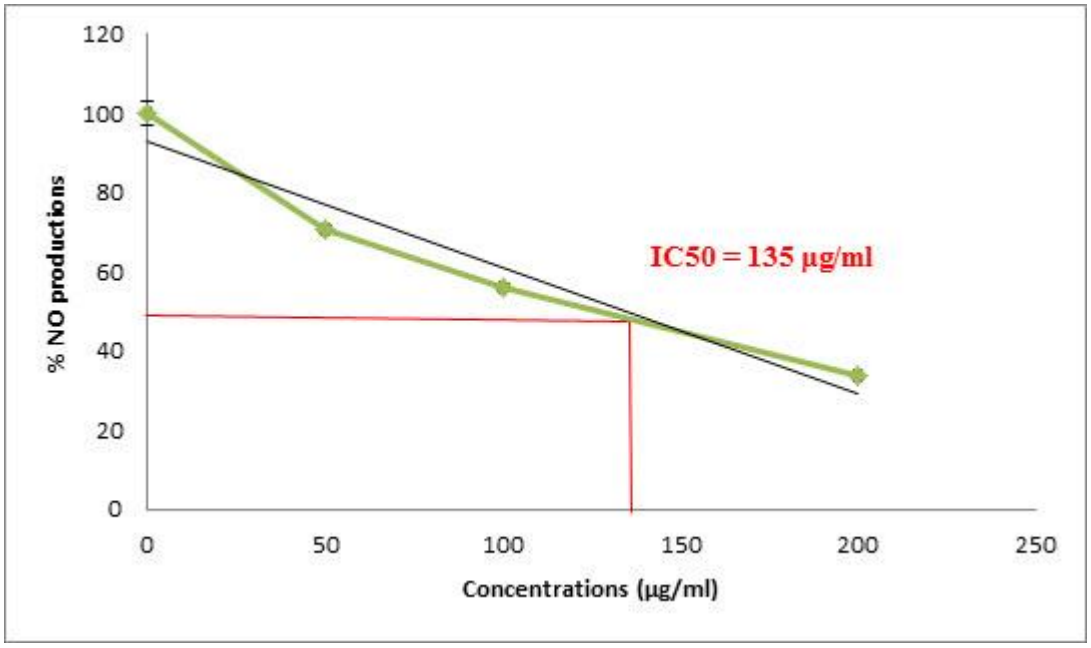


Figure 4.6 IC50 of nitric oxide reduction by (+)-goniofufurone in LPS-activated RAW264.7 macrophage cells (data taken from Figure 4.5)

4.5 Suppression of iNOS protein expression by (+)-goniofufurone in LPS activated RAW264.7 cells

From previous result, (+)-goniofufurone was shown to reduce NO production. To confirm that this reduction was a result from decreased expression of iNOS protein, the expression of iNOS protein was observed in LPS-activated RAW264.7 cells treated with (+)-goniofufurone (section 3.5). The result showed that (+)-goniofufurone at the concentration of 200 μg/ml could down regulate iNOS protein expression with 76.76% reduction in LPS-induced RAW264.7 cells compared with cells treated with LPS alone (Figure 4.7).

There results indicated that inhibition of NO production by (+)-goniofufurone (Figure 4.5) was have to the suppression of iNOS expression in LPS activated RAW264.7 cells.

Figure 4.7 Effect of (+)-goniofufurone on LPS-induced iNOS protein expression in RAW264.7 cells. RAW264.7 cells were pretreated with (+)-goniofufurone at various concentrations (0-200 μg/ml) for 2 h and activated with LPS for 22 h. Total protein was extracted and subjected to electrophoresis. Beta-actin was used as loading control and the relative amount of iNOS protein was normalized with β-actin protein.

4.6 Inhibition of prostaglandin E_2 production by (+)-goniofufurone in LPS activated RAW264.7 cells

Prostaglandins are signaling molecules in the immune system. They are derived from arachidonic acid, which are catalyzed by cyclooxygenase (COX) enzyme. Of two COX isoforms, COX-1 is expressed in most tissues, whereas COX-2 activated by specific cellular stimuli during inflammation. At the site of inflammation, this chemical mediators increase vasodilation, vascular permeability and chemotaxis for the accumulation of plasma proteins including cytokines and chemokines.

To measure the ability of compound to inhibit PGE_2 production, various concentrations of compound were pre-treated on RAW264.7 cells for 2 h befor co-treated with LPS of *E.coli* (100 ng/ml) for 22 h. The supernatant was collected to analyze PGE_2 productions using ELISA kit. PGE_2 production was determined by measuring absorption at 450 nm and converted absorbance reading to PGE_2 concentrations by comparing with standard curve (see appendix).

The results showed that (+)-goniofufurone alone did not activated PGE_2 production and cells treated with 200 μg/ml of (+)-goniofufurone. RAW264.7 cells activated with LPS could increase PGE_2 at 4,516.98 ± 413.05 pg/ml. Furthermore, PGE_2 production was decreased in concentrations-dependent in LPS-stimulated cells treated with (+)-goniofufurone. As shown in Figure 4.8, (+)-goniofufurone at 50, 100, and 200 μg/ml could reduce PGE_2 production in LPS-treated cells with 9.07%, 11.96% and 64.38%, respectively compared to PGE_2 production in LPS-treated cells without the compound. Furthermore, the inhibition concentration 50 (IC_{50}) of (+)-goniofufurone on LPS induced PGE_2 production in RAW264.7 macrophage cells was obtained at 175 μg/ml (0.7 mM) as shown in Figure 4.9.

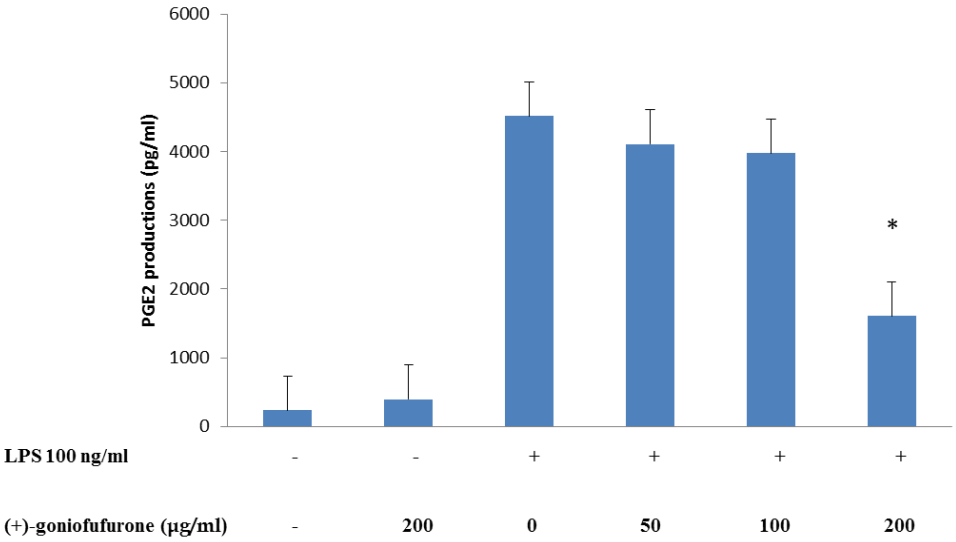


Figure 4.8 Effect of (+)-goniofufurone on LPS-induced PGE_2 production in RAW264.7 macrophage cells. PGE_2 production was expressed as PGE_2 concentration. * indicated $p<0.05$ as compared with LPS-alone group.

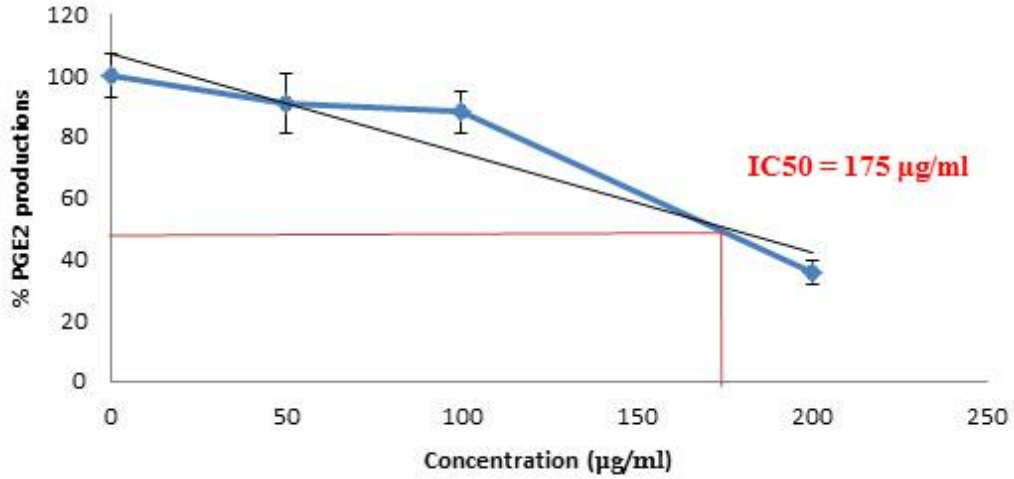


Figure 4.9 IC50 of PGE_2 reduction by (+)-goniofufurone in LPS-activated RAW264.7 macrophage cells (data taken from Figure 4.8)

CHAPTER V
DISCUSSION

Polyalthia crassa is known locally as Nom maeo noi in Thai. Several member of the genus *Polyalthia* have been widely used in traditional medicine in Asia. (+)-Goniofufurone, isolated from *Polyalthia crassa*, belongs to the chemical class of styryl lactones [16] that are shown to have the antiproliferative activity against cancer cells [75]. More recently, two stryl lactones, cheliensin and goniofufurone have been reported to exert cytotoxic and anti-proliferative activity in human cancer cells [98, 99]. Other styryl-lactone, goniothalamin from *Goniothalamus macrophyllus* was reported that it could inhibit TNF-α induced NF-κB activation in Jurkat and K562 leukemia cells at concentrations as low as 5 μM [100]. However, for (+)-goniofufurone, there are no reports in literature on its anti-inflammatory activity. Although, (+)-goniofufurone has a unique structure features and promising antitumour activities [99], understanding how this compound exerts its activity as anti-inflammatory process is essential for future development of this compound for human use.

In the present study, (+)-goniofufurone, the purified compound from leaves and twigs of *Polyalthia crassa* was tested for its cytotoxicity to RAW264.7 macrophage cell lines and its IC50 was obtained. The result from MTT assay showed that (+)-goniofufurone was not cytotoxic to RAW264.7 cells at concentrations upto 0.8 mM exhibiting the percentage of cell viability more than 80% and the morphology of cells was not changed. In addition, (+)-goniofufurone exhibited cytotoxicity to RAW264.7 cells with the IC_{50} at 520 μg/ml or 2.08 mM which was less than an anti-inflammatory or an immunosuppressant drug such as prednisone (a synthetic coticoisteroid) with IC_{50} values 3.81 ± 0.59 mM, reported previously by Gunawardena et. al (2014). However, comparing to various anti-inflammatory compounds extracted from medicinal plants, (+)-goniofufurone has less cytotoxicity. For example,

mitragynine extracted from *Mitragyna speciosa* inhibited the growth of RAW264.7 cells with the IC_{50} at 21.23 μg/ml [81].

Macrophage cell is one of immune cells that plays an important role in defense mechanism of host cells against pathogens or infections. LPS of Gram-negative bacteria is the most common cause of macrophage activation and therefore, it has been widely used to induce macrophages for studying inflammatory responses. In response to LPS, TLR signaling pathways are stimulated. TLR signaling consists of at least two distinct pathways : a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and MyD88-independent pathway associated with the IFN-β stimulation and the maturation of dendritic cells. During the inflammatory process, expression of the COX-2 and iNOS is induced through MyD88-dependent and MyD88-independent pathways, respectively [33, 36, 37]. Our results showed the induction of inflammatory genes (TNF-α, IFN-β, IL-1, IL-6 and IL12-p40), mediators (NO and PGE_2), and inducible enzymes (iNOS and COX-2) in RAW264.7 stimulated by LPS of *E.coli*. These results confirm that the expression of these molecules is a possible mechanism of signal transduction through MyD88-dependent and MyD88-independent pathways in LPS-activated RAW264.7. The activation of MyD88-dependent signaling pathways of TLR4 leads to the activation of IKK complex [70]. The activation of IKK complex allows migration of NF-κB to nucleus to turn on inflammatory genes. NF-κB is the transcription factor of pro-inflammatory enzymes including COX-2, iNOS, and pro-inflammatory cytokines [86] which is controlled by the signaling from MyD88-dependent and/or MyD88-independent pathway. Several natural compounds were found to have a specific anti-inflammatory potency such as activity of polyphenols from blueberries which inhibited the mRNA expression of inflammatory cytokines IL-1β, IL-6 and IL-12 in LPS-induced macrophages [85]. (-)-Epigallocatechin-3-gallate, flavonoids compound from green tea showed the suppression of the inflammatory gene through both MyD88- and TRIF-dependent signaling pathways of TLRs, which the targets were IKKβ and TBK1 in MyD88- and TRIF-dependent signaling pathway, respectively [87].

Tricin 4'-O-(threo-β-guaiacylglyceryl) ether, flavolignan compound from Njavara could inhibit LPS-induced NO and ROS generation in RAW264.7 cells and suppressed the expression of iNOS and COX-2 genes via NF-κB and STAT3. In

addition, tricin 4'-O-(threo-β-guaiacylglyceryl) also reduced the symptoms of ear edema in mice, which blocked the induction of iNOS and COX-2 through the regulation of NF-κB and STAT3 [88]. Alantolactone, a sesquiterpene lactone compound isolated from the root of *Aucklandia lappa* could suppress the expression of iNOS and COX-2 by down-regulating NF-κB, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW264.7 cells [89]. Irigenin, flavonoid compounds isolated from the rhizomes of *Belamcanda chinensis* could suppress the expression of iNOS and COX-2 and reduced the level of NF-κB in LPS-activated RAW264.7 cells [90].

In this study, (+)-goniofufurone was found to suppress the expression of proinflammatory marker including COX-2 mRNA, and the expression of the inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-12p40 mRNA in LPS-activated RAW264.7 cells. Therefore, this compound might modulate TLR-mediated MyD88 signaling pathway. In addition, LPS could also activate gene expression downstreams of MyD88-independent pathway or TRIF-dependent signaling pathways. The activation of TRIF-dependent signaling pathways of TLR4 leads to the phosphorylation of IRF3 and NF-κB resulting in the translocation into the nucleus, which regulated the expression of IFN-β and IFN-inducible genes such as iNOS [34]. Our results also demonstrated that the target of (+)-goniofufurone might be inhibited in MyD88-independent pathway because (+)-goniofufurone inhibited the expression of IFN-β and iNOS mRNA in RAW264.7 cells activated with LPS. As known that the inhibitory effect of (+)-goniofufurone on COX-2, iNOS, TNF-α, IL-1β, IL-6 and IL-12p40 expression is controlled by NF-κB. However, the inhibitory potency of (+)-goniofufurone on COX-2, IL-1β and IL-6 was weak. This may be due to the requirement of other transcription factors involved in these gene expressions such as AP-1 activated by MAPK pathway that may be (+)-goniofufurone-insensitive. Under pathological conditions, excessive inflammatory mediators and pro-inflammatory cytokines produced by activated macrophages cause inflammatory processes and act synergistically with other inflammatory mediators [91, 92, 93].

NO is a free radical synthesized from L-arginine by iNOS and it is involved in inflammation. Specifically, NO can react with superoxide leading to formation of the highly reactive peroxynitrite which can cause various kinds of

cellular damage and activate cell death pathway [94, 95]. Prostaglandin synthase, product of COX, is essential component of host innate immune and inflammatory responses [96, 97]. COX-2, which is inducible form in the inflammatory response, converts arachidonic acid into prostaglandin E_2. Therefore, inhibition of the excessive PGE_2 and NO production by blocking COX-2 and iNOS might be a useful strategy for the treatment of inflammatory disease.

From our results, (+)-goniofufurone could suppress the production of NO and PGE_2 in a dose dependent manner with the IC_{50} of 135 μg/ml (0.54 mM) and 175 μg/ml (0.7 mM), respectively. Although the IC_{50} value of NO production of (+)-goniofufurone from this experiment was less than that of other anti-inflammatory drug such as prednisone (with IC_{50} values 0.69 ± 0.26 mM) [100], (+)-goniofufurone was quite a weak suppressor of LPS-induced NO and PGE_2 production compared to many other anti-inflammatory substances that are effective at low micromolar [100, 111].

In this study, the mechanisms underlying the inhibitory effects of (+)-goniofufuron on inflammatory responses in LPS-activated RAW264.7 cells remains to be clearly elucidated. Blocking some signaling pathways may clearly explain the potent activity of (+)-goniofufurone as an inhibitor of inflammatory mediators and cytokines.

Chronic inflammation can lead to cancer and both have many similarities in the microenvironment, suggesting a role for inflammatory cells and cytokines in tumor progression and immunosuppression [101]. Key modulators, which drive inflammation to cancer, include various molecules such as NF-κB, TNF-α, IL-6, COX-2 and iNOS [102]. The active NF-κB, in normal physiology, controls the expression of many genes that regulate the immune, growth, and inflammation. By contrast, the excessive and improper activation of NF-κB can mediate inflammation and tumorigenesis. In addition, NF-κB acts as a linkage between inflammation and cancer [103, 104]. The microenvironment of tumors, as well as different inflammatory agents, carcinogens, and tumor promoters may activate the NF-κB [105]. In 2005, Abdel-latif et. al presented anticancer and anti-inflammatory activities of caffeic acid phenethyl ester in a gastric epithelial cell line, claiming that caffeic acid phenethyl

ester inhibits the production of TNF-α and IL-8 and suppresses the expression of NF-κB, AP-1 and COX-2 [106].

Recently, (+)-altholactone was reported to have activity against a variety of human tumor cell lines and also exhibited immune modulating activity by inhibiting several key downstream targets in the immune pathway (NF-κB, iNOS and COX-2) in RAW264.7 cell lines [107]. However, some styryl lactones such as (+)-goniothalmin and (+)-3-*O*-acetylaltholactone were reported to have activity against cancer cell line [108] but did not exhibit the activity of anti-inflammation. These results suggest the action of (+)-goniothalmin and (+)-3-*O*-acetylaltholactone involving their cytotoxicity against tumor cell lines appear to arise from mechanisms independent of targets in the NF-κB immune mediated pathway [107].

The derivatives of (+)-goniofufurone exhibits anti-proliferative activities against several human tumor cell lines as shown in Table 5.1. From the result, (+)-goniofufurone derivative was found to completely inactivate against HL-60 and MRC-5 cells [99]. Our study showed that (+)-goniofufurone had less cytotoxicity to RAW264.7 cells and could suppress inflammatory genes.

Table 5.1 Antiproliferative activities of (+)-goniofufurone derivative [99]

Compound	IC50 (μM)					
	K562	HL-60	Jurkat	Raji	HeLa	MRC-5
(+)-Goniofufurone	0.41	>100	32.45	18.45	8.32	>100

To our knowledge, this is the first study exhibiting the anti-inflammatory effect of (+)-goniofufurone in RAW264.7 cells. Possible mechanism by which (+)-goniofufurone may mediate its anti-inflammatory effect is by suppressing the signaling cascades up stream of NK-κB transcription factor and IRF-3. It may suppress the phosphorylation of IkB (inhibitor of NK-κB) so that IkB cannot be degraded out of transcription factor NF-κB. In addition it may suppress the phosphorylation of TBK1 leading to decreased expression of IRF-3. So when the expression of those transcription factors was decreased, the expression of

inflammatory related genes and mediators was also decreased. Furthermore, it was found that sterile alpha and HEAT/armadillo motif protein (SARM) which is a negative regulator of TRIF dependent pathway also up-regulated by the addition of (+)-goniofufurone in LPS-stimulated RAW264.7 cells (unreported). This result could confirm that (+)-goniofufurone could suppress the expression of inflammatory related genes in both MyD88-dependent and MyD88-independent pathways activated by LPS in RAW264.7 cells.

CHAPTER VI
CONCLUSION

Inflammation is a response of mammalian body to variety harmful stimuli and physical damage to tissue. Most of human population worldwide is getting affected by inflammation related disorders. It is believed that current drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) is not useful in all cases, because of their side effects like gastrointestinal irritation, liver dysfunction and many others [4,5]. Accordingly, therapeutic agents suitable for the treatment of chronic inflammatory diseases are highly desirable, which have resulted in an increased interest in complementary and alternative medicines.

The plant compound (+)-goniofufurone was isolated from leaves and twigs of *Polyalthia crassa* and it was tested for the cytotoxic and anti-inflammatory property in RAW264.7 macrophage cells activated with LPS from Gram negative bacteria, *E. coli*. The binding of LPS to TLR4 receptor on surface of macrophage leads to the activation of two signaling pathways, MyD88-dependent and MyD88-independent. The downstream signaling of MyD88 activates phosphorylation of IkB proteins. This phosphorylation leads to the degradation of IkB protein and the subsequent translocation of the transcription factor NF-kB, which controls the expression of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-12, and IL-1β, in addition to inducible enymes, including COX-2 [33]. For the downstream of MyD88-independent, TRIF activates downstream kinases, leading to the activation of IFN refulatory factor 3 (IRF3) [34]. The activation of TRIF pathway also induces delayed activation of NF-kB [35]. The representative target genes regulated through TRIF signaling pathways of TLR4 include IFNβ and IFN-inducible genes such as iNOS and IP-10 [36,37].

The plant compound, (+)-goniofufurone, was tested for its cytotoxicity to RAW264.7 macrophage cell. The concentration up to 200 μg/ml of (+)-goniofufurone was not toxic to cell and proper to be used to determine the anti-inflammatory effect of

this compound. It was found that the plant compound (+)-goniofufurone could suppress inflammatory related genes including TNF-α, IL-1β, IL-6, IL-12p40, COX-2, IFN-β and iNOS, and mediators including NO and PGE_2.

REFERENCES

1. Tapsell LC, Hemphill I, Cobiac L, et. al. Health benefits of herbs and spices: the past, the present, the future. Med J Aust 2006:185.
2. Lai PK, Roy J. Antimicrobial and chemopreventive properties of herbs and spices. Curr Med Chem 2004;11(11):1451-60.
3. Swain T. Plants in the development of modern medicine. Harvard University Press 1968.
4. Dutt V, Dutt R, Kumar S and Dhar V. Evaluation of analgesic activity of *Solanum platanifolium* Sims. Fruits Indian Drugs 2007;44(5):405-7.
5. Vasudevan MM, Gunnam KK and Parle M. Antinociceptive and anti inflammatory effects of *Thespesia populnea* bark extract. J Ethnopharmacol 2007;109(2):264-270.
6. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Enriron. Health Perspect 2001;109:69-75.
7. Meskin, Mark S. Phytochemicals in nutrition and health. CRC Press 2002:123.
8. Angiosperms: Division Magnoliophyta: General Features. Encyclopaedia Britannica 1993;13(15):609.
9. Karen S, Toni MK. Introduction to the different classes of natural products. Plant-derived natural products: synthesis, function, and application. Lanzotti, Virginia. Springer 2009:3.
10. Kanokmedhakul S, Kanokmedhakul K, Ohtani II, Isope M. Phytochemistry 1998;4: 131-3.
11. Connolly JD, Haque E, Kadir AA. Phytochemistry 1996;43:295-7.
12. Saepou S, Pohmakotr M, Reutrakul V, Yoosook C, Kasisit J, Napaswad C, et al. Anti-HIV-1 Diterpenoids from Leaves and Twigs of *Polyalthia sclerophylla*. Planta Med 2010;76:721-5.

13.Thangnipon W, Suwanna N, Kitiyanant N, Soi-ampornkul R, Tuchinda P, Munyoo B, et. al. Protective role of *N-trans*-feruloyltyramine against β-amyloid peptide-induced neurotoxicity in rat cultured cortical neurons. Neuro Lett 2012;513:229-32.

14. Tuchinda P, Pohmakotr M, Reutrakul V, Thanyachareon W, Sophasan S, Yoosook C, et. al. 2-Substituted Furans from *Polyalthia suberosa*. Planta Med 2001;67:572-5.

15. Bhattacharya AK, Chand HR, John J, Deshpande MV. Clerodane type diterpene as a novel antifungal agent from *Polyalthia longifolia* var. *pendula*. Eur J Med Chem 2015;94:1-7.

16. Tuchinda P, Munyoo B, Pohmakotr M, Thinapong P, Sophasan S, Santisuk T, et. al. Cytotoxic Styryl-Lactones from the Leaves and Twigs of *Polyalthia crassa.* J Nat Prod 2006;69:1728-33.

17. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol 2011;12:204-12.

18. Mirshafiey A, Jadidi-Niaragh F. Prostaglandins in pathogenesis and treatment of multiple sclerosis. Immunopharmacol Immunotoxicol 2010;32:543-54.

19. Cotran SR; Kumar V, Collins T. Robbins Pathologic Basis of Disease. Philadelphia: W.B Saunders Company 1999;6:845-901.

20. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et. al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes, J Exp Med 1989;170:827-45.

21. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, et. al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naïve CD4+ T cells. J Immunopathol 1995;154:5071-9.

22. Murtaugh MP, Foss DL. Inflammatory cytokines and antigen presenting cell activation. Vet Immunol Immunopathol 2002;87:109-21.

23. Dunne A, O'Neill LA. Adaptor usage and Toll-like receptor signaling specificity. FEBS Lett 2005;579(15):3330-5.

24. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent toll-like receptor signaling. Nat Immunol 2006;7:1074-81.

25. Stern AS, Podlaski FJ, Hulmmes JD, Pan YCE, Quinn PM, Wolitzky AG, et. al. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci USA 1990;87:6808-12.

26. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, et. al. The Toll-like receptor2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999;401:811-5.

27. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.

28. Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev 2002;82:97.

29. Wang SZ, Bao YX, Rosenberger CL, Tesfaigzi Y, Stark JM, Harrod KS. IL-12p40 and IL-18 modulate inflammatory and immune responses to respiratory syncytial virus infection. J Immunol 2004;173:4040-9.

30 Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, et. al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 1999;13:1015-24.

31. Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-including IFN-beta (TRIF)-dependent pathway in TLR signaling. J Immunol 2004;73:2913-7.

32. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et. al. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 2005;6:1087-95.

33. Srivastava SK, Ramana KV. Focus on Molecules: Nuclear factor-kappaB. Exp Eye Res 2009;88:2-3.

34. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et. al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491-6.

35. Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA. Ripl mediates the Trif-dependent toll-like receptor 3-and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem 2005;280:36560-6.

36. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, et. al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 2001;167:5887-94.

37. Gao JJ, Filla MB, Fultz MJ, Vogel SN, Russell SW, Murphy WJ. Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Statl alpha in mouse macrophages:pivotal role of Statlalpha in induction of the inducible nitric oxide synthase gene. J Immunol 1998;161:4803-10.

38. Entrez Gene: IL1B interleukin 1, beta [Internet]. 2015 [cited 2015 April 16]. Available from: http://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=3553

39. Robak T, Gladalska A, Stepien H. The tumour necrosis factor family of receptors/ligans in the serum of patients with rheumatoid arthritis. Eur Cytokine Netw 1998;9(2):145-54.

40. Hirano T. Interleukin 6 and its receptor:10 years later. Int Rev Immunol 1998;16(3-4):249-84.

41. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6 type cytokine signaling through the gp130/Jak/STAT pathway. Biochem J 1998;334(2):297-314.

42. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, et. al. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. Embo J 1990;9(6):1897-906.

43. Daeipour M, Kumar G, Amaral MC, Nel AE. Recombinant IL-6 activates p42 and p44 mitogen-activated protein kinases in the IL-6 responsive B cell line, AF-10. J Immunol 1993;150(11):4743-53.

44. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 1998;16:495-521.
45. Mocellin S, Rossi CR, Pilati P, Nitti D. Tumor necrosis factor, cancer and anticancer therapy. Cytokine Growth Factor Rev 2005;16(1):35-53.
46. Nurnberger W, Platonov A, Stannigel H, Beloborodov VB, Michelmann I, von Kries R, et. al. Definition of a new score for severity of generalized *Neisseria meningitides* infection. Eur J Pediatr 1995;154(11):896-900.
47. Mark KS, Trickler WJ, Miller DW. Tumor necrosis factor-alpha induces Cyclooxygenase-2 expression and prostaglandin release in brain Microvessel endothelial cells. J Pharacol Exp Therap 2001;297(3):1051-8.
48. Tak PP. IFN-beta in rheumatoid arthritis. Front Biosci 2004;9:3242-7.
49. Bruemmer-Smith S, Stuber F, Schroeder S. Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis. Intensive Care Med 2001;27(12):1835-41.
50. Belardelli F, Gresser I. Theneglected role of type I interferon in the T-cell response: implications for its clinical use. Immunol Today 1996;17(8):369-72.
51. Eloranta ML, Sandberg K, Ricciardi-Castagnoli P, Lindahl M, Alm GV. Production of interferon-alphabeta by murine dendritic cell lines stimulated by virus and bacteria. Scand J Immunol 1997;46(3):235-41.
52. Svensson H, Johannisson A, Nikkila T, Alm GV, Cederblad B. The cell surface phenotype of human natural interferon-alpha producing cells as determined by flow cytometry. Scand J Immunol 1996;44(2):164-72.
53. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et. al. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999;284(5421):1835-7.
54. Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, et. al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med 1999;5(8):919-23.
55. Bogdan C. The function of type I interferons in antimicrobial immunity. Curr Opin Immunol 2000;12(4):419-24.

56. Nickolaus P, Zawatzky R. Inhibition by interleukin-4 of constitutive beta interferon synthesis in mouse macrophages. J Virol 1994;68(10):6763-6.

57. Kaufmann SH. Heat shock proteins and the immune response. Immunol Today 1990;11:129-36.

58. Lydyard PM, van Eden W. Heat shock proteins:immunity and immunopathology. Immunol Today 1990;11:228-9.

59. Lange C, Hemmrich G, Klostermeier UC, Lopez-Quintero JA, Miller DJ, et. al. Defining the origins of the NOD-like receptor system at the base of animal evolution. Mol Biol Evol 2001;28:1687-702.

60. Proell M, Riedel SJ, Fritz JH, Rojas AM, Schwarzenbacher R. The Nod-like receptor (NLR) family: a tale of similarities and differences. Plos One 2008;3:e2199.

61. Gao JJ, Filla MB, Fultz MJ, Vogel SN, Russell SW, Murphy WJ. Autocrine/paracrine IFN-alpha/beta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macroohages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene. J Immunol 1998;161(9):4803-10.

62. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol 2002;3(4):392-8.

63. Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3(9):651-62.

64. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, et. al. TLR 4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol 2002;3(4):392-8.

65. Matsayapan P. *Burkholderia pseudomallei* induces mouse macrophage activation via MyD88-dependent pathway. Thesis 2006, M.Sc. (Microbiology), Mahidol University.

66. Archaroen S, Wikraiphat C, Pudla M, Limposuwan K, Wood DE, Sirisinha S, Utaisincharoen P, Fate of a Burkholderia pseudomallei lipopolysaccharide mutant in the mouse macrophage cell line RAW264.7: possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. Infect Immun. 2007;75:4298-304.

67. Green SJ, Scheller LF, Marletta MA, Seguin MC, Klotz FW, Slayter M, et. al. Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. Immunol.Lett. 1994;43(1-2):87-94.

68. Mungrue IN, Husain M, Stewart DJ. The role of NOS in heart failure: lessons from murine genetic models. Heart Fail Rev 2002;7(4):407-22.

69. Akira S, Yamamoto M, Takeda K. Role of adapters in Toll-like receptor signaling. Biochem Soc Trans 2003;31:637-42.

70. Hayashi F, Smith D, Ozinsky A, Hawn R, Yi C, Goodlett R, Eng K, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor-5. Nature 2001;410:1099-103.

71. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. Science 2003;300:1524-25.

72. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 2001;167:5887-94.

73. Han KJ, Yang Y, Xu LG, Shu HB. Analysis of a TIR-less splice variant of TRIF reveals an unexpected mechanism of TLR3-mediated signaling. J Biol Chem 2010;285:12543-50.

74. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays : the MTT assay. Methods Mol Biol 2011;731:237-45.

75. De Fatima A, Modolo LV, Conegero LS, Pilli RA, Ferreira CV, Kohn Lk, et. al. Curr Med Chem 2015;28(14):3371-84.

76. Fan XP, Anderson JE, Chang CJ, Fanwick PE, McLaughlin JL. Novel bioactive styryl-lactones: goniofufurone, goniopypyrone, and 8-acetylgoniotriol from *Goniothalamus giganteus* (Annonaceae). X-ray molecular structure of goniofufurone and of goniopypyrone. J Chem Soc Perkin Trans 1990;1:1655-61.

77.Fang XP, Anderson JE, Chang CJ, McLaughlin JL, Fanwick PE. Two new styryllactones, 9-deoxygoniopypyrone and 7-epi-goniofufurone, from Goniothalamus giganteus. J Nat Prod 1991;54:1034-43.

78. Wu YC, Duh CY, Chang FR, Chang GY, Wang SK, Chang JJ, et. al. J Nat Prod 1991;54:1077.

79. Talapatra SK, Basu D, Deb T, Goswami S, Talapatra B. Indian J Chem 1985;24B: 29-34.

80. Sam TW, Yeu CS, Matsjeh S, Gan EK, Razak D, Mohamed AL. Tetrahedron Lett 1987;28:2541.

81. Utar Z, Isa Abdul Majid M, Ilham Adenan M, Fadly Amar Jamil M, Mei Lan T. Mitragynine inhibits the COX-2 mRNA expression and prostaglandin E_2 production induced by lipopolysaccharide in RAW264.7 macrophage cells. J Ethnopharmacol 2011;136:75-82.

82. Nhiem NX, Thu Hien NT, Tai BH, Tuan Anh HL, Thuy Hang DT, Quang TH, et. al. New ent-kauranes from the fruits of *Annona glabra* and their inhibitory nitric oxide production in LPS-stimulated RAW264.7. Bioorg Med Chem Lett 2015;25:254-8.

83. Deng GG, Wei W,Yang XW, Zhang YB, Xu W, Gong NB, et. al. New coumarins from the roots of *Angelica dahurica* var. *formosana* cv. Chuanbaizhi and their inhibition on NO production in LPS-activated RAW264.7 cells. Fitoterapia 2015;101:194-200.

84. Jin M, Suh SJ, Yang JH, Lu Y, Kim SJ, Kwon S, et. al. Anti-inflammatory activity of bark of *Dioscorea batatas* DECNE through the inhibition of iNOS and COX-2 expression in RAW264 cells via NF-kB and ERK1/2 inactivation. Food Chem Toxicol 2010;48:3073-9.

85. Cheng A, Yan H, Han C, Wang W, Tian Y. Polyphenols from blueberries modulate inflammation cytokines in LPS-induced RAW264.7 macrophages. Int J Biol Macromol 2014;69:382-7.

86. Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001;107:7-11.

87. Youn HS, Lee JY, Saitoh SI, Miyake K, Kang KW, Choi YJ, et. al. Suppression of MyD88- and TRIF-dependent signaling pathways of toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochem Pharmacol 2006;72:850-9.

88. Jung YS, Kim DH, Hwang JY, Yun NY, Lee YH, Han SB, et. al. Anti-inflammatory effect of tricin 4'-O-(threo-β-guaiacylglyceryl) ether, a novel flavonolignan compound isolated from Njavara on in RAW264.7 cells and in ear mice edema. Toxicology and Applied Pharmacology 2014;277:67-76.

89. Chun J, Choi RJ, Khan S, Lee DS, Kim YC, Nam YJ, et. al. Alantolactone suppresses inducible nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-κB, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW264.7 cells. Int Immunopharmacol 2012;14(4):375-83.

90. Ahn KS, Noh EJ, Cha KH, Kim YS, Lim SS, Shin KH, et. al. Inhibitory effects of Irigenin from the rhizomes of *Belamcanda chinensis* on nitric oxide and prostaglandin E_2 production in murine macrophage RAW264.7 cells. Life Sci 2006;78:2336-42.

91. Libby P. Inflammation and cardiovascular disease mechanisms. Am. J Clin Nutr 2006;83:456S-60S.

92. Salvemini D, Kim SF, Mollace V. Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. Am J Physiol Regul Integr Comp Physiol 2013;304:R473-87.

93. Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J. Endotoxin Res 2000;6:453-7.

94. Ahmad R, Rasheed Z, Ahsan H. Biochemical and cellular toxicology of peroxynitrite: implications in cell death and autoimmune phenomenon. Immunopharmacol Immunotoxicol 2009;31:388-96.

95. Korhonen R, Lahti A, Kankaanranta H, Moilanen E. Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy 2005;4:471-9.

96. Hobbs AJ, Higgs A, Moncada S. Inhibition of nitric oxide synthase as a potential therapeutic target. Annu Rev Pharmacol Toxicol 1999;39:191-220.

97. Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, et. al. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem 1992;267:25934-8.

98. Deng X, Su J, Zhao Y, Peng LY, Li Y, Yao ZJ, et. al. Development of novel conformation-constrained cytotoxic derivatives of cheliensisin a by embedment of small heterocycles. Eur J Med Chem 2011;46(9):4238-44.

99. Popsavin V, Sreco B, Benedekovic G, Francuz J, Popsavin M, Kojic V, et. al. Design, synthesis and antiproliferative activity of styryl lactones related to (+)-goniofufurone. Eur J Med Chem 2010;45:2876-83.

100. Gunawardena D, Shanmugam K, Govindaraghavan S, Low M, Bennett L, Head R, et. al. Determination of anti-inflammatory activities of standardised preparations of plant and mushroombased foods. Eur J Nutr 2014;53(1):335-43.

101. Boveri T. Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris. J Cell Sci 2008;121:1-84.

102. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Cancer 2006;4:221-33.

103. Bharti AC, Aggarwal BB. Nuclear factor-kappa B and cancer: its role in prevention and therapy. Biochem Pharmacol 2002;64:883-8.

104. Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. Nat Rev Immunol 2004;4:641-8.

105. Aggarwal BB. Nuclear factor-κB: the enemy within. Cancer Cell 2004;6:203-8.

106. Abdel-Latif MM, Windle HJ, Homasany BS, et. al. Caffeic acid phenethyl ester modulates *Helicobacter pylori*-induced nuclear factor-kappa B and activator protein-1 expression in gastric epithelial cells. Br J Pharmacol 2005;146:1139-47.

107. Johnson TA, Sohn J, Ward AE, Cohen TL, Lorig-Roach ND, Chen H, et al. (+)-Altholactone exhibits broad spectrum immune modulating activity by inhibiting the activation of pro-inflammatory cytokines in RAW264.7 cell lines. Bioorg Med Chem 2013;21:4358-64.

108. de Fatima A, Marquissolo C, de Albuquerque S, Carraro-Abrahao AA, Pilli RA. Eur J Med Chem 2006;41:1210.

109. Lavault M, Guinaudeau H, Bruneton J. (-)-Thaipetaline, a tetrahydroprotoberberine from a Malayan annonaceae. Phytochemistry 1990;29:3845-7.

110. Paioti PHS, Coelho F. A Morita-Baylis-Hillman adduct allows the diastereoselective synthesis of styryl lactones. Tetrahedron lett 2011;52:6180-4.

111. Ho T, Wu, Yung-Yi C, Chao-Jung C, Lean-Teik Ng, Li-Chen C, et. al. Three new clerodane diterpenes from Polyalthia longifolia var. pendular. Molecules 2014;19(2):2049-60.

112. Majno G, Joris I. Cell, tissues and disease. Oxford Univ Press 2004.

113. Barton GM. A calculated response: control of inflammation by the innate immune system. J Clin Invest 2008;118:413-20.

114. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nature Immunol 2005;6:1191-7.

115. Chandrasoma P, Taylor CR. The acute inflammatory response, sub-section Cardinal Clinical Signs. Concise Pathology 2005;3.

116. Ma X, Trinchieri G. Regulation of interleukin-12 production in antigen-presenting cells. Adv Immunol 2001;79:55-92.

117. Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. Immunol Today 1992;13:77-8.

118. Lederer JA, Perez VL, DesRoches L, Kim SM, Abbas AK, Lichtman AH. Cytokine transcriptional events during helper T cell subset differentiation. J Exp Med 1996;184:397-406.

119. Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNFα in pulmonary pathophysiology. Resp Res 2006;7:125.

120. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011;34:637-50.

121. Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tomor necrosis factor-alpha and interferon-gamma by bronchoalveolar leukocytes from patients with bronchial asthma. Am Rev Resp Dis 1993;147(2):291-5.

122. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, et. al. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA 2005;102:9577-82.

123. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805-20.

124. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor κB is a molecule target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 2001;276:32008-15.

125. Miller MJ, Grisham MB. Nitric oxide as a mediator of inflammation? You had better believe it. Mediators Inflamm 1995;4:387-96.

126. Cochran FR, Selph J, Sherman P. Insights into the role of nitric oxide in inflammatory arthritis. Med Rev 1996;16:547-63.

127. Weisz A, Cicatiello I, Esumi H. Regulation of the mouse inducible type nitric oxide synthase gene promoter by interferon-gamma, bacterial lipopolysaccharide and NG-monomethyl-L-arginine. Biochem J 1996;316:209-15.

128. Janeway CA, Travers P, Walpot M, Shlomchik M. The Immune System in Health and Disease. Immunobiology 2001;5.

129. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et. al. Cyclooxygenase in biology and disease. FASEB J 1998;12:1063-73.

130. Nathan C. Points of control in inflammation. Nature 2002;420:846-85.

131. Bogdan C. Nitric oxide and the immune response. Nat Immunol 2001;2:907-16.

132. Ivanov AI, Romanovsky AA. Prostaglandin E_2 as a mediator of fever: synthesis and catabolism. Front Biosci 2004;9:1977-93.

133. Yanagawa R, Furukawa Y, Tsunoda T, Kitahara O, Kameyama M, Murata K, et. al. Genome-wide screening of genes showing altered expression in liver metastases of human colorectal cancers by cDNA microarray. Neoplasia 2001;3:395-401.

134. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, et. al. Frequent nitic oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res 1998;58:334-41.

135. Cianchi F, Cortesini C, Fantappie O, Messerini L, Schiavone N, Vannacci A, et. al. Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. Am J Pathol 2003;162:793-801.

136. O'Neill GP and Ford-Hutchinson AW. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Lett 1993;330(2):156-60.

137. Satoh H, Amagase K, Ebara S, Akiba Y, Takeuchi K. Cyclooxygenase (COX)-1 and COX-2 both play an important role in the protection of the duodenal mucosa in cats. J Pharmacol Exp Ther 2012;344(1):189-95.

138. Font-Nieves M, Sans-Fons MG, Gorina R, Bonfill-Teixidor E, Salas-Perdomo A, Marquez-Kisinousky L, et. al. Induction of COX-2 enzyme and down-regulation of COX-1 expression by lipopolysaccharide (LPS) control prostaglandin E_2 production in astrocytes. J Biol Chem 2012;287(9):6454-68.

139. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et. al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301:640-3.

140. Covert MW, Leung TH, Gaston JE, Baltimore D. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science 2005;309:1854-7.

141. Hibbs JB, Vavrin Z, Taintor RR. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J Immun1987;138:550-65.

142. McCarthy MM, Amateau SK, Mong JA. Steroid modulation of astrocytes in the neonatal brain: implications for adult reproductive function. Biol Reprod 2002;67(3):691-8.

143. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004;16:3-9.

APPENDIX

Reagent for cell culture

Unsupplemented DMEM medium

Advanced D-MEM 1X
With D-glucose at 4500 mg/l
With NEAA
With sodium pyruvate at 110 mg/l
Without L-glutamine

Supplemented DMEM medium

Unsupplemented DMEM medium	94	ml
Heat-inactivated Fetal Bovine Serum	5	ml
(FBS was heated at 56°C for 30 min)		
L-glutamine	1	ml

Phosphate-buffered saline (PBS) pH 7.0

NaCl	8	g
Na_2HPO_4	0.92	g
KH_2PO_4	0.2	g
KCl	0.2	g
Distilled water to a final volume of	1	liter

The solution was adjusted to pH 7.0 and sterilized by autoclaving

Reagent for ELISA mouse PGE_2

Wash buffer

Wash Buffer Concentrate	20	ml
Distilled water to a final volume of	480	ml

May be stored for up to 1 months (4°C storage)

Substrate solution-1:1 mixture of

Color reagent A (H_2O_2)

Color reagent B (Tetramethylbenzidine)

Freshly prepared and used within 15 minutes

2N H_2SO_4 Stop solution

Sample preparation require a 3-fold dilution-1:2 of

Samples	150	µl
Calibrator Diluent	300	µl

Reagents for PCR

Tris-base-EDTA buffer (TBE, 10X)

Tris base	108	g
Boric acid	55	g
Na_2EDTA	8.3	g
Distilled water to a final volume of	1	liter

Adjusting pH to 8.0

TBE running buffer (1X)

10X TBE buffer	100	ml
Distilled water	900	ml

Agarose gel preparation

1.5% agarose gel

Agarose (dry powder)	1.65	g
1X TBE buffer	110	ml

Dissolved by boiling in microwave and cool at RT

Reagents for SDS-PAGE

Stock acrylamide (30%w/v)

Acrylammide	150	g
N,N-bis-methylene acrylamide	4.5	g
Distilled water to a final volume of	500	ml

The solution was filtrated with Whatman filter paper No.1 and stored at 4°C

Gel buffer 1.875 M pH 8.8

Trizma base (MW 121.14)	113.57	g
Distilled water to a final volume of	500	ml

Adjusting pH to 8.8

Gel buffer 1.875 M pH 6.8

Trizma base (MW 121.14)	113.57	g
Distilled water to a final volume of	500	ml

Adjusting pH to 6.8

Electrophoresis buffer

Trizma base	3	g
Glycine	14.4	g
SDS	1	g
Distilled water to a final volume of	1	liter

Towbin's buffer

Trizma base	9.1	g
Glycine	43.2	g
Methanol	600	ml
Distilled water to a final volume of	3	liters

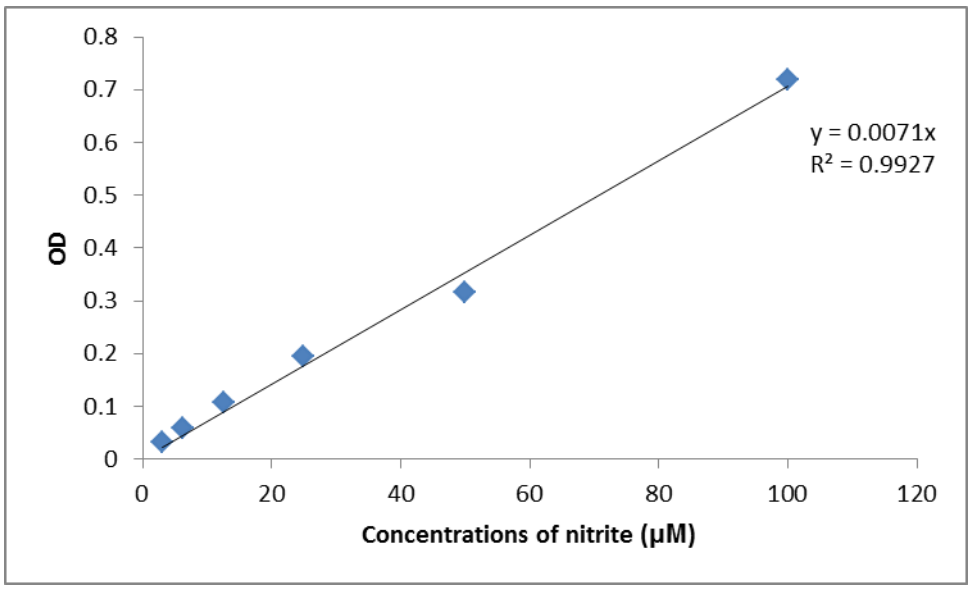
Phosphate-buffered saline (PBS)

NaCl	8.9	g
Na_2HPO_4	1.28	g
$Na_2HPO_42H_2O$	0.156	g
Distilled water to a final volume of	1	liter

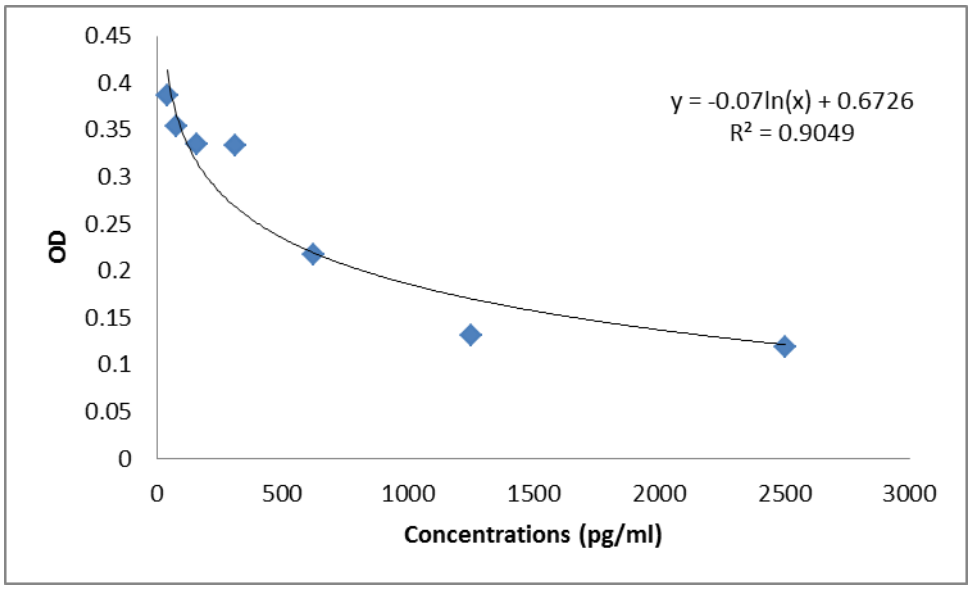
Lysis buffer (5X) pH 6.8

Trizma base	0.378	g
SDS	0.5	g
Glycerol	5	lm
2-mercaptoethanol (2-ME)	2.5	ml
1N HCl	1.5	ml
Bromophenol blue	0.005	g
Distilled water to a final volume of	10	ml

Standard curve of nitrite



Standard curve of PGE_2



BIOGRAPHY

NAME	Miss Wanichaya Khakhunmalee
DATE OF BIRTH	20 MAY 1989
PLACE OF BIRTH	Roi-et, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2008-2012 Bachelor of Science (Medical technology) Mahidol University, 2012-2015 Master of Science (Biotechnology)
HOME ADDRESS	372/6, Wang, Phontong, Roi-et, 45110 Thailand Tel. 061-5030090 E-mail : Wanichaya_mtmu55@hotail.com
PRESENTATION	Khakhunmalee W, Tuchinda P, Utaisincharoen P, Mungsantisuk I, Anti-inflammatory effects of compound from the rhizomes of *Zingiber mekongense.* MU Graduate Research Expo 2014, 2014 Dec 1-2; Ramathibodi school of nursing Building, Bangkok: Mahidol University; 2014.p. 53.