ฤทธิ์กระตุ้นระบบภูมิคุ้มกันของสารสกัดสมุนไพรไทยบางชนิด

นางสาว ชมพูนุท บุญอากาศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชเคมี ภาควิชาชีวเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-5568-6

IMMUNOSTIMULATION OF SOME THAI MEDICINAL PLANT EXTRACTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biomedicinal Chemistry
Department of Biochemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2003
ISBN 974-17-5568-6

Thesis Title	IMMUNOSTIMULATION OF SOME THAI MEDICINAL PLANT EXTRACTS
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Field of study	Biomedicinal Chemistry
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ชมพูนุท บุญอากาศ: ฤทธิ์กระตุ้นระบบภูมิคุ้มกันของสารสกัดสมุนไพรไทยบางชนิด.
IMMUNOSTIMULATION OF SOME THAI MEDICINAL PLANT EXTRACTS.
อาจารย์ที่ปรึกษา: รศ.ดร. ธิติมา เพ็งสุภาพ, อาจารย์ที่ปรึกษาร่วม: รศ.ดร. วิมลมาศ ลิปิพันธ์,
106 หน้า. ISBN 974-17-5568-6

วัตถุประสงค์ของการศึกษานี้ คือ การคัดกรองฤทธิ์กระตุ้นระบบภูมิคุ้มกันในหลอดทดลองของสารสกัดสมุนไพรไทย 15 ชนิดต่อการเพิ่มจำนวนของลิมโฟไซต์ที่ได้จากม้ามหนูขาว และการฟาโกไซโทซิสโดยเซลล์เพาะเลี้ยงมาโครฟาจชนิด J774A.1 นอกจากนี้ยังดูผลของสารสกัดต่อการผลิตซัยโตไคน์ด้วย จากผลการทดลองพบสมุนไพร 5 ชนิด ที่มีผลต่อการเพิ่มจำนวนของลิมโฟไซต์ที่ความเข้มข้น 12.5 ไมโครกรัมต่อมิลลิลิตร คือ หอมไกลดง *(Harpullia arborea)* ผักกระโฉม *(Limnophila rugosa)* นางแดง *(Mitrephora maingayi)* สังหยูใบขน *(Pseuduvaria setosa)* และงำเงาะ *(Stelechocarpus cauliflorus)* และยังพบว่าหอมไกลดงและสังหยูใบขนมีผลกระตุ้นการฟาโกไซโทซิสของเซลล์ J774A.1 สำหรับผักกระโฉมไม่ได้สกัดแยกสารบริสุทธิ์ เนื่องจากไม่สามารถหาต้นไม้ได้เพียงพอ อย่างไรก็ตามจากการทดสอบสารสกัดหยาบของผักกระโฉมพบว่า สามารถกระตุ้นการเพิ่มจำนวนของลิมโฟไซต์ได้ดีที่สุดโดยที่ไม่มีผลต่อการกระตุ้นการฟาโกไซโทซิส นอกจากนี้มันยังสามารถเหนี่ยวนำให้เซลล์ J774A.1 หลั่ง IL-12 ได้มากที่สุดด้วย ส่วนสารสกัดหยาบอื่นที่มีฤทธิ์ถูกนำไปสกัดแยกจนได้สารบริสุทธิ์ สารบริสุทธิ์ที่ได้จาก hexane fraction ของ นางแดงและงำเงาะคือ kaurenoic acid และ β-sitosterol ตามลำดับ จากการทดลองสารสกัดทั้งสองมีฤทธิ์กระตุ้นการเพิ่มจำนวนของลิมโฟไซต์มากที่สุดที่ความเข้มข้น 41.4 ไมโครโมลาร์ (37.7 ± 0.9 % stimulation) และ 60.4 ไมโครโมลาร์ (41.4 ± 6.8 % stimulation) ตามลำดับ ในขณะที่ quebrachitol ซึ่งได้จาก aqueous fraction ของหอมไกลดง มีฤทธิ์ต่อการเพิ่มจำนวนของลิมโฟไซต์มากที่สุดที่ความเข้มข้น 257.7 ไมโครโมลาร์ (45.7 ± 1.5 % stimulation) และยังกระตุ้นการฟาโกไซโทซิสของเซลล์ J774A.1 ดีที่สุดที่ความเข้มข้น 515.5 ไมโครโมลาร์ (40.1 ± 3.6 % phagocytosis) นอกจากนี้สารสกัดที่ได้จาก chloroform fraction ของ สังหยูใบขน คือ 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine, และ ouregidione มีฤทธิ์กระตุ้นการเพิ่มจำนวนของลิมโฟไซต์มากที่สุดที่ความเข้มข้น 18 ไมโครโมลาร์ (78.4 ± 5.6 % stimulation) และ 37.2 ไมโครโมลาร์ (88.1 ± 15.2 % stimulation) ตามลำดับ และสารบริสุทธิ์ทั้งสองยังสามารถเพิ่มการฟาโกไซโทซิสของเซลล์ J774A.1 ได้ที่ความเข้มข้นนี้ด้วย สารบริสุทธิ์ที่สกัดได้คือ kaurenoic acid, β-sitosterol, quebrachitol และ ouregidione สามารถเหนี่ยวนำให้เซลล์ J774A.1 หลั่ง IL-12 ได้ ยกเว้น 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine ที่ไม่สามารถเหนี่ยวนำให้เซลล์ J774A.1 หลั่ง IL-12 และมีเพียง kaurenoic acid เท่านั้นที่สามารถเหนี่ยวนำให้ลิมโฟไซต์หลั่ง IL-2 ได้เล็กน้อย การศึกษาครั้งนี้ได้ให้ข้อมูลเบื้องต้นเกี่ยวกับสมุนไพรไทยที่มีผลต่อระบบภูมิคุ้มกันซึ่งอาจมีประโยชน์สำหรับใช้กระตุ้นภูมิคุ้มกันในการรักษาโรคเอดส์ โรคติดเชื้อ และโรคมะเร็งได้

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเวชเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา.....2546.....ลายมืออาจารย์ที่ปรึกษาร่วม.....

4476564733: MAJOR BIOMEDICINAL CHEMISTRY
KEY WORDS: IMMUNOSTIMULATION / MEDICINAL PLANTS / LYMPHOCYTE PROLIFERATION / MACROPHAGE PHAGOCYTOSIS / INTERLEUKIN-2 / INTERLEUKIN-12

CHOMPUNUCH BOONARKART: IMMUNOSITMULATION OF SOME THAI MEDICINAL PLANT EXTRACTS. THESIS ADVISOR: ASSOC. PROF. THITIMA PENGSUPARP, Ph. D., THESIS CO-ADVISOR: ASSOC. PROF. VIMOLMAS LIPIPUN, Ph. D., 106 pages. ISBN 974-17-5568-6

The purpose of this study was to screen for *in vitro* immunostimulating activity of 15 Thai medicinal plant extracts on rat splenic lymphocyte proliferation and J774A.1 macrophage phagocytosis. The effects of plant extracts on cytokine production were also evaluated. Five crude ethanolic plant extracts were found to stimulate lymphocyte proliferation at the concentration of 12.5 μg/ml. These were the extracts of *Harpullia arborea*, *Limnophila rugosa*, *Mitrephora maingayi*, *Pseuduvaria setosa* and *Stelechocarpus cauliflorus*. Furthermore, *Harpullia arborea* and *Pseuduvaria setosa* showed phagocytosis enhancement at the same concentration. The ethanolic extract of *Limnophila rugosa* has not been further purified due to the limitation of the plant material. It demonstrated the highest stimulation activity on rat lymphocyte proliferation without an effect on macrophage phagocytosis. This plant extract also exhibited the highest inducing effect on IL-12 secretion from J774A.1 cells. The other active plant extracts were further submitted to fractionation and purification process to yield the immunostimulant compounds. Kaurenoic acid and β-sitosterol was obtained from the hexane fraction of *Mitrephora maingayi* and *Stelechocarpus cauliflorus*, respectively. Both compounds exhibited maximum stimulation effect to rat splenic lymphocytes at the concentration of 41.4 μM (37.7 ± 0.9 % stimulation), and 60.4 μM (41.4 ± 6.8 % stimulation), respectively. Quebrachitol, isolated from the aqueous fraction of *Harpullia arborea* showed maximum lymphocyte proliferation stimulation at the concentration of 257.7 μM with 45.7 ± 1.5 % stimulation and enhanced the phagocytic activity of J774A.1 cells at the concentration of 515.5 μM (40.1 ± 3.6 % phagocytosis). 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine and ouregidione obtained from the chloroform fraction of *Pseuduvaria setosa* maximum stimulated splenic lymphocyte proliferation at the concentration of 18 μM (78.4 ± 5.6 % stimulation) and 37.2 μM (88.1 ± 15.2 % stimulation), respectively. In addition, both compounds enhanced the phagocytic activity of J774A.1 cells at the same concentration. The isolated pure compounds, *i.e.*, kaurenoic acid, β-sitosterol, quebrachitol, and ouregidione were capable to enhanced IL-12 secretion from J774A.1 cells, except 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine could not induce the IL-12 production. Among all isolated compounds, only kaurenoic acid could weakly induced the IL-2 production from rat splenic lymphocytes. The results from this work could provide preliminary information on the immunomodulating effect of some Thai medicinal plants, which may be of benefit in increasing the immunity for the treatment of AIDS, infectious disease and cancer.

Department…….Biochemistry……Student's signature……..
Field of study……Biomedicinal Chemistry….Advisor's signature……
Academic year……2003…….……...Co-advisor's signature……

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to my advisor, Associate Professor Dr. Thitima Pengsuparp for her invaluable advice, attention and encouragement throughout this study. Her kindness and helpfulness are also really appreciated.

I am also very grateful to my the thesis co-advisor, Associate Professor Dr. Vimolmas Lipipun of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for her valuable advice and encouragement during my study.

I would like to thank the members of my thesis committee for their valuable time to critical review of this dissertation.

I would like to express my sincere gratitude and deep appreciation to Assistant Professor Dr. Rutt Suttisri and his graduate student, especially to Miss Lalita Wirasathien, Miss Khanittha Deepralard, and Miss Ratchanee Poovapatthachart of Department of Pharmaceutical Botany of this institute, for kindly supplying all plant extracts, isolated pure compounds, and their kindness and helpful suggestion for the completion of this thesis.

I also would like to give my special thanks to the personals of Department of Pharmacology, Faculty Pharmaceutical Sciences, Chulalongkorn University, for the animal facilities. I would like to express my appreciation to Dr. Poonlarp Cheepsunthorn of Department of Anatomy, Faculty of Medicine, Chulalongkorn University, for his kindly supplying J777A.1 macrophage cell line.

I also thank to the personals of Department of Biochemistry, Faculty Pharmaceutical Sciences, Chulalongkorn University, for their friendship and help during my thesis work.

This study was partly supported by the Graduate Research Funds from the Ministry of University Affairs and the Graduate School, Chulalongkorn University.

Finally, special gratitude is extended to my dear parents for their love, understanding, helpful and continuous support all my life.

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LIST OF ABBREVIATIONS

AIDS	autoimmune deficiency syndrome
ANOVA	analysis of variance
AP-1	activator protein-1
^{o}C	degree celsius (centigrade)
Con A	concanavalin A
CC_{50}	50 % cytotoxic concentration
CO_2	carbondioxide
DMSO	dimethylsulfoxide
DMEM	dulbecco's modified eagle's medium
e.g.	*exempli gratia,* for example
ELISA	enzyme-linked immunosorbent assay
et al.	*et alii,* and others
EtOH	ethanol
FBS	fetal bovine serum
Fig	figure
g	gram (s)
HCl	hydrochloric acid
HRP	Horse Radish Peroxidase
hr (s)	hours
i.e.	*id est,* that is
IFN	interferon
Ig	immunoglobulin
IL	interleukin

LIST OF ABBREVIATIONS (Cont.)

KOH	potassium hydroxide
LPS	lipopolysaccharide
MeOH	methanol
mg	milligram (s)
MHC	major histocompatibility complex
min	minute (s)
ml	millilitre (s)
MTT	3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide
NaCl	sodium chloride
NBT	nitro blue tetrazolium
ND	not determined
NF-κB	nuclear factor κB
nm	nanometre
NO	nitric oxide
NS	not stimulated
OD	optical density
PBS	phosphate buffer saline solution
pH	the negative logarithm of hydrogen ion concentration
PMS	Phenazine methosulfate
ROS	Reactive oxygen species
S.E.M.	Standard error of mean
SPSS	Statistical package for social sciences
T_C	T cytotoxic
T_H	T helper

LIST OF ABBREVIATIONS (Cont.)

TMB	5,5’-tetramethylbenzidine hydrochloride
TNF	tumor necrosis factor
U.S.A.	United State of America
XTT	3,3,-[(phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzensulfonic acid hydrate
μg	microgram (s)
μl	microlitre (s)
%	percentage

CHAPTER I

INTRODUCTION

Studies concerning the effects of alternative drugs on immunity became even more urgent with the onset of the worldwide epidemic of acquired immune deficiency syndrome (AIDS). AIDS is caused by human immunodeficiency virus (HIV) and results in a collapse of the immune system, making and individual highly susceptible to opportunistic microorganisms (Friedman *et al*., 2003). Moreover, therapeutic stimulation of immune response is desirable in cancer and inflammatory diseases. The availability of drugs that stimulate the immune response against infections and tumors is desirable, but many drugs are limited as a result of low efficacy and life-threatening toxicity.

Plants are recognized for their ability to produce a wealth of secondary metabolites and mankind had used many species for centuries to treat a variety of diseases. Secondary metabolites are biosynthesized in plants for different purposes including growth regulation, inter- and intra-specific interactions and defense against predators and infections. Many of these natural products have been shown to present interesting biological and pharmacological activities and are used as chemotherapeutic agents or serve as the starting point in the development of modern medicines. In mammals, the immune system plays a vital role as the main line of defense against infections. Its integrity and efficiency is important during chemotherapeutic intervention for the treatment of many diseases. In many parts of the world, there is a rich tradition in the use of herbal medicine for stimulating the immune system against viral and bacterial infections (Wilasrusmee *et al*., 2002). Furthermore, herbal products are being used increasingly in the U.S.A. and the rest of the world. In the U.S.A., 12-17% of the population was reported to use herbal medicines (Eisenberg *et al*., 1993; Astin, 1998). A survey from Ambulatory Research Clinics of the National Institute of Health (NIH) found that approximately one out of six patients took herbal products in addition to their prescribed treatment (Johnson *et al*., 2000). Consequently, NIH has established an institute devoted to research on Complementary and Alternative Medicine (NCCAM). This institute has recently

participated in an initiative to develop botanicals against infections bioterrorism agents (Wilasrusmee *et al*., 2002). Thus, medicinal plants may offer a new source of immunostimulating agents for boosting the immune system of patients with infection.

Immunological defense is a complicated interplay of many factors. Many researchers attempted to identify the pharmacological mechanism of the whole plants or the bioactive compounds on the immune system. Irrespective of the primary targets of the immunostimulant, which are T or B lymphocytes or the complement system or macrophages, the enhancement on phagocytosis by macrophages and proliferation of lymphocytes play a central role in immunostimulation. Therefore, in the present study, the *in vitro* lymphocyte proliferation and macrophage phagocytosis were used to screen for immunomodulatory activity of some Thai medicinal plants.

Rationale of the study

Most of Thai medicinal plant extracts used in this study were selected according to chemotaxonomic data, for example, *Hymenodictyon excelsum*, *Limnophila aromatica* and *Limnophila rugos*. In our experimental designs, the investigation of the toxic effects of 15 plant extracts to rat splenic lymphocytes and J774A.1 macrophage cell line were performed. Cytotoxicity was assessed by the method of Alamar blue assay and XTT assay for rat splenic lymphocytes and J774A.1 macrophage cells, respectively. Both methods are based on the dyes which were reduced by mitochondrial dehydrogenase in metabolic active cells and converted to reduce form or formazan, repectively. Then, plant extracts were tested for immunostimulant activity on rat splenic lymphocyte proliferation using Alamar Blue assay and macrophage phagocytosis using NBT reduction method. Finally, the effects of plant extracts on cytokine production were also determined. The main purpose of this study is to evaluate the immunostimulation effect of Thai medicinal plants, using rat splenic lymphocytes and J774A.1 macrophage cell line as represented cell of adaptive immunity and innate immunity, respectively. In addition, the preliminary mechanism of the active plant extracts which stimulated lymphocyte proliferation was investigated by measuring the effect on cytokine production, particularly IL-2 and IL-12.

Objectives

1. Evaluate the effect of 15 Thai medicinal plant extracts on rat splenic lymphocyte proliferation and macrophage phagocytosis.
2. Study the effect of the active Thai medicinal plant extracts on cytokine (particularly IL-2 and IL-12) secretion from lymphocytes and macrophages.

CHAPTER II

LITERATURE REVIEWS

Immunostimulating activity of some medicinal plants

Medicinal plants have been used to cure human illness for thousands of years. Some of these drugs are believed to promote positive health and maintain organic resistant against infection by re-establishing body equilibrium and conditioning the body tissues.

Glycyrrhizin (GL), isolated from an aqueous extract from liquorice root, consists of glycyrrhetic acid and two molecules of glucuronic acid. It is extensively used in Japan and is being tried in European countries in patients with active and chronic hepatitis (Suzuki *et al*., 1983; Tsubota *et al*., 1999; Van Rossum *et al*., 1999). Various effects of GL have been demonstrated, such as induction of IFN production, enhancement of NK cell activity and modulation of the growth response of lymphocytes through augmentation of IL-2 production (Abe *et al*., 1982; Zhang *et al*., 1993). Moreover, GL has been described as an anti-viral agent against human cytomegalovirus, herpes simplex virus type 1 and influenza virus (Pompei *et al*., 1979; Numazaki *et al*., 1994; Utsunomiya *et al*., 1997). GL has also been reported to inhibit HIV replication *in vitro* (Harada *et al*., 1998). This compound can prevent the progression of disease in mice with murine acquired immune deficiency syndrome (AIDS) and improve the clinical symptoms in HIV-infected patients (Ito *et al*., 1988; Watanoe *et al*., 1996). In addition, GL enhanced LPS-induced IL-12 production by peritoneal macrophages (Dai *et al*., 2001)

The genus *Echinacea* (purple coneflower) is a North American indigenous plant. Its extract has been traditionally used in the treatment of various conditions, such as bacterial/viral infections, cancer, seizures and AIDS (O'Hara *et al*., 1998). In recent years, it has become one of the popular immune promoters, particularly for the prevention and treatment of upper respiratory tract infections. According to many *in vitro* studies, *Echinacea*-induced immunomodulation appears to occur through stimulation of non-specific immune system (Stimpel *et al*., 1984; Leuttig *et al*., 1989).

It has, thus, been shown to stimulate the macrophages (Wangner and Jurcic, 1991; Stotzem *et al*., 1992), increase the production of interleukin-1 (IL-1), IL-6 and TNF-α from the macrophage (Burger *et al*., 1993), and enhance the natural killer function of human peripheral blood mononuclear cells (See *et al*., 1997). Furthermore, *Uncaria tomentosa* (Rubiaceae) is used in South America folk medicine. The extracts from various components of this plant have been shown to have cytostatic, anti-inflammatory, antiviral, enhancement of phagocytosis, and enhancement of normal human lymphocyte proliferation. Most of these studies were based on organic solvent extraction and attributed to the effects of indole alkaloids separated from the plant parts (Aquino *et al*., 1991; Sheng *et al*., 2000; Wagner *et al*., 1985; Wurm *et al*., 1998).

Chinese medicinal herbs (CMH) have been traditionally used to prevent and treat many kinds of diseases in China, especially autoimmune diseases and tumors. CMHs have many biological activities. The characterization of CMH recipes have been made by biochemical, immunological and pharmacological methods (Yoshida *et al*., 1997). The term ginseng means "the essence of man" in Chinese and *Panax ginseng* has been used as a revitalizing agent by Chinese medical practitioners for over 3,000 years (Liu and Xiao, 1992). The herb is still utilized in many Asian countries for a variety of conditions. In addition, there is evidence that the herb can stimulate cellular immune function. The extracts of ginseng augmented murine lymphocyte proliferation and natural killer (NK) cell function *in vitro*. One double-blinded, placebo-controlled study in normal human volunteers revealed an increase in neutrophil function, CD4 cell count and NK-function in individuals taking ginseng compared to those given placebo (Scaglione, 1990). *Acanthopanax giraldii*, *Astragalus membranaceus* and *Oldenlandia diffusa* are also reported to enhance the immune responses (Yoshida *et al*., 1997).

Furthermore, several plants employed in traditional medicine in Europe for rejuvenation therapy and for the treatment of chronic disorders have been shown to possess immunostimulatory activity. Immunostimulant activity has also been demonstrated in plants employed in the indigenous systems of medicine in China and India. For example, *Picrorhiza kurroa* (Scrophulariaceae), a perennial herb growing in the alpine Himalayas, has long been used in India and Sri Lanka as a tonic and for

the treatment of fever, jaundice, and other liver ailments (Puri *et al*., 1992). The aqueous extracts of *Picrorhiza kurroa* roots have been reported to inhibit *in vitro* the classical and alternate pathways of human serum complement and the generation of oxygen radicals by activated polymorphonuclear leucocytes (PMN). The extract also stimulated the production of macrophage inhibition factor (MIF) by pokeweed-induced human peripheral blood lymphocytes (Simonsa *et al*., 1989; Labadie *et al*., 1989). Ethanol extracts of the plant root have been found to modulate cell-mediated and humoral immune responses (Atal *et al*., 1986).

Perilla frutescens is one of the most common herbs in many Asian countries. *Perilla* leaves, usually accompanied with seafood, are believed to prevent food poisoning and to protect the digestive tract from inflammatory diseases. Also, they have been used as diuretic, a sedative, an antidote, and antifebrile in Japanese traditional medicine (Kwon *et al*., 2002).

Curculigo orchioides Gaerten (Amaryllidaceae) is a small herb found in India in the sub-tropical Himalayas from Kumaon eastwards and in the Western Ghats from Konkan soutwards. It is commonly known as Kalimusli in Hindi. Its tuberous roots or rhizomes are used as an alterative, demulcent, diuretic, restorative and for the treatment of jaundice. It is also the component of several Ayurvedic tonics. In China, it is being used for the treatment of decline in strength. The root powder is said to stop bleeding and heal wounds (Lakshmi *et al*., 2003). Methanolic extract of the roots has been shown to enhance phagocytic activity of macrophages. The active principle of the extract identified as a curculigoside (5-hydroxy-2-*O*-β-D-glucopyranosyl, benzyl 2,6-dimethoxy benzoate) has been reported to possess adjuvant activity (Saike *et al*., 1981). In addition, significant immunostimulant activity was found in purified glycoside-rich fraction isolated from the ethyl acetate fraction of this plant (Lakshmi *et al*., 2003).

Clausena excavata, a wild shrub of the Rutaceae family, is widely distributed in South Asia. It has been used as folk medicines for the treatment of cancer and several disorders in the East of Thailand. Its leaves and stems are also used for the ailment of colic, cough, headache, rhinitis, sore, wounds, yaws and detoxification in some countries. The main constituents of this plant have been revealed to be

carbazole alkaloids and coumarins (Manosroi *et al*., 2003). The ethanolic extract from its root bark has antibacterial activity with complete inhibitory effect against most of the tested microorganisms (Wu and Furukawa, 1982). The methanolic extract from its stem bark has inhibitory effect on rabbit platelet aggregation thus leading to vasoconstriction, while the aqueous extract from its wood has antimutagenic effect to both direct and indirect mutagen in Hepa ICIC7 murine hepatoma cell line (Puatanachokchai *et al*., 1998). The acetone extract from its leaves have several novel furanocoumarins with inhibitory effects on tumor-promotion against 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus early antigen activation in Raji cells (Chihiro *et al*., 2000). In addition, the aqueous extract and acetone extract exhibited the effect on phagocytic activity and proliferated B cell through T cell independent pathway (Manosroi *et al*., 2003).

The use of herbal medications has increased tremendously in recent years. Various types of medicinal plants have been reported to affect the immune responses, which they enhanced function of macrophages and lymphocytes. Thus, in this thesis an attempt was made to investigate the effect of Thai medicinal plants on the immune responses.

Immune system functions

The human immune system is very complex and consists of a number of organs, tissues, cell types, and molecules that are scattered throughout the body. The cells of the immune system synthesize and recognize a variety of molecules, including antibodies, complement proteins, cytokines, growth factors, and receptors for these molecules. Many of these molecules have pleiotropic and synergistic effects. Overall protection is provided by an interaction between the various cells and molecules of the immune system.

The main functions of the immune system are to defend the body against invading organisms, foreign antigens, and host cells that have become neoplastic. Immunity is a state of resistance or protection from pathogenic microorganisms. There are two types of immunity: acquired (specific) immunity, which responds to specific stimuli (antigens) and is enhanced by repeated exposure to the stimuli; and

innate (nonspecific) immunity, which does not require stimulation and is not enhanced by repeated exposure. The innate immune responses are the first line of defense against invading pathogens. They are also required to initiate specific adaptive immune responses. Innate immunity relies on the body's ability to recognized conserved features of pathogen that are not present in the uninfected host. The innate immune mechanisms consist of physical barriers, such as mucous membranes, and the phagocytic and cytotoxic functions of neutrophils, monocytes, macrophages, and lymphatic cells (NK cells). Acquired immunity can be classified into 2 types based on the response, *i.e.*, humoral immunity and cell-mediated immunity. Humoral immunity is mediated by immunoglobulins produced by bone marrow-derived lymphocytes (B lymphocytes) and is responsible for specific recognition and elimination of extracellular antigens. Cell-mediated immunity is mediated by cells of the immune system, particularly thymus-derived lymphocytes (T lymphocytes). Cell-mediated immunity is responsible for delayed-type hypersensitivity (DTH) reactions, foreign graft rejection, resistance to many pathogenic microorganisms, and tumor immunosurveillance. Macrophages are also important in cell-mediated immunity as antigen presenting cells and through the production of regulatory mediators such as cytokines and eicosanoids, in addition to their involvement in nonspecific immunity (Meydani and Ha, 2000; Delves and Roitt, 2000).

Macrophages

The mononuclear phagocytic system consists of monocytes circulating in the blood and macrophages in the tissues (Figure 1). Monocytes circulate in the bloodstream for about 8 hrs, during this time they enlarge, then migrate into the tissues and differentiate into specific tissue macrophages.

Differentiation of a monocyte into a tissue macrophage involves a number of changes: the cell enlarges five- to ten-fold; its intracellular organelles increase in both number and complexity; and it increases phagocytic ability; *e.g.*, produces higher levels of hydrolytic enzymes, and begins to secrete a variety of soluble factors. Macrophages are dispersed throughout the body. Some take up residence in particular tissues, becoming fixed macrophages, whereas, others remain motile and are called

free or wandering macrophages. Free macrophages travel by amoeboid movement throughout the tissues. Macrophage-like cells serve different functions in different tissues and are named according to their tissues locations; *e.g.*, Kupffer cells in the liver, microglial cells in the brain, *etc*.

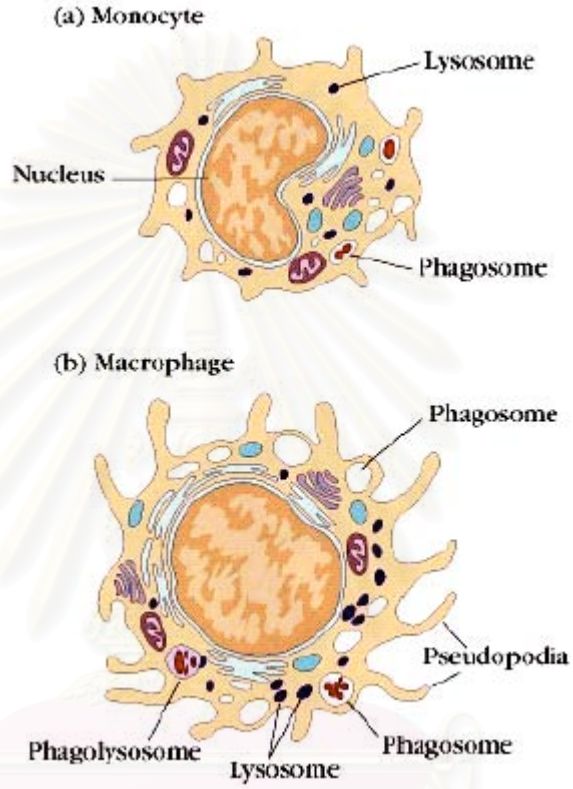

Figure 1 Typical morphology of a monocyte and a macrophage. Macrophages are five- to tenfold larger than monocytes and contain more organelles, especially lysosomes (Goldsby *et al*., 2001).

Macrophage has several functions in the body. This ubiquitous and mobile cell is constantly sampling the environment and responding to its various stimuli. The macrophage has diverse surface receptors that allow it to interact with a range of hormones, exogenous and endogenous proteins, polysaccharides, and lipids. A list of some of the macrophage surface receptors is shown in Table 1.

Table 1 Plasma membrane receptors of macrophages (Paul, 1989; Abbas *et al*., 2000).

Receptor	Ligand
Fc receptors (FcR)	
FcγRI	Monomers of IgG
FcγRII	Aggregates of IgG
FcγRIII	Aggregates of IgG
Complement receptors (CR)	
CR1	C3b, C4b, iC3b
CR3	iC3b, fibrinogen, factor X
C5a	C5a
Fibronectin receptor	Fibronectin oligomers
Mannose-fucose receptor	Oligosaccharides
Receptors for growth factors and cytokines	
Interferons α and β	
Interferons γ	
Interleukin-1	
Monocyte colony-stimulating factor	
Tumor necrosis factor	

The Fc receptors for different Ig heavy chain isotypes are expressed on many leukocyte populations and serve diverse functions in immunity. Of these Fc receptors, the ones that are most important for phagocytosis of opsonized particles are receptors for the heavy chains of IgG antibodies, called Fcγ receptors. The three types of Fcγ receptors have different affinities for the heavy chains of different IgG subclasses (Abbas *et al*., 2000). Sequential binding of Fc receptors to antibody-coated particles leads to engulfment of the particles and their internalization in phagocytic vesicles. These phagosomes fuse with lysosome and the phagocytosed particles are destroyed in the phagolysosomes.

Binding of opsonized particle to phagocyte Fc receptor, particularly FcγRI, also activates phagocytes by virtue of signals transduced by the FcR γ chain. These signals result in the activation of several tyrosine kinases in the phagocytes. One of the consequences is the activation of the enzyme phagocyte oxidase, which catalyzes the intracellular generation of reactive oxygen intermediates that are cytotoxic to phagocytosed microbes. Expression of FcγRI on macrophages is stimulated by the macrophage-activating cytokine interferon-γ (IFN-γ). The antibody isotypes that bind best to Fcγ receptors are also produced as a result of IFN-γ mediated isotype switching of B cells. In addition, IFN-γ directly stimulates the microbicidal activities of phagocytes. Thus, IFN-γ is an excellent example of a cytokine that has multiple actions, it functions cooperatively in one mechanism of host defense, namely, elimination of microbes by phagocytes.

Many of the biologic activities of the complement system are mediated by the binding of complement fragments to membrane receptors expressed on macrophages. CR1 receptor is a high-affinity receptor for C3b and C4b. CR1 transduces signals that activate the phagocytic mechanisms of macrophage. CR3 receptor is a receptor for the iC3b fragment generated by proteolysis of C3b and plays an important role in the phagocytosis of iC3b-coated particles. The mannose receptor is a lectin that binds terminal mannose and fucose residues of glycoproteins and glycolipids. These sugars are typically part of molecules found on microbial cell walls.

Phagocytosis

Macrophages are capable of ingesting and digesting exogenous antigens, such as whole microorganisms and insoluble particles; and endogenous matter, such as injured or dead host cells, cellular debris, and activated clotting factors. In the first step in phagocytosis, macrophages are attracted by and move toward a variety of substances generated in an immune response; this process is called chemotaxis. The next step in phagocytosis is adherence of the antigen to the macrophage cell membrane. Complex antigens, such as whole bacterial cells or viral particles, tend to adhere well and are readily phagocytosed; isolated proteins and encapsulated bacteria tend to adhere poorly and are less readily phagocytosed. Adherence induces membrane protrusions, called pseudopodia, to extend around the attached material.

Fusion of the pseudopodia encloses the material within a membrane-bounded structure called a phagosome, which then enters the endocytic processing pathway. In this pathway, a phagosome moves toward the cell interior, where it fused with a lysosome to form a phagolysosome. Lysosomes contain lysozyme and a variety of other hydrolytic enzymes, which digest the ingested material. The digested contents of the phagolysosome are then eliminated in a process called exocytosis (Figure 2).

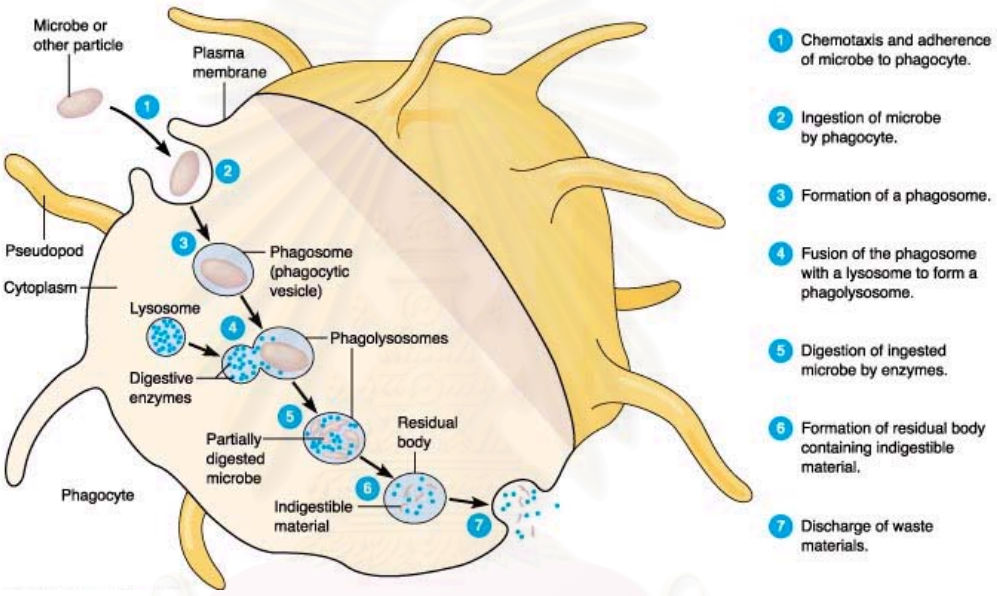

Figure 2 Phagocytosis and processing of exogenous antigen by macrophage (http://www.utc.edu/Faculty/Becky-Bell/210-Ex2-studyguide.html[2004, March 15]).

A number of antimicrobial and cytotoxic substances produced by activated macrophages can destroy phagocytosed microorganisms (Table 2). Many of the mediators of cytotoxicity listed in Table 1 are reactive forms of oxygen.

Table 2 Mediators of antimicrobial and cytotoxic activity of macrophages (Goldsby *et al*., 2001).

Oxygen-dependent killing	Oxygen-independent killing
Reactive oxygen intermediates	Defensins
O_2^- (superoxide anion)	Tumor necrosis factor α
$OH^•$ (hydroxyl radicals)	Lysozyme
H_2O_2 (hydrogen peroxide)	Hydrolytic enzymes
ClO^- (hypochloride anion)	
Reactive nitrogen intermediates	
NO (nitric oxide)	
NO_2 (nitrogen dioxide)	
HNO_2 (nitrous acid)	
$OONO^-$ (peroxynitrite)	
Others	
NH_2Cl (monochloramine)	

Oxygen-independent killing

Activated macrophages also synthesize lysozyme and various hydrolytic enzymes whose degradative activities do not require oxygen. In addition, activated macrophages produce a group of antimicrobial and cytotoxic peptides, commonly known as defensins. These molecules are cysteine-rich cationic peptides containing 29-65 amino acid residues. These circularized defensin peptides have been shown to form ion-permeable channels in bacterial cell membranes. Defensins can kill a variety of bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae*. Activated macrophages also secrete tumor necrosis factor α (TNF-α), a cytokine that has a variety of effects and is cytotoxic for some tumor cells.

Oxygen-dependent killing

Activated phagocytes produce a number of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates that have potent antimicrobial activity. Ligand binding of Fc receptors (on neutrophils, monocytes or macrophages) and mannose receptors (on macrophages) increases their oxygen (O_2) uptake, called the respiratory burst. These receptors activate a membrane-bound NADPH oxidase that reduces O_2 to O_2^-. Superoxide can be reduced to $OH^\bullet$ or dismutated to H_2O_2 by superoxide dismutase. O_2^-, $OH^\bullet$, and H_2O_2 are activated oxygen species that are extremely toxic to ingested microorganisms. As the lysosome fused with the phagosome, macrophages will induce hypochlorite (ClO^-) from H_2O_2 and chloride ions (Cl^-) through the action myeloperoxidase. Hypochlorite, the active agent of household bleach, is toxic to ingested microbes (Babior, 1984; Karnovsky and Badwey, 1986).

When macrophages are activated with bacterial cell wall components, such as lipopolysaccharide (LPS) or, in the case of mycobacteria, muramyl dipeptide (MDP), together with a T-cell derived cytokine (Interferon-γ, IFN-γ), they begin to express high levels of nitric oxide synthetase (NOS), an enzyme that oxidizes L-arginine to yield L-citrulline and a gas nitric oxide (NO) (Marletta, 1993; Nathan, 1992). Nitric oxide has potent antimicrobial activity; it also can combine with the O_2^- to yeild even more potent antimicrobial substances ($OONO^-$, $OH^\bullet$)

Lymphocytes

Lymphocytes are the primary cell types involved in the immune response. There are two types of lymphocytes, the B cells and the T cells. Both cells are derived from bone marrow lymphoid stem cells, but the T cells undergo through an additional maturation process in the thymus. Although the morphology of T lymphocytes and B lymphocytes is similar, their functions are distinct. Once exposure to foreign substances known as antigens, B cells develop into antibody producing plasma cells, whereas, T cells are divided into functional subtypes that possess distinct cell surface antigens. Lymphocytes are the only cells in the body capable of specifically recognizing and distinguishing different antigenic

determinants, therefore, are responsible for the two defining characteristics of the adaptive immune response, specificity and memory.

B lymphocytes

The B lymphocytes mature within the bone marrow and then migrate to secondary lymphoid organs. Each cell expresses a unique antigen-binding receptor on its membrane (Figure 3). The B-cell receptor is a membrane-bound immunoglobulin. Immunoglobulins are glycoproteins, each molecule consists of two identical heavy polypeptide chains and two identical light polypeptide chains. The amino terminal ends of the pairs of heavy and light chains form a Y-shaped cleft within which antigen binds. When a naive B cell, which has not previously encountered antigen, first encounters the antigen that matches its membrane-bound immunoglobulin, the binding of the antigen to the immunoglobulin causes the cell to divide rapidly; its progeny differentiate into memory B cells and effector B cells called plasma cells.

Memory B cells have longer life span than naïve cells; they continue to express the same membrane-bound antibody as their parent naive B cells. Plasma cells do not express membrane-bound antibody; instead they produce the antibody in a form that can be secreted. Although plasma cells live for only a few days, they secrete enormous amounts of antibody during this time. It has been estimated that a single plasma cell can secrete more than 200 molecules of antibody per second. Secreted antibodies are the major effector molecules of humoral immunity.

T lymphocytes

T lymphocytes also arise in the bone marrow. Unlike B lymphocytes, which mature within the bone marrow, T cells migrate to the thymus gland to mature. T cell encounters antigen combined with an MHC molecule on the cell, the T cell proliferates and differentiates into memory T cells and various effector T cells.

There are two well-defined subpopulations of T cells: T helper (T_H) and T cytotoxic (T_C) cells. Although a third type of T cell, called a T suppressor (T_S) cell, has been postulated, recent evidence suggests that it may not be distinct from the T_H and T_C subpopulations. T helper and T cytotoxic cells can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their

surfaces (Figure 3). T cells displaying CD4 generally function as T_H cells, whereas those displaying CD8 function as T_C cells.

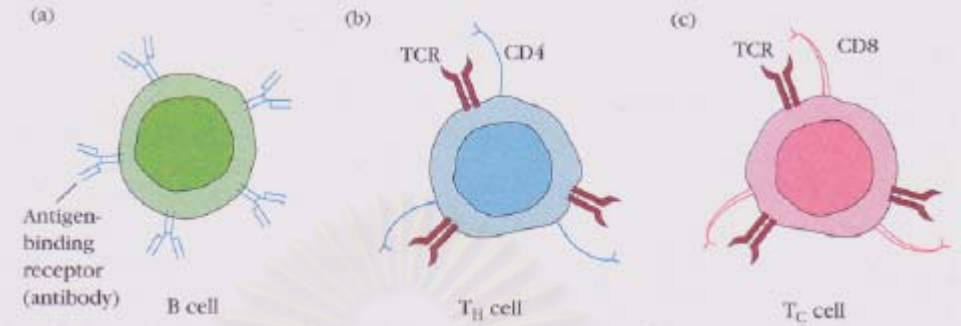

Figure 3 Distinctive membrane molecules on lymphocytes. (a) B cells have about 10^5 molecules of membrane-bound antibody per cell. (b) T cells bearing CD4 ($CD4^+$ cells) recognize only antigen bound to class II MHC molecules. (c) T cells bearing CD8 ($CD8^+$ cells) recognize only antigen associated with class I MHC molecules (Goldsby *et al*., 2001).

Most of the immune response occurring through $CD4^+$ cell can be subdivided into T_H1 and T_H2 responses. The T_H1 response predominantly results in T cytotoxic cells and stimulates IgG2a production. In contrast, a T_H2 response produces IgE and eosinophilic infiltration, and stimulates the production of IgG1 (Constant and Bottomly, 1997; Kang *et al*., 1999; Liblau *et al*., 1995; Romagnani, 1996.). These subtypes also produce unique sets of cytokines, that is, T_H1 cells generate IFN-γ and interleukin (IL)-2, and T_H2 cells produce IL-4, IL-6 and IL-10. In healthy organisms, there is delicate balance maintained between the activity of T_H1 versus T_H2 cells in that the activity of T_H1 cells is directly cross-regulated by the T_H2 cells and vice versa. However, under certain pathological conditions especially chronic viral and bacterial infections, the functioning of T_H1 cells may be superceded by that of the T_H2 cells hence leading to a humoral but non-protective immune response at the expense of the more protective cellular response. A similar imbalance exists in other chronic conditions such as allergics and autoimmune disorders. It is, thus, vital for us to maintain or to re-introduce the delicate balance in the functioning of the T_H1 and T_H2 cells. This possibly explains the difference between the use of immune boosters versus the use of delicate immune modulators: an immune booster will simply

enhance and already imbalanced response (O'Garra and Arai, 2000; Mackay, 2001). Immune modulators, on the other hand, attempt to bring back the balance and, therefore, correct the underlying immune abnormality. Many researchers are currently attempting to enhance the activity of T_H1 cells in order to eradicate latent and chronic pathogens by testing immune modulators (recombinant proteins/growth factors or whole bacteria, *etc*.) in HIV infection and tuberculosis (Chehimi *et al*., 1994; Erb *et al*., 1998; Fentuzzi *et al*., 1996; Kay, 2001; Wang and Rook, 1998; Walker and Zuany-Amorim, 2001).

After a T_H cell recognized and interacts with an antigen-MHC class II molecule complex, the cell is activated and becomes an effector cell that secretes various growth factors known collectively as cytokines. The secreted cytokines play an important role in activating B cells, T_C cells, macrophages, and various other cells that participate in the immune response. Differences in the pattern of cytokines produced by activated T_H cells result in different types of immune response.

Under the influence of T_H-derived cytokines, T_C cell that recognizes an antigen-MHC class I molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). In contrast to the T_H cell, the CTL generally does not secrete many cytokines, it exhibits cytotoxic activity, instead. The CTL has a vital function in monitoring the cells of the body and eliminating any cells that display antigen, such as virus infected cells, tumor cells, and cells of a foreign tissue graft. Cells that display foreign antigen complexed with a class I MHC molecules are called altered self-cells.

Lymphocyte activation

The term lymphocyte activation denotes an ordered series of events through which a resting lymphocyte is stimulated to divide and produce progeny, some of which become effector cells (Figure 4). Thus, the full response thus includes both the induction of cell proliferation (mitogenesis) and the expression of immunologic functions. Lymphocytes become activated when specific ligands bind to receptors on their surfaces. The ligands requirements are different for T cells and B cells, but the response itself is similar in many respects for all types of lymphocytes.

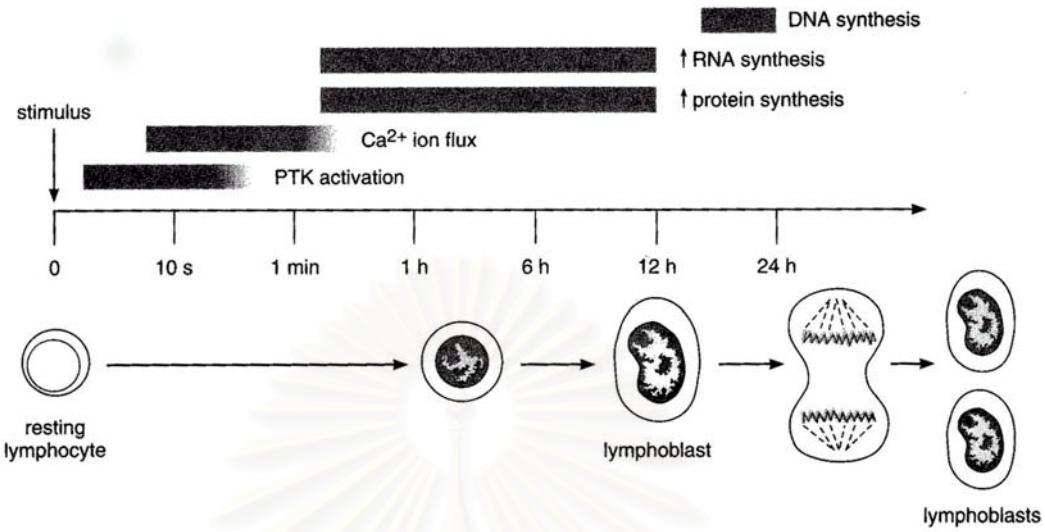

Figure 4 Major biochemical and morphologic events in lymphocytes activation (Parslow *et al*., 2001).

The earliest event of the T cell or B cell activation, is a marked increase in activity of cytoplasmic protein tyrosine kinases (PTKs), proteins that have the ability to catalyze the phosphorylation of tyrosine residues in other proteins. This process occurs within seconds and reflects the functional activation of numerous different PTKs. Several important types of lymphocyte surface receptors (including membrane immunoglobulin and T-cell receptor proteins) are physically linked to specific cytoplasmic PTK proteins. The ligand-induced clustering of receptors on the B- or T-lymphocyte surface appears to be a key event in triggering PTK activation. The receptor associated PTKs, in turn, may then activate other types of PTKs through phosphorylation, so that almost immediately a host of different PTKs are recruited into the response. By phosphorylating other types of substrate, such as proteins that control cytoskeletal organization, expression of specific genes, and entry into the cell cycle, these newly activated PTKs appear to be either directly or indirectly responsible for triggering all subsequent events in lymphocyte activation. At present, however, the functions of most individual PTKs are uncertained.

One almost immediate effect of the PTK cascade is to activate the cytosolic enzyme phospholipase C-γ1, which then acts to hydrolyze a specific class of phospholipids, called phosphatidylinositides,that are found in cellular membranes. The products of this hydrolysis include two small organic molecules, diacylglycerol (DAG) and inositol 1,4,5-triphophate (IP_3), which serve as second messengers to trigger additional changes in cellular physiology. DAG remains within the membrane of origin, where it binds and allosterically activates protein kinase C, a family of cytosolic enzymes that can phosphorylate other proteins at serine and threonine residues. IP_3 is released into the cytoplasm; binds to specific membrane receptors; and triggers a rapid, marked increase in the concentrations of intracellular free calcium ions, which flood into the cytosol from organelle storage pools, reaching maximal concentrations within 1 minute after contact with the activating stimulus. Like the PTK cascade, protein kinase C activation and these rapid calcium fluxes are thought to be critical for initiating the subsequent events in activation.

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Within the first hour after stimulation, the rates of oxidative metabolism and of overall protein and RNA synthesis in the lymphocyte rise. The chromatin becomes less dense as previously silent genes are transcribed and the cell prepares to undergo mitosis. After 2-4 hours, specific proteins that are thought to regulate cell proliferation, such as the product of the protooncogene *c-myc*, becomes detectable in the nucleus. In parallel with these biochemical events, the morphology of the cell changes in a process known as blast transformation: its overall diameter increase to 15-30 μm as both its nucleus and cytoplasm enlarge: the nuclear chromatin becomes loose and pale-staining; and the cell acquires a prominent nucleolus (reflecting a high rate of RNA synthesis). Within 8-12 hours, the changes are sufficiently marked that the cell can be recognized under the light microscope as a lymphoblast, a lymphocyte poised to begin mitosis. DNA synthesis takes place at around 18-24 hours after stimulation. The first cell division occurs 2-4 hours later depends on the conditions, and can be repeated five or more times in succession, at intervals of 6 hours. The effector cells, produced as a result of each division, mature completely within a few days and express the immune functions typical of their lineage for several days thereafter (Baniyash *et al*., 1988; Berridge, 1987; Graber *et al*., 1991; Janeway and Bottomly, 1994; Parslow *et al*., 2001; Weiss *et al*., 1986).

Cytokines

Cytokines are polypeptides produced in response to microbes and other antigens and play critical roles in regulating all aspects of immune responses, including lymphoid development, homeostasis, differentiation, tolerance and memory. This thesis will concentrate in only the IL-2 and IL-12.

Interleukin-2

Human IL-2 is a single chain polypeptide containing 133 amino acids. It is a glycoprotein which the carbohydrate component being attached via an *O*-linked glycosidic bond to threonine residue number 3. The mature molecule displays a molecular mass ranging from 15 to 20 kDa, depending upon the extent of glycosylation. The carbohydrate moiety is not required for biological activity.

IL-2 is an essential growth factor for T cell cycle progression and proliferation. IL-2 is important not only for T cell survival, but also for proliferation and differentiation of T cell into T_H1 or T_H2 predominance. Inadequate IL-2 synthesis may lead to T cell death or induction of clonal anergy. Furthermore, deficient IL-2 production has been associated with autoimmune and/or inflammatory states (Figure 5)

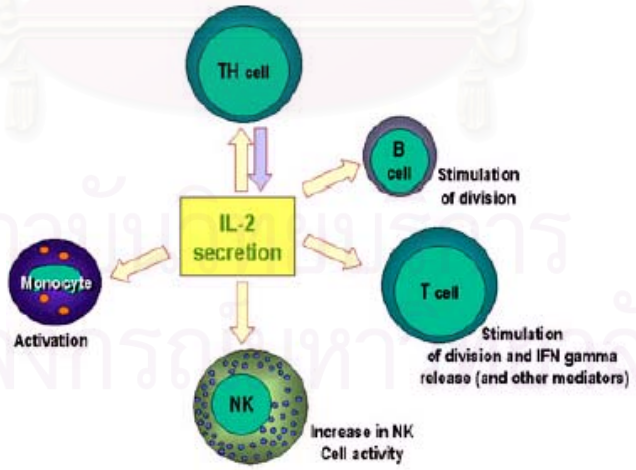


Figure 5 Biologic actions of interleukin-2. IL-2 stimulates the proliferation and differentiation of T and B lymphocytes and natural killer (NK) cells (http://www.med.sc.edu:85/bowers/imm-req.html[2004, March 15]).

Transcriptional regulation of the IL-2 gene occurs at multiple levels but is mediated primarily through cooperative interaction of positive-regulatory transcription factors, including NFκB, AP-1, nuclear factor of activated T cells (NF-AT). Binding of these factors to their specific transcription sites is necessary for optimal transcription of the IL-2 gene. Sufficient activation of T cell leads to transcription of IL-2, detected within 40 min of activation IL-2 mRNA levels peak at 6-8 hr and then decline by 24 hr (Hughes and Pober, 1996; Mosmann and Sad, 1996; 2001; Scott, 1993; Weiss *et al*., 1984; Weiss and Littman, 1994).

Interleukin-12

IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits designated p35 and p40. The p35 subunit is a 196-amino acid glycoprotein exhibiting a molecular mass in the 30-35 kDa range. The p40 subunit, containing 306 amino acids, is also glycosylated and exhibits a molecular mass in the 35-45 kDa range. IL-12 is produced by monocytes, macrophages, dendritic cells, neutrophils, and to a lesser extent B cells. One major action of IL-12 is its induction of other cytokines, particularly IFN-γ, which coordinate the ensuing immune response. IL-12 initially induces IFN-γ production in NK and T cells (Figure 6). IFN-γ stimulates the bactericidal activity of phagocytic cells, therefore, boosts the innate immune responses.

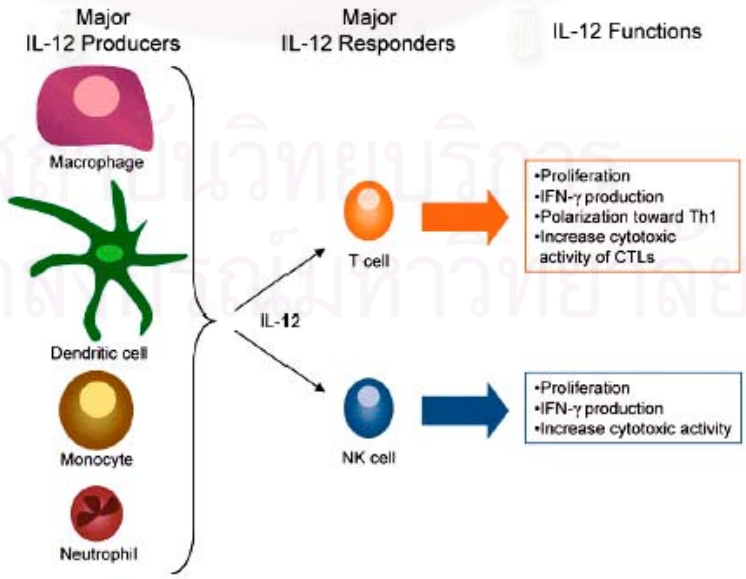


Figure 6 Cellular sources and responders of IL-12 (Watford *et al*., 2003).

A major function of IL-12 is the regulation of the adaptive immune response (Figure 7). Cytokines play a critical role in the developmental regulation of naïve $CD4^+$ cells into either T_H1 or T_H2 cell types. IL-12 is the main cytokine that regulates T_H1 differentiation and has a number of important actions that serve to promote cell-mediated immunity. IL-12 induces T cell proliferation; it preferentially acts on naïve $CD4^+$ T cells to induce their differentiation and expansion. Conversely, IL-12 and IFN-γ antagonize T_H2 differentiation and the production of IL-4, IL-5, and IL-10 (Gately *et al*., 1998; Trinchieri, 1993; Watford *et al*., 2003).

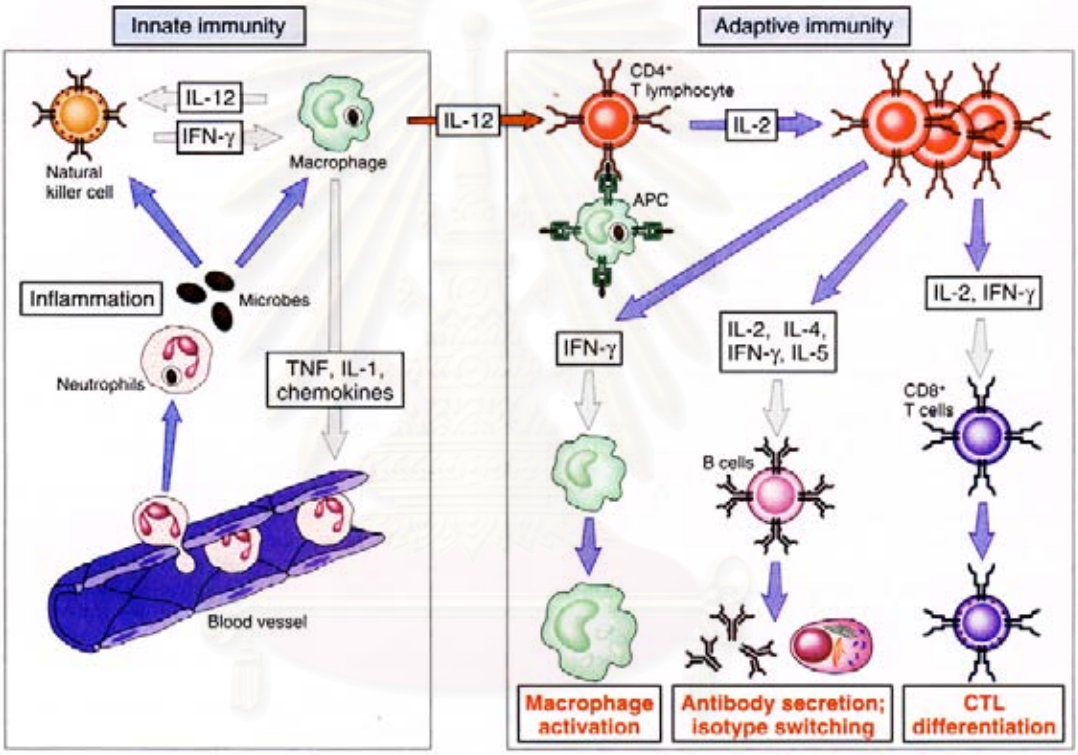


Figure 7 Functions of cytokines in host defense (Abbas *et al*., 2000).

In vitro indexes of immune function

To study immune functions *in vitro*, immune cells are first separated from whole blood, lymphoid tissues, and gut-associated immune cells. The cells are then maintained and cultured with and without various immune cell stimuli. To measure the activity of isolated phagocytes, the cells are incubated with bacteria or other engulfable materials with or without opsonin for a limited time and then stained for uptake of foreign bodies. Lymphocytes are usually stimulated for varying lengths of time by a variety of stimuli (mitogens, antigens, and other stimulator or target cells) for measurement of their proliferative or cytotoxic activity or release of immunologically active molecules such as antibodies, cytokines, and eicosanoids.

Phagocytic activity

The ability to perform phagocytosis and kill microbes, including bacterial pathogens, is a major effector function of macrophages. These properties of macrophages are particularly important for host defense against facultative intracellular organisms, which can replicate within macrophages. The pathogenesis of facultative intracellular bacteria is determined by their ability to survive within macrophages. Several organisms were used previously as targets to determine macrophage killing. These include *Saccharomyces cerevisiae, Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Candida albicans*.

A variety of methods have been developed for the measurement of phagocytosis. These techniques can be divided into two broad categories: (1) those which assess phagocytic ingestion per se; and (2) those assays which measure bactericidal activity of macrophages. In the former, the various techniques can themselves be grouped into two general classes: (1) The “microscopic category” in which the extent of particle uptake by individual cells is determined directly, and (2) the “macroscopic (or bulk) category” in which measurement of a certain parameter (*e.g.*, oxygen consumption) are made on samples containing large numbers of cells. Such techniques can only indirectly measure the extent of phagocytic ingestion. Both classes of methods provide extremely useful information and enhanced insight into the phagocytic process (Absolom, 1986).

Microscopy is the most direct approach when measuring phagocytosis however, disadvantage of light microscopy is that a lack of resolution makes it difficult to count small particles. An alternate approach is to use labeled particles that can be monitored by measuring their fluorescence, radioactivity or visible absorbance (Hampton and Winterbourn, 1999).

Measurement of phagocytic activity of macrophages was among the earliest techniques for determination of immunologic effects of plant extracts. This assay measures the ability of macrophages to phagocytose zymosan.

Zymosan A, a carbohydrate-rich cell wall preparation derived from yeast *Saccharomyces cerevisiae*, is known to elicit various early functions in monocytes and macrophages such as arachidonic acid mobilization, generation of reactive oxygen intermediates, and lysosomal enzyme release (Czop and Austen, 1985; Kelly and Carchman, 1987; Suzuki *et al*., 1985). In addition, zymosan particles induce an intracellular calcium rise and the hydrolysis of phosphoinositides in macrophages, and both events may activate serine/threonine kinases, such as PKC (Sanguedolce, 1993)

Zymosan A is mainly composed of mannan and β(1-3)-glucan (Di Carlo and Fiore, 1957), these particles are ingested nonopsonically by macrophages via complement receptor type 3 (CR3) (Cabec *et al*., 2000; Ross *et al*., 1985) and mannose receptor (Speert and Silverstein, 1985). Phagocytosis of zymosan through its glucan component has been shown to trigger the production of O_2^- (Astarie-Dequeker *et al*., 1999; Czop and Austen, 1985)

One way to measure respiratory burst activity is by reduction of nitro blue tetrazolium (NBT) to formazan. In the NBT test, the uptake of four electrons by one molecule of NBT results in the formation of a corresponding amount of formazan. NBT is a soluble yellow redox dye which, when present in the extracellular fluid is swept into phagocytic vacuole together with the zymosan particle. In the presence of superoxide anions the dye is chemically reduced, to yield a dark purple insoluble compound (formazan) which can be extracted from the macrophages with dioxane or dimethyl sulfoxide (DMSO). This dark purple formazan has an adsorption maximum at 580 nm, and can be clearly discerned microscopically (Absolom, 1986; Fernandez *et al*., 1999; Manosroi *et al*., 2003; Schopf *et al*., 1984).

In this study, we proposed to observe the effect of Thai medicinal plants on phagocytic activity and IL-12 production of the murine macrophage-like cell line, J774A.1.

This continuous cell line, J774A.1, originating from the cultured murine lymphoblastoid cells, have already been shown to have several characteristics of macrophages. The J774A.1 cells responded effectively to the chemotactic stimulus C5a, but they did not migrate toward lymphocyte-derived chemotactic factor (LDCF). They contained acid phosphatase in the level comparable to that of stimulated peritoneal macrophages (Snyderman *et al*., 1977). This macrophage-like cell line has been so far used in many investigations. The J774A.1 cells had been treated with compound and/or lipopolysaccharide (LPS) for evaluating the effects on phagocytic activity; *e.g.*, the production of nitric oxide and superoxide, the secertion of TNF-α, IL-1, IL-10 and IL-12 (Agostino *et al*., 2001; Blonska *et al*., 2004; Kim, *et al*., 2003; Mudiyanselage *et al*., 2003; Shah *et al*., 2002).

Lymphocyte proliferation assay

Measurement of the proliferative response of lymphocytes is the most commonly used technique for evaluating cell-mediated immune response. Quantitative analysis of proliferative response involves measuring the number of cells in culture in the presence and absence of a stimulatory agent such as an antigen or a mitogen. The most common polyclonal mitogens used to test the proliferation of lymphocytes are concanavalin A (Con A), phytohemagglutin, LPS, and pokeweed mitogen. Con A and phytohemagglutin stimulate T cells, LPS stimulates B cells, and pokeweed mitogen stimulated both T and B cells (Ruscetti and Chervenick, 1975; Novogrodsky and Katchalski, 1971; Perlmann *et al*., 1970; Miller *et al*., 1978).

Several assays have been reported to quantify cellular proliferation. These include: (1) incorporation of radioactive nucleotides, [^{3}H]thymidine or [^{125}I] iododeoxyuridine; (2) cleavage of tetrazolium salt, MTT (Mosmann, 1983) and XTT; (3) turnover of lysosomal hexodaminidase, *p*-nitrophenol-*N*-acetyl-*β*-D-gluxosaminide (NAG test) (Landegren, 1984) and (4) incorporation of 5-bromodeoxyuridine (BrdU), a pyrimidine analogue (Porstmann, 1985). Despite the availability of current non-radioactive assays, [^{3}H]thymidine incorporation assay is

still the most commonly employed assay because of its sensitivity and relative reliability. However, disadvantage of this assay is that radiation hazard to personnel, determination of radioactive counts of each well can take as much as 3-10 min per sample and toxicity due to xylene-based scintillation fluids. All the above radioactive and non-radioactive assays involve multiple steps or reagents and, thus, increase the chance of error. Ahmed *et al*. (1994) presented a new method one-step non-radioactive assay to determine the proliferation of murine lymphocytes, lymphoid tumor cells and hybridoma cells. This assay requires the addition of Alamar Blue dye to cell cultures and the degree of change in its color, which is reflective of the extent of cellular proliferation, can be determined by a microtiter plate reader.

When stimulated with polyclonal mitogen, lymphocytes rapidly enter the G_1 phase and progress through the cell cycle. Proliferation of lymphocytes can be determined by non-radioactive colorimetric assay. This technique employs Alamar Blue dye, which contains an oxidation-reduction (redox) indicator. Cellular proliferation induces chemical reduction of the media which results in a change in redox color from blue to red. The intensity of red color reflects the extent of cellular proliferation. Alamar Blue reduction can be monitored either spectrophotometrically or fluorimetrically. In measuring Alamar Blue reduction spectrophotometrically, absorbance must be read at two wavelengths due to the significant overlap of the absorption spectra of the oxidized (blue) and the reduced forms (red). Two suitable wavelengths are 570 nm (reduced forms) and 600 nm (oxidized forms) (Ahmed *et al*., 1994; Nakayama *et al*., 1997; Zhi-Jun *et al*., 1997).

Cytotoxicity assay

Colorimetric assays of cell viability, activation, and proliferation based on the use of the tetrazolium salt, such as MTT and XTT assays. MTT, commenced in the 1950s and is based on the fact that live cells reduce tetrazolium salts into colored formazan compounds. The biochemical procedure is based on the activity of mitochondria enzymes which are inactivated shortly after cell death. XTT, was first described by P.A. Scudiero in 1988 (Scudiero, 1988). Whilst the use of MTT produced a non-soluble formazan compound, which necessitated dissolving the dye in

order to measure it (Mosmann, 1983) the use of XTT produces a soluble dye. The use of XTT greatly simplifies the procedure of measuring proliferation.

The XTT method is based on the cleavage of the yellow tetrazolium salt, XTT, to form an orange formazan dye by mitochondrial dehydrogenase in metabolic active cells. When XTT entered to cells along with a secondary electron transfer reagent such as menadione or phenazine methosulfate (PMS), results in the formation of and aqueous, soluble formazan product. This eliminated the need for a solubilization step in the procedure. XTT forms an orange-colored water soluble product that can be directly measured by absorption spectrophotometry at wavelengths between 440-490 nm (Rochm, 1991). However, the XTT tetrazolium compound has the disadvantages of limited solubility and stability in solution, requiring preparation of fresh solutions prior to each assay.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Adult male Wistar rats of body weight between 250-300 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. They were allowed free access to food [F.E. Zeulig, Thailand] and water in the Central Animal House, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. Chemicals

Alamar blue [Serotec, England]

Antibiotic-antimycotic (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, 25 μg/ml fungizone) [Gibco Grand Island, U.S.A.]

Concanavalin A (Con A) (from *Canavalia ensiformis*) [Sigma, U.S.A.]

Dimethyl sulfoxide (DMSO) [Fisher Scientific, England]

Dulbecco's Modified Eagle's Medium (DMEM) [Gibco Grand Island, U.S.A.]

Ellipticine [Sigma, U.S.A.]

Fetal bovine serum (FBS) [Seromed, Germany]

Histopaque®-1077 (contains polysucrose 5.7 g/dl and sodium diatrizoate 9.0 g/dl) [Sigma, U.S.A.]

Hydrochloric acid (HCl) [J.T. Baker, U.S.A.]

Lipopolysaccharide (LPS) (from *Escherichia coli* serotype 055: B5) [Sigma, U.S.A.]

Methanol [J.T. Baker, U.S.A]

Nitro blue tetrazolium (NBT) [Sigma, U.S.A]

Potassium chloride (KCl) [Merck, Germany]

Potassium dihydrogen phosphate (KH_2PO_4) [Merck, Germany]

Potassium hydroxide (KOH) [Merck, Germany]

Phenazine methosulfate (PMS) [Sigma, U.S.A.]

RPMI 1640 medium [Gibco Grand Island, U.S.A.]

Sodium bicarbonate (Na_2HCO_3) [Merck, Germany]

Sodium chloride (NaCl) [Merck, Germany]

Disodium hydrogen phosphate (Na_2HPO_4) [Merck, Germany]

Trypan blue [Sigma, U.S.A.]

3,3,-[(phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT) [Sigma, U.S.A]

Zymosan A (from *Saccharomyces cerevisiae*) [Sigma, U.S.A]

3. ELISA kit

Interleukin-2 [(r) IL-2], Rat biotrak ELISA system assay range between 25 to 1,600 pg/ml and sensitivity < 4 pg/ml

Interleukin-12 Total [(1) IL-2], (p40 and p70) Mouse biotrak ELISA system assay range between 47 to 3,000 pg/ml and sensitivity < 12 pg/ml

All ELISA kits were purchased from Amersham biosciences, England.

4. Material for cell culture

96-well flat bottom sterile tissue culture plates [Nunc, Denmark]

15-ml and 50-ml polypropylene conical tubes [Nunc, Denmark]

25-cm^2 and 75-cm^2 tissue culture flasks [Nunc, Denmark]

Acrocap filter and acrodisc filters (0.2 μm) [Gelman Sciences, U.S.A.]

Cell scrapers

Eppendorf vials

5. Instruments

Analytical balance, Autoclave [Hirayama, Japan], CO_2 incubator [Forma Scientific, U.S.A.], Haemacytometer, Hot air oven, Inverted microscope [Olympus® CK30/CK40], Laminar flow hood [Issco model BV 225, U.S.A.], Microplate reader [Anthos htl, Australia], Peristaltic pump, pH meter, Pipette aid, Refrigerated centrifuge [EBA12R Hettich Centrifuge, U.S.A], Refrigerator -20 °C, Refrigerator -80 °C, Single channel and eight-channel micropipettes, Sonicator [Elma, Germany],

Vortex mixer, and Water bath were supplied by Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

6. Plant materials (Table 3)

Plants were collected from Khao Chong, Trang in March, 2002. Except for *Harpullia arborea, Hymenodictyon excelsum and Limnophila rugosa* were collected from Mae wong National park, Kampangpet in May, 2001. *Ellipeiopsis cherrevensis* was collected from Sakaerat, Nakhon Ratchasima in October, 2002. *Cleome viscosa, Euphorbia hirta* and *Eurycoma longifolia* were collected from Bangkok in June, 2003.

Plants were extracted by Assistant Professor Dr. Rutt Suttisri's group, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each plant was extracted with 95% ethanol (crude ethanolic extract; code 001). The active plant extracts were fractionated and further purified. Briefly, ethanolic extract was partitioned with hexane (code 002), chloroform (code 003), and aqueous (code 004). The extraction scheme was shown in Figure 20 in the appendix. The scheme was used for all active plant extracts, except for *Stelechocarpus cauliflorus.*

The dried leaves of *Stelechocarpus cauliflorus* were separately macerated with hexane (code 002), ethyl acetate (code 003) and methanol (code 004). The extraction scheme of *Stelechocarpus cauliflorus* was shown in Figure 21 in the appendix.

The extract, which showed strong immunostimulant activity, was fractionated through silica gel column and eluted with mixture of hexane-ethylacetate-methanol led to the isolation of pure compound (see details in the appendix).

All plant extracts and pure compounds were prepared into stock solutions at the concentration of 40 mg/ml in DMSO. The stock solutions were stored at -20 °C until used.

Table 3 Plant materials used in this study.

Number	Code	Thai name	Scientific name	Family	Part of use
1	AK001	ลางสาดดง	*Aglaia korthalsii*	Meliaceae	Leaves
2	CV001	ผักเสี้ยนผี	*Cleome viscosa*	Capparidaceae	Stems
3	EC001	นมแมวป่า	*Ellipeiopsis cherrevensis*	Annonaceae	Leaves
4	EH001	น้ำนมราชสีห์	*Euphorbia hirta*	Euphorbiaceae	Stems
5	EL001	ปลาไหลเผือก	*Eurycoma longifolia*	Simaroubaceae	Roots
6	HA001	หอมไกลดง	*Harpullia arborea*	Sapindaceae	Leaves
7	HE001	อุโลก	*Hymenodictyon excelsum*	Rubiaceae	Leaves
8	LA001	ผักแขยง	*Limnophila aromatica*	Scrophulariaceae	Stems
9	LR001	ผักกระโฉม	*Limnophila rugosa*	Scrophulariaceae	Leaves
10	MM001	นางแดง	*Mitrephora maingayi*	Annonaceae	Stems
11	OB001	พริกแดง	*Orophea brandisii*	Annonaceae	Leaves
12	PR001	สังหยูดำ	*Pseuduvaria rugosa*	Annonaceae	Leaves
13	PS001	สังหยูใบขน	*Pseuduvaria setosa*	Annonaceae	Leaves
14	RA001	กริม	*Rinorea anguifera*	Violaceae	Stems
15	SC001	งำเงาะ	*Stelechocarpus cauliflorus*	Annonaceae	Leaves

Methods

1. Cell preparation

1.1 Splenic lymphocyte preparation

Spleens were obtained from Wistar rat under sterile condition and submerged in RPMI 1640 with 1% antibiotic-antimycotic and stored at 4 °C until use. Spleens were grinded with syringe and gently teased on nylon mesh to obtain single cell suspension in a sterile disposable 60-mm Petri dish containing 5 ml of RPMI 1640. The cell suspensions were then layered onto histopaque-1077 and were centrifuged at 5,500 rpm for 20 min at 25 °C to isolate mononuclear cells. The buffy coat containing lymphocytes was collected and suspended in RPMI 1640 and centrifuged at 5,500 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in complete RPMI 1640 medium supplement with 10% heat inactivated-fetal bovine serum. The viability of the isolated splenic lymphocytes determined by trypan blue exclusion test, was >95%.

1.2 J774A.1 cell culture

The murine macrophage-like cell line, J774A.1 (ATCC Number TIB-67), was obtained from Dr. Poonlarp Cheepsunthorn, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. The cells were maintained in 75-cm^2 plastic culture flasks in complete DMEM medium containing 10% heat inactivated-fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were harvested by gentle scarping and were passaged every 3-4 days. The cell viability was assayed by trypan blue exclusion method (Kim *et al*., 2003).

2. *In vitro* cytotoxicity assay

2.1 *In vitro* cytotoxicity to splenic lymphocytes determined by using Alamar Blue method

The splenic lymphocyte suspension was adjusted to 2.5×10^6 cells/ml in complete RPMI 1640 medium. One hundred microlitres of the suspension were placed in 96-well sterile culture plate containing 10 μl of two-fold dilution of 12.5 – 200 μg/ml of plant extract or compound or 0.5% DMSO as vehicle control or 5 μg/ml of Ellipticine as positive control, and then 90 μl of complete RPMI 1640 medium were added. The plate was incubated in 5% CO_2 under humidified conditions at 37 °C for 48 hrs.

After the incubation period of 48 hrs, 20 μl/well of Alamar Blue were added and the plate was re-incubated for 24 hrs. Since Alamar Blue contained a oxidation-reduction (Redox) indicator, and cellular proliferation induced chemical reduction of the media which resulted in a change in Redox color from blue to red. The intensity of red color reflected the extent of cellular proliferation. The plate was then measured the absorbance at 570 nm (reduced form) and 600 nm (oxidized form) using microplate reader. Specific absorbance (Specific OD), obtained by subtracting the absorbance at 600 nm from that of 570 nm, was used in the calculation for % cytotoxicity.

$$\% \text{ cytotoxicity} = \left(\frac{\text{specific OD (control)} - \text{specific OD (sample)}}{\text{specific OD (control)}} \right) \text{x } 100$$

2.2 *In vitro* cytotoxicity to J774A.1 cell line determined by using XTT reduction assay

The J774A.1 macrophage cells were adjusted to 5×10^5 cells/ml in complete DMEM medium. One hundred and ninety microlitres of the suspension were placed in 96-well sterile culture plate containing 10 μl of two-fold dilution of 12.5 - 200 μg/ml of plant extract or compound or 0.5% DMSO as vehicle control or 5 μg/ml of Ellipticine as positive control. The plate was incubated in 5% CO_2 under humidified conditions at 37 °C for 24 hrs.

Macrophages were treated for 24 hrs and then washed by aspirating with PBS. Cells were incubated with 1 mg/ml of XTT and 25 μM of PMS. Incubation was continued for 4 hrs, and the colorimetric determination of XTT formazan product, which was soluble in aqueous solution, was measured spectrophotometrically at 450 nm. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ cytotoxicity} = \left(\frac{OD_{450nm} \text{ (control)} - OD_{450nm} \text{ (sample)}}{OD_{450nm} \text{ (control)}} \right) \times 100$$

3. *In vitro* lymphocyte proliferation assay determined by using Alamar Blue method

The splenic lymphocyte suspension was adjusted to 2.5×10^6 cells/ml in complete RPMI 1640 medium. One hundred microlitres of the suspension were placed in 96-well sterile culture plate containing 10 μl of two fold dilution of 1.6 - 200 μg/ml of plant extract or compound or 0.5% DMSO as vehicle control or 5 μg/ml of Con A or 50 μg/ml of LPS as positive control, and then 90 μl of complete RPMI 1640 medium were added. The plate was incubated in 5% CO_2 under humidified conditions at 37 °C for 48 hrs.

After the incubation period of 48 hrs, 20 μl/well of Alamar Blue were added and the plate was re-incubated for 24 hrs. Since Alamar Blue contained an oxidation-reduction (Redox) indicator, and cellular proliferation induced chemical reduction of the media which resulted in a change in Redox color from blue to red.

The intensity of red color reflected the extent of cellular proliferation. The plate was then measured the absorbance at 570 nm (reduced form) and 600 nm (oxidized form) using microplate reader. Specific absorbance (Specific OD), obtained by subtracting the absorbance at 600 nm from that of 570 nm, was used in the calculation for % stimulation.

$$\% \text{ stimulation} = \left(\frac{\text{specific OD (sample)} - \text{specific OD (control)}}{\text{specific OD (control)}} \right) \text{ x } 100$$

4. *In vitro* phagocytosis assay on NBT reduction of J774A.1 macrophage cell line

The J774A.1 cells were adjusted to 5×10^5 cells/ml in complete DMEM medium. One hundred and ninety microlitres of the suspension were placed in 96-well sterile culture plate containing 10 μl of two-fold dilution of 1.6 - 200 μg/ml of plant extract or compound or 0.5% DMSO as vehicle control or 5 μg/ml of LPS as positive control. The plate was incubated in 5% CO_2 under humidified conditions at 37 °C for 24 hrs.

Macrophages were treated for 24 hrs and then washed twice with DMEM medium. Cells were incubated with 800 μg/ml of zymosan and 600 μg/ml of NBT. The cell culture was incubated for further 60 min and the adherent macrophages were rinsed vigorously with DMEM, followed by three times washing with 200 μl methanol to eliminate the unreduced NBT dye. The cell pellets were air-dried. An amount of 120 μl of 2 M KOH and 140 μl of DMSO were added, consecutively. The absorbance of the turquoise blue solution was measured at 570 nm by microplate reader. The percentage of NBT reduction or % phagocytosis was calculated by the following equation:

$$\% \text{ phagocytosis} = \left(\frac{OD_{570nm} \text{ (sample)} - OD_{570nm} \text{ (control)}}{OD_{570nm} \text{ (control)}} \right) \text{ x } 100$$

5. Measurement of cytokine production by ELISA method

5.1 Measurements of IL-2

The splenic lymphocytes (2.5×10^6 cells/ml) were treated with crude extracts or pure compounds at concentrations which exhibited the highest percent stimulation on lymphocyte proliferation for 24 hrs and then the supernatants were harvested and any cells in the supernatants were removed by centrifugation at 1,000 rpm for 5 min at 4 °C. The supernatant was collected and stored at –80 °C in small aliquots until use for cytokine assay. IL-2 production by splenic lymphocytes was assayed by using commercial rat IL-2 ELISA kits.

5.2 Measurements of IL-12

The J774A.1 cells (5×10^5 cells/ml) were treated with crude extracts or pure compounds at concentrations which exhibited the highest percent stimulation on lymphocyte proliferation for 24 hrs. Then, the culture supernatants were collected and stored at –80 °C in small aliquots. The IL-12 production by J774A.1 cells was assayed by using commercial mouse IL-12 ELISA kits.

5.3 ELISA procedure

The 50 µl of standard diluent was pipetted into each well, followed by the addition of 50 µl of standard or supernatant to each well in duplicate. IL-2 ELISA assay plate was incubated in 5% CO_2 under humidified conditions at 37 °C for 1.5 hrs, while IL-12 ELISA assay plate was incubated in room temperature (20-25 °C) for 1 hr. At the end of the incubation period, the plate was washed three times with washing buffer using automatic washer. 100 µl of the biotinylated antibody reagent were added to all wells and incubated at the same condition. Then, the plate was washed and 100 µl of streptavidin-HRP solution were added. The assay plate was further incubated for 30 min in 5% CO_2 under humidified conditions at 37 °C for IL-2 ELISA assay, while IL-12 ELISA assay was incubated in room temperature. Then, the plate was washed and 100 µl of TMB substrate solution were added. The enzymatic color reaction was allowed to develop at room temperature for 30 min. The substrate reaction yielded a blue solution that turned yellow when stop solution

was added. After 30 min, the reaction was stopped by adding 100 μl of stop solution to each well. The plate was then measured the absorbance at 450 nm using a microplate reader.

6. Statistical analysis

All results were presented as the mean values ± S.E.M. from two or three independent cultures, with duplicate or triplicate or quadruplicate replications in each experiment. Differences among means were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA followed by Scheffe test. All statistical analysis was performed according to the statistical program, SPSS. A value of $p < 0.01$ or $p < 0.05$ was considered to be significant.

CHAPTER IV

RESULTS

1. *In vitro* Cytotoxicity assay

The maximal concentration of DMSO that was not cytotoxic to cell culture was 0.5% (Keattikunpairoj, 2002). Therefore, in this study, the final concentration of DMSO in all plant extract solution was 0.5%.

The rat splenic lymphocytes and J774A.1 macrophage cells were treated with two-fold dilution of crude plant extract, concentrations vary between 12.5 to 200 μg/ml. The 50% cytotoxic concentration (CC_{50}) values of all plant ethanolic extracts (list in Table 3) were showed in Table 4. AK001, CV001, EL001 and PR001 were most cytotoxic to splenic lymphocytes with the CC_{50} values between 1 to 50 μg/ml. EH001, HA001, LA001, OB001 and PS001 possessed moderate cytotoxic activity with the CC_{50}, values between 50 to 100 μg/ml. RA001 was weakly cytotoxic to splenic lymphocytes, its CC_{50} value was between 100 to 200 μg/ml. On the other hand, HE001, LR001, MM001 and SC001 were not cytotoxic at the concentration of 200 μg/ml.

Most of the tested plant extracts were not toxic to J774A.1 cells. They had high CC50 values (> 200 μg/ml). Except for AK001, EC001 and HA001, the CC_{50} values were 77.7 ± 2.0 μg/ml, 138.3 ± 6.1 μg/ml, and 165.8 ± 8.3 μg/ml; respectively. The plant extracts were further tested in lymphocyte proliferation assay and macrophage phagocytosis assay at the non-cytotoxic concentration.

Table 4 50% cytotoxic concentration (CC_{50}) of crude extracts on splenic lymphocytes and J774A.1 macrophage cell line.

Sample	CC_{50} (μg/ml)[a]	
	J774A. 1 cell line	**Splenic lymphocytes**
Ellipticine	< 5	< 5
Con A	> 20	> 20
LPS	> 100	> 100
AK001	77.7 ± 2.0	38.4 ± 2.3
CV001	> 200	26.2 ± 0.7
EC001	138.3 ± 6.1	< 12.5
EH001	> 200	51.7 ± 9.2
EL001	> 200	42.8 ± 2.0
HA001	165.8 ± 8.3	55.3 ± 4.1
HE001	> 200	> 200
LA001	> 200	87.4 ± 6.4
LR001	> 200	> 200
MM001	> 200	> 200
OB001	> 200	65.5 ± 9.4
PR001	> 200	41.2 ± 3.2
PS001	> 200	99.8 ± 13.1
RA001	> 200	143.4 ± 8.2
SC001	> 200	> 200

[a]The 50% cytotoxic concentration (CC_{50}) expressed as the mean ± S.E.M. of two or three independent experiments, with triplicate replication in each experiment (n=2, n=3).

2. *In vitro* lymphocyte proliferation assay

2.1 Effect of Con A and LPS on rat splenic lymphocytes

Representative T cell and B cell mitogen used in this study is Con A and LPS, respectively. Concentration-dependent curve of the two mitogens are shown in Figure 8 and Figure 9. Con A at a concentration of 20 μg/ml and LPS at a concentration of 100 μg/ml induced minimal effect on lymphocyte proliferation. Both mitogens dose-dependently stimulated the splenic lymphocyte proliferation. In all following experiments, 5 μg/ml of Con A and 50 μg/ml of LPS were chosen as positive control based on the previous reports (Zheng et al., 1998; Miller et al., 1978).

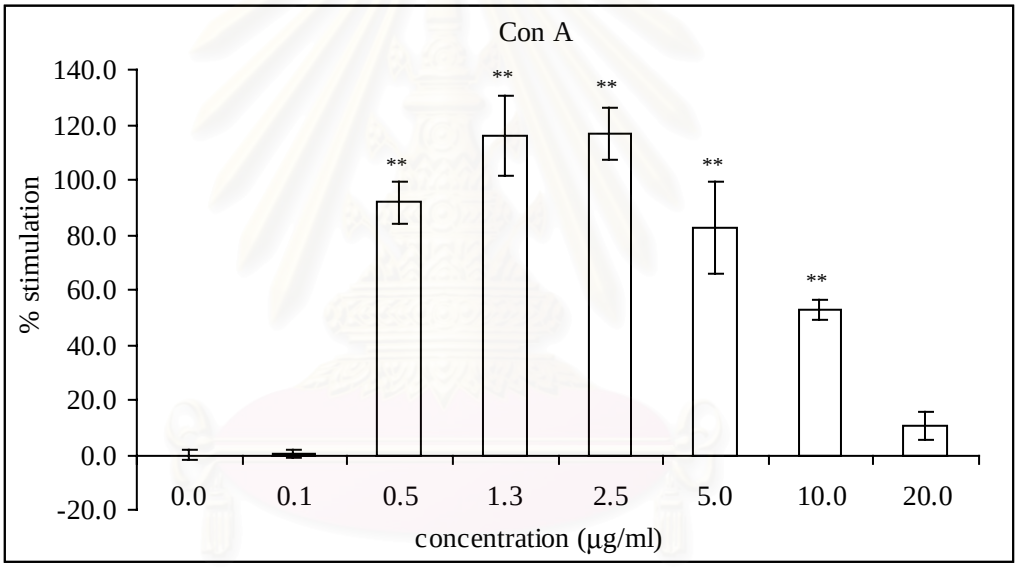


Figure 8 Concentration-dependent curve of Con A-induced proliferation of lymphocytes. The splenic lymphocytes were incubated with 0.01-20 μg/ml Con A for 96 hrs before determination of Alamar Blue reduction assay. Each column with vertical bar expressed as mean ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

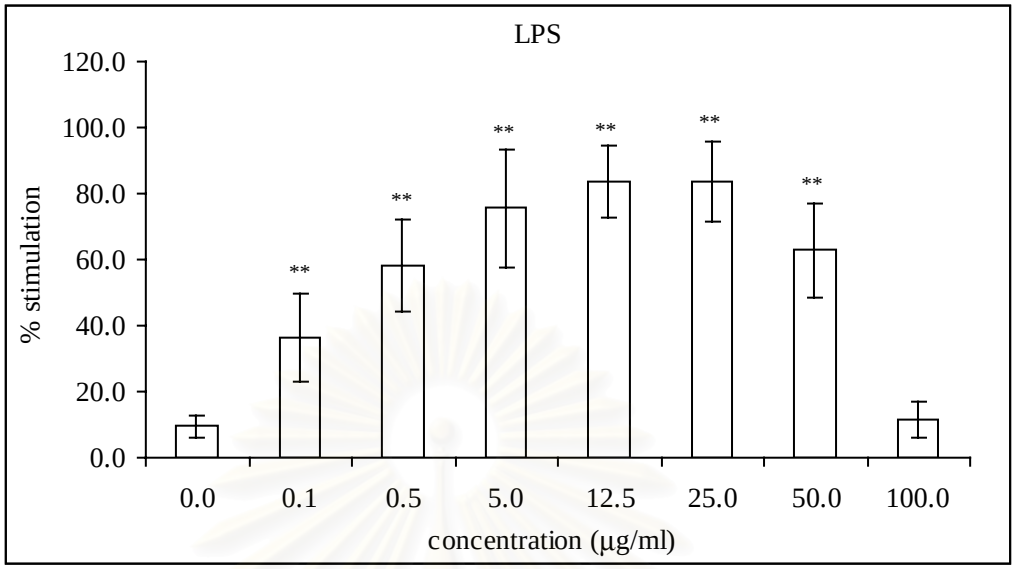


Figure 9 Concentration-dependent curve of LPS-induced proliferation of lymphocytes. The splenic lymphocytes were incubated with 0.01-100 μg/ml LPS for 96 hrs before determination of Alamar Blue reduction assay. Each column with vertical bar expressed as mean ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

2.2 Screening of crude plant extracts on the proliferation of splenic lymphocytes

All crude ethanolic extracts were tested at the concentration which was not cytotoxic to the splenic lymphocytes. Five plant extracts (LR001, HA001, MM001, PS001 and SC001) markedly stimulated the proliferation of spleen cells at the concentration of 12.5 μg/ml (Figure 10). LR001 showed the highest stimulation activity at this concentration (44.5 ± 2.4 %). In contrast, EC001 was very toxic to the splenocytes at 12.5 μg/ml. Nine plant extracts did not enhanced the proliferation of the spleen cells at this concentration (Table 5).

Four crude ethanolic extracts, which found to possess strong stimulation effect on lymphocyte proliferation, were further fractionated into three fractions by changing polarity of the solvent. The LR001 extract was not fractionated because of the limitation of this plant material. Thus, LR001 was only determined for dose-dependent effect which were showed in Figure 11. The highest stimulatory activity was at the concentration of 12.5 μg/ml (44.5 ± 2.4 %).

The plant extracts gave similar dose-response pattern to those of the positive compounds, i.e., Con A (Figure 8) and LPS (Figure 9). The higher concentrations appeared to inhibit the splenic lymphocyte proliferation.

Table 5 Effect of the crude ethanolic extracts on splenic lymphocyte proliferation.

Sample code	Tested concentration (μg/ml)[a]	% stimulation[a]	% stimulation at 12.5 μg/ml
Con A	5	82.3 ± 16.7**	ND
LPS	50	62.9 ± 14.4 **	83.6 ± 10.9**
AK001	12.5	NS	NS
CV001	12.5	NS	NS
EC001	12.5	Toxic	Toxic
EH001	12.5	NS	NS
EL001	12.5	NS	NS
HA001	12.5	20.6 ± 4.5*	20.6 ± 4.5*
HE001	50	NS	NS
LA001	25	NS	NS
LR001	200	16.9 ± 1.6	44.5 ± 2.4**
MM001	25	6.22 ± 2.17	14.0 ± 4.5*
OB001	25	NS	NS
PR001	12.5	NS	NS
PS001	50	4.06 ± 7.64	43.3 ± 13.1**
RA001	50	NS	NS
SC001	50	18.3 ± 4.8*	39.3 ± 2.7**

[a]All crude extracts were tested at concentration which was not cytotoxic to spleen lymphocytes. After 72 hrs of incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each data represented the mean value with S.E.M. of three different experiments (n=3). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. ND is not determined; NS is not stimulated.

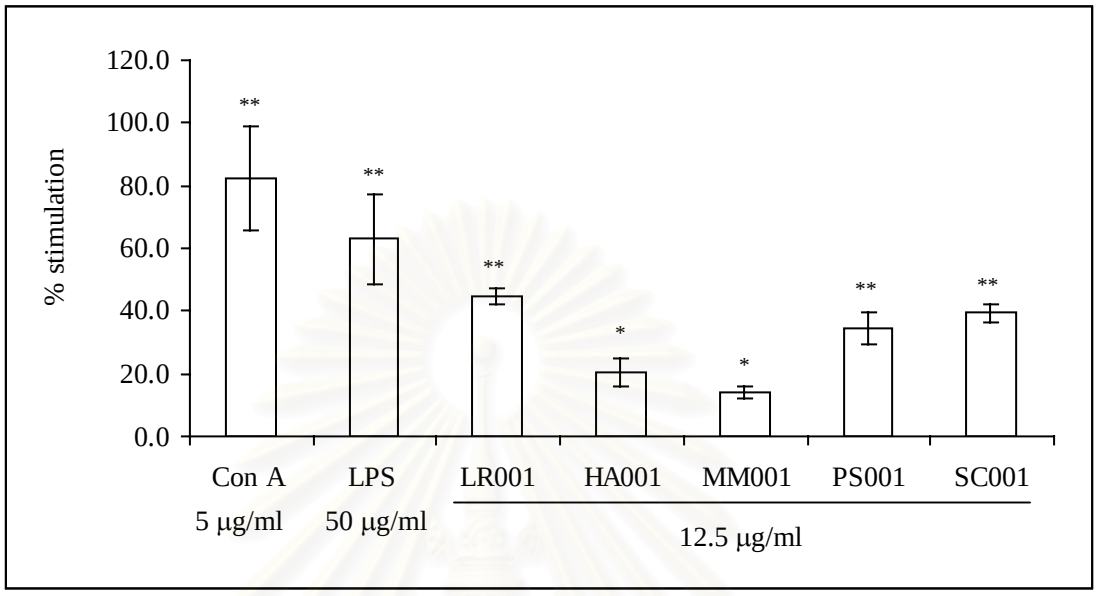


Figure 10 Effect of crude ethanolic extracts on the proliferation of lymphocytes. All crude extracts were tested at concentration 12.5 μg/ml for 72 hrs. After incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each value represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$), compared to the vehicle control.

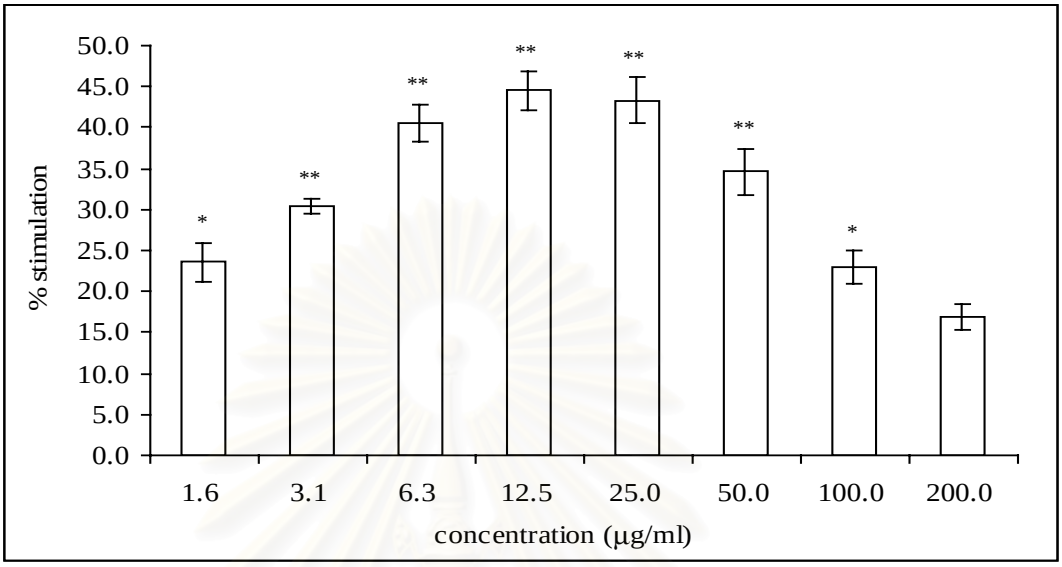


Figure 11 Concentration-dependent curve of *Limnophila rugosa*. LR001 was tested at concentrations ranging between 1.56-200 μg/ml. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each column represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$), compared to the vehicle control.

2.3 Effect of *Harpullia arborea* extracts on rat splenic lymphocytes

The cytotoxic effect and lymphocyte proliferation stimulating effect of samples from Harpullia arborea were shown in Table 6 and Figure 12. Crude ethanolic extract (HA001) was found to have stimulating effect on lymphocyte proliferation. Therefore, this ethanolic extract was further fractionated by partition to yield hexane (HA002), chloroform (HA003) and aqueous (HA004) fractions.

The HA003 fraction, and HA004 fraction were not toxic to J774A.1 cells, their CC_{50} values were higher than 200 μg/ml. In contrast, the crude ethanolic extract (HA001) and HA002 fraction were weakly toxic, their CC_{50} values were 165.8 ± 8.3 μg/ml and 135.3 ± 24.8 μg/ml, respectively. While HA001, HA002, and HA003 showed strong cytotoxicity against splenic lymphocytes, the aqueous fraction (HA004) was not toxic. In addition, the HA004 fraction showed dose-dependent enhancement of lymphocyte proliferation. Repeated chromatography of this aqueous fraction over silica gel led to the isolation of quebrachitol (Me_5) structure is showed in Figure 22 in the appendix. Quebrachitol also demonstrated concentration-dependently stimulation of splenic cell proliferation, without any toxic toward both splenic lymphocytes and J774A.1 cells. The maximal enhancing activity (45.7 ± 1.5 %) of this compound was found at the concentration of 50 μg/ml (257.7 μM) (Figure12).

Table 6 The cytotoxic effect and the stimulation of lymphocyte proliferation effect of *Harpullia arborea*.

Code	Extract	CC_{50} (μg/ml)[a]		Tested concentration (μg/ml)[b]	% stimulation (lymphocyte proliferation)
		J774A.1 Cell line	Splenic lymphocytes		
HA001	Crude EtOH	165.8 ± 8.3	55.3 ± 4.1	12.5	20.6 ± 4.5*
HA002	Hexane	135.3 ± 24.8	< 12.5	12.5	Toxic
HA003	$CHCl_3$	> 200	46.9 ± 5.9	12.5	NS
HA004	Aqueous	> 200	> 200	200	9.83 ± 5.77
Me_5	HA004	> 100	> 100	100	43.1 ± 6.8**

[a]The 50 % cytotoxic concentration (CC_{50}) data represented the mean ± S.E.M. of two or three independent experiments, with triplicate replication in each experiment (n=2, n=3).

[b]All plant extracts were tested at concentration which were not cytotoxic to the splenic lymphocytes. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** $p < 0.01$, * $p < 0.05$ determined by One-way ANOVA. NS is not stimulated.

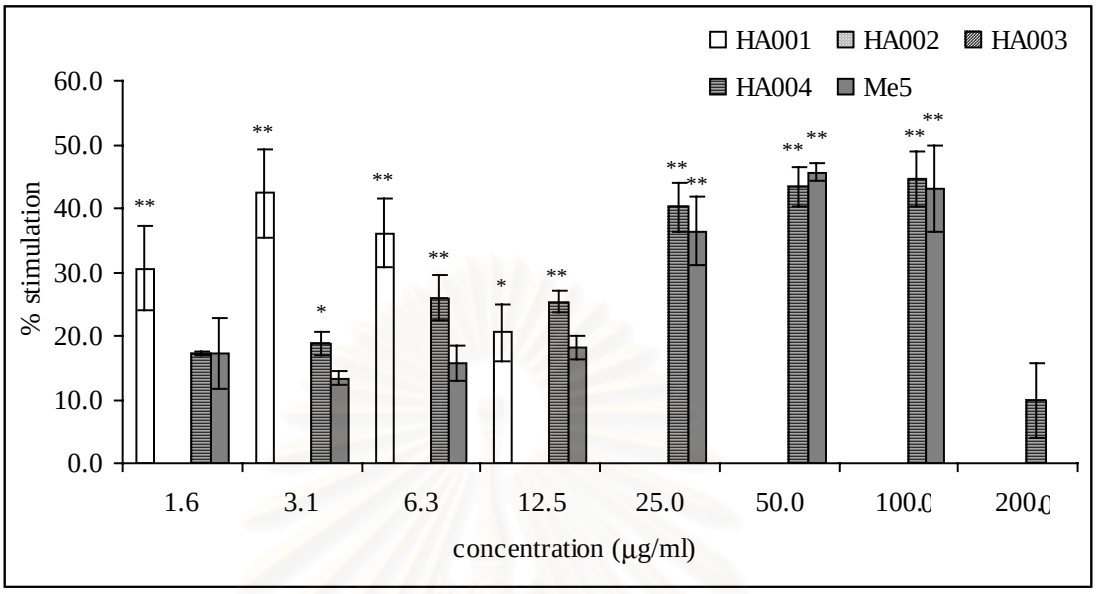


Figure 12 Concentration-dependent curve of samples from *Harpullia arborea*. All plant extracts and a pure compound were tested at various non-toxic concentrations ranging between 1.56-200 µg/ml for 72 hrs. Except for HA002 and HA003 were tested at concentration between 12.5-200 µg/ml for 72 hrs. After incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each column with a vertical bar represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Significant was determined by One-way ANOVA (** $p < 0.01$, *$p < 0.05$), compared with vehicle control.

2.4 Effect of *Mitrephora maingayi* extracts on rat splenic lymphocytes

Effects of plant extracts on splenocyte and J774A.1 cell survival and lymphocyte proliferation were shown in Table 7 and Figure 13. All samples were not cytotoxic to J774A.1 cells but have some effect on the spleen cells. From previously screening for promoting lymphocyte proliferation, the crude ethanolic extract *Mitrephora maingayi* (MM001) was shown to be active. Therefore, this ethanolic extract was fractionated into three fractions by partition with solvent to yield the hexane (MM002), chloroform (MM003) and aqueous (MM004) fractions.

From Table 7, MM002 and MM003 fractions were weakly toxic to splenic lymphocytes. The MM002 and MM003 fractions, at the concentration of 12.5 μg/ml, were exhibited strong stimulatory effect on lymphocyte proliferation (Figure 13). Therefore, MM002 was further purified by column chromatography, which yielded a kaurane-type diterpene, identified as kaurenoic acid (MHF) structure is showed in Figure 23 in the appendix. Kaurenoic acid demonstrated dose-dependently lymphocyte proliferation stimulation. The maximum stimulation effect (37.7 ± 0.9 %) of this compound was detected at the concentration of 12.5 μg/ml (41.4 μM) as showed in Figure 13.

Table 7 The cytotoxic effect and the enhancement of lymphocyte proliferation of *Mitrephora maingayi*.

Code	Extract	CC_{50} (μg/ml)[a]		Tested concentration (μg/ml)[b]	% stimulation (lymphocyte proliferation)
		J774A.1 cell line	Splenic lymphocytes		
MM001	Crude EtOH	> 200	> 200	25	6.22 ± 2.17
MM002	Hexane	> 200	91.3 ± 5.3	50	6.56 ± 2.23
MM003	$CHCl_3$	> 200	151.4 ± 12.0	25	0.41 ± 0.15
MM004	Aqueous	> 200	> 200	25	2.82 ± 0.71
MHF	MM002	> 100	> 100	25	32.1 ± 1.4**

[a]The 50 % cytotoxic concentration (CC_{50}) data represented the mean ± S.E.M. of two or three independent experiments, with triplicate replication in each experiment (n=2, n=3).

[b]All plant extracts were tested at concentrations which were not cytotoxic to spleen lymphocytes. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** $p < 0.01$, *$p < 0.05$ determined by One-way ANOVA.

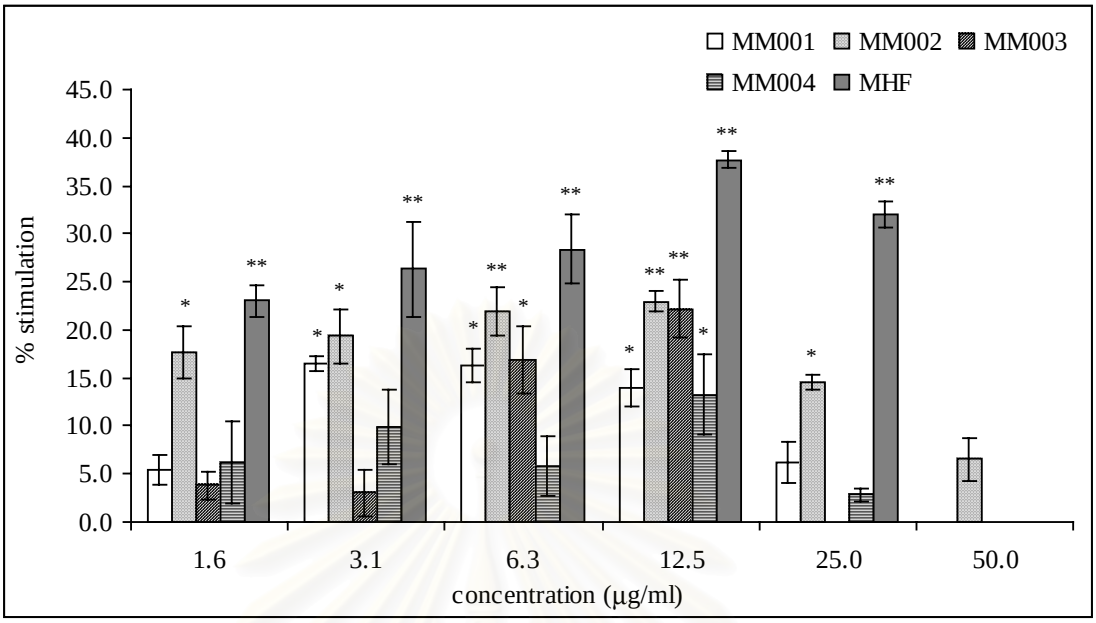


Figure 13 Concentration-dependent curve of samples from *Mitrephora maingayi*. All plant extracts and a pure compound were tested at various concentrations between 1.56-200 µg/ml. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each column with a vertical bar represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Significant was determined by One-way ANOVA (** $p < 0.01$, * $p < 0.05$), compared with vehicle control.

2.5 Effect of *Pseuduvaria setosa* extracts on rat splenic lymphocytes

Effects of extract and pure compounds prepared from Pseuduvaria setosa on rat splenic lymphocytes and J774A.1 cells were shown in Table 8 and Figure 14. All samples were not cytotoxic to J774A.1 cells but have some effect on rat splenocytes. The crude ethanolic extracts (PS001) were found to stimulate lymphocyte proliferation. Therefore, this ethanolic extract (PS001) was fractionated into three fractions by partition to get hexane (PS002), chloroform (PS003) and aqueous (PS004) fractions.

Although the chloroform fraction (PS003) was moderately cytotoxic to splenic lymphocytes, it markedly stimulated lymphocyte proliferation at the concentration of 6.25 μg/ml with % stimulation value of 69.0 ± 15.6 % (Figure 14). Thus, the PS003 fraction was further purified by column chromatography, which yielded two oxoaporphines, identified as 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (PS-1) and ouregidione (PS-2). Their structures are showed in Figure 24 in the appendix. Both compounds demonstrated concentration-dependent enhancement of lymphocyte proliferation (Figure 14b). The maximum stimulating activity of the PS-1 was detected at 6.3 μg/ml (18.0 μM) (69.0 ± 5.6 %) while that of PS-2 was detected at 12.5 μg/ml (37.2 μM) (88.1 ± 15.2 %).

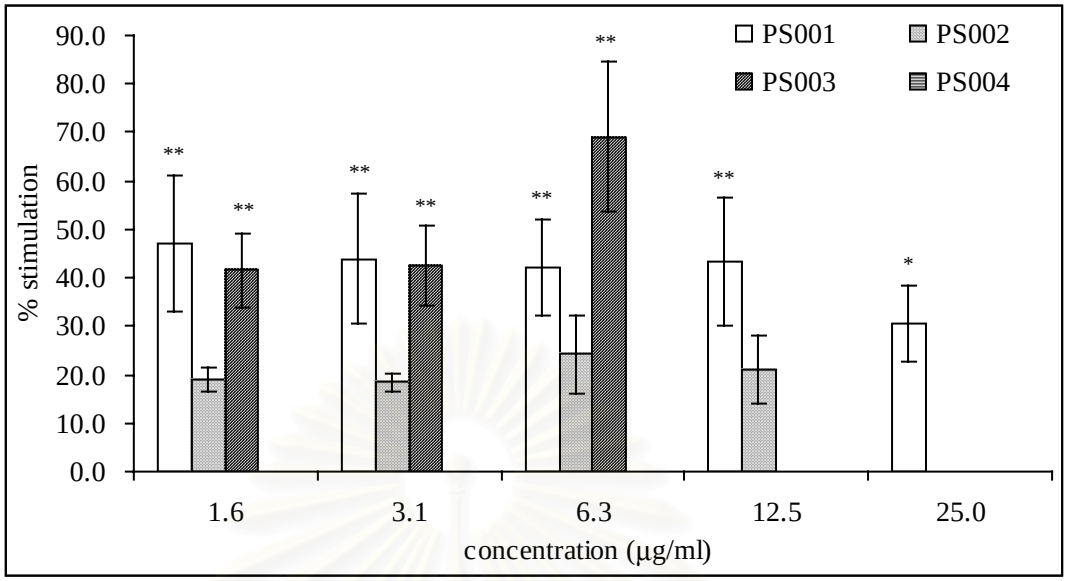
Table 8 The cytotoxic effect and the lymphocyte proliferation stimulation of *Pseuduvaria setosa*.

Code	Extract	CC_{50} (μg/ml)[a]		Tested concentration (μg/ml)[b]	% stimulation (lymphocyte proliferation)
		J774A.1 cell line	Splenic lymphocytes		
PS001	Crude EtOH	> 200	99.8 ± 13.1	50	4.06 ± 7.64
PS002	Hexane	> 200	76.6 ± 8.1	12.5	21.1 ± 7.1
PS003	$CHCl_3$	> 200	41.0 ± 3.1	6.25	69.0 ± 15.6**
PS004	Aqueous	> 200	> 200	200	4.26 ± 0.59
PS-1	PS003	> 100	> 100	50	17.7 ± 1.4
PS-2	PS003	> 100	> 100	100	38.4 ± 0.6**

[a]The 50 % cytotoxic concentration (CC_{50}) value represented the mean ± S.E.M. of two or three independent experiments, with triplicate replication in each experiment (n=2, n=3).

[b]All plant extracts were tested at concentrations which were not cytotoxic to spleen lymphocytes. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** $p < 0.01$, * $p < 0.05$ determined by One-way ANOVA.

(a)



(b)

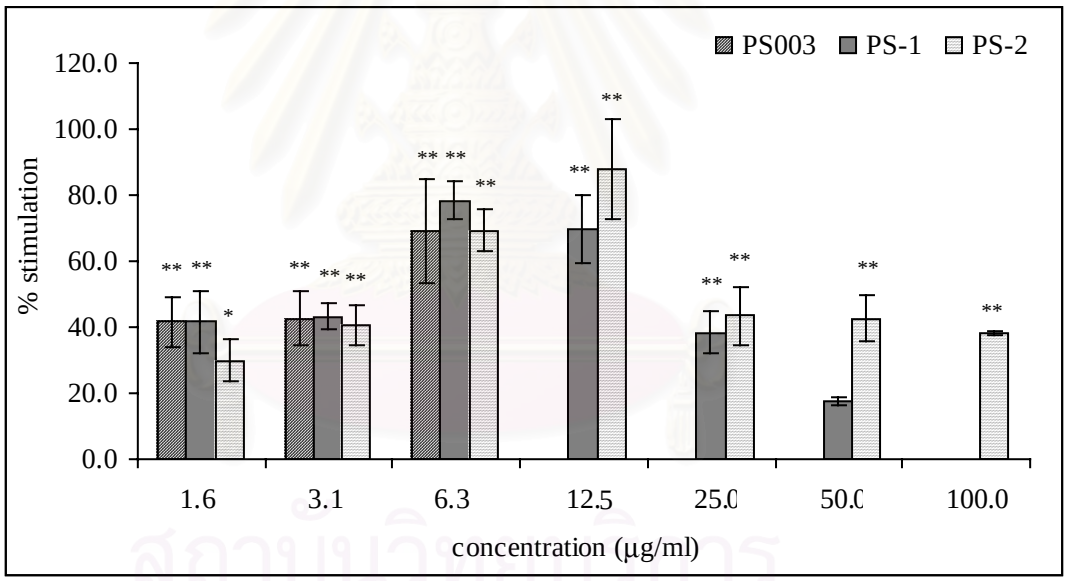


Figure 14 Concentration-dependent curve of samples from *Pseuduvaria setosa*. (a) Fraction of *Pseuduvaria setosa*. (b) isolated pure compounds. All plant extracts and isolated pure compound were tested at various concentrations ranging between 1.56-200 µg/ml. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each column with a vertical bar represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Significant was determined by One-way ANOVA (** $p < 0.01$, * $p < 0.05$), compared with vehicle control.

2.6 Effect of *Stelechocarpus cauliflorus* extracts on rat splenic lymphocytes

Effects of plant extracts on splenic lymphocyte and murine macrophage cell survival, and lymphocyte proliferation were shown in Table 9 and Figure 15. All samples were not cytotoxic to J774A.1 cell but has some effect on the spleen cells. Crude ethanolic extract of Stelechocarpus cauliflorus (SC001) was found to enhance lymphocyte proliferation. Therefore, this ethanolic extract was fractionated into three fractions by maceration method to yield hexane (SC002), chloroform (SC003) and methanol (SC004) fractions.

As shown in Table 9, the SC002 and SC003 fractions possessed moderate cytotoxic activity with the CC50 values between 50 to 100 μg/ml, while the SC004 fraction is weakly toxic to splenic lymphocytes, its CC50 value of 124.6 ± 24.4 μg/ml. The SC002 and SC004 fractions enhanced lymphocytes proliferation at the non-toxic concentration. The SC004 fraction has not been fractionated yet. The SC002 fraction was further purified by column chromatography, which yielded a β-sitosterol (SCL-2) (Figure 25 in the appendix). SCL-2 was also capable to stimulated splenic lymphocyte proliferation in a concentration-dependent manner (Figure 15). The maximal activity of this compound (41.35 ± 6.8 %) was observed at the concentration of 25 μg/ml (60.4 μM).

Table 9 The cytotoxic effect and the lymphocyte proliferation enhancement of *Stelechocarpus cauliflorus.*

Code	Extract	CC_{50} (μg/ml)[a]		Tested concentration (μg/ml)[b]	% stimulation (lymphocyte proliferation)
		J774A.1 cell line	Splenic lymphocytes		
SC002	Hexane	>200	85.5 ± 9.5	25	18.7 ± 2.1*
SC003	EtOAc	> 200	66.7 ± 6.3	12.5	13.0 ± 0.8
SC004	MeOH	> 200	124.6 ± 24.4	50	30.2 ± 13.9 **
SCL-2	SC002	> 100	> 100	100	20.6 ± 3.1*

[a]The 50 % cytotoxic concentration (CC_{50}) data represented the mean ± S.E.M. of two or three independent experiments, with triplicate replication in each experiment (n=2, n=3).

[b]All plant extracts were tested at concentrations which were not cytotoxic to spleen lymphocytes. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** $p < 0.01$, * $p < 0.05$ determined by One-way ANOVA.

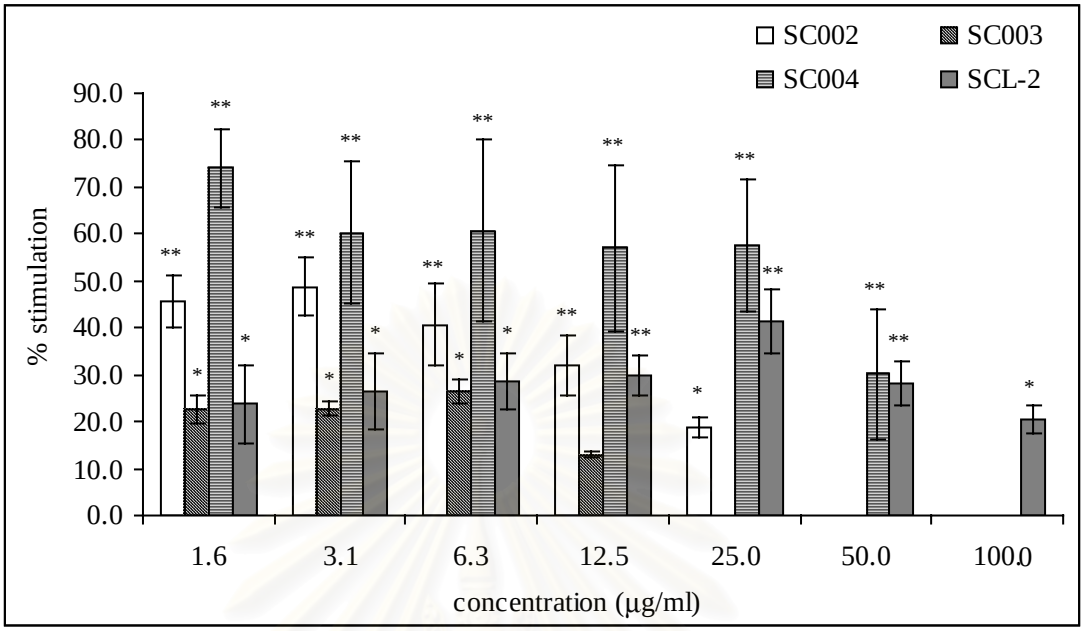


Figure 15 Concentration-dependent curve of *Stelechocarpus cauliflorus*. All plant extracts and an isolated pure compound were tested at various concentrations ranging between 1.56-200 µg/ml. After 72 hrs incubation, the degree of the lymphocyte proliferation was measured by Alamar Blue assay. Each column with a vertical bar represented the mean value ± S.E.M. of three independent experiments, with triplicate replication in each experiment (n=3). Significant was determined by One-way ANOVA (** $p < 0.01$, *$p < 0.05$), compared with vehicle control.

3. *In vitro* phagocytosis assay

3.1 Effect of LPS on the phagocytosis of zymosan particle by J774A.1 cells

An attempt to isolated peritoneal macrophage from Wistar rat has been made in our preliminary work according to the method of Dai's group (Dai et al., 2001). Peritoneal macrophages were harvested by lavage PBS at 10 min following intraperitoneal injection and were subsequently cultured in complete DMEM. The viability of macrophage was checked by trypan blue dye exclusion method. The amount and viability of the isolated peritoneal macrophages were inadequate to use for macrophage phagocytosis assay. Since J774A.1 cells have been reported as cellular model for macrophage phagocytosis (Agostino et al., 2001; Blonska et al., 2004; Kim, et al., 2003; Mudiyanselage et al., 2003; Shah et al., 2002). Therefore, this macrophage-like cell line (J774A.1) was chosen as representative phagocytic cells for assessment of macrophage phagocytosis assay.

The in vitro phagocytic assay on NBT reduction was carried out according to the method described in the materials and methods section. LPS was used as a positive control for induced phagocytic activity of macrophage. J774A.1 cells were treated with LPS at concentrations ranging from 1.25-50 μg/ml. Dose response curve of LPS effects were shown in Figure 16. LPS at a concentration of 5 μg/ml induced maximal phagocytic activity (30.6 ± 3.7 %).

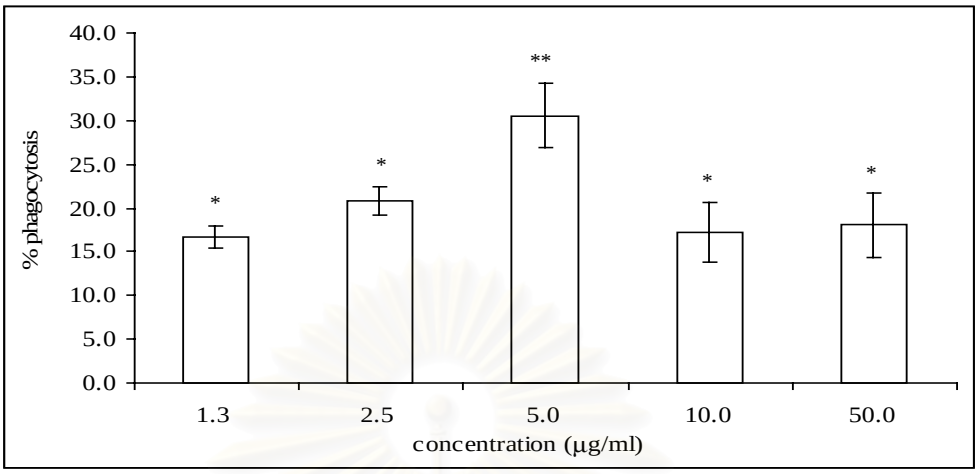


Figure 16 LPS-induced phagocytosis of macrophage. Macrophage (J774A.1) cells were incubated with LPS at concentrations ranging from 1.25-50 μg/ml for 24 hrs. Thereafter, 600 μg/ml of NBT and 800 μg/ml of zymosan were added and the cells were re-incubated for 60 min followed by measurement of formazan at 570 nm. Each value represented the mean ± S.E.M. of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

3.2 Screening of crude extract on phagocytic activity of J774A.1 macrophage cell line

From previous study, all plant extract were tested for their cytotoxic effects to J774A.1 macrophage cells, the CC50 values were showed in Table 4. Many of crude extracts were not toxic to the cells, they exhibited high CC50 values (> 200 μg/ml). Except for AK001, EC001, and HA001 exhibited the CC50 values of 77.7 ± 2.0, 138.3 ± 6.1, and 165.8 ± 8.3 μg/ml, respectively. However, EH001 and PR001 at concentration of 200 μg/ml were weakly toxic to macrophage cell line with the % cytotoxicity of 16.9 ± 3.7 % and 18.1 ± 4.2 %, respectively. Thus, in this phagocytosis experiment, AK001 and EC001 were tested at 50 μg/ml while EH001, HA001, and PR001 were used at 100 μg/ml.

All crude extracts were tested at non-cytotoxic concentrations toward macrophages, non of them showed any stimulating effects on J774A.1 phagocytosis at the maximal non-cytotoxic concentration. After testing at the diluted concentrations until 12.5 μg/ml, the maximal stimulating effects of some crude extracts were revealed (Table 10). Two plant extracts, HA001, and PS001 significantly enhanced the phagocytic activity of macrophages at the concentration of 12.5 μg/ml (Table 10 and Figure 17), while the other plants did not stimulated the phagocytic activity of macrophage cells.

Two crude ethanolic extracts, which possessed strong of phagocytosis enhancement property, were fractionated into three fractions by changing polarity of solvent and pure compounds were isolated as described in the next section.

Table 10 Effect on macrophage phagocytosis of the crude extract[a]

Code	Tested concentration (μg/ml)[a]	% phagocytosis[a]	% phagocytosis at 12.5 μg/ml
LPS	5	30.6 ± 3.7	ND
AK001	50	NS	NS
CV001	200	NS	NS
EC001	50	NS	NS
EH001	100	NS	NS
EL001	200	NS	NS
HA001	100	NS	13.8 ± 1.6*
HE001	200	NS	NS
LA001	200	NS	NS
LR001	200	NS	NS
MM001	200	NS	NS
OB001	200	NS	NS
PR001	100	NS	NS
PS001	200	NS	28.7 ± 4.3**
RA001	200	NS	NS
SC001	200	NS	NS

[a]All crude extracts were tested at concentration which was not cytotoxic to macrophages. Macrophage cells were exposed to crude extracts for 24 hrs. Then, 600 μg/ml of NBT and 800 μg/ml of zymosan were added and the cells were re-incubated for 60 min, followed by the measurement of formazan at 570 nm. Each value represented mean ± S.E.M. of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. ND is not determined; NS is not stimulated.

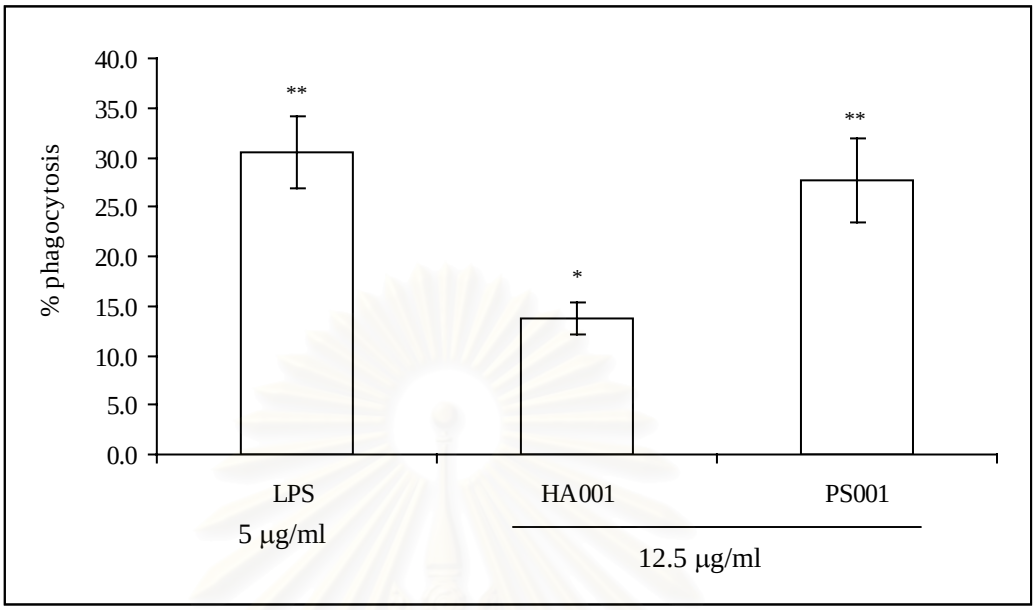


Figure 17 Effect of crude extract on phagocytic activity of J774A.1 macrophages. Macrophage cell line was incubated with 5 μg/ml of LPS or 12.5 μg/ml of crude extracts for 24 hrs. After the incubation period, 600 μg/ml of NBT and 800 μg/ml of zymosan were added and re-incubated for 60 min, followed by measurement of formazan at 570 nm. Each column represented the mean ± S.E.M. of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

3.2 Effect of *Harpullia arborea* extracts on phagocytic activity of J774A.1 macrophage cell line

The cytotoxic effects of plant extracts on macrophages were evaluted and then, the effect on phagocytic activity of J774A.1 cells were detected as shown in Table 11 and Figure 18. The ethanolic extract of Harpullia arborea (HA001) was found to possess stimulating effect on macrophage phagocytosis. Therefore, the HA001 extract was fractionated by partition to yield hexane (HA002), chloroform (HA003) and aqueous (HA004) fractions.

The hexane fraction (HA002) and the chloroform fraction (HA003) showed no potentiating effect on macrophage phagocytosis. In contrast, the aqueous fraction (HA004) was capable to enhance the phagocytosis at various concentrations (Figure 18). Then, repeated chromatography of aqueous fraction over silica gel was performed and led to the isolation of quebrachitol (Me5). Quebrachitol demonstrated stimulating effect on macrophage phagocytosis with maximum effect (40.1 ± 3.6 %) at concentration 100 μg/ml (515.5 μM).

Table 11 50% cytotoxic concentration (CC50) and phagocytic activity on J774A.1 macrophage cell line of samples from Harpullia arborea.

Code	Extract	CC_{50} on J774A.1 cell line (μg/ml)[a]	Tested concentration (μg/ml) [b]	% phagocytosis
HA001	Crude EtOH	165.8 ± 8.3	12.5	13.8 ± 1.6*
HA002	Hexane	135.3 ± 24.8	12.5	NS
HA003	$CHCl_3$	> 200	12.5	NS
HA004	Aqueous	> 200	100	10.6 ± 0.1
Me_5	HA004	> 100	100	40.1 ± 3.6**

[a]Data were presented as the mean CC_{50} values ± S.E.M. of two independent experiments, with triplicate replication in each experiment (n=2).

[b]All plant extracts were tested at concentrations which were not cytotoxic to macrophages. The value represented mean ± S.E.M.of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. NS is not stimulated.

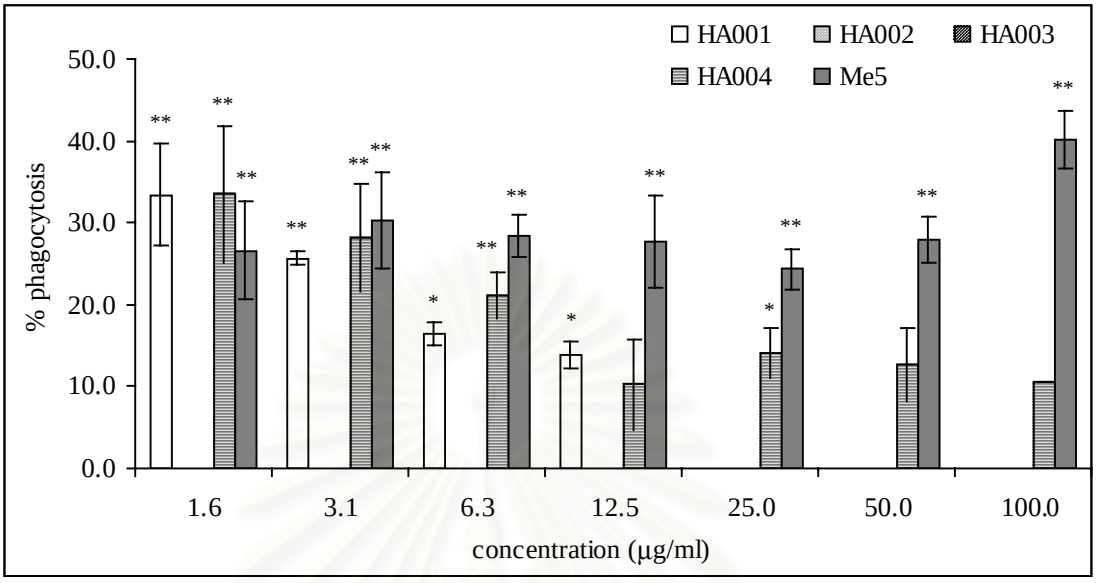


Figure 18 Effect of samples obtained from *Harpullia arborea* on J774A.1 macrophage cells. Macrophage cells were incubated with two-fold dilution of plant extracts at concentrations ranging from 1.56-200 μg/ml for 24 hrs. Then, 600 μg/ml of NBT and 800 μg/ml of zymosan were added and the cell were re-incubated for 60 min, followed by the measurement of formazan at 570 nm. Each value represented mean ± S.E.M. of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

3.3 Effect of *Pseuduvaria setosa* extracts on phagocytic activity of J774A.1 macrophage cell line

Effects of plant extracts on macrophage cell viability were determined and the stimulating effects on phagocytic activity of J774A.1 cells were shown in Table 12 and Figure 19. From previously screening of crude ethanolic extracts, the PS001 ethanolic extract were found to enhance macrophage phagocytosis. Therefore, the PS001 was fractionated into three fractions by partition to get hexane (PS002), chloroform (PS003) and aqueous (PS004) fractions.

The PS003 fraction demonstrated the phagocytosis enhancement activity with dose-response relationship as shown in Figure 19. Therefore, repeated column chromatography of this chloroform fraction over silica gel was performed and led to the isolation of two oxoaporphines, identified as 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (PS-1) and ouregidione (PS-2). Both compounds (PS-1 and PS-2) demonstrated stimulating activity on J774A.1 cell phagocytosis at various concentrations as shown in Figure 19.

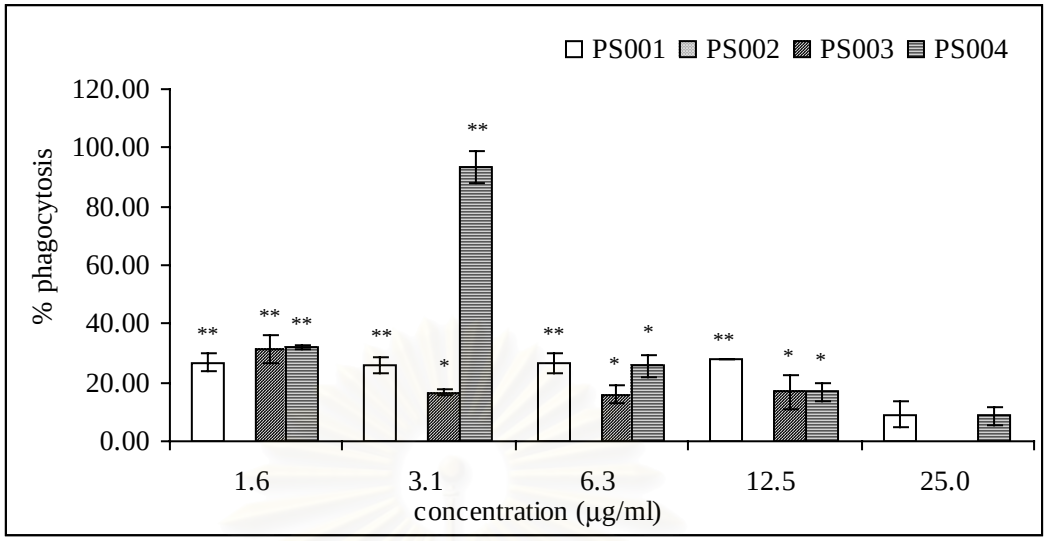
Table 12 50% cytotoxic concentration (CC_{50}) and phagocytic activity on J774A.1 macrophage cell line of samples from *Pseuduvaria setosa*.

Code	Extract	CC_{50} on J774A.1 cell line (μg/ml)[a]	Tested concentration (μg/ml)[b]	% phagocytosis
PS001	Crude EtOH	> 200	12.5	25.7 ± 4.3**
PS002	Hexane	> 200	12.5	NS
PS003	$CHCl_3$	> 200	12.5	16.8 ± 5.9*
PS004	Aqueous	> 200	200	NS
PS-1	PS003	> 100	100	21.0 ± 0.5*
PS-2	PS003	> 100	100	27.1 ± 4.8**

[a]Data were presented as the mean CC_{50} values ± S.E.M. of two independent experiments, with triplicate replication in each experiment (n=2).

[b]All plant extracts were tested at concentrations which was not cytotoxic to macrophages. The value represented mean ± S.E.M.of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. NS is not stimulated.

(a)



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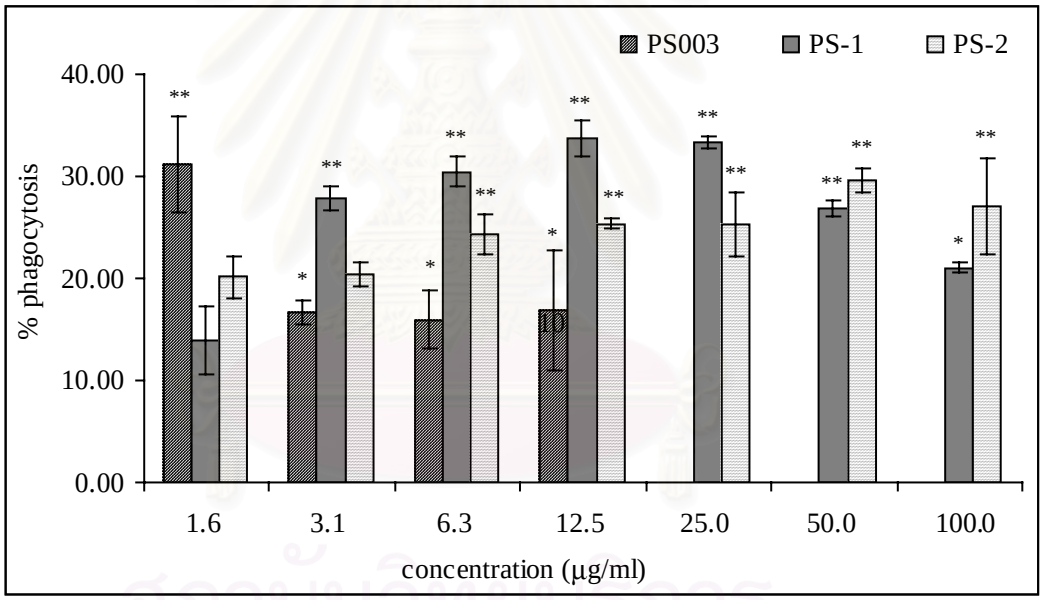

Figure 19 Effect of samples obtained from *Pseuduvaria setosa* on J774A.1 macrophage cells. (a) Fraction of *Pseuduvaria setosa.* (b) isolated pure compounds. Macrophage cells were incubated with two-fold dilution of plant extract at concentrations ranging between 1.56-200 µg/ml for 24 hrs. After the incubation period, 600 µg/ml of NBT and 800 µg/ml of zymosan were added and the cells were re-incubated for 60 min, followed by the measurement of formazan at 570 nm. Each value represented mean ± S.E.M. of two independent experiments, with quadruplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control.

4. Cytokine production

4.1 IL-2 production from splenic lymphocytes

In the previous section, several plant extracts and pure compounds were shown to stimulate rat splenic lymphocyte proliferation. Therefore, the effects of these samples on IL-2 secretion from splenic lymphocytes were determined by using ELISA test kit. Only kaurenoic acid (MHF), the diterpene type compound isolated from the hexane fraction of *Mitrephora maingayi*, exhibited weakly induction effect on the IL-2 production, compared to those of Con A and LPS. The IL-2 production data were presented in Table 13.

4.2 IL-12 production from J774A.1 macrophage cells

Several plant extracts and pure compounds demonstrated enhancing activity on the phagocytosis of J774A.1 cells, as shown previously. Therefore, the effects of these samples on the IL-12 production of J774A.1 cells were determined at the concentrations that ever capable to stimulate lymphocyte proliferation, determined in the previous section. The results were showed in Table 14. The ethanolic extract from *Limnophila rugosa* (LR001) exhibited the highest inducing effect on IL-12 secretion at the concentration of 12.5 μg/ml. The aqueous fraction from *Harpullia arborea* (HA004) and the isolated pure compound (Me_5) could enhance IL-12 secretion with stimulating effect on J774A.1 macrophage cell phagocytosis. In addition, the isolated compounds from *Pseuduvaria setosa* and *Stelechocarpus cauliflorus* (PS-2, and SCL-2, respectively) also enhanced IL-12 secretion from J774A.1 cells at the concentration of 12.5 μg/ml. On the other hand, the isolated compounds and aqueous fraction from *Pseuduvaria setosa* (PS-1, and PS004, respectively) have effect on macrophage phagocytosis but could promote the IL-12 secretion from the J774A.1 cells.

Table 13 IL-2 production from splenic lymphocytes.

Sample	Tested concentration (μg/ml)[a]	% stimulation	IL-2 (pg/ml)[b]
Con A	5	82.3 ± 16.7	3,184.6**
LR001	12.5	44.5 ± 2.4	NS
HA004	100	44.7 ± 4.3	NS
Me_5	100	43.1 ± 6.9	NS
MM002	12.5	22.9 ± 1.1	NS
MHF	12.5	37.7 ± 0.9	9.1 ± 0.9*
PS003	6.25	69.0 ± 15.6	NS
PS-1	12.5	78.4 ± 5.6	NS
PS-2	12.5	88.1 ± 15.2	NS
SC002	12.5	40.7 ± 8.8	NS
SCL-2	25	41.3 ± 6.9	NS

[a]Concentration of sample which exhibited the highest percent stimulation on lymphocyte proliferation.

[b]The amount of IL-2 secretion from splenic lymphocytes after incubation for 24 hrs with tested samples. Data were presented as the mean values ± S.E.M. of two independent experiments, with duplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. NS is not stimulated.

Table 14 IL-12 production from J774A.1 macrophage cell line.

Sample	Tested concentration (μg/ml)[a]	% phagocytosis	IL-12 (pg/ml)[b]
LPS	5	30.6 ± 3.7	6,537.3**
LR001	12.5	NS	1,065.3 ± 582.0*
HA004	100	10.6 ± 0.1	901.9 ± 61.4**
Me_5	100	40.1 ± 3.6	930.91 ± 31.8**
MM002	12.5	ND	ND
MHF	12.5	NS	317.4 ± 90.9**
PS003	6.25	15.9 ± 2.8	ND
PS004	3.25	93.3 ± 5.4	178.2 ± 24.4
PS-1	12.5	33.8 ± 1.8	151.9 ± 1.4
PS-2	12.5	25.4 ± 0.4	272.8 ± 8.2*
SC002	12.5	ND	ND
SCL-2	25	NS	653.7 ± 373.2*

[a]Concentration of sample which exhibited the highest percent stimulation on lymphocyte proliferation.

[b]The amount of IL-12 secretion from J774A.1 cells after incubation for 24 hrs with tested samples. Data were presented as the mean values ± S.E.M. of two independent experiments, with duplicate in each experiment (n=2). Statistical significance was determined by One-way ANOVA (**$p < 0.01$; *$p < 0.05$) in the comparison with vehicle control. ND is not determined. NS is not stimulated.

CHAPTER V

DISCUSSION AND CONCLUSION

The main objective of this study was to determine the *in vitro* immunostimulatory activity of some Thai medicinal plant extracts. An effective immunostimulating agent should be non-toxic to the cell at the stimulating concentration. Therefore, cytotoxicity test of crude extracts was primarily investigated.

A colorimetric XTT assay was used for cytotoxicity assessment of all plant materials on J774A.1 macrophage cell line, since the cleavage of XTT has several desirable properties for assaying cell survival and proliferation. This cell survival measurement using the XTT-microculture tetrazolium assay was first described by Scudiero's group in 1988 (Scudiero *et al.,* 1988). XTT is cleaved by all metabolically active cells and the amount of XTT formazan generated is directly proportional to the viable cell number (Rochm *et al*., 1991). However, tetrazolium salt XTT assay cannot be used to detect proliferation of splenic lymphocytes because of the nature of this suspension cells. Therefore, a one-step assay to measure cell proliferation was chosen to measure the viability of splenic lymphocytes. This is a very simple assay which only requires the addition of Alamar Blue solution to the cultures. Alamar Blue solution contains Redox indicator, the oxidized form of which is dark blue and absorbance at 600 nm. When the cells grow, they will induce the chemical reduction of media, cause the indicator change to red color (the reduced form) which its absorbance at 570 nm. Ahmed's group (1994) found that the spontaneous proliferation curves of a wide-range of cell lines (*e.g*., YCD3-1) and mitogen-induced proliferation of lymphocytes, assessed by Alamar Blue, were closely correlated to that of [^{3}H]thymidine incorporation (Ahmed *et al*., 1994; Zhi-Jun *et al*., 1997).

The concentrations of all plant extracts used in the immunostimulating activity determination were at non-cytotoxic concentrations. As a first step towards understanding the immunostimulant activity of plants, their effect of on rat splenic lymphocyte proliferation and J774A.1 macrophage phagocytosis were investigated. These cells represented the effectors of the adaptive immunity and the innate

immunity, respectively. Since cytokines play a prominent role in the development of immune responses, the effect of plant extracts and isolated compounds on the production of IL-2 and IL-12 of the immune cells were also investigated.

Con A was used as a positive control for stimulating T cell proliferation, whereas LPS was used for enhancing B cell proliferation (Ruscetti and Chervenick, 1975; Novogrodsky and Katchalski, 1971; Perlmann *et al*., 1970; Miller *et al*., 1978). Concentration-dependent responses of Con A and LPS (Figure 8 and Figure 9) were similar. Con A gave the maximal enhancing effect at the concentration of 2.5 μg/ml while LPS exhibited maximal effect at the concentration of 25 μg/ml. However, at the higher concentrations, they appeared to show inhibitory effects on rat splenic lymphocyte proliferation.

Fifteen Thai medicinal plants were selected based on chemotaxonomic data. Almost all of them have not been previously reported for their biological activities on the immune system. By using rat splenocyte to evaluate the immunomodulatory activity, five crude ethanolic plant extracts were found to stimulate lymphocyte proliferation. These are the extracts of *Limnophila rugosa* belongs to the family Scrophulariaceae; *Harpullia arborea* belongs to the family Sapindaceae; and *Mitrephora maingayi, Pseuduvaria setosa* and *Stelechocarpus cauliflorus* belong to the family Annonaceae. In the previous work of our group, the crude ethanolic extract from the stem bark of *Harpullia arborea* and those from leaves of *Limnophila rugosa* were reported to promote human peripheral blood lymphocyte proliferation, determined by [^{3}H]thymidine incorporation method (Chunhacha, 2001). Therefore, the proliferation of lymphocytes assessed by Alamar Blue in this study was closely correlated to that of [^{3}H]thymidine incorporation method.

Among 15 tested plant extracts, crude ethanol extract of *Limnophila rugosa*, at the concentration of 12.5 μg/ml, were found to have the highest stimulatory activity on lymphocyte proliferation. Unfortunately, this plant material was not available for further fractionation and purification. Although the present study did not give the complete view of the extract from the leaves of *Limnophila rugosa* on murine immune system, however, it has suggested that this plant may contain compounds which had immunomodulatory activity particularly on the cells of the acquired

immunity. Mukherjee and coworkers (2003) isolated a minor flavonoid constituent of *Limnophila rugosa* from its petrol extract and identified as 5'7-dihydroxy-8,3',5'-trimethoxyflavone (Mukherjee *et al*., 2003). The flavonoid presented in the extract might be responsible for the immunostimulant activities found in this study. Phenolic compounds, such as furanoconmarins and flavonoids, have been reported to possess immunomodulating activities (Rudie, 1993). The phenolic compounds can stimulate or suppress the immune system due to the hydroxyl groups in the structures.

In addition, the crude ethanolic extracts from *Mitrephora maingayi* and *Stelechocarpus cauliflorus* were revealed to have stimulation effect on the rat splenic lymphocyte proliferation. The further purification of these active samples led to isolate the immunostimulant compounds, identified as kaurenoic acid and β-sitosterol, respectively. Although, these two compounds were reported to found in several plants, this is the first time to be isolated from *Mitrephora maingayi* and *Stelechocarpus cauliflorus* and identified as immunostimulant.

Although kaurenoic acid, a diterpene type compound, have not been reported to have an effect on the lymphocyte proliferation, it has been demonstrated to contain biological activities as a selective antibacterial activity against Gram-positive bacteria (Wilkens *et al*., 2002) and anti-inflammatory potential in acetic acid-induced colitis (Paiva *et al*., 2002). In addition, kaurenoic acid at a concentration of 78 μM produced growth inhibition of CEM leukemic cells by 95%, MEF-7 breast and HCT-8 colon cancer cells by 45% each. Moreover, this compound induced a dose-dependent hemolysis of mouse and human erythrocytes with an EC_{50} value of 74.0 and 56.4 μM (Costa-Lotufo *et al*., 2002). In the present study, kaurenoic acid (MHF) was isolated from the hexane extract of *Mitrephora maingayi*. This compound stimulated splenic lymphocyte proliferation, augmented secretion of IL-2 from this cell and promoted secretion of IL-12 from J774A.1 cells but has no enhancement effect on the macrophage phagocytosis at the concentration of 12.5 μg/ml (41.4 μM).

According to the report from Bouic's group, the β-sitosterol (BSS) and its glycoside (BSSG) could enhance the *in vitro* proliferative response of T-cell stimulated by sub-optimal concentration of phytohaemagglutinin (Bouic *et al*., 1996; Bouic and Lamprect, 1999). In animals, BSS and BSSG have been shown to exhibit

anti-inflammatory (Yamamoto *et al*., 1991), anti-neoplastic (Raicht *et al*., 1980), anti-pyretic (Gupta *et al*., 1980), and immunomodulating activity (Yamada *et al*., 1987). A mixture of BSS:BSSG (termed essential sterolin formulation, ESF) showed higher stimulation effect than the individual sterols. In addition, ESF was able to significantly enhance the expression of CD25 and the class II human leukocyte antigen (HLA-Dr) activation antigens on T-cells, increased the secretion of IL-2 and IFN-γ, but inhibited the IL-4 and IL-6 secretion. A proprietary BSS:BSSG mixture has demonstrated promising results in a number of studies, including *in vitro* studies, animal models, and human clinical trials. This phytosterol complex seems to target specific T-helper lymphocytes, the T_H1 and T_H2 cells, helping to normalize their functioning and resulting in improved T-lymphocyte and natural killer cell activity. NK-cell activity was also increased by BSS, BSSG, and the mixture (Bouic *et al*., 1996). In the present study, β-sitosterol (SCL-2) was isolated from the hexane extract of *Stelechocarpus cauliflorus*. This compound could stimulate splenic lymphocyte proliferation, but has no effect on the secretion of IL-2 at the concentration of 12.5 μg/ml (60.4 μM). In contrast, β-sitosterol has no effect on macrophage phagocytosis but could promote the IL-12 secretion from the J774A.1 cells.

Since macrophages play an important role in the defense mechanism against host infection and the killing tumor cells, the modulation of antitumor properties of macrophages by various biological response modifiers is an area of active interest for cancer chemotherapy (Kang *et al*., 2002), which is closely related to immunomodulating activity. The higher reduction in the NBT assay represented higher activity of oxidase enzyme, this reflected the stimulation of phagocytes in proportional to intracellular killing (Rainard, 1986).

Among all 5 active crude plant extracts tested in this study, the ethanolic extracts from *Harpullia arborea* (HA001) and *Pseuduvaria setosa* (PS001) were exhibited stimulating activity on both rat splenic lymphocyte proliferation and J77A.1 macrophage cell phagocytosis. The fraction from *Harpullia arborea* which contained activity was the aqueous fraction (HA004) and the compound isolated from this fraction, quebrachitol, also possessed the ability to enhance both splenic lymphocyte proliferation and J774A.1 macrophage phagocytosis with 43.1 ± 6.8 % lymphocyte proliferation stimulation and 40.1 ± 3.6 % phagocytosis enhancement at the

concentration of 100 μg/ml (515.5 μM). The compound was able to stimulate macrophage phagocytosis at the concentration as less as 1.56 μg/ml (8.04 μM). On the other hand, the active fraction of *Pseuduvaria setosa* which stimulated both lymphocyte proliferation and macrophage phagocytosis was the chloroform fraction. The active compounds isolated from this fraction (PS003) were dioxoaporphine-type compounds, 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine and Ouregidione. All these isolated pure compounds have not been previously reported to have effects on the immune system.

From Literature review, quebrachitol has been extracted from the active n-BuOH fraction of *Artemia iwayomosi* (Kim *et al*., 2004). It has been used as a laxative substance in European countries since long time ago (Aurousseau *et al*., 1964). It was also used as a precursor to synthesize conduriol B epoxide, a glycosidase inhibitor, potentially used for the treatment of diabetes and cancer (Falshaw *et al*., 2000). In addition, aporphine-type alkaloids, isolated from various plants, have been reported to possess various biological activities. For examples, 6a,7-dehydroaporphine alkaloids, dehydrostephanine and dehydrocrebanine, were identified to have potent antimalarial activity (Likhitwitayawuid *et al*., 1999). Several aporphine-type alkaloids have been reported to be mutagen using Ames test (Nozaka *et al*., 1990). Ouregidione exhibited larvicidal activity against mosquito (*Aedes aegypti*) larvae and cytotoxic activity (Lee *et al*., 1999). Moreover, Chang's group isolated new 7-dehydroaporphine alkaloid, identified as 7-hydroxy-dehydrothalicsimidine. This compound exhibited significant inhibition of platelet aggregation induced by collagen, arachidonic acid, platelet activating factor, or thrombin (Chang *et al*., 1998).

The immune system is an intricate network of cells and soluble factors released by these cells. It is accepted that cytokines are major factors involved in the regulation of the immune response to antigens and infectious agents. T cells are made up of two distinct subsets, the CD4 helper cell and the CD8 cytotoxic/suppressor cells. T helper cells are divided in TH1 cells and TH2 cells from the profile of cytokine secretion (Moamann and Coffman, 1989). It is known that TH1 cells are able to produce IL-2 and IFN-γ, whereas TH2 cells can produce IL-4, IL-6 and IL-10.

The TH1 cells upregulate mainly cell-mediated immunity and downregulate humoral immunity, whereas TH2 cells act oppositely (Hino and Nariuchi, 1996).

IL-2 is one of the important cytokines to modulate the responses of immune cells such as T cells, B cells, and NK cells. IL-2 was one of the first cytokines to be identified and plays a central role in the clonal expansion of activated T cells as autocrine or paracrine (Liu et al., 1989; Smith and Cantrell, 1985). We examined the active plant extracts for their effects on the IL-2 production of rat splenic lymphocyte to elucidate the change in intrinsic activities of lymphocytes. In the present study, most of the tested plant extracts and isolated pure compounds, possessed immunostimulating activity toward splenic lymphocyte proliferation, did not induce the IL-2 secretion from the cells. Therefore, the stimulation of lymphocyte proliferation of active plant extracts may not cause by IL-2 effect. Since the isolated splenic lymphocyte population used in this study may contain monocytes and B lymphocytes together with T lymphocytes and these cell types were capable to secrete other cytokines e.g., IFN-γ, IL-4, IL-6 and IL-10 which could then induce T cell or B cell proliferation and expansion. IL-4, the representative cytokine of TH2 cells, is essential for the differentiation of naive TH cells into TH2 cells; IFN-γ, the representative cytokine of TH1 cells, is known to suppress this TH2 cell differentiation (Mosmann et al., 1986; Coffman and Carty, 1986). Therefore, the stimulating activity of these samples on lymphocyte proliferation may due to the effect of IFN-γ, IL-4, IL-6, and IL-10 rather than IL-2. In contrast, kaurenoic acid (MHF) could weakly induce IL-2 secretion from lymphocytes that resulted in promoting T cell proliferation. Thus, further investigations of immunomodulatory mechanism of these pure compounds should be on the effect on IFN-γ, IL-4, IL-6, and IL-10 secretion.

IL-12 is produced by monocytes, macrophages, dendritic cells, neutrophils, and to a less or extent B cells (Watford et al., 2003). A major function of IL-12 is its regulation of the adaptive immune response. Cytokines play a critical role in the developmental regulation of naive CD4+ T cells into either TH1 or TH2 cells. Appropriate T helper cell development is essential for an effective adaptive immune response. IL-12 is and important factor in controlling the differentiation of TH cells (Hsieh et al., 1993), favoring the expansion of TH1 cells and suppressive the

differentiation of TH2 cells (Seder et al., 1993; Manetti et al., 1993), an effect probably mediated by IFN-γ (Gately et al., 1994). In addition, a deficiency in endogenous IL-12 production in HIV-infected individuals has been shown to contribute to the progression of immunodeficiency (Chehimi et al., 1994). Several studies have demonstrated that IL-12 recovered various functions of effector cells from HIV-infected patients (Chehimi et al., 1992). Research for pharmacological agents that promote endogenous IL-12 production may provide new immunomodulating therapy for AIDS, and allergic diseases such as asthma. In this study, most of the active plant extracts and isolated pure compounds which possessed immunostimulating activity toward splenic lymphocyte proliferation enhanced IL-12 production in J774A.1 macrophage cells. These findings suggested that these compounds may be used as immunotherapeutic agents for the induction of IL-12, which were potentially applicable for infectious disease and allergic disease.

In this experiment, 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (PS-1), isolated from chloroform fraction of Pseuduvaria setosa (PS003), and the aqueous fraction of this plant (PS004) were found to enhance macrophage phagocytosis without the effect on IL-12 secretion from the cells. Several cytokines are able to augment macrophage phagocytosis, for example, IL-1 and TNF-α are produced by macrophages, neutrophils, epithelial cells (e.g., keratinocytes) and endothelial cells. Both cytokines are pro-inflammatory cytokines which stimulated the recruitment of neutrophils and macrophages to sites of infection and activated these cells to eradicate microbes. In addition, they cause vascular endothelial cells to express new surface receptors, called adhesion molecules, that make the endothelial surface become adhesive for leukocytes, and stimulated endothelial cells and macrophages to secrete cytokines called chemokines that induce leukocyte chemotaxis and recruitment (Abbas et al., 2000). The further study of these pure compounds should be evaluating the effect on IL-1, and TNF-α secretion.

In conclusion, five out of fifteen Thai medicinal plant extracts tested in the present study possessed immunostimulation activity, determined by in vitro rat splenic lymphocyte proliferation assay. Only two of the five active plant extracts could augment phagocytosis of the J774A.1 macrophage cells. Some of the bioactive compounds have been isolated from these active plant extracts. All active samples

found to promote IL-12 secretion, some of them were capable to induce IL-2 production.

Although this study was performed only with an in vitro assay system, the information obtained from the present work may provide a rational basis for the efficacy of these medicinal plants. For example, they may use as immunopotentiators in the cancer patient, HIV-infected patients or the patient with other diseases related to immune disorders. The detail mechanism involved in the enhancement of lymphocyte proliferation and macrophage phagocytosis of all active plants discovered in this study should be further investigated. In addition, further purification of the active components and examination of their effects in vivo seem to be required for clinical applications.

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APPENDICES

APPENDIX I

PREPARATION OF PLANT MATERIALS

Crude extraction

Plants were extracted by Assist. Prof. Dr. Rutt Suttisri's group, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each plant was chopped into small pieces and extracted with 95% ethanol for 3-7 days. Then, the extract was evaporated under reduced pressure at 40 °C and the evaporate (ethanol extract; code 001) was prepared into a stock solution at a concentration of 40 mg/ml in DMSO. The stock solution was stored at -20 °C until used. Ethanol extract was tested in lymphocyte proliferation assay and macrophage phagocytosis assay.

Purification of active plant extract

The active plant extracts were fractioned and further purified by Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Briefly, ethanolic extract was partitioned with hexane. The combined hexane extract was evaporated under reduce pressure to dryness to give a hexane extract (code 002). The aqueous layer was then extracted with chloroform. The combined chloroform extract was dried by the same process as the hexane extract to give a chloroform extract (code 003). The aqueous fraction was also evaporated to give aqueous extract (code 004). Each fraction was placed in an evaporating dish and all solvents were then removed by evaporating on the water bath before used. The extraction scheme was shown in Figure 20. The scheme was used for all active plant extracts, except for *Stelechocarpus cauliflorus.*

The dried leaves of *Stelechocarpus cauliflorus* were separately macerated with hexane (code 002), ethyl acetate (code 003) and methanol (code 004). The extraction scheme of *Stelechocarpus cauliflorus* was shown in Figure 21.

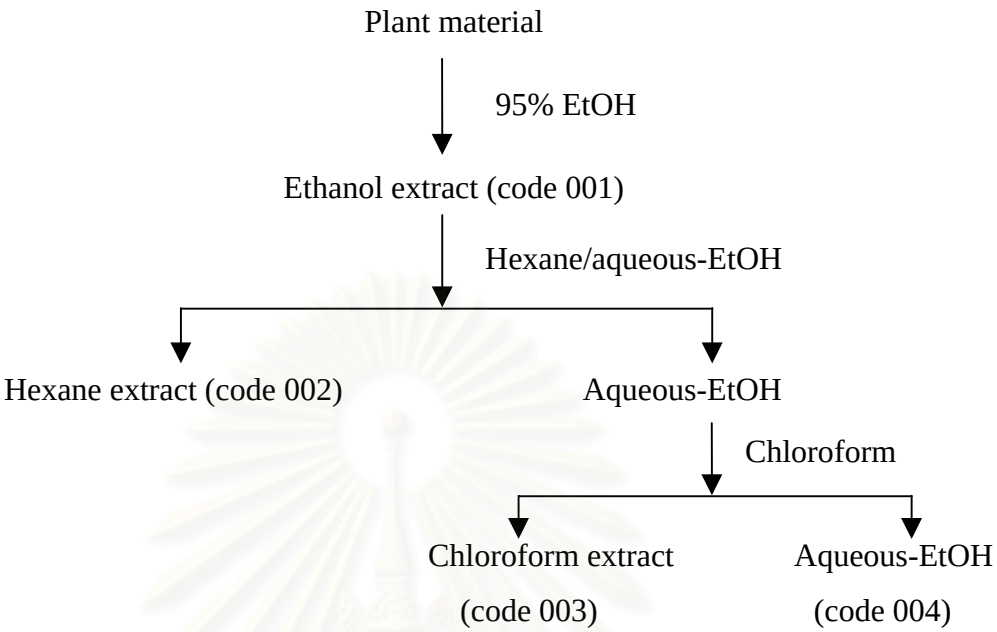

Figure 20 Extraction scheme of active plant extracts.

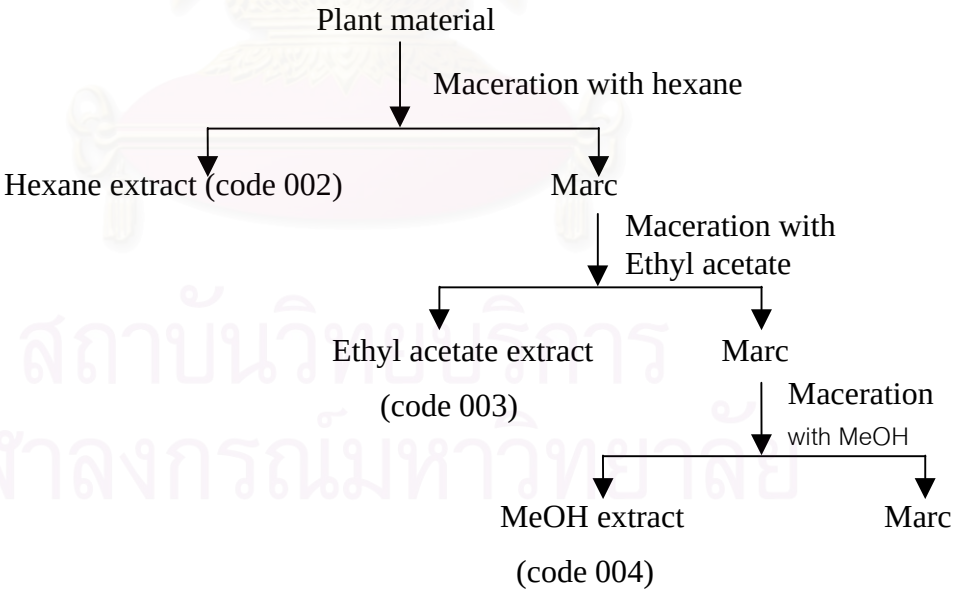

Figure 21 Extraction scheme of *Stelechocarpus cauliflorus.*

The extract, which showed strong immunostimulant activity, was fractionated through silica gel column and eluted with mixture of hexane-ethylacetate-methanol led to the isolation of pure compound as follow:

- ***Harpullia arborea***

Quebrachitol (Me_5) was isolated from the aqueous-methanol extract (HA004), the structure is showed in Figure 22.

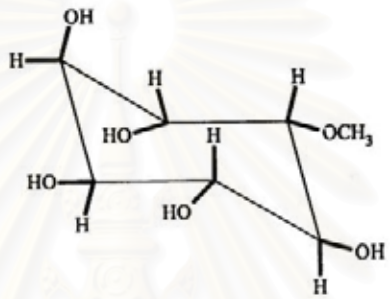

Figure 22 Quebrachitol (M.W. 194)

- ***Mitrephora maingayi***

Kaurenoic acid (MHF) was isolated from the hexane extract (MM002), the structure is showed in Figure 23.

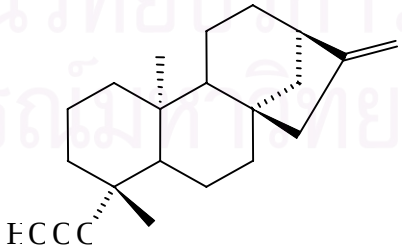

Figure 23 Kaurenoic acid (M.W. 302)

- ***Pseuduvaria setosa***

Two oxoaporphine (PS-1 and PS-2) was isolated from the chloroform extract (PS003), the structures are showed in Figure 24.

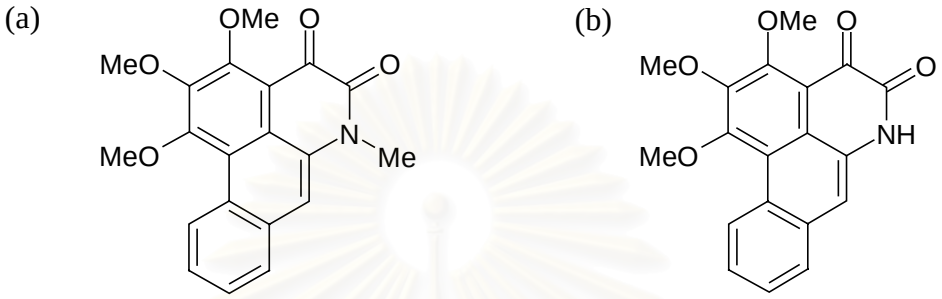

Figure 24 (a) 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (PS-1) [M.W. 350]
(b) Ouregidione (PS-2) [M.W. 336]

- ***Stelechocarpus cauliflorus***

β-sitosterol (SCL-2) was isolated from the hexane extract (SC002), the structure is showed in Figure 25.

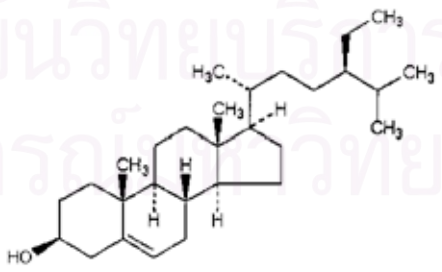

Figure 25 β-sitosterol (M.W. 414)

APPENDIX II

PREPARATION OF REAGENTS

Growth medium of J774A.1 macrophage cell line

DMEM powder was dissolved with deionized distilled water and the 3.7 g/l sodium bicarbonate was added. The solution was mixed well and adjusted pH to 7.2 with 2N HCl. Then, the solution was adjusted volume to 1,000 ml. This solution was sterilized by filtration (0.2 μm millipore filter membrane). Before use, this solution was supplemented with 10% FBS.

Growth medium of splenic lymphocyte

RPMI powder was dissolved with deionized distilled water and the 2 g/l sodium bicarbonate was added. The solution was mixed well and adjusted pH to 7.2 with 2N HCl. Then, the solution was adjusted volume to 1,000 ml. This solution was sterilized by filtration (0.2 μm millipore filter membrane). Before use, this solution was supplemented with 10% FBS and 1% antibiotic-antimycotic agents.

Heat-inactivated FBS

The desired amount of FBS was thawed at ambient temperature or 2-8 °C. The bottle of FBS was placed into the water bath which was adjusted to 56 ± 2 °C so that the entire contents of the bottle are immersed in water. The bottle was heated for 30 minutes, and swirled periodically. The bottle was removed from the water bath, and allowed to cool. FBS was aliquoted in sterile bottles and stored at –20 °C or 2-8 °C.

Phosphate buffered saline (PBS)

To make 1 liter of PBS, 950 ml deionized distilled water was added to the container. The ingredients including, 8.00 g NaCl, 0.20 g KCl, 0.20 g KH2PO4, and 1.15 g Na2HPO4, were dissolved with continuously stirring. The pH of solution was adjusted to 7.2 with 1 N NaOH. Then, the solution was adjusted to 1,000 ml with deionized distilled water and autoclaved for 20 min at 121 °C.

XTT solution

XTT solutions were freshly made each day by dissolving XTT in hot DMEM (60 °C) to make the final XTT concentration of 1 mg/ml. Prior to each experiment, the XTT was diluted with PBS.

PMS solution

PMS was made up as 100 mM solution in PBS and stored at 4 °C for periods up to 1 month. Prior to each experiment, the PMS was diluted with PBS.

Zymosan A particles

Zymosan A particles, suspended in 0.15 M sodium chloride at the concentration of 4 mg/ml, were placed in a boiling water bath for 30 min. Then they were centrifuged for 30 min at 4,000 rpm. The supernatant was discarded and the residue was resuspended in DMEM at 4 mg/ml and stored at –20 °C in small aliquots for at least a month.

Nitro blue tetrazolium

NBT was dissolved in distilled water at 2 mg/ml. A stock solution at 2 mg/ml is stable for 1-2 weeks in the dark at –20 °C. Prior to each experiment, the NBT was diluted with an equal volume of buffer containing 270 mM NaCl, 10.4 mM Na2HPO4, and 3.16 mM KH2PO4 (NBT working solution).

APPENDIX III

TABLES OF EXPERIMENTS RESULTS

Table 15 The percentage of stimulation of Con A and LPS on rat splenic lymphocytes.

Sample	Concentration (μg/ml)	% stimulation[a]
Con A	0.01	0.00 ± 0.30
	0.1	0.21 ± 1.60
	0.5	91.7 ± 7.5**
	1.2	115.9 ± 14.6**
	2.5	116.6 ± 9.6**
	5.0	82.3 ± 16.7**
	10.0	52.9 ± 3.7**
	20.0	10.5 ± 5.4
LPS	0.01	9.42 ± 3.49
	0.1	36.4 ± 13.5**
	0.5	58.2 ± 14.1**
	5.0	75.5 ± 17.9**
	12.5	83.6 ± 10.9**
	25.0	83.9 ± 12.1**
	50.0	62.9 ± 14.4**
	100.0	11.4 ± 5.4

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** $p < 0.01$, *$p < 0.05$ determined by One-way ANOVA.

Table 16 The percentage of stimulation of *Limnophila rugosa* on rat splenic lymphocytes.

Concentration (μg/ml)	% stimulation[a]
1.6	23.5 ± 2.5*
3.1	30.4 ± 0.9**
6.3	40.6 ± 2.3**
12.5	44.5 ± 2.4**
25.0	43.3 ± 2.8**
50.0	34.6 ± 2.8**
100.0	23.0 ± 2.1*
200.0	16.9 ± 1.6

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA.

Table 17 The percentage of stimulation of *Harpullia arborea* on rat splenic lymphocytes.

Concentration (μg/ml)	% stimulation of plant extract[a]		
	HA001	HA004	Me_5
1.6	30.6 ± 6.7**	17.1 ± 0.3	17.2 ± 5.5
3.1	42.3 ± 6.9**	18.8 ± 1.8*	13.3 ± 1.1
6.3	36.2 ± 5.3**	26.0 ± 3.4**	15.6 ± 2.8
12.5	20.6 ± 4.5**	25.3 ± 1.6**	18.1 ± 1.8
25.0	NS	40.2 ± 3.8**	36.4 ± 5.3**
50.0	NS	43.4 ± 3.2**	45.7 ± 1.5**
100.0	NS	44.7 ± 4.3**	43.1 ± 6.8**
200.0	NS	9.83 ± 5.7	37.5 ± 4.6

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated.

Table 18 The percentage of stimulation of *Mitrephora maingayi* on rat splenic lymphocytes.

Concentration (μg/ml)	% stimulation of plant extract[a]			
	MM001	**MM002**	**MM003**	**MHF**
1.6	5.43 ± 6.7	17.6 ± 2.6*	3.79 ± 1.44	23.0 ± 1.7**
3.1	16.4 ± 6.9*	19.4 ± 2.8*	3.05 ± 2.42	26.3 ± 4.9**
6.3	16.3 ± 5.3*	21.9 ± 2.5**	16.9 ± 3.4*	28.4 ± 3.6**
12.5	14.0 ± 4.5*	22.9 ± 1.1**	22.2 ± 3.0**	37.7 ± 0.9**
25.0	6.22 ± 2.17	14.5 ± 0.8*	0.41 ± 0.15	32.1 ± 1.4**
50.0	NS	6.56 ± 2.23	NS	NS
100.0	NS	NS	NS	NS
200.0	NS	NS	NS	ND

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated; ND is not determined.

Table 19 The percentage of stimulation of *Pseuduvaria setosa* on rat splenic lymphocytes.

Concentration (μg/ml)	% stimulation of plant extract[a]				
	PS001	**PS002**	**PS003**	**PS-1**	**PS-2**
1.6	47.2 ± 14.0**	18.8 ± 2.5	41.6 ± 7.6**	41.5 ± 9.2**	29.9 ± 6.4**
3.1	43.9 ± 13.3**	18.5 ± 2.0	42.6 ± 8.3**	43.2 ± 4.0**	40.6 ± 5.8**
6.3	42.2 ± 9.9**	24.2 ± 8.1	69.0 ± 15.6**	78.4 ± 5.6**	69.3 ± 6.4**
12.5	43.3 ± 13.1*	21.1 ± 7.1	NS	69.7 ± 10.4**	88.1 ± 15.2**
25.0	30.7 ± 7.9*	NS	NS	38.5 ± 6.5**	43.4 ± 9.6**
50.0	4.06 ± 7.65	NS	NS	17.7 ± 1.4	42.7 ± 7.0**
100.0	NS	NS	NS	NS	38.4 ± 0.5**
200.0	NS	NS	NS	ND	ND

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated; ND is not determined.

Table 20 The percentage of stimulation of *Stelechocarpus cauliflorus* on rat splenic lymphocytes.

Concentration (μg/ml)	% stimulation of plant extract[a]			
	SC001	**SC002**	**SC004**	**SCL-2**
1.6	18.4 ± 2.3*	45.6 ± 5.5**	74.0 ± 8.4**	23.8 ± 8.4*
3.1	21.9 ± 6.3*	48.8 ± 6.4**	60.2 ± 15.2**	26.5 ± 8.0*
6.3	23.2 ± 5.6*	40.7 ± 8.8**	60.7 ± 19.5**	28.5 ± 6.1*
12.5	39.3 ± 2.7**	32.1 ± 6.4**	57.1 ± 17.7**	29.9 ± 4.2**
25.0	31.9 ± 5.4**	18.7 ± 2.1*	57.4 ± 14.1**	41.3 ± 6.8**
50.0	18.3 ± 4.8*	NS	30.2 ± 13.9**	28.1 ± 4.6**
100.0	NS	NS	NS	20.6 ± 3.1*
200.0	NS	NS	NS	ND

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated; ND is not determined.

Table 21 The percentage of phagocytosis of LPS on J774A.1 macrophage cell line.

Concentration (μg/ml)	% phagocytosis[a]
1.3	16.7 ± 1.2*
25	20.8 ± 1.6*
5.0	30.5 ± 3.7**
10.0	17.3 ± 3.4*
50.0	18.0 ± 3.6*

[a]Each value represented the mean value with S.E.M. of two different experiments (n=2). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA.

Table 22 The percentage of phagocytosis of *Harpullia arborea* on J774A.1 macrophage cell line.

Concentration (μg/ml)	% phagocytosis of plant extract[a]		
	HA001	**HA004**	**Me_5**
1.6	33.4 ± 6.2**	33.5 ± 0.3**	26.6 ± 6.0**
3.1	25.7 ± 0.7**	28.1 ± 1.8**	30.3 ± 6.0**
6.3	16.4 ± 1.4*	21.2 ± 3.4*	28.4 ± 2.6**
12.5	13.8 ± 1.6*	10.2 ± 1.6*	27.8 ± 5.6**
25.0	NS	14.1 ± 3.8*	24.3 ± 2.5**
50.0	NS	12.7 ± 3.2	27.9 ± 2.7**
100.0	NS	10.6 ± 4.3	40.1 ± 3.6**
200.0	NS	NS	ND

[a]Each value represented the mean value with S.E.M. of two different experiments (n=2). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated; ND is not determined.

Table 23 The percentage of phagocytosis of *Pseuduvaria setosa* on J774A.1 macrophage cell line.

Concentration (μg/ml)	% phagocytosis of plant extract[a]				
	PS001	**PS003**	**PS004**	**PS-1**	**PS-2**
1.6	26.8 ± 3.3**	31.2 ± 4.7**	32.0 ± 0.6**	13.9 ± 3.4	20.1 ± 2.0
3.1	25.9 ± 2.4**	16.7 ± 1.2*	93.3 ± 5.3**	27.8 ± 1.2**	20.4 ± 1.2
6.3	26.5 ± 3.3**	15.9 ± 2.8*	25.6 ± 3.8*	30.5 ± 1.5**	24.3 ± 2.0**
12.5	27.7 ± 0.0**	16.8 ± 5.9*	17.0 ± 3.1*	33.8 ± 1.8**	25.4 ± 0.4**
25.0	9.08 ± 0.17	NS	8.53 ± 2.89	33.3 ± 0.6**	25.3 ± 3.1**
50.0	0.75 ± 2.12	NS	4.83 ± 0.07	26.9 ± 0.8**	29.7 ± 1.2**
100.0	NS	NS	4.35 ± 2.02	21.0 ± 0.5*	27.0 ± 4.8**
200.0	NS	NS	NS	ND	ND

[a]Each value represented the mean value with S.E.M. of three different experiments (n=3). Asterisks denote significant differences from the vehicle control group: ** p< 0.01, *p < 0.05 determined by One-way ANOVA. NS is not stimulated; ND is not determined.

VITA

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