ACTIVITY OF LONGEVITY REMEDY WATER EXTRACT ON MURINE MACROPHAGES

Miss Kullanan Jongnimitphaiboon

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ฤทธิ์ของสารสกัดน้ำจากตำรับยาอายุวัฒนะต่อเซลล์แมคโครฟาจของหนู

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กุลนันทน์ จงนิมิตไพบูลย์ : ฤทธิ์ของสารสกัดน้ำจากตำรับยาอายุวัฒนะต่อเซลล์แมคโครฟาจของหนู (ACTIVITY OF LONGEVITY REMEDY WATER EXTRACT ON MURINE MACROPHAGES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ภก. ดร.ชัยศักดิ์ จันศรีนิยม, หน้า.

ยาอายุวัฒนะ เป็นตำรับยาไทยแผนโบราณที่ใช้บำรุงร่างกาย ให้มีชีวิตยืนยาว ประกอบด้วยสมุนไพร 6 ชนิด คือ หัวแห้วหมู เมล็ดพริกไทย เมล็ดข่อย เถาบอระเพ็ด เปลือกตะโกนา และเปลือกทิ้งถ่อน ในสัดส่วนที่เท่ากัน งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์กระตุ้นเซลล์แมคโครฟาจ J774A.1 ของสารสกัดน้ำจากตำรับยาอายุวัฒนะและสมุนไพรที่เป็นองค์ประกอบในตำรับ ในการศึกษาฤทธิ์ของสารสกัดต่อการสร้างไนตริกออกไซด์ที่เป็นสารสื่อที่สำคัญในกระบวนการจับกินสิ่งแปลกปลอมและกระบวนการอักเสบของเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยวิธี Griess reaction หลังเซลล์ได้รับสารสกัดเป็นเวลา 24 ชม. พบว่าสารสกัดน้ำจากตำรับ หัวแห้วหมู และเมล็ดพริกไทยที่ 1.56-25 ไมโครกรัม/มิลลิลิตร มีฤทธิ์กระตุ้นการสร้างไนตริกออกไซด์ในเซลล์แมคโครฟาจ ตามความเข้มข้นของสารสกัด ส่วนสารสกัดน้ำจากเมล็ดข่อย เถาบอระเพ็ด และเปลือกตะโกนาที่ 12.5-100 ไมโครกรัม/มิลลิลิตร กระตุ้นการสร้างไนตริกออกไซด์ได้ต่ำกว่าสารสกัดข้างต้น ส่วนสารสกัดจากเปลือกทิ้งถ่อนไม่มีฤทธิ์กระตุ้นการสร้างไนตริกออกไซด์ สารสกัดทุกตัวในความเข้มข้นที่ใช้ทดสอบไม่มีความเป็นพิษต่อเซลล์แมคโครฟาจ ส่วนการศึกษาฤทธิ์ของสารสกัดต่อการแสดงออกในระดับ mRNA ของโปรตีนที่ใช้เป็นตัวชี้บ่งของเซลล์แมคโครฟาจที่ถูกกระตุ้นด้วยวิธีอาร์ทีพีซีอาร์ หลังเซลล์ได้รับสารสกัดเป็นเวลา 4 และ 24 ชม. โดยใช้สารสกัดของตำรับ หัวแห้วหมู และเมล็ดพริกไทยที่ 3.13, 6.25 และ 12.5 ไมโครกรัม/มิลลิลิตร สารสกัดจากเมล็ดข่อย เถาบอระเพ็ด และเปลือกตะโกนาที่ 25, 50 และ 100 ไมโครกรัม/มิลลิลิตร พบว่า สารสกัดจากตำรับและสารสกัดจากสมุนไพรองค์ประกอบเพิ่มการแสดงออกในระดับ mRNA ของ IL-1β, IL-6, TNF-α และ iNOS ที่ใช้เป็นตัวบ่งชี้ของเซลล์แมคโครฟาจที่ถูกกระตุ้นชนิด M1 นอกจากนี้สารสกัดเหล่านี้ยังกระตุ้นการแสดงออกของ IL-10 และ argenase-1 ที่เป็นตัวบ่งชี้ของเซลล์แมคโครฟาจที่ถูกกระตุ้นชนิด M2 ส่วนสารสกัดจากเปลือกทิ้งถ่อนไม่มีผลต่อการแสดงออกของตัวบ่งชี้ของเซลล์แมคโครฟาจที่ถูกกระตุ้น

ผลจากการศึกษานี้แสดงให้เห็นว่าสารสกัดน้ำของตำรับยาอายุวัฒนะและสมุนไพรที่เป็นองค์ประกอบเกือบทุกตัวมีฤทธิ์กระตุ้นเซลล์แมคโครฟาจด้วยความแรงที่แตกต่างกัน สารสกัดจากตำรับและหัวแห้วหมูมีความแรงสูงสุด ตามด้วยสารสกัดจากเมล็ดพริกไทย ส่วนสารสกัดเมล็ดข่อย เถาบอระเพ็ด และเปลือกตะโกนามีฤทธิ์ต่ำกว่าสารสกัดข้างต้น ฤทธิ์กระตุ้นเซลล์ในระบบภูมิคุ้มกันอาจเป็นส่วนหนึ่งของประโยชน์ของการใช้ตำรับยาอายุวัฒนะ

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KULLANAN JONGNIMITPHAIBOON: ACTIVITY OF LONGEVITY REMEDY WATER EXTRACT ON MURINE MACROPHAGES. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., CO-ADVISOR: CHAISAK CHANSRINIYOM, Ph.D., pp.

Longevity remedy is a Thai traditional remedy which has been used to promote and restore health for longevity. This remedy consists of six herbs including *Cyperus rotundus* root, *Piper nigrum* fruit, *Streblus aspera* seed, *Tinospora crispa* stem, *Diospyros rhodocalyx* bark, and *Albizia procera* bark, in the same proportion. This study aimed to investigate the stimulatory activities of the water extracts of longevity remedy and its components on macrophage J774A.1 cells. Effects of the water extracts on nitric oxide (NO) production were measured by Griess reaction assay after 24 h of treatment. The water extracts of longevity remedy, *C. rotundus* and *P. nigrum* at 1.56–25 μg/ml significantly increased NO production in a concentration-dependent manner. The extracts of *S. aspera*, *T. crispa* and *D. rhodocalyx* at 12.5–100 μg/ml demonstrated lower stimulatory effects on NO production than the extracts above. The extract of *A. procera* did not stimulate NO production. All water extracts at all concentrations used in this study had no effects on J774A.1 cell viability. Effects of all water extracts on mRNA expression of several markers of macrophage activation were also evaluated by RT-PCR after 4 and 24 h of treatment. The water extracts of longevity remedy, *C. rotundus* and *P. nigrum* at 3.13, 6.25 and 12.5 μg/ml as well as the extracts of *S. aspera*, *T. crispa* and *D. rhodocalyx* at 25, 50 and 100 μg/ml were used. These extracts increased mRNA expression of IL-1β, IL-6, TNF-α, and iNOS which are often used as the markers of M1 activated macrophages. The extracts also up-regulated mRNA expression of IL-10 and argenase-1 which are the markers of M2 activated macrophages. The extract of *A. procera* had no effect on mRNA expression of all markers.

The results from this study demonstrated that the water extracts of the longevity remedy and almost all of its components can activate macrophage J774A.1 cells with different potencies. The extracts of the remedy and *C. rotundus* had the highest potency, followed by the extract of *P. nigrum*. The other three extracts including *S. aspera*, *T. crispa* and *D. rhodocalyx* had lower potencies on macrophage activation than the extracts of the remedy, *C. rotundus*, and *P. nigrum*. This immunostimulatory activity may be a part of beneficial effects of the use of this longevity remedy.

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LIST OF ABBREVIATIONS

%	Percent
/	Per
<	Less than
µl	Microliter (s)
µM	Micromolar
°C	Degree Celsius
APCs	Antigen presenting cells
Arg	Arginase
ATCC	American Type Culture Collection
Ca^{2+}	Calcium
CCL	Chemokine (C-C motif) ligand
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
Con-A	Concanavalin-A
COX-2	Cyclo-oxygenase-2
CXCL	Chemokine (C-X-C motif) ligand
DMEM	Dulbeco’s Modified Eagle’s Medium
dNTP	Deoxyribonucleotide triphosphate
dsRNA	double-stranded ribonucleic acid
FBS	Fetal bovine serum

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GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hour
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
LTs	Leukotrienes
M	Molarities
$MgCl_2$	Magnesium chloride
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter(s)
MMPs	Matrix metalloproteinase
NED	N-1-napthylethylenediamine dihydrochloride
NF-kB	Nuclear factor-kappa B
ng	Nanogram (s)
NK cell	Natural killer cell
nm	Nanometer

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NO	Nitric oxide
NRAM1	Natural resistance-associated macrophage protein 1
O_2	Oxygen
OD	Optical density
ODC	Ornithine decarboxylase
PAMPs	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PHSC	Pluripotent haematopoietic stem cell
PRR	Pattern recognition receptor
mRNA	Massager ribonucleic acid
RBL	Rat basophilic leukemia
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reverse transcription
S.D.	Standard deviation
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
T_H	Helper T cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell

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CHAPTER I

INTRODUCTION

Background and Rationale

The immune system is classified generally by specificity and characteristic into innate and adaptive immunities. Innate immunity is the first line of host defense against microbes. Its cellular and biochemical defense mechanisms are in place before microbial infection and response rapidly to the infection. These mechanisms respond to repeated infections in the same magnitude as primary infection. Cells in innate immunity specifically recognize common structures of groups of related microbes. They cannot differentiate fine differences among microbes in the same group. Adaptive immunity is the immunity develops after microbial infection and adapts to the infection of each type of microbes. It is the second line defense initiated by innate immunity. It has an extraordinary ability to distinguish between different microbes and molecules as it is also called specific immunity. Characteristics of adaptive immunity are high specificity for distinct molecules and ability to remember and respond more effectively to repeated infections of the same microbe. Both innate and adaptive immunities always cooperatively work for fully effective defense [1].

Macrophages are essential immune cells which play roles in both innate and adaptive immunity. In innate immunity, these cells function as phagocytes for removing pathogens and cell debris [2]. They act as inflammatory and anti-inflammatory cells during tissue injury and wound healing process. In adaptive immunity, they are antigen presenting cells for activating T cell to generate adaptive immunity. They also act as effector cells in both humoral and cell mediated immune responses to eliminate pathogens [3]. All of these macrophages functions mostly

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generated by activated macrophages. Several molecules are used as the markers for macrophage activations. Nitric oxide (NO) and inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), prostaglandin E2 (PGE2), and cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-12] are the markers of activated macrophages (M1 phenotype) during phagocytosis and inflammatory process. IL-10 and transforming growth factor (TGF)-β which are anti-inflammatory cytokines are usually the markers of activated macrophages (M2 phenotype) during healing process.

Macrophages recognize pathogen and destroy it by toxic agent including NO by iNOS or hydrolytic enzyme in phagocytic process [2]. They also synthesize and secret many mediators - TNF-α, IL-1, IL-6 and IL-12 - while they present pathogenic peptides as antigen to adaptive immune cells, resulted in activation of adaptive immunity. Moreover, macrophages play as effector cell, occurred in phagocytosis and opsonization, by positive signaling from adaptive immune cells (T and B cells) [3]. As previous, macrophages exhibit antimicrobial, anti-proliferative and cytotoxic activity following inflammation that is classically activated macrophages or M1 macrophages function. In post-inflammatory phase of inflammatory process, these M2 macrophages induce arginine–arginase pathway to replace iNOS pathway for collagen formation in tissue remodeling [4]. These two phenotypes of activated macrophages are dynamically changed in a same cell depending to distinct microenvironment [5]. Agents that can modulate macrophage functions may have some beneficial effect on human health. Several Thai traditional medicines have been used for health promotion without any scientific prove. A longevity remedy which consists of six herbs including *Cyperus rotundus*, *Piper nigrum*, *Streblus aspera*, *Tinospora crispa*, *Diospyros rhodocalyx*, and *Albizia procera* has been used for health promotion and restoration. It is used by boiling with water, macerating with

alcohol or as crude powder in tablets [6]. Some herbs from this remedy were reported immunomodulatory activities such as anti-allergic and immunostimulatory properties. In previous study, the water extract from the remedy also had anti-allergic property by inhibiting the release of β-hexosaminidase on antigen-induced mast cells [7]. However, the remedy has never been investigated on macrophage function. This study aimed to study immunomodulatory activity of the longevity remedy on J774A.1 macrophages. The results from this study should reveal some beneficial evidences for the use of this remedy in health promotion.

Objective

This study intended to investigate immunomodulatory activities of the water extracts of the longevity remedy and its components which are *C. rotundus*, *P. nigrum*, *S. aspera*, *T. crispa*, *D. rhodocalyx*, and *A. procera* on murine macrophage J774A.1 cells.

Hypothesis

The water extracts of the longevity remedy and its components have immunostimulatory effects on macrophages.

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Keywords

Longevity remedy, macrophages, phagocytosis, cytokines, nitric oxide, inducible nitric oxide synthase, arginase 1

CHAPTER II
LITERATURE REVIEWS

Immune system

The immune system is the main defense system of the body against microbes, cancer or foreign particles. In human, the immunity is divided according to rapidity and specificity into innate and adaptive immunity. Innate immunity is initial, steady and non-specific immune response. Adaptive immunity is generated after innate immune response. It is highly specific response. It can memorize repeated encounter antigens and immediately react to these antigens more effectively than the first encounter. Both types of immunity collaborate to each other as a complex network for fully immune response [1].

Innate immunity

Innate immunity is the first line defense for preventing the host body from infection. It has broad-spectrum response without memorial capacity. This defense includes physical and chemical barriers, and immune components. Physical barriers are the acidic pH skin or serum from the dermis and mucus membranes in the gastrointestinal, respiratory and urogenital system. Chemical barriers include hydrolytic enzymes in saliva and small intestine or low pH of stomach and vaginal. Immune components are cells as monocytes, macrophages, eosinophils, basophils, neutrophils, mast cells and natural killer (NK) cells in circulation or in tissues and proteins as complements and cytokines. These immune cells can recognize, internalize or opsonize microorganism and produce several mediators consisting cytokine and chemokine that is a part of chemical barriers [8].

Adaptive immunity

Adaptive immunity is the other type of immunity that occurs after pathogen invasion into the body. It is the specific protection with memory for each microbe. The adaptive immunity is divided to humoral and cell-mediated immunity. T lymphocytes or T cells play role to generate cell-mediated response. B lymphocytes or B cells involve in humoral immune response by generating antibodies which can specifically recognized microorganisms or antigens [8].

The innate and adaptive immunity co-operatively work together for fully effective immune response. The innate immunity induces adaptive immunity by informing and generating mediators that stimulate adaptive immunity from the cells of innate immunity. In the same way, adaptive immunity can amplify and control the innate immunity to strongly damage foreign particles and maintain a balance of the immunity. Macrophages are one the important immune cells which function in both innate and adaptive immunity.

Macrophages

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Macrophages are mononuclear phagocytes originated from pluripotent haematopoietic stem cells (PHSC) in bone marrow. They are derived from myeloid progenitor cells to monoblast, promonocytes and monocytes, respectively. After monocytes in blood circulation migrate into various tissues, they become macrophages that have different names according to tissues they reside. They are osteoblasts in bone, microglia in central nervous system, alveolar macrophages in lungs, Kupffer cells in liver and other cells (Fig.1) [9].

Activated macrophages are classified according to phenotypes into two groups: classically activated macrophages (M1) and alternatively activated macrophages (M2) [10].

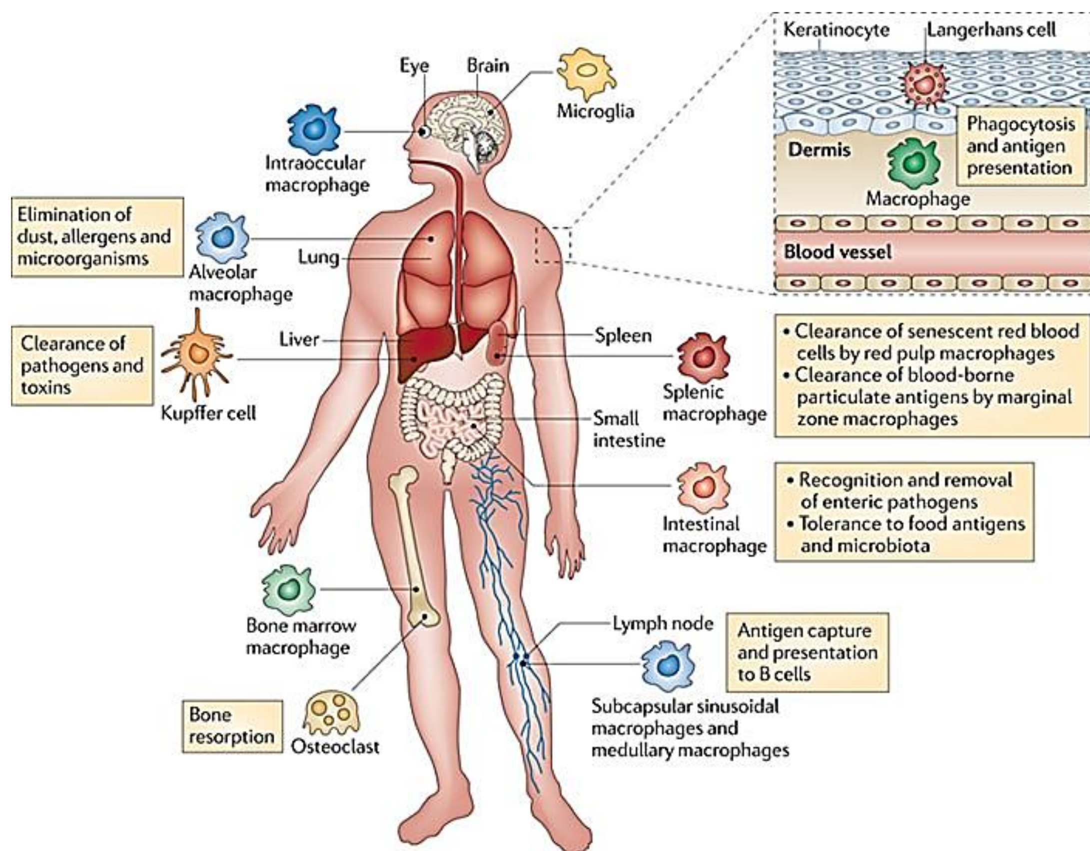

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Figure 1: **Tissue-specific macrophages.** Monocytes spread and differentiate to macrophages in various tissues of the body for immune balance [11].

Classically activated macrophages: M1 macrophages

Classically activated macrophages are stimulated by pathogen associated molecular patterns (PAMPs), IFN-γ from T_H 1 cell and NK cell, TNF-α or granulocyte macrophage colony-stimulating factor (GM-CSF) from other immune cells. They play a role in microbicidal activity, antigen presentation and T_H 1 polarization resulting inflammation and tissue injury[12].

Alternatively activated macrophages: M2 macrophages

Alternative macrophages are activated by cytokines as IL-4 or IL-13 produced by T_H 1 cells and other granulocytes (mast cells, eosinophils, basophils). These M2 phenotype cells function as T_H 2 supporter, exert immunosuppressive activity, involve in resolution of inflammation, tissue remodeling, angiogenesis and wound repair [13].

Functions of Macrophages

Macrophages are phagocytic cells that play roles in both innate and adaptive immunity. They have several functions in the immune system including phagocytosis, source of immune mediators, antigen presentation cells (APCs), and effector cells in adaptive immunity. Most of these functions are generated when the cells are in the stage of activated macrophages.

1. Phagocytosis

Macrophages are tissue phagocytes which use pattern recognition receptor (PRRs) to recognize pathogen-associated molecule patterns (PAMPs) of pathogens, such as LPS, lipoproteins, double-stranded RNA (dsRNA), lipoteichoic acid and other stimuli. After pathogen recognition, macrophages engulf the pathogen in phagosome which subsequently fuses with lysosome to become phagolysosome [14]. Pathogen

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in phagolysosome is destroyed via oxygen-dependent and oxygen-independent mechanisms as presented in Fig. 2.

Oxygen-dependent mechanism

After engulfing pathogen, reactive oxygen species (ROS) and NO were generated by NADPH oxidase enzyme and iNOS, respectively. ROS include superoxide anion (O_2^-), hydroxyl radicals (OH•), singlet oxygen, hypochlorite anion (OCl^-) and hypochlorous acid (HOCl) [2]. Both ROS and NO are the strong toxic agents. They react and damage components of microorganism such as thiols, metal centers, protein tyrosine residues, nucleic acids and lipids [15].

Nitric oxide (NO) is a diatomic molecule which is the product of L-arginine catalyzed by nitric oxide synthase (NOS) in many cell types. NO in macrophages is generated by iNOS. This enzyme is inducible and generates large amount of NO in micromolar concentrations for hours or days or longer in activated macrophages [16, 17]. iNOS is induced by pro-inflammation mediators such as pro-inflammatory cytokines in activated macrophages. NO reacts and destroys pathogen in phagolysosome. It also reacts with ROS to form peroxynitrite which is also labile toxic mediator for pathogen.

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Oxygen-independent mechanism

Pathogens in phagolysosome are also destroyed by antimicrobial proteins, peptides and enzymes such as lactoferrin, natural resistance-associated macrophage protein 1 (NRAMP1), defensins, cathelicidins, lysozyme, assorted lipases and proteases [2].

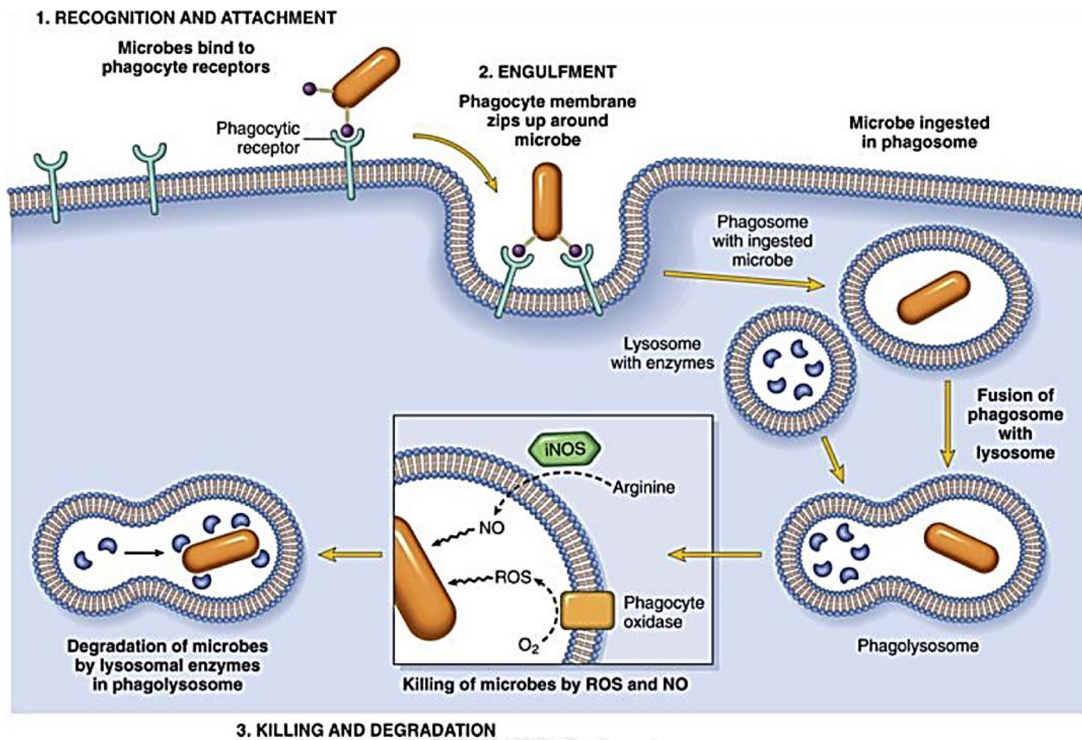

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Figure 2: Phagocytic process. In phagolysosome, microbes are killed by ROS and RNS (NO) and degraded by lysosomal enzymes [18].

2. Cytokine production

Activated macrophages are sources of several cytokines and mediators as presented in Fig. 3. They produce and release both pro- and anti-inflammatory cytokines, depend on their phenotypes.

Pro-inflammatory cytokines

M1 macrophages are activated macrophages that generate various cytokines as well as pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, IL-12, IL-23) and chemokines (CXCL9, CXCL10). These cytokines play roles in inflammatory process, in T cell activation and in effector phase of adaptive immune response.

Interleukin-1 (IL-1) is a pro-inflammatory cytokine. There are two subtypes, IL-1α and IL-1β. IL-1α always acts locally as autocrine mediator while IL-1β release into systemic circulation and act as endocrine mediator [19]. IL-1α is synthesized by various cells especially keratinocytes and endothelial whereas IL-1β is mostly created by monocyte and macrophage [20]. Both subtypes have the same activities. IL-1 induces neutrophil chemotaxis, increases monocytes activity, induces macrophage cytokine synthesis, activates mast cell and basophil degranulation, and stimulates prostaglandin E_2 (PGE_2) synthesis during inflammation [21].

Interleukin-6 (IL-6) is a pleiotropic cytokine. It plays a pivotal role for transiting from innate to adaptive immunity. Similar to TNF-α and IL-1, IL-6 attracts neutrophils in the initial phase of inflammation and then it suppresses neutrophils attraction following the apoptosis of neutrophil. It stimulates the release of monocyte-attracting chemokine lead to monocyte recruitment. It induces monocyte to differentiate to macrophages and stimulates cytokine production in macrophage. It activates T cells and rescue T cell from apoptosis. IL-6 also promotes B cell differentiation and stimulates antibody production [19, 22].

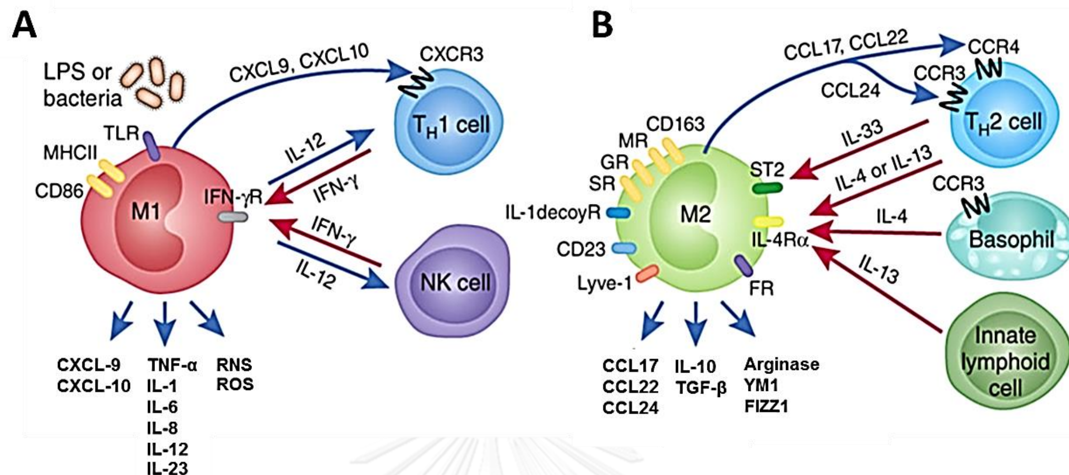

Figure 3: Mediators generated by activated M1 and M2 macrophages. Macrophages can generate both pro- and anti-inflammatory mediators. (A) Pro-inflammatory mediators are generated by inflammatory macrophages. (B) Anti-inflammatory mediators are generated by alternative macrophages.

CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; FIZZ1, resistin-like molecule-α; FR, folate receptor; GR, galactose receptor; IFN-γR, IFN-γ receptor; IL-1decoyR, IL-1 decoy receptor; Lyve-1, lymphatic vessel endothelial hyaluronan receptor 1; NK cell, natural killer cell; MHCII, major histocompatibility complex class II; MR, mannose receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SR, scavenging receptor; ST2, Interleukin 1 receptor-like 1; T_H cell, helper T cell; Ym1, chitinase-like molecules.

Tumor necrosis factor- α (TNF-α) has many biological activities similar to IL-1 and IL-6. It activates macrophages to generate and release several cytokines and various immune mediators. It has some properties different from IL-1 and IL-6. First, TNF-α is released predominantly by activated macrophage and T cells. Second, TNF-α is cytotoxic to divert types of tumor cells. Third, TNF-α directly activates neutrophil activation, not via chemoattractant but it indirectly stimulates T and B cell function by stimulating adhesion molecules. Lastly, TNF-α induces peroxide production in neutrophils. This activity is not found in IL-1 activity [21, 23].

Anti-inflammatory cytokines

If macrophages are activated into M2 phenotype, they generate and secrete anti-inflammatory cytokines (TGF-β, IL-10) and chemokines (CCL17, CCL22, CCL24). All these mediators reduce formation of pro-inflammatory mediators and activation of phagocyte and lymphocyte.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine which is released from several immune cells, as dendritic cells, eosinophils, monocytes, macrophages, mast cells, NK cells, T_H 2 cells and B cells. It suppresses major histocompatibility complex II (MHC class II) expression, inhibits co-stimulatory signaling and synthesis of pro-inflammatory cytokines and chemokines, and inhibits T cell through suppressing function of antigen presenting cells. However, IL-10 enhances B cell, granulocyte, mast cell and keratinocyte proliferation and differentiation and also stimulates NK cell and cytotoxic T cell activities on tumor cells [24, 25].

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3. Antigen presenting cells and effector cells in adaptive immunity

Macrophages are also act as antigen presenting cells similar to dendritic cells and B cells. They present antigenic peptides from destroyed pathogen to activate T cells for generating adaptive immune response against the pathogen as presented in Fig. 4.

After macrophages engulf and digest microorganism in phagolysosomes, the pathogen is destroyed in some parts to antigenic peptides. Macrophages present these peptides via MHC class II to T cell receptor (TCR) which involve the first signal for T_H cell (or $CD4^+$ cells) activation. For complete naïve T cell activation, T cells require co-stimulatory signal such as CD28 on T cells to interact with B7 on APCs [8].

Macrophages are also activated by cytokines (IL-2 and IFN-γ) form activated T_H cells or from antibody to become activated macrophages during effector phase of adaptive immunity. These activated macrophages also have phagocytosis and cytokine production for pathogen elimination the same as when they function in innate immunity [26] as presented in Fig. 4.

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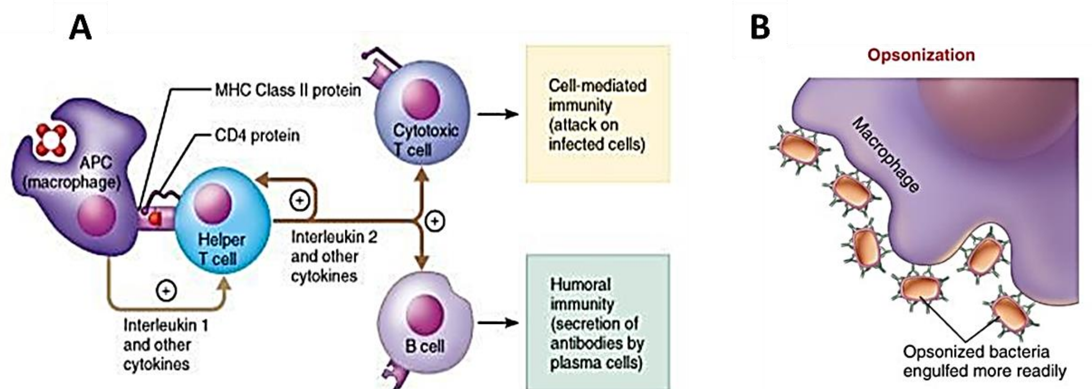

Figure 4: Innate and adaptive immune relation. (A) Macrophages serve as antigen presenting cell, informing antigen to adaptive immunity. (B) Macrophages serve as effector cell, conducting phagocytosis or opsonization [13].

APC, antigen presenting cell; MHC class II, major histocompatibility complex class II.

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Macrophages in resolution of inflammation

In resolution phase of inflammation, activated macrophages polarize themselves to M2 macrophages which have alternative actions for reducing inflammation. They decrease neutrophil infiltration to inflammatory site, remove apoptotic neutrophils at the inflammatory site, and produce anti-inflammatory cytokines, IL-10 and TGF-β, to inactivate T cells and other immune cells [27] as presented in Fig. 5.

M2 macrophages eliminate apoptotic neutrophils using their scavenger receptor. They secret selective chemokines, CCL17 and CCL22, involved recruitment of fibroblasts and regulatory T cells. They produce several proteins (resistin-like molecule-α; RELMα or FIZZ1, chitinase-like molecules; Ym1 and Ym2), involved in immunosuppression [11, 28, 29]. They also generate arginase-1 enzyme which plays in collagen formation and wound healing.

Arginase-1 (Arg-1) is an enzyme that hydrolyzes L-arginine to ornithine and urea. Ornithine is used in synthesis pathways of polyamine and proline which are important for cellular proliferation and tissue repair [28, 30]. Arg-1 decreases arginine availability for NO production by iNOS because both enzymes catalyze the same precursor L-arginine. Affinity with substrate of Arg-1 is higher than iNOS, but catalytic rate of Arg-1 is faster than iNOS. Arg-1 and iNOS generate biological mediators which can control each other as a negative feedback (Fig.6) [30].

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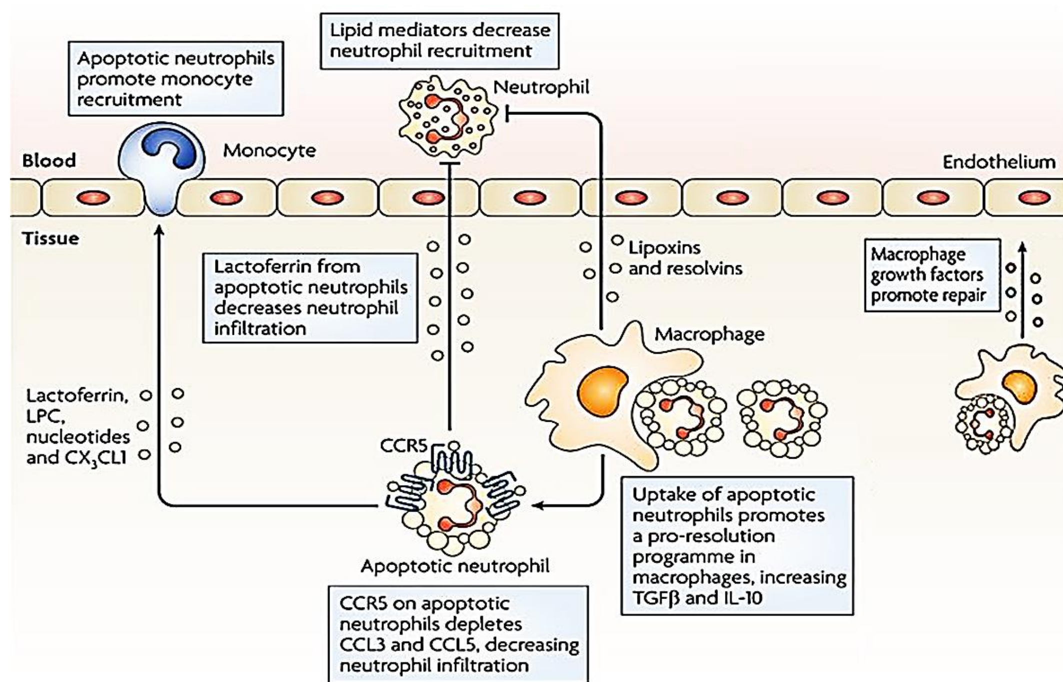

Figure 5: Inflammatory resolution by macrophages. Macrophages eliminate apoptotic neutrophils by phagocytosis in initial phase of resolution and also produce anti-inflammatory cytokines (IL-10 and TGF-β) and several mediators to promote tissue remodeling [27].

LPC, Lysophosphatidlycholine; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CX_3CR1, CX_3C-chemokine receptor 1

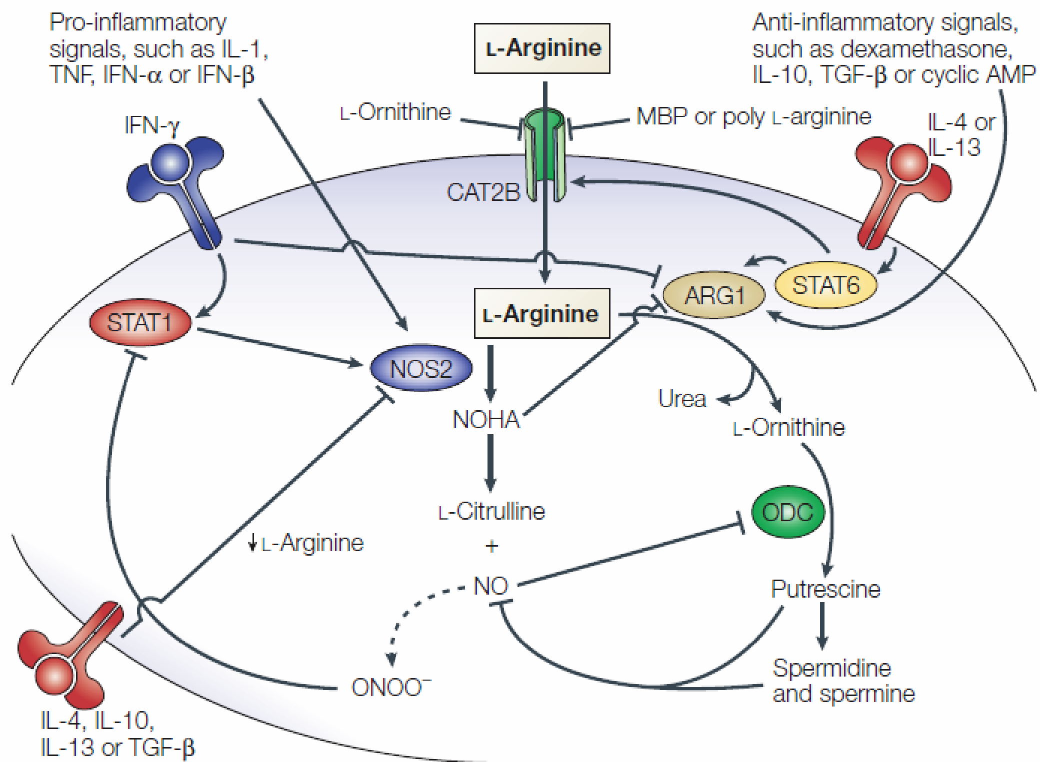

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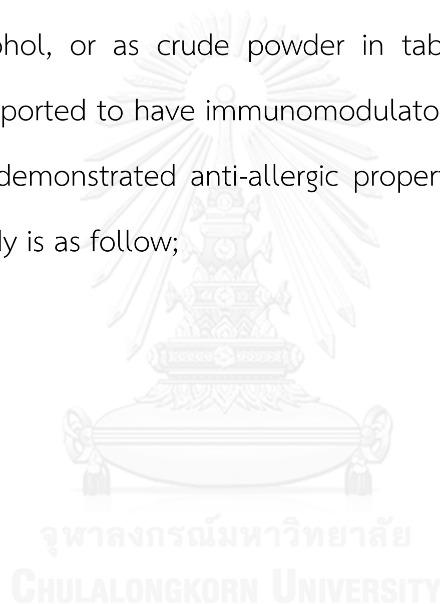
Figure 6: L-arginine metabolism. Pro- and anti-inflammatory signals influence to balance iNOS and Arg-1 pathway through negative-feedback biochemical circuits [30].

ARG1, arginase 1; CAT2B, cationic amino-acid transporter 2B; cyclic AMP, cyclic adenosine monophosphate; IFN, interferon; IL, interleukin; MBP, major basic protein; NO, nitric oxide; NOHA, N^G-hydroxy-L-arginine; NOS2, nitric oxide synthase 2; ODC, ornithine decarboxylase; $ONOO^-$, peroxynitrite; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TNF, tumor-necrosis factor.

Longevity remedies

Longevity remedies are commonly used as Thai traditional medicines for health promotion and health restoration. These remedies contain similar basic mixture of herbal plants, but they can differently contain other additional plants in their formulas. The basic formula of longevity remedies consist of *Cyperus rotundus*, *Piper nigrum*, *Streblus aspera*, *Tinospora crispa*, *Diospyros rhodocalyx*, and *Albizia procera* in equal proportion [31]. This formula is used for health promotion and restoration. It is also used as longevity medicine. It is used by boiling with water, macerating with alcohol, or as crude powder in tablets [6]. Some herbs in this remedy have been reported to have immunomodulatory activities. The water extract of the remedy also demonstrated anti-allergic property [7]. Detail of each herb in basic longevity remedy is as follow;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1. *Cyperus rotundus*

Figure 7: ***Cyperus rotundus*** (A) appearance (B) rhizomes (C) rhizome powder.

Family name: CYPERACEAE

Scientific name: *Cyperus rotundus*

Common names: nut grass, nutsedge, purple nutsedge

Thai name: แห้วหมู (Haeo mu)

Traditional uses: rhizome part is used as cardiotonic, diaphoretic, or diuretic agent

Chemical compositions: essential oils (α-copaene, cyperene, β-selinene, α-cyperone, β-cyperone, monoterpenes, sesquiterpenes, and norsesquiterpene), alkaloids (steroid glycosides), saponins, flavonoids, tannins, fructose-amino acid conjugate, isocurcumenol, starch, and carbohydrates [32]

Pharmacological properties:

- Anti-bacterial activity: against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhosa,* and *Shigella sonnei* [33, 34]
- Anti-HIV activity: The water extract inhibited HIV-1 integrase [35].

- Anti-cancer activity: The 95% ethanol extract inhibited proliferation of the human estrogen receptor negative breast cancer cells (MDA-MB-231) [36].
- Immunomodulatory activities :

Extract	Immunomodulatory activities
water	suppress allergy by inhibit the release of antigen-induced β-hexosaminidase from RBL-2H3 mast cells [7].
70% ethanol and sesquiterpene	- Inhibit 5-lipoxygenase-catalyzed LTs production on rat basophilic leukemia-1 (RBL-1) cells. - Inhibit β-hexosaminidase degranulation on IgE-stimulated RBL-2H3 cells. - Inhibit delayed-type hypersensitivity in mice [37].
water	- Reduce carrageenan-induced paw edema in rat. - Reduce acetic acid-induced peritonitis in mice [38].
methanol	Inhibit NO production on IFN-γ and LPS-stimulated RAW 264.7 macrophage cells by suppression of iNOS protein and mRNA expression [39].
80% ethanol and essential oils	Inhibit NO and PGE_2 production on LPS-induced RAW 264.7 macrophage cells [40].
α-cyperone (essential oils)	Inhibit PGE_2 production by suppression of COX-2 mRNA expression and decrease the release of IL-6 by suppression of IL-6 gene expression through the NFκB signaling on LPS-induced RAW 264.7 macrophage cells [40].
70% ethanol	Reduce carrageenan-induced edema and formaldehyde-induced arthritis in albino rats [41].

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2. *Piper nigrum*

Figure 8: ***Piper nigrum*** (A) appearance (B) fruits (C) fruit powder.

Family name: PIPERACEAE

Scientific name: *Piper nigrum L.*

Common names: pepper, black pepper, white pepper

Thai name: พริกไทย (Prik thai)

Traditional uses: Fruits are used as antiflatulent, element tonic, appetizer, diaphoretic, and diuretic agent

Chemical compositions: alkaloids (piperine, piperidine, piperettine, piperanine) [42]; phenolic compounds; flavonoids [31]

Pharmacological properties:

- Anti-bacterial activity: against *Staphylococcus aureus, Bacillus subtilis, Bacillus sphaericus, Klebsiella aerogenes, Chromobacterium violaceum, Escherichia coli, Salmonella thypii, Shigella dysenteriae, Candida albicans* [42-45]
- Anti-oxidant activity: The methanol extract [46], ethanol extract [47], essential oil and oleoresins [48] demonstrated anti-oxidant activities.
- Anti-cancer activity:

- The ethanol extract inhibited proliferation of the human PC-3 prostate cancer cells [49].
- Piperine had cytotoxic effect against the human promyelocytic leukemia HL60 cells [50], human rectal tumor-18 cells [51], human *HER2*-overexpressing breast cancer cells [52], human prostate cancer cells [53], and human fibrosarcoma HT-1080 cell [54].
- Piperidine inhibited proliferation of epithiloma in the human larynx carcinoma HEp2 cells [42].

- Immunomodulatory activities :

Extract	Immunomodulatory activities
water and 95% ethanol	suppress allergy by inhibit the release of antigen-induced β-hexosaminidase from RBL-2H3 mast cells [7].
water	- Increase proliferation of splenocyte. - Stimulate T_H 1 cytokine release and inhibit T_H 2 cytokine release. - Increase NO production on macrophages. - Increase the cytotoxic activity of NK cells [55].
piperine	- Inhibit production of IL-6, MMPs-13, COX-2 and PGE2 in human IL-1β-stimulated fibroblast-like synoviocytes. - Reduce carrageenan-induced arthritis in rat [56].
	- Reduce nitrite levels in LPS- or Con-A-induced mice. - Reduce TNF-α level in LPS-induced mice [57].
DMSO	Inhibit the release of IL-6, TNF-α, IL-10 and suppress COX-2 and iNOS mRNA expression in LPS-induced RAW 264.7 macrophage cells [58].

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3. *Streblus aspera*

Figure 9: ***Streblus aspera*** (A) flower and leaves (B) seeds (C) seed powder.

Family name: MORACEAE

Scientific name: *Streblus aspera* Lour.

Common names: Siamese rough bush, tooth brush tree

Thai name: ข่อย (Khoi)

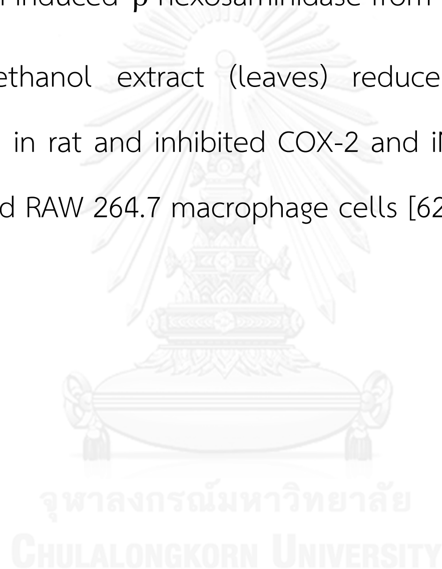
Traditional uses: Seeds are used as element tonic, appetizer, carminative, longevity agent

Chemical compositions: cardiac glycosides (kamloside, asperoside, strebloside, indroside, cannodimemoside, strophalloside, strophanolloside); volatile oil (phytol 45.1%, α-farnesene 6.4%, *trans*-farnesyl acetate 5.8%, caryophyllene 4.9%, and *trans-trans*-α-farnesene 2.0%) [59]; phenolic compounds; flavonoid [31]

Pharmacological properties:

- Anti-bacterial activity: against *Streptococcus mutans, Porphyromonas gingivalis, Peptostreptococcus micros* [59].
- Cardiotonic activity: The ethanol extract induced positive ionoptropic effect and a systolic response on isolated frog heart [59].

- Anti-oxidant activity: The methanol extract demonstrated anti-oxidant both in vitro and in vivo [60].
- Anti-cancer activity: The 95% ethanol extract inhibited proliferation of the human estrogen receptor negative breast cancer cells (MDA-MB-231) and the lung cancer CHAGO cells [36, 61].
- Immunomodulatory activities :
    - 95% ethanol extract suppressed allergy by inhibit the release of antigen-induced β-hexosaminidase from RBL-2H3 mast cells [7].
    - 50% ethanol extract (leaves) reduced carrageenan-induced paw edema in rat and inhibited COX-2 and iNOS mRNA expression in LPS-induced RAW 264.7 macrophage cells [62].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4. *Tinospora crispa*

Figure 10: ***Tinospora crispa*** (A) appearance (B) stems (C) stem powder.

Family name: MENISPERMACEAE

Scientific name: *Tinospora crispa* (L.) Miers ex Hook.f. & Thomson

Thai name: บอระเพ็ด (Bora phet)

Traditional uses: Stem is used as antipyretic, diaphoretic, relieve thirst, bitter element tonic, and appetizer

Chemical compositions: adenosine, uridine, salsolinol, higenamine, tyramine [63]; phenolic compounds [21]

Pharmacological properties:

- Cardiovascular activity: The n-butanol extract decreased blood pressure and heart rate in anesthetized rat [63, 64].
- Anti-cancer activity: The 95% ethanol extract inhibited proliferation of the lung cancer CHAGO cells and the human PC-3 prostate cancer cells [49, 61].
- Immunomodulatory activity: The water extract suppressed allergy by inhibit the release of antigen-induced β-hexosaminidase from RBL-2H3 mast cells [7].

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5. *Diospyros rhodocalyx*

Figure 11: ***Diospyros rhodocalyx*** (A) leaves (B) stem barks (C) bark powder.

Family name: EBENACEAE

Scientific name: Diospyros rhodocalyx Kurz

Common name: ebony

Thai name: ตะโกนา (Tako na)

Traditional uses: Stem bark is used as tonic, element tonic, and longevity medicine. It is used for impotence treatment.

Chemical compositions: tannin (catechin, gallocatechin) [65]; phenolic compounds; flavonoid [31]

Pharmacological properties:

- Anti-cancer activity: The 95% ethanol extract inhibited proliferation of the human estrogen receptor negative breast cancer cells (MDA-MB-231) and the human PC-3 prostate cancer cells [36, 49]

- Immunomodulatory activity: The water extract suppressed allergy by inhibit the release of antigen-induced β-hexosaminidase from RBL-2H3 mast cells [7].

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6. *Albizia procera*

Figure 12: ***Albizia procera*** (A) leaves and flowers (B) stem barks (C) stem bark powder.

Family name: LEGUMINOSAE-MIMOSOIDEAE

Scientific name: Albizia procera (Roxb.) Benth.

Common names: white siris, sit

Thai name: ทิ้งถ่อน, ถ่อน (Thing thon)

Traditional uses: Stem bark is used as antiasthmatic, antidiarrheal, longevity medicine

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Chemical compositions: tannins; phenolic compounds [31]; isoflavonoid (biochanin A, formonetin, genistein, daidzein) [66]; saponin [67]

Pharmacological properties:

- Anti-oxidant activity: The 95% ethanol extract demonstrated antioxidant activity [31].
- Anti-HIV activity: The water extract inhibited HIV-1 integrase [35].
- Anti-cancer activity: The 95% ethanol extract inhibited proliferation the human PC-3 prostate cancer cells [49].

CHAPTER III
MATERIALS AND METHODS

Materials

1. Water extracts of a longevity remedy and its components

All herbs in a longevity remedy including *C. rotundus*, *P. nigrum*, *S. aspera*, *T. crispa*, *D. rhodocalyx* and *A. procera* were purchased from Chao Krom Poe herbal dispensary (Bangkok) and confirmed morphology by Associate Professor Dr. Nijsiri Ruangrungsi, College of Health Sciences, Chulalongkorn University. These herbs were made as crude powders and mixed together in equal weight to become a longevity remedy. Crude powders of the remedy and each herb were heated at 75°C in distilled water with the ratio crude powder: solvent at 1:5 for 15 min. The supernatants were collected and the remnant crude powders were heated twice for collecting the supernatants. The supernatants were filtered through 11 microfilter papers, lyophilized to lyophilized powder, and 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 all extract were showed in Table 1. The lyophilized extracts were kept in closed container at -20°C until used.

In all experiments, the lyophilized extracts were dissolved as the stock solutions and diluted to 1.56–100 μg/ml in Dulbeco's Modified Eagle's Medium (DMEM) for testing their activities.

Table 1: The yields of six lyophilized water extracts compared to crude powders used for extraction

Test compounds	Percentage of yields
Longevity remedy	14.13
Cyperus rotundus	14.34
Piper nigrum	3.60
Streblus aspera	10.98
Tinospora crispa	16.04
Diospyros rhodocalyx	11.56
Albizia procera	11.93

2. 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, 1% penicillin-streptomycin at 37°C in 5% CO_2 and 95% humidity. They were sub-cultured every 2 days and determined their cell viability by 0.4% trypan blue.

In all experiments, the cells were used at 4×10^5 cells/ml with more than 90% cell viability. DMEM and 100 ng/ml lipopolysaccharide (LPS) were used as the negative and the positive controls, respectively.

3. Chemicals and reagents

The following chemicals and reagents were used in this study; Dulbecco's Modified Eagle's Medium; DMEM (Gibco, USA), fetal bovine serum; LPS (Gibco, USA), penicillin-streptomycin (Gibco, USA), Griess reagent (Promega, USA), resazurin sodium

chloride (Sigma, USA), lipopolysaccharide; LPS (Sigma, USA), 0.4% trypan blue dye, TRIzol® reagent (Invitrogen, UK), chloroform (Sigma, USA), DEPC (Molekula, UK), ImProm-II™ reverse transcription kit (Promega, USA), Primer (Bio Basic, Canada; Integrated DNA Technologies, Singapore), Taq polymerase (Vivantis, USA)

4. Equipments and instruments

The following equipments and instruments were used in this study; filter papers (Whatman®, USA), biohazard laminar flow hood (ESSCO, USA), light microscope (Nikon, USA), CO_2 Incubator (Thermo, USA), autopipette (Gilson, USA), vortex mixer (Scientific industries, USA), hemocytometer (Brand, Germany), 96 and 24-well plates (Corning, USA), T25 tissue culture flasks (Corning, USA), centrifuge machine (Hettich, USA), thermal cycler machine (Eppendorf, USA), Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, Singapore), gel electrophoresis apparatus (Bio-Rad, USA), scrappers (Greiner, UK), microplate reader machine (Labsystems Multiskan, USA), Molecular Imager® Gel Doc™XR+ System (Bio-Rad, USA), Nanodrop (Thermo scientific, USA)

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Methods

1. Study the effects of the water extracts of longevity remedy and its components on nitric oxide production in macrophages J774A.1 cells

Concentrations used for determining effect of all extracts on NO production were chosen from a preliminary study. Previously, all extracts at 1.56 – 100 µg/ml were screened for their effects on NO production. The extracts of the remedy, *C. rotundus* and *P. nigrum* maximally increased NO production at 25 µg/ml, while the other four extracts started to increase NO production at 100 µg/ml. In this study, the extracts of the remedy, *C. rotundus* and *P. nigrum* were used at 1.56-25 µg/ml, while the extracts of *S. aspera*, *T. crispa*, *D. rhodocalyx* and *A. procera* were used at 12.5-

100 µg/ml in order to determine effects of these extracts on NO production by Griess reaction as in the following procedure:

1. Incubate J774A.1 cells at 4×10^5 cells/ml in sterile 96-well plates, at 37°C, 5% CO_2 and 95% humidity for 24 h.

2. Treat the cells with each water extracts at 1.56–100 µg/ml and incubate for 24 h.

3. Collect 100 µl supernatant of the treated cells from each well into a 96-well plate

4. Add 20 µl sulfanilamide from Griess reagent, mix, and incubate in the dark at room temperature for 10 min

5. Add 20 µl N-1-napthylethylenediamine dihydrochloride (NED), mix, and incubate in the dark at room temperature for 10 min

6. Measure the absorbance of each well using a microplate reader at 540 nm.

7. Calculate NO concentrations in nitrite form by using a standard nitrite solution curve.

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2. Study the effects of the water extracts of longevity remedy and its components on J774A.1 cell viability

Viability of the treated cells from NO determination was evaluated by rezasurin reduction assay as in the following procedure:

1. Add 50 µl of 50 µg/ml rezasurin in DMEM into each well.

2. Incubate at 37°C for 2 h.

3. Measure the plate at at 570 and 600 nm using microplate reader.

4. Calculate the percentage of cell viability by comparing with vehicle control using the following formula.

$$\% \text{ Cell viability} = \frac{[OD_{570} - OD_{600}]_{sample}}{[OD_{570} - OD_{600}]_{control}} \times 100$$

3. Study the effects of the water extracts of longevity remedy and its components on mRNA expression of mediators in macrophage J774A.1 cells

mRNA expression of mediators and enzymes associated with macrophage activation was determined by reverse transcription polymerase chain reaction (RT-PCR). These enzymes and mediators were iNOS, arginase-1, anti-inflammatory cytokine markers (IL-10) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) at 4 and 24 h. The assays were performed at least three-independent experiments as in the following procedure:

1. Incubate J774A.1 cells at $4x10^5$ cells/ml in sterile 24-well plates, at 37˚C, 5% CO_2 and 95% humidity for 24 h.

2. Treat the cells with 6 water extracts at 3.13–100 μg/ml and incubate for 4 and 24 h.

3. Remove supernatant and isolate total RNA from the treated cells using TRIzol reagent as follow

- Lyse the treated cells with 1 ml TRIzol® reagent for 5 min.

- Transfer each lysate sample into eppendorf tube.

- Add 200 µl/tube chloroform, vigorously shake for 15 sec, and incubate at room temperature for 3 min.

- Separate solvents by centrifugation at 12,000 g, 4ºC for 15 min.

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- Carefully collect the aqueous layer from each sample into a new eppendorf tube.

- Add 500 µl/tube isopropanol, shake, and incubate at room temperature for 30 min.

- Precipitate the RNA pellet by centrifugation at 12,000 g at 4°C for 10 min.

- Remove the supernatant, wash the pellet with 1 ml of 75% ethanol by shortly mixing with vortex and then centrifuge at 7,500 g at 4°C for 5 min.

- Air dry the RNA pellet, dissolve it in DEPC treated water, and determine the total RNA concentration and contamination by measuring at 230, 260 and 280 nm using Nanodrop machine.

4. Reverse transcribe the total RNA for each sample to cDNA using ImProm-II™ reverse transcription kit as follow;

- Mix 1.5 µg total RNA with 1 µl oligo $(dT)_{15}$ primer in DEPC treated water in the 200 µl tube, heat at 70°C for 5 min, and immediately keep on ice for 5 min.

- Prepare reverse transcription mixture containing 25 mM $MgCl_2$, 10 mM mixed dNTP, ribonuclease inhibitor and reverse transcriptase.

- Add 15 µl mixture solutions in to each tube.

- Generate cDNA in a thermal cycler machine using the following conditions; 25°C for 5 min, then 42°C for 1 h and 30 min, and finally 70°C for 15 min.

- Store each cDNA sample at -20°C until use.

5. Synthesize the PCR product from each cDNA with specific primers by PCR as follow;

- Prepare PCR reaction mixture containing 50 mM $MgCl_2$, 10 mM mixed dNTP, specific primers (Table 2), PCR buffer and Taq DNA polymerase 1 unit.

- Mix 24 µl PCR mixture solution with 1 µl cDNA products in 200 µl tube.

- Amplify PCR product of each cDNA in the thermo cycler machine using the condition presented in Table 3.

- Determine the PCR product of each sample by running 1.5% agarose gel electrophoresis at 100 volt for 50 min in TBE buffer, staining the gel with ethidium bromide for 15 min, de-staining the gel with TBE buffer for 30 min, identifying and analyzing the density of PCR product by gel documentation, and calculating amount of PCR product as a ratio of PCR product density of each sample to the PCR product density of β-actin gene.

Statistic analysis

The data from at least three independent experiments were showed as mean ± standard deviation (mean ± SD) values. Statistical evaluation were determined by one-way analysis of variance (ANOVA) followed by Turkey's post hoc test. The p-value less than 0.05 were considered as statistically significance.

Table 2: Specific primers for RT-PCR of interested genes identified in this study

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

Table 3: The PCR condition of each gene

Gene	Denature	Annealing	Extension	Cycles
TNF-α	94°C, 30 s	55°C, 45 s	72°C, 1 min	21
IL-1β	94°C, 30 s	57°C, 45 s	72°C, 1 min	30
IL-6	94°C, 30 s	57°C, 45 s	72°C, 1 min	30
IL-10	94°C, 30 s	60°C, 45 s	72°C, 1 min	35
iNOS	94°C, 30 s	60°C, 45 s	72°C, 1 min	24
Arg1	94°C, 1 min	54°C, 1 min	72°C, 1 min	45
β-actin	94°C, 30 s	57°C, 45 s	72°C, 1 min	30

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CHAPTER IV
RESULTS

1. Effects of the water extracts of the longevity remedy and its components on nitric oxide production in macrophage J774A.1 cells.

NO is the main reactive nitrogen species generated by M1 activated macrophages. Its functions involve in phagocytosis and inflammatory processes. Effects of the water extract of the longevity remedy and its components on NO production in J774A.1 cells were determined by Griess reaction assay. The concentrations of NO in nitrite form were calculated from the standard curve of reference nitrite solutions. The extracts at 1.56-100 μg/ml were evaluated.

The water extracts of the remedy, *P. nigrum, C. rotundus, T. crispa,* and *S. aspera* significantly stimulated NO production in J774A.1 cells when compared to the solvent control. The extract of the remedy significantly increased NO production at all concentrations used in the study (1.56-25 μg/ml) as presented in Fig.13 A. The extract of *C. rotundus* significantly increased NO production at concentrations 6.25 μg/ml and higher (Fig.13 B). The extract of *P. nigrum* significantly increased NO production at concentrations 12.5 μg/ml and higher (Fig.13 C). The extract of *S. aspera* and *T. crispa* significantly increased NO production only at the highest concentration used in this study (100 μg/ml) as presented in Fig.13 D-E. The extracts of *D. rhodocalyx* and *A. procera* did not significantly stimulate NO production (Fig.13 F-G).

The effects of these extract on NO production were compared to each other by using the ratio of NO concentration produced by each extract to NO concentration produced by LPS stimulation. The extracts of the remedy and *C. rotundus* had the highest stimulatory activities, followed by *P. nigrum* (Fig.14).

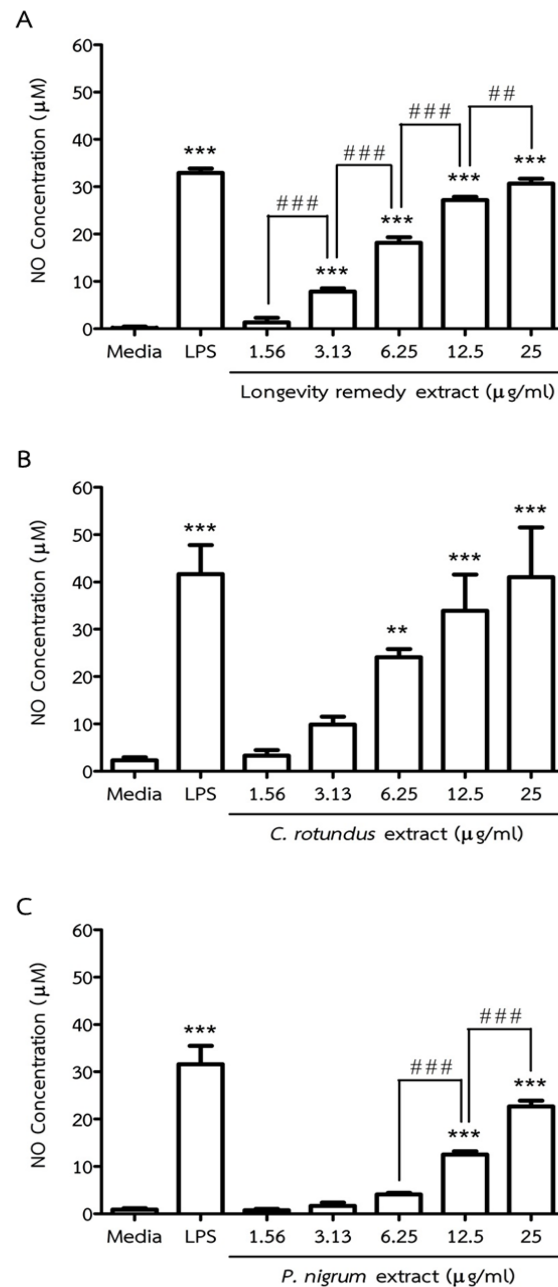

Figure 13: Effects of the water extract of the longevity remedy and its components on NO production in macrophage J774A.1 cells after 24 h of treatment. Concentrations of NO produced from the cells by (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* are represented as means ± S.D. from three independent experiments (n=3). ** $p<0.01$, *** $p<0.001$ compared to the solvent control. $^{##}$ $p<0.01$, $^{###}$ $p<0.001$ compared among concentrations.

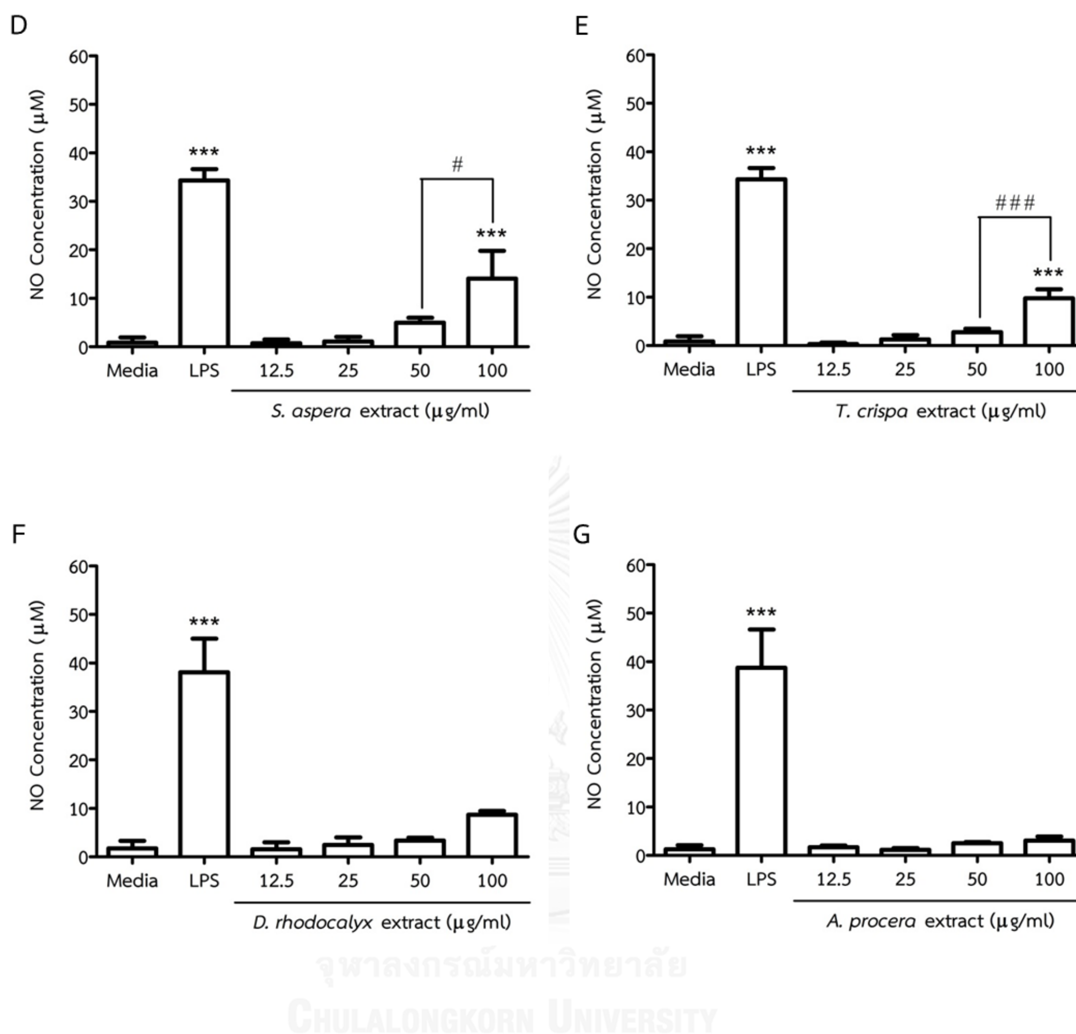

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Figure 13(cont.): Effects of the water extract of the longevity remedy and its components on NO production in macrophage J774A.1 cells after 24 h of treatment. Concentrations of NO produced from the cells by (D) *S. aspera,* (E) *T. crispa*, (F) *D. rhodocalyx* and (G) *A. procera* are represented as means ± S.D. from three independent experiments (n=3). *** $p<0.001$ compared to the solvent control. # $p<0.05$, ### $p<0.001$ compared among concentrations.

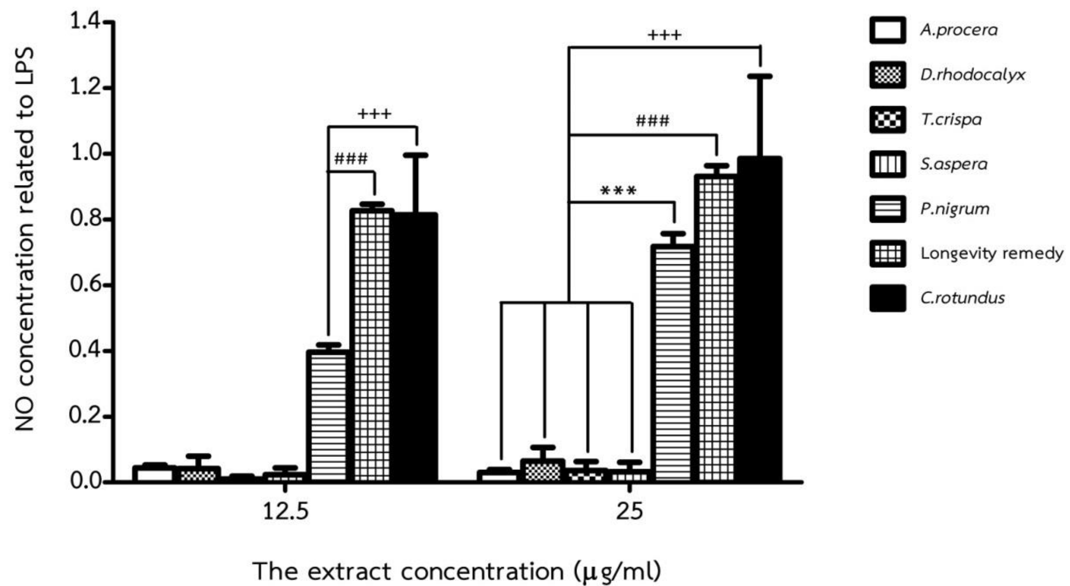

Figure 14: Comparison of NO stimulation effects among the water extracts of the remedy and its components. The ratio of NO concentration produced by each extract to NO concentration produced by LPS stimulation are represented as means ± S.D. from three independent experiments (n=3). $^{+++}$ p<0.001 when *C. rotundus* was compared among extracts, $^{\#\#\#}$ p<0.001 when the remedy was compared among extracts, and *** p<0.001 when *P. nigrum* was compared among extracts.

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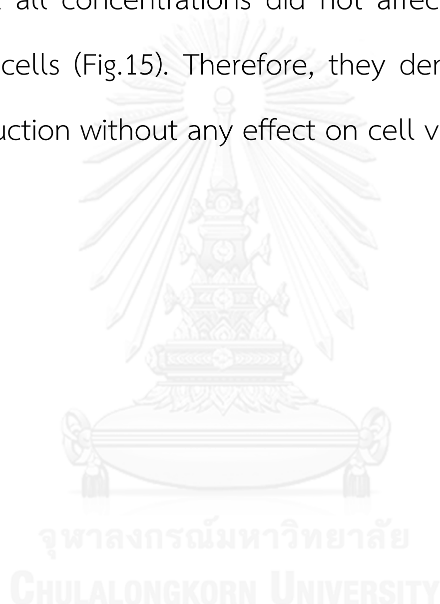
2. Effects of the water extracts of the longevity remedy and its components on macrophage J774A.1 cell viability.

In order to ensure that the stimulatory effects on NO production of the extracts did not affect cell viability of J774A.1 cells. The remained treated cells from NO production determination were evaluated for their viability by resazurin reduction assay. Viable cells turn blue resazurin to red resorufin product by mitochondrial reductase enzyme.

All extracts at all concentrations did not affect cell viability of the treated macrophage J774A.1 cells (Fig.15). Therefore, they demonstrated direct stimulatory activities on NO production without any effect on cell viability.



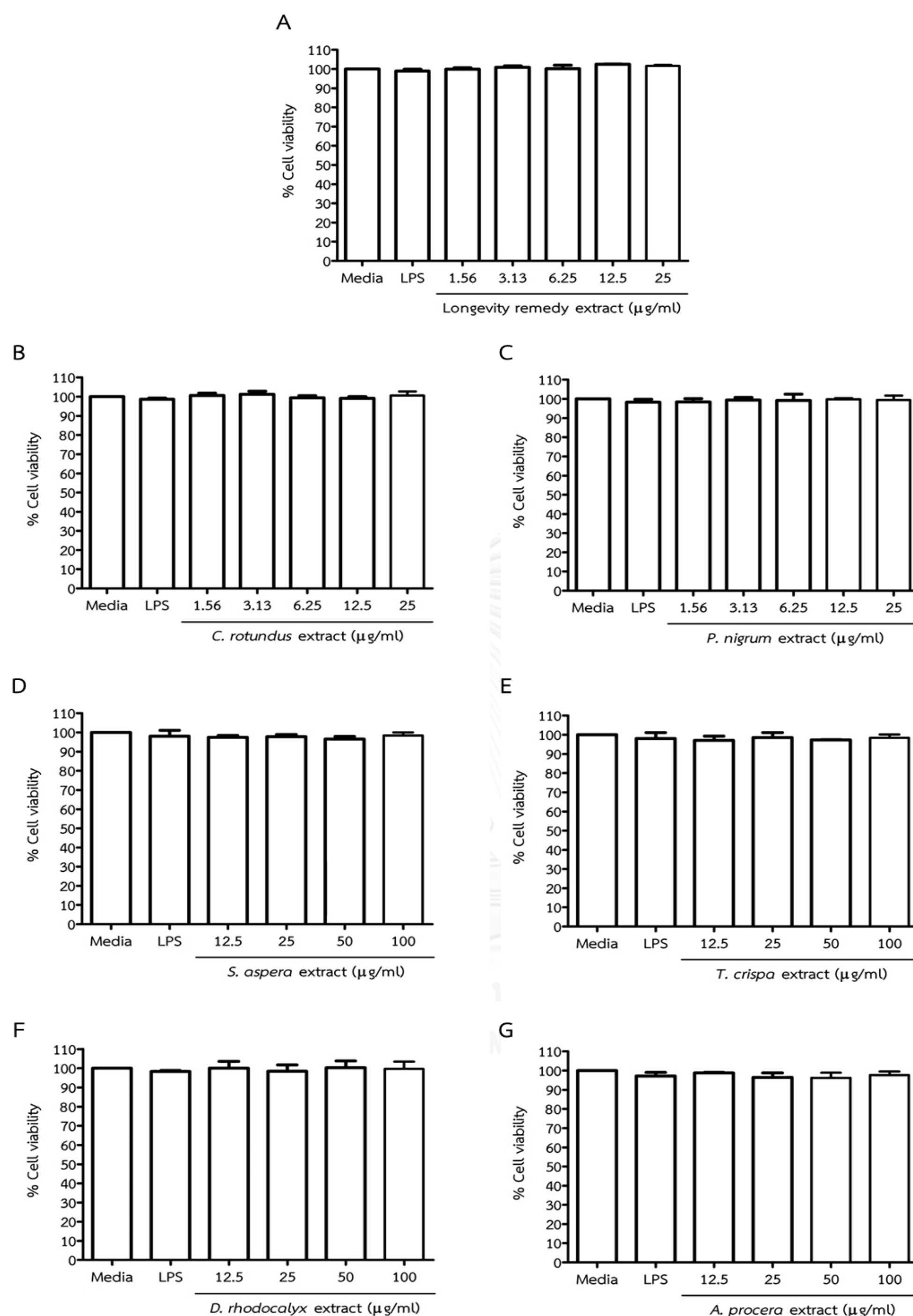


Figure 15: Effects of the water extract of longevity remedy and its components on cell viability in treated cells. The results are showed as percentage of cell viability after treating with the water extract of (A) the remedy, (B) *C. rotundus*, (C) *P. nigrum*, (D) *S. aspera*, (E) *T. crispa*, (F) *D. rhodocalyx* and (G) *A. procera*. The data represent as means ± S.D. from three independent experiments (n=3).

3. Effects of the water extracts of the longevity remedy and its components on iNOS mRNA expression in macrophage J774A.1 cells.

In order to confirm the stimulatory effects of the water extract of the longevity remedy and its components on NO production, the effects of these extract on iNOS were also evaluated. iNOS is an inducible nitric oxide synthase which catalyzes L-arginine to NO at micromolar levels during phagocytosis and inflammation. It is known to be a marker of activated macrophages especially M1 macrophages.

Three concentrations of all extracts were selected for determining their effects on mRNA expression of all markers of activated macrophages according to their effects on NO production. The extracts of the remedy, *C. rotundus* and *P. nigrum* were determined at 3.13, 6.26 and 12.5 μg/ml. The extracts of *S. aspera*, *T. crispa* and *D. rhodocalyx* were examined at 25, 50 and 100 μg/ml. The extract of *A. procera* which had no effect on NO production was not examined in this experiment.

All the water extracts except the extract of *A. procera* up-regulated mRNA expression as presented in Fig. 16. The extract of the remedy and *P. nigrum* significantly increased mRNA expression of iNOS at 24 h of treatment at 3.13, 6.25 and 12.5 μg/ml. They had higher stimulatory effects at 24 h of treatment than at 4 h of treatment. The extract of *C. rotundus* significantly activated iNOS mRNA expression at both 4 and 24 h of treatment at 6.25 and 12.5 μg/ml. The extract of *S. aspera* significantly activated iNOS mRNA expression at both 4 and 24 h of treatment at 25, 50 and 100 μg/ml. The extract of *D. rhodocalyx* significantly activated iNOS mRNA expression at 24 h of treatment at 25, 50 and 100 μg/ml. It did not increase the expression of iNOS at 4 h of treatment. The extract of *T. crispa* significantly increased iNOS mRNA expression at 4 h of treatment at 100 μg/ml, and at 24 of treatment at 50 μg/ml.

Effects of three extracts with high activities including the remedy, *C. rotundus* and *P. nigrum* extracts on iNOS expression were compared to each other in the term of the ratio of iNOS expression induced by each extract to iNOS expression induced by LPS at 24 h of treatment. The extract of the remedy had the trend to have the highest activity (Fig.17).

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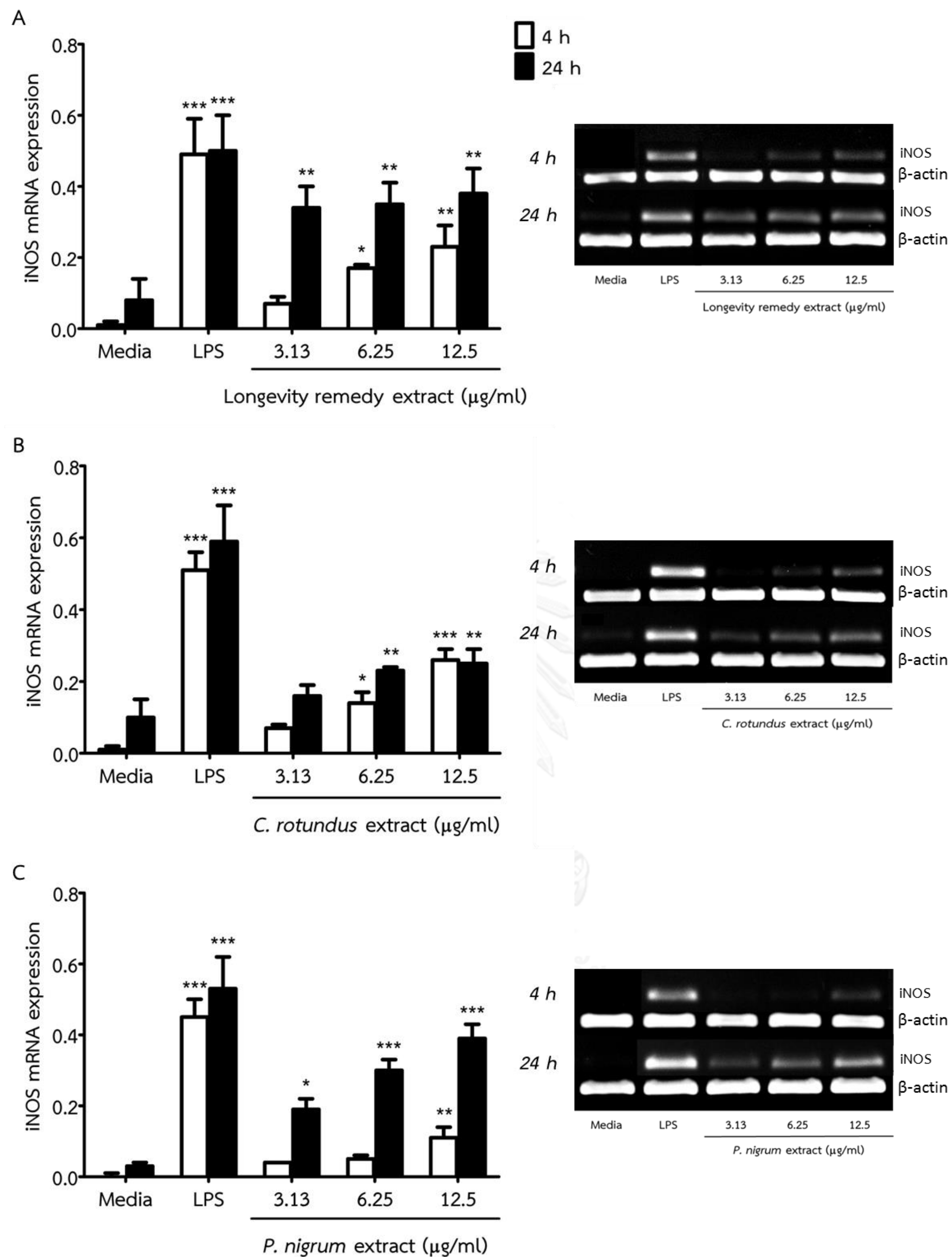

Figure 16: Effects of the water extracts of the longevity remedy and its components on iNOS mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of iNOS to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

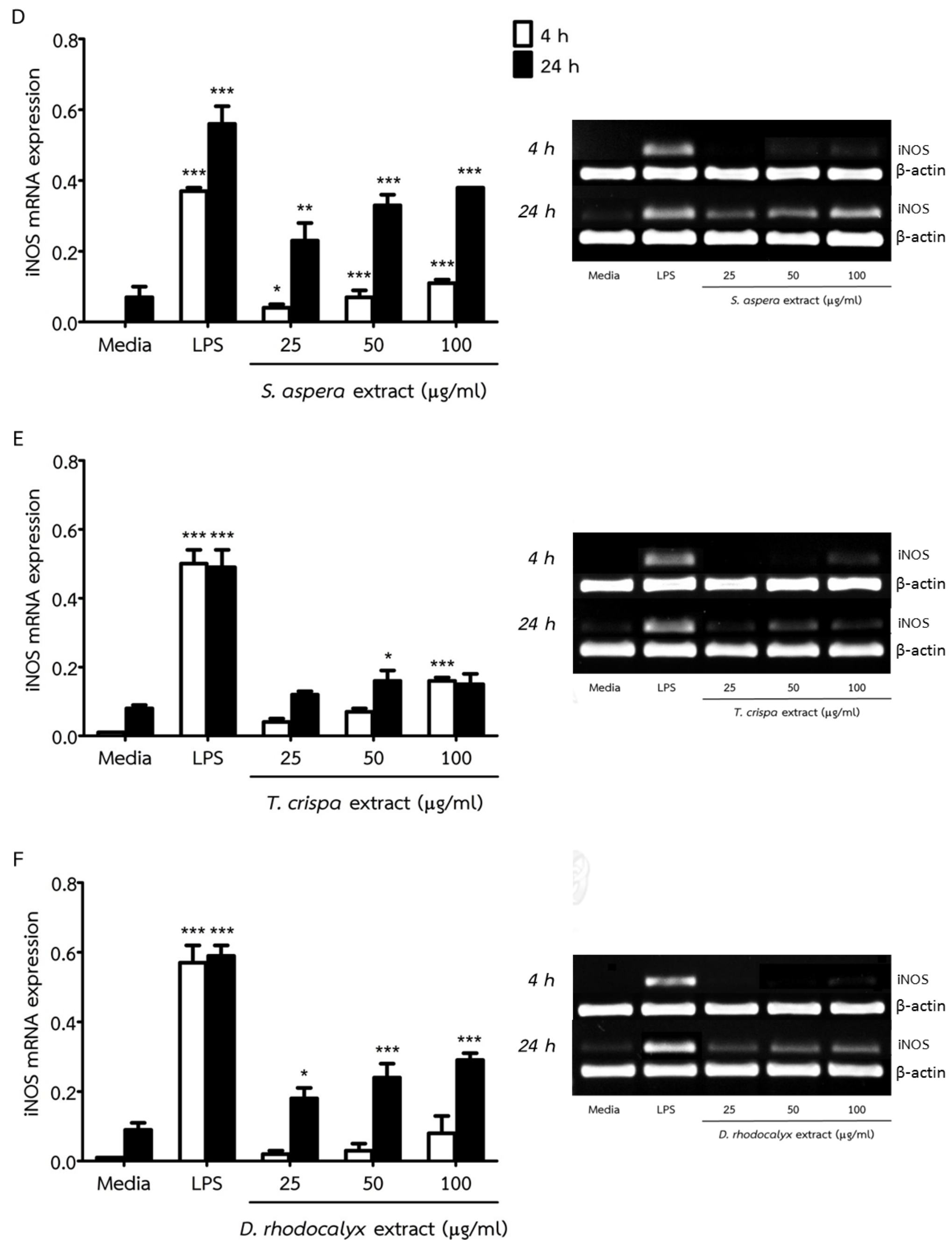

Figure 16(cont.): Effects of the water extracts of the longevity remedy and its components on iNOS mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of iNOS to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

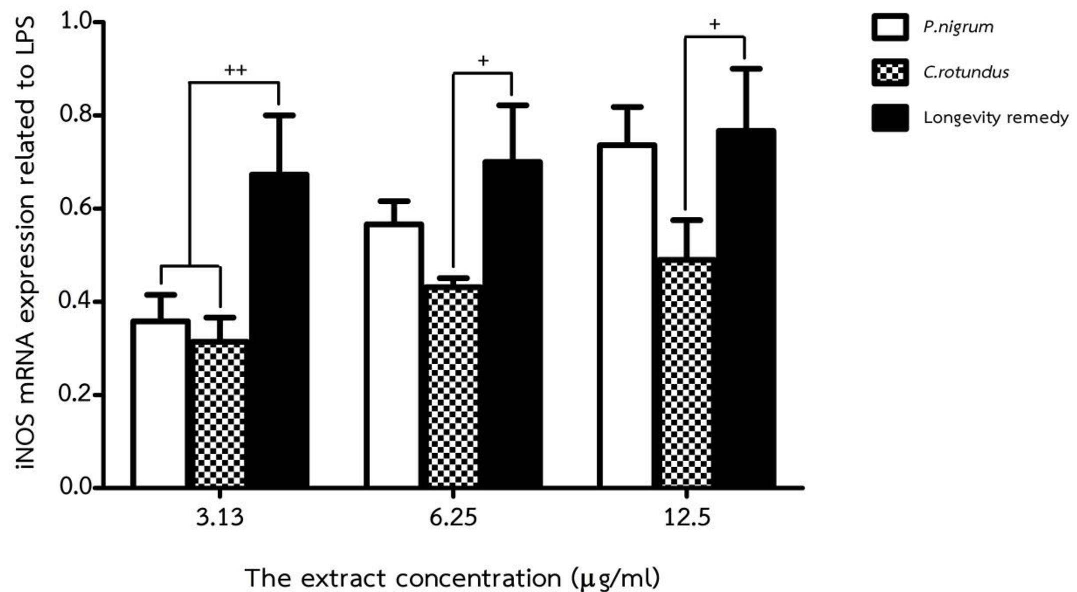

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Figure 17: Comparison of iNOS stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 24 h of treatment. The ratio of iNOS mRNA expressed by each extract to iNOS mRNA expressed by LPS stimulation are represented as means ± S.D. from three independent experiments (n=3). $^{+}$ $p<0.05$, $^{++}$ $p<0.01$ when the remedy was compared to the other extracts.

4. Effects of the water extracts of the longevity remedy and its components on Arg-1 mRNA expression in macrophage J774A.1 cells.

In resolution of inflammation, tissue remodeling and cell proliferation are mediated by Arg-1 enzyme. This enzyme catalyzes L-arginine which is the same substrate of iNOS to L-ornithine. L-ornithine is the precursor for collagen production. Arg-1 is known to be produced in M2 activated macrophages.

The extracts of the remedy and *C. rotundus* significantly increased mRNA expression of Arg-1 at both 4 h and 24 h of treatment at 3.13, 6.25, and 12.5 μg/ml (Fig.18 A-C). The extract of *P. nigrum* significantly increased mRNA expression of Arg-1 at both 4 h and 24 h of treatment at 6.25 and 12.5 μg/ml. The extracts of *S. aspera* and *D. rhodocalyx* significantly increased mRNA expression of Arg-1 at 24 h of treatment at 25, 50, and 100 μg/ml (Fig.18 D-F). The extract of *T. crispa* had no effect on Arg-1 mRNA expression at both 4 and 24 h of treatments.

Effects of three extracts with high activities including the remedy, *C. rotundus* and *P. nigrum* extracts on Arg-1 expression were compared to each other in the term of the ratio of Arg-1 expression induced by each extract to Arg-1 expression induced by LPS at 24 h of treatment. All three extracts demonstrated similar stimulatory effects (Fig.19).

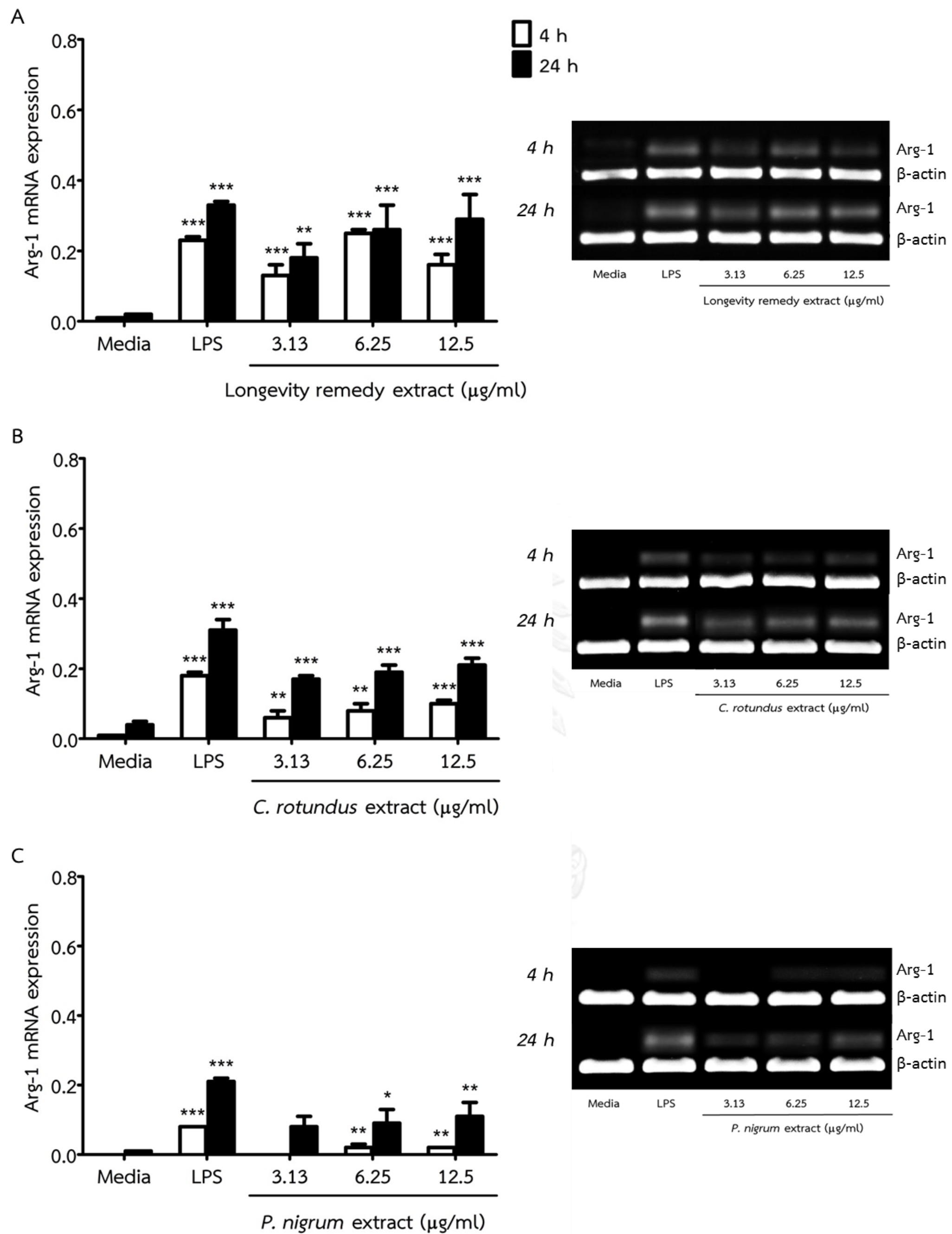

Figure 18: Effects of the water extracts of the longevity remedy and its components on Arg-1 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of Arg-1 to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

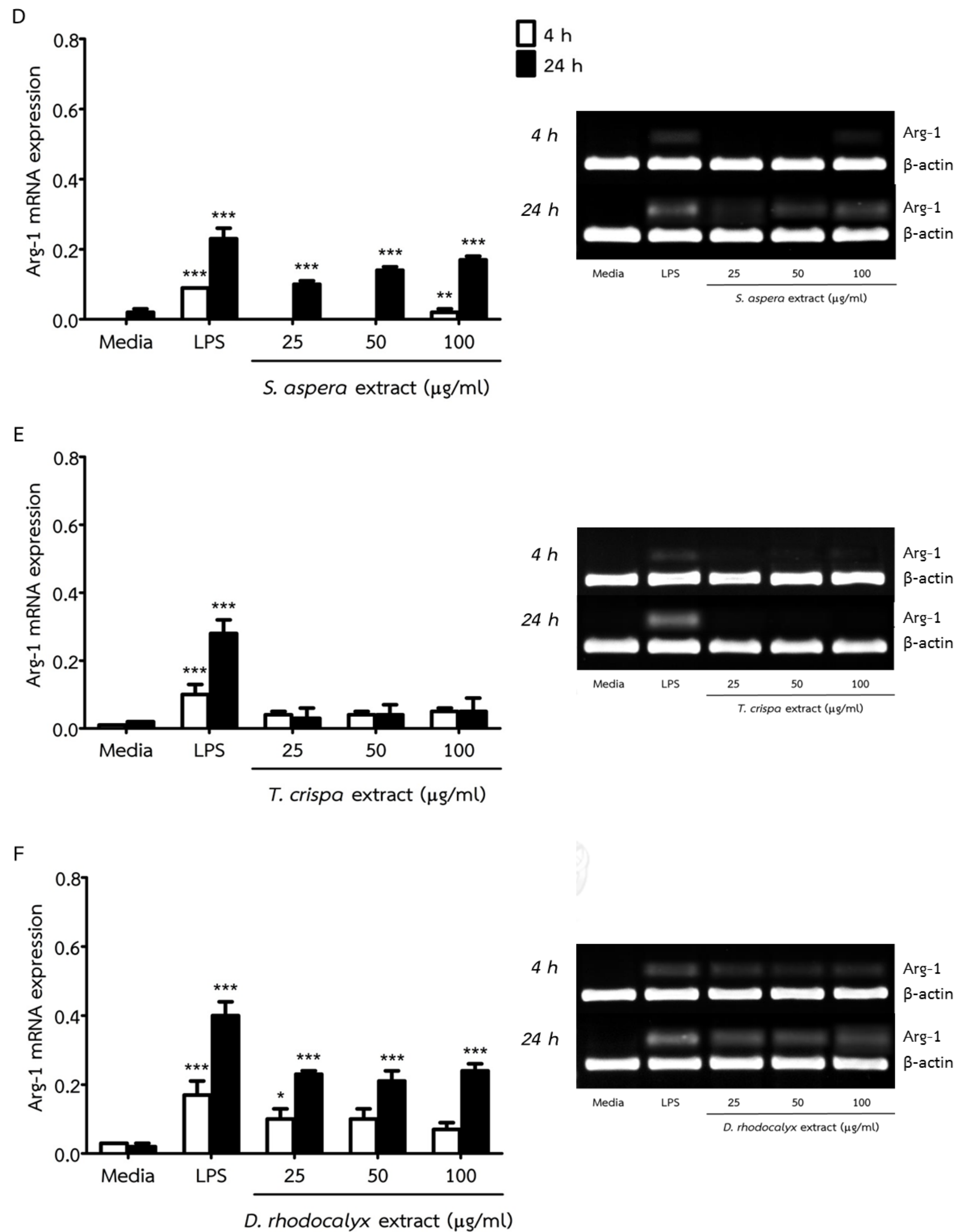

Figure 18(cont.): Effects of the water extracts of the longevity remedy and its components on Arg-1 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of Arg-1 to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

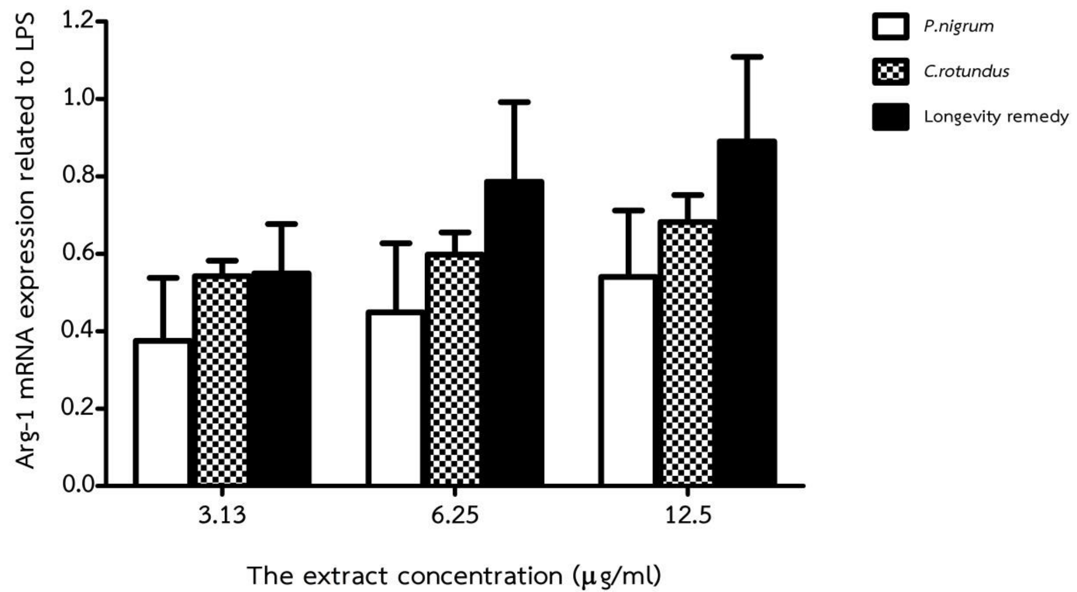

Figure 19: Comparison of Arg-1 stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 24 h of treatment. The ratio of Arg-1 mRNA expressed by each extract to Arg-1 mRNA expressed by LPS stimulation are represented as means ± S.D. from three independent experiments (n=3).

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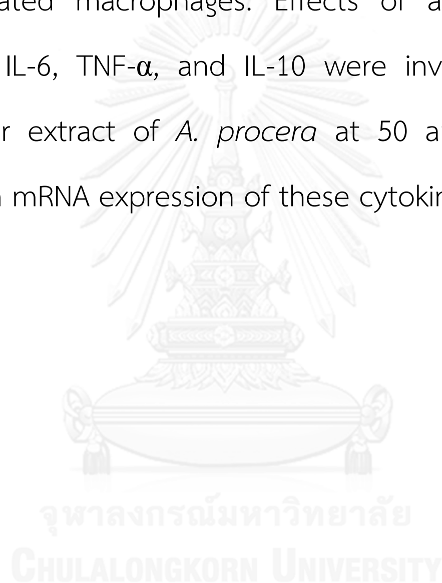
5. Effects of the water extracts of the longevity remedy and its components on mRNA expression of cytokines (IL-1β, IL-6, TNF-α, and IL-10) in macrophage J774A.1 cells.

Cytokines are often used as indicators of macrophage activation. Activated macrophages up-regulate expression of many pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α, involve in innate immunity and inflammatory process. These cytokines are highly generated in M1 activated macrophages. Expression of anti-inflammatory cytokine IL-10 is also up-regulated in activated macrophages. It is a marker of M2 activated macrophages. Effects of all water extracts on mRNA expression of IL-1β, IL-6, TNF-α, and IL-10 were investigated at 4 and 24 h of treatment. The water extract of *A. procera* at 50 and 100 μg/ml did not have stimulatory effects on mRNA expression of these cytokines (data not shown).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5.1 Effects of the water extracts on mRNA expression of IL-1β

The extract of the longevity remedy, *C. rotundus* and *P. nigrum*, at 3.13-12.5 μg/ml, significantly up-regulated IL-1β expression at 4 h of treatment (Fig.20 A-C). Most of stimulatory effects of these extracts on IL-1β expression at 24 h were decreased. The extract of *P. nigrum*, at 6.25 and 12.5 μg/ml, also significantly increased IL-1β expression at 24 h of treatment. Stimulatory effect of the remedy extract was significantly demonstrated at 12.5 μg/ml. The extracts of *C. rotundus* did not have effect on IL-1β expression at 24 h.

The water extract of *S. aspera, T. crispa* and *D. rhodocalyx* demonstrated lower stimulating activities on IL-1β mRNA expression than the extracts of the remedy, *C. rotundus* and *P. nigrum* (Fig.20 D-F). The water extract of *S. aspera,* at 25, 50 and 100 μg/ml, significantly increased IL-1β expression at 4 h of treatment. The water extract of *T. crispa* at 50 and 100 μg/ml significantly increased IL-1β expression at 4 h of treatment. The water extract of *D. rhodocalyx* at 100 μg/ml significantly increased IL-1β expression at 4 h of treatment. These three extracts did not stimulated IL-1β expression at 24 h of treatment, except the extract of *S. aspera* at 100 μg/ml.

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Effects of three extracts with high activities including the remedy, *C. rotundus* and *P. nigrum* extracts on IL-1β expression were compared to each other in the term of the ratio of IL-1β expression induced by each extract to IL-1β expression induced by LPS at 4 h of treatment. The extract of the remedy had the highest stimulatory effect, followed by *C. rotundus* and *P. nigrum* respectively (Fig.21).

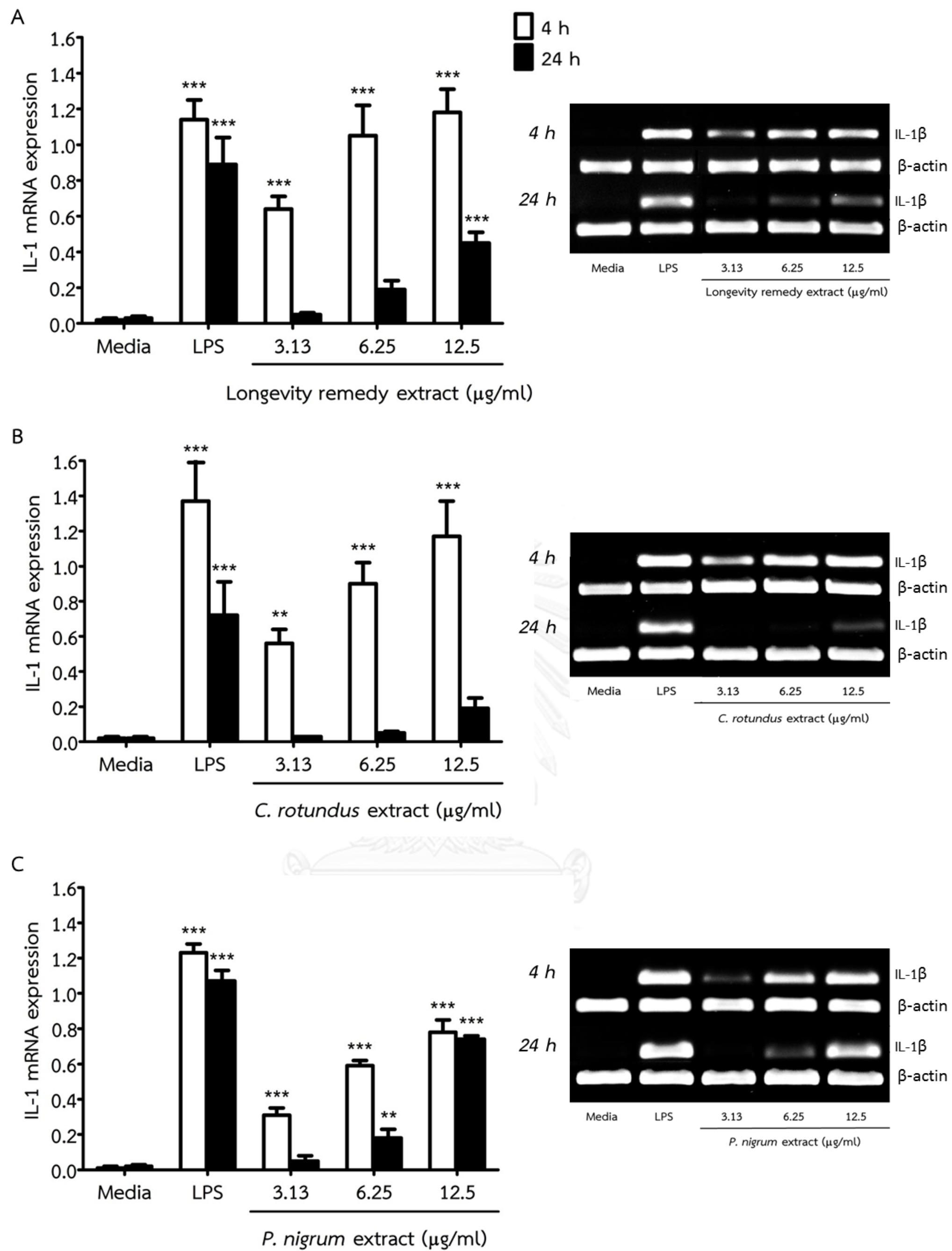

Figure 20: Effects of the water extracts of the longevity remedy and its components on IL-1β mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-1β to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). ** $p<0.01$, *** $p<0.001$ compared to the media control.

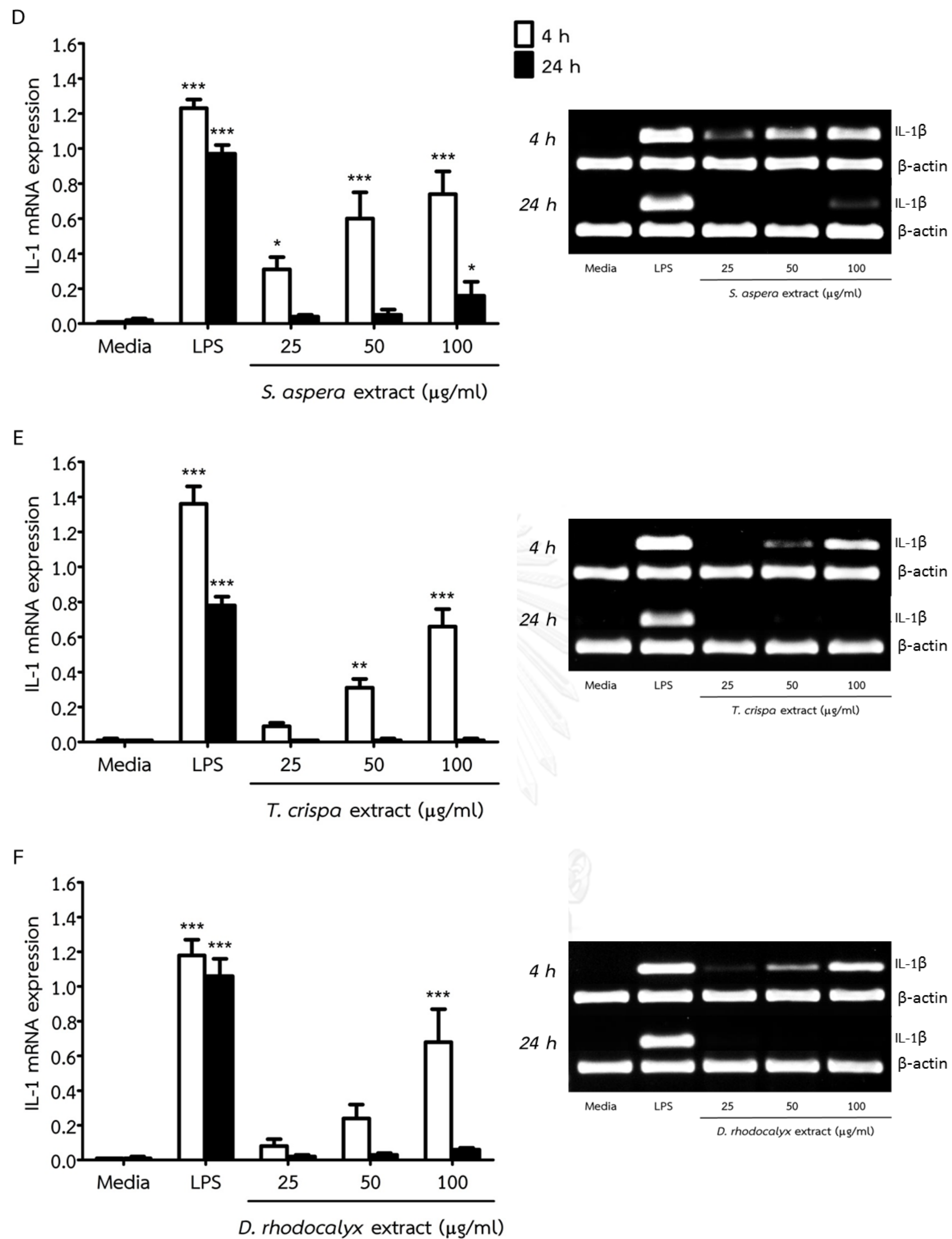

Figure 20(cont.): Effects of the water extracts of the longevity remedy and its components on IL-1β mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-1β to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

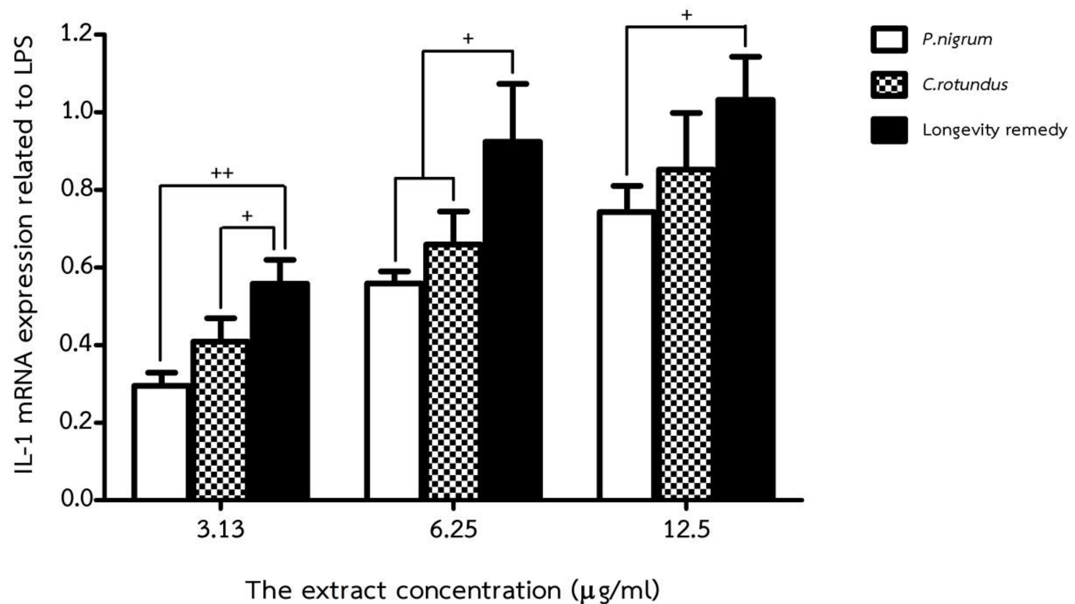

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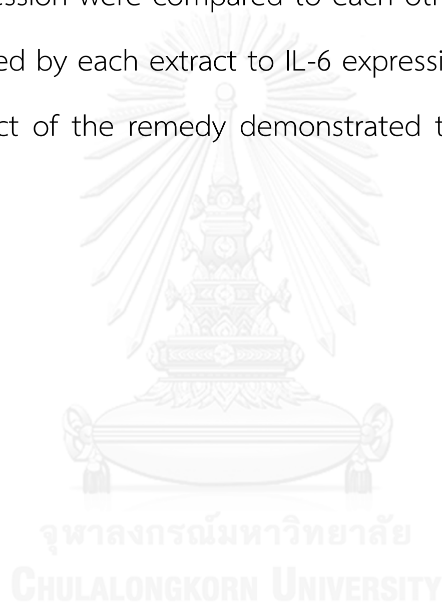
Figure 21: Comparison of IL-1β stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 4 h of treatment. The ratio of IL-1β expression induced by each extract to IL-1β expression induced by LPS are represented as means ± S.D. from three independent experiments (n=3). $^{+}$ p<0.05, $^{++}$ p<0.01 when the remedy was compared to the other extracts.

5.2 Effects of the water extracts on mRNA expression of IL-6

Almost all extracts did not have effect on IL-6 mRNA expression at 4 h of treatment (Fig.22). The extract of the remedy at 12.5 μg/ml was the only extract that significantly increased mRNA expression of IL-6. At 24 h of treatment, the extracts of the remedy (6.25-12.5 μg/ml), *C. rotundus* and *P. nigrum* (12.5 μg/ml) significantly increased mRNA expression of IL-6.

Effects of three extracts including the remedy, *C. rotundus* and *P. nigrum* extracts on IL-6 expression were compared to each other in the term of the ratio of IL-6 expression induced by each extract to IL-6 expression induced by LPS at 24 h of treatment. The extract of the remedy demonstrated the highest stimulatory effect (Fig.23).



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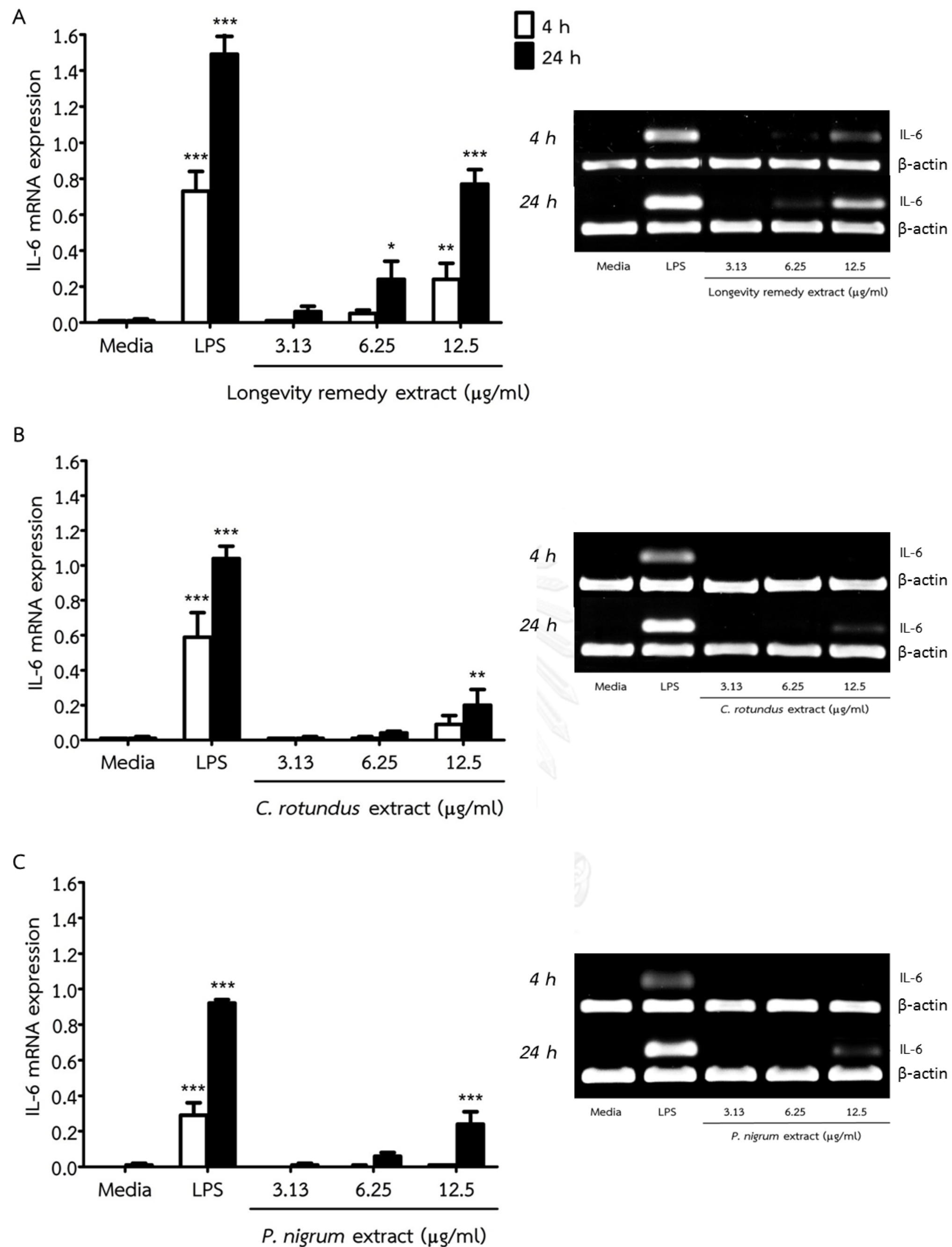

Figure 22: Effects of the water extracts of the longevity remedy and its components on IL-6 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-6 to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). * p<0.05, ** p<0.01, *** p<0.001 compared to the media control.

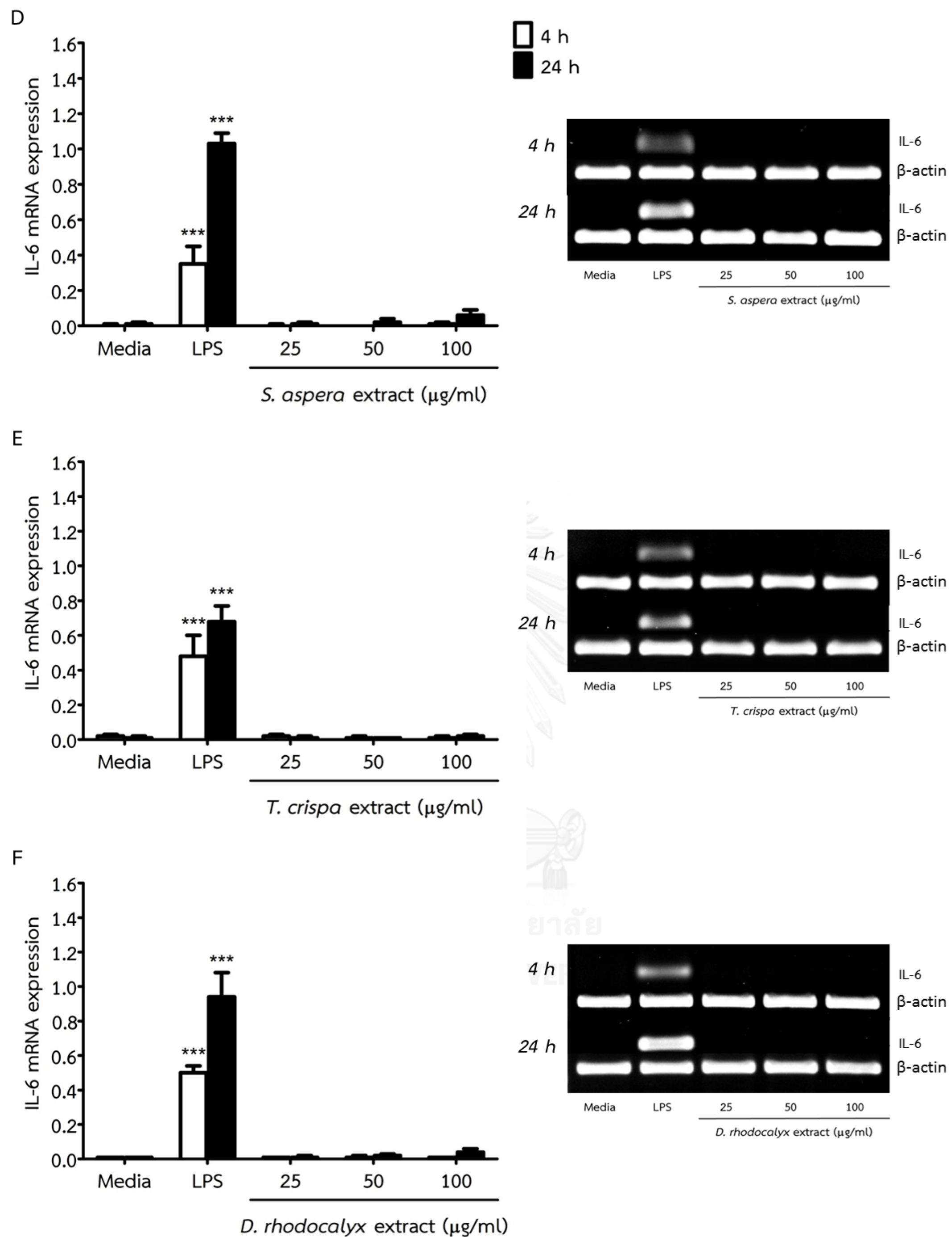

Figure 22(cont.): Effects of the water extracts of the longevity remedy and its components on IL-6 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-6 to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). *** $p<0.001$ compared to the media control.

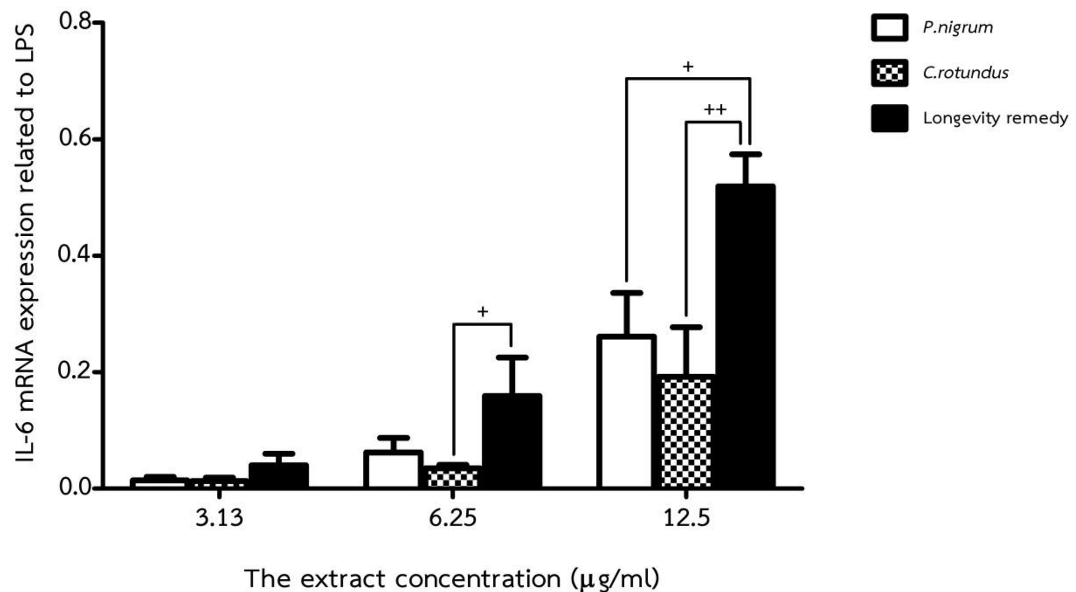

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Figure 23: Comparison of IL-6 stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 24 h of treatment. The ratio of IL-6 expression induced by each extract to IL-6 expression induced by LPS are represented as means ± S.D. from three independent experiments (n=3). $^{+}$ p<0.05, $^{++}$ p<0.01 when the remedy was compared to other extracts.

5.3 Effects of the water extracts on mRNA expression of TNF-α

All the water extracts except the extract of *A. procera* demonstrated stimulatory effects on mRNA expression of TNF-α at 4 h of treatment. They had very low stimulatory effects at 24 h of treatment. All concentrations of these extracts used in the study significantly increased mRNA expression of TNF-α at 4 h of treatment (Fig.24). The stimulatory effects of *C. rotundus* and *P. nigrum* extracts at 24 h of treatment were demonstrated only at 12.5 μg/ml.

Effects of three extracts with high activities including the remedy, *C. rotundus* and *P. nigrum* extracts on TNF-α expression were compared to each other in the term of the ratio of TNF-α expression induced by each extract to TNF-α expression induced by LPS at 4 h of treatment. These three extracts had similar stimulatory effect. The extract of the remedy and *P. nigrum* at 6.25 μg/ml demonstrated higher activities than the extract of *C. rotundus* (Fig.25).

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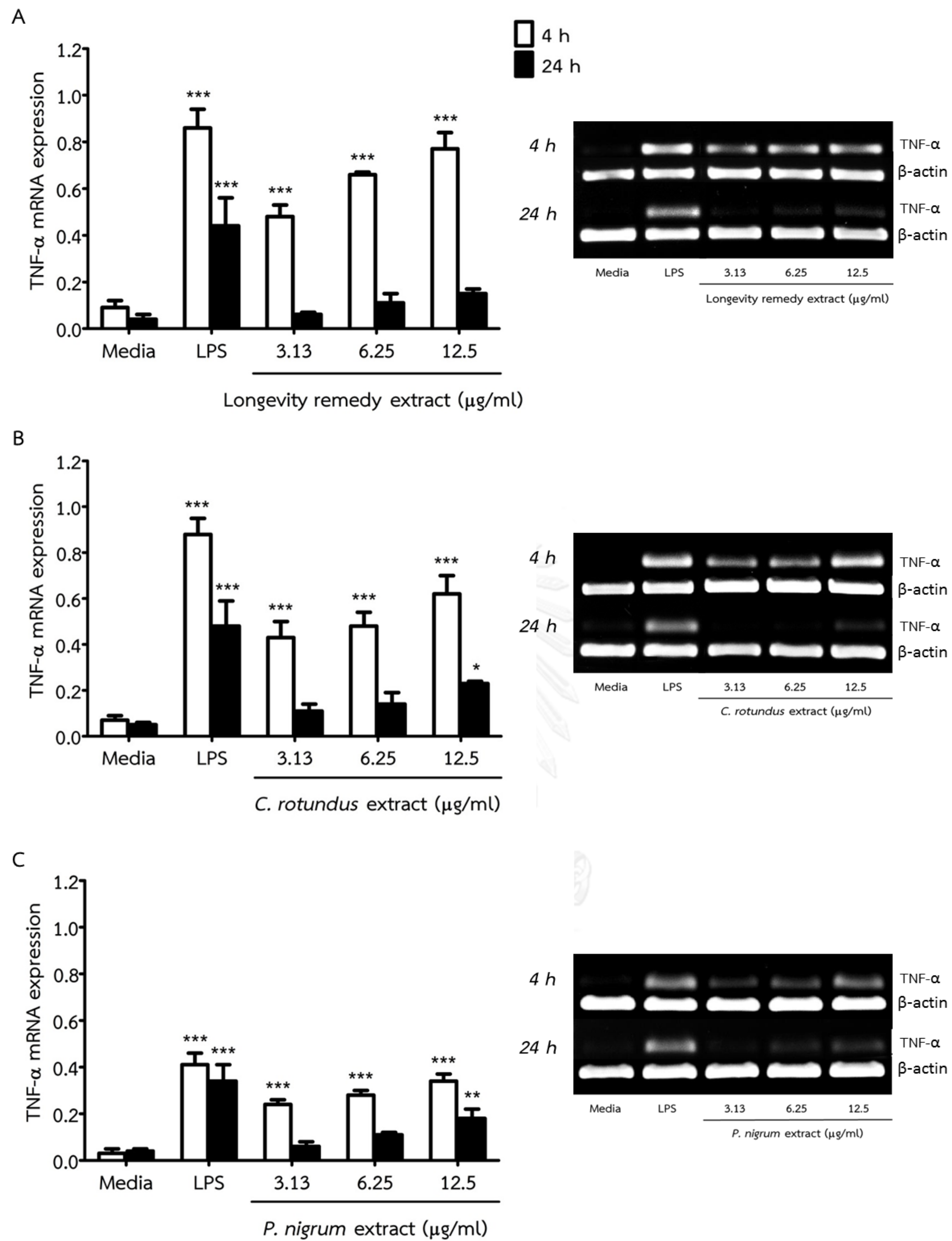

Figure 24: Effects of the water extracts of the longevity remedy and its components on TNF-α mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of TNF-α to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

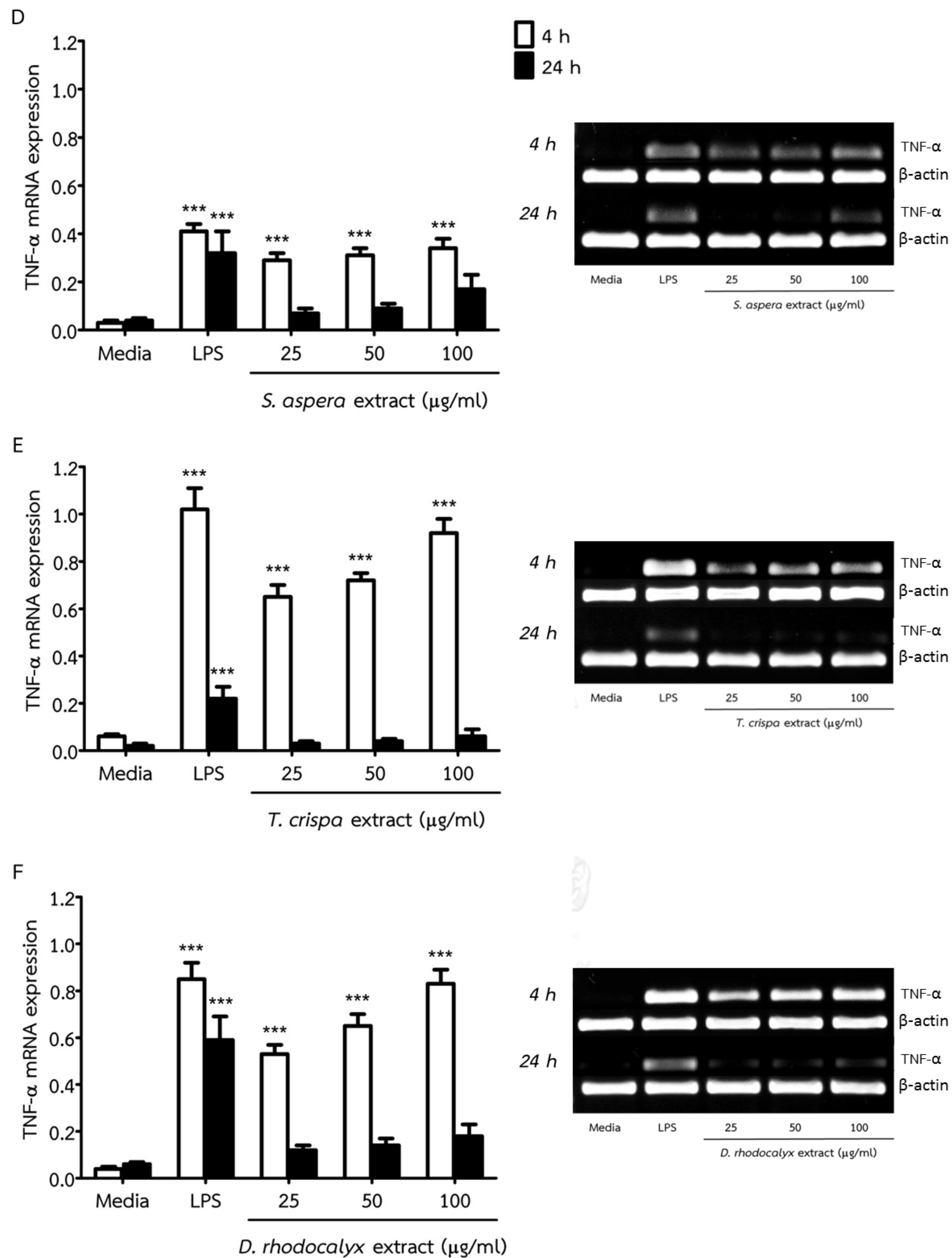

Figure 24(cont.): Effects of the water extracts of the longevity remedy and its components on TNF-α mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of TNF-α to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

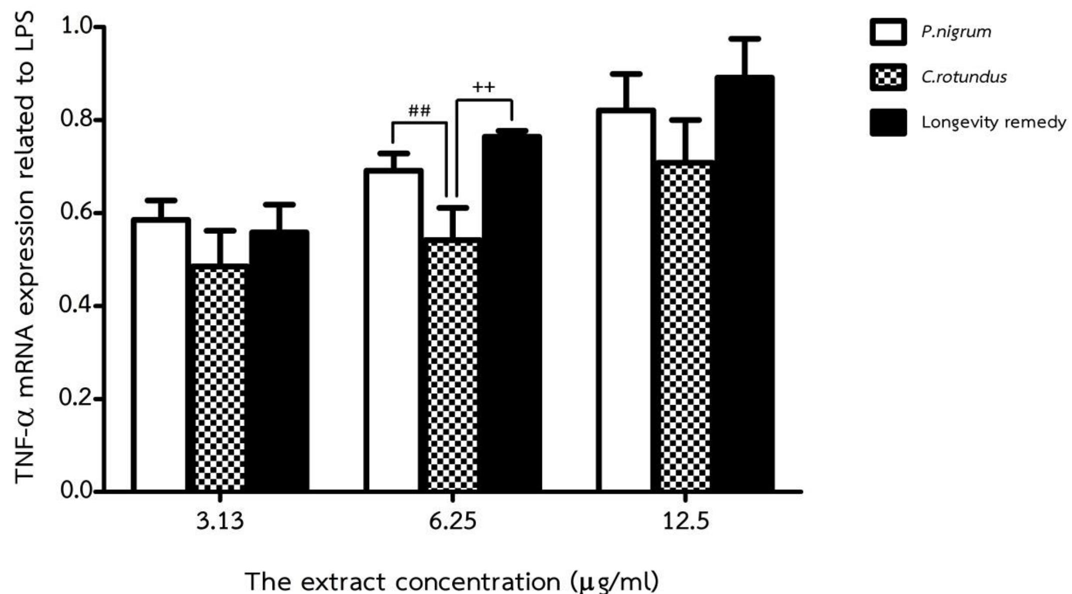

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Figure 25: Comparison of TNF-α stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 4 h of treatment. The ratio of TNF-α expression induced by each extract to TNF-α expression induced by LPS are represented as means ± S.D. from three independent experiments (n=3). $^{++}$ p<0.01 when the remedy was compared to *C. rotundus*, and $^{\#\#}$ p<0.01 when *P. nigrum* was compared to *C. rotundus*.

5.4 Effects of the water extracts on mRNA expression of IL-10

The effects of the extracts of the remedy and its components on IL-10 expression were similar to their effects on IL-1 mRNA expression. The extract of the longevity remedy, *C. rotundus* and *P. nigrum,* at 3.13-12.5 μg/ml, significantly up-regulated IL-10 expression at 4 h of treatment (Fig.26 A-C). Most of stimulatory effects of these extracts on IL-10 expression at 24 h were decreased. Stimulatory effect of the remedy extract on IL-10 expression at 24 h of treatment was significantly demonstrated at 3.13, 6.25 and 12.5 μg/ml. The extract of *C. rotundus* and *P. nigrum* significantly increased IL-10 expression at 24 h of treatment only at 12.5 μg/ml.

The water extracts of *D. rhodocalyx, S. aspera,* and *T. crispa* also increased mRNA expression of IL-10 at 4 and 24 h of treatment (Fig.26 D-F). The extracts of *S. aspera,* and *T. crispa* at 25, 50 and 100 μg/ml increased mRNA expression of IL-10 at 4 h of treatment. Their stimulatory effects were decreased at 24 h of treatment. The extract of *D. rhodocalyx* increased IL-10 mRNA expression at 4 h of treatment at 100 μg/ml. It increased the IL-10 expression at 24 h at 50 and 100 μg/ml.

Effects of three extracts with high activities including the remedy, *C. rotundus* and *P. nigrum* extracts on IL-10 expression were compared to each other in the term of the ratio of IL-10 expression induced by each extract to IL-10 expression induced by LPS at 4 h of treatment. All three extracts had similar stimulatory effect (Fig.27).

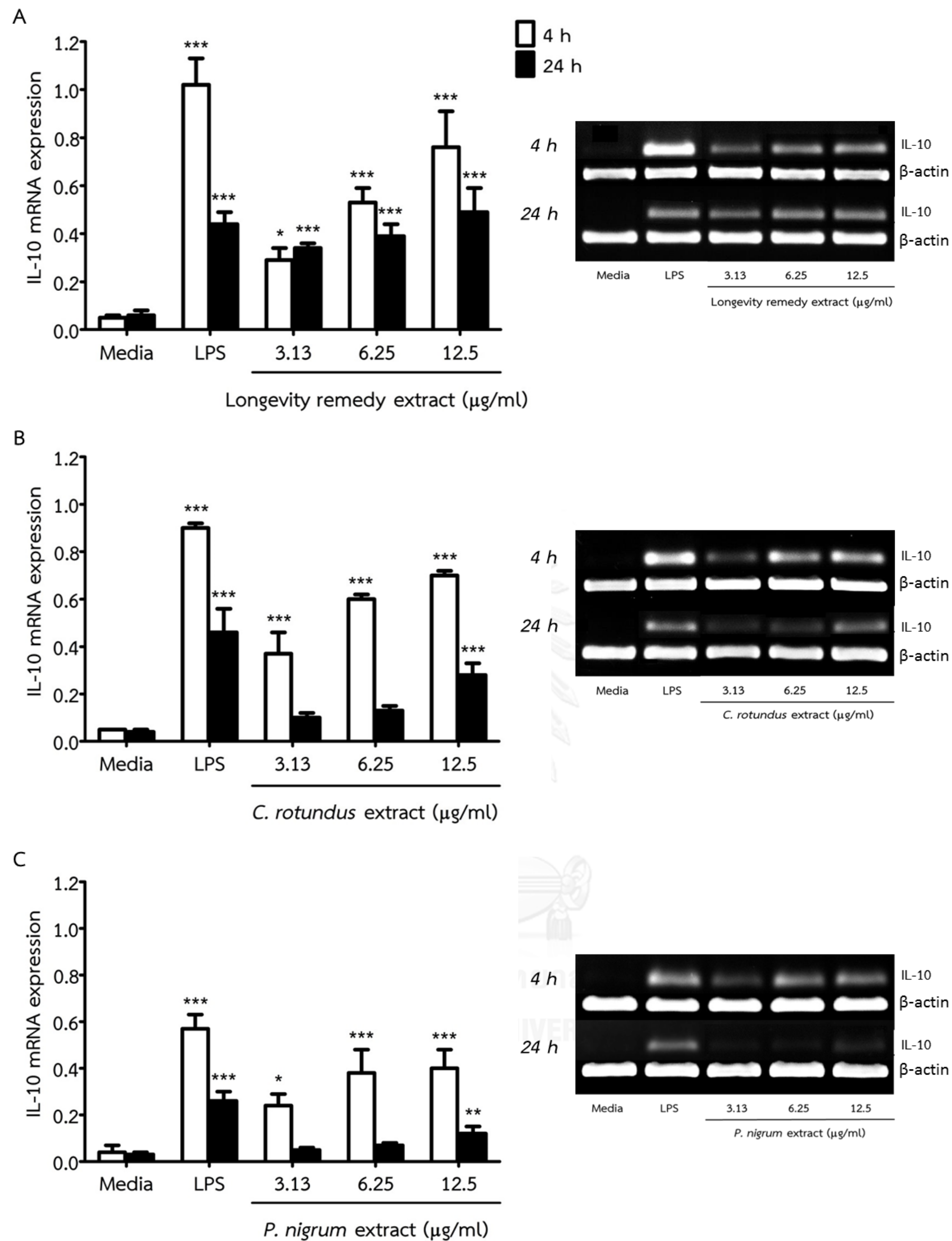

Figure 26: Effects of the water extracts of the longevity remedy and its components on IL-10 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-10 to β-actin from each treated condition of (A) the remedy (B) *C. rotundus* and (C) *P. nigrum* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

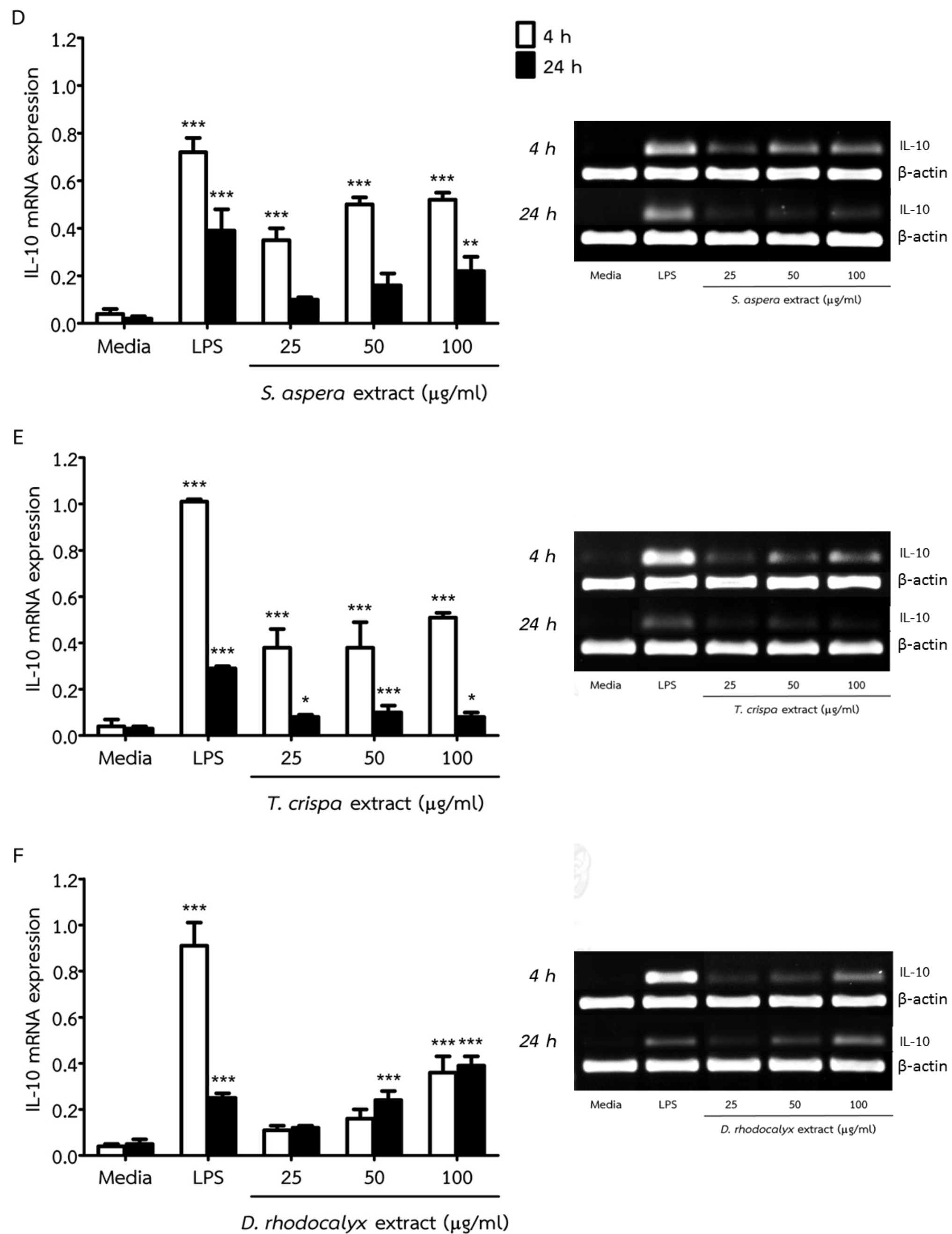

Figure 26(cont.): Effects of the water extracts of the longevity remedy and its components on IL-10 mRNA expression in macrophage J774A.1 cells at 4 and 24 h of treatment. The data are presented as means ± S.D. of the ratio of IL-10 to β-actin from each treated condition of (D) *S. aspera*, (E) *T. crispa* and (F) *D. rhodocalyx* from three independent experiments (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the media control.

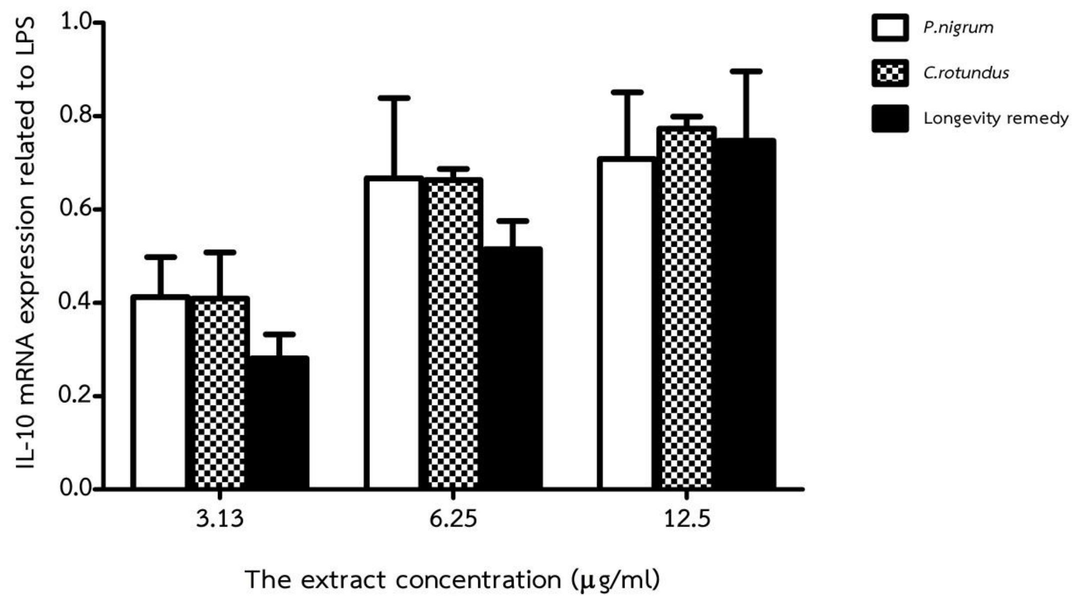

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Figure 27: Comparison of IL-10 stimulation effects among the water extracts of the remedy, *C. rotundus* and *P. nigrum* at 4 h of treatment. The ratio of IL-10 expression induced by each extract to IL-10 expression induced by LPS are represented as means ± S.D. from three independent experiments (n=3).

CHAPTER V
DISCUSSION AND CONCLUSION

Macrophages are known to have multi-functions in the immune system, both in innate and in adaptive immune responses [68]. Many pharmacological agents that can modulate macrophage functions have been clinically used as modern medicines and as alternative medicines. The best examples of modern medicines are corticosteroid drugs and non-steroidal anti-inflammatory drugs (NSIADs) which inhibit macrophage activation and production of various inflammatory mediators [69]. Imiquimod is an immunomodulatory drug that activates macrophage via TLR 7 for boosting immune response against human papilloma virus in patients with warts [69]. Numerous herbal medicines traditionally used for treatment of inflammation or health promotion have been reported to modulate macrophages both *in vivo* and *in vitro*. However, a lot of traditional medicines used for anti-inflammation or health promotion still have not been investigated for their pharmacological activities on macrophages. This study intended to investigate the longevity remedy which is traditionally used for health promotion and restoration whether it may have immunomodulatory activity on macrophages or not. The remedy is recommended to administer in aqueous, alcoholic, or powder forms. This study determined to investigate the water extracts of this remedy and its components including *Cyperus rotundus*, *Piper nigrum*, *Streblus aspera*, *Tinospora crispa*, *Diospyros rhodocalyx*, and *Albizia procera*. This remedy was chosen because it contains only basic combination herbs which are also in several longevity remedies containing other additional herbs.

Macrophages can be activated into broadly classified as M1 and M2 macrophages, depending on stimuli and the environment [70, 71]. Activated M1 macrophages are potent phagocytes and effectors cells in both innate and adaptive

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immunity. They express and generate receptors and mediators involve in inflammation and T cell activation [12]. M2 macrophages produce low amount of inflammatory mediators but they express and generate cell surface molecules and mediators involve in anti-inflammation and tissue repair [13]. Some markers of activated macrophages were used to determined immunomodulatory activity of the remedy and its six herbs. TNF-α, IL-1, IL-6, iNOS, and NO were used as the markers of activated macrophages with M1 phenotypes. IL-10 and Arg-1 were used as the markers of activated macrophages with M2 phenotypes.

Effects of the water extract of the remedy and its six components were firstly evaluated on NO production in macrophage J774A.1 cells. NO is a mediator of activated macrophages involved in killing pathogens during phagocytosis [16]. It is also an inflammatory mediator secreted from activated macrophages to kill extracellular pathogens and to cause vascular relaxation during inflammatory process. The extracts demonstrated different stimulatory activities to induce NO production. The extracts of the remedy, *C. rotundus*, and *P. nigrum* had much higher potencies than *S. aspera,* and *T. crispa*. The extract of *A. procera* and *D. rhodocalyx* did not significantly induce NO production in J774A.1 cells. These results were confirmed by evaluating mRNA expression of iNOS, an inducible enzyme for large amount of NO production in activated M1 macrophages. The water extracts of the remedy and almost its entire components except *A. procera* also significantly stimulated iNOS expression in J774A.1 cells after 24 h of treatment. Most of these extracts had stimulatory effects on iNOS expression after 24 h of treatment higher than after 4 h of treatment. The extract of the remedy trended to have the highest activity on INOS expression. These results demonstrated for the first time that the water extract of a longevity remedy had stimulatory effect on iNOS expression and NO production in macrophages. Some herbal plants in this remedy demonstrated

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immunomodulatory activity on NO production in macrophages. The water extract of *P. nigrum* demonstrated stimulatory effect on iNOS expression and NO production in J774A.1 cells [55]. The methanol extract from *C. rotundus* Inhibited iNOS expression and production, and NO production in IFN-γ plus LPS-induced macrophages. The ethanol extract and essential oil from this plant also had similar effects as the methanol extract [39, 41]. The ethanol extract from *S. aspera* leaves also suppressed iNOS expression on LPS-induced macrophages [62]. The results from previous reports and this study suggest that different solvents used for extraction the same plant may yield different active compounds with opposite activities.

iNOS is induced to express by several stimuli as well as by LPS and pro-inflammatory cytokines which are usually expressed and generated in activated M1 macrophages. Effects of the extracts of the remedy and its components on expression of cytokines in activated M1 macrophages were also evaluated. Cytokines highly expressed and generated in activated M1 macrophages including TNF-α, IL-1β, IL-6 were determined. Almost all of the extracts, except the extract of *A. procera,* significantly stimulated mRNA expression of TNF-α and IL-1β in macrophage J774A.1 cells after 4 h of treatment better than after 24 h of treatment. However, only the extracts with high stimulatory activities on NO production were able to stimulate mRNA expression of IL-6. These extracts were from the remedy, *C. rotundus* and *P. nigrum.* They demonstrated less stimulatory effects on IL-6 expression than on expression of TNF-α and IL-1β. The stimulatory activities of the extracts were different from the effect of LPS control. LPS could potently stimulate expression of TNF-α, IL-1β, and IL-6. Effects of some plants in this remedy on expression or production of these inflammatory cytokines have been reported. Ethanol extract, methanol extract, and essential oil from *C. rotundus* demonstrated inhibitory effects on expression or production of these cytokines [40]. Piperine which is an active

compound in *P. nigrum* also had inhibitory effect on these cytokines [56]. It is postulated that the stimulatory effect of the water extract from *P. nigrum* should come from other active compound(s) in *P. nigrum* seeds.

Anti-inflammatory cytokines, IL-10 and TGF-β are known to be up-regulated in activated M2 macrophages. These cytokines have inhibitory effects on activated M1 macrophages and T cell activation, and promote tissue repair. Effects of the water extracts from the remedy and its components on expression of IL-10 were investigated in this study. Almost entire extracts, except the extract of *A. procera*, stimulated mRNA expression of IL-10, similar pattern to the effect of the LPS control. Most of these extracts induced IL-10 expression after 4 h of treatment higher than after 24 h of treatment. The extract of *D. rhodocalyx* is the only extract that had similar stimulatory effect after 4 and 24 h of treatment. Arg-1, the other marker of activated M2 macrophages, was also used to evaluate the effects of all water extracts. Most of the extracts, except *A. procera* and *T. crispa*, increased mRNA expression of Arg-1 after 24 h higher than after 4 h of treatment. The effects of the remedy and its components on mediators of M2 macrophages have never been reported before.

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For the first time, this study revealed the immunostimulatory activity of the water extract of a basic longevity remedy. The extract potently activated macrophages J774A.1 cells to express and to produce enzymes and mediators which are the markers of activated macrophages. These markers are also used to identify M1 and M2 subtypes of activated macrophages. This study could not point out whether the extract of the remedy stimulates macrophages to which activated macrophage subtypes. Further investigation in vivo is needed and may clarify this question. The immunostimulatory effect of the water extract of the remedy is suggested to come from the synergistic effects of five from six herbal plants

containing in the remedy in equal amount. The remedy demonstrated the highest potency (at 3.13-12.5 μg/ml) to induce almost all markers of activated macrophages evaluated in this study. The extracts of *C. rotundus* and *P. nigrum* were the only two components which also had high potency. The extracts from *D. rhodocalyx*, *S. aspera*, and *T. crispa* demonstrated lower potency than the remedy, *C. rotundus* and *P. nigrum* on macrophage activation. The extract from *A. procera* had no effect on macrophages. The synergism of components in the remedy on macrophages activation can be explained by the similar potency between *C. rotundus* or *P. nigrum* and the remedy which contains one sixth of each component. If no synergism, the remedy should have less potency than these two plants. This study also demonstrated a good example of traditional use of combination herbs for their synergistic effects in the recipe or formulation. *A. procera* was the only one without immunomodulatory activity in this study. However, it demonstrated the most potent antioxidant activity when compared to the remedy and the other herbs in this remedy [31]. This herb should have other beneficial activity for the use of this remedy.

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In summary, the results from this study demonstrated the immunostimulatory effect of the water extract of a longevity remedy containing six herbs on macrophages. This effect likely comes from synergistic effects of the components in the remedy. Immunostimulatory activity may be a part of pharmacological properties of the remedy. This immunostimulatory activity should also be beneficial information for selecting preparation of the remedy which is able to use as aqueous solution, alcoholic solution, or powder. However, further investigation on other immune cells and *in vivo* is needed to confirm immunostimulatory activity of the remed

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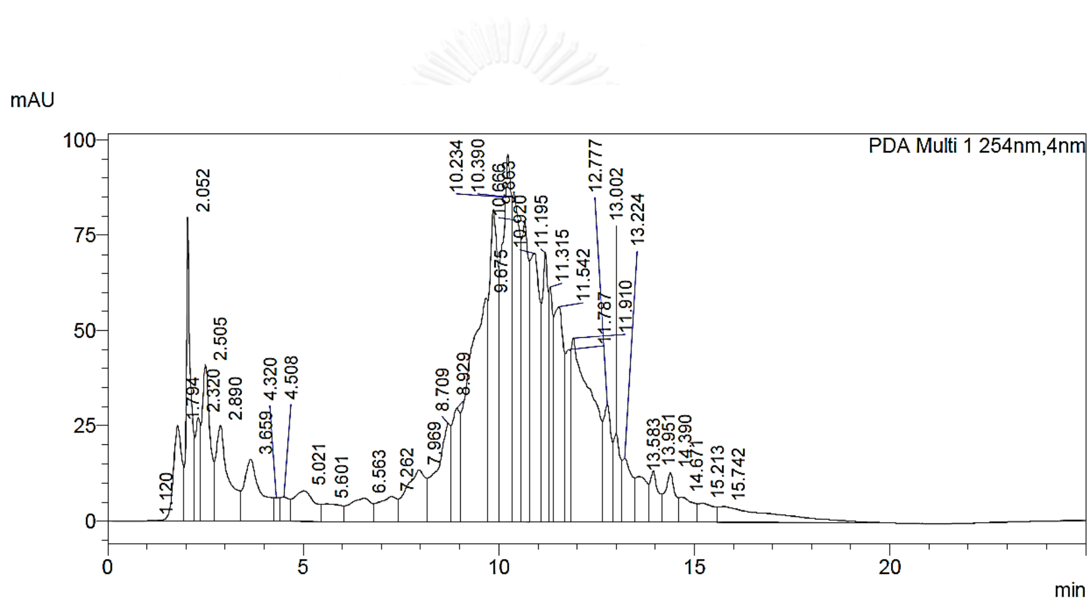
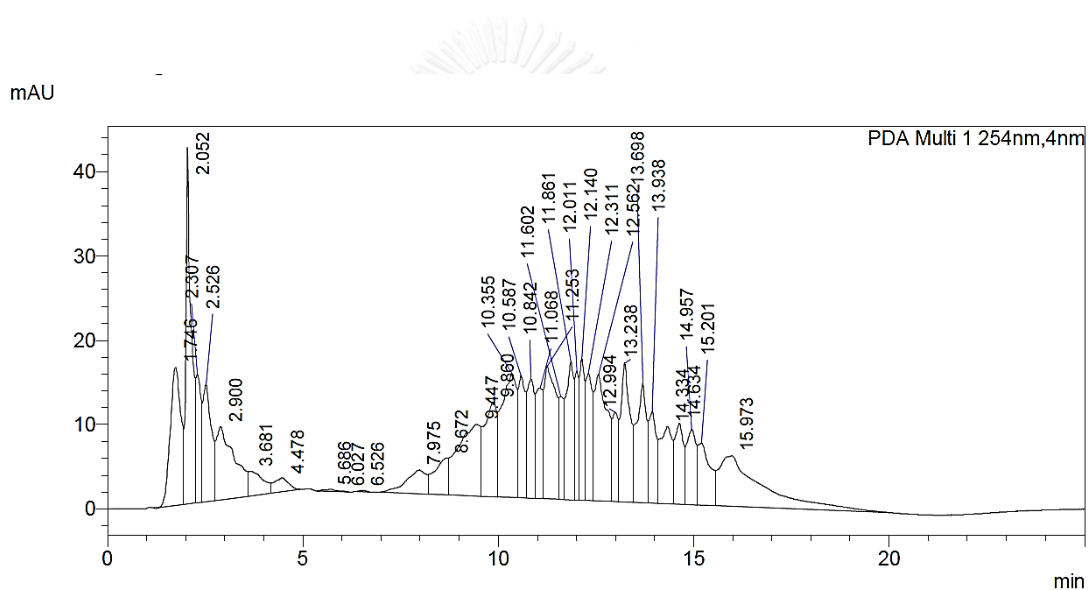
APPENDIX

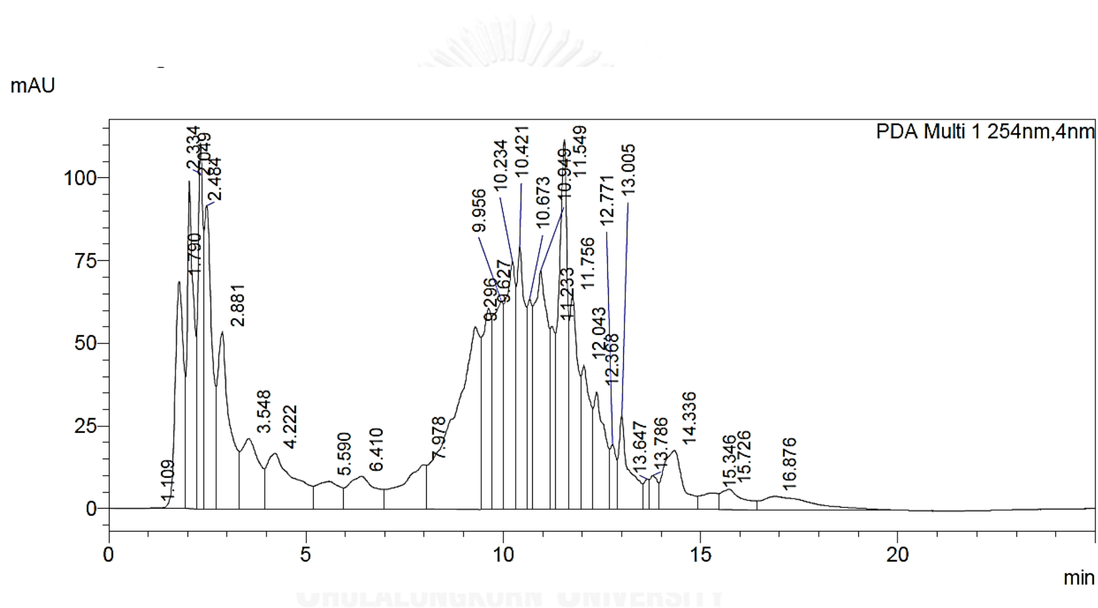
Appendix A: The HPLC chromatogram of the water extract of the longevity remedy [column: Inertsustain® C18 (2.0 mm), 2.1 mm´ 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



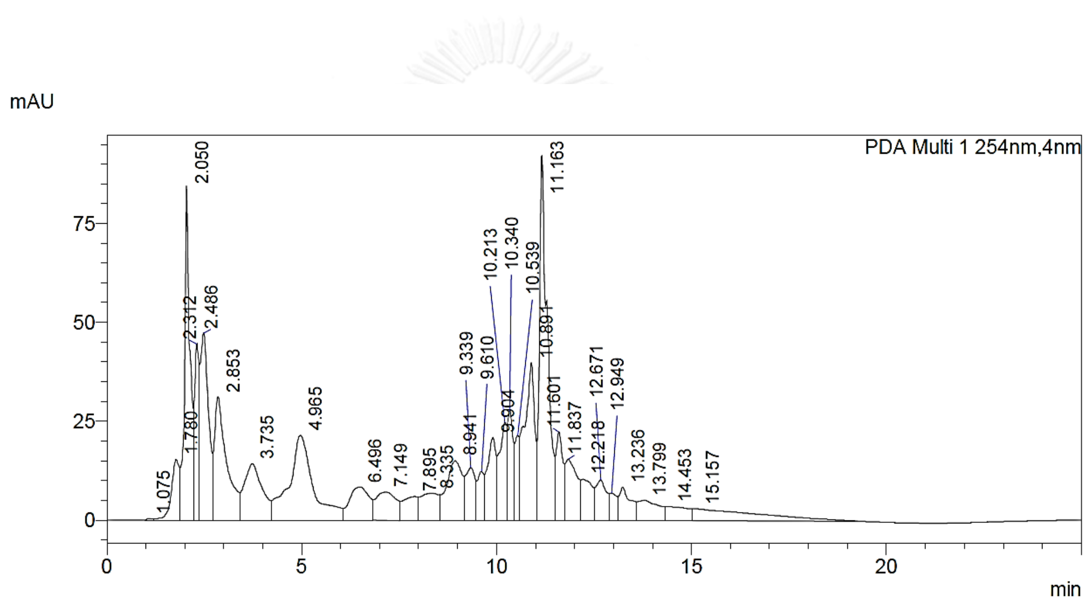
Appendix B: The HPLC chromatogram of the water extract of *C. rotundus* [column: Inertsustain® C18 (2.0 mm), 2.1 mm´ 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



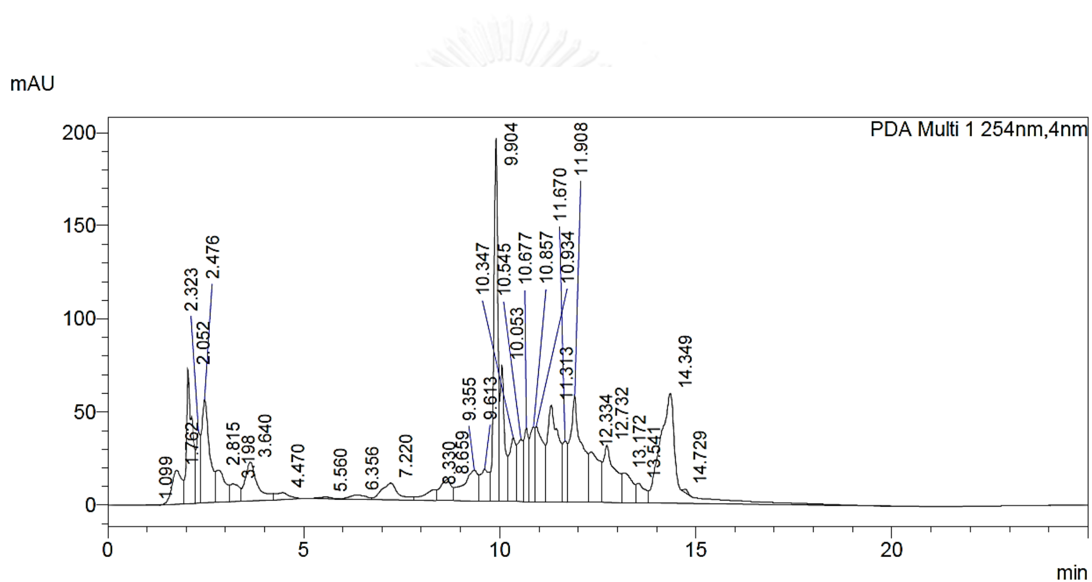
Appendix C: The HPLC chromatogram of the water extract of *P. nigrum* [column: Inertsustain® C18 (2.0 mm), 2.1 mm´ 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



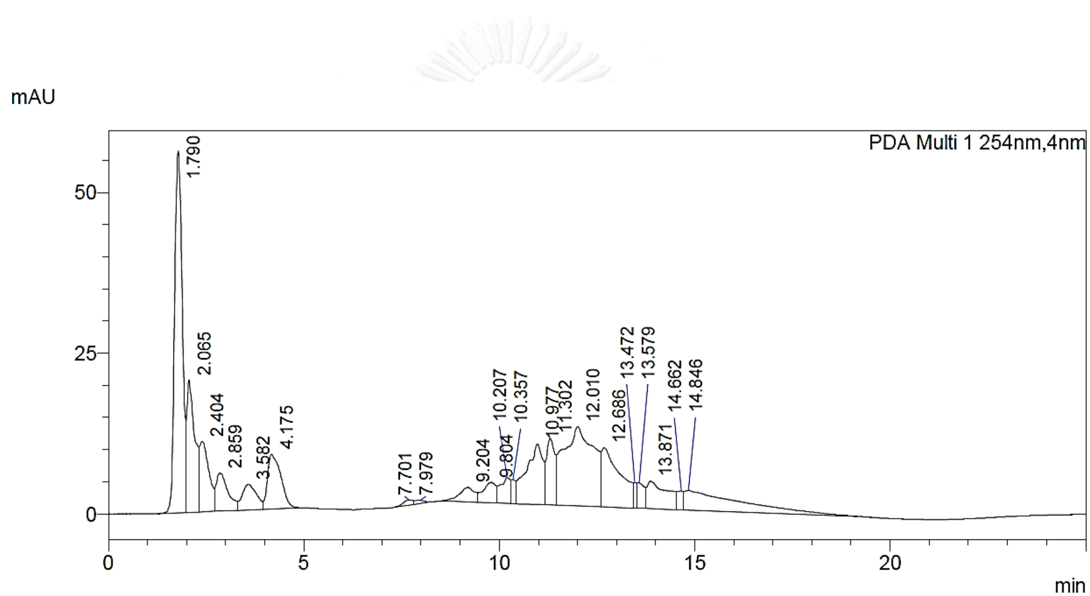
Appendix D: The HPLC chromatogram of the water extract of *S. aspera* [column: Inertsustain® C18 (2.0 mm), 2.1 mm´ 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



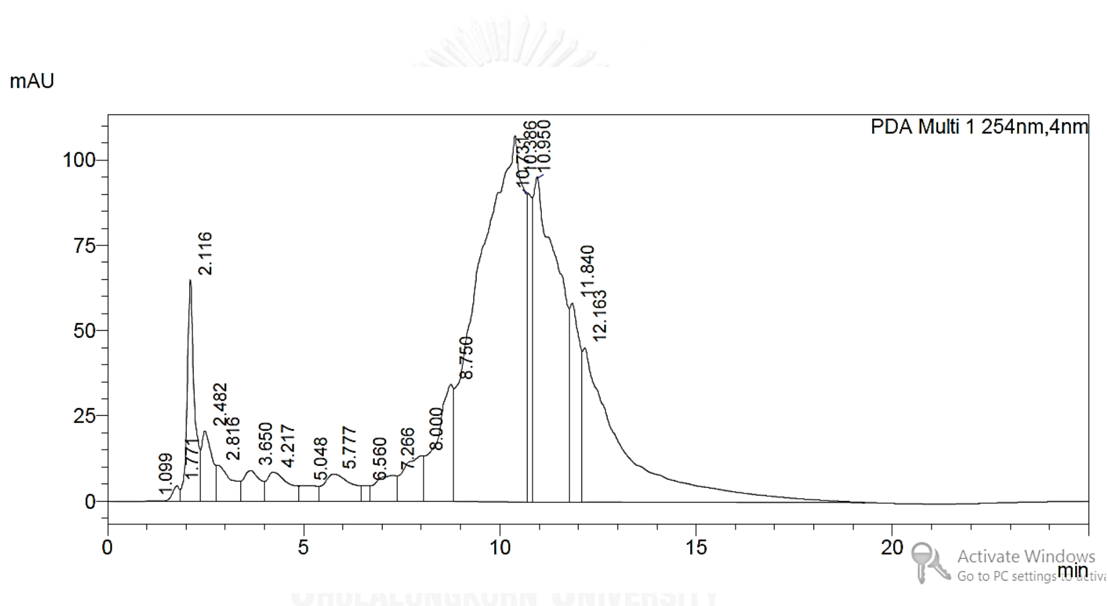
Appendix E: The HPLC chromatogram of the water extract of *T. crispa* [column: Inertsustain® C18 (2.0 mm), 2.1 mm´ 100 mm; mobile phase: 0.1% orthophosphoric acid: MeOH, gradient; flow rate: 0.2 ml/min, detector: PDA 254 nm; oven temperature: 30°C].



Appendix F: The HPLC chromatogram of the water extract of *D. rhodocalyx* [column: Inertsustain® 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 G: The HPLC chromatogram of the water extract of *A. procera* [column: Inertsustain® 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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