STUDY OF IMMUNOMODULATORY EFFECT OF WATER EXTRACTS OF FIVE-FLOWER REMEDY AND ITS COMPOSITIONS ON MACROPHAGES

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การศึกษาฤทธิ์ปรับเปลี่ยนภูมิคุ้มกันของสารสกัดน้ำพิกัดเกสรทั้งห้าและองค์ประกอบของพิกัดต่อเซลล์แมคโครฟาจ

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พิกัดเกสรทั้งห้าเป็นพิกัดยาไทยแผนโบราณที่ใช้ บำรุงร่างกายและลดไข้ พิกัดนี้ประกอบด้วยดอกมะลิ ดอกพิกุล ดอกบุนนาค ดอกสารภี และเกสรบัวหลวงในสัดส่วนน้ำหนักที่เท่ากัน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดน้ำของพิกัดและองค์ประกอบทั้ง 5 ชนิดต่อเซลล์แมคโครฟาจ J774A.1 ทำการทดสอบผลของสารสกัดต่อการสร้างไนตริกออกไซด์ด้วยวิธี Griess reaction พบว่า สารสกัดของพิกัด ดอกมะลิ และ เกสรบัวหลวง ที่ 6.25-100 ไมโครกรัม/มิลลิลิตร มีฤทธิ์แรงในการเพิ่มการสร้างไนตริกออกไซด์ ส่วนสารสกัดของดอกบุนนาค ดอกพิกุล และดอกสารภีออกฤทธิ์กระตุ้นได้ต่ำกว่า นอกจากนี้ได้ทำการทดสอบฤทธิ์ของสารสกัดทั้งหกชนิดต่อตัวชี้บ่งของเซลล์แมคโครฟาจที่ถูกกระตุ้นเป็นแบบ M1 และ M2 ด้วยวิธี RT-PCR โดยใช้สารสกัดของพิกัดและดอกมะลิที่ 6.25 – 25 ไมโครกรัม/มิลลิลิตร สารสกัดของเกสรบัวหลวงที่ 25 – 100 ไมโครกรัม/มิลลิลิตร สารสกัดของดอกบุนนาค ดอกพิกุล และดอกสารภีที่ 50 – 200 ไมโครกรัม/มิลลิลิตร สารสกัดทั้งหมดเพิ่มการแสดงออกในระดับอาร์เอนเอของตัวชี้บ่งของเซลล์แมคโครฟาจที่ถูกกระตุ้นชนิด M1 คือ TNF-α, IL-1 และเอนไซม์ iNOS สารสกัดของพิกัด ดอกมะลิ เกสรบัวหลวง และ ดอกบุนนาค เพิ่มการแสดงออกของ IL-6 ซึ่งเป็นตัวชี้บ่งของเซลล์ชนิด M1 เช่นกัน นอกจากนั้นสารสกัดทั้งหมดยังเพิ่มการแสดงออกของตัวบ่งชี้ของเซลล์ชนิด M2 คือ IL-10 สารสกัดของพิกัด ดอกมะลิ เกสรบัวหลวง และ ดอกบุนนาค กระตุ้นการแสดงออกของเอนไซม์ Arg-1 ที่เป็นตัวบ่งชี้ของเซลล์ชนิด M2 ด้วย สารสกัดกระตุ้นเซลล์ J774A.1 ในลักษณะที่คล้ายกับแอลพีเอสที่ทราบอยู่แล้วว่ากระตุ้นเซลล์ให้เป็นชนิด M1 สารสกัดของพิกัดและดอกมะลิมีฤทธิ์สูงสุดในการกระตุ้นแมคโครฟาจ ตามด้วยสารสกัดของเกสรบัวหลวงและดอกบุนนาค ส่วนสารสกัดของดอกพิกุลและดอกสารภีมีฤทธิ์กระตุ้นต่ำสุด

ผลจากการศึกษานี้แสดงว่าสารสกัดน้ำของพิกัดเกสรทั้งห้าและองค์ประกอบอาจกระตุ้นแมคโครฟาจให้เป็นชนิด M1 ด้วยความแรงที่ต่างกัน คาดว่าสารสกัดเมื่อรวมกันในพิกัดมีฤทธิ์เสริมกันในการกระตุ้นเซลล์ และการกระตุ้นแมคโครฟาจอาจเป็นฤทธิ์ทางเภสัชวิทยาของพิกัดในการบำรุงร่างกาย

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NARUMON POONPAIBOONROTE: STUDY OF IMMUNOMODULATORY EFFECT OF WATER EXTRACTS OF FIVE-FLOWER REMEDY AND ITS COMPOSITIONS ON MACROPHAGES. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., CO-ADVISOR: CHAISAK CHANSRINIYOM, Ph.D., 83 pp.

Five-flower remedy is a traditional Thai remedy used for promoting health and reducing fever. It consists of the flowers of *Jasminum sambac, Nelumbo nucifera, Mesua ferrea, Mimusops elengi* and *Mammea siamensis*, in equal weight. This study aimed to investigate effects of the water extracts of the remedy and its components on macrophage J774A.1 cells. Effects of these extracts on production of nitric oxide (NO) were determine by Griess reaction. The extracts of the remedy, *J. sambac* and *N. nucifera*, all at 6.25-100 µg/ml, potently increased NO production. The extracts of *M. ferrea, M. elengi* and *M. siamensis* had less stimulating activities than the extracts mentioned before. Effects of all extracts on mRNA expression of some markers of M1 and M2 activated macrophages were also evaluated by RT- PCR. The extracts of the remedy and *J. sambac* at 6.25 – 25 µg/ml, the extract of *N.nucifera* at 25 – 100 µg/ml, and the extracts of *M. ferrea, M. elengi* and *M. siamensis* at 50 – 200 µg/ml were used. These extracts up-regulated mRNA expression of TNF-α, IL-1, and inducible nitric oxide synthase (iNOS) which are markers of M1 macrophages and IL-10 which is a marker of M2 macrophages. The extracts of the remedy, *J. sambac, N. nucifera,* and *M. ferrea* stimulated the expression of a M1 macrophage marker IL-6 and Arg-1, a marker of M2 macrophages. All six extracts activated macrophage J774A.1 cells in similar manner to LPS which is known to activate M1 macrophages. The extracts of remedy and *J. sambac* had the highest activating activity, followed by the extracts *N. nucifera* and *M. ferrea*, respectively. The extracts of *M. elengi* and *M. siamensis* demonstrated the lowest stimulating effects.

These results suggest that the water extracts of five-flower remedy and its four components may stimulate macrophage J774A.1 cells to M1 activated phenotype with different potencies. It is predicted that all components synergistically activate macrophages when they are combined as the remedy. Activation of macrophages may be a part of pharmacological properties of this five-flower remedy for health promotion.

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Student's Signature .....
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LIST OF ABBREVIATIONS

%	Percent
/	Per
<	Less than
µl	Microliter (s)
µM	Micromolar
µg	Microgram (s)
°C	Degree Celsius
APCs	Antigen presenting cells
Arg	Arginase
ATCC	American Type Culture Collection
cDNA	Complementary DNA
CO_2	Carbon dioxide
COX-2	Cyclo-oxygenase-2
DMEM	Dulbeco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage-colony stimulating factor
h	Hour
HPLC	High performance liquid chromatography
HSV	Herpes simplex virus

IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolyssaccharide
LSD	Least significant difference
M	Molarities
MHC	Major histocompatibility complex
min	Minute
ml	Mililiter (s)
mg	Miligram (s)
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NED	N-1-napthylethylenediamine dihydrochloride
ng	Nanogram (s)
NK cell	Natural killer cell
nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
O_2	Oxygen
OD	Optical density
PAMPs	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PRRs	Pattern recognition receptor

RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reverse transcription
S.E.M.	Standard error of mean
TGF-β	Transforming growth factor-beta
T_h	T helper cell
TNF	Tumor necrosis factor
U	Unit

CHAPTER I
INTRODUCTION

Background and rational

Macrophages are immune cells which perform multi-functions in both innate and adaptive immunity to eliminate pathogens, tissue debris, apoptotic cells, and cancer cells [1]. They are main tissue phagocytes in innate immunity responsible for eliminating tissue invading pathogens by phagocytosis which is the process that macrophages engulf, digest, and kill pathogen in phagolysosomes by oxygen-dependent and oxygen-independent mechanisms [2]. Activated macrophages play role in acute inflammatory process by ingesting pathogens and cell debris at inflammatory site by phagocytosis and by secreting several pro-inflammatory cytokines. These cytokines act on vascular endothelial cells to enhance the recruitment of more leukocytes from the blood into injury or infection sites, thereby amplifying the protective response [3]. Macrophages also function in wound healing process by secreting cytokines and growth factors to remodel tissue during wound repair [4]. Macrophages are a type of antigen presenting cells (APCs) which present antigenic peptides of digested pathogens to activate T helper lymphocytes. T helper cells recognize antigenic peptides in the context of antigenic peptide-MHC class II complexes on (APCs). These T cells are then activated to initiate cell mediated immune response [5]. Macrophages are also effector cells of humoral and cell mediated immunity against pathogens and tumor cells. In effector phase of adaptive immunity, macrophages are activated by antigen-antibody complexes and by cytokines from T cell lymphocytes. Macrophages are activated to perform these functions by various types of pathogen-associated molecular patterns (PAMPs) of microbes and by several host stimuli such as cytokines which are produced in response to injury or infection. These stimuli can generate activated macrophages with different functions [6]. Activated macrophages are classified into M1 and M2 macrophages. Classically M1 macrophages display pro-inflammatory phenotype to protect the body from injury or infections. They eliminate invading pathogens by phagocytosis, secret pro-

inflammatory cytokines [such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12] and many inflammatory mediators in inflammatory process, act as APCs, and function as effector cells in adaptive immune response. Alternatively M2 macrophages suppress immune and inflammatory responses in late-phase inflammation. They inhibit immune response by secreting anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF)-β] and producing arginase (Arg)-1 enzyme which involves in collagen synthesis for wound healing and tissue repair. These two macrophage phenotypes are plasticity. They can change their phenotype depending on their microenvironment and stimuli [7, 8].

Modulation of macrophages should have impact on host response against pathogens and cancer cells. Activation of macrophages is known to be a pharmacological activity of clinically used immunomodulating agents such as aluminum (adjuvant) in vaccines, hematopoietic growth factor GM-CSF, anticancer IL-2, and antiviral drug imiquimod [9]. Many polysaccharides from mushrooms have been reported to have immunostimulating on macrophages and complement proteins [10]. They are widely used as food supplements for health promotion. It is possible that tonic herbs and remedies which are traditionally used to restore health for preventing infection and cancer may also have stimulatory effects on macrophages.

Five-flower remedy is a traditional Thai remedy which used for long time for maintain a good health, promote hearth function, decrease tiredness/dizziness and reduce fever. This remedy is widely used as a part of other traditional remedies such as aromatic remedies for health promotion [11]. The remedy consists of flowers of five plants including *Jasminum sambac*, *Mimusops elengi, Mesua ferrea, Mammea siamensis* and *Nelumbo nucifera,* in equal weight. Some components of the remedy have been reported to have immunomodulatory effects. There is no evidence of immunomodulatory activity of the remedy. This study aimed to investigate immunomodulatory activity of five-flower remedy and its components on macrophage J774A.1 cells. The results from this study may reveal pharmacological evidences for using this remedy in health promotion.

Objective

This study proposed to investigate immunomodulatory effects of the water extracts of five-flower remedy and its components on macrophage J774A.1 cells

Hypothesis

The water extracts of five-flower remedy and its components can activate macrophages.

Research design

Experimental research

Keywords

Five-flower remedy, macrophages, nitric oxide, cytokines, iNOS, Arg-1

CHAPTER II
LITERATURE REVIEWS

The immune system

The immune system is the body's defense system against pathogenic organisms and foreign antigens. This system generates a series of immune response which involves the functions of a network of organs, tissues, cells and mediators, that work together to protect the body. Immune response is broadly classified into innate and adaptive immunity (Fig.1) [6]. Innate immunity is an immune response that generates general protection with less-specific response against antigens. Adaptive immunity develops after appearance of pathogen. It is highly effective and specific response against antigens. Both types of immune response work together as a complicated network for eliminating invading antigens [12].

Innate immunity

Innate immunity is present in the body before exposing antigens. It has ability to generate an immediate response within hours of an antigen encounter in the body. Components of this type of immunity recognize only patterns of antigens that differ from self-antigens. They have no memory, thus they response to each invading antigen in the same way as the first encounter [13]. The main components of the innate immune system are physical and chemical barriers, and immune components. Epithelial surface of the skin works as the physical barrier for protecting entry of pathogens into the body. Saliva hydrolytic enzymes and acidity of the stomach are chemical barriers of the body against pathogens that pass the physical barriers. Immune components of innate immunity are cells and various mediators. Innate immune cells in circulation and tissues are neutrophils, eosinophils, basophils, monocytes, macrophages, natural killer (NK) cells and mast cells. Innate immune mediators include proteins in the complement systems, cytokines from activated innate immune cells, nitric oxide, reactive oxygen species (ROS), antimicrobial peptides and enzymes [6].

Adaptive immunity

Adaptive immunity develops after exposure to an antigen. It takes longer time than innate immunity to generate immune response against invading antigens. It also needs activation signals from the components of innate immunity for generating specific immune response. The main properties of adaptive immunity are highly specific to numerous foreign antigens, more effective in secondary immune response than primary immune response against the same antigens by generating long-life memory lymphocytes after the first time of antigen encounter, and self-tolerance to host tissues [1]. There are two types of adaptive immunity: humoral and cell mediated immunity. Humoral immunity is mediated by antibodies produced from plasma cells which are final development of activated B lymphocytes. Cell mediated immunity is mediated by T lymphocytes. Both humoral and cell mediated immunity need components of innate immunity in effector phase to generate immune response against antigens. Examples of these components are the complement system, NK cells, and macrophages [6].

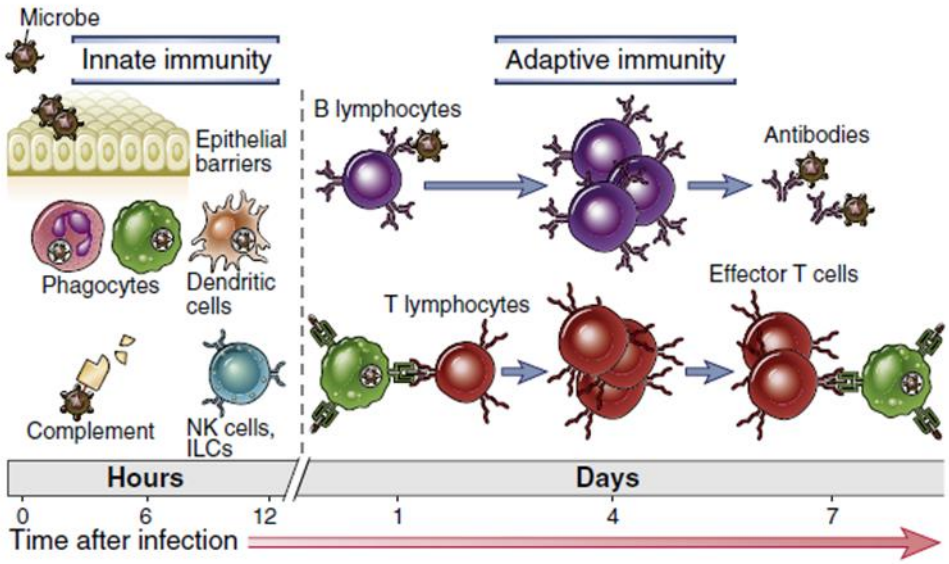

Figure 1: The immune system. Innate immunity is the first immunity against invading microbes. Adaptive immunity develops later by activation of T and B lymphocytes [6].

Macrophages

Macrophages are tissue mononuclear phagocytes developed from circulating monocytes that derived from bone marrow progenitor cells. When monocytes from the blood circulation enter to tissues, they differentiate to tissue macrophages. These cells reside in various tissues of the body. They are differently named according to their location, such as microglia in central nervous system, Kupffer cells in liver, and osteoblasts in bone. When macrophages are stimulated by antigens, they become activated macrophages with different functional phenotypes depending on stimuli and environment. There are two main subsets of activated macrophages: classically (M1) or alternatively (M2) activated macrophages (Fig.2) [14, 15].

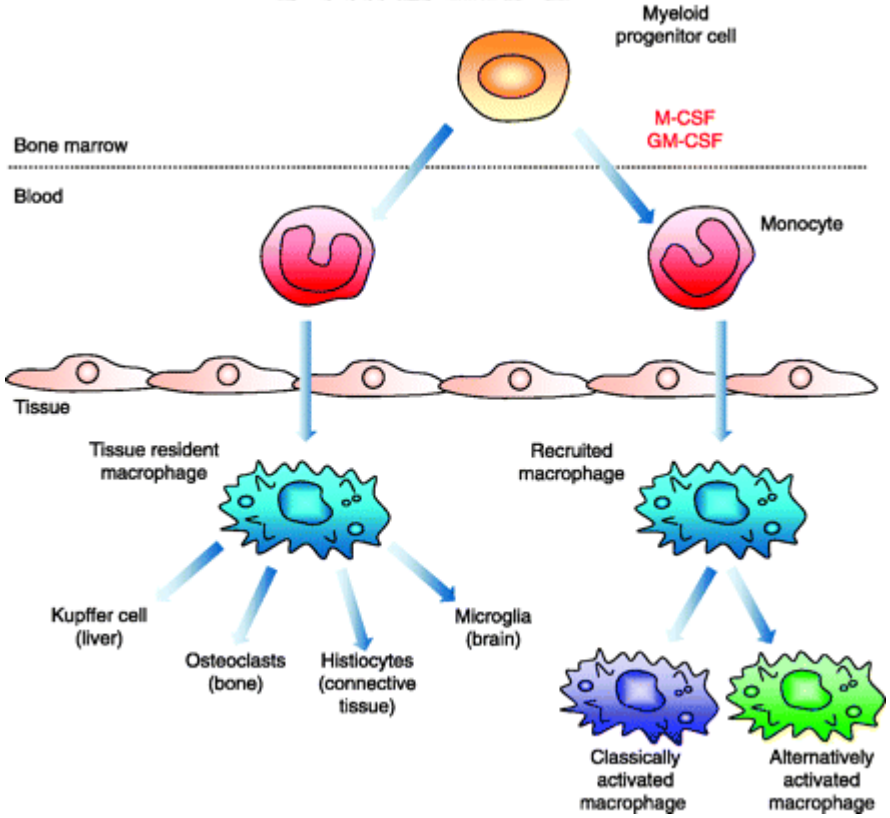

Figure 2: Development and differentiation of macrophages [14].

Classically activated macrophages (M1 macrophages)

M1 macrophages are immune effector cells with acute inflammatory phenotype. They are highly effective cells against pathogens. They perform phagocytosis for eliminating pathogens and produce many cytokines and mediators for inflammatory process and for T cell activation. Stimuli that can generate M1 activated macrophages are IFN-γ from T_H1 cells and NK cells, TNF-α or granulocyte macrophage colony-stimulating factor (GM-CSF) from other immune cells [8].

Alternatively activated macrophages (M2 macrophages)

M2 macrophages are activated macrophages that can counter-balance the actions of M1 macrophages. They accumulate at injury sites in the late phase of inflammatory process. They play roles in tissue protection by suppressing immune and inflammatory responses and promoting wound repair and angiogenesis. They are activated by cytokines such as IL-4, IL-13 from T_H2 cells [16].

Functions of macrophages

Macrophages are multifunction cells that play essential roles in both innate and adaptive immunity. These cells have many functions in the immune system including phagocytosis, antigen presenting cells (APCs) and cytokine production. These functions are mostly performed by activated macrophages.

Phagocytosis

Macrophages are tissue phagocytes that can eliminate invading pathogens and foreign particles by phagocytosis. Macrophages recognize pathogen-associated molecule patterns (PAMPs) of pathogens by using pattern recognition receptors (PRRs) of the cells. The pathogens are engulfed into phagosomes which then fuse with lysosomes to become phagolysosomes (Fig.3). The pathogen in phagolysosomes are destroyed by oxygen-dependent and oxygen independent mechanisms [2, 17].

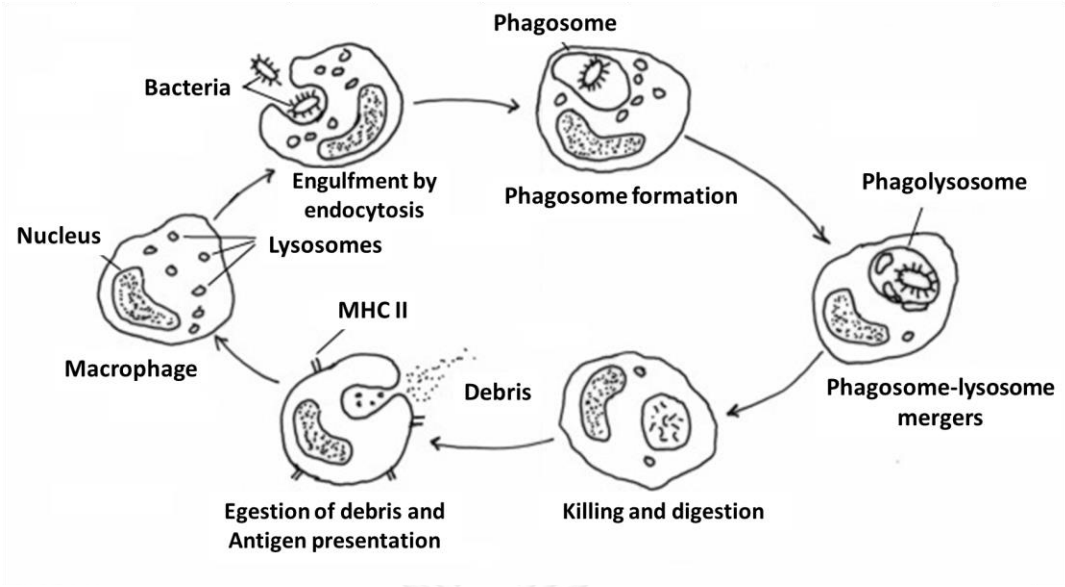

Figure 3: Phagocytosis of a bacterium by a macrophage [17].

Oxygen-dependent mechanism

Pathogens in phagolysosomes are destroyed by oxygen- and nitrogen-free radical. Oxygen uptake is increased in macrophages after binding to pathogens. Molecular oxygen (O_2) is converted to oxygen reactive spcicies (ROS) such as superoxide anions (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) and hypochlorite anion (OCl^-) by NADPH oxidase (Fig.4). These free radicals can react with several components of microbes and cause microbial cell damage and death. In addition, peroxynitrite ($ONOO^-$) generated from superoxide and nitric oxide (NO) is the other free radical involves in destruction of pathogens in phagolysosome [13].

Nitric oxide (NO) is reactive nitrogen species (RNS) that involves in phagocytosis via oxygen dependent mechanism. NO is a gas produced by many cell types in the body. Normally, NO at physiological concentration functions as a vasodilation agent and neurotransmitter. Endothelial and neuronal nitric oxide synthases (eNOS and nNOS) play role in NO production by converting L-arginine to NO at low concentration at physiological condition. During phagocytosis, macrophages produce NO in large amount by inducible NOS (iNOS) (Fig.4). iNOS generates NO to destroy pathogen during phagocytosis and during inflammatory process. This enzyme generally does not express in resting macrophages [18, 19].

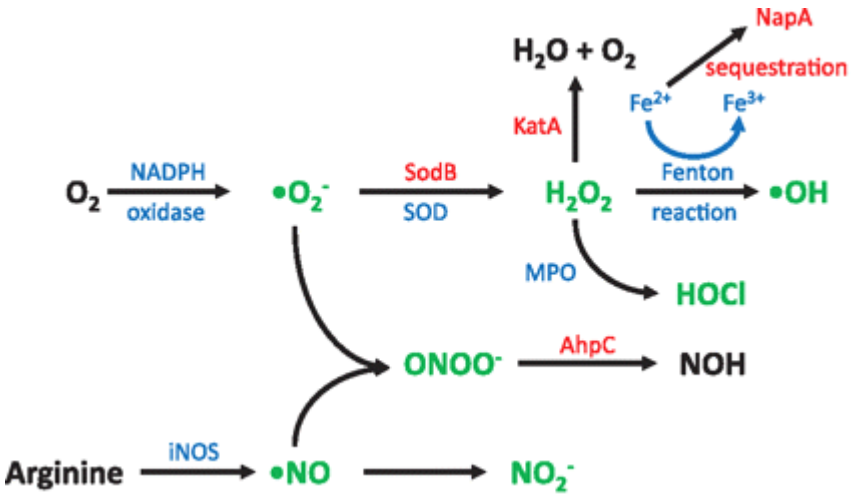

Figure 4: Reactive oxygen and nitrogen species (ROS and RNS) from molecular oxygen and arginine in oxygen-dependent mechanism [19].

Oxygen-independent mechanism

Macrophages can also destroy pathogens in phagolysosomes by using lysosomal antimicrobial peptides and hydrolytic enzymes such as proteases, nucleases, lipases, phosphatases, esterases, lysozyme and lactoferrin. In addition, acidic condition of lysosomes also causes acidity of phagolysosomal environment. This acidic environment can inhibit microbial growth [20].

Antigen presenting cells (APCs)

Cell-mediated immune response is initiated by recognition of antigenic peptides on APCs of T lymphocytes. Dendritic cells, macrophages and B cells are professional APCs that can present antigens to T lymphocytes. APCs usually take up antigens and process them into antigenic peptides. Macrophages generate antigenic peptides from pathogen destruction in phagolysosomes during phagocytosis process. These antigenic peptides can be presented to T helper cells through major histocompatibility complex (MHC) class II on cell surface of macrophages (Fig.5). The signaling cascades from binding of MHC-antigenic peptide complex with T cell receptor and from costimulatory molecules on both macrophages and T cells initiates to T cell activation for generating cell mediated immune response in adaptive immunity [5, 21].

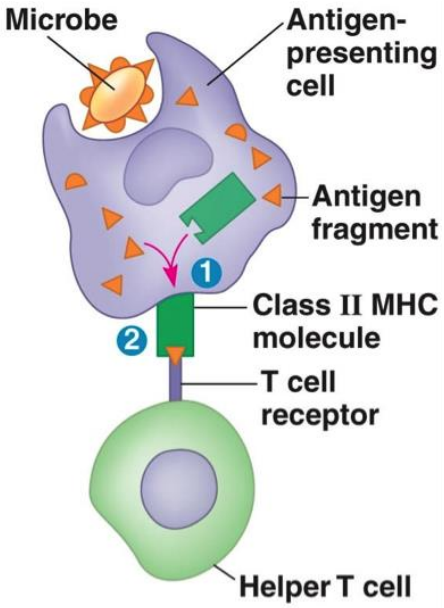

Figure 5: Macrophage function as antigen presenting cell [21].

Cytokine Production

Cytokines are key mediators of both M1 and M2 activated macrophages. Expression and production of these mediators mostly occur in activated macrophages. Resting macrophages produce none or very few amount of cytokines. Many cytokines are used as the markers of activated macrophages.

Cytokines of M1 activated macrophages

M1 activated macrophages produced several cytokines such as IL-1, IL-6, IL-8, IL-12, TNF-α which have many functions in the immune system such as in inflammatory process, in leukocyte migration and in T cell activation. IL-1, IL-6, and TNF-α are usually used as the markers of M1 activated macrophages, IL-10 is the marker of M2 activated macrophages.

Interleukin-1 (IL-1) is an important cytokine in innate and adaptive immunity. It is also one of pro-inflammatory cytokines which play roles in inflammatory process. This cytokine has two isoforms which are IL-1α and IL-1β. These isoforms are encoded from different genes [22]. IL-1 is produced by many cells including macrophages, keratinocytes, fibroblast, T and B lymphocytes. IL-1α acts locally while IL-1β acts

systemically. Both isoforms of IL-1 have similar functions. They are a co-stimulator of T cell activation, stimulate T cell polarization, increase recruitment of immune cells, induce macrophage cytokine production, and stimulate PGE_2 and NO production [23].

Interleukin-6 (IL-6) is the other key pro-inflammatory cytokines in acute inflammatory responses. It is produced by many different cell types including monocytes, macrophages, T cells, endothelial cells and fibroblasts. In homeostatic conditions, IL-6 levels are very low. The stimuli that trigger increase the level of IL-6 include TNF-α and IL-1, bacterial products, viral infections and necrotic cells. IL-6 is the main stimulator of the production of most acute phase proteins in the liver as well as the production of neutrophils in the bone marrow. It supports proliferation of B cells and induces B cells to plasma cells. It also stimulate T cell activation and differentiation [24, 25].

Tumor necrosis factor-α (TNF-α) is mainly produced by activated macrophages. It is also produced by other cells such as T cells, NK cells, neutrophils, mast cells and eosinophils. It has broad range of biological activities, include antitumor, antiviral activity, proliferative and growth-promoting effects. It is able to induce fever, cell apoptosis and inflammation. TNF-α is also an important pro-inflammatory cytokine in inflammatory process. It stimulates recruitment of neutrophils, monocyte and leucocyte to infectious site. It increases the production of other pro-inflammatory cytokines of activated macrophages including IL-1 and IL-6 [3].

Cytokines of M2 activated macrophages

During infection, inflammatory processes are critical to remove pathogen. However, inflammation is associated with damaging effects for the tissue environment, and must thus be repressed to allow complete healing. M2 macrophages play a major role in the resolution of inflammation by decreasing pro-inflammatory cytokine production, increasing production of anti-inflammatory cytokines, IL-10 and TGF-β. These anti-inflammatory inhibit phagocyte and lymphocyte activation. IL-10 is used as the marker of M2 macrophages [26].

Interleukin-10 (IL10) functions as an important regulator of the immune system. It is mainly produced by the T_H2 cells. It is also produce by monocytes, macrophages, dendritic cells, mast cells, NK cells, eosinophils and neutrophils. IL-10 has many roles in immune system. It inhibits cytokine production by macrophages and downregulates the expression of T_H1 cytokines, MHC class II and co-stimulatory molecules in macrophages. It enhances B cell survival, proliferation, and antibody production [27, 28].

In the late phase of inflammatory process, M2 macrophages highly express argenase-1 (Arg-1), an enzyme which plays role in collagen synthesis for wound healing. It is also used as the marker of M2 activated macrophages. Arg-1 catalyzes arginine to ornithine and urea. Ornithine is an essential precursor for the production of collagen and polyamines which are required for wound healing and tissue repair. In macrophages, arginine is catalyzed by iNOS to produce NO and by Arg-1 to collagen and polyamines. Arg-1 and iNOS generated substrate competition for balancing of inflammatory response (Fig.6) [29, 30].

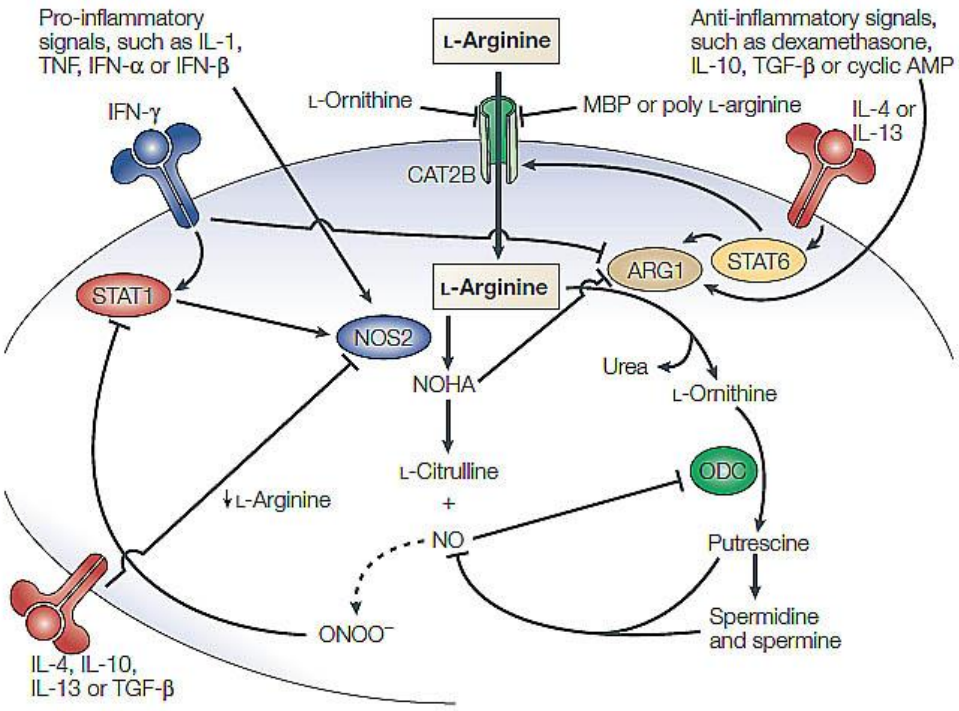

Figure 6: The balance between inducible enzymes arginase-1 and nitric oxide synthase. Arg-1 and iNOS directly activate several biochemical circuits that negatively regulate each other [29].

Five-flower remedy

Five-flower remedy is a traditional Thai remedy which is used for promoting heart function, decreasing tiredness/dizziness and reducing fever. It is also widely added to other remedies. It is a component in aromatic remedy which is used as cardio tonic and used to relieve vertigo. It is also a component in tonic remedy used as health promoter [11]. Five-flower remedy consists of the flowers of *Jasminum sambac*, *Nelumbo nucifera*, *Mesua ferrea*, *Mimusops elengi* and *Mammea siamensis* in equal weight. The remedy is consumed as herbal tea, boiled solution, or in tablets mixing with other herbs. Some herbs in this remedy have been reported immunomodulatory activities but no evident of the water extract of this remedy. The detail of each herb in five-flower remedy is as follow;

1) *Jasminum sambac*

Figure 7: Flower of *Jasminum sambac*

Family name: OLEACEAE

Scientific name: *Jasminum sambac* Linn.

Common name: Arabian jasmine, jusmine, kampopot

Thai name: มะลิ

Medicinal uses: Tonic for heart, reducing fever, relieving stress and anxiety, cooling medicine

Chemical compositions:

Linalool, benzyl alcohol, benzyl acetate, jasmine lactone, methyl jasmonate, geraniol, jasmine, jasmone, methyl benzoate, caryophyllene, cadinene, hexenyl benzoate, irridoid glycoside, rutin, kaempferol, and quercetin [31]

Pharmacological properties:

- **Anti-bacterial:** against *Escherichia coli, Streptococcus pyogenes, Salmonella enterica. Shigella dysenteria, Listeria innocua, Enterococcus faecalis* [32, 33].
- **Anti-fungal:** against *Malassezia sympodialis, Malassezia dermatitis, Malassezia furfur* [34].
- **Anti-oxidant:** The essential oil and methanol extract demonstrated anti-oxidant activities [32].

- **Analgesic activity:** The ethanol extract had writhing inhibition activity in acetic acid-induced writhing in mice [35].
- **Anti-cancer activity:** The methanol extract inhibited proliferation of HeLa and mouse fibroblast cells [36].
- **Immunomodulatory activities:**
    - The methanol extract reduced carrageenan-induced paw edema in rat [37].
    - The methanol extract reduced cotton pellet induced granuloma formation in rat [38].
    - The essential oil promoted the growth of mouse lymphocytes [39].

2) *Nelumbo nucifera*

Figure 8: Flower of Nelumbo nucifera

Family name: NELUMBONACEAE

Scientific name: *Nelumbo nucifera* Gaertn.

Common name: Sacred lotus, pink lotus-lily

Thai name: บัวหลวง

Medicinal uses: Tonic for heart, element tonic, restoration of power, diuretic agent, relieving stress and anxiety, improving mental condition, component in aromatic remedy

Chemical compositions:

Pentadecanoic acid, octadecatrienoic acid, methyl ester, heptadecanoic acid, ethyl α –d-glucopyranoside, campesterol, stigmasterol, β-sitosterol, β-amyrin, anonaine, armepavine, asimilobine, ginnol, liensinine, lirinidine, liriodenine, nuciferine, neferine, isoquercetin, quercetin, roemerine, linalool, and N-nonadecane [40]

Pharmacological properties:

- **Anti-oxidant:** The organic solvent extracts of lotus seed and leaf, and the extract from lotus germ demonstrated anti-oxidant activities [41-43].
- **Anti-viral activity:** NN-B-5 purified from *N. nucifera seed* ethanolic extract blocked HSV-1 multiplication in HeLa cells without apparent cytotoxicity [44].
- **Anti-cancer activity:**
    - The water extract of *N. nucifera* leaves reduced tumor volume and tumor weight in mice inoculated with MCF-7 cells compared to the control samples [45].
    - Neferine purified from *N. nucifera* exhibited cytotoxicity against HCC Hep3B cells [46].
- **Immunomodulatory activities:**
    - NN-B-4 and (S)-armepavine purified from *N. nucifera* ethanolic extract suppressed T cells proliferation and decreased IL-2, IL-4, IL-10, and IFN-γ production in peripheral blood mononuclear cells [47, 48].
    - Lotus plumule decreased the levels of pro-inflammatory cytokine TNF-α but increased the levels of anti-inflammatory cytokine IL-10 produced by peritoneal macrophages from LPS-challenged mice [49].
    - The ethanol extract of *N. nucifera* rhizome protected mast cells from degranulation, inhibited lipopolysaccharide (LPS)-induced activation of macrophages by decreasing the expression of co-stimulatory molecules and suppressed NO production [50].

- The methanol extract of lotus leaf reduced mRNA expression of nitric oxide synthase (iNOS) and COX-2, as well as the mRNA expression and level of IL-6 and TNF-**α** in LPS induced activated of murine macrophage cell line RAW264.7 [51].

3) *Mesua ferrea*

Figure 9: Flower of *Mesua ferrea*

Family name: CLUSIACEAE

Scientific name: *Mesua ferrea* Linn.

Common name: Iron wood, Indian rose chestnut

Thai name: บุนนาค

Medicinal uses: Cardio tonic, element tonic, restoration of power and health, relieving dizziness, diuretic and antipyretic agent

Chemical compositions:

Mesuol, mesuone, mammeisin, mesuagin, mammeigin, mesuabixanthone A and B, mesuaferrol A and B, mesuaxanthone A and B, euxanthone, mesuaferrone, and mesuanic acid [52]

Pharmacological properties:

- **Anti-oxidant:** The methanolic extract demonstrated anti-oxidant activity [52-54].
- **Analgesic activity:** The n-hexane, ethyl acetate and methanol extracts exhibited analgesic activity in acetic acid induced writhing response in mouse [55].
- **Anti-microbial activity:** against *Bacilllus* spp., *Salmonella* spp., *Pseudomonas* spp., *Streptococcus pneumonia, Staphylococcus aureus, Sarcina lutea, Proteus mirabilis, Lactobacillus arabinosus, Bordetella bronchiseptica, Micrococcus luteus*, *Staphylococcus epidermidis, Escherichia coli*, *Klebsiella pneumoniae, Enterococcus faecalis*, *Candida albicans, Aspergillus niger* and *Saccharomyces cerevisiae* [53, 56, 57].
- **Hepatoprotective activity:** The essential oil had protective activity against paracetamol induced hepatotoxicity in rats [58].
- **Anti-cancer activities:**
    - The methanol extract inhibited growth of Ehrlich's ascites carcinoma in mice [59].
    - The ethanol extract exhibited cytotoxicity activity against human cholangiocarcinoma CL-6, human laryngeal (Hep-2), and human hepatocarcinoma (HepG2) cell lines [60].
    - The essential oil exhibited anticancer activity against KB human oral carcinoma, MCF-7 breast cancer and small cell lung cancer NCI-H187 cell lines [61].
- **Immunomodulatory activities:**
    - Mesuaxanthone A and mesuaxanthone B reduced carrageenan induced hind paw edema and cotton pellet induced granuloma formation in albino rats [62].
    - Mesuol potentiated percentage neutrophil adhesion in neutrophil adhesion test in rats and phagocytosis in carbon clearance assay [63].

4) *Mimusops elengi*


Figure 10: Flower of *Mimusops elengi*

Family name: SAPOTACEAE

Scientific name: *Mimusops elengi* L.

Common name: Spanish Cherry, tanjong tree, bullet wood

Thai name: พิกุล

Medicinal uses: Tonic for heart and lung, restoration of power, curing biliousness, treating liver complaints, relieving headache

Chemical compositions:

Thymol, cadinol, taumuurolol, hexadecanoic acid, diisobutyl phthalate, octadecadienoic acid, phenyl propyl gallate, quercitol, ursolic acid, dihydro quercetin, quercetin, mimusops acid, mimusopsic acid, mimusopane, mi-saponin A, 16-alpha-hydroxy mi-saponin, mimusopside A and B, taxifolin, and alpha-spinasterol glucoside [64]

Pharmacological properties:

- **Anti-oxidant:** The methanol extract of bark, leaves and flower of *M. elengi* had antioxidant activities [65, 66].
- **Anti-Bacterial:** The aqueous and ethanolic extracts had anti-bacterial activities against *Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Bacillus cereus, Alcaligenes faecalis, Salmonella typhimurium* [67].

- **Anti-cancer activity:** The ethanolic extract had cytotoxic effect on the cholangiocarcinoma CL- 6, human laryngeal carcinoma cell line Hep-2, and human hepatocarcinoma HepG2 cell lines [60].
- **Gastroprotective effects:** The ethanol extract reduced ethanol-induced, pylorus-ligated and water-immersion plus stress-induced gastric ulcerations in rats [68].

5) *Mammea siamensis*

Figure 11: Flower of *Mammea siamensis*

Family name: CLUSIACEAE

Scientific name: *Mammea siamensis* Kosterm.

Thai name: สารภี

Medicinal uses: Tonic for heart, restoration of power and health, component in aromatic remedy

Chemical composition:

Mammea coumarins, β-sitosterol, stigmasterol, campesterol, C-glucosyl flavone, mammea acetamide [69]

Pharmacological properties:

- **Anti-oxidant:** The water extract of *M. siamensis* demonstrated anti-oxidant activities [70].
- **Anti-bacterial:** The chloroform and methanol extracts of *M. siamensis* had antibacterial effects on *Staphylococcus aureus* and *Bacillus subtilis* [69].
- **Anti-cancer activities:**
    - The coumarins isolated from *M. siamensis* showed the strongest inhibitory activity on cell proliferation in colon cancer DLD-1, breast adenocarcinoma MCF-7, human cervical cancer HeLa, and human lung cancer NCI-H460 cell lines [71].
    - The *n*-hexane extracts of *M. siamensis* exhibited very strong cytotoxic effect against human CCRF-CEM leukemia cells [72].
- **Immunomodulatory activities:**
    - Coumarin from methanol extract of *M. siamensis* inhibited nitric oxide (NO) production in lipopolysaccharide-activated RAW264.7 cells [73].

CHAPTER III

MATERIALS AND METHODS

Materials

Water extracts of five-flower remedy and its compositions

The herbal compositions of five-flower remedy including *J. sambac*, *M. elengi, M. ferrea, M. siamensis and N. nucifera* were purchased from Chao Krom Poe herbal dispensary Bangkok. These herbs were confirmed their morphology by Associate Professor Dr. Nijsiri Ruangrungsi, College of Health Sciences, Chulalongkorn University. They were grounded into crude powder. The remedy was prepared by mixing crude powder of all herbs together in equal weight. The powder of the remedy and each herb were extracted by heating at 80°C in distilled water with the ratio of powder: solvent at 1: 5, for 15 minutes. The supernatant was collected. The remnant powder was extracted in distilled water twice. The supernatant from each extraction was pooled together, filtered through 11 micron filter paper, and lyophilized to become the solid powder extract. All the extracts were kept in closed container at -20°C until used. These extracts were characterized by high performance liquid chromatography (HPLC) method by Dr. Chaisak Chansriniyom, National Nanotechnology Center, National Science and Technology Development Agency (NSTDA). The yields of these extracts were presented in Table 1. The extracts were dissolved in distilled water as the stock solutions, filtered with 0.2 µm steriled syringe filter and diluted to 1.56 – 200 µg/ml in incomplete Dulbeco's Modified Eagle's Medium (DMEM) for using in all experiments of this study.

Table 1: The percentage yields of all water extracts of the five-flower remedy and its compositions compared to their crude powders before extraction.

Test compounds	% yield	% in the remedy	Ratio in the remedy
Five-flower remedy	26.25	-	-
Jasminum sambac	25.56	19.50	0.975 / 5
Nelumbo nucifera	19.00	14.50	0.725 / 5
Mesua ferrea	21.05	16.06	0.803 / 5
Mimusops elengi	27.20	20.75	1.037 / 5
Mammea siamensis	38.25	29.19	1.460 / 5

Cell Culture

Murine macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin and incubated at 37ºC in 5% CO_2. They were sub-cultured three times weekly during use. The viability of cells was determined by 0.4% trypan blue staining. In all experiments, the cells with their viability not less than 90% were used at $4x10^5$ cells/ml. DMEM and 100 ng/ml lipopolysaccharide (LPS) were used as the negative and the positive controls, respectively.

Chemicals and reagents

The chemicals and reagents used in this study were Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), penicillin/ streptomycin (Gibco, USA), 0.4% trypan blue dye (Sigma, USA), resazurin (Sigma, USA), lipopolysaccharide (Sigma, USA), Griess reagent (Promega, USA), TRIzol Reagent (Invitrogen, USA), chloroform (Sigma, USA), dietyl pyrocarbonate (DEPC) (Molekula, UK), absolute ethanol (Merck, Germany), ImProm-II™ reverse transcription system (Promega, USA), primers (Bio Basic, Canada), and Taq polymerase (Gibco, USA),

Equipments and instruments

The following equipments and instruments used in this study were autopipettes (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge (Hettich, USA), CO_2 incubator (Thermo, USA), light microscope (Nikon, USA), hemocytometer (Brand, Germany), 96 and 24 muti-well plate (Corning, USA), scrapper (Greiner, UK), T-25 Tissue Culture flasks (Corning, USA), filter paper (Whatman®, USA), thermal cycler machine (Eppendorf, USA), vortex mixer (Scientific industries, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), Molecular Imager® Gel Doc^{TM}XR+ Systtem (Bio-Rad, USA), and Nanodrop (Thermo Scientific, USA)

Methods

1. **To study the effect of the water extracts of five-flower remedy and its compositions on nitric oxide production and cell viability of macrophage J774A.1 cells**

The effects of the extracts on NO production were determined by Griess reaction assay as follows;

1. Incubate J774A.1 cells at $4x10^5$ cells/ml in 96-well plate for 24 h at 37ºC, 5% CO_2 and 95% air.
2. Treat the cells with the water extracts at 1.56 – 200 µg/ml for 24 h at 37ºC.
3. Transfer 100 µl supernatant from each well of treated cells to 96-well plate for measuring NO concentration in nitrite form, as follows;
    a. Add 20 µl of sulfanilamide to each well, incubate for 10 min, then add 20 µl of NED (N-1-napthylethylenediamine dihydrochloride) and incubate for 10 min in the dark at room temperature.
    b. Measure the absorbance at 540 nm by a microplate reader.
    c. Calculate the NO concentration by using sodium nitrite standard curve.

4. Determine viability of the remaining treated cells by resazurin reduction assay as follows;

    a. Add 50 µl of 50 µg/ml resazurin in DMEM into each well and incubate for 2 h at 37ºC, 5% CO_2 and 95% air.

    b. Determine the reduction of resazurin to resorufin in viable cells by measuring the absorbance of the treated cells at 570 and 600 nm by a microplate reader.

    c. Calculate the percentage of viable cells using the following formula.

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

2. To study the effects of the water extracts of five-flower remedy and its compositions on mRNA expression of mediators in macrophage J774A.1 cells

The effects of the water extracts on expression of several markers of activated macrophages including inducible nitric oxide synthase (iNOS), arginase-1, anti-inflammatory cytokine (IL-10) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) were determined by reverse transcription polymerase chain reaction (RT-PCR) as follows;

1. Incubate J774A.1 cells at $4x10^5$ cells/ml in 24-well plate for 24h at 37ºC, 5% CO_2 and 95% air.
2. Treat the cells with the water extracts at 3.13 – 200 µg/ml at 37ºC for 4 and 24 h.
3. Use the treated cells for RT-PCR by isolating total RNA, reversing the mRNA to cDNA and amplifying cDNA with specific primers of all studied markers.

Isolation of total RNA from the treated cells

1. Remove all supernatant from the treated cells.
2. Add 500 µl of TRIzol® reagent into each well for 5 minutes at room temperature to lyse the treated cell.
3. Transfer the lysates to 1.5 ml eppendorf tube.
4. Add 200 µl of chloroform to each tube, vigorously shake for 15 sec, and incubate for 3 min at room temperature.
5. Centrifuge the samples at 12,000 g for 15 min at 4 ºC
6. Carefully collect the aqueous layer from each tube to a new eppendorf tube.
7. Add 500 µl of isopropanol to each tube, shake and incubate at -20 ºC for 1 h.
8. Centrifuge the samples at 12,000 g at 4 ºC for 10 minutes to separate the total RNA.
9. Remove the supernatant, wash the RNA pellet with 1 ml of 75% ethanol in DEPC treated water, vertex shortly, and centrifuge at 7,500 g at 4 ºC for 5 minutes.
10. Remove the supernatant, air dry the RNA pellet, and dissolve it in DEPC treated water
11. Determine the total RNA concentration and contamination of each sample using Nanodrop machine at absorbance 230, 260 and 280 nm.
12. Store the RNA samples at -80 ºC until use.

Reversion of RNA samples to cDNA using ImProm-II™ reverse transcription system

1. In each 200 µl tube, mix 1.5 µg total RNA sample with 1 µl of oligo dT_{15} primer, heat the mixture at 70 ºC for 5 minutes, and then keep on ice for 5 minutes.
2. Add 15 µl of reverse transcription mixture solution (25 mM MgCl2, mixed dNTP, ribonuclease inhibitor and reverse transcriptase) in each sample tube.
3. Generate cDNA in a thermocycler machine using the following conditions; 25ºC for 5 minutes then 42ºC for 1 h and 30 minutes, and follow by 70ºC for 15 minutes.
4. Store the cDNA samples at -20ºC until use.

Amplification of cDNA samples by PCR with specific primers

1. In each 200 µl tube, mix 1 µl of cDNA sample with 24 µl PCR reaction mixture solution (containing 10 mM mixed dNTP, 50 mM MgCl2, primer (Table 2) and Taq polymerase in PCR buffer).
2. Generate PCR products in thermocycler machine using the condition described in the Table 3.
3. Identify the PCR products by running on 1.5% agarose gel electrophoresis at 100 volt for 50 min in TBE buffer, staining the gel with 0.5 µg/ml ethidium bromide in TBE buffer for 15 min, and de-staining with TBE buffer for 30 min.
4. Analyze the densities of the PCR products by gel-documentation and normalize these densities with the density of the β-actin PCR product.

Table 2: The specific primers for generating PCR products from cDNA samples

Gene	Primer sequences	Fragment size (bp)
IL-1β	F 5'- CAGGATGAGGACATGAGCACC-3' R 5'- CTCTGCAGACTCAAACTCCAC-3'	447
IL-6	F 5'- GTACTCCAGAAGACCAGAGG-3' R 5'- TGCTGGTGACAACCACGGCC-3'	308
TNF-α	F 5'- TTGACCTCAGCGCTGAGTTG-3' R 5'- CCTGTAGCCCACGTCGTAGC-3'	364
IL-10	F 5'-GGACTTTAAGGGTTACTTGGGTTGCC-3' R 5'-CATTTTGATCATCATGTATGCTTCT-3'	313
iNOS	F 5'- CCCTTCCGAAGTTTCTGGCAGCAGC-3' R 5'- GGCTGTCAGAGCCTCGTGGCTTTGG-3'	496
Arg1	F 5'-ATGGAAGAGACCTTCAGCTAC-3' R 5'-GCTGTCTTCCCAAGAGTTGGG-3'	224
β-actin	F 5'-GTGGGCCGCCCTAGGCACCAG-3' R 5'-GGAGGAAGAGGATGCGGCAGT-3'	603

Table 3: The conditions for generating PCR products from cDNA samples

Gene	Denature	Annealing	Extension	Cycles
IL-1β	T 94°C 30 sec	T 57°C 45 sec	T 72°C 1 min	30
IL-6	T 94°C 30 sec	T 57°C 45 sec	T 72°C 1 min	30
TNF-α	T 94°C 30 sec	T 55°C 45 sec	T 72°C 1 min	20
IL-10	T 94°C 30 sec	T 60°C 45 sec	T 72°C 1 min	35
iNOS	T 94°C 30 sec	T 60°C 45 sec	T 72°C 1 min	23
Arg1	T 94°C 1 min	T 54°C 1 min	T 72°C 1 min	45
β-actin	T 94°C 30 sec	T 57°C 45 sec	T 72°C 1 min	30

Statistic analysis

The data are present as means ± standard error of means (S.E.M) from at least three independent experiments. One-way ANOVA with least significant difference (LSD) post hoc test was used to determine the statistical significance of difference of the data. The *p*-values less than 0.05 were regarded as statistically significant.

CHAPTER IV

RESULTS

1. Effects of the water extracts of five-flower remedy and all its compositions on nitric oxide production in macrophage J774A.1 cells

NO is a free radical produced by activated macrophages. It is commonly used as one of the markers for macrophage activation. In this study, the effects of the water extracts of the five flower remedy and its five components on NO production in macrophage J774A.1 cells were investigated using Griess reaction assay to determine amount of nitrite in the supernatants of the treated cells. In a preliminary study, the extracts of the remedy, *J. sambac and N. nucifera* were more potent than the extracts of *M. ferrea*, *M. elengi* and *M. siamensis* for activating NO production. In order to determine the concentration dependent effects of these extracts, the extracts of the remedy, *J. sambac* and *N. nucifera* were used at 6.25 – 100 µg/ml. The extracts of *M. ferrea*, *M. elengi* and *M. siamensis* were used at 12.5 – 200 µg/ml.

All of the extracts significantly stimulated NO production in J774A.1 cells when compared to the solvent control. The untreated cells generated NO at 0.45 $\pm$ 0.78 µM. LPS at 100 ng/ml significantly stimulated NO production in the treated cells to 33.72 $\pm$ 0.44 µM. The extract of the remedy at 6.25 – 100 µg/ml stimulated NO production to 12.98 $\pm$ 1.53 – 30.30 $\pm$ 0.84 µM (Fig.12). The extract of *J. sambac* at 6.25 – 100 µg/ml stimulated NO production to 13.96 $\pm$ 2.64 µM - 30.64 $\pm$ 1.24 µM (Fig.13). The extract of *N.nucifera* at 12.5 – 100 µg/ml significantly increased NO production to 4.01 $\pm$ 1.76 µM – 26.58 $\pm$ 0.55 µM (Fig.14). The extract of *M. ferrea* at 50 – 200 µg/ml significantly increased NO production to 4.58 $\pm$ 2.17 µM – 15.49 $\pm$ 2.60 µM (Fig.15). The extracts of *M. elengi* and *M. siamensis* at 200 µg/ml significantly increased NO production to 4.94 $\pm$ 2.34 *µM* and 5.13 $\pm$ 1.91 µM, respectively (Fig.16 and Fig.17).

The stimulatory effects of the remedy and its five components on NO production were divided into two groups when their effects were compared with the effect of LPS (Fig.18). The high stimulatory effect group included the extracts of the

remedy, *J. sambac*, and *N.nucifera*. The low stimulatory effect group included the extracts of *M. ferrea, M. elengi,* and *M. siamensis*. For the next experiments, three concentrations of each extract were selected. The selected concentrations of the extracts with the highest stimulatory activities (the remedy and *J. sambac)* were based on the minimum concentration which stimulated NO production. The concentrations of the other extracts were chosen based on their maximum stimulatory effect. The extracts of the remedy and *J. sambac* were used at 6.25, 12.5, and 25µg/ml. The extract of *N. nucifera* was used at 25, 50, and 100µg/ml. The extracts of *M. ferrea, M. elengi*, and *M. siamensis* were used at 50, 100, and 200 µg/ml.

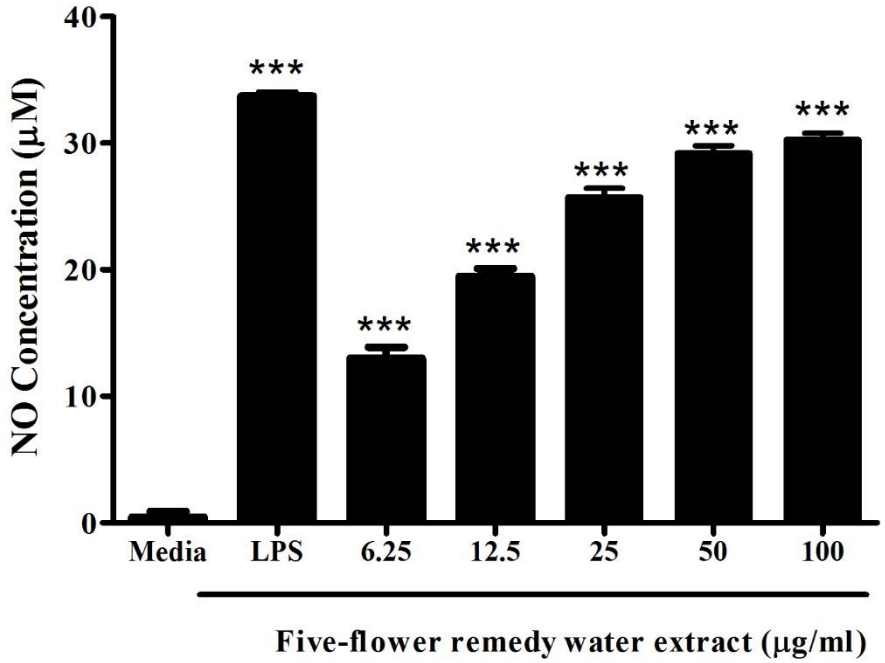

Figure 12: Effect of the water extract of five-flower remedy on NO production in macrophage J774A.1 cells. The cells were treated with 6.25 – 100 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). *$^{***}p < 0.001$* compared to DMEM media control.

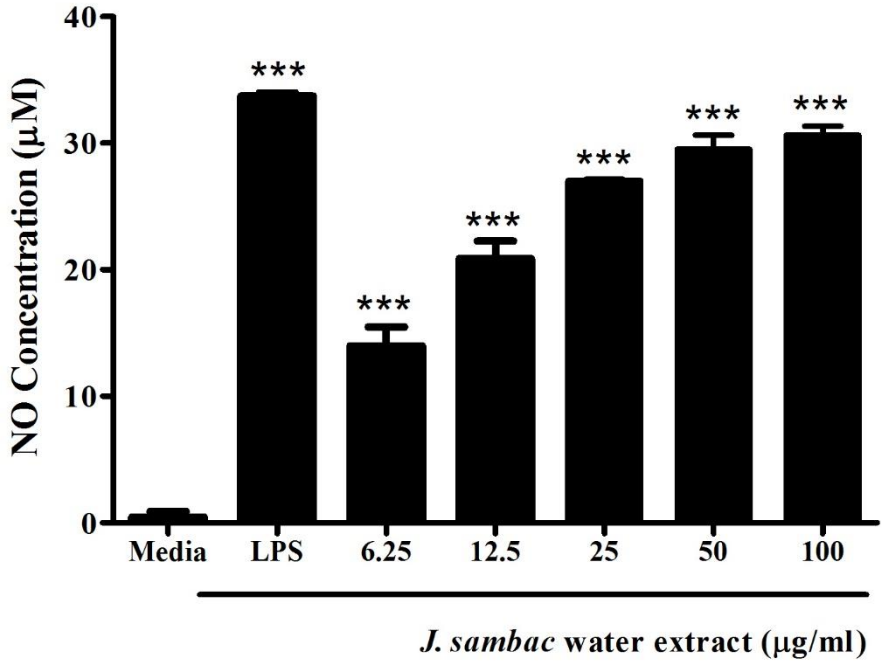

Figure 13: Effect of the water extract of *J. sambac* on NO production in macrophage J774A.1 cells. The cells were treated with 6.25 – 100 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). ****p < 0.001* compared to DMEM media control.

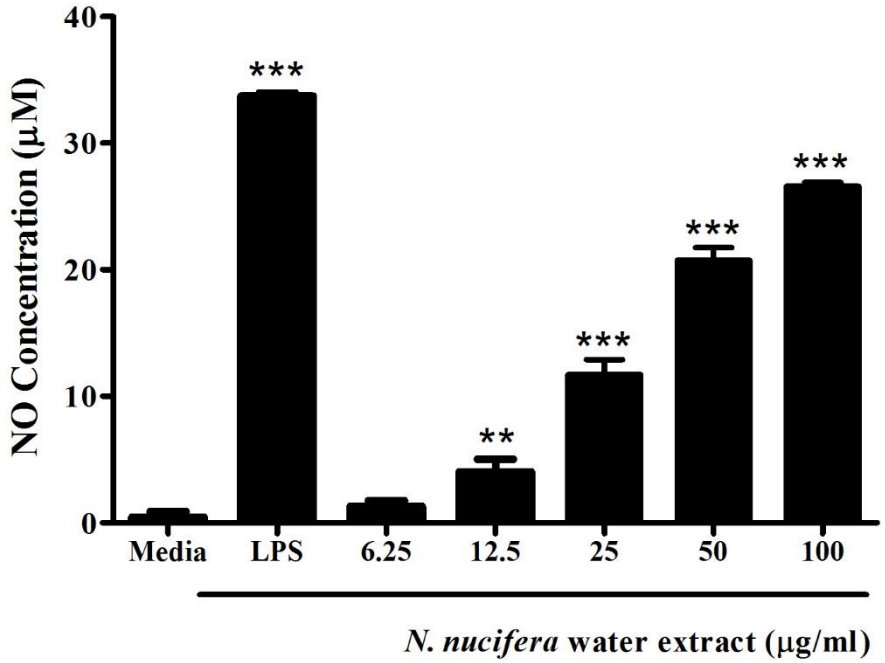

Figure 14: Effect of the water extract of *N. nucifera* on NO production in macrophage J774A.1 cells. The cells were treated with 6.25 – 100 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). ***p < 0.01, ***p < 0.001* compared to DMEM media control.

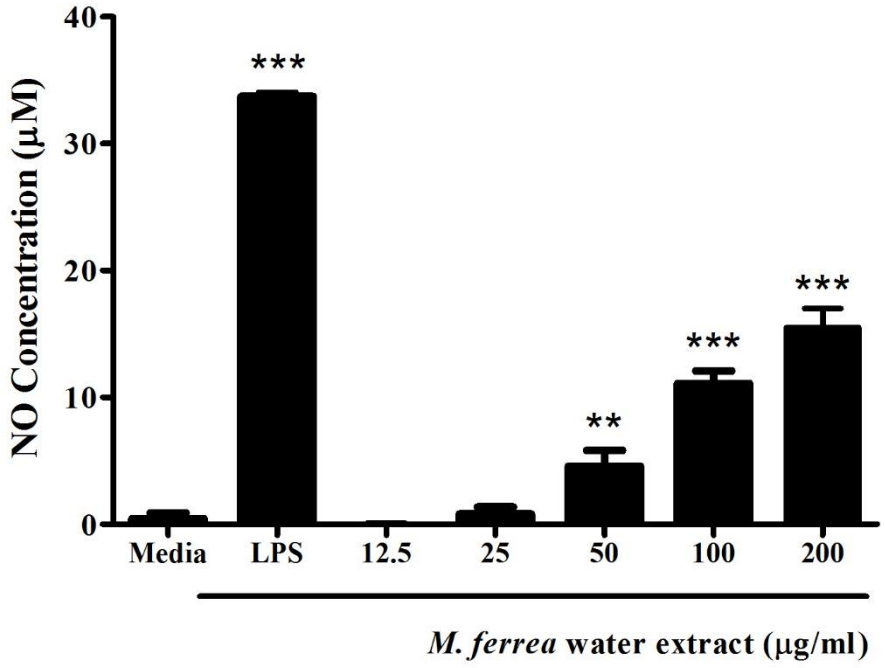

Figure 15: Effect of the water extract of *M. ferrea* on NO production in macrophage J774A.1 cells. The cells were treated with 12.5 – 200 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). *$^{**}p < 0.01$, $^{***}p < 0.001$* compared to DMEM media control.

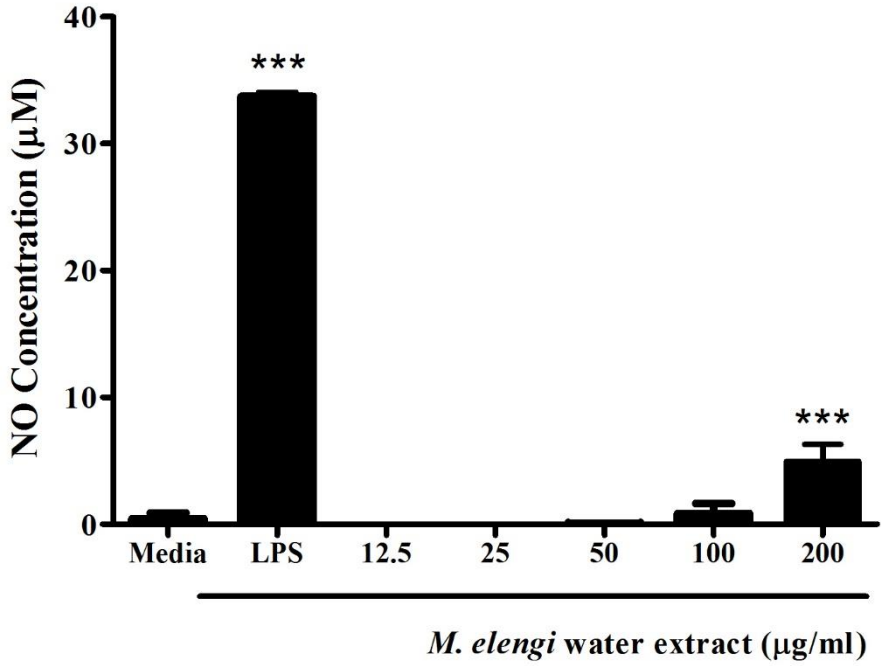

Figure 16: Effect of the water extract of *M. elengi* on NO production in macrophage J774A.1 cells. The cells were treated with 12.5 – 200 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). ****p < 0.001* compared to DMEM media control.

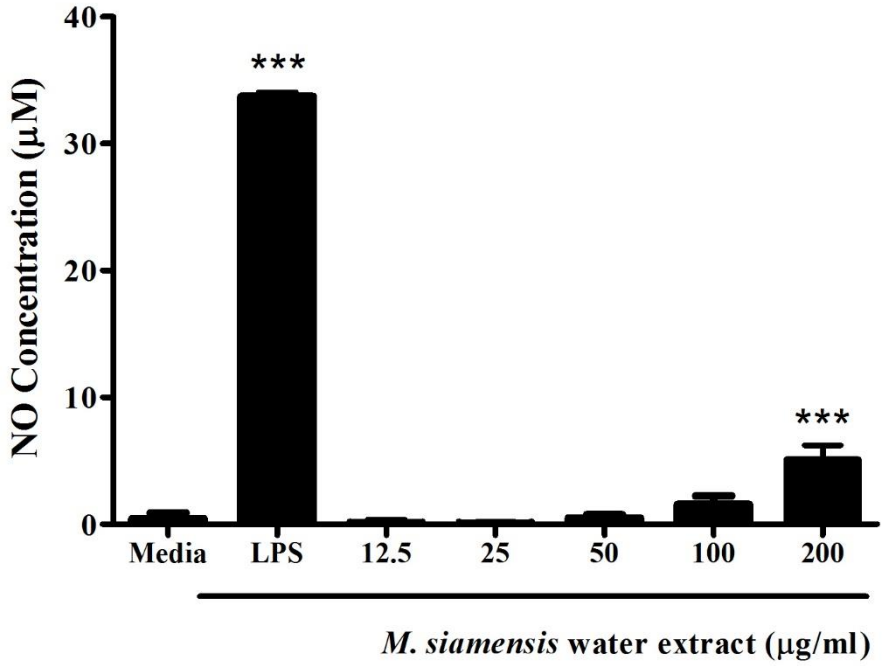

Figure 17: Effect of the water extract of *M. siamensis* on NO production in macrophage J774A.1 cells. The cells were treated with 12.5 – 200 µg/ml of the extract for 24 h. The amount of NO production in the supernatant of the treated cells was determined by Griess reaction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3). ****p < 0.001* compared to DMEM media control.

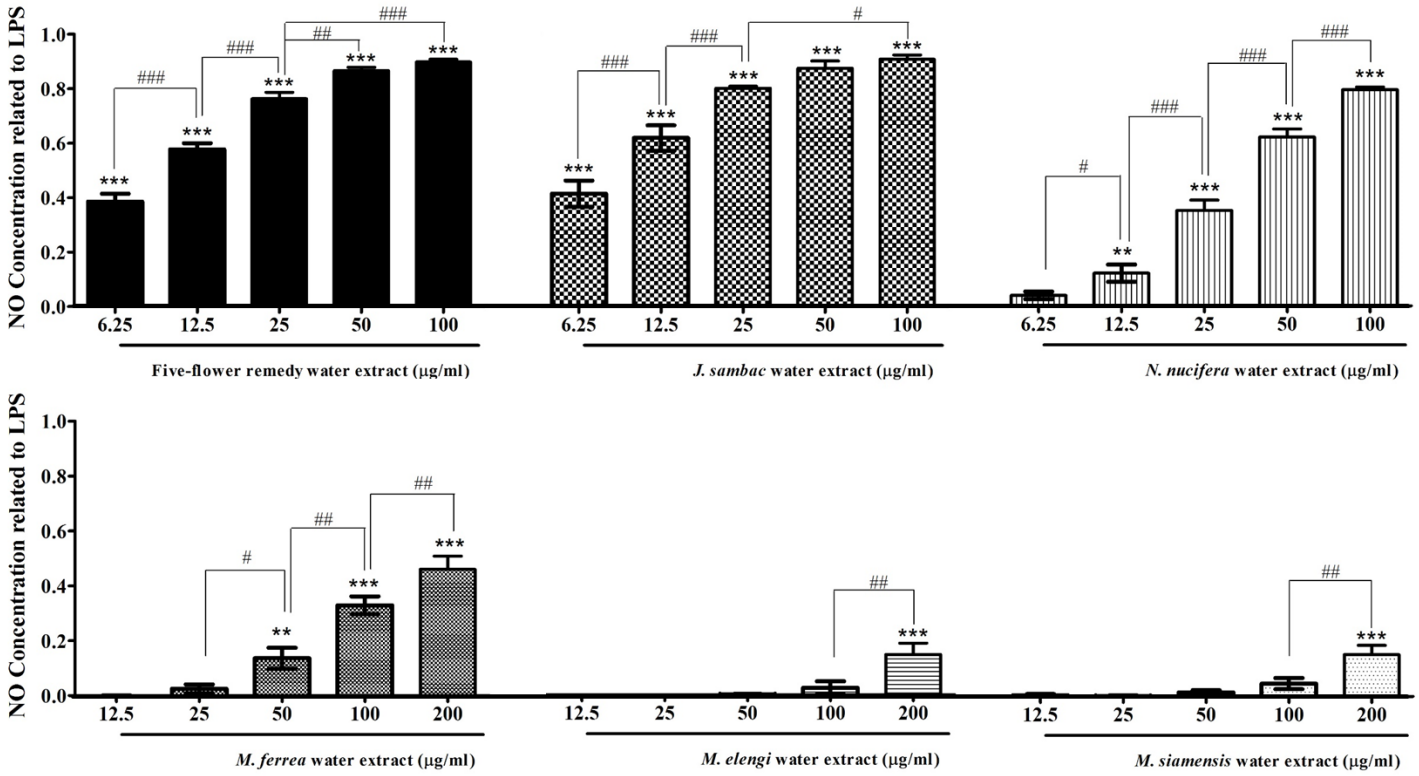

Figure 18: Comparison of the stimulatory effects on NO production of all six extracts to the effect of LPS control. The data are represented as mean ± S.E.M. from three independent experiments (n=3). *$^{**}p < 0.01$, $^{***}p < 0.001$* compared to DMEM media control. *$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$* compared to other concentrations.

2. Effects of the water extracts of five-flower remedy and all its compositions on macrophage J774A.1 cell viability

All six extracts were evaluated whether their stimulatory effects on NO production were from their proliferative effects on these cells or not. Resazurin reduction assay was used to determine the viability of the cells treated with these extracts. More viable cells can change resazurin (blue color) by mitochondrial reductase enzyme to higher resorufin product (pink color) than less viable cells.

The results showed that all extracts at all concentrations used in this study had no effect on the viability of macrophage J774A.1 cells (Fig.19). So, these extracts stimulated NO production in the cells without any effect on cell viability.

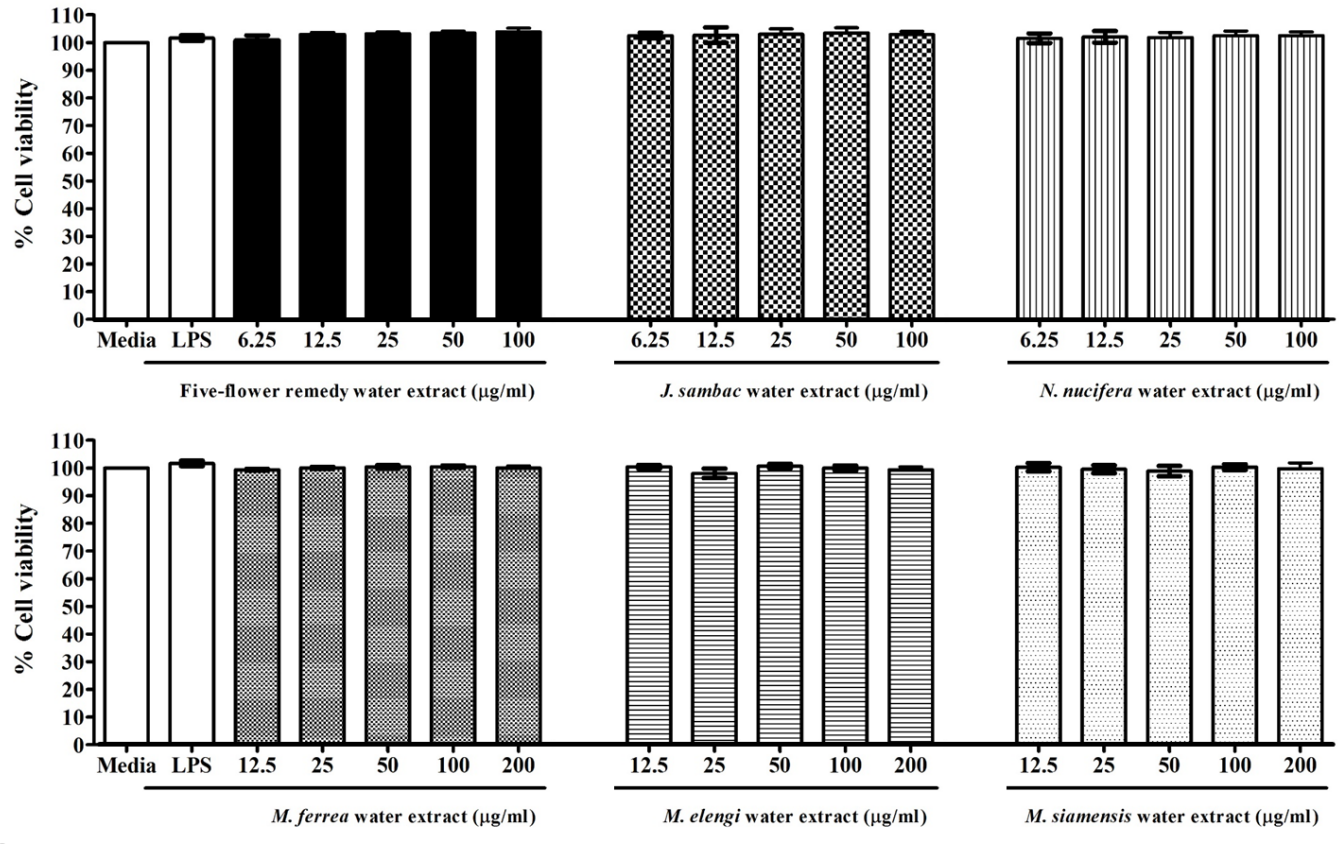

Figure 19: Effects of the water extracts of the remedy and its composition on cell viability of macrophage J774A.1. Cells were treated *with* the extracts for 24 h. Viability of the treated cells was determined by resazurin reduction assay. The data are represented as mean ± S.E.M. from three independent experiments (n=3).

3. Effect of the water extracts of five-flower remedy and all its compositions on some markers of activated macrophages

Activated macrophages can be differentiated from their resting stage by using several inducible enzymes, cytokines, and cell surface proteins as the markers of activated macrophages. Some of these markers were used in this study to determine the stimulatory effects of the water extracts of the remedy and its compositions on macrophage J774A.1 cells.

3.1. Effects of the water extracts on mRNA expression of cytokine (IL-1β, IL-6, TNF-α and IL-10) in macrophage J774A.1 cells

Several cytokines are used as the markers of macrophage activation. This study evaluated the effects of the extracts on the expression of cytokines which are the markers of M1 activated macrophages (IL-1β, IL-6, and TNF-α) and of M2 activated macrophages (IL-10).

J774A.1 cells were treated with the extracts for 4 and 24 h to determine the mRNA expression of IL-1β, IL-6, TNF-α and IL-10. The extracts of the remedy and *J. sambac* were used at 6.25 – 25 µg/ml. The extracts of *N. nucifera* was used at 25 – 100 µg/ml. The extracts of *M. ferrea*, *M. elengi* and *M. siamensis* were used at 50 – 200 µg/ml.

3.1.1 Effect of the water extracts on mRNA expression of IL-1β

Resting macrophages did not express IL-1β mRNA. All of the water extracts significantly increased IL-1β expression when compared to the media treated control. The extracts of the remedy and *J. sambac* at 6.25 – 25 μg/ml (Fig.20 A-B), the extract of *N. nucifera* at 25 – 100 μg/ml (Fig.21 A), and the extract of *M. ferrea* at 50 – 200 μg/ml (Fig.21 B) significantly up-regulated IL-1β mRNA expression. Each of these extracts increased the same amount of IL-1 expression at both 4 and 24 h of treatment. The extracts of *M. elengi* and *M. siamensis* had weaker stimulatory activities on IL-1β expression than the other four extracts (Fig.22 A-B). The extracts at 100 and 200 μg/ml significantly increased IL-1β expression at 4 h of treatment. Their stimulatory effects were not observed at 24 h of treatment. Only the extract of *M. elengi* at 200 μg/ml significantly increased IL-1β expression at 24 h.

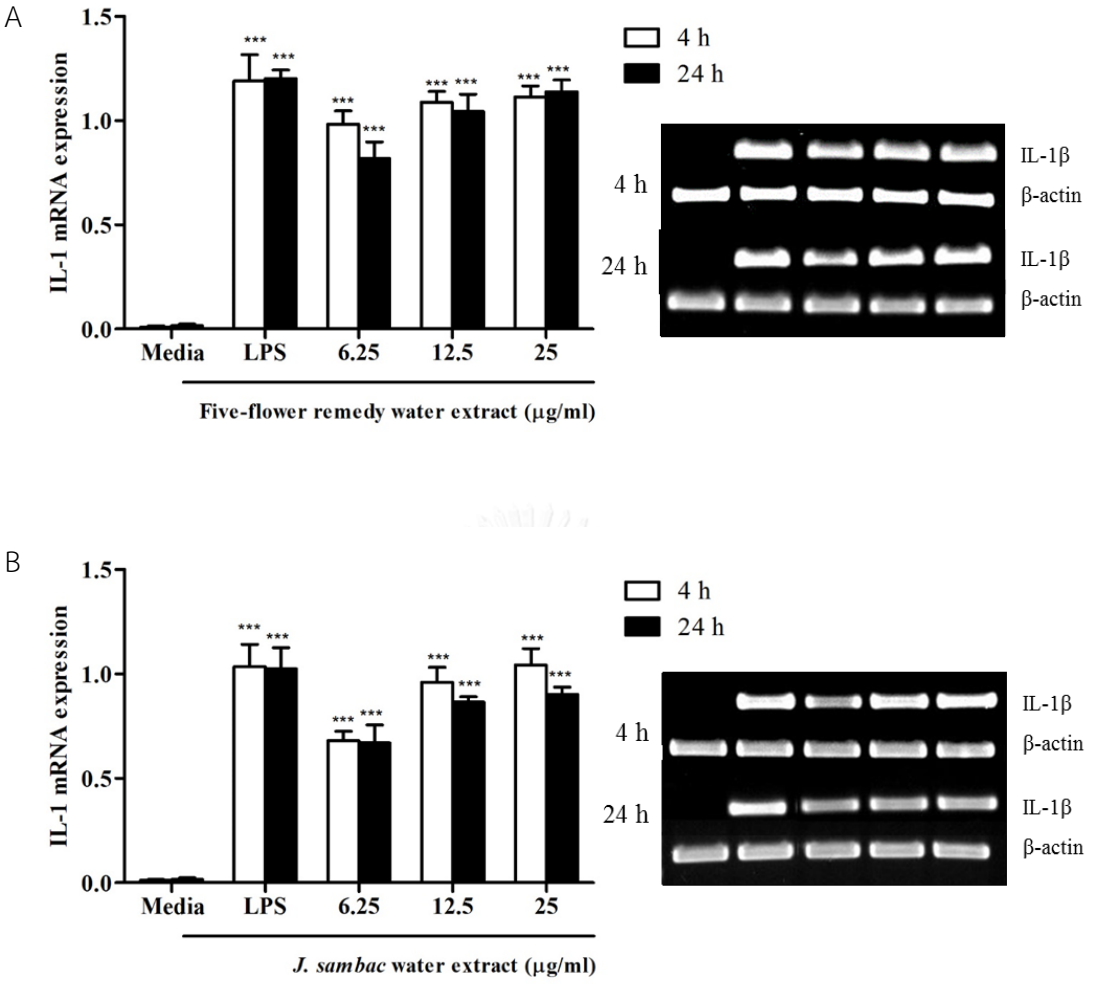

Figure 20: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on IL-1β mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-1β to β-actin from three independent experiments (n=3) of each treated condition. ****p < 0.001* compared to DMEM media control.

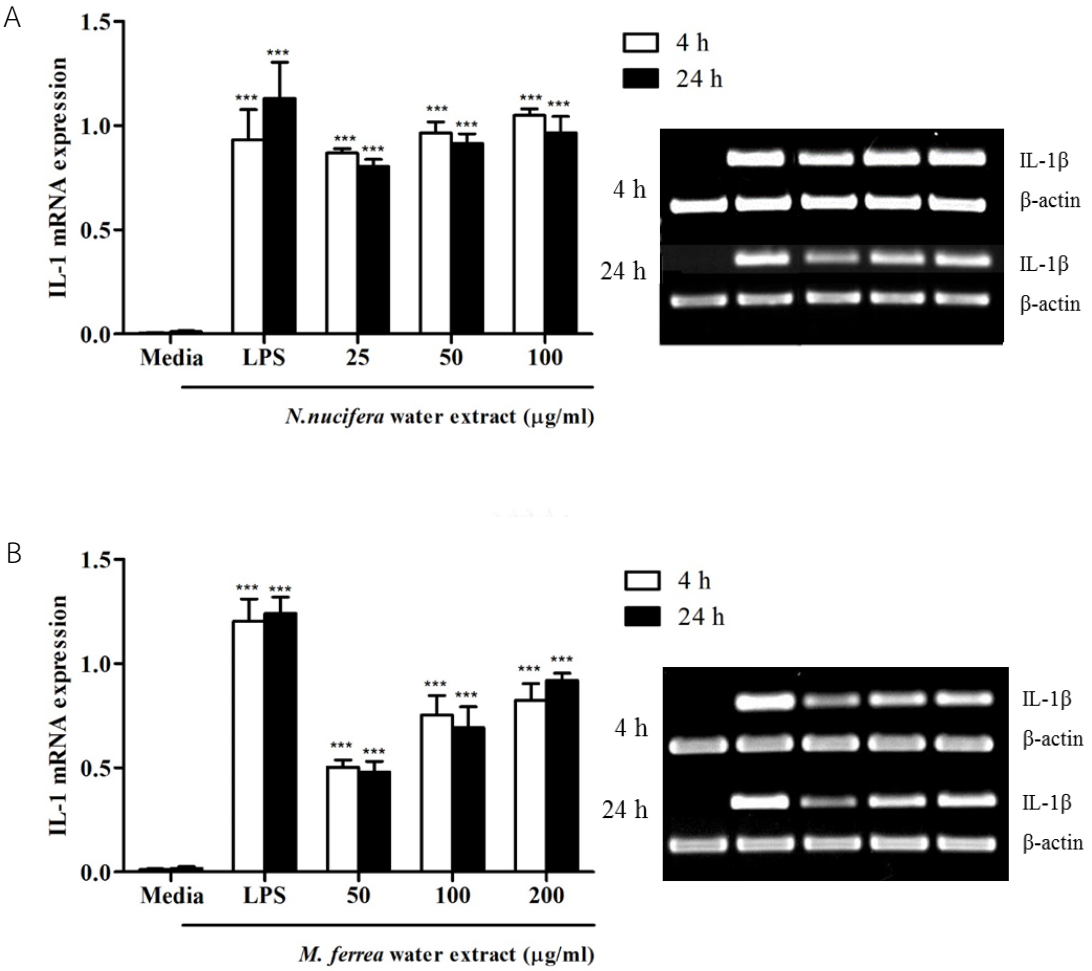

Figure 21: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on IL-1β mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-1β to β-actin from three independent experiments (n=3) of each treated condition. ****p < 0.001* compared to DMEM media control.

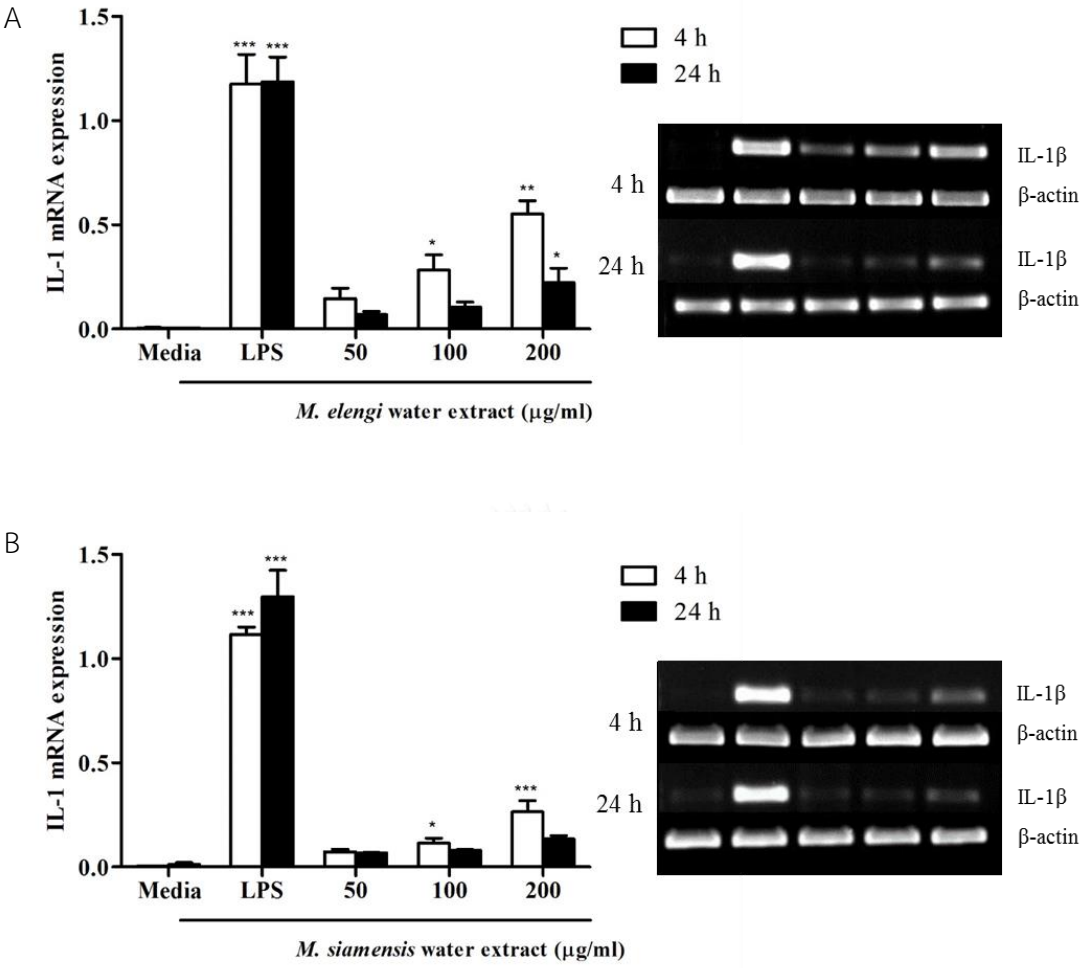

Figure 22: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on IL-1β mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-1β to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control.

3.1.2 Effect of the water extracts on mRNA expression of IL-6

Four of all six extracts had stimulatory effects on IL-6 expression in J774A.1 cells. These four extracts were the extracts of the remedy, *J. sambac, N. nucifera,* and *M. ferrea.* They trended to have higher stimulatory effects on IL-6 expression at 24 h than at 4 h. After 4 h of treatment, the water extracts of the remedy at 6.25 – 25 µg/ml (Fig.23 A*), J. sambac,* at 12.5 – 25 µg/ml (Fig.23 B), *N. nucifera*, at 50 – 100 µg/ml (Fig.24 A), and *M. ferrea*, at 100 – 200 µg/ml (Fig.24 B) significantly up-regulated IL-6 mRNA expression. After 24 h of treatment, these extracts at all concentrations used in the study significantly stimulated IL-6 expression. The other two extracts, *M. elengi* and *M. siamensis* (Fig.25 A-B) did not have effect on IL-6 mRNA expression at both 4 and 24 h of treatment.

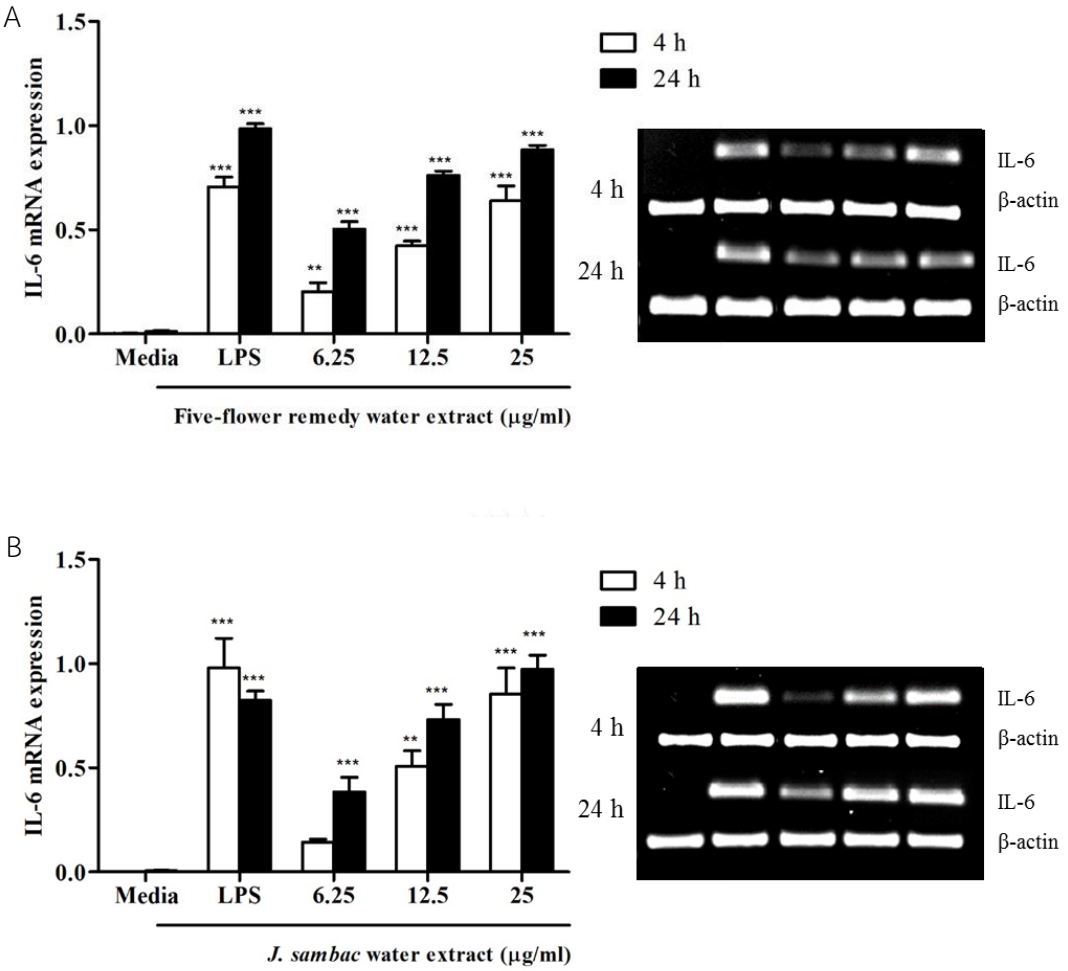

Figure 23: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on IL-6 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-6 to β-actin from three independent experiments (n=3) of each treated condition. ***p < 0.01, ***p < 0.001* compared to DMEM media control.

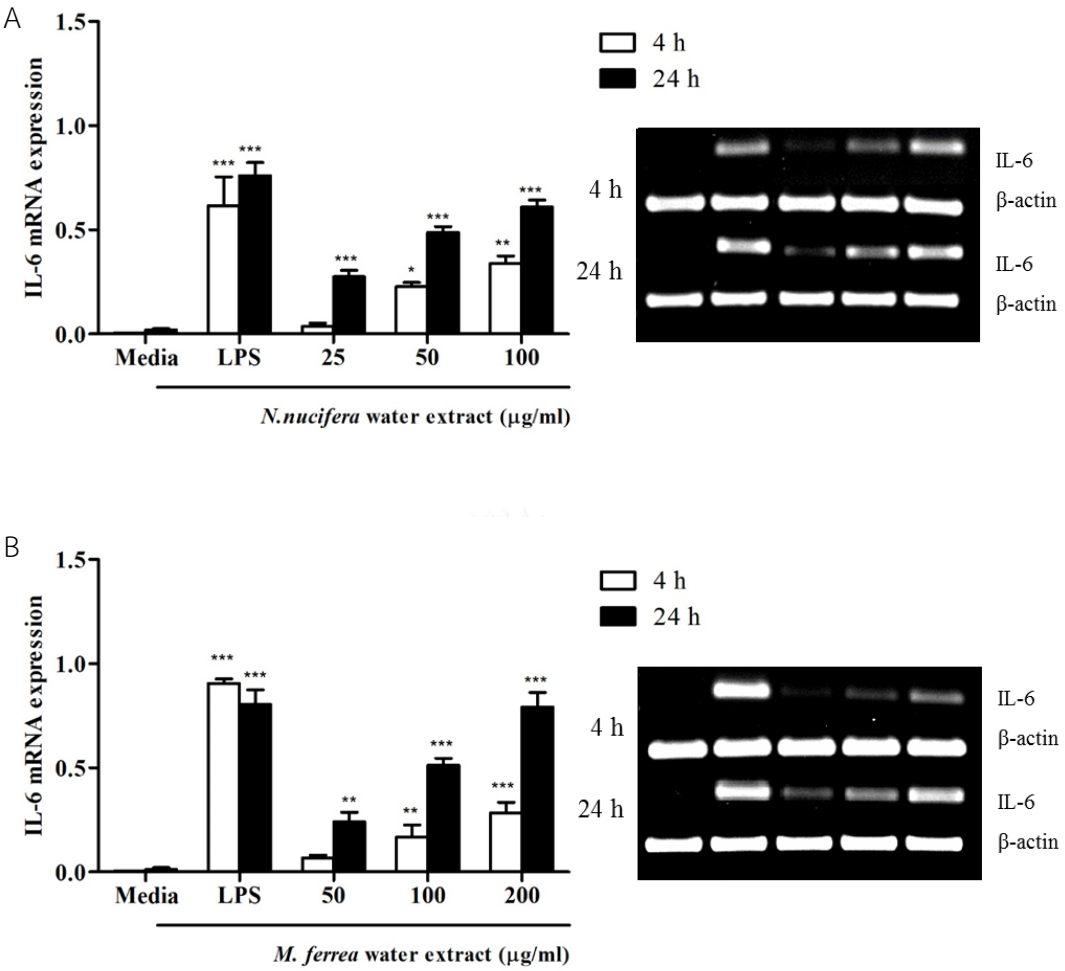

Figure 24: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on IL-6 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-6 to β-actin from three independent experiments (n=3) of each treated condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to DMEM media control.

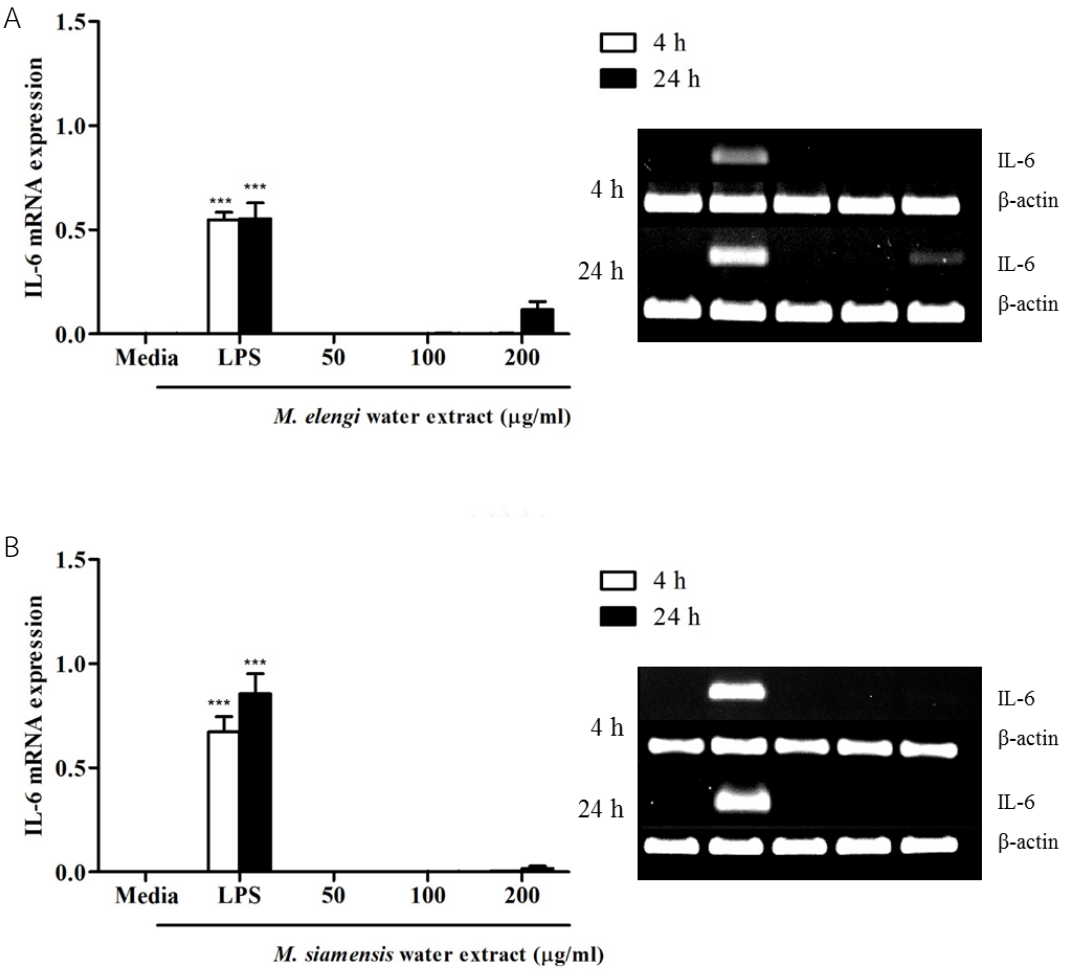

Figure 25: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on IL-6 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-6 to β-actin from three independent experiments (n=3) of each treated condition. ****p < 0.001* compared to DMEM media control.

3.1.3 Effect of the water extracts on mRNA expression of TNF-α

All the water extract had stimulatory effects on mRNA expression of TNF-α in J774A.1 cells. Their stimulatory effects at 4 h were higher than at 24 h. The water extract of the remedy (Fig.26 A), *J. sambac* (Fig.26 B), *N. nucifera* (Fig.27 A), *and M. ferrea* (Fig.27 B) at all concentrations used in this study significantly increased the TNF-α mRNA expression at 4 and 24 h of treatment. The extract of *M. elengi* and *M. siamensis* at all concentration (50- 200 µg/ml) significantly increased the expression of TNF-α at 4 h (Fig.28 A-B). The extract of *M. elengi* at 200 µg/ml and *M. siamensis* at 100 – 200 µg/ml significantly stimulated the expression of TNF-α at 24 h.

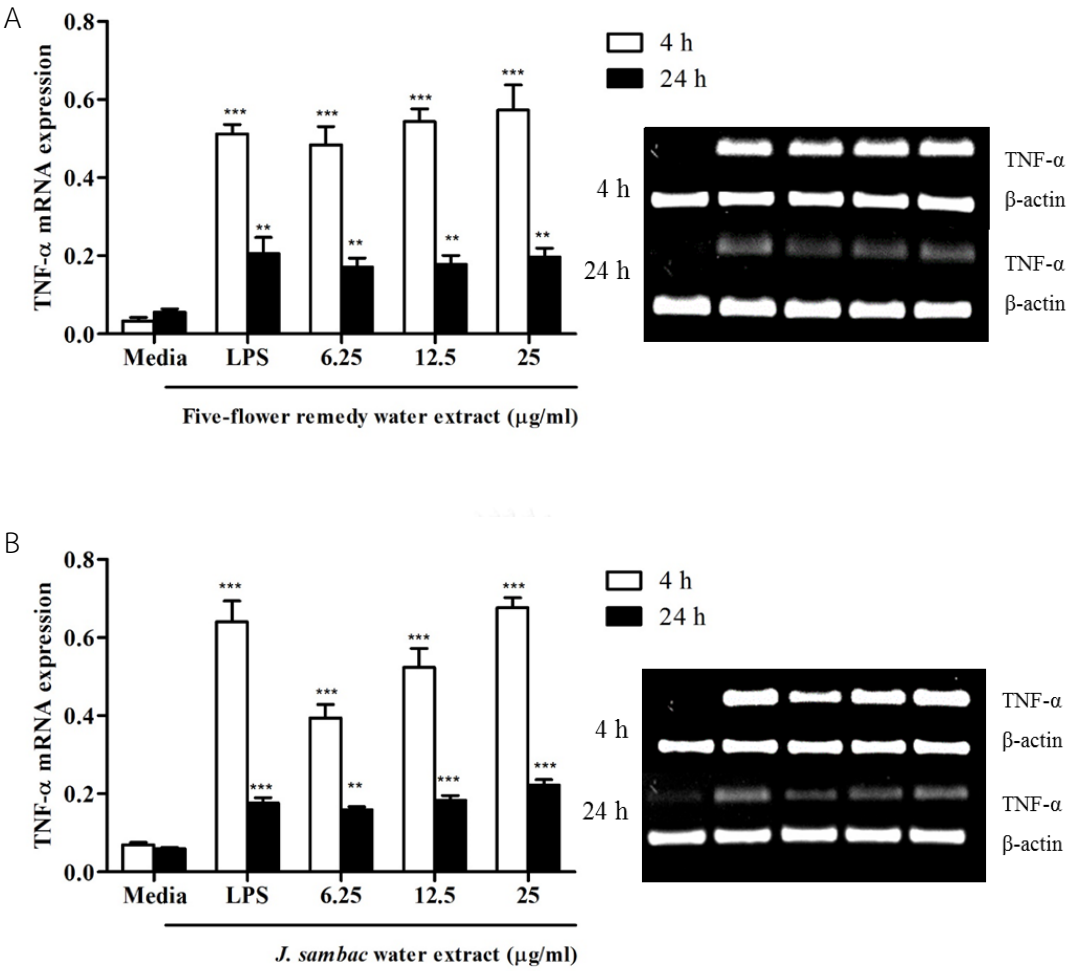

Figure 26: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on TNF-α mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of TNF-α to β-actin from three independent experiments (n=3) of each treated condition. ***p < 0.01, ***p < 0.001* compared to DMEM media control.

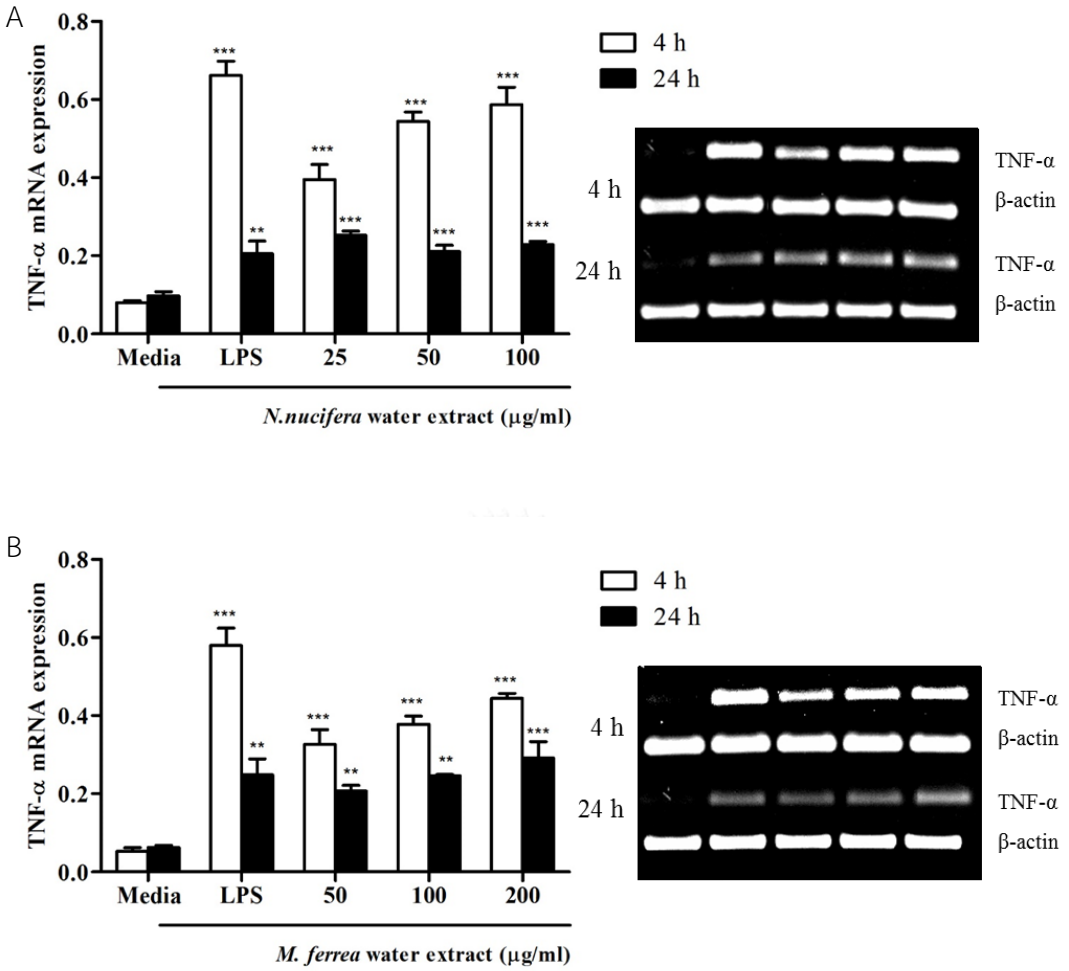

Figure 27: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on TNF-α mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of TNF-α to β-actin from three independent experiments (n=3) of each treated condition. ***p < 0.01, ***p < 0.001* compared to DMEM media control.

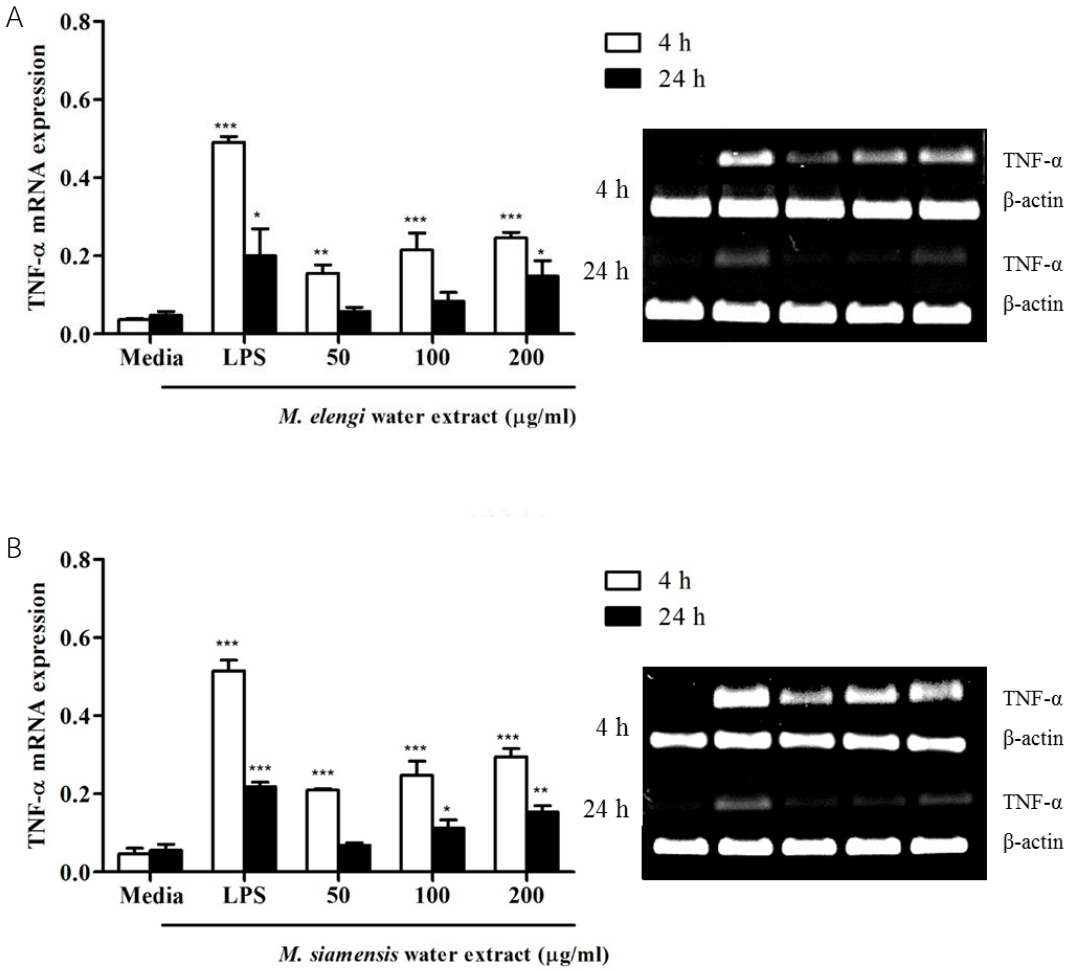

Figure 28: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on TNF-α mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of TNF-α to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control.

3.1.4 Effect of the water extracts on mRNA expression of IL-10

All six extracts had stimulatory effects on IL-10 expression in J774A.1 cells (Fig.29-31). Their stimulatory effects on IL-10 expression at 4 h were much higher than at 24 h. After 4 h of treatment, the extract of the remedy (Fig.29 A), *J. sambac* (Fig.29 B)*, N. nucifera* (Fig.30 A), *and M. ferrea* (Fig.30 B) at all concentration used in this study significantly increase IL-10 mRNA expression at 4 h of treatment. The extracts of *M. elengi (*Fig.31 A) *and M. siamensis (*Fig.31 B) significantly increased the IL-10 mRNA expression only at 200 µg/ml. After 24 h of treatment, the extract of the remedy at all concentration still significantly increased the IL-10 expression in the much lesser amount than at 4 h. Stimulation of IL-10 expression was also observed when J774A.1 cells were treated with 25 µg/ml *J. sambac* extract and 200 µg/ml *M. ferrea* extract. The extracts of *N. nucifera, M. elengi,* and *M. siamensis* did not stimulated IL-10 expression at 24 h of treatment.

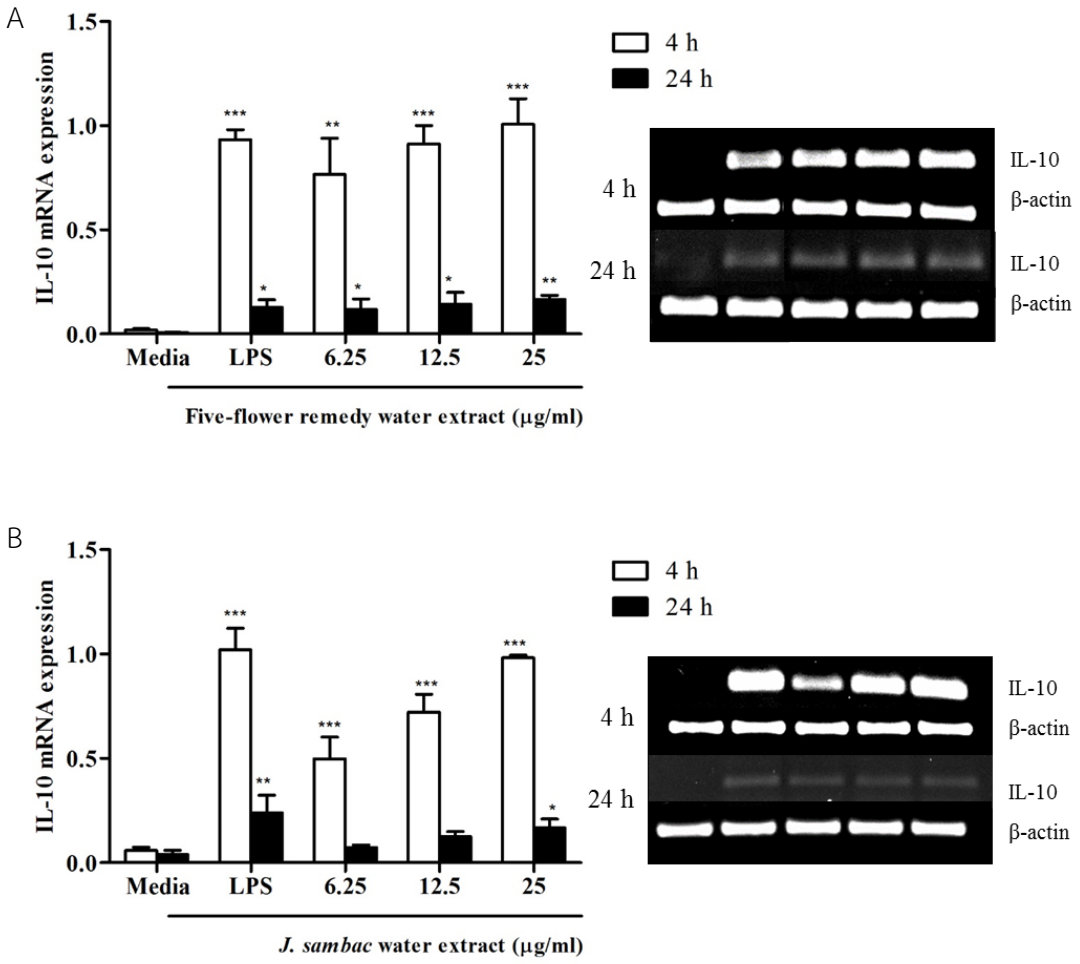

Figure 29: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on IL-10 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-10 to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control.

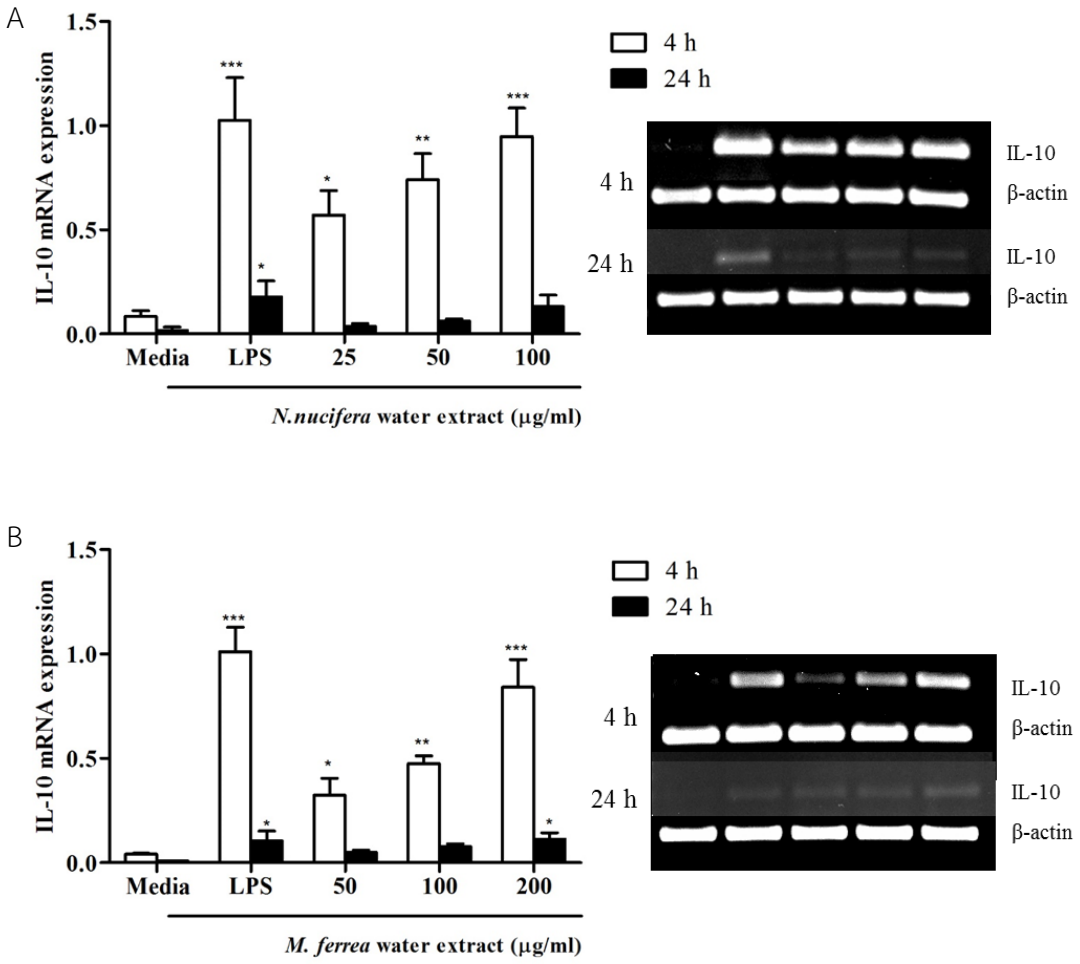

Figure 30: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on IL-10 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-10 to β-actin from three independent experiments (n=3) of each treated condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to DMEM media control.

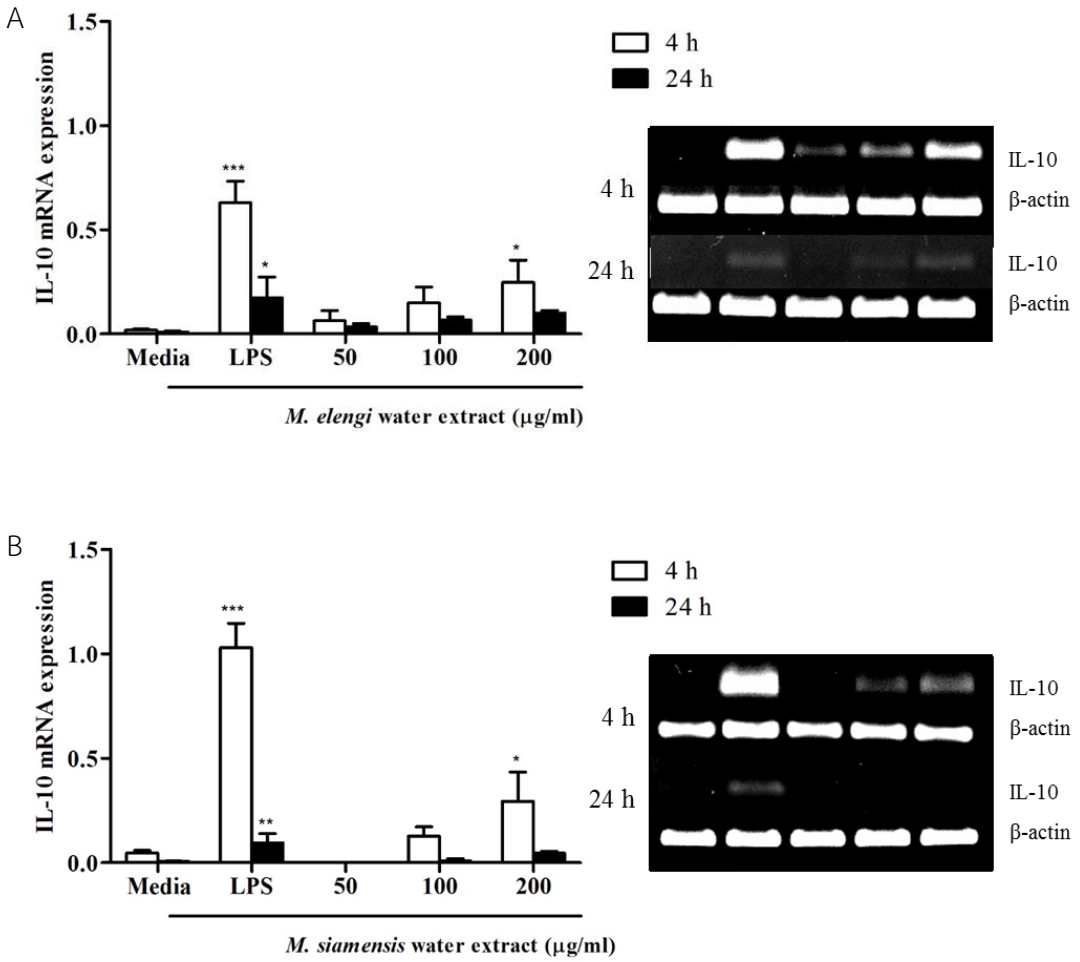

Figure 31: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on IL-10 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of IL-10 to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control

3.2. Effect of the water extracts on iNOS mRNA expression in macrophage J774A.1 cells

iNOS is an inducible enzyme which generates NO from L-arginine in activated macrophages. NO plays roles in phagocytosis and acts as an inflammatory mediator in inflammatory process. Both iNOS and NO are commonly used as ones of the markers of activated macrophages.

All extracts significantly increased iNOS mRNA expression in J774A.1 cells after 24 h of treatment. They had much lesser stimulatory effects on iNOS mRNA expression at 4 h. The extracts of the remedy (Fig.32 A), *J. sambac* (Fig.32 B), *N. nucifera* (Fig.33 A), *and M. ferrea* (Fig.33 B) at all concentrations increased iNOS mRNA expression at 24 h of treatment. The extract of *M. elengi* significantly activated iNOS mRNA expression at 100 and 200 µg/ml (Fig.34 A). The extract of M. siamensis at 200µg/ml significantly stimulated NO production at 24 h of treatment (Fig.34 B).

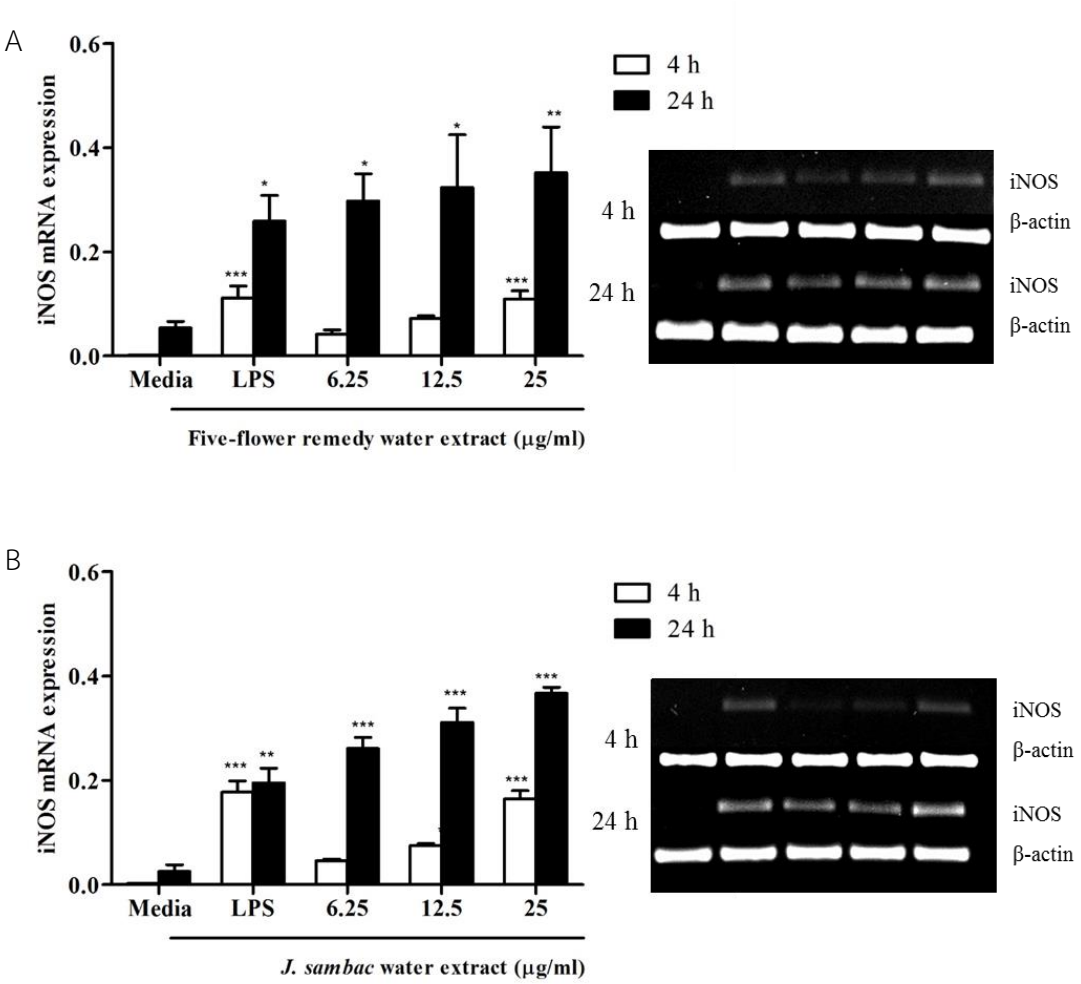

Figure 32: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on iNOS mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of iNOS to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control.

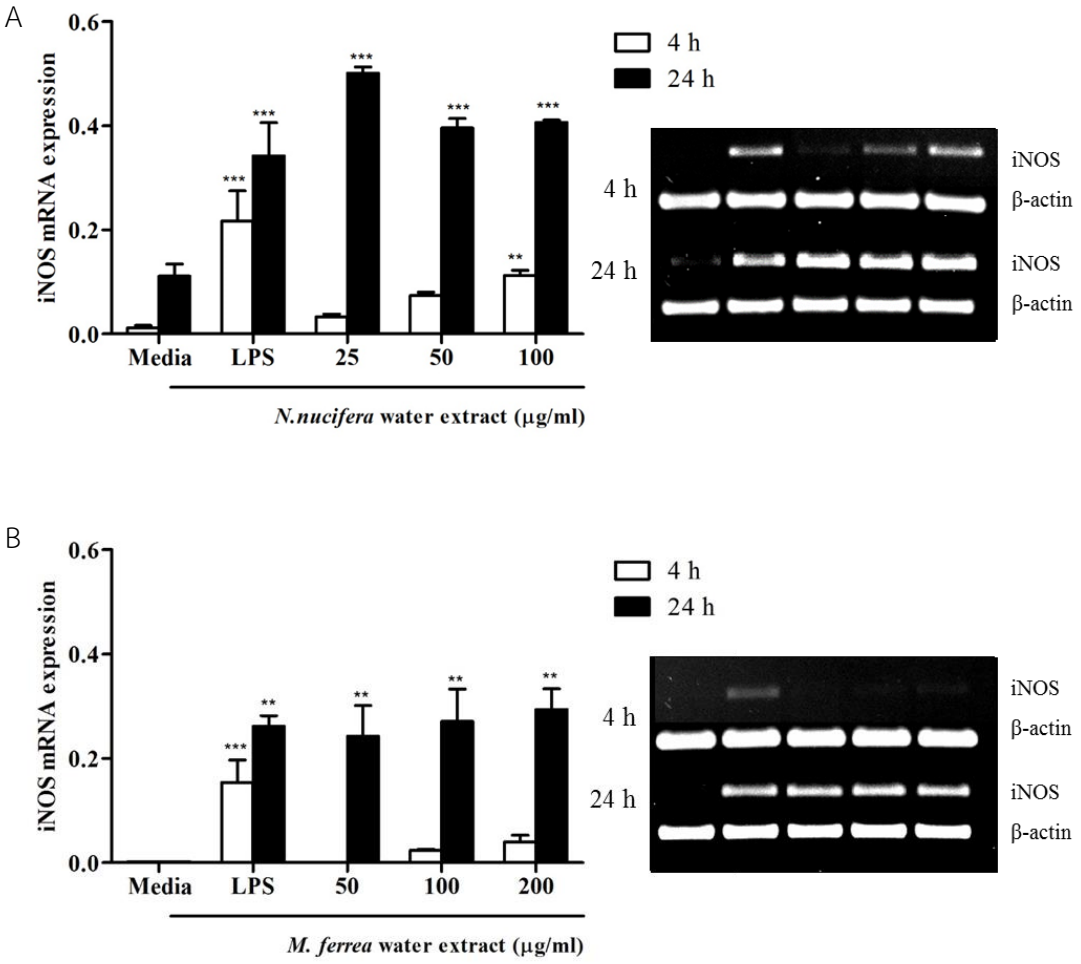

Figure 33: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on iNOS mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of iNOS to β-actin from three independent experiments (n=3) of each treated condition. ***p < 0.01, ***p < 0.001* compared to DMEM media control.

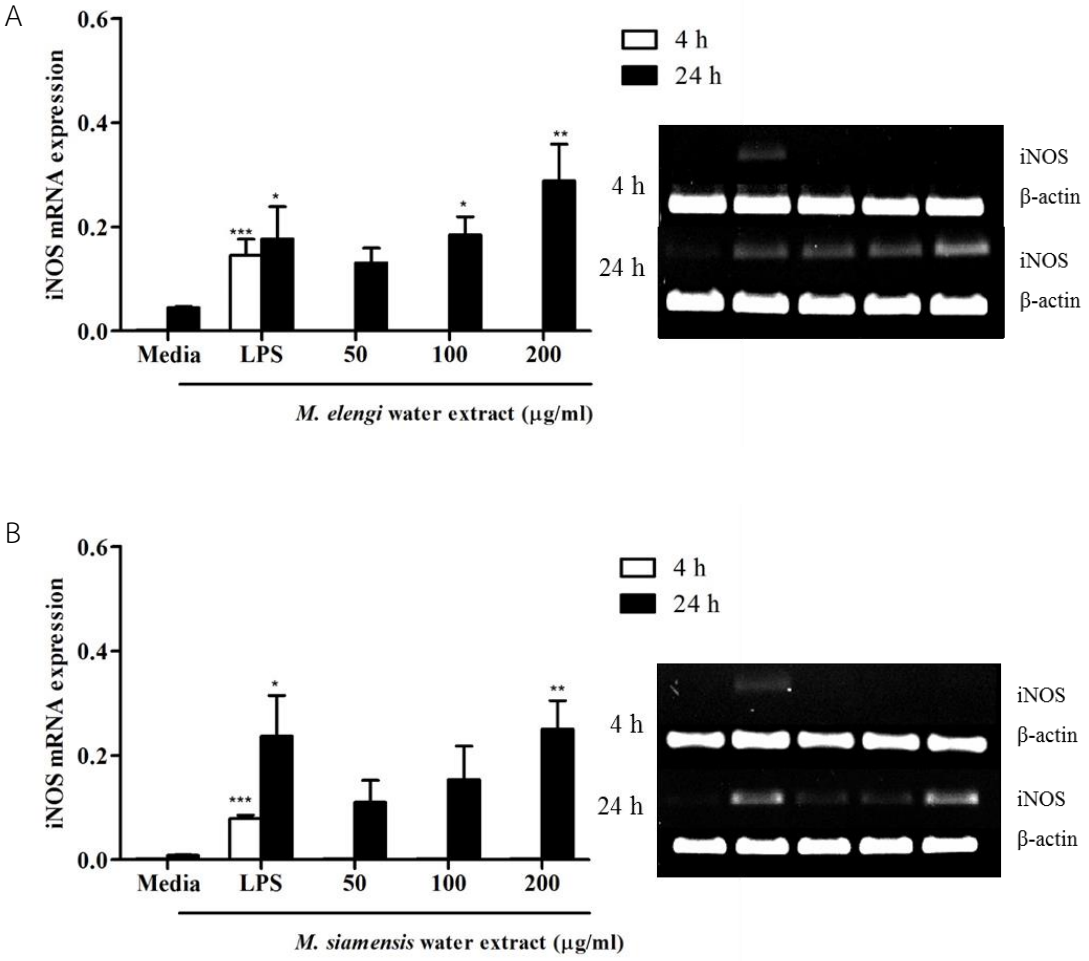

Figure 34: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on iNOS mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of iNOS to β-actin from three independent experiments (n=3) of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control

3.3. Effect of the water extracts on Arg-1 mRNA expression in macrophage J774A.1 cells

Argenase-1 (Arg-1) metabolizes L-arginine to L-ornithine which is essential precursor for collagen synthesis that required for wound healing process. It is recently used as one of the marker of M2 activated macrophages which play important roles in regulation of immune response and tissue repair in inflammatory process. Effects of all six water extracts on Arg-1 expression in J774A.1 cells at 4 and 24 h were evaluated.

All six extracts did not have effect on Arg-1 mRNA expression at 4 h of treatment (Fig.35-37). Four of six extracts significantly increased Arg-1 expression at 24 h of treatment. These four extracts were the remedy extract at 12.5 – 25 µg/ml (Fig.35 A), *J. sambac* extract at 6.25 – 25 µg/ml (Fig.35 B), *N. nucifera* extract at 50 – 100 µg/ml (Fig.36 A), and *M. ferrea* extract at 100 – 200 µg/ml (Fig.36 B). The extracts of *M. elengi* (Fig.37 A) and *M. siamensis* (Fig.37 B) did not have effect on Arg-1 expression at both 4 and 24 h of treatment.

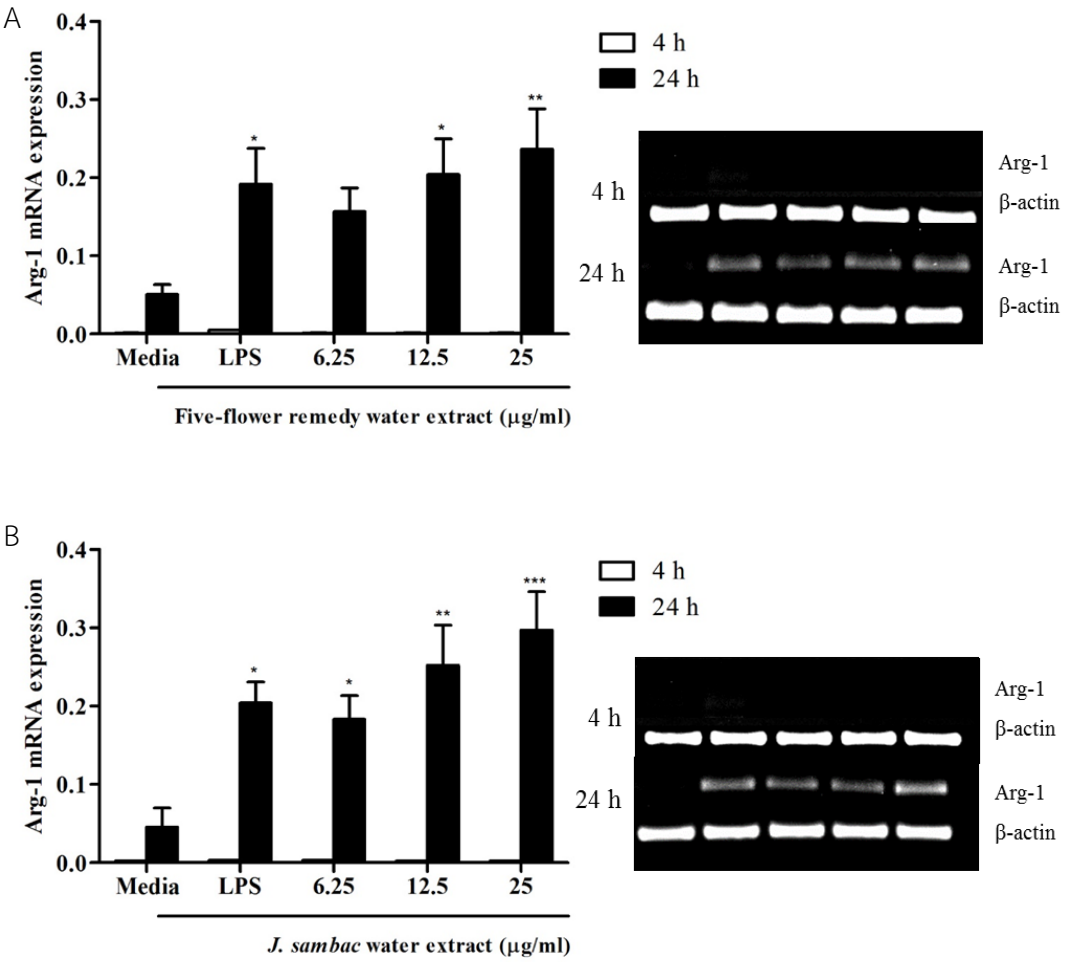

Figure 35: Effects of the water extracts of five-flower remedy (A) and *J. sambac* (B) on Arg-1 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of Arg-1 to β-actin from three independent experiments (n=3) at 24 h and one experiments (n=1) at 4 h of each treated condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to DMEM media control.

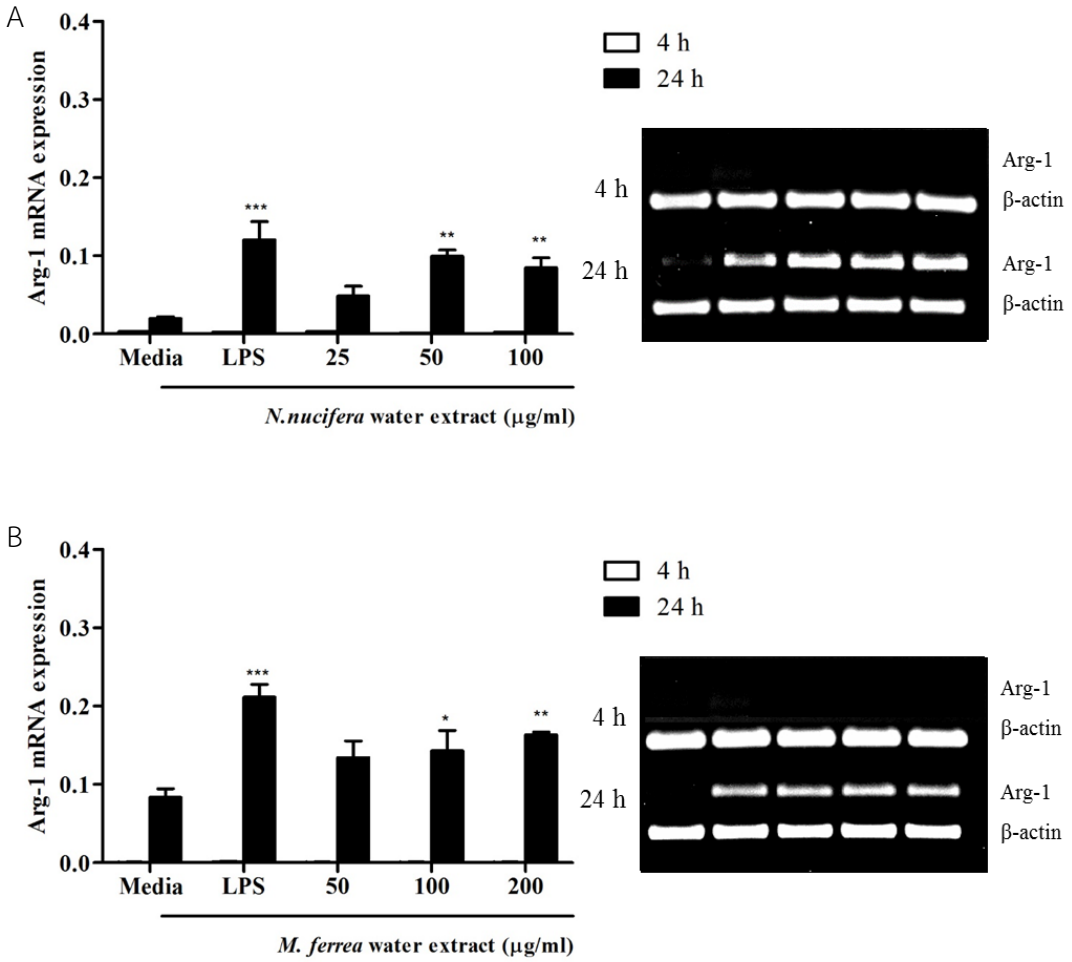

Figure 36: Effects of the water extracts of *N. nucifera* (A) and *M. ferrea* (B) on Arg-1 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of Arg-1 to β-actin from three independent experiments (n=3) at 24 h and one experiments (n=1) at 4 h of each treated condition. **p < 0.05, **p < 0.01, ***p < 0.001* compared to DMEM media control.

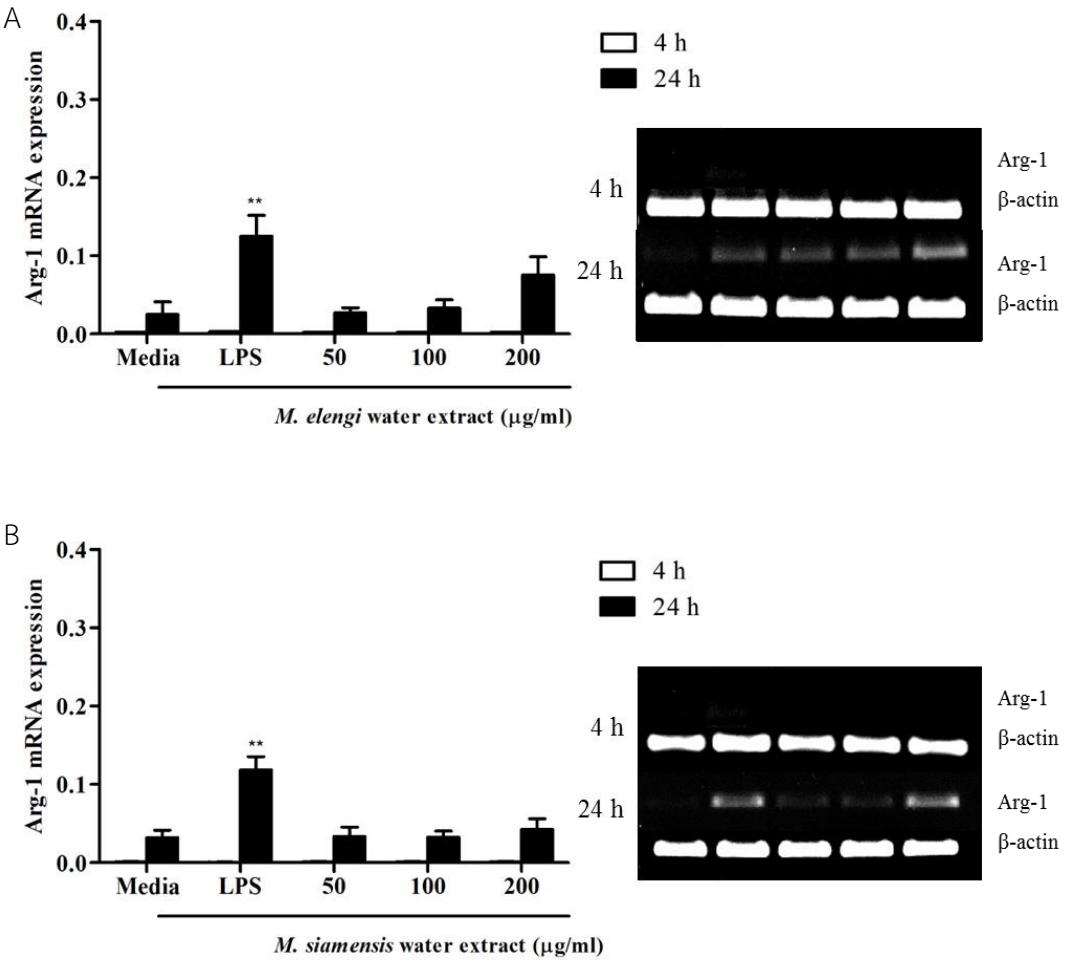

Figure 37: Effects of the water extracts of *M. elengi* (A) and *M. siamensis* (B) on Arg-1 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are represented as mean ± S.E.M of the ratio of Arg-1 to β-actin from three independent experiments (n=3) at 24 h and one experiments (n=1) at 4 h of each treated condition. ***p < 0.01* compared to DMEM media control

CHAPTER V
DISCUSSION AND CONCLUSION

Decrease in immune response leads to health problem because immunity is very crucial host defense against pathogens and cancer cells. Many factors can cause decline in immune functions such as chronic illnesses, stress condition, aging, and medications. Boosting the immune system by immunomodulating agents is a potential strategy to strengthen host immunity. Most of immunostimulating agents which are clinically and traditionally used have immunomodulating effects on macrophage functions [10, 74]. Since macrophages play multiple roles in both innate and adaptive immunity for host defense against infection and cancer, they were used in this study to evaluate the potential immunomodulating activities of the water extracts of five-flower remedy and its five components which are flowers of *Jasminum sambac, Nelumbo nucifera, Mesua ferrea, Mimusops elengi* and *Mammea siamensis*. Several markers of activated macrophages, both M1 and M2 macrophages were investigated. These included NO and iNOS, pro-inflammatory cytokines (IL-1, IL-6, and TNF-α), anti-inflammatory cytokine IL-10 and Arg-1.

NO is reactive nitrogen intermediate generated by iNOS in activated macrophages. It destroys pathogen in phagocytosis process by oxygen-dependent pathway. It also acts as an inflammatory mediator in acute inflammation [18]. The water extracts of five-flower remedy and its components increased NO production in macrophage J774A.1 cells. They demonstrated different degree of stimulatory effects when using the effect of 100 ng/ml LPS control as the basis. Among five components of the remedy, *J. sambac* extract had the highest potency, followed by *N. nucifera* extract and *M. ferrea* extract. *M. elengi* extract and *M. siamensis* extract had the lowest stimulatory activities when compare to the other extracts. The extract of the remedy had highest stimulatory effect similar to the effect of *J. sambac* extract. These results demonstrated that the extract of five flowers synergistically activated NO production when they were used as the remedy. The stimulatory effects of these six extracts on NO production came from their effects on expression of iNOS which catalyzes large

amount of NO production. iNOS is an enzyme that usually does not express in resting macrophages. It is induced during phagocytosis and after cytokine production in activated macrophages. NO and iNOS are ones of the markers of M1 macrophages. These extracts induced much higher iNOS expression at 24 h than at 4 h of treatment. The stimulatory effect of the water extracts of the remedy and its five components on NO production and iNOS expression on macrophages has not been documented. However, effects of other solvents extracts of some components of the remedy were studied. The methanol extract of *N. nucifera* reduced mRNA expression of nitric oxide synthase (iNOS) and COX-2 [51]. The ethanol extract of *N. nucifera* inhibited NO production in LPS-activated macrophages [50]. Coumarin from the methanol extract of *M. siamensis* inhibited NO production in LPS-activated RAW264.7 cells [73]. It is possible that active compounds of the same herbs in water extracts in this study are different from active compounds in other solvents used in previous studies.

Activated macrophages not only destroy pathogens by phagocytosis process. They also generate several enzymes and mediators to function in both innate and adaptive immunity. Pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, are key mediators of M1 macrophages [3]. They not only function in inflammatory process but also initiate T cell activation and perform other functions in immune response. These cytokine are commonly used as the marker of M1 macrophage activation [7]. In this study, all six water extracts significantly activated IL-1β and TNF-α mRNA expression. They stimulated IL-1β expression in the same level at 4 and 24 h of treatment. They stimulated TNF-α expression at 4 h higher than at 24 h of treatment. The orders of stimulatory activity of the extracts on these cytokine expressions were similar to the way that they stimulated NO production. The extracts of remedy and *J. sambac* had the highest stimulatory activity. Four out of six water extracts stimulated IL-6 mRNA expression. These extract were the extracts of remedy, *J. sambac, N. nucifera,* and *M. ferrea.* The extracts of *M. elengi and M. siamensis* had no effect on IL-6 expression. The water extracts in this study stimulated these pro-inflammatory cytokine expressions in similar patterns to the effect of LPS. LPS in well known as activator of M1 macrophages. So, it is possible that these water extracts are also M1

macrophage activators. The results also demonstrated that the extract of five flowers synergistically activated pro-inflammatory cytokine expression when they were used as the remedy. These results were comparable to their effects on NO production. Previously, other solvent extracts of a component of the remedy demonstrated inhibitory effect on pro-inflammatory cytokine production. The methanol extract of *N. nucifera* reduced production of IL-6 and TNF-α in LPS-activated of murine macrophage RAW264.7 cells [51]. Active compounds in the water extract of *N. nucifera* in this study may different from active compounds in the methanol extract of the previous study.

This study also investigated the effects of the water extracts on markers of alternative or M2 activated macrophages. IL-10 and Arg-1 were chosen to determine. All six water extracts stimulated IL-10 expression in similar manner to the effect of LPS control. Their stimulatory effects at 4 h were higher than at 24 h of treatment. Previously, S-armepavine purified from *N. nucifera* ethanolic extract demonstrated inhibitory effect on IL-2, IL-4, IL-10, and IFN-γ production in peripheral blood mononuclear cells [47, 48]. Four of six water extracts, the extracts of remedy, *J. sambac, N. nucifera,* and *M. ferrea*, stimulated the expression of Arg-1 which is the other marker of M2 macrophage activation. Their stimulatory effects were observed at 24 h of treatment. The extracts of *M. elengi* and *M. siamensis* did not have effects on Arg-1 expression. The effects of these five flower extracts on Arg-1 have not been reported.

Five-flower remedy containing the flowers of *J. sambac, N. nucifera, M. ferrea, M. elengi* and *M. siamensis* has long been used as traditional Thai medicine. However, its immunomodulating activity has not been scientifically reported. There are also very few information about effects of these five components on immune functions, both *in vitro* and *in vivo*. This study revealed the effect of the remedy on the immune system for the first time. The water extract of the remedy activated macrophage J774A.1 cells to express or produce cytokines, enzymes, and NO in similar manner to the effect of LPS control. This suggests that the extract may activate macrophages to M1 phenotypes. It is postulated that the stimulatory effect of the remedy comes from the synergistic effects of its components. The water extract of the remedy contained

around one fifth (0.727/5 – 1.46/5) of each component which had different levels of stimulating activities. The remedy had the highest stimulating activity, similar to *J. sambac* extract, to activate all markers of macrophage activation evaluated in the study. The extracts of *M. elengi* and *M. siamensis* demonstrated the lowest stimulating activity on macrophages. According to the yield of each extract after extraction, it is estimated that the remedy extract contained almost 50% (20.75% plus 29.19% = 49.94%) of *M. elengi* and *M. siamensis.* The synergism of components in the remedy on macrophage activation can be explained by the similar potency between the remedy and *J. sambac* extract which was about one fifth of the remedy extract. If no synergism, the remedy should have less potency than *J. sambac* extract. This study also reveals that different solvent extraction on the same herb may yield different active compounds in each solvent extract. The water extracts of the components in the remedy demonstrated immunostimulating effects on macrophages but methanol or ethanol extracts of some components had inhibitory effects [47, 48, 50, 51, 73]. This study also represented a good example of traditional use of combination herbs for their synergistic effects in the formulation.

In conclusion, this study initially revealed the immunostimulating activities of the water extracts of five-flower remedy and its components on macrophages which usually have multi-immune functions. This immunostimulating effect on macrophages may be a part of tonic effect of the remedy. It is highly possible that the components in the remedy synergistically activated macrophages when they were combined as the remedy. The results from this study may be used as useful information for selecting the preparation of this remedy. Herbal tea and boiled solution may contain active components similar to the water extract of the remedy in this study. Further studies, both *in vivo* and *in vitro*, are needed to evaluate more immunomodulating effects of the remedy.

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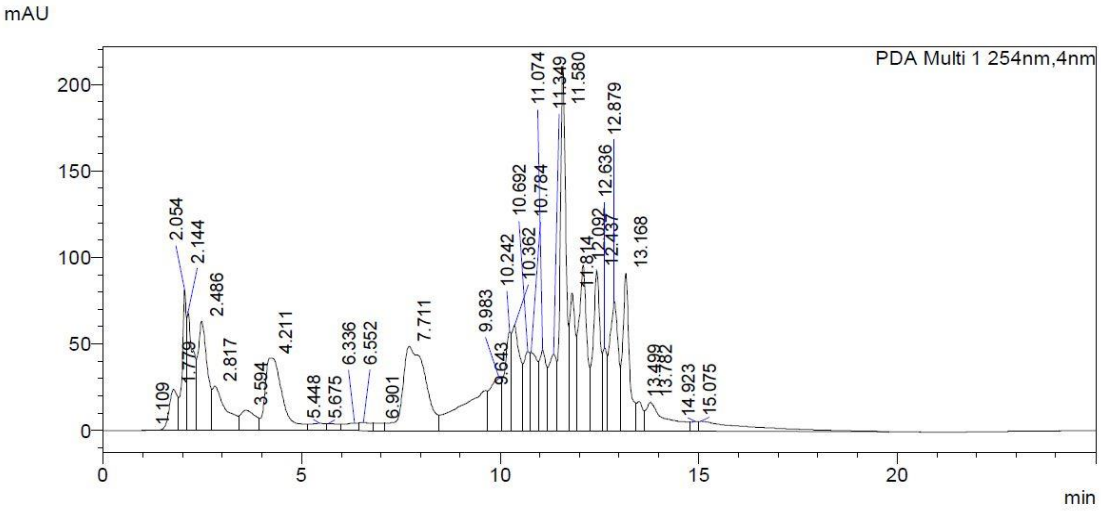
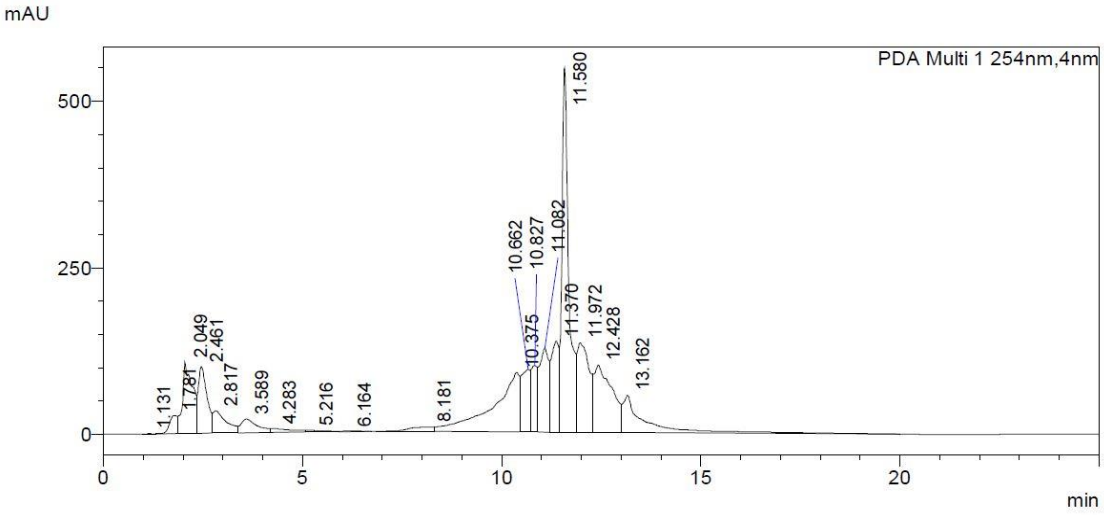
APPENDIX

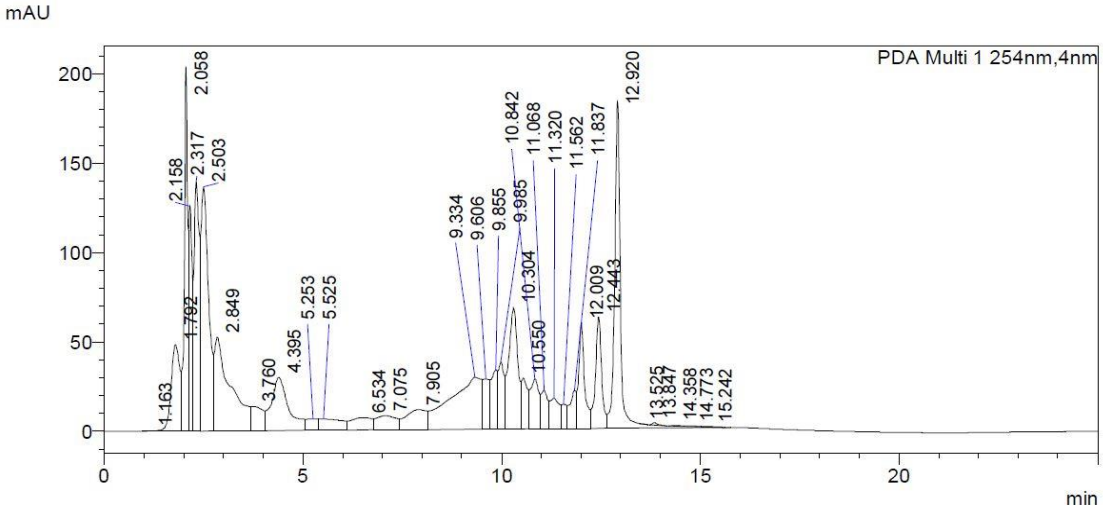
Appendix 1: The HPLC chromatogram of the water extract of five-flower remedy [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30˚C].



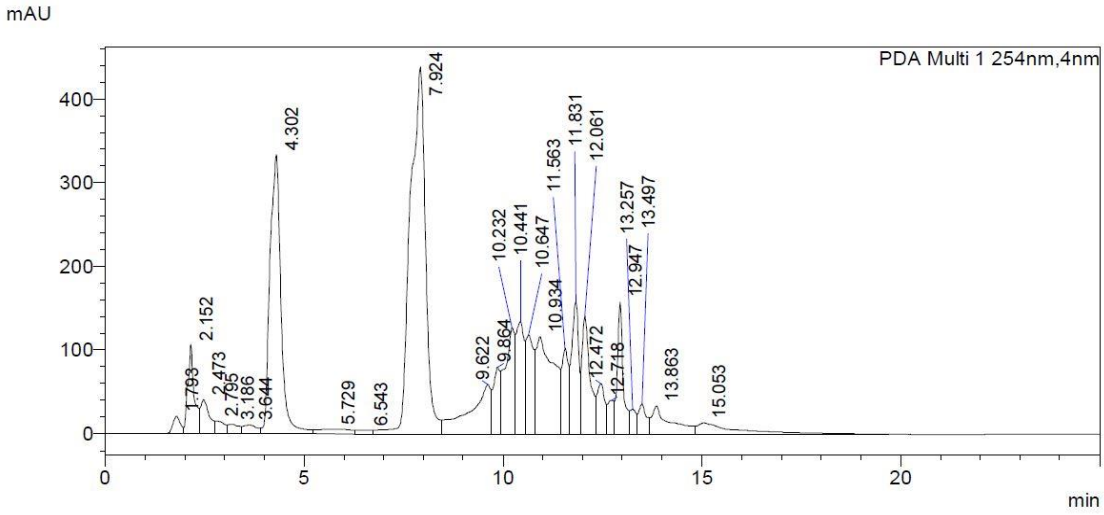
Appendix 2: The HPLC chromatogram of the water extract of *Jasminum sambac* [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30˚C].



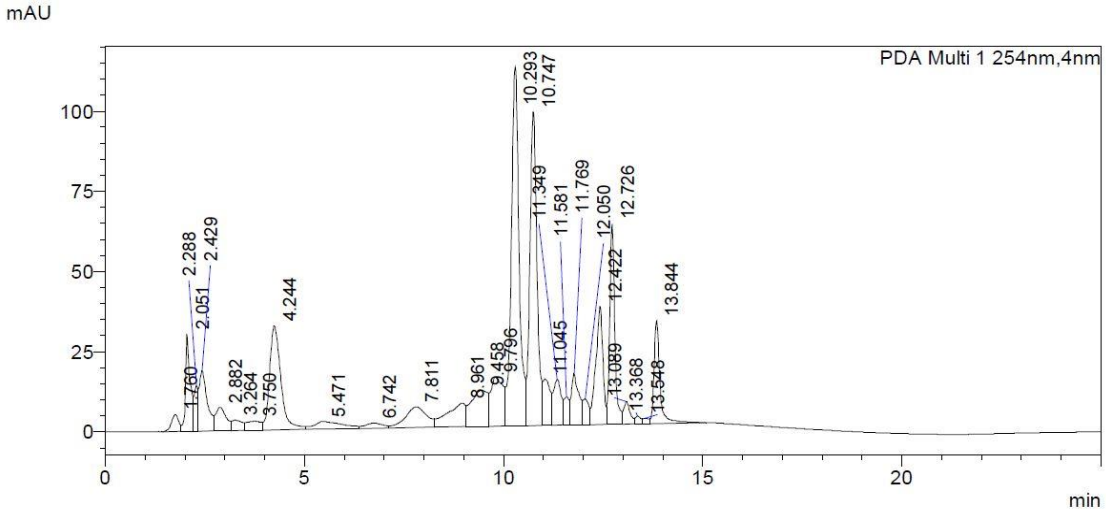
Appendix 3: The HPLC chromatogram of the water extract of *Nelumbo nucifera* [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30˚C].



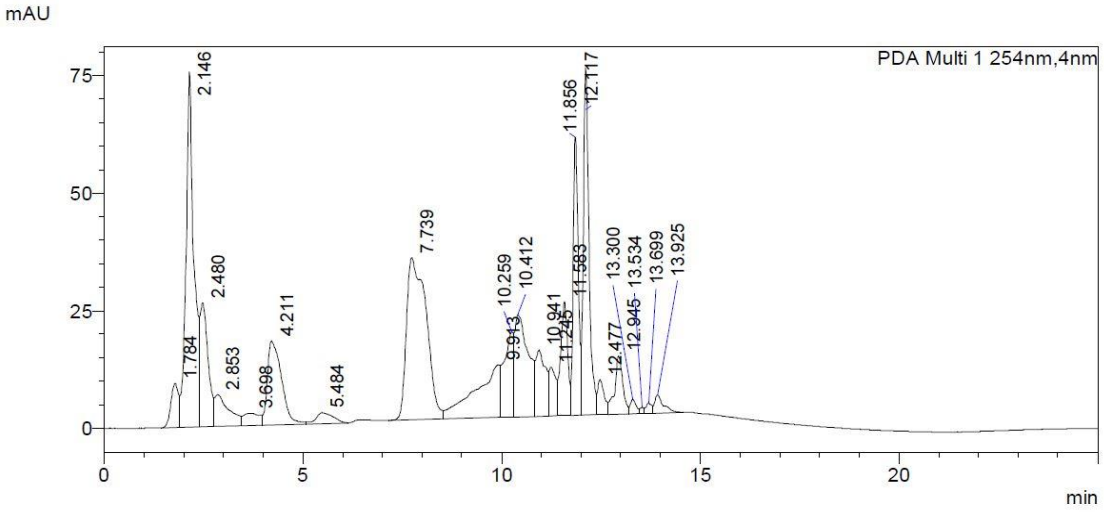
Appendix 4: The HPLC chromatogram of the water extract of *Mesua ferrea* [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



Appendix 5: The HPLC chromatogram of the water extract of *Mimusops elengi* [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30˚C].



Appendix 6: The HPLC chromatogram of the water extract of *Mammea siamensis* [column: Inertsustrain® C18 (2.0 mm), 2.1 mm 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30˚C].



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