Effects of Artesunate and Dihydroartemisinin on LPS-Activated Macrophages

Miss Thin Sandi Htun

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ฤทธิ์ของอาร์ติซูเนตและไดไฮโดรอาร์ติมิซินินต่อเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยแอลพีเอส

นางสาวติน ซานดิ ตัน

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ติน ชานดิ ตัน : ฤทธิ์ของอาร์ติซูเนตและไดไฮโดรอาร์ติมิซินินต่อเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยแอลพีเอส (Effects of Artesunate and Dihydroartemisinin on LPS-Activated Macrophages) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ปิยนุช วงศ์อนันต์, 78 หน้า.

การอักเสบแบบเรื้อรังเป็นกระบวนการที่ซับซ้อน เกิดจากการทำงานของเซลล์ในระบบภูมิคุ้มกันหลายชนิดโดยเฉพาะเซลล์แมคโครเฟจ การหากลยุทธใหม่ๆ ในการยับยั้งการทำหน้าที่ของเซลล์แมคโครเฟจที่ถูกกระคุ้นซึ่งเกี่ยวข้องกับการอักเสบแบบเรื้อรังจึงเป็นสิ่งที่ได้น่าสนใจ อาร์ติซูเนตและไดไฮโดรอาร์ติมิซินินเป็นอนุพันธุ์ของอาร์ติมิซินินที่มีฤทธิ์ทางเภสัชวิทยาอื่นนอกเหนือจากฤทธิ์ต้านมาลาเรีย การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาและเปรียบเทียบผลของอาร์ติซูเนตและไดไฮโดรอาร์ติมิซินินต่อเซลล์แมคโครเฟจ J774A.1 ที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ ผลการศึกษาพบว่ายาทั้งสองที่ความเข้มข้น 5-50 ไมโครโมลาร์ยับยั้งการสร้างไนติกออกไซด์ได้แบบขึ้นกับความเข้มข้นของยา โดยมีค่า IC50 ของอาร์ติซูเนตเท่ากับ 28.3 ± 3.5 ไมโครโมลาร์ และของไดไฮโดรอาร์ติมิซินินเท่ากับ 13.12 ± 2.3 ไมโครโมลาร์ ซึ่งสอดคล้องกับฤทธิ์ของยาทั้งสองในการยับยั้งการแสดงออกระดับ mRNA ของเอนไซม์ iNOS ที่ใช้สร้างไนตริกออกไซด์ ยาทั้งสองตัวที่ความเข้มข้น 10-50 ไมโครโมลาร์มีฤทธิ์คล้ายกันในการยับยั้งการแสดงระดับ mRNA ของสารสื่อต่างๆ และเอนไซม์ที่เกี่ยวข้องกับการอักเสบ ได้แก่ ไซโตคายน์ (TNF-α), IL-1 และ IL-6), คีโมคายน์ (MCP-1 and MIP-1a), โพลสตาแกลนดิน E2 (PGE2), cyclooxygenase-2 (COX-2), และ microsomal prostaglandin E synthase-1 (mPGES-1). เมื่อทำการเปรียบเทียบฤทธิ์ของยาสองตัวนี้โดยอาศัยค่า IC50 พบว่า ยาทั้งสองตัวมีความแรงคล้ายกันในการยับยั้งการแสดงออกของ IL-1, MIP-1α, iNOS, COX2, mPGES1, และการยับยั้งการสร้าง PGE_2 พบว่า ไดไฮโดรอาร์ติมิซินินออกฤทธิ์แรงกว่าอาร์ติซูเนตในการยับยั้งการสร้างไนตริกออกไซด์และการยับยั้งการแสดงออกของ TNF-α และ MCP-1 ในทางตรงข้ามอาร์ติซูเนตออกฤทธิ์ยับยั้งการแสดงออกของ IL-6 ได้ดีกว่าไดไฮโดรอาร์ติมิซินิน ยาทั้งสองเพิ่มการแสดงออกของ IL-10 ที่มีฤทธิ์ต้านการอักเสบ การศึกษานี้แสดงให้เห็นว่าอาร์ติซูเนตและไดไฮโดรอาร์ติมิซินินเป็นสารต้านอักเสบที่ออกฤทธิ์แรง ผลจากการศึกษานี้สนับสนุนความเป็นไปได้ในการจะนำยาทั้งสองไปใช้เป็นยาต้านอักเสบในอนาคต

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THIN SANDI HTUN: Effects of Artesunate and Dihydroartemisinin on LPS-Activated Macrophages. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., CO-ADVISOR: PIYANUCH WONGANAN, Ph.D., 78 pp.

Chronic inflammation is a complicated process mediated by the actions of many types of immune cells, notably, macrophages. Thus, there is a great deal of interest in launching new strategies to inhibit functions of activated macrophages associated with chronic inflammation. Artesunate (AS) and dihydorartemisinin (DHA), the artemisinin derivatives, have been shown to have pharmacological actions beyond anti-malarial effects. This study aimed to investigate and compare the effects of AS and DHA on lipopolysaccharide (LPS) activated macrophage J774A.1 cells. The results showed that AS and DHA markedly inhibited nitric oxide (NO) production in a concentration dependent manner with IC50 at 28.3 ± 3.5 μM for AS and at 13.12 ± 2.3 μM for DHA. The activities of AS and DHA on NO production were consistent with their inhibitory effects on mRNA expression of iNOS. AS and DHA at 10-50 μM significantly down-regulated mRNA expressions of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) chemokines (MIP-1α and MCP-1), COX2, mPGES1, and decreased PGE_2 production. By comparing between their IC50 values, AS and DHA had similar inhibitory effects on mRNA expression of IL-1, MIP-1α, iNOS, COX2, mPGES1, and on PGE_2 production. DHA was more potent than AS on inhibiting NO production and on down-regulating mRNA expression of TNF-α and MCP-1. In contrast, AS showed higher inhibitory effect on IL-6 expression than DHA. Both AS and DHA increased mRNA expression of anti-inflammatory cytokine, IL-10 in LPS-activated macrophages. The results in this study demonstrated potent anti-inflammatory activities of AS and DHA. These results supported the potential use of artemisinin derivatives as anti-inflammatory agents in the future.

Field of Study: Medical Science
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Background and rationale

Inflammation is the body defense against harmful stimuli (infective agents, physical, chemical, immunological agents). There are two main types of inflammation, acute and chronic inflammation. Acute inflammation is a short duration of inflammatory process lasting for hours to days. It is a protective process which responds to harmful stimuli with the cardinal signs of inflammation (redness, swelling, heat, and pain). During acute inflammation, there are massive migration of neutrophils from blood circulation into injured tissue followed by monocytes which later develop into inflammatory macrophages that subsequently proliferate and thereby contribute the roles of resident tissue macrophages [1]. Chronic inflammation is a long-run inflammatory process due to the persistence of harmful stimuli or unresolved immune response of acute inflammation. It can last for weeks to even years.

Macrophages play crucial roles in chronic inflammatory process. In the presence of a stimulant, they become activated macrophages which release plenty of pro-inflammatory cytokines, chemokines and other inflammatory mediators. These mediators can damage normal tissues or cells nearby and lead to chronic inflammatory conditions associated with several diseases such as pulmonary disease,

type II diabetes mellitus, Alzheimer disease and even cancer. Emergence of various diseases with chronic inflammation is a challenging global health problem. As chronic inflammatory diseases usually have high morbidity, they can impair the quality of people's lives. To treat many chronic inflammatory diseases, several anti-inflammatory agents have pharmacological activities that can inhibit the production and functions of inflammatory mediators. It is an important issue to take action for the development of newer, effective and safer anti-inflammatory agents. Conventionally used anti-inflammatory drugs are potent and effective but dangerous due to common adverse effects especially in long term use. They have severe adverse effects like gastric irritation, ulcerogenic action, gastrointestinal bleeding, nephropathy for NSAIDs, salt and water retention, hypertension, steroid psychosis, peptic ulceration for corticosteroids [2] [1], visual disturbances, corneal opacities, irreversible retinal damage for chloroquine. Given these impediments to the widespread application of established anti-inflammatory drugs, significant efforts have been made to develop alternative strategies. And thus, safer anti-inflammatory drugs with scientifically testified efficacy for potential use should be found out.

Several compounds from natural sources have been proven to be valuable as effective therapeutic drugs. As macrophages are major sources of many inflammatory mediators, targeting macrophages and their mediators are important strategy for controlling inflammatory diseases. Artemisinin is a well-known

antimalarial compound isolated from sweet woodworm, *Artemisia annua L* [3]. Artemisinin derivatives are currently recommended by WHO as first line antimalarial drugs for falciparum malaria [4]. According to previous studies, artemisinin and its derivatives exhibited a rich assortment of biological activities beyond antimalarial action which include antiviral, antischistosomal, anthelmintic, antiprotozoal, antifungal, antiangiogenic, anticancer, antiallergic and anti-inflammatory activities [5]. *In vivo*, artemisinins including artesunate are universally converted to the active metabolite, dihydroartemisinin (DHA), whose clinical efficacy is at least the same as parent compounds [6]. Some studies have revealed effects of these drugs on macrophages. And very few studies about effects of AS and DHA on anti-inflammatory mediators of macrophages have been reported. This study aims to investigate and compare immune-modulating effects of artesunate (AS) and its active metabolite, DHA on LPS-activated macrophage J774A.1 cells.

Research Objectives

- To determine the inhibitory activities of AS and DHA on inflammatory mediators and enzymes involve in inflammatory and anti-inflammatory mediators production in LPS-activated J774A.1 cells.
- To compare the inhibitory effects of AS and DHA on LPS-activated J774A.1 cells.

Hypothesis

- AS and DHA have inhibitory effects on the production and expression of pro-inflammatory cytokines, chemokines and enzymes involved in inflammation of LPS-activated J774A.1 cells.
- DHA has higher activity than AS.

Research Questions

1. What are the effects of artesunate (AS) and dihydroartemisinin (DHA) on inflammatory and anti-inflammatory parameters in LPS-activated macrophage J774A.1 cells?
2. How are the differences in the inhibitory effects between AS and DHA on LPS-activated macrophage J774A.1 cells?

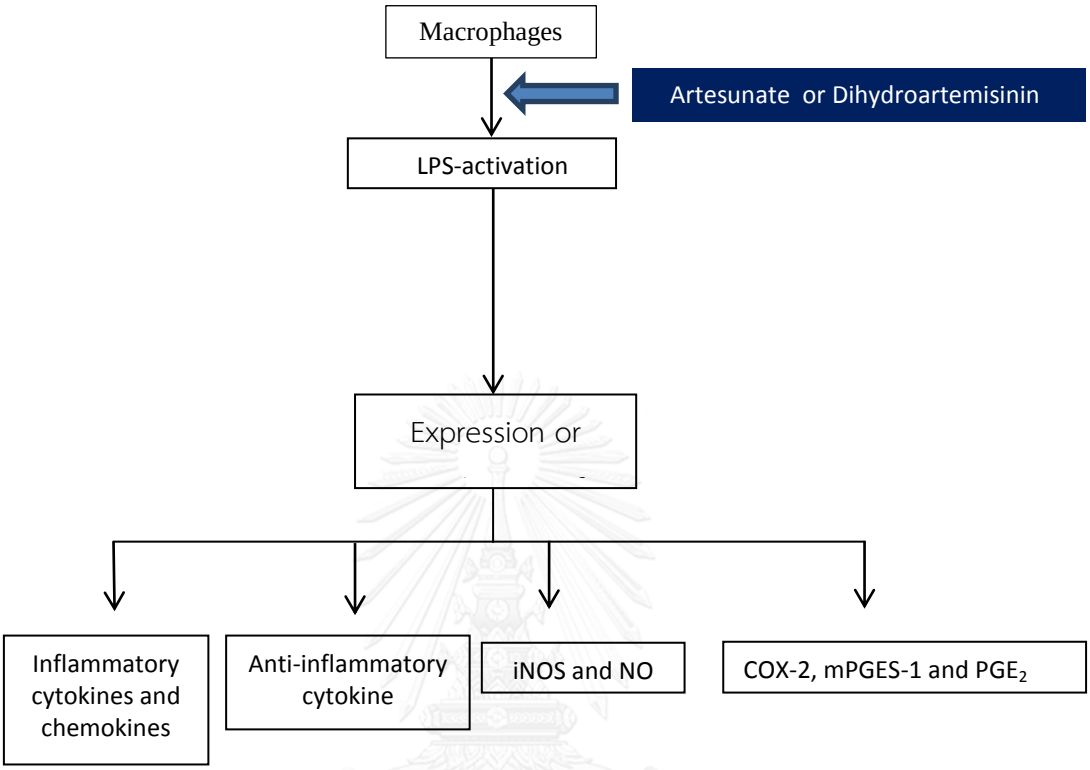
Research Design

Experimental study

Key Words

Artesunate, dihydroartemisinin, macrophage, LPS, inflammatory mediators

Conceptual Framework



CHAPTER II

LITERATURE REVIEW

Inflammation is a complex biological response to infection or injury with main cardinal signs of redness, pain, swelling, and organ dysfunction, aimed at eliminating the threats and re-establishing homeostasis. It had been believed to implicate in the pathogenesis of stroke, cancer, arthritis, cardiovascular and neurodegenerative diseases [1]. It is classified into two major types, acute and chronic inflammation [7] [8] [9] [10].

Acute Inflammation

Acute inflammation is a short duration process, lasting from a few hours to a few days. When a harmful stimulus affects part of the body, there is a biological response which tries to remove it. The signs and symptoms of inflammation, typically acute inflammation, indicate that the body is combating and trying to restore normal physiological processes [11]. This process evokes the typical features of inflammation: calor (heat), rubor (redness), dolor (pain) and tumor (swelling) [9]. At the site of an infection or injury, mast cells, platelets, nerve endings, endothelial cells, and other resident cells release signaling molecules and chemo attractants that recruit leukocytes to the affected area. The acute phase of inflammation is recognized by the rapid migration of blood granulocytes, neutrophils, followed by

blood monocytes to the injury site. These blood monocytes then mature into inflammatory macrophages [9] [12] [10].

Acute inflammation is a self-limiting process. The usual result is effective resolution and improvement of tissue damage, rather than persistence of the inflammatory response and dysfunction of the cells and tissues [1]. If the initiating factor or stimulus is removed by the body, the inflammatory process can slow down and subside. Termination of granulocyte functions, returning of macrophages and lymphocytes to physiological numbers and phenotypes are the essential steps of the resolution of inflammation. If the stimulus persists and cannot be eradicated, acute inflammation can progress to chronic inflammation, autoimmunity and excessive tissue damage [9] .

Chronic Inflammation

Chronic inflammation can last for weeks, months and even years, and is primarily mediated by tissue macrophages and lymphocytes [10]. Macrophages participate in the chronic inflammatory processes with the production and release of large amount of several inflammatory mediators, free radicals and protease enzymes that destroy the cause of inflammation as well as the body's own tissues. Principally, tissue damage is a hallmark of chronic inflammation [7]. Failure of resolution and chronic inflammation lead to tissue damage, scarring and loss of organ

functions, and are implicated in the pathogenesis of many inflammatory diseases [10].

Macrophages and Inflammation

Macrophages are major components of the mononuclear phagocyte system. They are immune cells, found in almost all the tissues of the body. Macrophages originate from bone marrow stem cells which develop to promonocytes and turn into monocytes in the blood circulation. The majority of monocytes localizes in different tissues and transform into macrophages which have particularized functions depending on their locating tissues. These macrophages have specific name like Kupffer cell (liver), osteoclasts (bone), microglia (brain) and histiocytes (connective tissues) (Figure 1) [7] [13].

Pattern recognition receptors (PRR) on macrophages and DC play crucial roles on antigen recognition. They recognize and bind to certain pathogen-associated molecular patterns or epitopes consisting of bacterial DNA, viral double-stranded RNA, lipopolysaccharide (LPS), peptidoglycan and flagellin [14]. These pathogen-associated molecular patterns of pathogens can stimulate macrophages to become activated macrophages by initiating innate immune response. TLR-4 is pattern recognition receptor for LPS-induced inflammatory responses with the assistance of the adaptor protein, myeloid differentiation factor 88 (MyD88). Subsequently, MyD88 recruits IL-1 receptor-associated kinase (IRAK), and activates IkappaB kinase, (IKK)-beta

and nuclear factor kappaB (NF-kB) resulting in the release of various inflammatory mediators (Figure 2). Transcription factor NF-kB is one of the key players of the immune response, and its activation is a universal phenomenon for inflammation responsible for induction of a variety of inflammatory mediator expression in most cell types [15]. It has also been reported that LPS can also stimulate anti-inflammatory cytokine production [16] . Literally, LPS induces the production of anti-inflammatory cytokines (IL-10 and TGF-β) by TLR4 signaling from endosomal compartments [17] .

Macrophages at inflammatory sites are usually in activated state. Excess amount of pro-inflammatory and cytotoxic mediators from activated macrophages can result in the creation of pathophysiological responses in chronic inflammation [13]. It is important to remove or deactivate the mediators and inflammatory effector cells in order to inhibit inflammation which allows the body to repair tissue damage. Anti-inflammatory cytokines (IL-10 and TGF-β) and cytokine antagonists that are mainly expressed by activated macrophages can deactivate the activated macrophages. Hence, macrophages can manage the auto regulation of the inflammatory reactions. In this concern, therapeutic interventions targeting macrophages and their products are the concepts for controlling inflammatory diseases [7].

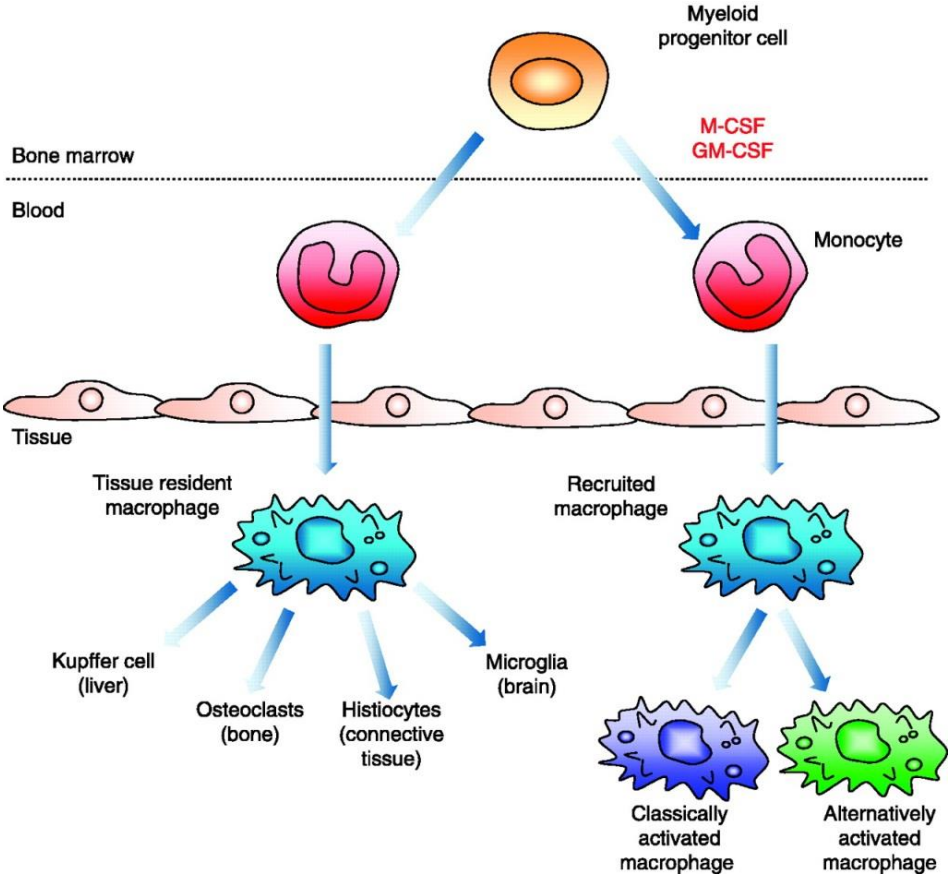


Figure 1: Macrophage maturation from bone marrow precursor cells [18]

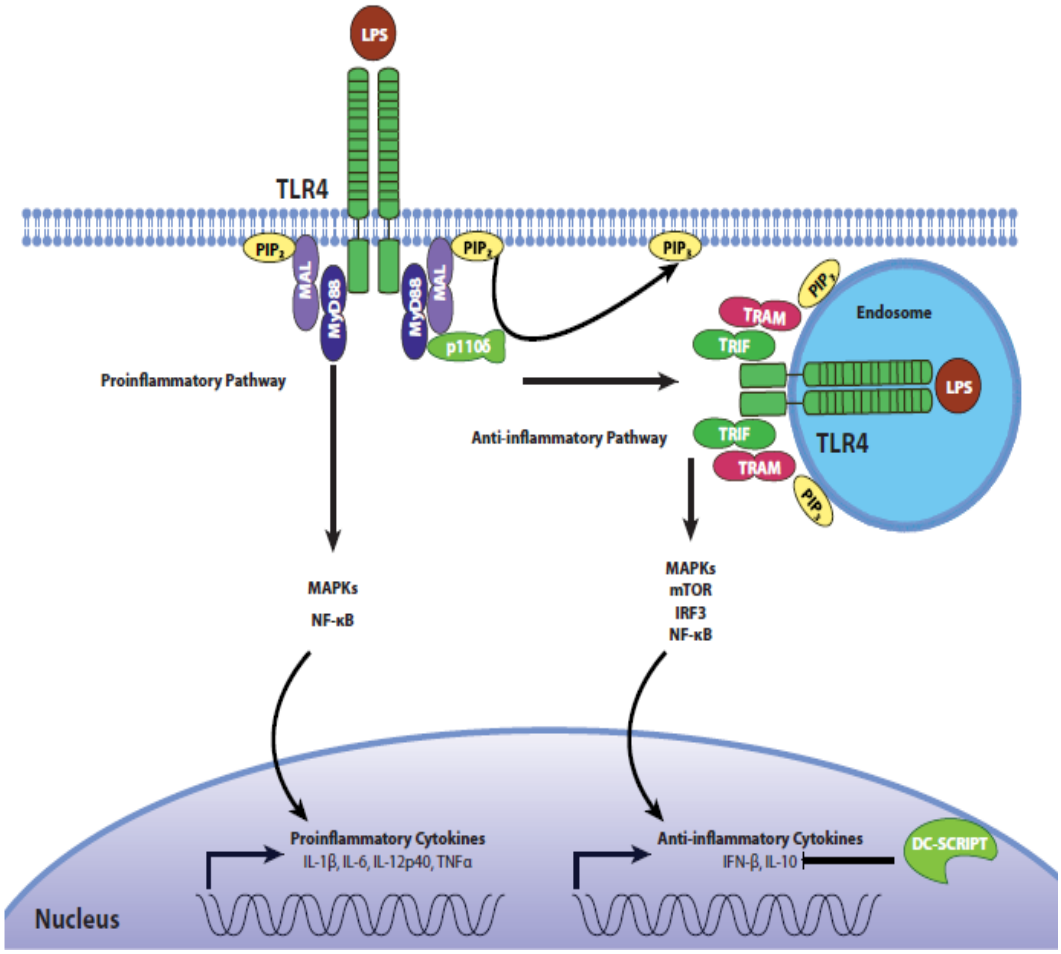


Figure 2: Pro- and anti-inflammatory signaling pathways of LPS-TLR4 [16]

Inflammatory mediators of activated macrophages

During inflammation, activated macrophages produce and release plethora of enzymes and mediators with diverse functions in inflammatory response. These enzymes and mediators are pro-inflammatory cytokines, chemokines, inducible nitric oxide synthase (iNOS) for nitric oxide (NO) production, cyclooxygenase 2 (COX-2) and microsomal prostaglandin E synthase 1 (mPGES-1) for prostaglandin E_2 (PGE_2) production and other several mediators. Most of these enzymes and inflammatory mediators are expressed and produced only in activated macrophages (Figure 2).

Cytokines

During both acute and chronic inflammatory processes, a variety of extracellular soluble materials mediate in the regulation of the resident cells such as mast cells, endothelial cells, tissue macrophages and fibroblasts [19]. The systemic responses to the inflammatory process (*e.g.* fever, hypotension, leukocytosis) attribute to the newly recruited inflammatory cells (monocytes, lymphocytes, neutrophils, and eosinophils), and some of the mediators unleashed from them. NO and a group of small proteins, known as cytokines, involve in four main categories of these soluble factors that mediate inflammatory responses [8].

Cytokines are small proteins that are released by various cells in the body, usually in response to an activating stimulus. They are key players in orchestrating inflammation with the regulatory effects on various target cells [8] [19]. In the

process of inflammation, pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and chemokines are usually generated very early from activated macrophages in response to many stimuli such as microorganisms or LPS. These cytokines and chemokines induce many events of inflammation for eliminating microorganisms. They have both beneficial and detrimental effects depending on their concentration, duration of expression, levels of their receptors and concentration of their inhibitors [20].

Tumor necrosis factor α (TNF-α)

It is a potent inflammatory mediator that is central to the implementation of inflammatory action of the innate immune system, including induction of cytokines, chemokines and other inflammatory mediators, activation or expression of adhesion molecules, and growth stimulation. It is a key cytokine in orchestrating the inflammatory response that includes both systemic and local responses [19]. The main source of TNF-α is activated mononuclear phagocytes. The most potent stimuli for initiating TNF-α production are LPS and other microbial products. TNF-α acts on receptors of TNF-R family [20].

Interleukin-1 (IL-1)

IL-1 is also a potent pro-inflammatory cytokine in the inflammatory response [21]. It is produced by multiple cell types, mainly monocytes and macrophages.

Gene expression of IL-1 can be induced by stimulating pattern receptors (PRRs) including Toll-like receptors (TLRs) [19]. IL-1 shares important inflammatory property with TNF-α in increasing synthesis of inflammatory mediators and promoting inflammation. It also triggers fever by increasing PGE_2 production [19] [8].

Interleukin-6 (IL-6)

IL-6 is a pleiotropic inflammatory cytokine produced by monocytes, macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. IL-6 involves in a series of biologic processes, dealing with T cell activation and differentiation, stimulation of the growth factor for B cell maturation to plasma cell [22]. Liver synthesis of acute phase protein is also stimulated by IL-6. In addition, IL-6 induces changes in the levels of complement proteins, fibrinogen, C-reactive protein, and serum amyloid A [19] .

Table 1: Properties of IL-1, IL-6 and TNF-α

	IL-1	IL-6	TNF-α
Producers	Macrophages, keratinocytes, endothelial cells, T cells, B cells, astrocytes, microglia, fibroblasts	Macrophages, T cells, B cells, endothelial cells, fibroblasts, astrocytes, mesangial cells	Macrophages,T cells, NK cells
Biological activities	Induction of : PGE_2 synthesis Growth of fibroblasts Fever Sleep Anorexia Synthesis of collagenase Growth and differentiation of T and B cells	Induction of : B cell differentiation Acute phase protein synthesis T cell activation and differentiation Macrophage differentiation Mesangial cell growth	Tumoricidal activity Inhibition of lipoprotein lipase Induction of bone resorption Growth and differentiation of B cells

Interleukin-10 (IL-10)

IL-10, an anti-inflammatory cytokine, is produced by a variety of cell types, including B cells, T cells, monocytes and macrophages [23]. The special function of this cytokine includes prevention and controlling of over-reacting immune reactions and subsequent tissue damage. It has been proved to suppress a wide range of inflammatory responses and shown to be as an important mediator in balancing overall immune responses [24]. It is both produced by, and inhibits macrophage functions showing an excellent example of a negative feedback regulator. IL-10 gene transcription of macrophages is induced after exposure with a variety of endogenous and exogenous mediators such as bacterial lipopolysaccharide (LPS). It has been observed that all monocytes and macrophages are the main target cells of the inhibitory IL-10 effects [25] [24]. Generally, IL-10 can inhibit many functions of macrophages including secretion of pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 [26] [23].

Chemokines

Chemokines are a class of cytokines that have chemo attractant properties inducing cells to migrate towards the source of chemokines (Figure 3) [27]**.** Several chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are produced by activated macrophages. The

main stimuli for production of such chemokines are bacterial products such as LPS [28].

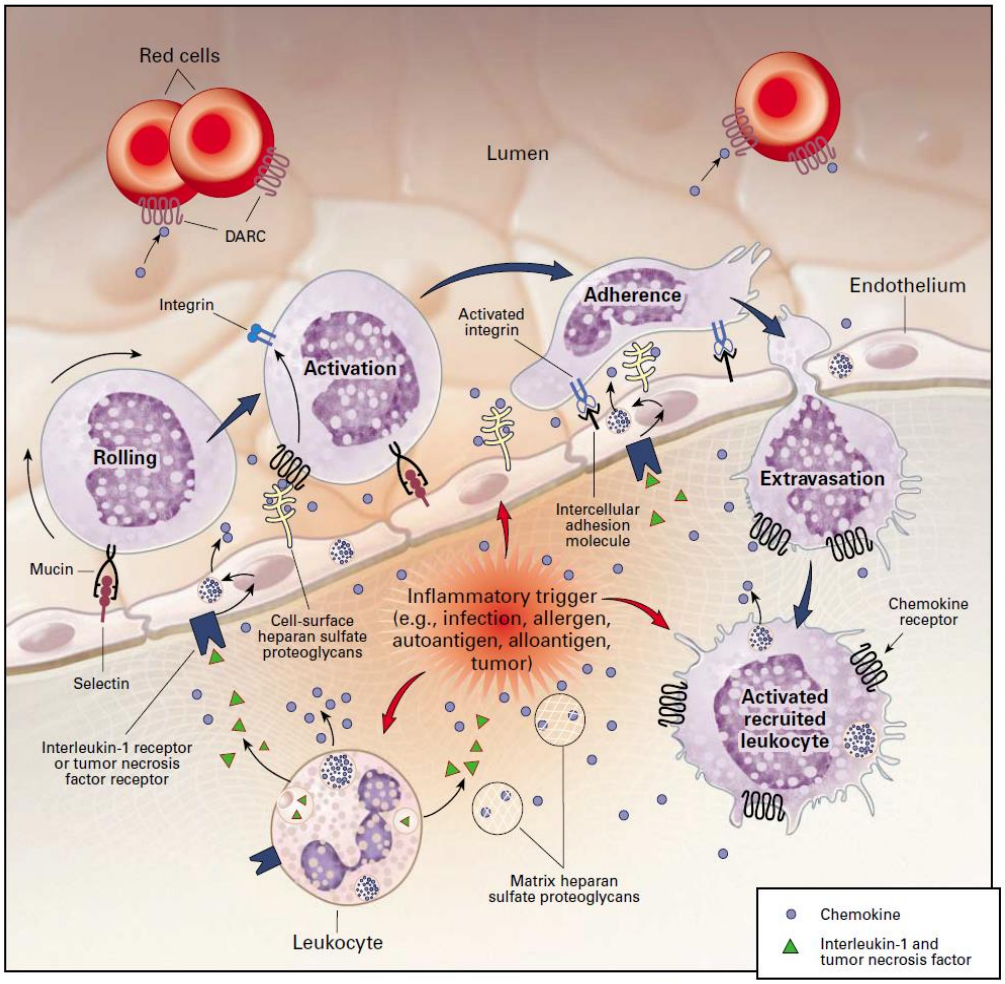


Figure 3: Leukocyte movement by chemotaxis [28]

Macrophage chemoattractant protein-1 (MCP-1)

Migration and infiltration of monocytes and macrophages are adjusted by one of the key chemokines named monocyte chemoattractant protein-1 (MCP-1) [29]. Resident macrophages in inflamed tissues release MCP-1 and cytokines to activate vascular endothelial cells. The activated endothelial cells express adhesion molecules to interact with the ligands on the surface of leukocytes, resulting in increased recruitment of monocyte to site of infection. Nevertheless, the significant sources of MCP-1 are monocytes and macrophages. A variety of cell types such as mesangial, endothelial, epithelial cells and microglial cells also generate MCP-1 [29] [30] [31] [32] [33]. Induction of MCP-1 in various disease conditions, specifically, allergic asthma, inflammatory bowel disease and rheumatoid arthritis have been demonstrated [34].

Monocyte inflammatory protein-1α (MIP-1α)

MIP-1α has ability of chemotaxis induction to eosinophils and macrophages to inflammatory site. The release of chemokines like MIP-1α is a crucial step in cell recruitment necessary during initiation and maintenance of inflammatory responses. MIP-1 is produced in a variety of cell types such as monocytes, macrophages, T and B lymphocytes , NK cells, mast cells and dendritic cells [35] [36]. In macrophages, its production can be induced by LPS, TNF-α, IFNγ, IL-1α/β. In contrast, MIP-1

expression can be down-regulated by IL-4, IL-10, dexamethasone or other anti-inflammatory signals [35].

Nitric oxide and inducible nitric oxide synthase

NO is an important intracellular and intercellular signaling molecule involved in the management of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. The production of NO in the body is catalyzed by a family of enzymes called nitric oxide synthases (NOSs). Currently, at least three distinct isoforms of NOS have been isolated and cloned: endothelial NOS (eNOS, NOS I), inducible NOS (iNOS, NOS II) and neuronal NOS (nNOS, NOS III). Two enzymes, eNOS and nNOS isoforms, designated as constitutive NOSs, are constantly present in resting cells. The third isoform, inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory cytokines, bacterial products (LPS) or infection. After a two-step oxidative reaction of L- arginine, NO and L-citrulline are formed (Figiure 4) [37] [38]. The amount of NO produced by iNOS is micromolar level which is much higher than the nanmolar level synthesized from the constitutive eNOS and nNOS [39] [40]. Transcription factors (NF-kB, STAT-1α and AP-1) participate at the transcription level of production of this mediator [37].

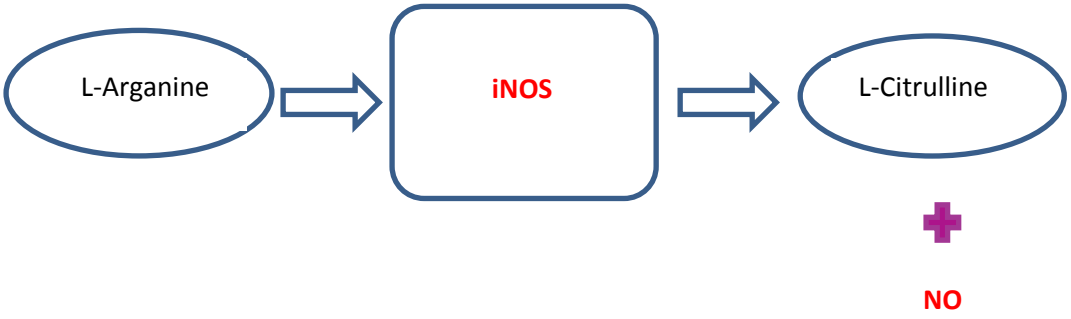


Figure 4: Formation of NO by the reaction catalyzed by NOS (modified from [41])

NO is a strong pro-inflammatory mediator that increases vascular permeability in inflamed tissue. On the other hand, it is a nitrogen free radical which can act as a cytotoxic mediator in pathological processes, particularly in inflammatory disorders. It can also combine with super oxide to become peroxynitrite which also has detrimental effect to the cells [42] . High level of NO not only is toxic to undesired microbes, parasites or malignant cells but may also damage healthy tissues [37]. Therefore, inhibition of iNOS which in turn can inhibit production of NO may be beneficial for the treatment of inflammatory diseases [43].

Cyclooxygenase 2, microsomal prostaglandin E synthase 1, and prostaglandin E_2

PGs are bioactive signaling molecules derived from a 20-carbon unsaturated fatty acid, arachidonic acid [1]. They are both responsible for sustaining homeostatic functions and mediating pathogenic mechanisms which include the inflammatory response. Generally, amount of PGs are increased immediately in inflammation

before the leukocytes are recruited and other immune cells are infiltrated [1]. Among the prostanoids, PGE_2 is the most common and abundant prostanoid which has potent bioactivities [44] [45]. PGE_2 can regulate the activities of many cell types including macrophages and dendritic cells leading to modification of inflammatory responses and the cardinal signs of inflammation (pain, redness and swelling) [1] [46]. Deregulated PGE_2 synthesis is believed to have a link with a wide extent of pathological conditions [47]. Thus, pharmacological interventions along the PGE_2 synthesis pathway may serve as therapeutic strategies for various inflammatory diseases [48].

Along the pathway of PGE_2 synthesis, COX-2 and mPGES1 are major rate limiting enzymes actively producing PGs according to body's needs [49]. Classically, these enzymes are responsible for the generation of PGE_2 and are up regulated in many inflammatory conditions. Thus, blocking of such enzymes may be useful as useful anti-inflammatory agents with minimal adverse effects [48].

Two different COX enzymes existed had been found by the researchers, now known as cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2). Both of them are targets of non-steroidal anti-inflammatory drugs (NSAIDs) [1]. Of the two COX isoforms, COX-1, expressed constitutively in most tissues, is main source of prostanoids that perform housekeeping functions, such as protection of gastric epithelial cells and homeostasis. Unlike COX1, COX-2 is inducible in response to

cellular transformation, mitogens and cytokines. It is the more essential source of PG formation in inflammatory and proliferative diseases, such as arthritis, other chronic inflammatory diseases and cancer [1]. In inflammation and clinically relevant biological systems, COX2 and mPGES-1 expressions are induced by various inflammatory stimuli in cells and tissues originated from patients and animal disease models, consistent with the requirements of PGE_2 synthesis [50]. During inflammatory process, amount of PGE_2 expressed by COX-2 and mPGES-1 of activated macrophages is higher than that is generated by COX-1 in other cell types under physiological condition [49].

Microsomal prostaglandin E synthase-1 (mPGES-1) is the end-stage enzyme in eicosanoid biosynthesis catalyzing prostaglandin H_2 (PGH_2) to PGE_2 [51] [50]. It functions at the one-step downstream of COX2 in the prostaglandin E_2 (PGE_2) biosynthesis pathway (Figure 5). Thus far, there are three PGES enzymes, mPGES-1, mPGES-2 and cytosolic PGES (cPGES) [52]. Cytokines and growth factors can significantly induce the production of mPGES1 whereas anti-inflammatory glucocorticoids can down regulate it, as in the case of COX-2 [53] [54].

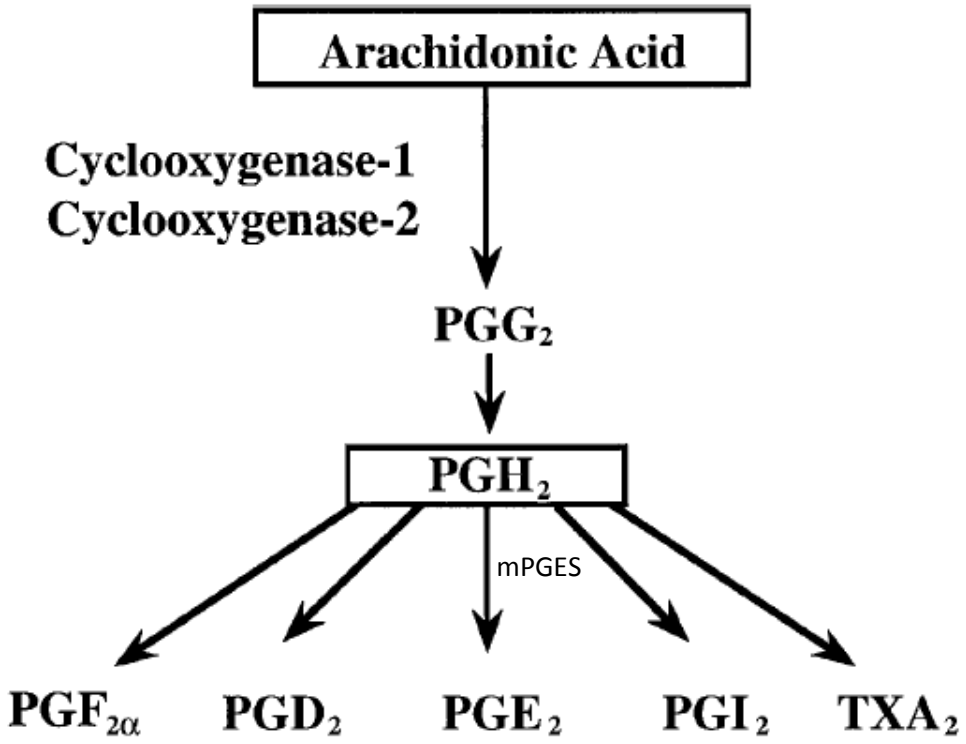


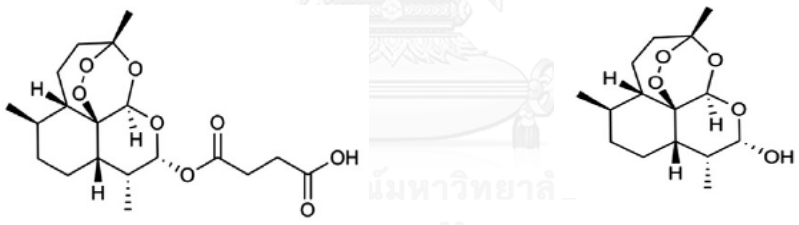
Figure 5: Formation of prostaglandins and other eicosanoids [2]

Artemisinin and its derivatives

Artemisinin was isolated from sweet woodworm, *Artemisia annua L* [3]. This plant has long been used in traditional Chinese medicine to treat chills and fever for more than 2000 years [55]. Researchers have special interest on artemisinin and its derivatives because of their outstanding anti-malarial activity [3]. Artemisinins (artemisinin and all the related derivatives) have been considered as an important neoclassical class of qinghaosu-based highly beneficial, frontline antimalarial agents [56]. Artemisinins, with the established records of safety, were clinically used as potent anti-malarial agents against *Plasmodium falciparum* since few decades ago [5]. They are currently recommended by WHO as first line antimalarial drugs for falciparum malaria as artemisinin based combination therapy (ACT). In these days, many semi-synthetic and synthetic artemisinins such as artesunate (Figure 6), arteether, artemether, dihydroartemisinin (Figure 6), artelinic acid have been developed [4, 5].

Artemisinin and its derivaives demonstrated many pharmacological activities beyond antimalarial action which include antiviral, antischistosomal, anthelmintic, antiprotozoal, antifungal, antiangiogenic, anticancer, antiallergic and anti-inflammatory [57]. It was reported that the monocytes/ macrophage-mediated immune functions were modulated by artemisinins [57]. A transcriptional pathway, NF-kB system, participated in the expression of genes related to inflammation,

adhesion molecules, cytokine production and apoptosis is the most crucial system affected by artemisinins [58]. Among the family of artemisinins, artesunate (AS), a water-soluble hemisuccinate derivative of artemisinin, is the most studied analog due to high oral bioavailability, resulting in a more favorable pharmacological profile [59] [60]. In human body, artesunate is universally converted to the active metabolite, dihydroartemisinin (DHA), which has clinical efficacy of anti-malarial action at least equivalent to its parent compound. Metabolism and pharmacokinetic studies have shown that artesunate (estimated $t_{1/2}$ is 20minutes) is rapidly hydrolyzed to DHA [6]. Due to the rapid conversion, artesunate is often considered as pro-drug of DHA [55].



Artesunate Dihydroartemisinin

Figure 6: Chemical structures of artesunate (AS) and dihydroartemisinin (DHA) [56]

Some of the studies have been reported that artesunate and dihydroartemisinin had anti-inflammatory activity and inhibitory effects on inflammatory mediators both *in vivo* and *in vitro*. An investigational study by Li had shown that there was a dose-dependent inhibition of heat-killed *Staphylococcus*

aureus and peptydoglycan-induced TNF-α release from murine peritoneal macrophage cell lines which were pretreated by artesunate (AS) [61] . A prior study done by the same research team also reported similar findings that there was a dose dependent suppression of AS on CpG oligodeoxynucleotide (CpGODN)-, LPS-, or heat-killed *E. coli*-stimulated TNF-α and IL-6 release from murine peritoneal macrophages by reducing the expression of TLR4 and NF-kB [59]. Konkimalla also demonstrated that artemisinin and five of its derivatives including AS showed a significant inhibition of NO production and iNOS mRNA expression in murine macrophage RAW 264.7 cells [62].

AS was reported to have a protective effect on LPS-induced human umbilical vein endothelial cell (HUVEC) activation and injury, which might be related to the inhibition of TNF-α mRNA expression [63]. A decrease in the secretion of in IL-1, IL-6, and IL-8 in TNF-α stimulated rheumatoid arthritis (RA) or fibroblast-like synoviocytes (FLS) had shown to be related to the actions of AS through inhibition of NF-kB pathway [64]. In both collagen induced rat models and RAW 264.7 cells, AS was found to block the degradation of IkB and significantly inhibit the phosphorylation of ERK1/2 and JNK of mitogen activated protein kinases [65]. AS suppressed the production of NO, iNOS, IL-1 in LPS induced BV2 microglial cells by inhibiting the expression of TLR4, MyD88 and NF-kB [66].The expressions of PGE_2, COX2 and mPGES1 by interfering with NF-kB and p38 MAPK signaling in microglial BV2 cells were

also shown to be significantly decreased by AS [67]. Besides, AS concentrations between 0.1-1.5 μg/mL showed that there was a dose dependent reduction in lymphoproliferation in PHA-stimulated PBMC and WB cultures whereas inhibition by DHA with the same concentrations showed similar effects [68].

The main active metabolite of artemisinin derivatives, DHA was shown to have dose-dependent inhibition on NF-kB, IL-8 and COX-2 in pancreatic BxPC-3 and PANC-1 cells [69]. Moreover, DHA had been reported to have potent inhibitory action on TNF-α production in the culture supernatant of peritoneal macrophages of BXSB mice by decreasing expression of NF-kB, blockage of NF-kB activation and translocation to nucleus, and IkappaB degradation [4] [70]. Furthermore, 12.5-100 μmol/L DHA inhibited the release of inflammatory factors TNF-α, IL-6 and NO by down-regulating iNOS protein in LPS induced RAW264.7 cells [71]. COX2 expression and PGE_2 production in phorbol ester activated RAW 264.7 cells were found to be significantly inhibited by DHA by down-regulating AKT and MAPK pathway [72].

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Cells

Murine macrophage J774A.1 cells from the American Type Culture Collection (Manassa, VA) were used in this study. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 10µg/mL streptomycin. They were maintained at 37°C in a humidified atmosphere of 5% CO_2.

1.2 Chemicals and Reagents

Artesunate (AS) (Sigma-Aldrich Co., Ltd, USA)

Dihydroartemisinin (DHA) (kindly provided by Dr. Myo Myint, Director of Development Center for Pharmaceutical Technology, Ministry of Industry, Yangon, Myanmar)

Dexamethasone (Sigma-Aldrich Co., Ltd, USA)

Disposable cell scrapers (Greiner bio-one, USA)

Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA)

Fetal bovine serum (Gibco, USA)

Penicillin/streptomycin (Gibco, USA)

Sodium bicarbonate (Baker, USA)

Sodium hypochlorite (Clorox, USA)

Potassium hydroxide (Sigma, USA)

Hydrochloric acid (Merck, Germany)

Dimethyl sulfoxide (DMSO) (Sigma, USA)

Ethanol (Merck, Germany)

Lipopolysaccharide (Sigma, USA)

Griess reagent kit (Promega, USA)

Resazurin (Sigma, USA)

Trizol reagent (Invitrogen, USA)

Chloroform (Merck, Germany)

Isopropanol (Merck, Germany)

Diethyl pyrocarbonate (DEPC) (Molekula, UK)

ImProm-II™ reverse transcription system (Promega, USA)

Primer (Bio Basic, Canada)

Sybr green and rox reference dye (Invitrogen, UK)

PGE_2 ELISA kit (Thermo scientific, USA)

1.3 Equipments and Instruments

Autopipette (Gilson, USA)

Biohazard laminar flow hood (ESSCO, USA)

Centrifuge machine (Hettich, USA)

CO_2 Incubator (Thermo, USA)

Filter papers (Whatman®, USA)

Hemocytometer (Brand, Germany)

Light microscope (Nikon, USA)

Microplate reader (Labsystems Multiskan, USA)

Nanodrop (Thermo scientific, USA)

Ph meter (Metler Toledo, Switzerland)

Scrappers (Greiner, UK)

Stepone plus^{tm} real time PCR system (Applied Biosystems, USA)

T25 tissue culture flasks (Corning, USA)

Thermalcycler machine (Eppendorf, USA)

Vortex mixer (Scientific industries, USA)

96 and 24-well plates (Corning, USA)

2. Methods

2.1 Preparation of stock and working solutions

Artesunate (AS) was dissolved in 50% ethanol at a concentration of 25 mM and stored at -20ºC. Working solutions with concentrations of 5, 10, 25, 50 µM at final constant 0.2% ethanol were used to treat the cells. Preparation of stock solution of dihydroartemisinin (DHA) was done by dissolving in 100% DMSO and working solutions with the same concentrations as AS done by diluting the stock solution in 2% DMSO. The final concentration of DMSO was 0.2%. Dexamethasone 2 mg was dissolved in 500 µL of absolute ethanol and then 500 µL of distilled water was added. 20 µM of Dexamethasone was used as a positive control in the final constant 0.2% ethanol.

2.2 Determination of nitric oxide production by Griess reaction assay

J774A.1 cells ($4x10^5$ cells/mL) were cultured in 96-well plate for 20 h. Afterwards, the cells were treated with LPS alone or with AS (5, 10, 25, 50 µM) and DHA (5, 10, 25, 50 µM) for 1 h and then inflammatory response was initiated with 100 ng/mL of LPS for 24 h. In order to determine the percentage of inhibition of NO by using Griess reaction assay kit, supernatants of the treated cells were collected. The left over treated cells were used to evaluate the effects of the test compounds on cell viability by resazurin assay. One hundred (100) µL of the culture supernatant was

reacted with 20 µL of 1% sulfanilamide for 10 min with the subsequent reaction with 20 µL of 0.1% N-[naphthyl] ethylene diamine dihydrochloride for 10 min at room temperature in the dark. OD was measured at 540 nm using a microplate reader.

The percentage of nitric oxide inhibition was calculated in comparison to LPS-stimulated condition as follows:

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

2.3 Determination of cell viability by resazurin reduction assay

After removing the supernatant, cell viability assay was succeeded to assess the effects of AS and DHA on survival of the cells. The assay was based on the reduction of blue resazurin dye to red resorufin product by action of microsomal reductase in viable cells. Non-viable cells rapidly lost the metabolic capacity to reduce resazurin and thus no resorufin was produced. Then, 70 µL of 50 µg/mL resazurin was added to the 24 h treated cells. After that, the cells were incubated at 37°C for 3-4 h. Then plates were read using a microplate reader set to 570 nm wavelength with reference wavelength of 600 nm. The percentage of cell viability was figured out by using the following equation.

$$\% \text{ Cell viability} = (\triangle OD_{sample} / \triangle OD_{control}) \times 100$$

$$\triangle OD = OD_{570nm} - OD_{600nm}$$

2.4 Effects of AS and DHA on mRNA expression of cytokines, chemokine, iNOS and COX-2 in LPS stimulated J774A.1 cells

To determine the mRNA expressions of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-10), chemokines (MIP-1α, MCP-1) and enzymes (mPGES1, iNOS and COX2), 4 x 10^5 cell/mL J774A.1 cells were incubated in 24 well culture plate at 37ºC for 20h. Then the cells were treated with 10-50 µM AS and DHA for 4h and 24h. After that, the treated cells were processed for total mRNA isolation, cDNA transformation and evaluation of gene expressions by qPCR.

Isolation of total RNA from the treated cells

Firstly, all the supernatants were harvested. After that, the cells were lysed by adding 500 µL of Trizol to each well and thoroughly mixed by pipetting up and down. The homogenized samples were incubated at room temperature for 5 min. The samples were transferred to microcentrifuge tubes and subsequently reacted with 200 µL of chloroform. The tubes were forcefully shaken for 15 sec and incubated for 2-3 min at room temperature. After centrifugation at 12,000g for 15 min at 4ºC, the aqueous phase was carefully collected and transferred to new microcentrifuge tubes. 500 µL of isopropanol was added and incubated at 4ºC for an hour to precipitate RNA pellets. Then, RNA pellets were collected by centrifugation at 12,000g at 4ºC for 10 min. The collected RNA pellets were washed by gentle vortexing with 75% ethanol. The washed RNA pellets were air-dried for 5-10 min,

dissolved in DEPC treated water to evaluate RNA concentration and contamination of each sample by Nanodrop at 260 and 280 nm.

Reversion of mRNA to cDNA by reverse transcriptase

Complementary cDNA products were prepared from total RNA samples by using Improm II™ reverse transcription kit by the following procedures:

4 µL of 250 µg/mL of each RNA sample was mixed with 1 µL of oligo (dT) and heated at 70ºC for 5 min and then immediately chilled on ice for 5 min. A mixture of nuclease free water (15µL), 25 mM MgCl2, 10 mM dNTP, ribonuclease inhibitor and reverse transcriptase was added to each tube and cDNA was generated by using thermocycler machine. The conditions used for thermocycler machine were 25ºC for 5 min, 42ºC for 90 min and 70ºC for 15 min. The products of reverse transcription were kept at -20ºC until use.

Determination of gene expression by real time PCR

PCR was performed by using primers of interested genes (Table 1) for pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-10), chemokines (MIP-1α, MCP-1 and enzymes (mPGES-1, COX2 and iNOS) and GAPDH as housekeeping gene. Real-time reactions were set up on StepOnePlus™ Real Time PCR (Therrmo Fisherr Scientific, USA) with the cycling conditions in Table 2. All PCR conditions were adjusted to assure that product formation was on the linear portion of a cycle curve.

Table 2: Sequence and Tm of specific primers, and lengths of PCR products identified in this study

Gene	Primer sequences	Tm°C	Production Length (bp)
TNF-α	F 5'-TTGACCTCAGCGCTGAGTTG-3' R 5'-CCTGTAGCCCACGTCGTAGC-4'	55	364
IL-1	F 5'-CAGGATGAGGACATGAGCADD-3' R 5'CTCTGCAGACTCAAACTCCAC-3'	60	447
IL-6	F 5'GTACTCCAGAAGACCAGAGG-3' R 5'TGCTGGTGACAACCACGGCC-3'	56	308
IL-10	F 5'-GGACTTTAAGGGTTACTTGGGTTGCC-3' R 5'-CATTTTGATCATCATGTATGCTTCT-3'	56	313
MIP-1α	F 5'-GCCCTTGCTGTTCTTCTCTGT-3' R 5'-GGCAATCAGTTCCAGGTCAGT-3'	60	488
MCP-1	F 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3' R 5'-TTCCTTCTTGGGGTCAGCACAGAC-3'	60	618
mPGES-1	F 5'- ATGCCTGCCCACAGCCTG-3' R 5' - TCACAGGTGGCGGGCCGC -3'	56	129
iNOS	F 5'-TGGAGCGAGTTGTGGATT -3' R 5'-GGTCGTAATGTCCAGGAAGT -3'	49	496
COX-2	F 5'-CACTACATCCTGACCCACTT-3' R 5'-ATGCTCCTGCTTGAGTATGT-3'	60	659
GAPDH	F 5'- AAGGTCGGAGTCAACGGATTTGGT-3' R 5'- ATGGCATGGACTGTGGTCATGAGT-3'	60	530

Table 3: The conditions used for qPCR

Gene	Denature	Annealing	Extension	Cycles
TNF-α	50°C for 2 min 95°C for 2 min	95°C for 30s 55°C for 30s 72°C for 30s	95°C for 3 min	40
IL-1	50°C for 2 min 95°C for 2 min	95°C for 30s 60°C for 30s 72°C for 30s	95°C for 3 min	40
IL-6	50°C for 2 min 95°C for 2 min	95°C for 30s 56°C for 30s 72°C for 30s	95°C for 3 min	40
IL-10	50°C for 2 min 95°C for 2 min	95°C for 30s 56°C for 30s 72°C for 30s	95°C for 3 min	40
MIP-1 α	50°C for 2 min 95°C for 2 min	95°C for 30s 60°C for 30s 72°C for 30s	95°C for 3 min	40
MCP-1	50°C for 2 min 95°C for 2 min	95°C for 30s 60°C for 30s 72°C for 30s	95°C for 3 min	40
mPGES-1	50°C for 2 min 95°C for 2 min	95°C for 30s 56°C for 30s 72°C for 30s	95°C for 3 min	40
iNOS	50°C for 2 min 95°C for 2 min	95°C for 30s 49°C for 30s 72°C for 30s	95°C for 3 min	40
COX-2	50°C for 2 min 95°C for 2 min	95°C for 30s 60°C for 30s 72°C for 30s	95°C for 3 min	40
GAPDH	50°C for 2 min 95°C for 2 min	95°C for 30s 60°C for 30s 72°C for 30s	95°C for 3 min	40

2.5 Determination of PGE_2 production by ELISA

J774A.1 cells (4 x 10^5 cells/mL) were grown in 24 well plates for 20 h at 37ºC. Then, cells were treated with either LPS alone or with AS and DHA. Supernatants were harvested and stored in a freezer (-20ºC) until use. Amount of PGE_2 in the supernatants was measured by PGE_2 ELISA kit (Thermo Scientific) according to the following steps.

1. ELISA wells were originally coated with captured antibody which is specific for PGE_2.
2. 100 µL of standard diluent was added into the appropriate wells.
3. 100 µL of the samples and standards were added into the appropriate wells.
4. 50 µL of the reagent diluent and PGE_2-AP conjugate were added into the appropriate wells.
5. Then, the following steps were done by adding 50 µl of PGE_2 antibody into each well.
6. The plate was incubated at room temperature on a plate shaker for 2 h approximately at 500 rpm.
7. The plate was washed for 4 times with 400 µL of the 1X buffer.
8. The plate was firmly tapped on a paper towel to empty any remaining wash buffer after the final wash.
9. Then, 200 µL of the substrate solution was added to each well, and the plate was kept at room temperature for 45 min without shaking.

10. For the final step, 50 µL of stop solution was added to each well in order to stop the reaction and the plate was read immediately by using optical density at 405 nm preferably with correction 570 and 590 nm.

Statistical analysis

All experiments were performed in of 3 independent experiments (n=3). Results were presented as mean ± S.E.M. Data were analyzed by using One-way Analysis of Variance (ANOVA) with Turkey's post hoc test. Statistical significance was assigned for p value < 0.05.

CHAPTER IV

RESULTS

1. Effects of AS and DHA on NO production in LPS stimulated J774A.1 cells

Nitric oxide (NO) is one of inflammatory mediators of activated macrophages at inflammatory site. To investigate the inhibitory effects of AS and DHA on NO production in activated macrophages, LPS-activated J774A.1 cells were treated with 5-50μM AS and DHA for 24 h. AS and DHA significantly decreased NO production in LPS-activated J774A.1 cells in a concentration dependent manner. When compared to the LPS control, AS at 5, 10, 25 and 50 μM significantly decreased NO production by 40.1%, 51.3% and 58% respectively (Figure 7). DHA at 5, 10, 25 and 50 μM significantly decreased NO 47.5 %, 69 % and 83.2 % respectively (Figure 7). The maximal half inhibitory concentrations (IC50) values of AS and DHA were 28.3 ± 3.5 μM and 13.13 ± 2.3 μM respectively. DHA significantly had higher potency than AS for inhibiting NO production in LPS-activated macrophages ($p < 0.05$).

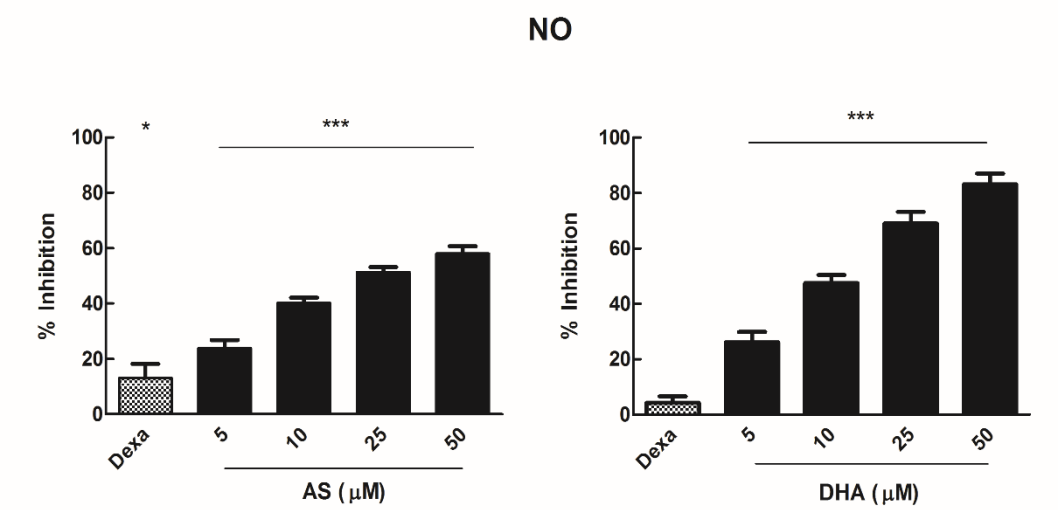


Figure 7: Effects of AS and DHA on NO production in LPS-activated J774A.1 cells. Cells were treated with 100 ng/mL LPS and 5-50 μM AS and DHA for 24 h. NO in the supernatant of the treated cells was determined by Griess reaction assay. The percentage of NO inhibition of AS and DHA was compared to the LPS control. Dexamethasone at 20 μM was used as the positive control. All data are expressed as mean ± S.E.M. of 3 independent experiments. *$p < 0.05$ and ***$p < 0.001$ indicate significance compared to the LPS control.

2. Effects of AS and DHA on viability of J774A.1 cells

Resazurin assay was performed to determine whether the inhibitory effect of the drug is due to the toxic effect on the cells or not. Cells were treated with AS and DHA up to 50 μM concentrations in the presence or absence of LPS. All the samples were compared to the untreated control. There was no significant toxicity of the test compounds in a range of 5-50 μM (Figure 8).

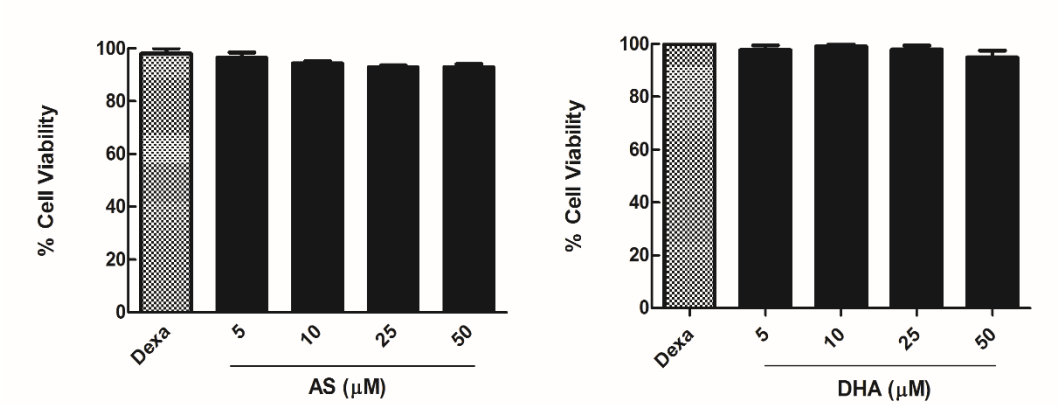


Figure 8: Effects of AS and DHA on viability of J774A.1 cells. Cells were treated with 100 ng/mL LPS and 5-50μM AS and DHA for 24 h. The viability of the treated cells was determined by resazurin assay. The percentage of cell viability was calculated and compared to the untreated control. All data are presented as mean ± SEM for 3 independent experiments.

3. Effects of AS and DHA on mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) in LPS-stimulated J774A.1 cells

Macrophages express and generate pro-inflammatory cytokines when they become activated macrophages. These cytokines are usually not expressed in the resting stage of macrophages. In the present study TNF-α, IL-1 and IL-6 were used as inflammatory markers for evaluating inhibitory effects of AS and DHA on activated macrophages. J774A.1 cells were treated with AS or DHA in the presence of 100 ng/mL of LPS for 4 hr. The expressions of mRNA of the pro-inflammatory cytokines were determined by qPCR. AS and DHA at 10, 25, and 50 μM significantly inhibited mRNA expression of all the studied pro-inflammatory cytokines in LPS activated J774A.1 cells (Figure 9, 10 and 11). DHA was more potent than AS on inhibiting the expression of TNF-α ($p < 0.05$), with IC50 at 30.06 ± 0.29 μM for AS and at 14.83 ± 3.1 μM for DHA. AS and DHA had similar potency for inhibiting IL-1 gene expressions, with IC 50 at 16.85 ± 3.42 μM for AS and 22.59 ± 0.43 μM for DHA. AS was more potent than DHA on inhibiting IL-6 gene expression ($p < 0.05$) with IC50 at 2.89 ± 0.41 μM μM for AS and at 6.53 ± 0.7 μM μM for DHA.

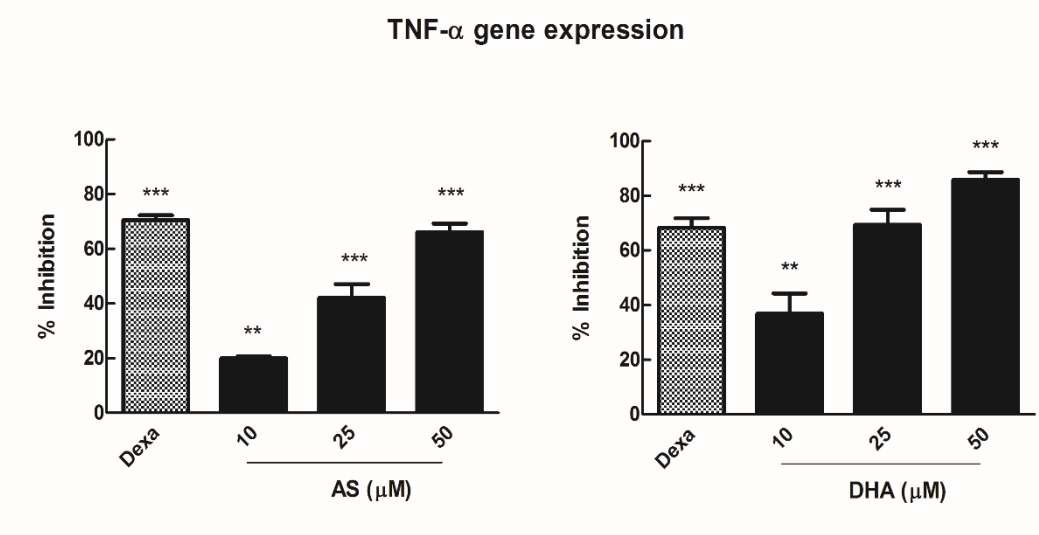


Figure 9: Effects of AS and DHA on the mRNA expression of TNF-α in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as a positive control. The expression of TNF-α was detected by qPCR. The results were presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significance when compared to the LPS control.

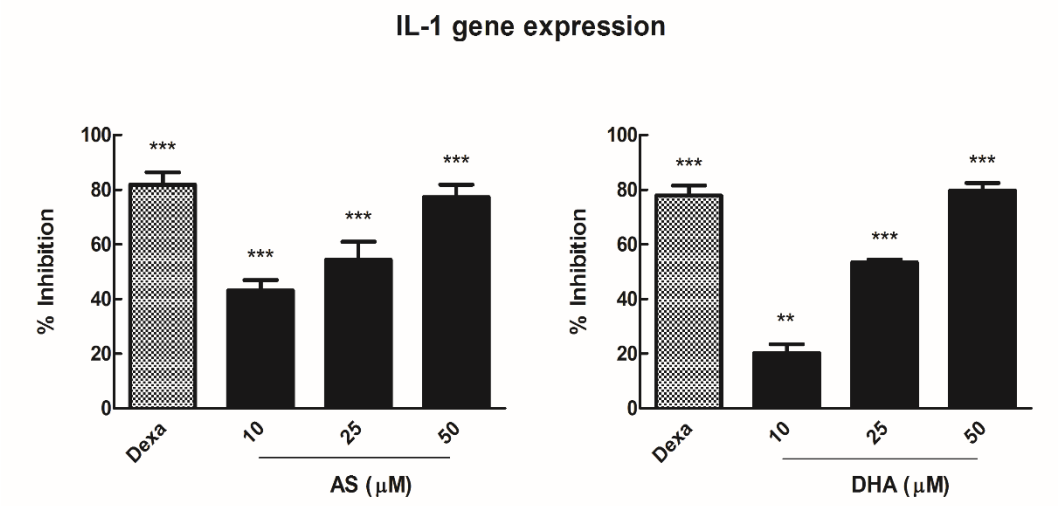


Figure 10: Effects of AS and DHA on the mRNA expression of IL-1 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as positive control. The expression of IL-1 was detected by qPCR. The results were presented as percentage inhibition of gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$) indicate significance when compared to the LPS control.

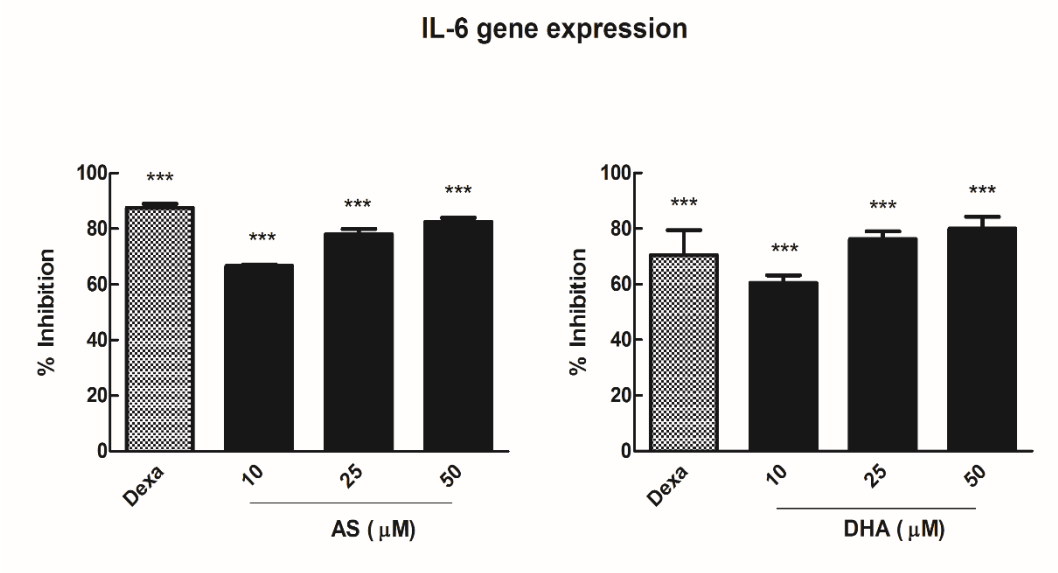


Figure 11: Effects of AS and DHA on the mRNA expression of IL-6 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as positive control. The expression of IL-6 was detected by qPCR. The results were presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. ***$p < 0.001$ indicates significance when compared to the LPS control.

4. Effects of AS and DHA on mRNA expression of anti-inflammatory cytokine (IL-10) in LPS-stimulated J774A.1 cells

IL-10 is an anti-inflammatory cytokine, also produced by activated macrophages as the negative feedback mechanism. Induction of anti-inflammatory cytokine production may be beneficial to reduce chronic inflammatory process. Effects of AS and DHA on the production of this cytokine were also evaluated. J774A.1 cells were treated with AS or DHA in the presence of 100 ng/mL LPS for 4 hr. The expression of mRNA of the IL-10 was determined by qPCR. AS and DHA at 10, 25, and 50 μM stimulated mRNA expression of this cytokine compared to the untreated samples (Figure 12). The stimulatory effects of AS and DHA on IL-10 expression at low concentration were more remarkable than that of high concentrations.

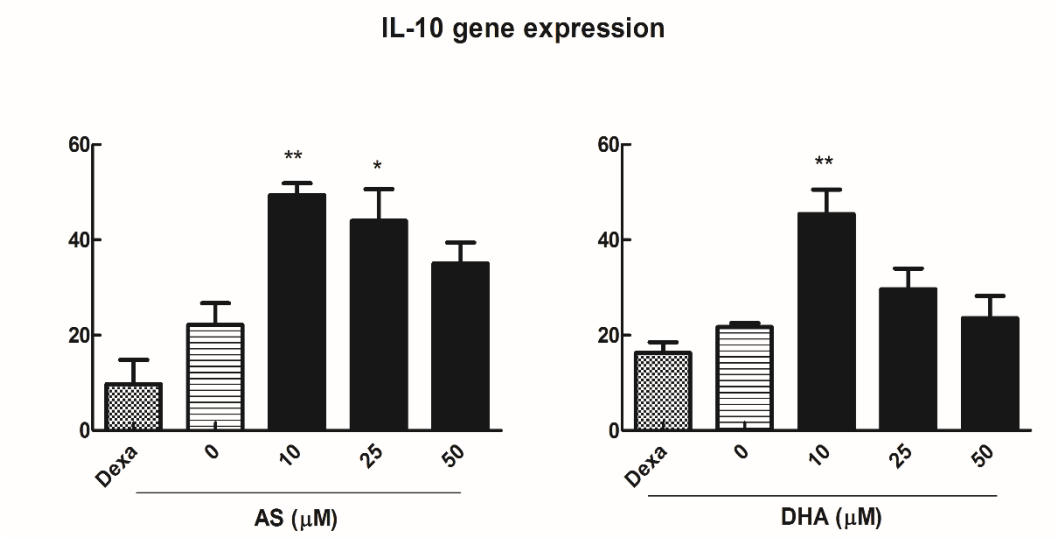


Figure 12: Effects of AS and DHA on the mRNA expression of IL-10 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as positive control. The expression of IL-10 was detected by qPCR. The results were presented as IL-10/GAPDH gene expression compared to the untreated control. All data are representative of 3 independent experiments. $*p < 0.05$ and $**p < 0.01$ indicate significance when compared to the LPS control.

5. Effects of AS and DHA on mRNA expression of pro-inflammatory chemokines (MIP-1α and MCP-1) in LPS-stimulated J774A.1 cells

Production of several chemokines are also induced in activated macrophages during inflammatory process for attracting more leukocytes from the blood circulation to inflammatory site. In the present study, MIP-1α and MCP-1 were used as the representative chemokines from activated macrophages. J774A.1 cells were treated with AS or DHA in the presence of 100ng/mg for 4 hr. The expressions of mRNA of the pro-inflammatory cytokines were determined by qPCR. The results showed that both 10-50 μM of AS and DHA down-regulated the expression of MIP-1α (Figure 13) and MCP-1 (Figure 14) in a concentration dependent manner. AS and DHA had similar inhibitory effects on MIP-1α gene expression with IC50 at 24.25 ± 2.76 μM for AS and at 21.87 ± 3.5 μM for DHA. DHA was more potent than AS for inhibiting MCP-1 gene expression with IC50 at 39 ± 3.84 μM for AS and 7.15 ± 2.26 μM for DHA ($p < 0.05$).

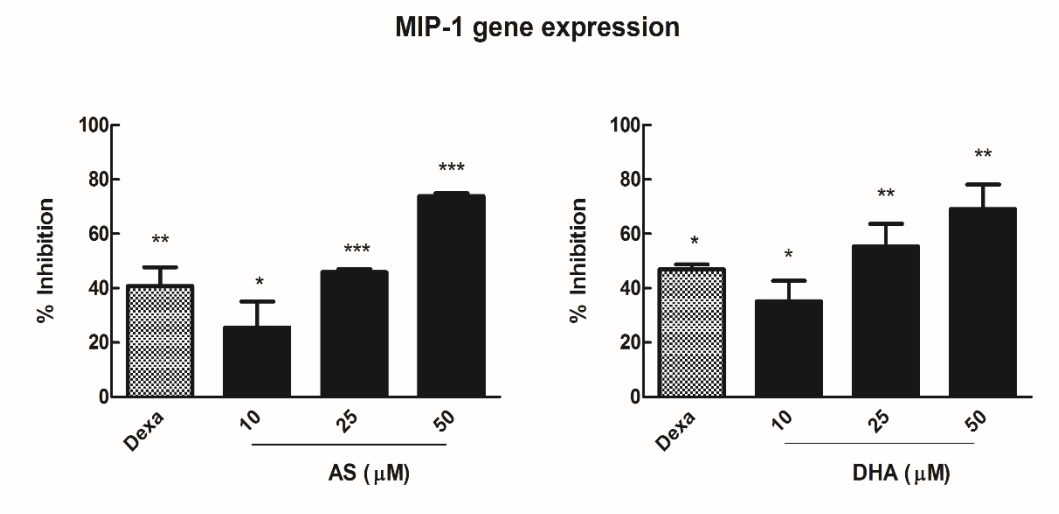


Figure 13: Effects of AS and DHA on the mRNA expression of MIP-1α in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as a positive control. The expression of MCP-1α was detected by qPCR. The results were presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) indicate significance when compared to the LPS control.

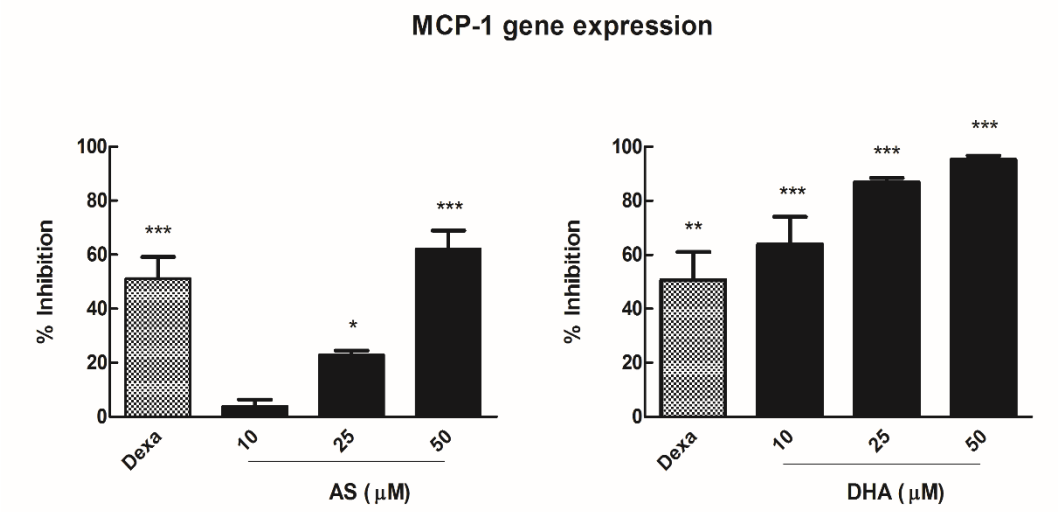


Figure 14: Effects of AS and DHA on the mRNA expression of MCP-1 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 4 h. Dexamethasone at 20 μM was used as a positive control. The expression of MCP-1 was detected by qPCR. The results were presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. *p <0.05, **p < 0.01, and ***p < 0.001 indicate significance when compared to the LPS control.

6. Effects of AS and DHA on mRNA expression of COX2 and mPGES1 in LPS-stimulated J774A.1 cells.

COX2 and mPGES1 are inducible enzymes for massive amount PGE_2 synthesis in activated macrophages. These enzymes were also used as the markers of activated macrophages in the present study in order to explore the inhibitory effects of AS and DHA on activated macrophages. The expressions of COX2 and mPGES1 of J774A.1 cells were assessed after 24 h treatment with 10-50 μM of AS and DHA in the presence of 100 ng/ mL LPS. Both AS and DHA similarly inhibited LPS-induced COX2 (Figure 15) and mPGS1 expression (Figure 16) in activated macrophages. They inhibited COX2 gene expression with IC50 at 29.68 ± 5.31 μM for AS and at 37.22 ± 6.73 μM for DHA, and mPGES1 gene expression with IC50 at 13.48 ± 2.4 μM for AS and at 14.57 ± 2.28 μM for DHA.

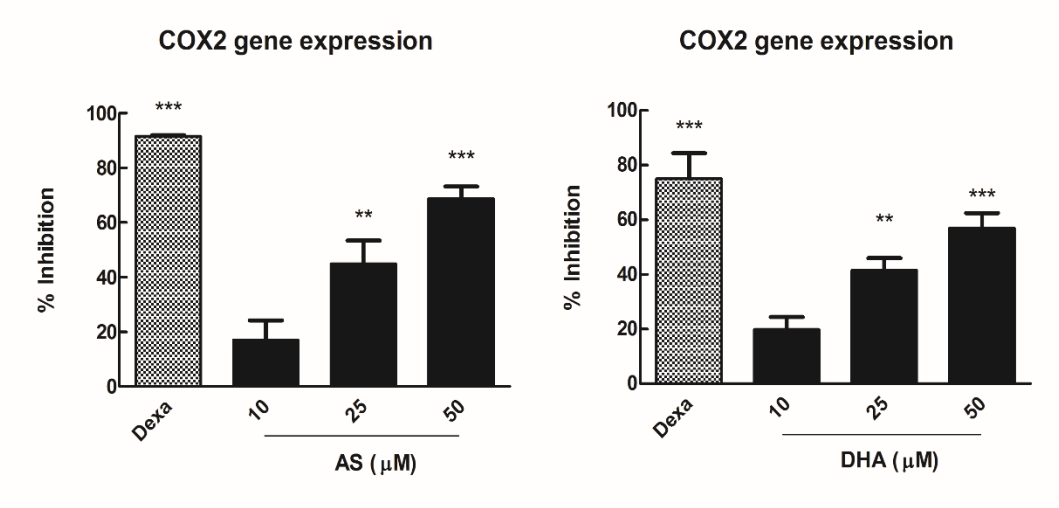


Figure 15: Effects of AS and DHA on the mRNA expression of COX2 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 24 h. Dexamethasone at 20 μM was used as a positive control. The expression of COX2 was detected by qPCR. The results are presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significance when compared to the LPS control.

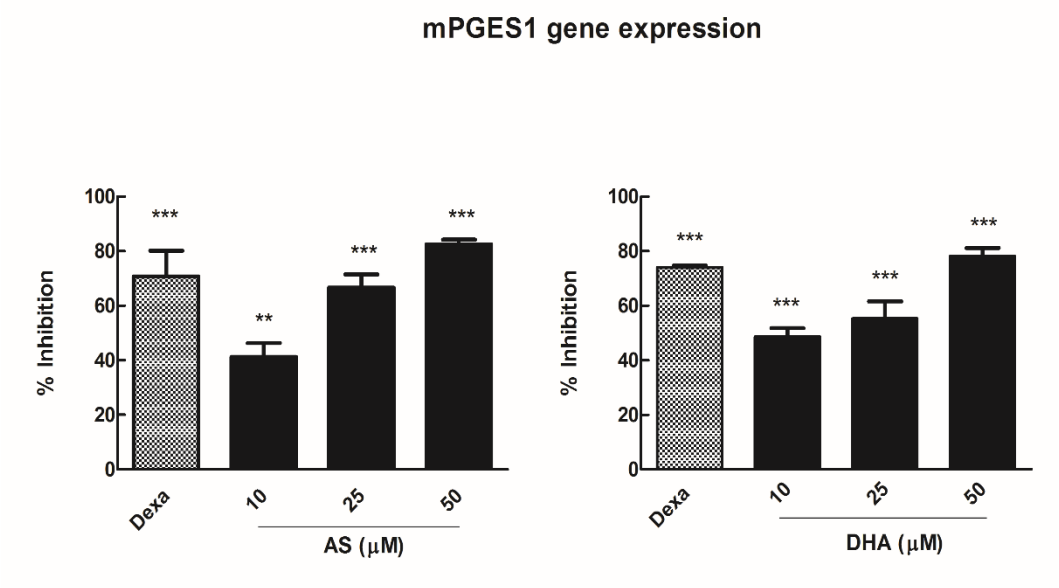


Figure 16: Effects of AS and DHA on the mRNA expression of mPGES1 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 24 h. Dexamethasone 20 μM was used as a positive control. The expression of mPGES1 was detected by qPCR. The results are presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. $^{**}p$ <0.01 and $^{***}p$ < 0.001 indicate significance when compared to the LPS control

7. Effects of AS and DHA on mRNA expression of iNOS in J774A.1 cells

iNOS is an inducible enzyme accountable for the production of large amount of NO production in activated macrophages. The inhibitory effect of AS and DH on NO production in LPS activate macrophages was confirmed by evaluating their effects on iNOS gene expression. The expression of iNOS of J774A.1 cells were assessed after 24 h treatment with 10-50 μM AS and DHA in the presence of 100 ng/ml LPS. Both AS and DHA attenuated iNOS expression in LPS activated macrophages (Figure 17). The effects of AS and DHA on iNOS were correlated with their effects on NO production in LPS activated macrophages. AS and DHA had similar inhibitory effects on iNOS gene expression with IC50 at 6.79 ± 3.92 μM for AS and at 6.14 ± 2.74 μM DHA.

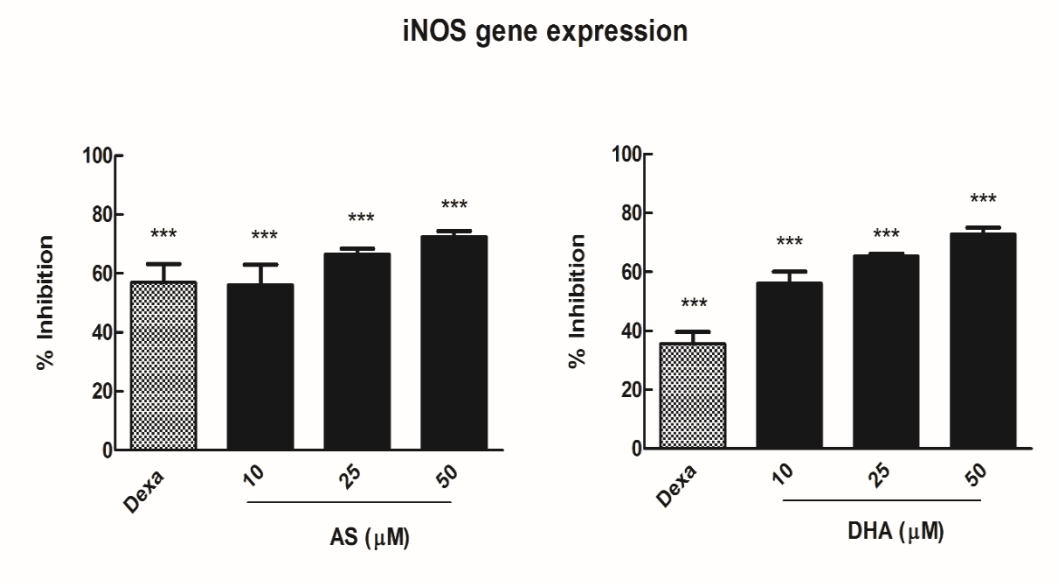


Figure 17: Effects of AS and DHA on the mRNA expression of iNOS1 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 24 h. Dexamethasone at 20 μM was used as a positive control. The expression of iNOS was detected by qPCR. The results are presented as the percentage of inhibition in gene expression by AS and DHA compared to the LPS control. All data are representative of 3 independent experiments. ***$p < 0.001$ indicates significance when compared to the LPS control.

8. Effects of AS and DHA on PGE_2 production in LPS-stimulated J774A.1 cells

PGE_2 is one of the most well-known inflammatory mediators from activated macrophages. It is biologically active in varieties of inflammatory conditions exhibiting cardinal features of inflammation. The inhibitory effects of AS and DHA on PGE_2 production in LPS activated macrophages were evaluated by ELISA. The levels of PGE_2 of J774A.1 cells were assessed after 24 h treatment with 10-50 μM of AS and DHA in the presence of 100 ng/mL LPS. AS and DHA had similar potentcy on inhibiting PGE_2 production in activated macrophages (Figure 18) with IC50 at 3.64 ± 2.32 μM for AS and at 1.77 ± 0.79 μM DHA. These results were correlated to their inhibitory effects on the gene expressions of COX2 and mPGES1, the enzymes involved in PGE_2 production.

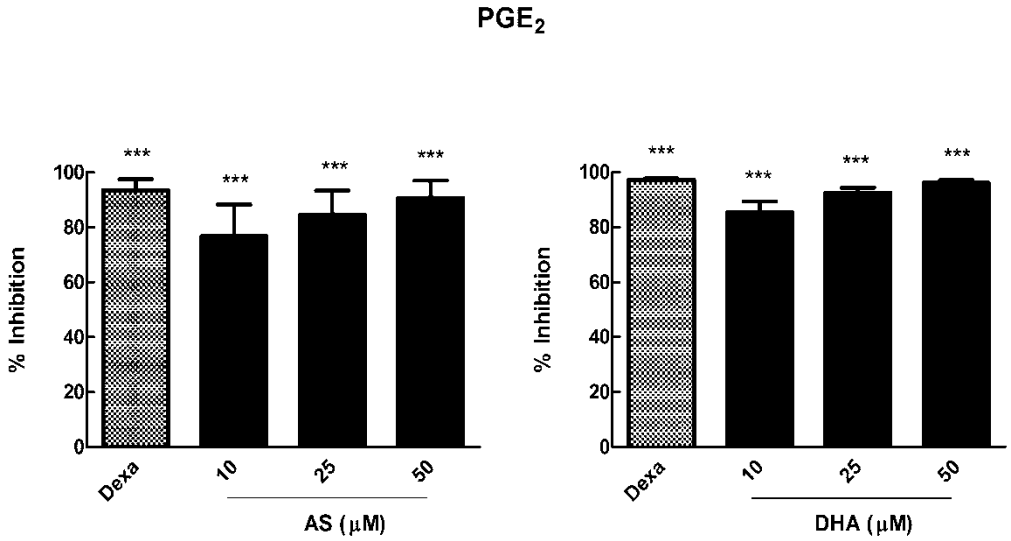


Figure 18: Inhibitory effects of AS and DHA on the protein expression of PGE_2 in J774A.1 cells. Cells were treated with 10-50 μM AS and DHA in the presence of LPS 100 ng/mL for 24 h. Then protein levels of PGE_2 were detected by ELISA. Dexamethasone at 20 μM was used as a positive control. The results were presented as the percentage of inhibition in protein expression by AS and DHA compared to the LPS control. All data are presented as mean ± SEM for 3 independent experiments. ***$p < 0.001$ indicates significance when compared to the LPS control.

9. **Comparison of the potency of AS and DHA on LPS-stimulated J774A.1 cells**

The comparison between the inhibitory effects of AS and DHA on the inflammatory mediators and the enzymes determined in this study, compared by using their IC50 values, was summarized in Table 4.

Table 4: Comparison between the potency of AS and DHA

	Potency (IC50)
Cytokines and chemokines	
IL-1 and MIP-1α	AS = DHA
TNF-α, MCP-1	DHA > AS
IL-6	AS > DHA
iNOS	AS = DHA
NO	DHA > AS
COX2 and mPGES1	AS = DHA
PGE_2	AS = DHA

CHAPTER V

DISCUSSION AND CONCLUSION

Inflammation is a complex biological response characterized by redness, pain, swelling leading to body's system or organ dysfunction. Chronic inflammation contributes to many serious chronic diseases such as septicaemic shock, cancer, diabetes, atherosclerosis and its consequent diseases. It is a major global health problem which causes increased prevalence of morbidity and mortality [73]. Suppression of inflammatory process is an interesting field of research for treating various chronic inflammatory diseases. Activated macrophages are the major target cells for suppressing chronic inflammation. They play a crucial role in chronic inflammation by generating various cytokines and mediators involve in inflammation and many other homeostatic processes. Suppression of inflammatory process is an interesting field of research for treating various diseases. Many researchers have reported that lipopolysaccharide (LPS) is a powerful bacterial factor that can trigger inflammation, especially in Gram-negative infections [74] [20].

Inhibition of the production and activities of the inflammatory cytokines and mediators is the main strategy for preventing and treatment of inflammatory diseases. For searching novel anti-inflammatory agents, LPS-stimulated macrophages have been commonly used as the representatives of activated macrophages at inflammatory sites for determining anti-inflammatory activities of the test agents [75].

In this study, artesunate and its active metabolite dihydroartemisinin were interesting test compounds for searching their potential roles as inflammatory agents. Their effects on various inflammatory mediators and enzymes were determined in LPS-activated macrophage J774A.1 cells.

Pro-inflammatory cytokines, IL-1, IL-6, and TNF-α, are key mediators of activated macrophages. They have several beneficial activities in acute inflammation but large amount of these cytokines for a long time in chronic inflammation causes tissue damage in several chronic inflammatory conditions [19] [75]. Therefore, the suppression on the production these cytokines should be a potential strategy for treating inflammation. In this study, AS and DHA significantly inhibited the mRNA expression of these pro-inflammatory cytokines in LPS activated J774A.1 cells. These two compounds had similar inhibitory effects on the expression of IL-6. DHA had higher inhibitory effect than on the mRNA expression of TNF-α. AS demonstrated higher inhibitory effect on the expression of IL-1 than the effect of DHA. The inhibitory effects on pro-inflammatory cytokines of AS and DHA in this study were correlated to previous studies. It has been reported that AS suppressed CpG oligodeoxynucleotide, LPS, or heat-killed E. coli-stimulated TNF-α and IL-6 release from murine peritoneal macrophages [59]. DHA significantly reduced TNF-α level in the sera of SLE model BXSB mice. It also inhibited TNF-α production from the peritoneal macrophages of these mice [70]. Another study revealed that DHA at the

concentrations 12.5 - 100 μM inhibited TNF-α and IL-6 production in LPS-stimulated macrophage RAW 264.7 cells [71]. AS decreased IL-1 level in rat hind paws of collagen induced arthritis rats. And the anti-arthritic effect of artesunate was associated with suppression of NF-kB and MAPK signaling pathway [65]. AS inhibited LPS induced IL-1 expression in a concentration-dependent manner in murine microglial cells [66]. The results from this study confirmed anti-inflammatory effects of AS and DHA.

In addition to pro-inflammatory cytokines, chemokines from also participate in inflammatory response. They are usually released from activated immune cells as well as activated macrophages at inflammatory sites. They induce the migration of neutrophils, monocytes, and lymphocytes form blood circulation to accumulate at tissue injury sites for generating local inflammatory response [28]. This study demonstrated the inhibitory effects of AS and DHA on the mRNA expression of two chemokines, MCP-1 and MIP-1α, in LPS-activated J774A.1 cells. AS and DHA significantly and similarly inhibited the expression of MIP-1α in a concentration dependent manner. DHA had inhibitory effect on the expression of MCP-1 higher than the effect of AS. The effects of AS and DHA on these chemokines have not been documented. The results from this study suggested that AS and DHA may be able to decrease the recruitment of more leukocytes from the blood circulation to inflammatory sites supporting their anti-inflammatory activities.

This study also investigated the effects of AS and DHA on NO production and iNOS expression in LPS activated J774A.1 cells. AS and DHA significantly inhibited NO production in the activated macrophages, in a concentration dependent manner. Evidently, DHA demonstrated higher inhibitory activity on the production NO than AS. It is well known that iNOS is the key enzyme for large amount of NO production in activated macrophages [66] [76].

In this concern, the inhibitory effects of AS and DHA on NO production in activated macrophages were confirmed by determining their effect on iNOS expression. NO generated by iNOS has several roles in the immune system. It involves in pahgocytosis for killing micro-organism in phagolysosomes and in inflammatory process by acting as one of inflammatory mediators [77] [37] [78]. Sustained iNOS expression and NO production contribute to persistent inflammation and tissue destruction in chronic inflammatory conditions [79]. The inhibition of iNOS expression and NO production in activated macrophages should be a potential strategy for attenuating chronic inflammatory diseases. The inhibitory effects on NO production and iNOS expression of AS and DHA in this study supported the results from previous studies. DHA inhibited NO production in LPS-activated RAW264.7 cells and decreased iNOS level in LPS treated RAW264.7 cells [71]. Among other derivatives of Artemisinin, AS had maximum inhibitory effects on iNOS mRNA expression and NO production in macrophage RAW 264.7 cells [62]. It also

suppressed LPS-induced NO production and iNOS level in murine BV2 microglial cells in a dose dependent manner [66].

The effects of AS and DHA on the most well-known inflammatory mediator, PGE_2, were also evaluated in this study. Both AS and DHA potently inhibited PGE_2 production in LPS-activated J774A.1 cells, starting from lowest concentration (10 μM) in this study. In activated macrophages, large amount of PGE_2 is generated by the inducible enzymes COX-2 and mPGES-1. Expression of these enzymes is induced by microbial products and pro-inflammatory cytokines at inflammatory sites. COX2 expression and production are the targets of clinically used anti-inflammatory drugs, corticosteroids, non-steroid anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors [80]. In this study, LPS activated mRNA expression of both COX-2 and mPGES-1. AS and DHA significantly and similarly inhibited the expression of these two enzymes, in a concentration dependent manner. mPGES-1 is the end-stage enzyme in PGE_2 biosynthesis in activated macrophages. It catalyzes the conversion of PGH_2 to PGE_2 [51] [50]. This enzyme is expected to be a new target as anti-inflammatory agent in order to overcome the cardiovascular adverse effects of COX2 inhibitors [81] [82]. The inhibitory effects of AS and DHA on COX2 expression and PGE_2 production were correlated to previous reports. DHA effectively attenuated PMA-induced COX2 mRNA expression and protein level, in a dose dependent manner, via down-regulation of AKT and MAPK pathway in RAW 264.7 cells. It's inhibitory action on

PGE_2 production had also been evaluated in the same study [72]. AS significantly inhibited PGE_2 production in LPS and IFNγ-activated BV2 microglia cells. This effect was found to be mediated by the reduction in COX-2 and mPGES-1 proteins and the interference with NF-kB and MAPK signaling pathways [67].

Effects of AS and DHA on mRNA expression of IL-10 were also investigated. Both AS and DHA at lower concentration significantly increased the expression of IL-10 in LPS-activated J774A.1 cells. IL-10 is an anti-inflammatory cytokine produced from activated macrophages as the negative feedback regulator in acute inflammation. It inhibits many functions of activated macrophages as well as the release of inflammatory mediators [26] [23]. AS enhanced the production of IL-10 in splenocytes stimulated by concanavalin A at the concentration less than 30 μM. But it inhibited the production of IL-10 proteins at concentration more than 30 μM [83].

Any compound that can inhibit pro-inflammatory mediators as well as stimulate anti-inflammatory mediators should have potential to become anti-inflammatory agent. They should be able to attenuate chronic inflammatory response. This study demonstrated that AS and DHA had these properties.

This study also proved that the inhibitory effects of AS and DHA on the expression and production of inflammatory mediators and involving enzymes did not come from their cytotoxic effects on J774A.1 cell. The compounds (AS and DHA) at

the concentrations used in the study (5-50 μM) did not significantly change the viability of the treated cells.

In summary, this study demonstrated similar anti-inflammatory effects of AS and DHA on LPS activated J774A.1 cells. They inhibited mRNA expressions of the inflammatory cytokines IL-1, IL-6, TNF-α and chemokines MIP-1α and MCP-1 in LPS-activated macrophages. They decreased NO production by inhibiting the expression of iNOS. They decreased PGE_2 production by reducing the expression of COX-2 and mPGES-1. They increased mRNA expression of anti-inflammatory cytokine IL-10. The results from this study supported the potential use of artemisinin derivatives as anti-inflammatory agents in the future.

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APPENDIX

VITA

I am Thin Sandi Htun, a master student from Myanmar. I was born and graduated my bachelor degree (M.B.,B.S) in Yangon, Myanmar. I was awarded a scholarship from Chulalongkown University. I spent 2 and a half years in Chulalongkorn University for studying master in medical science program.