ฤทธิ์ปรับเปลี่ยนภูมิคุ้มกันร่างกายของสารสกัดน้ำจากรากเหงือกปลาหมอ

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ปีการศึกษา 2552

IMMUNOMODULATORY ACTIVITY OF THE WATER EXTRACT FROM ACANTHUS EBRACTEATUS VAHL. ROOT

MISS JANTANA YAHUAFAI

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	IMMUNOMODULATORY ACTIVITY OF THE WATER EXTRACT FROM ACANTHUS EBRACTEATUS VAHL. ROOT.
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จันทนา ยะหัวฝาย : ฤทธิ์ปรับเปลี่ยนภูมิคุ้มกันร่างกายของสารสกัดน้ำจากรากเหงือกปลาหมอ. (IMMUNOMODULATORY ACTIVITY OF THE WATER EXTRACT FROM ACANTHUS EBRACTEATUS VAHL. ROOT.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. ผ่องพรรณ ศิริพงษ์, 67 หน้า.

เหงือกปลาหมอ (*Acanthus ebracteatus*) เป็นสมุนไพรไทยที่มีสรรพคุณรักษาโรคมะเร็ง โรคผิวหนัง บำรุงร่างกาย และเป็นยาอายุวัฒนะ จากการศึกษาก่อนหน้าไม่พบพิษเรื้อรังของสมุนไพรในสัตว์ทดลอง พบว่ามีสารโพลีแซคคาไรด์เป็นองค์ประกอบ ซึ่งมีรายงานการศึกษาเกี่ยวกับฤทธิ์ของโพลีแซคคาไรด์จากพืชหลายชนิดในการกระตุ้นภูมิคุ้มกัน แต่ยังไม่พบรายงานเกี่ยวกับการออกฤทธิ์ของสมุนไพรเหงือกปลาหมอต่อระบบภูมิคุ้มกัน การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดน้ำของสมุนไพรเหงือกปลาหมอต่อภูมิคุ้มกันในเซลล์เพาะเลี้ยง โดยทำการศึกษาฤทธิ์ในการกระตุ้นการแบ่งตัวของเซลล์ลิมโฟไซต์ในเม็ดเลือดขาวของมนุษย์ที่แยกได้จากโลหิต ฤทธิ์ต่อการทำงานของเซลล์ในภูมิคุ้มกันแบบไม่จำเพาะ คือ เซลล์แมคโครฟาจ และเซลล์ natural killer (NK)

ผลการศึกษาพบว่าสารสกัดน้ำจากรากสมุนไพรเหงือกปลาหมอกระตุ้นการทำงานเซลล์ของแมคโครฟาจ J774A.1 โดยเพิ่มความสามารถในการจับกินสาร zymosan ได้ชัดเจนทุกความเข้มข้น และได้อย่างมีนัยสำคัญทางสถิติที่ความเข้มข้น 62.5, 125, 250 และ 500 ไมโครกรัม/มิลลิลิตร สารสกัดกระตุ้นให้เซลล์ J774A.1 สร้างไนตริกออกไซด์อย่างมีนัยสำคัญทางสถิติทุกความเข้มข้นที่ใช้ทดสอบ ผลที่ได้นี้สอดคล้องกับผลของสารสกัดที่กระตุ้นการแสดงออกในระดับอาร์เอนเอของเอนไซม์ inducible nitric oxide synthase (iNOS) ซึ่งใช้ในการสร้างไนตริกออกไซด์ นอกจากนี้สารสกัดยังกระตุ้นการแสดงออกในระดับอาร์เอนเอของไซโตคายน์ที่บ่งถึงเซลล์แมคโครฟาจถูกกระตุ้น คือ Tumor necrosis factor-α (TNF-α) และ interleukin-1 (IL-1) ได้อย่างชัดเจนและมีนัยสำคัญทางสถิติ ไม่พบว่าสารสกัดสามารถกระตุ้นการทำงานของเซลล์ NK ในการทำลายเซลล์เป้าหมายแบบต้องอาศัยแอนติบอดี และสารสกัดมีฤทธิ์ต่ำมากในการกระตุ้นการแบ่งตัวของลิมโฟไซต์ในเซลล์เพาะเลี้ยง

การศึกษานี้แสดงให้เห็นว่าสารสกัดน้ำจากรากของสมุนไพรเหงือกปลาหมอมีฤทธิ์กระตุ้นภูมิคุ้มกันร่างกายแบบไม่จำเพาะ ซึ่งยังไม่เคยมีรายงานมาก่อน ผลการศึกษานี้นับว่าเป็นข้อมูลพื้นฐานเพื่อนำไปใช้ในการศึกษาฤทธิ์ของสมุนไพรต่อระบบภูมิคุ้มกันทั้งในเซลล์เพาะเลี้ยงและในสัตว์ทดลองต่อไป ซึ่งอาจนำไปสู่การพัฒนาสมุนไพรนี้ให้เป็นยาที่สามารถใช้ในการกระตุ้นภูมิคุ้มกันในทางการแพทย์ในอนาคต

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5087120420 : MAJOR PHARMACOLOGY

KEYWORDS : INNATE IMMUNITY/ACANTHUS EBRACTEATUS/ IMMUNOMODULATORY

JANTANA YAHUAFAI : IMMUNOMODULATORY ACTIVITY OF THE WATER EXTRACT FROM ACANTHUS EBRACTEATUS VAHL. ROOT. THESIS ADVISOR : ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., THESIS CO- ADVISOR : DR. PONGPUN SIRIPONG, Ph.D., 67 pp.

Acanthus ebracteatus (Ngueak Plaa Mo) is a Thai medicinal plant used for treatment of cancer and skin diseases, and used in health promotion and anti-aging. Previous study report that the water extract of *A. ebracteatus* (AE) roots contained polysaccharides and had no chronic toxicities in animals. Several polysaccharides have been demonstrated to exhibit immunomodulatory activities. Since there has been no evidence on the immunomodulatory activities of *A. ebracteatus*, this study aimed to study the *in vitro* immunomodulating effects of the water extract of *A. ebracteatus* root. The effects of the extract on proliferation of lymphocytes in human PBMCs isolated from whole blood and on functions of cells in the innate immunity including macrophages and natural killer (NK) cells were evaluated. The results demonstrated that the water extract activated macrophage, J774A.1, functions. It clearly increased zymosan phagocytosis of these cells, at all concentration use, and significantly at 62.5-500 µg/ml. It significantly stimulated NO production, at all concentrations (31.25-500 µg/ml). This result was correlated with the increase of the mRNA expression of inducible nitric oxide synthase (iNOS), which involves in NO production, by the extract. The extract also significantly induced the mRNA expression of activated macrophage markers, TNF-α and IL-1. It didn't stimulate NK cell function to kill target cells via antibody-dependent cellular cytotoxicity and had very weak mitogenic activity on lymphocyte proliferation in culture. This study reveals that the water extract of *A. ebracteatus* root exhibited the immunostimulatory effect on the innate immunity. The results from this study provide basic information for further studies about the immunomodulating activities of this plant on immune cells both *in vivo* and *in vitro* which may lead to development this medicinal plant to immunomodulating agents in the future.

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

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
CFSE	Carboxyfluoroscein succinimidyl ester
CO_2	Carbondioxide
^{o}C	Degree celsius
DMSO	Dimethylsulfoxide
DMEM	Dublbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
iNOS	inducible nitric oxide synthase
KOH	potassium hydroxide
LPS	Lipopolysaccharide
MHC	major histocompalibity complex
IL	Interleukin
MeOH	methanol
NO	Nitric oxide
NBT	Nitroblue tetrazolium
OD	optical density
PBMCs	peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PBS	Phosphate buffered saline
PI	Propidium Iodide
S.E.M.	Standard error of mean
SPSS	Statistical package for social science
T_H	T helper
T_C	T cytotoxic
TNF-α	tumor necrosis factor
μg/ml	Microgram per milliliter
μl	Microliter
μM	Micromolar

CHAPTER I

INTRODUCTION

1.1 Background and rationale

The immune system plays an important role in prevention and destruction of foreign antigens in the body. These antigens include microbes, toxin, dying cells, as well as harmful or cancer cells. Body defense mechanisms composed of innate immunity, which is initial protection against foreign antigens, and adaptive immunity, which timely develops and mediates the later but more effective defense against antigens.

The immune response requires interplays of multiple cell types and many components of both innate and adaptive immunity responses for the protective role against antigens. Defects in the functions of the immune system lead to high susceptibility to infections and increase incidence of some cancers. Immunodeficiencies may result from genetic abnormalities, infections, malnutrition, and drugs that targets on immune components. Common immunodeficiencies are acquired immunodeficiency syndromes (AIDS) from human immunodeficiency viruses (HIV) infection and bone marrow suppression by cancer chemotherapeutic agents. Treatment of AIDS patients with anti-HIV drugs results in decrease of viral load and relief from opportunistic infections. Immunoglobulin and cytokines are immunomodulating agents and clinically used for treatment of various immunodeficiencies. Human immunoglobulin is used for many immunodeficiency diseases caused by genetic defects. Neutropenia from anticancer drugs is alleviated by using human colony stimulating factors, G-CSF and GM-CSF.

Drugs derived from medicinal plants make a large contribution to drug discovery up to now, despite advanced scientific and technology progress in molecular biology. Many compounds from botanical sources have been reported to exhibit immunomodulating activities. Polysaccharides from various traditional medicinal plants have been demonstrated to potentiate immune response both *in vivo* and *in vitro* (Ando

I. *et al*. 2002; Gao Y. *et al*. 2003; Han SB. *et al*. 1998; K-S A. *et al.* 1998; Liu M. *et al.* 1998; Sonoda Y. *et al*. 1998; Zhao J. *et al.* 1990) and have potential as immunomodulators for clinical use as alternative medicines. For examples, polysaccharides from certain mushrooms have anti-tumor activities via macrophage activation (Yoon YD. *et al*. 2003; Han SB. *et al*. 2003). Beta-glucans from the cell walls of plants exhibit anti-cancer and anti-infective activities (Patwardhan B. and Gautam M. 2005). The water extract of *Scaphium scaphigerum*, which contains polysaccharide, has been reported to activate macrophage function and exhibited weak mitogenic activity on human lymphocytes in vitro (Piyarat 2005). It also activate humoral immune response against sheep red blood cells and attenuated immunosuppressive effect of cyclophosphamide in mice (Siriarchavatana P. 2007). *Acanthaceae ebracteatus* Vahl. is a plant in the family Acanthaceae. It had been used as a traditional medicine for treatment of cancer and skin diseases and used for health promotion. Previous study revealed that the solvent extract of its root exhibited mitogenic activity on human lymphocytes (Masathien C. *et al*. 1991). This study aimed to further investigated *in vitro* immunomodulatory activities of the water extract of *A. ebracteatus* root on macrophage and NK cell functions as well as mitogenic activity on lymphocytes. The result from this study may provides some useful scientific information about this extract in immunological aspects, which may be useful for clinical application in the future.

1.2 Objective

To study *in vitro* immunostimulatory effect of *A. ebracteatus* (AE) root extract on immune cell functions and proliferation.

1.3 Hypothesis

The water extract of *A. ebracteatus* (AE) root exhibits immunostimulating activities on immune cell functions and proliferation.

CHAPTER II

LITERATURE REVIEWS

Immune responses

The physiologic function of the immune system is to protect the body against infections and to eradicate establishing infections, malignant cell and death cells.

The immune system is a complicate network of specialized tissues, organs, cells, and mediators. In mammalian, immune responses consists of innate and adaptive immunity (Fig 2.1). Innate immunity is the first line of defense, mediated by cells and molecules that are always present and ready to eliminate pathogen and other foreign antigens within hours. Adaptive immunity is the second line of defense which develops more slowly and mediates the later immune response. It is the form of immunity that is stimulated by foreign antigens, has high specificity for the antigens, and responds more effectively against each successive exposure to the same antigen. The cooperation between innate and adaptive immunity is requied to protect individuals from infections and other foreign sunstances (Zhao J. *et* al. 2009*;* Akira S. *et al*. 2006).

Innate immunity (Abbas A. K. and Lichtman A. H. 2005)

Innate immunity provides the first line of defense against infectious microbes. Most components of this immunity are present before the onset of infection and rapidly response to infections within hours. It constitutes a set of defense mechanisms that are broad reactivity to all members of microbes in the same species. It recognizes patterns of molecules present on various types of microbes by receptors encode in the germ line, and the responses against the same microbes are not enhanced by repeat exposures to microbes. Innate immunity consists of the following major components;

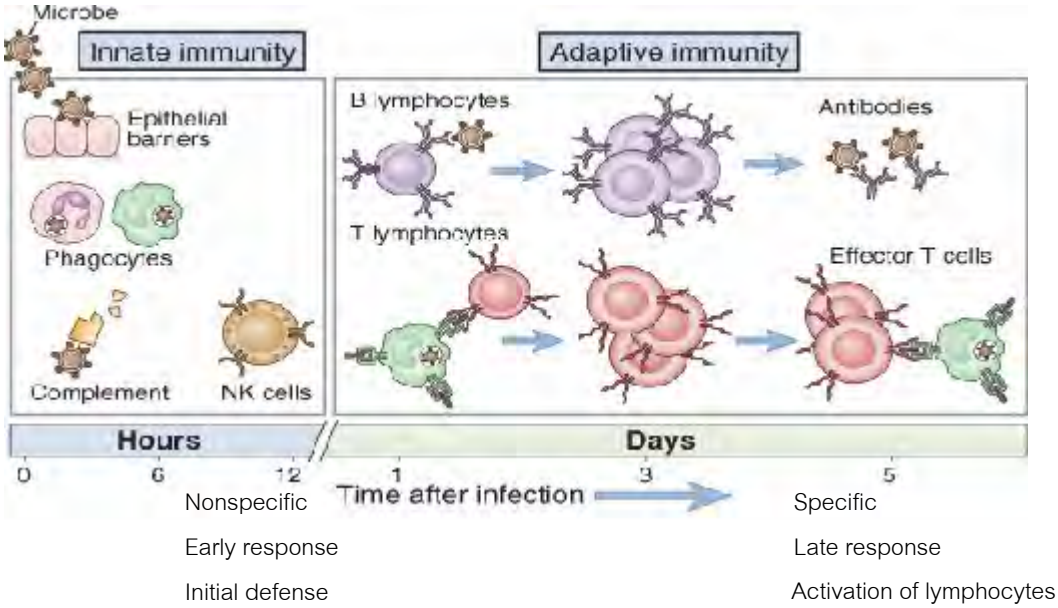

Figure 2.1 Nonspecific (innate) immunity and specific (adaptive) immunity (Available from: http://medic.med.uth.tmc.edu/edprog/immuntbl.htm. [2008, September]

1. Anatomical, mechanical and chemical barriers:

Skin and mucous membrane are anatomical barriers which are effective against the entry of most microorganisms. Intact skin acts as a barrier and the sticky mucous on mucous membrane as a trap for the microbes. Examples of mechanical barriers are coughing, sneezing, vomiting and diarrhea. These barriers can expel microbes which invade the physical barriers although they are not pleasant. Chemical barriers, such as tears, saliva, acid and enzymes in the stomach, can directly kill microbes. Most of them are weak acids that prevent microbes from entering the body as well as washing them away.

2. Phagocytes and natural killer cells

They are the second line defense of innate immunity when microbes can past through the first line barriers mentioned above.

- Phagocytes: Neutrophils and monocytes/ macrophages are white blood cells that are recruited to infection sites and able to recognize microbes by various types of receptors. They engulf microbes to intracellular digestion and destruction. This process called phagocytosis. These phagocytes also secrete many cytokines and mediators to eliminate microbes.
- Natural killer cells (NK cells) are primitive lymphocytes that nonspecifically kill microbial infected cells and cancer cells. They induce target cell lysis by similar mechanisms to cytotoxic T lymphocytes via perforin and granzymes as well as via death receptor. They also produce IFN-γ, which activates macrophages to kill phagocytosed microbes

3. The complement system:

The complement system consists of many plasma and membrane proteins play role in immunity against microbes. In this system, complement proteins (C1-C9) play key roles to generate immune responses. Complement activation is initiated by three distinct pathways, the classical or antibody-dependent, the alternative, and the lectin pathways. This activation initiates a proteolytic cascade of complement proteins (C1-C5) at the initial phase. All three pathways merge into activation of C3 and then of C5 by cleaving into active fragments by highly specific enzymatic complexes called convertases. In the common terminal pathway downstream of C5, C6 to C9 are nonproteolytic activated and assembled into the membrane attack complex (MAC), a pore-like structure, that cause cell lysis. In addition to direct killing of microbes through MAC formation, complement activation also mediates other host defense mechanisms

including opsonization of microbes, activation of inflammation, and clearance of immune complexes.

4. Inflammation

Inflammation is the physiological process occurs in vascularized tissue in response to injury or infection. It is a complex series of events fundamental to the ability of the human body to protect itself against injurious and infectious agents. The inflammatory process requires the co-ordinated interaction of soluble mediators and cellular components systemically work together in the attempt to contain and to eliminate the microbes or the cause of tissue injury. Inflammation is an innate immune response which functions to neutralized or eliminate inflammatory stimuli, limit and localize the response of the process, stimulate adaptive immunity and promote healing. The hallmark of inflammation are: redness (rubor), swelling (tumor), heat (calor), pain (dolor), and loss of function The inflammatory process, when poorly controlled, can result in massive tissue destruction

5. Cytokines

Cytokines are soluble proteins produced and secreted by immune cells and other cells in the body in response to a number of stimuli. They play role in a various biological activities including innate immunity, adaptive immunity, inflammation, and hematopoiesis. In innate immunity, activated macrophages after microbial recognition are the principal sources of cytokines are macrophages activated. TNF-α, IL-1, and chemokines are major cytokines involved in recruiting neutrophils and monocytes to sites of infection.

In this study, macrophage and NK cell functions were used to evaluate immunomodulatory activities of the water extract of *A. ebracteatus* root. Functions of macrophages and NK cells are described in detail below.

Macrophage activation

Macrophages are mononuclear phagocytic cells differentiated from monocytes in blood circulation. A tissue macrophage is different from a monocyte in many aspects. The cell is five- to tenfold larger; increases in number and complexity of intracellular organelles; increases phagocytic ability, produces higher levels of lysosomal enzymes, and is able to secrete a variety of soluble factors. Macrophages are distributed throughout the body and are named according to their tissue locations. They are called alveolar macrophages in the lung, histiocytes in connective tissues, kupffer cells in the liver, mesangial cells in the kidney, microglial cells in the brain and osteoclasts in bone. Macrophages recognize microbes by surface receptors that are specific for microbial products. Signal transduction from these receptors leads to activation of transcription factors and subsequently stimulates expression of cytokines, enzymes, and other proteins involved in the antimicrobial functions of activated phagocytes. Macrophages also express receptors that recognize other microbial structures and that promote phagocytosis and killing of the microbes.

Various stimuli can activate macrophages in the course of an immune response. Phagocytosis of foreign antigen particles, cytokines from activated TH1 cells, mediators of the inflammatory response, and components of bacterial cell walls such as lipopolysaccharide (LPS) are able to initiate macrophage activation. Activated macrophages are more effective than resting cells in eliminating microbes. They exhibit greater phagocytic activity, increase ability to kill ingested microbes, increase secretion of inflammatory mediators, increase ability to activate T cells, as well as secrete various cytotoxic proteins required for eliminating viral infected cells, tumor cells, and intracellular bacteria. They also express higher levels of class II MHC molecules, allowing them to function more effectively as antigen-presenting cells. Thus, macrophages and TH cells facilitate each other's activation during the immune response.

Phagocytosis

Macrophages recognized microbes by certain types of receptor can lead to phagocytosis of the microbes and activation of the phagocytes to kill the ingested microbes. Phagocytosis is a process in which the phagocyte extends its plasma membrane around the recognized microbe, the membrane closes up and pinches off, and the particle is internalized in a membrane-bound vesicle, phagosome. The phagosomes fuse with lysosomes to form phagolysosomes. Several enzymes in the phagolysosomes are activated to generate a number of antimicrobial and cytotoxic substances required for microbial killing via oxygen-dependent and –independent mechanisms. The digested contents in the phagolysosome are then eliminated in a process called exocytosis. All microbicidal substances which produced mainly within lysosomes and phagolysosomes act on the ingested microbes without damaging the phagocytes. In strong reactions, these microbicidal substances may be release into the extracellular space and may injure host tissues. This is the reason why inflammation, normally a protective host response to infections, may cause tissue injury as well. (Zhang X-f. *et al*. 2007). Figure 2.2

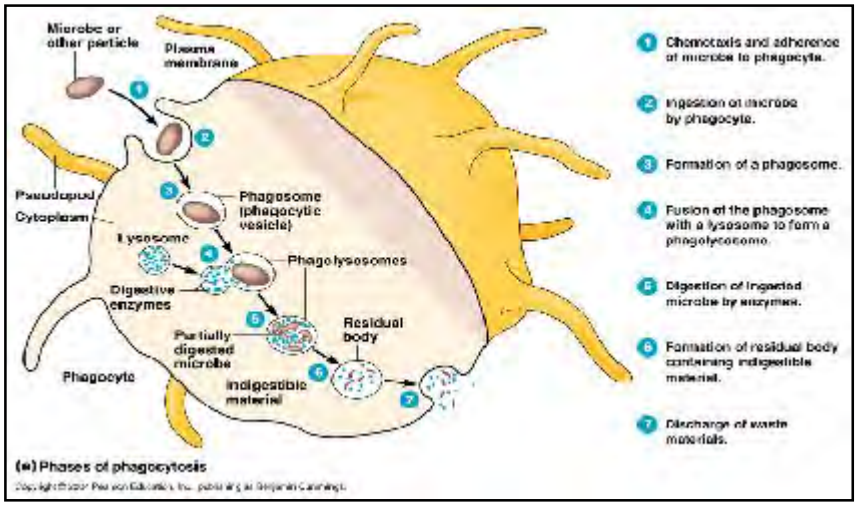

Figure2. 2 phagocytosis and processing of exogenous antigen by macrophage (http://www.utc.edu/Facculty/Becky-Bell/210-Ex2-studyguide-studyguide.htm [2008, 20 September]

Oxygen-dependent killing mechanisms: phagocyte oxidase in phagolysosome of activated macrophages converts molecular oxygen into reactive oxygen intermediates (ROIs), superoxide anion and free radicals, which are toxic to the ingested microbes. Inducible nitric oxide synthase (iNOS) in the activated cells catalyzes the conversion of arginine to high level of nitric oxide (NO) that sustains for hours or longer. NO is also a microbicidal substance. NO at high levels is very rapidly oxidized to $OONO^-$, that induce neighboring cell toxicity by nitrosating DNA and tyrosine residues, and inducing lipid peroxidation.

Oxygen-independent killing mechanisms: Activated macrophages also kill phagocytosed microbes by synthesize lysozyme, various hydrolytic enzymes, defensins, and TNF-α.

In addition to killing microbes by phagocytosis, activated macrophages also perform other functions to eliminate microbes. phagocytosed antigen is digested within the phagolysosome into antigenic peptides that associate with class II MHC molecules. These peptide–class II MHC complexes then move to the macrophage membrane. Activated macrophages also increased expression of both class II MHC molecules and the co-stimulatory B7 family of membrane molecules, thereby rendering the activated macrophages more effective as antigen presenting cell (APC) in activating helper T (TH) cells.

Activated macrophages are major sources cytokines in innate immunity. These include a wide range of cytokines, such as interleukin 1 (IL-1), TNF-α and interleukin 6 (IL-6), that promote inflammatory responses and fever. Typically, each of these agents has a variety of effects. For example, IL-1 activates lymphocytes; and TNF- α can kill a variety of cells as well as tumor cells. Activated macrophages also generate interferon-α and –β (IFN- α and –β) that induces an antiviral state; increases MHC class I expression and activates NK cells and colony stimulating factors (G-CSF, GM-CSF, M-CSF) that stimulate inducible hematopoiesis. These activated cell also secrete growth

factors and enzymes involve in remodeling injured tissue and replacing it with connective tissue.

In this study, stimulating effect of the water extract of *A. ebracteatus* root on macrophage functions was investigated by determining phagocytosis, NO production and expression of iNOS and cytokines, IL-1 and TNF-α. Phagocytosis was performed by using zymosan phagocytosis assay. Amount of NO production was determined by analyzing nitrite concentration using Griess reaction. The expression of inducible proteins, iNOS and cytokines, in activated macrophages was determined by detecting the mRNA of these proteins using RT-PCR.

NK cell activation

NK cell are lymphoid cells derived from bone marrow but distinct from T and B lymphocytes. They are large granular lymphocytes that do not rearrange TCR or membrane immunoglobulin genes. These cells constitute 5%–10% of lymphocytes in human peripheral blood. NK cells employ several types of stimulatory receptors specific for molecules associated with infected, stress, or transformed cells. They are also regulated by inhibitory receptors specific for class I MHC molecules. The distinctive specificity of the cells, along with their fast response and lack of a memory stage, is the basis for considering the cells part of the innate immunity.

NK cells can recognize tumor and viral infected cells in two different ways. They employ inhibitory receptors to distinguish a reduction of class I MHC molecules and activating receptors specific to the unusual profile of surface antigens on some tumor cells and viral infected cells. These target cell recognitions are independent on antibodies. Natural killer cells also express CD16 molecules which are receptors for the Fc portions of some IgG antibodies (FcγIIIa receptor) and use these receptors to bind to tumor or viral infected cells coated with antibodies. This binding leads to NK cell activation and subsequently destroys the targeted cells. This process is called antibody-

dependent cellmediated cytotoxicity (ADCC) (Christine *et al.* 1999; Bryceson YT. *et al.* 2006; Smyth MJ. *et al*. 2005). Figure 2.3

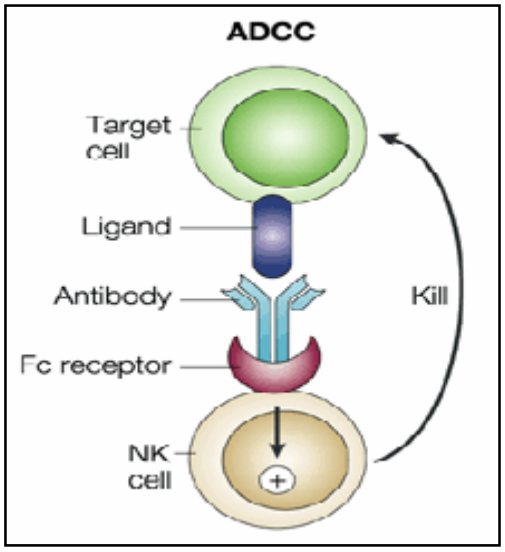

Figure 2.3 NK cells cytotoxicity *via* Antibody-dependent cellular cytotoxicity (ADCC)

Once NK cell is activated either via ADCC or via non-ADCC as mentioned before, the activated NK cell can kill tumor or viral infected cells by two distinct mechanisms.

- Perforin-dependent cytotoxicity: This mechanism is predominantly used by NK cells to destroy target cells. Activated NK cells secrete lysosomal contents, perforin and granzyme molecules, by exocytosis into the immunological synapse formed between the NK cell and the target cell. Perforins are pore-forming molecules while granzymes are serine protease that can induce target cell apoptosis similar to caspase enzymes which are key enzymes in apoptosis.

- Death receptor pathways: Activated NK cells secreted IFN-γ to induced death receptor, Fas, expression on target cells. Fas on the target cells interacts with Fas

ligand on NK cells and leads to Fas signaling, caspase activation and finally apoptosis induction. Another death receptor pathway mediating natural killing involves TNF-α related apoptosis-inducing ligand (TRAIL), expressed by natural killer cells. Binding of TRAIL to death receptor (DR)4 and DR5 on target cells results in a caspase activation and apoptotic induction similar to Fas-Fas ligand interaction.

Further more, activated NK cells produce and secrete the cytokine, IFN-γ which induces macrophages to activated cells with more effective at killing phagocytosed microbes. NK cells and macrophages function cooperatively to eliminate intracellular microbes: macrophages phagocytose microbes and generate 1L-12 which activates NK cells to secrete IFN-γ which turn to activates the macrophages to kill the ingested microbes.

Antibody-dependent cellular cytotoxicity (ADCC)

Not only NK cells, macrophages, monocytes, neutrophils, and eosinophils have cytotoxic potential to kill their target cells in antibody-dependent mechanisms called **antibody-dependent cell-mediated cytotoxicity (ADCC)**. These cytotoxic cells express membrane receptors for the Fc region of the antibody molecule (Fc receptors). When antibody is specifically bound to a target cell, these Fc receptor-containing cells can bind to Fc region of the antibody, and thus to the target cells, and subsequently cause the target cell lysis. Target-cell killing by ADCC appears to involve a number of different cytotoxic mechanisms depend on various substances (e.g., lytic enzymes, TNF, perforin, granzymes) secreted by these nonspecific cytotoxic cells. Even though these cells are nonspecific for antigen, the specificity of the antibody directs them to specific target cells Figure.2.4. .

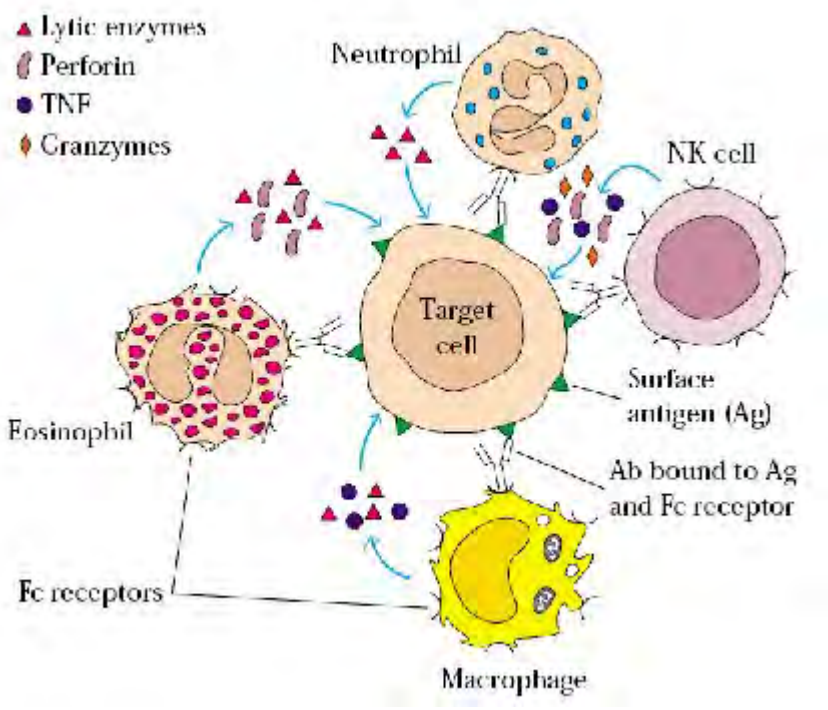

Figure.2.4. Antibody-dependent cell-mediated cytotoxicity (ADCC) (Richard, A., *et al*. 2003).

Adaptive immunity

Adaptive immunity generates immune responses that are highly specific to different types of foreign antigens. Unlike innate immune responses, adaptive immune responses recognize selectively eliminate specific foreign microorganisms and molecules. Adaptive immunity exhibits four major characteristics: antigenic specificity, diversity, immunologic memory and self/nonself recognition. There are two types of adaptive immunity, humoral and cell-mediated, that are mediated by different cells, T and B lymphocytes, and molecules to provide immune response, antibodies and range of cytokines against extracellular microbes and intracellular microbes, respectively (Figure 2.5)

Humoral immunity

Humoral immunity is the adaptive immunity mediated by antibodies. Recognition of specific antigen by the membrane-bound immunoglobulin on a mature naive B cell, as well as interactions with T cells and macrophages, selectively induces the activation and differentiation of antigen-specific B-cell clones. The antigen-specific B cell divides repeatedly and differentiates to a population of plasma cells and memory cells. Plasma cells, which have much less membrane-bound immunoglobulins than B cells, produce and secrete soluble immunoglobulin or called antibody. Antibodies are important immune mediators for protection against extracellular microbes and their toxins. They use several effector mechanisms to eliminate their specific antigens. Antibody-mediated effector functions include neutralization of microbes and microbial toxins by binding to and interfering with the ability of these microbes and toxins to attach to host cells.; activation of the complement system lead to the formation of the cytolytic membrane attack complex on target cells; opsonization of antigens for enhanced phagocytosis by binding to Fc receptors on phagocytes.; and antibody-dependent cell-mediated cytotoxicity (ADCC), by which antibodies target microbes for lysis by cells of the innate immunity such as NK cells. (Storni T. *et al.* 2005).

Cell-mediated immunity:

Cell-mediated immunity is the other arm of the adaptive immune response mediated by T lymphocytes to destroy intracellular microbes. The responses of T lymphocytes consist of sequential phases: recognition of specific antigenic peptides on antigen presenting cells by naive T cells, expansion of the antigen-specific T cells by proliferation, and differentiation into effector cells and memory cells. Effector T cells are generated mainly in lymph nodes draining sites of microbe entry and cells are able to migrate to a site of infection. There are two functionally distinct effector T cells; helper T cells (TH) and cytotoxic T lymphocytes (CTL).

Helper T cells (TH) or $CD4^+$ T cells

TH cells recognize the antigens of microbes ingested by antigen presenting cells including dendritic cells or macrophages. They play role as regulatory cell and mediate effector functions via their cytokines. There are two subsets of effector TH cells, TH1 and TH2. TH1 cells secrete IFN-γ to activate macrophages. Activated macrophages produce a range of substances, including reactive oxygen intermediates, nitric oxide, and lysosomal enzymes, that kill ingested microbes. Macrophages also produce cytokines that induce inflammation and other cytokines that promote fibrosis and tissue repair. TH2 cells secreted IL-4 and IL-5 that can stimulate eosinophilic functions and inhibit macrophage activation. Eosinophils are important in host defense against helminthic parasites. The balance between activation of TH1 and TH2 cells determines the outcomes of many infections.

Cytotoxic T lymphocytes (CTLs) or $CD8^+$ T cells

CTLs are direct effector cells that can kill their specific target cells such as infected cells and cancer cells. Antigen recognition by effector CTLs leads to the release of granules to the region of contact with the targets. CTLs kill target cells mainly

as a result of their granule contents, perforins and granzymes. Perforins create pores in target cell membranes and introducethe granzymes into the target cells. Granzymes activate caspase enzymes which induce DNA fragmentation and apoptosis, in the cytoplasm of the target cells substances that induce. Activated CTLs also express a membrane death receptor ligand called Fas ligand, which binds to a death receptor, called Fas on target cells. Signaling of Fas-Fas ligand interaction leads to caspase activation in the target cells and apoptotic induction.

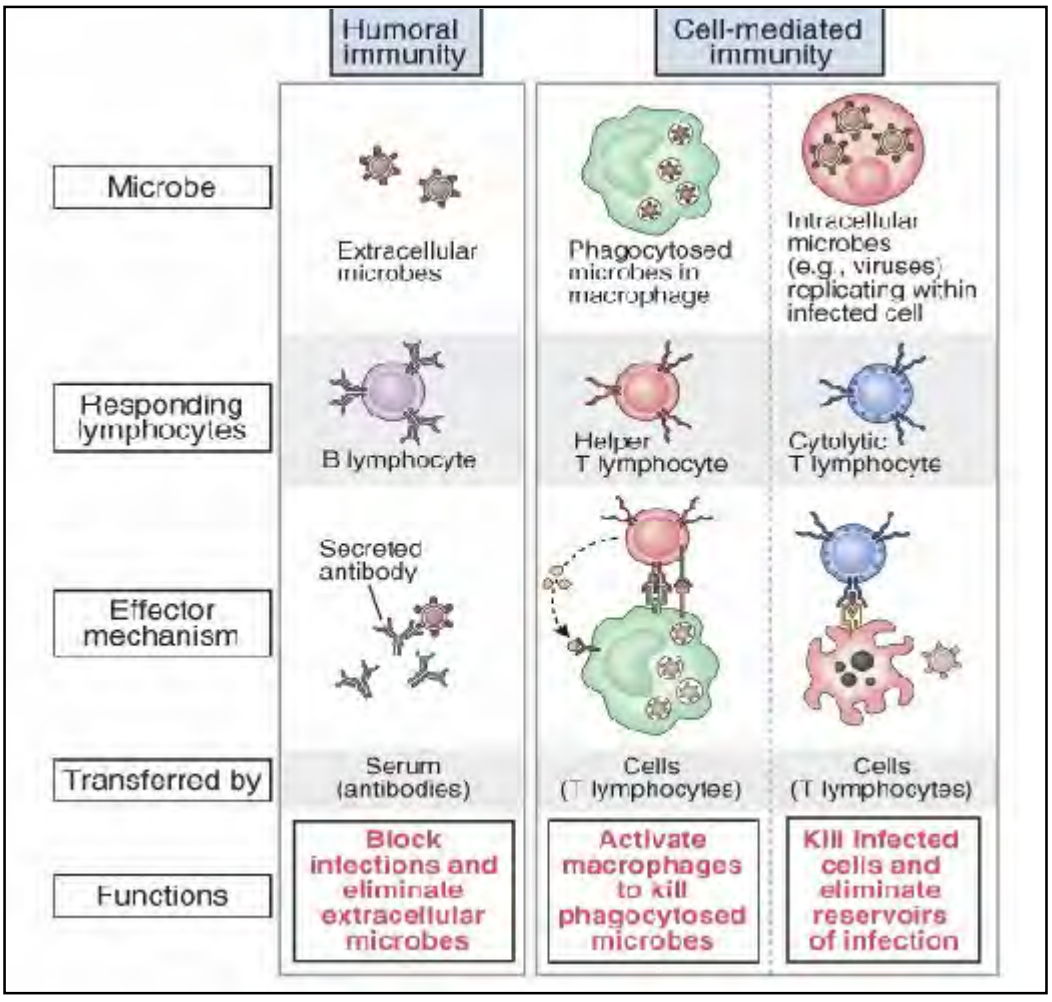

Figure 2.5 Adaptive immunity: Humoral immunity and Cell-mediated immunity. (Available from: http://medic.med.uth.tmc.edu/edprog/immuntbl.htm. [2008, September]

Immunostimulation and cancer

The involvement of the immune system in control of cancer diseases has been support by several evidences. Cancer is detected more frequently in immunodeficiency individuals than healthy ones (Whiteside TL. 2003). Most of common risk factors for cancer, stress, age, poor nutrition, stress, and smoking are also associated with abnormalities in the immune responses (Reiche EMV. *et al.* 2004; Perera FP. 1997; Pawelec G. *et al*. 2002). It has been suggested that immune responses against cancer cells is competent but absence of a strong "danger" signal contributes to the ability of newly forming tumors to avoid recognition by the host immune system. Immune responses against tumor-associated antigens (TAA), whether cellular or humoral, are weak and difficult to measure because TAA are self-antigens. Innate immunity, which should be responsible for early recognition and elimination of cancer cells, may be inefficient in patients who develop malignancy (Matzinger P. 1998). Several mechanisms of tumor evasion have been identified. In general, tumors use two strategies to evade recognition: they either hide from immune cells thus avoiding recognition or they directly interfere or eliminate immune cells. Cancer immunotherapy has been employed one of modalities for cancer treatment which including surgery, irradiation, chemotherapy and immunotherapy. Immunotherapy is used to activate both innate immunity and adaptive immunity in cancer patients (Abbas A. K. and Lichtman A. H. 2005). Adjuvants, including Bacillus Calmette-Guerin (BCG) which is the attenuated strains of *Mycobacterium bovis* called and *Corynebacterium parvuum,* have been used to boost tumor immunity. These adjuvants activate macrophages, increasing their expression of various cytokines, class II MHC molecules, and the B7 co-stimulatory molecule. These activated macrophages are better activators of TH cells, resulting in generalized increases in both humoral and cell-mediated responses. Human recombinant cytokines, including interferon-α (IFN-α) and interleukin-2 (IL-2), are also used in hematologic malignancies and some solid tumor based on their immunostimulatory activities. Several therapeutic antibodies against TAAs have been approved for treatment of many

cancers. These antibodies act as passive immunization against pathogens. They employ several effector mechanisms the same as endogenous antibodies to eliminate tumor. Immunostimulation is also use as the adjuvant in cancer therapy in order to boost immune cells during chemotherapy. Both G-CSF and GM-CSF are clinical used to activate neutrophil production as well as functions for restoring immune response against infection because many chemotherapeutic drugs exhibit bone marrow suppression as a common adverse drug reaction. Poor clinical responses, parental administrations, adverse drug reactions, as well as cost are problems of these immunotherapeutic agents. Development of new agents for alleviating these problems is still needed. Drug discovery from medicinal plant is also one of strategies to solve the problems.

Many medicinal plants exhibit immunomodulatory activities. They have been reported to modulate adaptive immune response, lymphocyte proliferation, antibody production, cytokine secretion, nitric oxide production, phagocytosis, and etc., both *in vitro and in vivo*. These plants contain a wide range of active constituents including isoflavonoids, indoles, phytosterols, sesquiterpenes, alkaloids, glucans, and polysaccharides with immunomodulating activities. These plant products might be used as immunomodulating agents to provide an alternative to expensive immunotherapeutics which are clinically used (Plaeger SF. 2003; and Patwardhan B. 2005). *Acanthus ebracteatus* Vahl., a medicinal plants which contain polysaccharide as one of its constituents, was in focus to evaluate its immunomodulating potential in this study.

Acanthus ebracteatus Vahl.

Acanthus ebracteatus Vahl. is a spiny plant in the family Acanthaceae distributed in the mangrove of Southeast Asia as well as Southern Thailand. It has the common names as sea holly or holly mangrove. The local names in Thai are Ngueak-Pla-Mor, Nang-kreng, Ja-kreng or Gam-mor. It is an erect and shrubby plant, grows up

to 1.5 meters tall, with many stems. Its leaves are dark green, thick, shiny, waxy, may have sharp spines at the end of each deep lobe. Flowers are white, in a cluster at the branch tip. The fruits are shiny green pods in a cluster. Seeds are off-white, and flat (Figure 2.1). This plant consists of various active components including glycosides, triterpinoids and polysaccharides. All parts of this plant have long been used in traditional folk medicine for treatment of various diseases. This plant is used to relieve cough, used to treat hepatitis, lymphoma, asthma and infections, and use for health promotion and longevity. In Thailand, the root and stem of *A. ebracteatus* are used to treat skin disease and cancer, and used for longevity. (Hokputsa S. *et al.* 2004; and Kanchanapoom T. *et al*. 2001).

Various biological activities of *A. ebracteatus* were investigated. The organic extracts were reported to have anti-mutagenic (Rojanapo W. *et al*. 1990) from water root extract and the aqueous extract was reported to inhibit eicosanoid synthesis (Laupattarakasem P. *et al*. 2003). The water extract of *A. ebracteatus* root have been reported to exhibit anti-tumor activity by inhibiting *in vivo* diethylnitrosamine-induce hepatic foci (Tiwawech D. *et al.* 1993), and has weak antitumor activity of against Sarcoma 180 ascites cells *in vivo* (Siripong P. *et al.* 1998) and reduce splenic size and prolong survival time of friend leukemia virus induced erythroleukemia swiss mice (Srivatanakul P. 1981). However, it did not have anti-proliferative effect against two cancer cell lines, human epidermoid carcinoma (KB) and P-388 mouse lymphocytic leukemia (P-388) *in vitro* (Siripong P. *et al.* 1998). It exhibited no chronic toxicity in Wistar rats when 0.06-3 g/kg/day extract was orally administered in these animals for 12 month (Siripong P. *et al.* 2001).

From the previous studies, it has been suggested that the water extract of *A. ebracteatus* might have a indirect anticancer effect *in vivo*. It was very interesting to investigate other activities of this extract that can link to anticancer activity. Therefore, this study was undertaken to assess the immunomodulating activities of the water extract of *A. ebracteatus* (AE) root which may relate to its folklore medicinal property as cancer remedy.

Figure 2.6 *Acanthus ebracteatus* Vahl. (Ngueak-Pla-Mor)

(Available from: http://www.mytho-fleurs.com/images/Fleurs_du_Vietnam/page_01.htm)

[2008, 20 September]

CHAPTER III

MATERIALS AND METHODS

Materials

Plant extract

The roots of *A. ebracteatus* (AE) Vahl. were collected from Samut Songkhram province, Thailand. This plant was identified by Dr. Pongpun Siripong National Cancer Institute, Thailand and compared with authentic specimen at the Forest Herbarium, Royal Forest Department. The voucher specimens have been deposited in the Herbarium of Natural Products Research Section, Research Division, National Cancer Institute, Thailand.

Preparation of the crude aqueous extract

The dried coarsely powder roots of the water extract *A. ebracteatus* Vahl. (AE, 600 gm) was refluxed for 2 hours with distilled water (8 lit) and filtered. The refluxing process was then repeated twice. The combined filtrate was concentrated under reduce pressure and lyophilized. The dark brownish lyophilized residue (140 gm, yield=23.33%) was stored as dry powder until use. It was freshly dissolved in double distilled water to required concentrations when it was used to treat cells.

Cells

The following cells were used in this study;

- Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of 20-35 year-old male (15 subjects) blood donors from National Blood bank, Thai Red Cross Society for Blood Donation with informed consent. The cells were maintained in the completed RPMI 1640 medium at 37 ^{o}C, 97 % humidity, 5% CO_2. They were used within 24 h after isolation.
- J774A.1 cells were murine macrophage-like cells purchased from ATCC. The cells were maintained in the completed DMEM medium at 37 ^{o}C, 97 % humidity, 5% CO_2. They were subcultured 3 timed weekly during use.
- Ramos cells were human Burkitt's lymphoma cells purchased from ATCC. The cells were matintained in the completed RPMI 1640 medium. They were subcultured 3 timed weekly during use.

Equipment and Instruments

The followings equipments and instruments were used in this study; CO_2 incubator (Thermo, USA), sterile laminar flow hood (ESSCO, USA), flow cytometer (Beckman Coulter, USA), centrifuge (Hettich, USA), inverted microscope (Nikon, USA), microplate reader (Labsystem, USA), spectrophotometer (V-530 UV/VIS Jasco, Japan), centrifuge (Hettich, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), analytical balance (GMPH, Satorius (Germany and UMT2, Mettler Toledo, Switzerland), 96 and 24 multi-well plate (Corning, USA), thermocycler machine (Eppendorf, USA), autopipette (Gilson, USA), T25 tissue culture flask (Corning, USA), 24-well tissue culture plate (Corning, USA), 96-well tissue culture plate (Corning, USA), cell scrapper (Greiner, UK), eppeddorf (corning, USA), sterile polypropylene centrifuge tube : 15 ml, 50 ml, T25 tissue culture flask (Corning, USA)

Chemicals and reagents

The following reagents were used in this study: phytohemagglutinin (PHA) (Sigma, USA), monoclonal anti-CD20 antibody (Roche, Switzerland), nitroblue tetrazolium (NBT) (Sigma, USA), lipopolysaccharide (LPS) (Sigma, USA), rezasurin (Sigma, USA), carboxyfluoroscein succinimidyl ester (CFSE) (Dojindo, Japan), propidium iodide (PI) (Santa Cruz, USA), histopaque (Sigma, USA), zymosan A from *Saccharomyces cervisiae*) (Sigma, USA), monoclonal anti-CD20 antibody (Roche, Switzerland), DEPC (Molekula, UK), dimethyl sulfloxide (DMSO) (Sigma, USA), ImProm-IITM Reverse Transcription system (Promega, USA), nitric oxide assay kit (Promega, USA), Taq polymerase (Invitrogen, UK) , trypan blue dye (Sigma, USA), TRiZol reagent (Invitrogen, UK), dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), RPMI 1640 medium (sigma, USA),sodium bicarbonate (Baker, USA)

Mix the blood sample 1:1 with Hanks' balanced salts solution (HBSS) containing 2 µl/ml heparin at room temperature.

Methods

1. Preparation of peripheral blood mononuclears cells

PBMCs were isolated from 10 ml whole blood of healthy male blood donors in the following procedures;

1. Ten Militer of whole blood was collected into a tube containing 1% heparin.
2. Centrifuge at 3200xg, 4C^{o} for 10 minutes.
3. Collect the cells at the Buffy coat layer after removing plasma on the top layer.
4. Dilute the cells with 5 ml incomplete RPMI 1640 medium before slowly layering on the top of 4 ml ficoll solution in steriled 15 ml polystyrene tube.
5. Centrifuge at 400xg for 30 minutes at room temperature.

6. Remove the supernatant and wash the pellet twice with incomplete RPMI1640 medium by centrifugation at 250xg for 10 min at room temperature.
7. Remove the supernatant and resuspend the pellet in 5 ml complete RPMI 1640 medium
8. Determine cell viability by staining with 0.4% trypan blue dye solution at the ratio 1:1 and counting on hemocytometer.
9. Adjust the cells to the required density with complete RPMI 1640 medium.

2. Effect of the water extract on functions of cells in the innate immunity

The effect of the water extract on the innate immunity was investigated by studying its effects on the functions of macrophages (J774A.1 cells) and natural killer (NK) cells in PBMCs as in the following approaches;

2.1 The effect of the water extract on macrophage activation

Effect of the water extract on nitric oxide production, phagocytosis, and expression of iNOS and cytokines was evaluated in murine macrophage like cell, J774A.1.

2.1.1 Effect on NO production

1. Incubate 500 μl/well of $4x10^5$ cells/ml J774A.1 cells in a 24-well plate for 24 hour at 37C°, 97% humidity, 5%CO_2.
2. Remove the supernatant, add 500 μl/well of 31.25-500 μg/ml the water extract, and further incubate for 24 h. Complete DMEM and 100 ng/ml LPS were used as the negative and the positive control.

3. Collect the supernatant for determining NO production in nitrite form by Griess reaction assay, while the cells were used to evaluate the cytotoxicity of the extract on these cells using resazurin reduction assay.

Griess reaction assay (Amano F. and Noda T., 1995)

1. Aliquot 100 μl of the supernatant from each well into 96-well plate
2. Add 20 μl of conc. sulfanilamide solution into each well and incubate for 10 min at room temperature in the dark.
3. Add 20 μl of conc. NED solution to each well and further incubate for 10 min at room temperature in the dark.
4. Measure the absorbance at 540 mm using microplate reader.
5. Determine the amount of nitrite generation, a representative form of NO production, from nitrite standard curve using 1.56-100 μM standard nitrite solution in two-fold dilution.

2.1.2 Effect of the extract on phagocytosis

Zymosan phagocytosis assay was used to evaluate the effect of the extract on phagocytosis activity of J774A.1 cells as in the following procedures (Manosroi A. *et al*. 2003);

1. Incubate 180 μl/well of $4x10^5$ cell/ml J774A.1 cell in of a 96-well plate for 24 h at $37C^o$, 97% humidity, 5%CO_2.
2. Add 20 μl/well the water extract to the final concentrations 31.25-500 μg/ml and incubate for 24 h at $37C^o$, 97% humidity, 5%CO_2. Complete DMEM and 100 ng/ml LPS were used as the negative and the positive control, respectively.
3. Carefully remove supernatant and wash the cells twice with DMEM.

4. Add 800 μg/ml of zymosan and 600 μg/ml of NBT in each well and incubate further for 60 minutes at 37C°, 97% humidity, 5%Co_2.
5. Wash the cells with 200 μl methanol three times.
6. Add 120 μl 2M KOH and 140 μl DMSO.
7. Measure the absorbance at 570 nm using microtiter plate
8. Calculate the percentage of phagocytosis of J774A.1 cells by using the following equation

$$\% \text{ Phagocytosis} = \frac{[OD570nm(sample) - OD570nm(control)]}{OD570nm(control)} \times 100$$

2.1.3 The effect of the water extract on the gene expression of iNOS and cytokine

RT-PCR was used to determine the expression of iNOS and cytokines (TNF-α and IL-1) after the J774A.1 cells were treated with the water extract as the following procedures;

1. Incubate 500 μl/well 4x10^5 cells/ml J774A.1 in a 24-well plate for 24 h at 37C°, 97% humidity, 5%CO_2.
2. Remove the supernatant, add 500 μl/well of 31.25-500 μg/ml the water extract, and incubate for 24 hour at 37C°, 97% humidity, 5%CO_2. Complete DMEM and 100 ng/ml LPS were as the negative and the positive controls, respectively.
3. Remove all the supernatant, leave the treated cells to perform total RNA preparation, cDNA production, and PCR for the expression of TNF-α, Il-1β, and iNOS.

- **Total RNA preparation**

1. Lyses and homogenate the treated cells in each well with 1 ml Trizol® reagent at room temperature for 5 min.

2. Transfer the homogenized samples to eppendorf tubes.
3. Add 200 µl chloroform into each tube, vigorously shake by hand for 15 seconds, incubated at room temperature for 3 min, centrifuge at12,000g for 15 min at 4°C.
4. Carefully collect the supernatants into a fresh eppendorf tubes.
5. Add 0.5 ml isopropanol into each tube and incubate at room temperature for 10 min.
6. Separate the RNA pellets by centrifugation 12,000g for 10 min. at 4 °C.
7. Remove the supernatant and wash the pellets twice with 1 ml of 75% ethanol by centrifugation at 7,500g for 5 min. at 4°C.
8. Air-dry the pellets and dissolve in RNase free-water.
9. Measure the absorbance of the RNA content in each tube at 260 nm by spectrophotometer.
10. Calculated the RNA concentration in each tube by the following formula;

$$RNA(\mu g) = OD_{260} \times 40 \times \text{dilution factor}$$

11. Store the RNA solution at -20°C until use

- **cDNA production by reverse transcription**

1. Preheat 1.5 µg total RNA in each tube with 1 µl oligo dT15 primer in nuclease-free water for 5 min at 70 °C.
2. Immediately chill the tubes on ice for 5 min.
3. Prepare reverse transcription mixture containing; 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor, and reverse transcriptase (Appendix X).
4. Add 15 µl of the mixture into each tube in (2).
5. Incubate the tubes in a thermocycler machine at 25 °C for 5 min, then at 42 °C for 1 hour and 30 min, and finally at 70 °C for 15 min.
6. Store the cDNA samples at -20 °C until use.

Amplification of iNOS and cytokine cDNA by polymerase chain reaction (PCR)

1. Mix 1 µl of cDNA sample in each tube with 24 µl PCR reaction mixture containing primer, mixed dNTP, Taq polymerase in PCR buffer.
2. Perform the PCR in the thermocycler machine using the following conditions; denaturation for 5 sec at 94 ˚C, annealing for 45 sec at 55˚C, extension for 60 sec at 72 ˚C, and final extension for 7 min at 72 ˚C at the end of 30^{th} cycles.
3. Run the 6 µl PCR products mixed with 2 µl loading dye on 1.5% agarose gel electrophoresis at 100 volt for 45 min in TBE buffer.
4. Stain the agarose gel with 0.5 µg/ml ethidium bromide in TBE buffer for 4 min and destained with the TBE buffer for 30 min.
5. Analyze the PCR products using a gel documentation.

2.2 Effect of the water extract on NK cell activity

Antibody-dependent cellular cytotoxicity (ADCC) of NK cells was used to investigate the effect of the water extract on these cells. NK cells in PBMCs were used as effector cells while human Burkitt's lymphoma cells, Ramos cells, were used as target cells. CD20 molecules on Ramos cell surface were also used as the target molecules of anti-CD20 monoclonal antibody, Rituximab, to induce ADCC(Meerten TV. *et al* 2006)

- **Preparation of effector cells** (PBMCs)

1. Incubate 450 µl of $1x10^6$ cell/ml PBMCs in 5 ml tissue culture tubes for 24 h at $37C^{o}$, 97% humidity, 5%CO_2.
2. Add 50 µl/tube of the water extract to the final concentration 250-1000 µg/ml and incubate for 24 hours at $37C^{o}$, 97% humidity, 5%CO_2.

Complete RPMI 1640 was used as the negative control. These treated PBMCs became target cells for ADCC assay.

- **Preparation of target cells (**Ramos cells**)**

1. Stain 9-11x10^6 Ramos cells in 1 ml RPMI 1640 medium with 5μM CFSE in the PBS for 5 min at room temperature in the dark.
2. Stop the staining with 10 ml complete RPMI medium, wash the cells twice with the complete medium by centrifugation at 250g for 10 min, and adjust the cell to 2x10^5 cells/ml with the medium.
3. Pipette 450 μl of the stained cells into 5 ml test tubes.
4. Add 50 μl/tube of 10 μg/ml anti CD-20 antibody (Rituximab) and incubate at 37 ^{o}C, 97% humidity, 5% CO_2 for 1 hours. Complete RPMI 1640 was use as the negative control.

Antibody-dependent cellular cytotoxicity (ADCC) assay

1. Incubate Labeled target cells were incubated with effectors (E:T ratio of 10:1 in duplicate for 4 hr at 37 ^{o}C with 5% CO_2 for 4 hours.
2. After incubation, centrifuge the tubes at 2200 rpm for 10 minutes at room temperature.
3. Add 100 μl of PBS and 5 μl of PI (ug/ml), the sample were incubated at roomtemp for 15 min.
4. Add PBS 400 μl and analyzed immediately by Flowcytometer.
5. The percentage of % cytotoxicity was calculate by the following equation

$$\% \text{ Cytotoxicity} = \left(\frac{\text{Number of dead tar get cells}}{\text{Number total target cells}} \right) \times 100$$

3. Mitogenic activity of the water extract on PBMCs

The mitogenic effect of the water extract from A. *ebracteatus* root was determine by resazurin reduction and CFSE staining assays.

Resazurin reduction assay

1. Incubate 90 µl/well of $1x10^6$ cell/ml PBMCs in a 96-well plate for 24 h at $37C^o$, 97% humidity, 5% CO_2.
2. Add 10 µl of 250-1000 µg/ml the water extract into each well. Ten µl of complete RPMI medium and 5µg/ml PHA were used as the negative and positive control respectively.
3. Incubate for 72 hour at $37C^o$, 97% humidity, 5% CO_2.
4. Add 5 µg/ml of 10 mg/ml resazurin solution into each well and further incubate for 10 hour at $37C^o$, 97% humidity, 5% CO_2.
5. Measure the absorbance at 570 nm and 600 nm using a microplate reader.
6. Calculate the percentage of stimulation of the extract compare to the solvent control by using the following formula;

$$\% \text{ Stimulation} = \frac{[OD(\text{sample}) - OD(\text{control})]}{OD(\text{control})} \times 100$$

CFSE staining assay

1. Stain 1ml of $10x10^6$ PBMCs in incomplete RPMI 1640 medium with 5µM CFSE in PBS for 5 min at room temperature in the dark.
2. Stop the staining with 9 ml complete RPMI 1640 medium and wash the cells twice with 10 ml complete RPMI medium by centrifugation at 250xg for 10 min at room temperature

3. Incubate 450 µl/well of $1x10^6$ cells/ml CFSE-stained PMBCs in complete RPMI 1640 medium in a 24 well-plate for 24 h at 37C°, 97% humidity, 5% CO_2.
4. Add 50 µl/well the water extract to the final concentrations 250-1000 µg/ml and further incubate for 72 h. Fifty Microliter of complete RPMI medium and 5µg/ml PHA
   were used as the negative and positive control respectively.
5. Remove the supernatant after centrifugation at 250g for 10 min.
6. Resuspend the cells in PBS and analyze fluorescent intensity of CFSE in the cells using flow cytometer.
7. Determine the proliferation of the cells from histogram compare to the negative and the positive control.

Statistical analysis

Data were presented as mean plus or minus standard error (mean ± S.E.). Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 17. Any *p*-value < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

4.1 Effects of the water extract from *A. ebracteatus* root on macrophage functions

4.1.1 Effect on nitric oxide production

Nitric oxide production is the other parameter used to determine the effect of the water extract on macrophage functions. Activated macrophages express iNOS which catalyzes L-arginine to large amount of NO. Once release from the cells, labile NO turns to nitrate or nitrite in the cell culture medium in vitro. In this study, only the amount of nitrite in the medium was use as the representative of NO concentration. Nitrite was determined by Griess reactions. J774A.1 cells were treated with 31.25-500 μg/ml the extract for 24 h. The amount of NO production was determined from the supernatant by Griess reaction.

The water extract significantly increased NO production in J774A.1 cells after 24 h exposure. The concentration response curve demonstrated that the extract activated the NO production in a concentration manner (Fig. 4.1). It increased the concentrations of NO (nitrite concentrations) to 4.3, 13.51, 20.32, 25.51 and 27.09 μM at the concentrations of 31.25, 62.50, 125, 250 and 500 μg/ml, respectively. The amount of nitrite was undetectable in the untreated J774A.1 while 100 ng/ml LPS activated the NO production of the cells up to 34.61 μM.

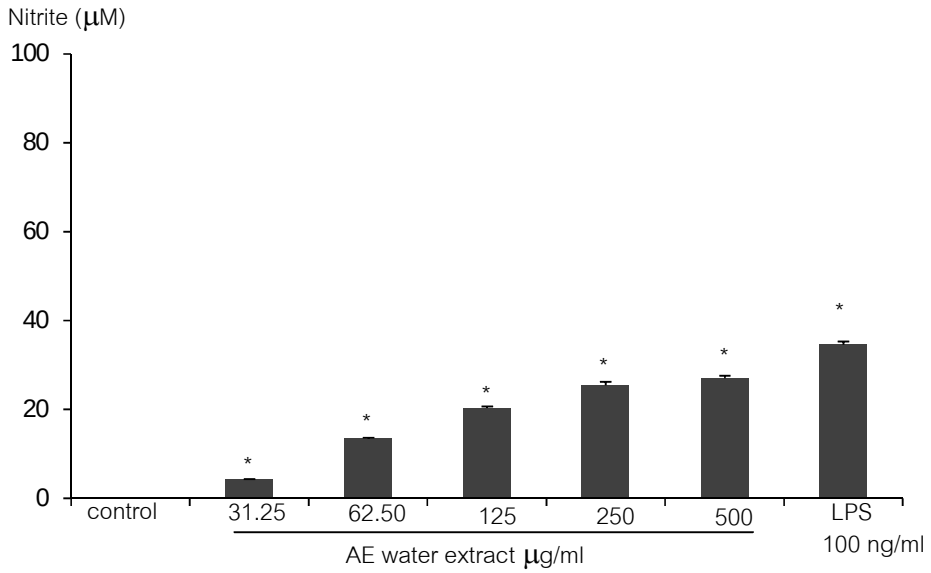

Figure 4.1: Effect of the water extract *A. ebracteatus* root on NO production from J774A.1 cells (n=3).

Cells were treated with 31.25-500 μg/ml extract for 24 hours and nitrite (NO_2) in the medium was evaluated as amount of NO production by Griess reaction assay. The data is expressed as mean ± S.E. of three independent experiments (n=3). * $p < 0.05$ compare to the untreated control.

4.1.2 Effect on phagocytosis

Effect of the AE extract on macrophage phagocytosis was determined by zymosan assay using J774A.1 cells. Nitroblue tetrazolium (NBT) mixed with zymosan was used as a dye marker for colorimetric detection of zymosan engulfment and 100 ng/ml LPS was used as the positive control. J774A.1 cells were treated with 31.25-500 μg/ml the AE extract for 24 h, then activated with zymosan and NBT for 1 h, and finally washed and lyses for detecting formazan product of NBT. The results demonstrate that 62.5, 125, 250 and 500 μg/ml of water extracts significantly activate phagocytosis activity of J774A.1. The AE extract at the lowest concentration, 31.25 μg/ml, also potentially activated the phagocytosis (Fig. 4.2). It activated the zymosan-NBT phagocytosis of J774A.1 cells to 16.08%, 29.91%, 34.70%, 47.43%, and 43.90% from the untreated control at the concentration of 31.25, 62.50, 125, 250 and 500 μg/ml, respectively. This effect of the AE extract was not as potent as 100 ng/ml LPS which activated the phagocytic activity to 51.23%. The concentration-response curve revealed that the maximum effect of the extract was at 250 μg/ml.

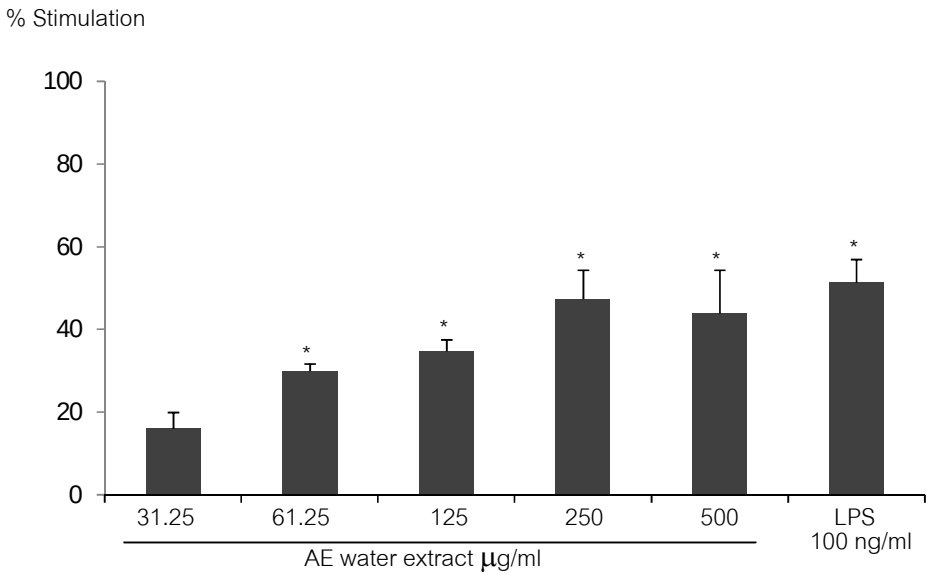

Figure 4.2: Effect of the water extract of *A. ebracteatus* root on phagocytotic activity of J774A.1 cells (n=3).

Cells were treated with 31.25-500 μg/ml of extract for 24 hours. The treated cells were determined for their phagocytic activity by zymosan and NBT assay. The percentage of phagocytosis stimulation was calculated from NBT reduction in the treated cells compare to the untreated control. The data is expressed as mean ± S.E. of three independent experiments (n=3).

*$p < 0.05$ compare to the untreated control.

4.1.3 Effect on the expression of iNOS and cytokines

The activation of the AE extract on the NO production in J774A.1 was confirmed by determining the expression of iNOS, the enzyme mentioned above. The cells were treated with 31.25-500 μg/ml the extract for 24 h. The total RNA was prepared from the treated cells and subjected to reverse transcription to cDNA for amplifying iNOS transcript by PCR. The AE extract from *A. ebracteatus* root, at all concentrations used in the study, induced the mRNA expression of iNOS (Fig. 4.3). Semi-quantitation of PCR product by gel documentation revealed that the AE extract increased the ratio of iNOS to β-actin gene expression to be 0.69, 0.84, 0.94, 0.97 and 1.00 at concentrations of 31.25, 62.5, 125, 250 and 500 μg/ml, respectively.There was no iNOS expression in the untreated cells while the LPS-treated cells also overexpressed iNOS with the ratio 0.884. This result was correlated with the activating effect of the extract on the NO production described before.

The mRNA expression of two cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), was also used to confirm the stimulatory activity of the extract on macrophage functions. These cytokines are known as markers of activated macrophages. J774A.1 cells were treated with 31.25-500 μg/ml the extract for 4 h before their total RNA being subjected to RT-PCR for amplifying their transcripts. The extract significantly activated the expression of IL-1 even at the lowest concentration used in the study after 4 h of treatment. It induced the ratio of IL-1 to β-actin gene expression to 1.08, 1.06, 1.01, 1.13 and 0.92 at concentrations of 31.25, 62.5, 125, 250 and 500 μg/ml, respectively (Fig.4.4). The AE extract also activated the mRNA expression of in similar pattern to TNF-α. It is clearly activated the expression at all concentrations of it, even though the statistically significance was observed only at 250 and 500 μg/ml (Fig.5). The expression of both IL-1 and TNF-α demonstrated that the AE extract from *A. ebracteatus* root activated macrophages.

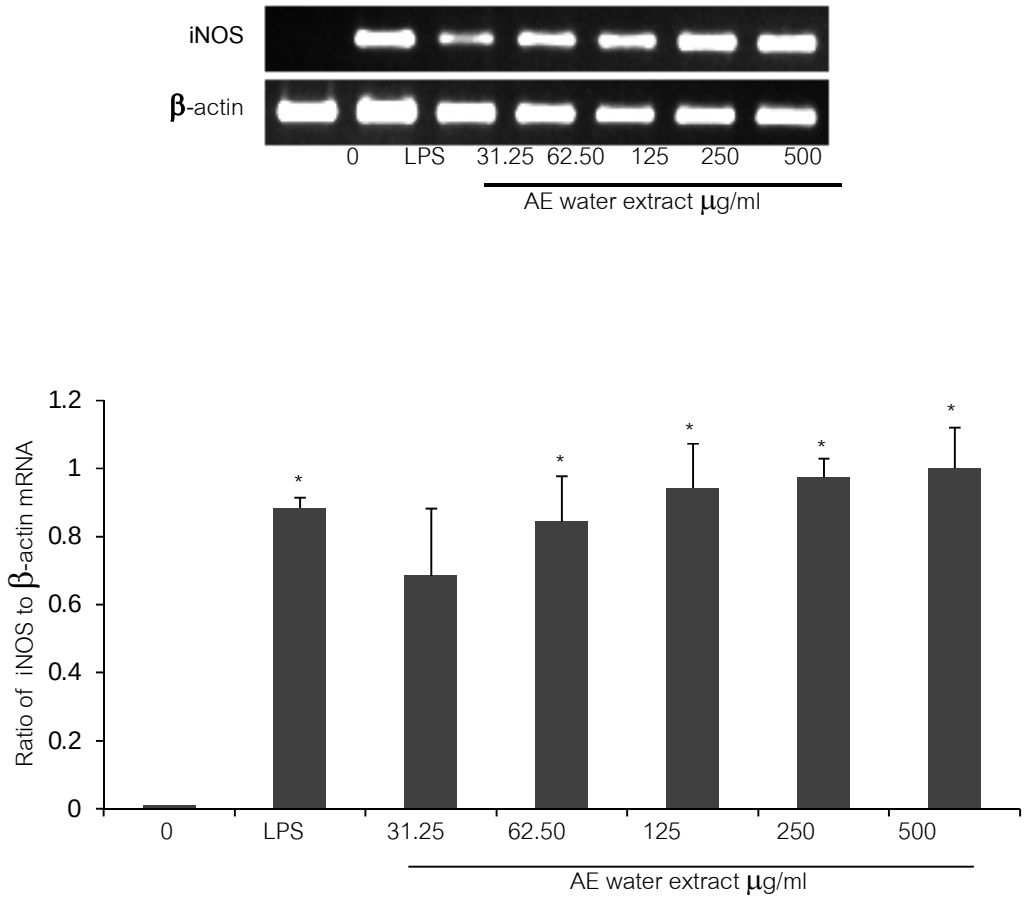

Figure4.3: Effect of the water extract of *A. ebracteatus* root on the mRNA expression of iNOS in J774A.1 cells (n=2).

Cells were treated with 31.25-500 μg/ml for 24 h. The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

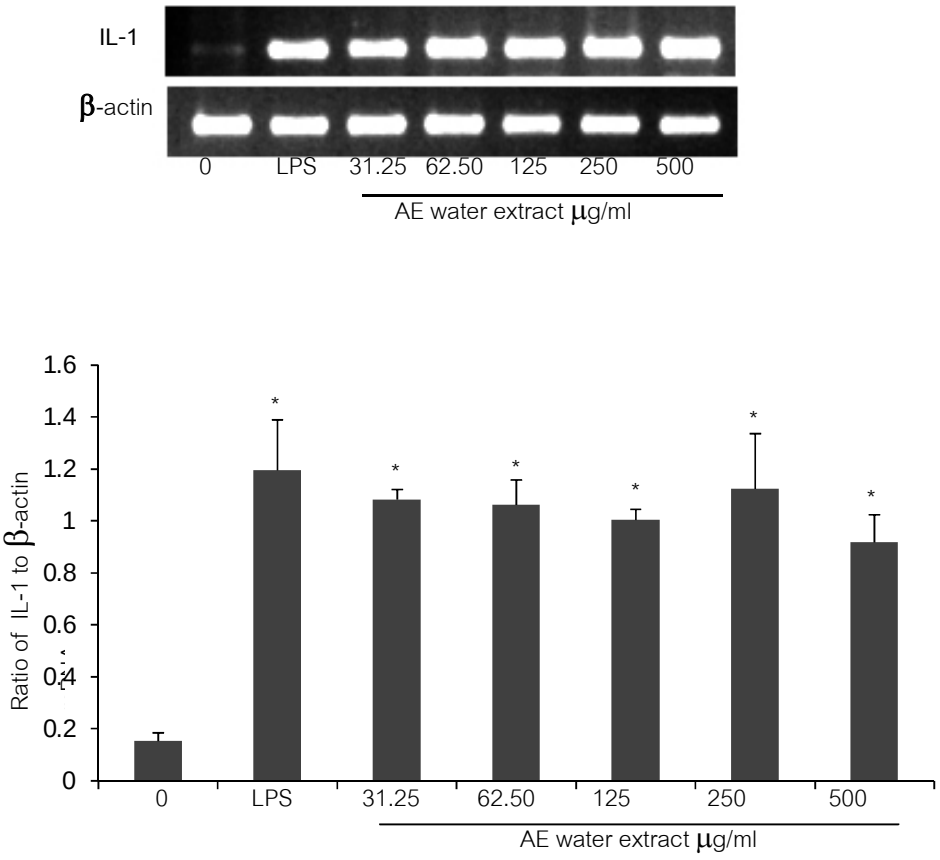

Figure 4.4: Effect of the water extract of *A. ebracteatus* root on the mRNA expression of IL-1 in J774A.1 cells (n=2).

Cells were treated with 31.25-500 μg/ml for 6 h. The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

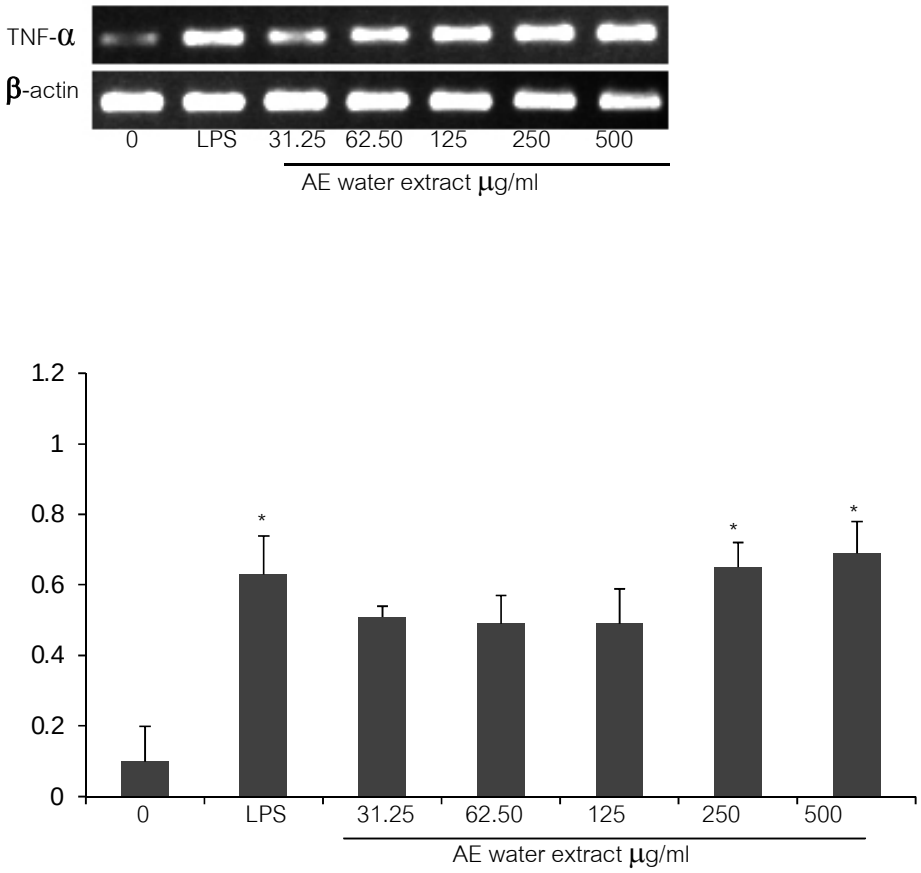

Figure4.5: Effect of the water extract of *A. ebracteatus* root on the mRNA expression of TNF-α inJ774A.1 cells (n=2).

Cells were treated with 31.25-500 μg/ml for 6 h. The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

4.2 Effect of the extract on NK cell function

It was clearly revealed that the AE extract could activate macrophage functions including NO production and iNOS expression, phagocytosis, as well as the expression of TNF-α and IL-1. Effect of the extract on the function of NK cells,the other important cells in the innate immunity, was also evaluated. Activated NK cells can kill target cells by either antibody-dependent or -independent cellular cytotoxicity (ADCC or non-ADCC). Only the ADCC was determined in this work by using NK in human PBMCs as effector cells, anti-human CD20 antibody as antibody to initiate the reaction, and human B cell lymphoma cells, Ramos cells, as target cells which express CD20 molecules on their cell surface. The PBMCs containing NK cells were treated with the water extract for 24 h, while the effector cells or Ramos cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFDA SE) for separating from PBMCs when monitored by flow cytometer and then incubated with anti-CD20 antibody. Both the extract-treated PBMCs and the CFDA SE-stained Ramos cells treated with anti-CD20 antibody were co-incubated at the ratio 10:1 (effector: target ratio) for 4 h followed by PI staining and flow cytometer monitoring. The cytotoxicity of target cells were detected as the $CSFE^{+}/PI^{+}$ cells. A representative data from flow cytometer was demonstrated in Fig.4.6. Only cells in the upper right quadrant were counted as target cell death. The water extract, at all concentrations used, didn't have any potentiating effect on ADCC leaded by anti-CD20 antibody (Fig.4.7). The antibody activated ADCC at the same degree in both conditions, with and without the extract. Effect of the extract on non-ADCC activity of NK cells is still need to be elucidated before conclusion.

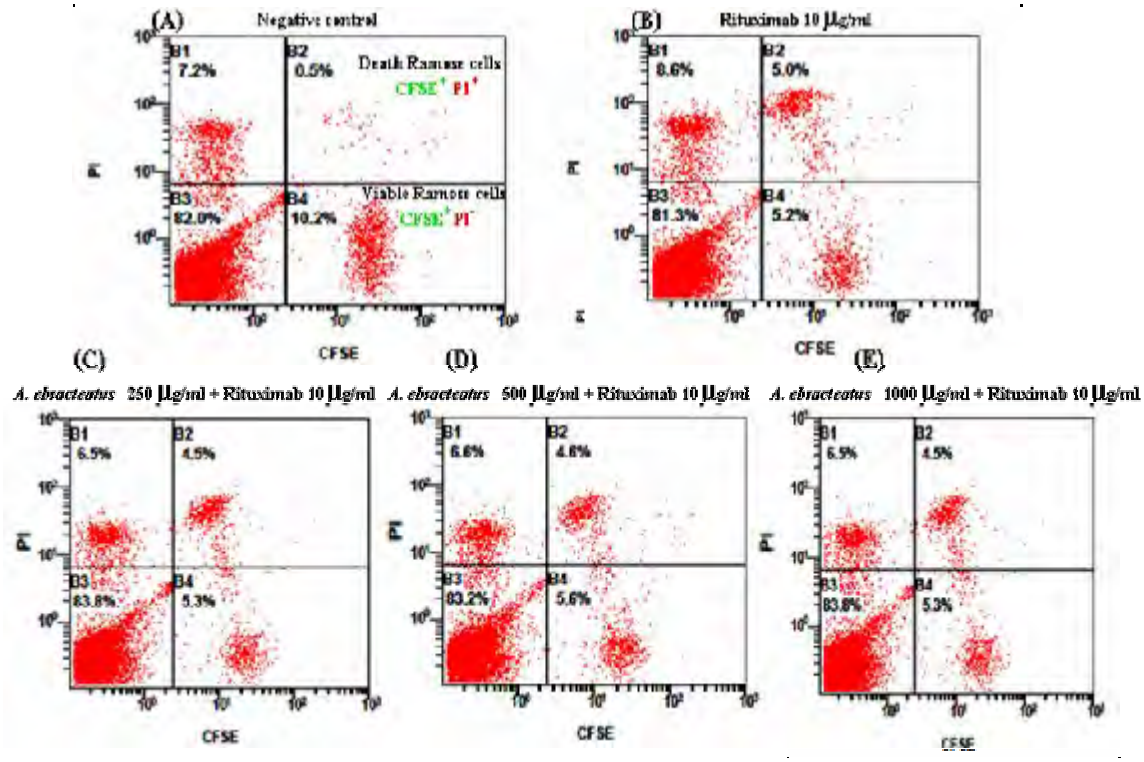

Figure 4.6: Representative histograms of determination of NK cell cytotoxic function against CFSD SE-labeled target cells.

Human PBMCs (effector cells) were treated with the extract (250, 500 and 1000 µg/ml) for 24 hours and mixed with CFDA SE-labeled Ramos cells (target cells) treated with anti-CD20 antibody (ratio 10:1) for 4 hours. The cytotoxicity of the target cells was determined by PI staining and monitored with flow cytometer.

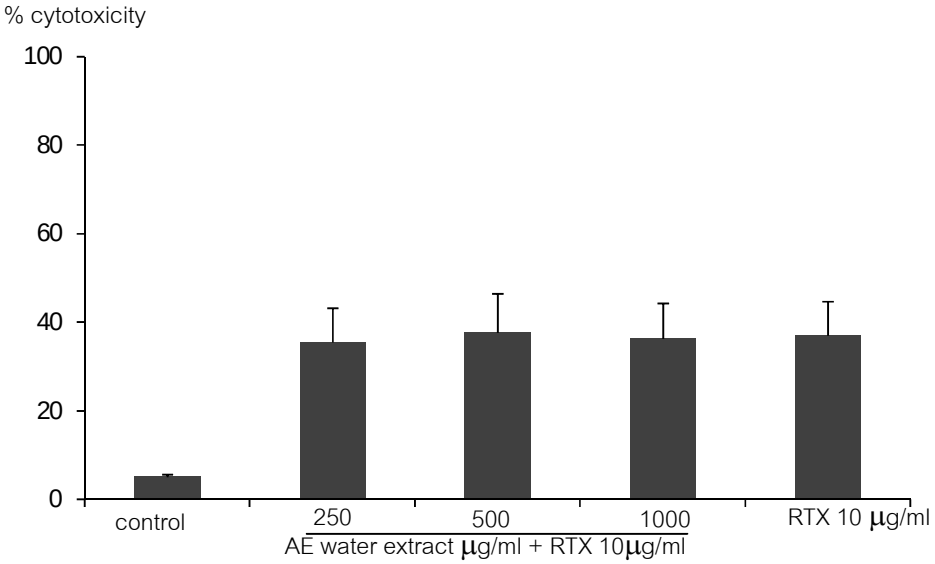

Figure 4.7: Effect of the water extract of *A. ebracteatus* root on NK cell cytotoxic function via ADCC (n=5).

Human PBMCs (effector cells) were treated with 100, 500 and 1000 μg/ml AE extract for 24 hours and mixed with CFDA SE-labeled Ramos cells (target cells) treated with anti-CD20 antibody (ratio 10:1) for 4 hours. The cytotoxicity of the target cells was determined by PI staining and monitored with flow cytometer. The data is expressed as mean ± S.E. of five independent experiments.

4.3 Mitogenic activity of the AE extract

Effect of the AE extract of *A. ebracteatus* root on human PBMC lymphocyte proliferation was used to evaluate the mitogenic activity of the extract. The mitogenic activity of the extract was studies by rezasurin reduction assay and CFSE staining assay.

Human PBMCs were treated with 250, 500 and 1000 µg/ml of AE extract for 72 h. PHA at 5µg/ml, was used as the positive control. The treated viable cells were measured by staining with rezasurin, the redox dye which is reduced from blue rezasurin to pink resorufin by mitochondrial reductase in viable cells. The water extract slightly increased viable PBMCs when compared to the untreated cells. Its activation was only 10.51% at 1000 µg/ml while 5µg/ml could activate cell proliferation to 35.97% (Fig.4.8). The mitogenic effect of the extract was very weak.

The very weak mitogenic effect of the AE extract was confirmed by CFSE staining assay. Human PBMCs were stained with CFDA SE and left overnight. The stained cells were treated with 250, 500 and 1000 µg/ml the water extract or 5µg/ml PHA for 72 h before monitoring the intensity of fluorescent product, CFSE, by flow cytometer. The histograms from the assay revealed that the extract did not cause any sequential halving of fluorescent vital dye which is indicated as cell proliferation as seen in the histogram of PHA treated cells (Fig. 4.9).

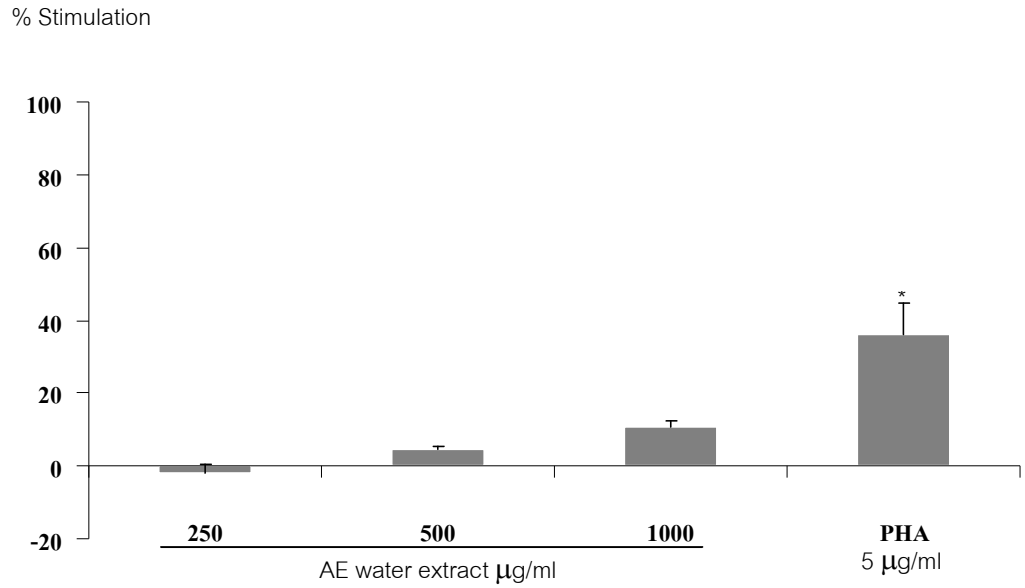

Figure 4.8: Mitogenic effect of the water extract of *A. ebracteatus* root on human PBMCs (n=5).

Cells were treated with 250, 500 and 1000 μg/ml AE extract for 72 hours. Viability of the treated cells was determined by resazurin assay. Percentage of stimulation was calculated by comparing to the untreated cells. The data is expressed as mean ± S.E. of five independent experiments. Five μg/ml PHA was used as a mitogen control. *$p<0.05$ compared to the untreated control.

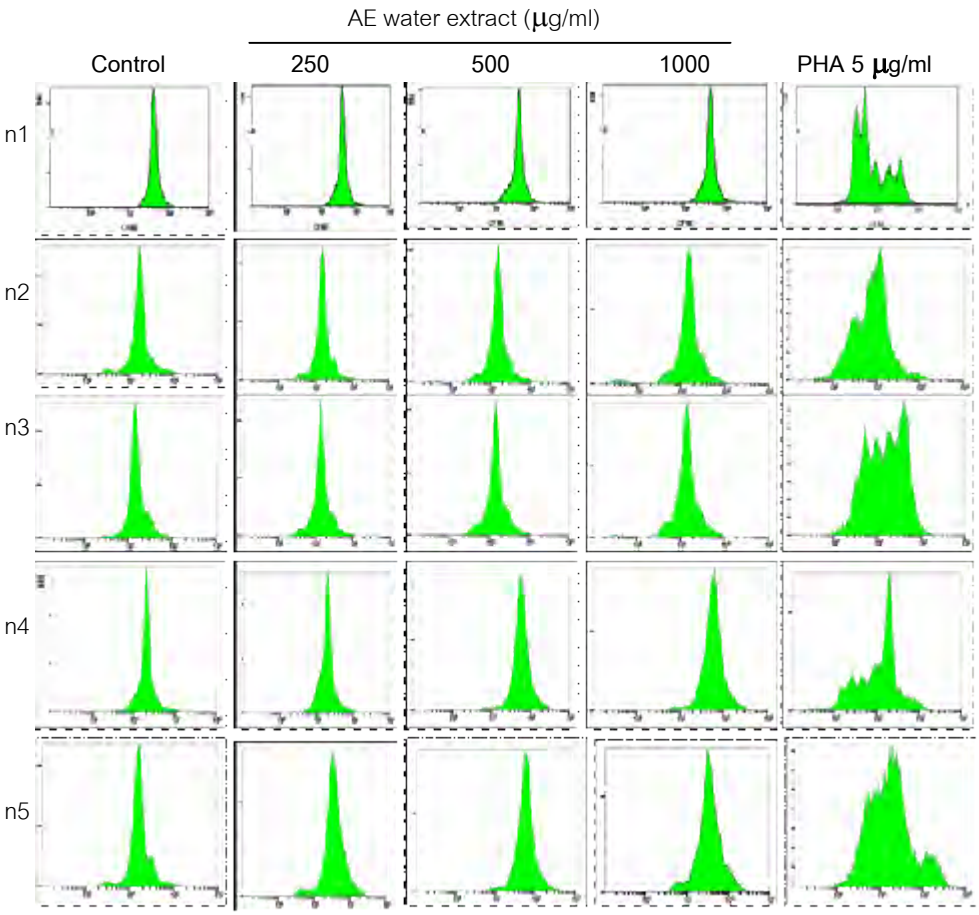

Figure 4.9: Mitogenic activity of the water extract of *A. ebracteatus* root on human PBMCs (n=5).

CFDA SE-labeled PBMCs were treated with 250, 500 and 1000 μg/ml AE extract for 72 hours. Proliferation of the treated cells were monitored by the series of halving fluorescent intensity in labeled cells using flow cytometer.

CHAPTER V

DISCUSSION AND CONCLUSION

It has been previously investigated both *in vivo* and *in vitro* that *Acanthus ebracteatus* exhibited antibacterial and antimutagenic activities, inhibited prostaglandin synthesis, and potentiated complement activity. Many constituents from each part of this plant have been identified. Sulphur, stigmasterol and β-sitosterol, lupeol, and polysaccharide were isolated from *A. erbacteatus* root. Polysaccharide have identified as one of active constituents of stem part. Megastigmane, aliphatic alcohol, benzoxazinoid glycosides and polysaccharides have been isolated from aerial part. Recently polysaccharides from microbial and botanical sources have been reported to exhibit a wide range of therapeutic properties as immunomodulators, anticancer, anti-inflammation and anti-microbial actions (Jae *et al*., 2002; Jue *et al*., 2008: 266-271; Xiaoming *et al*., 2008: 252-256; Mohammad saeem., 2009,55). It has been suggested that these activities of many polysaccharides may occur via macrophage activation (Chihara, 1992; Wang et al., 1997; Beutler, 2004). This study intended to investigate *in vitro* immunomodulatory activities of the water extract of *Acanthus ebracteatus* root. Effects of the extract on macrophage and NK cell activation, as well as proliferation activity on lymphocytes were evaluated *in vitro*.

Activated macrophages play crucial roles in host defense against microbes, death cells and cancer cells. They function as phagocytes to eliminate particulate foreign antigens, as antigen presenting cells to activate adaptive immune response, and as the sources of a diversity of cytokines, free radicals and other mediators to modulate immune responses as well as inflammatory reactions (Birk *et al*., 2001; Lingen, 2001, Klimp, *et al*., 2002). The results in this study demonstrated that the water extract of *A. ebracteatus* root activated macrophage functions. It increased phagocytic activity of murine macrophages, J774A.1 cells. The phagocytosis of macrophages is an essential process in host defense system for eliminating microbes, apoptotic cells and cancer

cells and for digesting microbes into antigenic peptides for T cell recognition (Blander JM., et al., 2004). Several mediators involved in immune response are also expressed when macrophages are activated, for example; iNOS, the enzyme that play role in NO production and several cytokines such as proinflammtory cytokines, TNF-α, IL-1, IL-6, and IL-8. It was revealed in the study that the AE extract significantly increased NO production in J777A.1 cells. It's effect of the AE extract was correlated to the increase in mRNA expression of iNOS, the enzyme involves in NO synthesis. NO is one of essential mediators in activated macrophages that plays role in degradation of ingested microbes or cancer cells in phagolysosomes (Abbas and Lichtman, 2005). The potentiating effect of the AE extract on macrophage was confirmed by detecting the expression of cytokines, TNF-α and IL-1. Results in this study showed that the AE extract activated J774A.1 to express the mRNA of both cytokines. These cytokines are expressed and produced only in activated macrophages. TNF-α has several properties as well as antitumor and immunomodulatory activities. It binds to TNF receptors, which are death receptors, on tumor cells and induces the extrinsic pathway of caspase activation followed by tumor cell apoptotic induction. It also increases the expression of MHC molecules on tumor cells thus increases the immune response against these cells (Terlikowski, 2002; Asano, 1997; Kim, et al.,2004). IL-1 plays roles in inflammatory response and activate adaptive immunity (Abbas and Lichtman, 2005). These results demonstrated that the water extract activates macrophage functions that play roles in eliminating foreign antigens.

Activated NK cells are also the other immune cell in innate immunity that plays major roles in killing viral infected cells and tumor cells. They are innate effector lymphocytes, functionally distinct from B and T lymphocytes in target cell recognition. They can recognize target cells both by antibody-dependent (ADCC) and –independent (non ADCC) pathways. Both pathways leads to NK cell activation and subsequently destruction of target cells. Activated NK cells kill targets mainly by two mechanisms, perforin/granzyme-dependent cytotoxicity and death receptor pathways (Abbas and

Lichtman, 2005). The results in this study demonstrated that the water extract did not activate NK cell via ADCC pathway. Activation by non ADCC pathway should be also investigated in the future.

Mitogen are non-specific polyclonal activators of lymphocytes. They induce polyclonal lymphocyte proliferation. A number of common mitogens are lectins such as concavalin A, pokeweed mitogen and phytohemagglutinin. They bind to specific carbohydrate groups on the surface of the lymphocytes and cause activation. The water extract exhibits very weak mitogenic activity on human lymphocyte proliferation in this study.

In summary, the results in this study reveal the immunostimulating activities of the water extract of *Acanthus ebracteatus* root, especially on macrophages which plays several significant roles on host defense against a vast array of pathogens as well as cancer cells. This study provides preliminary information for further investigation of the immunostimulating effects of *Acanthus ebracteatus*. Effect of the water extract of *A. ebracteatus* root on macrophage activation may contribute to immunomodulation, anti-microbial activity, and anti-cancer effect. Other immune cells and immune components may also be modulated by this extract. Future *in vitro* and *in vivo* studies are necessary to identify the active constituents that exhibit immunostimulating activities, and to analyze the cellular and molecular mechanisms of the immunomodulating effect of the extract.

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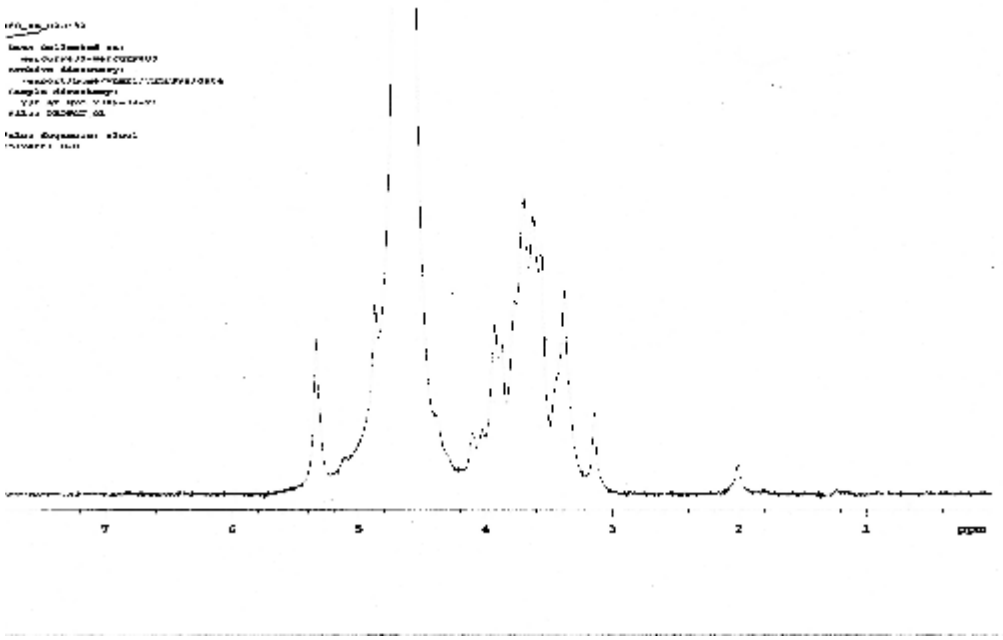
APPENDICES

Appendix A



Appendix A : NMR-spectrum of the water extract of *A. ebracteatus* root.

APPENDIX B

EXPERIMENTS RESULTS

Table A Effect of the water extract *A. ebracteatus* root on nitric oxide production release from J774A.1 cells by Griess reaction (n=3).

Sample	concentration (μg/ml)	NO_2 (μM)
A. ebracteatus	31.25	4.30 ±0.12*
	62.50	3.51±0.25*
	125	20.32±0.44*
	250	25.51±0.74*
	500	27.09±0.58*
LPS	100 ng/ml	34.61±0.78*

Figure1. Representative of NO_2 standard curve of J774a.1 cells by Griess reaction.

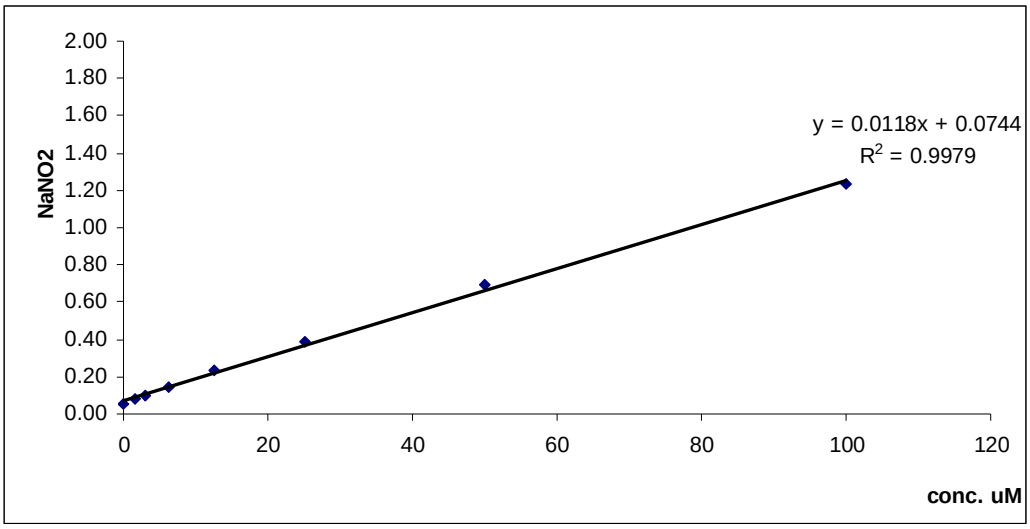


Table B Effect of the water extract *A. ebracteatus* root on phagocytosis of J774A.1 cells by phagocytosis zymosan assay.

The results were expressed as the percentage of stimulation over untreated control (mean±S.E.M.) ,(n=3). *P<0.05 compared with the untreated control.

Sample	concentration (μg/ml)	% stimulation
A. ebracteatus	31.25	16.08 ±3.88
	62.50	29.91±1.75*
	125	34.70±2.77*
	250	47.43±6.97*
	500	43.90±10.45*
LPS	100 ng/ml	51.23±5.74*

Table C Effect of the water extract *A. ebracteatus* root on macrophage iNOS, IL-1 and TNF-α expression.

The results were expressed as bane intensity analysed using Quality One 1-D analysis software, and the level of different sample was assessed relative to β-actin value are the mean±S.E.M (n=2). P* , 0.05 vs control.

Sample	concentration (μg/ml)	Ratio of iNOS, IL-1,TNF-α to β-actin mRNA		
		iNOS	IL-1	TNF-α
A. ebracteatus	31.25	0.69±0.19	1.08±0.03*	0.51±0.03
	62.50	0.84±0.13*	1.06±0.09*	0.49±0.08
	125	0.94±0.13*	1.00±0.04*	0.49±0.10
	250	0.97±0.05*	1.12±0.2*	0.65±0.07*
	500	1.00±0.12*	0.92±0.1*	0.69±0.09*
LPS	100 ng/ml	0.88±0.03*	1.19±0.19*	0.63±0.11

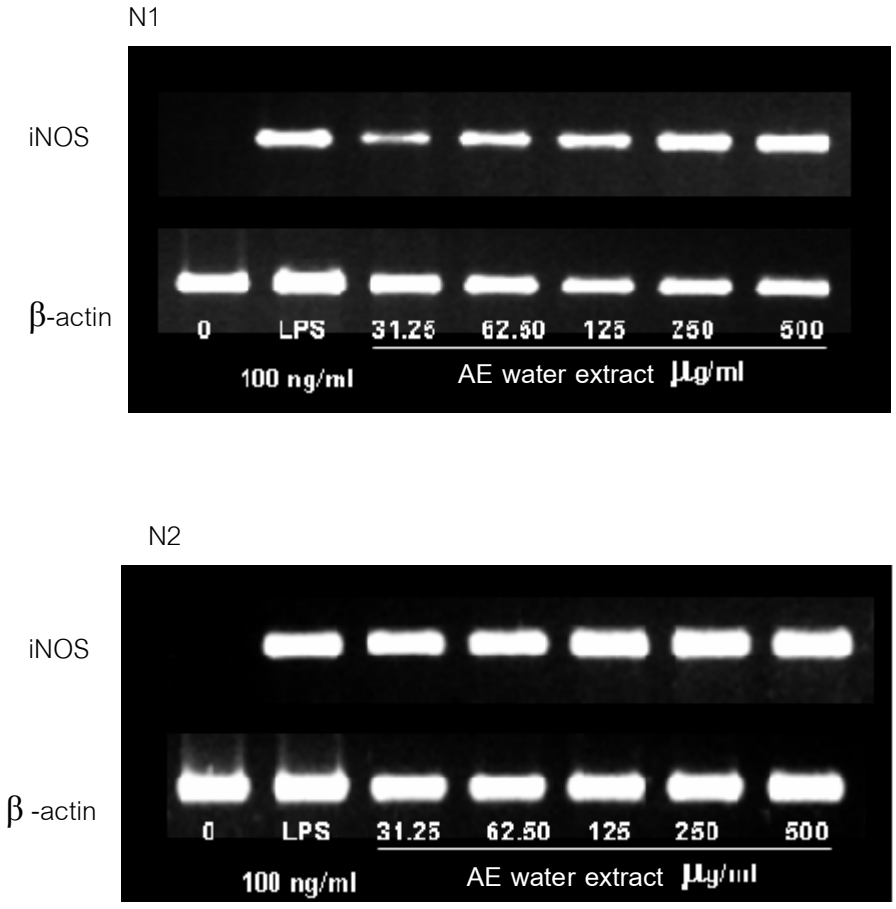


Figure2. Effect of the water extract *A. ebracteatus* root on the mRNA expression of iNOS in J774A.1 cells (n1,n2).

Cells were treated with 31.25-500 μg/ml for 24 h. The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

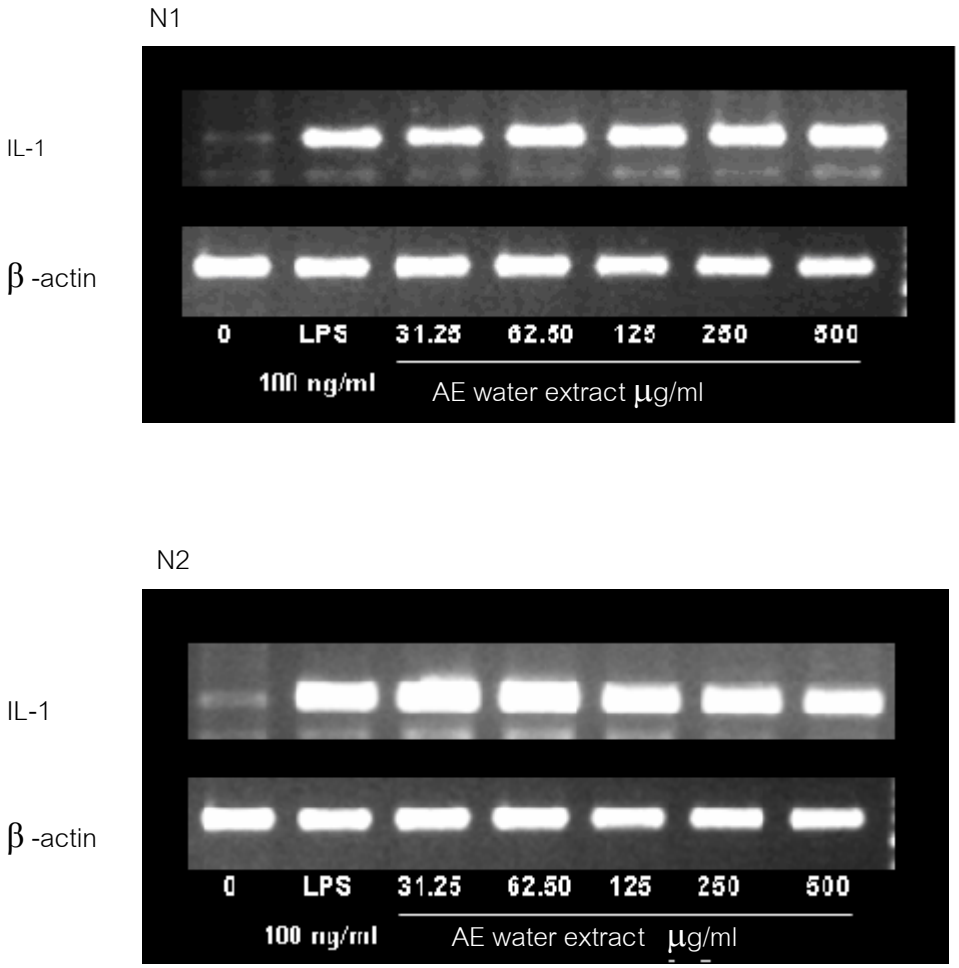


Figure3: Effect of the water extract *A. ebracteatus* root on the mRNA expression of IL-1 in J774A.1 cells(n1,n2).

Cells were treated with 31.25-500 μg/ml for 6 h. The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

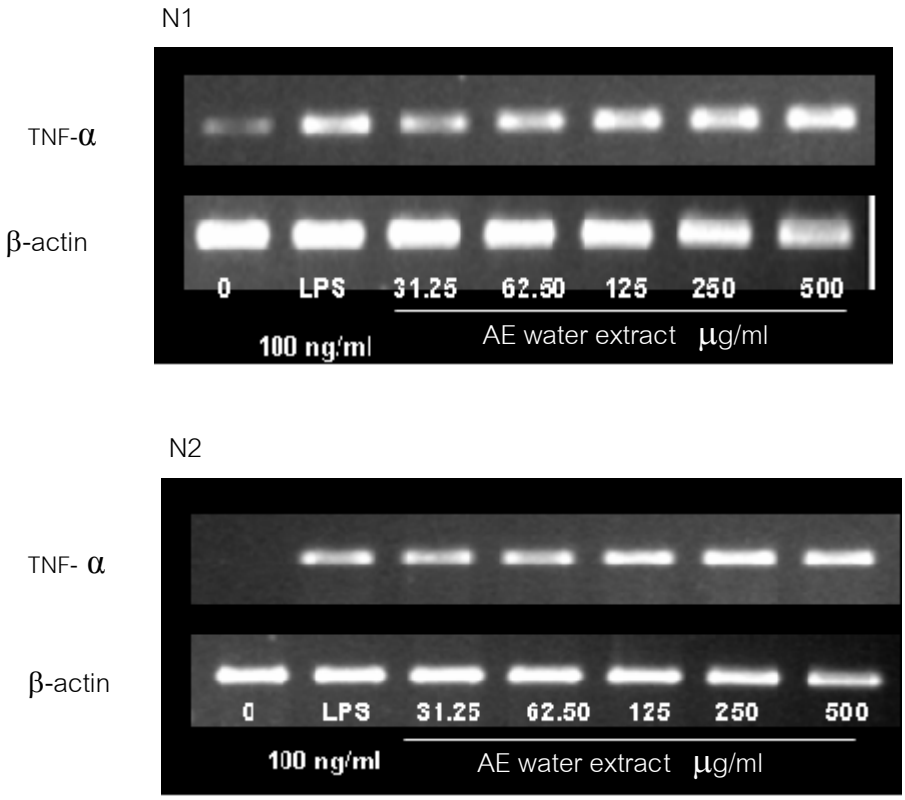


Figure 4. Effect of the water extract *A. ebracteatus* root on the mRNA expression of TNF-α in J774A.1 cells(n1,n2).

Cells were treated with 31.25-500 μg/ml for 6 h.The total RNA of treated cells was isolated, reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. The data is expressed as mean ± S.E. of two independent experiments. *$p<0.05$ compared to the untreated control.

Table D Effect of the water extract *A. ebracteatus* root on NK cell activity by ADCC (n=5).

Sample	concentration (μg/ml)	% cytotoxic
A. ebracteatus	100	35.48 ±7.77
	500	37.64±8.84
	1000	36.27±7.97
Rituximab	10 μg/ml	36.94±7.70

Table E Effect of the water extract *A. ebracteatus* root on human PBMCs proliferation by resazurin assay.

The results were expressed as the percentage of stimulation over untreated control (mean±S.E.M.) , (n=5). *P<0.05 compared with the untreated control.

Sample	concentration (μg/ml)	% stimulation
A. ebracteatus	250	-1.98 ±2.35
	500	2.57±2.39
	1000	10.51±1.75
PHA	5	35.97±8.71*

BIOGRAPHY

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