IMMUNOMODULATORY EFFECT OF THAI MEDICINAL PREPARATION FOR TREATMENT OF CANCER PATIENTS

BY

MISS SUMALEE PANTHONG

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
THE DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES
FACULTY OF MEDICINE
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was approved as partial fulfillment of the requirements for
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ABSTRACT

Benjakul and Hua-Khao-Yen are Thai medicinal preparations which have long been used for cancer treatment. Benjakul is used as adaptogenic drug before using other cancer drugs. This preparation consists of five plants; the fruits of *Piper chaba* Hunt., the roots of *Piper sarmentosum* Roxb., the stems of *Piper interruptum* Opiz, the roots of *Plumbago indica* Linn. and the rhizomes *Zingiber officinale* Rosc. Benjakul preparation and its plant components have been reported for cytotoxic activity on cancer cell lines but they have not been reported on immunomodulatory activity. Five species of Hua-Khao-Yen have been found five species in Thailand, but two species of Hua-Khao-Yen are popularly used as drugs for cancer patients, namely *Smilax corbularia* Kunth and *Dioscorea membranacea* Pierre. Two species of Hua-Khao-Yen have been reported regarding some immunomodulatory pathways. Thus, the objectives of this study were to investigate the immunomodulatory activity of Benjakul, two types of Hua-Khao-Yen and their isolated compounds. The finding results may be used for the basic data of Benjakul preparation and Hua-Khao-Yen for immunomodulatory aspects of cancer treatment.

Benjakul and Hua-Khao-Yen were extracted by two methods. Decoction method in water to obtained as water extract and maceration in 95% ethanol to obtain on ethanolic extract. Bioassay guided fractionation was used to guide isolation of

active compounds. The immunomodulatory assay of extracts and compounds were performed to study the effect of NK cells activity and lymphocyte proliferation. The stimulatory effect on cytokine productions, IL-2 and IFN-γ, were investigated by ELISA technique. The inflammatory and anti-inflammatory cytokines activation by LPS-stimulated RAW264.7 cells also were detected by ELISA. Finally, safety studies of component plants in Benjakul preparation were investigated by in vitro and in vivo experiments. *Piper* species were investigated for their effect on TRPV1 and TRPA1 activation, adjuvant effects, the stimulation of trafficking dendritic cells and cytokine production in FITC sensitized mice.

The ethanolic extract of *S. corbularia* showed significant effects on NK cells activity and lymphocyte proliferation at low concentration (0.01 and 0.1 μg/ml) and it decreased NK cells activity and lymphocyte proliferation at high concentration. Moreover, the ethanolic extract of *S. corbularia* significantly increased IFN-γ production (1-10 μg/ml). The water extract did not significantly affect stimulation of NK cells, lymphocyte proliferation and IL-2 and IFN-γ production. For cytokine production from macrophage, the ethanolic and water extracts of *S. corbularia* had no effect on inflammatory and anti-inflammatory cytokines. Two isolated compounds from the ethanolic extract of *S. corbularia*, quercetin and astilbin, had no significant effect on NK cells activity. Quercetin also showed no significant stimulation on lymphocyte proliferation.

The ethanolic extract and water extract of *D. membranacea* were investigated for their immunomodulatory activity. The results showed that the ethanolic extract of *D. membranacea* significantly increased NK cells activity at low concentration (0.1 μg/ml). Moreover, the ethanolic extract of *D. membranacea* showed inhibitory effect on IL-1β and IL-6 production (IC_{50} = 46.82 and 0.90 μg/ml) and activated IL-10 production (concentration in range 10-50 μg/ml to be 1.10-1.34 times when compare with condition in the presence of LPS) by LPS-stimulated RAW264.7 cells. Futhermore, the water and ethanolic extracts of *D. membranacea* had significant effect on IL-2 and IFN-γ production by PHA-stimulated PBMCs. Two compounds namely, dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene were isolated from the ethanolic extract of *D. membranacea*. These compounds showed no significant stimulation on NK cells and lymphocyte

proliferation. 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene also showed inhibitory effect on IL-1β and IL-6 production with IC_{50} value of 4.38 and 9.30 μg/ml. Dioscorealide B showed inhibitory effect on IL-6 production with IC_{50} value of 8.59 μg/ml. However, dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene had no stimulatory effect on IL-10 production from LPS-stimulated RAW264.7 cells.

The ethanolic extract of Benjakul caused an increase of NK cells activity, lymphocyte proliferation, IL-2 and IFN-γ production at low concentrations (concentration in range 0.01-10 μg/ml). It also showed inhibitory effect on IL-1β and IL-6 with IC_{50} value of 62.93 and 51.83 μg/ml, respectively. Piperine which is an isolated compound from the ethanolic extract of Benjakul, showed no significant effect on NK cells activity. However, piperine inhibited IL-6 with IC_{50} value of 16.71 μg/ml. From these results, the ethanolic extract of Benjakul showed anti-inflammatory effect but it had less effect on NK cells and lymphocyte proliferation. The ethanolic extract of *P. chaba*, *P. interruptum* and *P. sarmentosum* and piperine were chosen to safety study. Three *Piper* species are component plants of Benjakul preparation. The results showed that piperine and the ethanolic extracts of *P. chaba* and *P. interruptum* activated TRPV1 and TRPA1 receptor which affect to burning pain and stomachache. Furthermore, piperine and *P. chaba* extract showed adjuvant effect and induced contact hypersensitivity in mouse ear swelling test. The ethanolic extract of *P. chaba* enhanced dendritic cells-trafficking to draining lymph nodes cells and induced IL-4 and IFN-γ production in FITC-sensitized mice. Even though piperine induced adjuvant effect, it could not activated dendritic cells-trafficking and cytokines production (IL-4 and IFN-γ) in FITC-sensitized mice.

These results can be summarized in that Hua-Khao-Yen-Tai or *D. membranacea* showed the strongest effect on immune system when compared with Hua-Khao-Yen-Nua or *Smilax corbularia* and Benjakul preparation. Hua-Khao-Yen-Tai showed stimulatory effect on NK cells and lymphocyte proliferation. Moreover, Hua-Khao-Yen-Tai also inhibited inflammatory cytokine (IL-1β and IL-6) and activated anti-inflammatory cytokine (IL-10). However, Hua-Khao-Yen-Nua also showed some pathway of immunomodulatory activity by activation on NK cells, lymphocyte proliferation and IFN-γ production. For Benjakul preparation, it showed

no effect on NK cells activity and lymphocyte proliferation. However, Benjakul showed anti-inflammatory activity which inhibited IL-1β and IL-6. Thus, Benjakul can be used as an anti-inflammatory drug for cancer patients. However, the side effect of Benjakul should be concern because the component plant in Benjakul preparation, *P. chaba*, activated TRP channel and contact hypersensitivity. Thus, Benjakul preparation may show side effect similar to *P. chaba* extract. However, the ingredients of all extracts exhibited less immunological activity than their crude extract. Thus, the ethanolic extract of Hua-Khao-Yen and Benjakul should be promoted for their immunomodulatory and anti-inflammatory effects on cancer treatment.

Keywords: Thai traditional preparation, Benjakul, Hua-Khao-Yen, *D. membranacea*, *S. corbularia*, Immunomodulatory effect, NK cells activity, Lymphocyte proliferation, Cytokine production, Contact hypersensitivity, Adjuvant effect

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Miss Sumalee Panthong

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
Ca^{2+}	Calcium ion
CHO cells	Chinese hamster ovary cells
CO_2	Carbondioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
^{3}H-thymidine	Thymidine, [Methyl-^{3}H]
HEK cells	Human Embryonic Kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
hrs	Hours
IC_{50}	The half maximal inhibitory concentration
IFN- γ	Interferon-gamma
IL-1β	Interleukin-1 Beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
LPS	lipopolysaccharide
MHC	Major histocompatibility complex
ml	Millilitre
mM	Millimolar

Symbols/Abbreviations	Terms
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
$Na_2{}^{51}CrO_4$	Chromium radionuclide
NK cells	Natural killer cells
nm	Nanometre
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPV1	The transient receptor potential cation channel subfamily V member 1
UV	Ultraviolet
°C	Degree celsious
%	Percentage
µ	Micro
µg/ml	Microgram per millilitre

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

The immune system is an important system of body which destroys pathogens, such and bacteria and viruses. The immune response consists of innate immunity and adaptive immunity. The innate immunity is the first line of defense against foreign substances while the adaptive immunity plays a second line of defense. Innate immune response consists of a number of physical barriers (intact skin), a number of cellular components (macrophage and natural killer cells) and secretions accompanied by a number of serum factors such as interferon and complement (O'Gorman & Donnenberg, 2008). Response of the adaptive immune system, depends on antigen-specific reactions through T cells and B cells. The antigen is presented by the antigen presenting T or B cells leading to T or B cells activation, and differentiation. Then, the activated T cells leaved the lymphoid tissue and move to the site of tissue injure, or due to the secretion of antibody from activated B cells into blood and tissue fluids, and then to the infective focus (Parkin & Cohen, 2001). Besides pathogens, the immune system playes a critical role in opposing cancer.

Cancer is a major human health problem worldwide and the leading cause of death in Thailand. The death rate from cancer in Thailand followed an upward trend in 2004-2011 (95.17 per 100,000 populations in 2011)(Office of the Permanent Secretary for Public Health, 2011). Cancers are caused by the progressive growth of transformed cells. Transformed cells develop to malignant tumors which are determined in large part by the proliferative capacity of tumor cells and by the ability of these cells to evade host tissues and metastasize to distant sites (Dzivenu & O'Donnell-Tormey, 2003). In addition, the development of tumors involves the inflammatory response. Inflammation is a physiologic process in response to microbial pathogen infection, chemical irritation, and wounding which plays a critical role in tumorigenesis (especially in chronic inflammation). Chronic inflammation involved with infections or autoimmune disease leads to tumor development and can

induce oncogenic mutations, genomic instability, early tumor promotion, and activate angiogenesis. The inflammatory process can increase neoangiogenesis, stimulate tumor development and metastatic spread, cause local immunosuppression, and further augment genomic instability (Grivennikov, Greten, & Karin, 2010). Currently, the popular treatments for cancer patients are surgery, chemotherapy and radiotherapy which lead to bad side effects (Price, Sikora, & Illidge, 2008).

In fact, tumor cells can be destroyed by innate and adaptive immune response. For innate immune response, natural killer cells (NK cells) and macrophages play the important function of killing tumor cells. NK cells respond to the lack of class I MHC molecules, because some tumor cells lose MHC class I and NK cells may act against them (Abbas, Litchman, & Pillai, 2010). The tumoricidal ability of NK cells is increased by cytokines secretion during innate immune response. The role of cytokine is to regulate lymphoid development, differentiation, homeostasis, tolerance and memory (Khan, 2008). Many of cytokines are secreted in immune response to tumor cells including IL-2, IL-12, IL-4, IL-10, IL-6, GM-CSF IFN-γ (Dranoff, 2004). Nowadays, cytokines are developed to use as immunomodulatory agent in cancer immunotherapy (S. Lee & Margolin, 2011). IL-2 and IFN-γ is an important cytokine which were reported as involved with immune response to tumor (Dranoff, 2004). IL-2 is produced by activated CD4+ T cells, naïve CD8+ T cells, dendritic cells and thymic cells (Granucci et al., 2001; Malek, 2008). IL-2 stimulates NK cells and they become lymphokined-activated killer cells (LAK cells) that express a strong cytotoxic effect (Becknell & Caligiuri, 2005). For IFN-γ, it plays an important role in activation of innate and adaptive immune responses. IFN-γ was reported cytotoxic to some malignant cells and regulates other cytokines such as IL-2 and IL-12 (S. Lee & Margolin, 2011). Furthermore, previous studies showed that IFN-γ plays the role of immunoediting of the immune defense system and the oncogenic cells are recognized and eliminated by the immune system (Zaidi & Merlino, 2011).

Even though the immune system kills and destroys tumor cells, some immune response can activate tumor cell development. The inflammation is the response of immune system to damaged or injured tissue, to destroy the pathogen, cell debris or any cause of injuring. Inflammation shows four cardinal symptoms

including redness, heat, swelling and pain. The early phase of inflammation, acute inflammation, is regularly a short-term process which attacks foreign substances and stops within a few hours. However, acute inflammation can develop to chronic inflammation. Chronic inflammation, long-term inflammation is a severe and aggressive response which is associated with cancer development. Chronic inflammatory mechanism produces many free radical products such as reactive oxygen and nitrogen species which lead to oxidative damage and increased DNA mutations (Hussain, Hofseth, & Harris, 2003). Furthermore, many inflammatory cytokines and chemokines in chronic inflammation can stimulate tumor cell proliferation (Balkwill & Mantovani, 2001). Thus, much research has focused on finding some substances to inhibit the inflammatory process to prevent bad efoutcomes (Aravindaram & Yang, 2010).

Pro-inflammatory cytokines are proteins involved in the stimulation of inflammatory reactions. Many cytokines are involved in the pain process, including IL-1β and IL-6. IL-1β is produced at early stage of inflammatory process. IL-1β can increase the pain sensitivity and prostaglandin E_2 (Zhang & An, 2007). Moreover, IL-1β has been reported to increase the risk of cancer (Gatti, Burbano, de Assumpcao, Smith Mde, & Payao, 2004). IL-6 is produced from the stimulation of monocytes and macrophages. It is the important cytokine in acute inflammatory process which activated in B cells, CD4+ T cells, CD8+ T cells and NK cells, through stimulate acute phase proteins (Mire-Sluis & Thorpe, 1998). IL-6 play support role in mononuclear cell accumulation at the damage site and stimulate monocyte chemoattractant protein-1, through angioproliferation and anti-apoptotic functions on T cells. These all lead to increasing of the chronic inflammatory process (Gabay, 2006). Though inflammatory cytokines trigger the response of inflammation, anti-inflammatory cytokines suppress the inflammation and immune response. IL-10 is the most important anti-inflammatory cytokine. IL-10 down-regulates the synthesis of pro-inflammatory cytokines from monocytes or macrophages. It also inhibits cytokine production from neutrophils and NK cells. Moreover, IL-10 suppresses nuclear factor κB translocation and increases mRNA degradation of pro-inflammatory cytokines (Opal & De Palo, 2000).

At present, many researchers are interested in some agents to increase immune responses, called immunomodulators. Immunomodulators are substance which can suppress and stimulate components of the immune system (Agarwal & Singh, 1999; Enshasy, 2010) such as cyclosporine A, which is used as immunosuppressive drug for transplant patients, inhibits T-cell growth factor gene expression (Kronke et al., 1984; Uchida et al., 2004). Currently, many researches have highlighted the ability of substances from plants to increase immune response and play an important role in preventing of human diseases. Many plants improve the ability of immune system such as to increase cytokine secretion, stimulate T cell, B cell and NK cell and enhance phagocytic capability (C. F. Huang, Lin, Liao, Young, & Yang, 2008; Spelman et al., 2006). Moreover, immunomodulators can combine with a specific antigen to improve the immune response more than using the antigen alone, thus they are called adjuvants. Many natural products have been reported that showe adjuvant effect for example the bark extract of *Quillaja saponaria*, ginseng and astragalus (Rajput, Hu, Xiao, & Arijo, 2007).

In Thailand, Benjakul and Hua-Khao-Yen are Thai traditional preparations used for the treatment of cancer. Benjakul consists of parts from five plants in equal amounts; fruit of *Piper chaba* Hunt. (Piperaceae), root of *Piper sarmentosum* Roxb. (Piperaceae), stem of *Piper interruptum* Opiz (Piperaceae), root of *Plumbago indica* Linn. (Plumbaginaceae) and rhizome of *Zingiber officinale* Rosc. (Zingiberaceae). Benjakul is used to balance health in Thai traditional medicine. Selection interviews with folk doctors of Southern Thailand revealed that Benjakul was used as an adaptogenic drug, given to cancer patients before other drugs (Itharat, Sinchangchai, & Ratanasuwan, 1998). Benjakul has been used for the treatment of lung cancer patients at Arokayasala, Kumpramong Temple, Sakolnakorn Province in Thailand for more than ten years. Benjakul is also used for treatment of dyspepsia in The National Drug List of Herbal Medicine Products A.D. 2006 (Bureau of Drug Control, 2012).

Hua-Khao-Yen is Thai name of medicinal plant which found in many preparations of Thai traditional medicine detailed in textbooks (Ancient Medicine Association, 1962, 1978; Mutita, 1989; Pongbunrod, 1976; Traditional Lanna Thai Medicine, 1982). Hua-Khao-Yen are used to treat lymphopathy, dermopathy, leprosy,

venereal diseases, and cancer as well as inflammatory states involved with other diseases such as infectious diseases, rheumatism and other pain-causing conditions. Moreover, they are used as ingredients in Thai traditional drug preparation for cancer (Vimolkhunakorn, 1979). From the reported of Itharat (1998), folk doctors of Southern Thailand used Hua-Khao-Yen as an ingredient in their drug formulae for cancer in 60% of the 30 cancer drug formulae of drugs for cancer listed (Itharat et al., 1998). In fact, it is the most commonly used medicinal plants for this purpose. In a survey where 40 samples were collected from traditional pharmacies both in Bangkok and other provinces throughout the country, the samples of 'Hua-Khao-Yen' were identified as four species, namely *Smilax glabra* Roxb., *Smilax corbularia* Kunth; *Dioscorea birmanica* Prain et Burkill; and *Pygmaeopremna herbacea* (Roxb.) Mold. (Boonyaratanakornkit & Chantarateptawan, 1993). Moreover, Itharat (2002) found that five species of Hua-Khao-Yen such as *Dioscorea birmanica*, *Smilax corbularia*, *Smilax glabra*, *Pygmaeopremna herbacea* and *Dioscorea membranacea* have been used to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat, 2002). The extraction method of these Hua-Khao-Yen are usually prepared by decoction with water or marceration with ethanol (Pongbunrod, 1976; Tungtrongjit, 1978). Two species such as *Dioscorea membranacea* Pierre and *Smilax corbularia* Kunth are popularly used as anti-cancer drugs to treat cancer patients. *Dioscorea membranacea* Pierre or Thai name Hua-Khao-Yen-Tai showed the high cytotoxic activity against human lung cancer cells (A549, COR-L23 and H226) but was less effect against keratinocyte normal cells (SVK-14) (Ruangnoo, 2012). Moreover, its ethanolic extract showed strongly cytotoxic activity against lung, breast and colon cancer cell lines (IC_{50} = 6.2, 12.0 and 16.7 µg/ml, respectively) while its water extract that be effective against breast and colon cancer cell lines (MCF-7 and LS-174T) (IC_{50} = 5.5 and 15.6 µg/ml, respectively). In addition, the water extract of this plant also showed no cytotoxic activity against keratinocyte normal cell line (SVK-14) (IC_{50} > 70 µg/ml) which also showed the specificity of *D. membranacea* extracts to cancer cell lines (Itharat et al., 2004). *Smilax corbularia* Kunth showed less cytotoxic activity against breast cancer (MCF-7) and lung cancer (A549) cell lines. However, the ethanolic extract of *S. corbularia* showed high antioxidant activity by lipid peroxidation of liposome assay and DPPH assay with the EC_{50} of 8.10 and 3.91 µg/ml, respectively

(Itharat, 2010; Ruangnoo, 2012). In addition, *S. corbularia* rhizome showed that oral administration of the ethanol extract of *S. corbularia* rhizomes (1,600 mg/kg) significantly inhibited the paw edema induced by carrageenin in rats (Reanmongkol, Itharat, & Bouking, 2007). However, there is no report about immunomodulatory activity of *Dioscorea membranacea* Pierre and *Smilax corbularia* Kunth and its compounds on NK cell or cytokine-related immunomodulatory effect.

Currently, the clinical study of Benjakul tablet showed that some volunteers had stomachache after taking Benjakul tablet (Amorndoljai &Kietinun &Somparn, 2011). Benjakul may activate sensory receptor which involve in burning sensation. Moreover, the main pure compound of Benjakul is piperine which is found in *Piper* species. Previous study showed that piperine activated TRPV1 and TRPA1 receptors in sensory neurons which leave burning sensation after taste (McNamara et al., 2005; Okumura et al., 2010). Benjakul consist of three *Piper* species namely *P. chaba*, *P. sarmentosum* and *P. interruptum*. Thus, three *Piper* species may activate TRP channel. TRPA1 and TRPV 1 receptors are member of transient receptor potential cation channels (Rosenbaum & Simon, 2007). The activation of TRPA1 and TRPV1 are involved in the adjuvant effect during contact hypersensitivity (Shiba et al., 2009). Contact hypersensitivity is the IV type hypersensitivity or delayed type of hypersensitivity reaction which show the inflammatory response within 24-48 hours after reexposure with hapten. Signs of contact hypersensitivity are rash, itching, dry skin and swelling (Gaspari & Kats, 2001). However, piperine and three *Piper* species have not been reported about contact hypersensitivity. Thus, three *Piper* species were investigated on effect on TRP channel and contact hypersensitivity to use as basic data about side effect of traditional plants.

Therefore, the objectives of this study were to investigate the immunomodulatory activity related with immune response to cancer of Benjakul, two types of Hua-Khao-Yen and their isolated compounds. This study focused on the immunostimulatory effect, including lymphocyte proliferation activity, NK cells activity, IL-2, IL-10 and IFN-γ production. Futhermore, the immunosuppression effect is foucused on anti-inflammatory activity such as, the inhibitory effect on IL-1β and IL-6 production. Lastly, the safety study was investigated by in vitro and in vivo

experiment. The finding may be used as basic data of Benjakul preparation and Hua-Khao-Yen for immunomodulatory activity in cancer patients.

1.2 Objectives of this study

1.2.1 Overall objectives of this study

Overall objectives of this study are to investigate immunomodulatory activities of Hua-Khao-Yen, Benjakul and theirs pure compounds to confirm the capability of the medicinal plants for development into immunomodulatory agents.

1.2.2 Specific objectives of this study

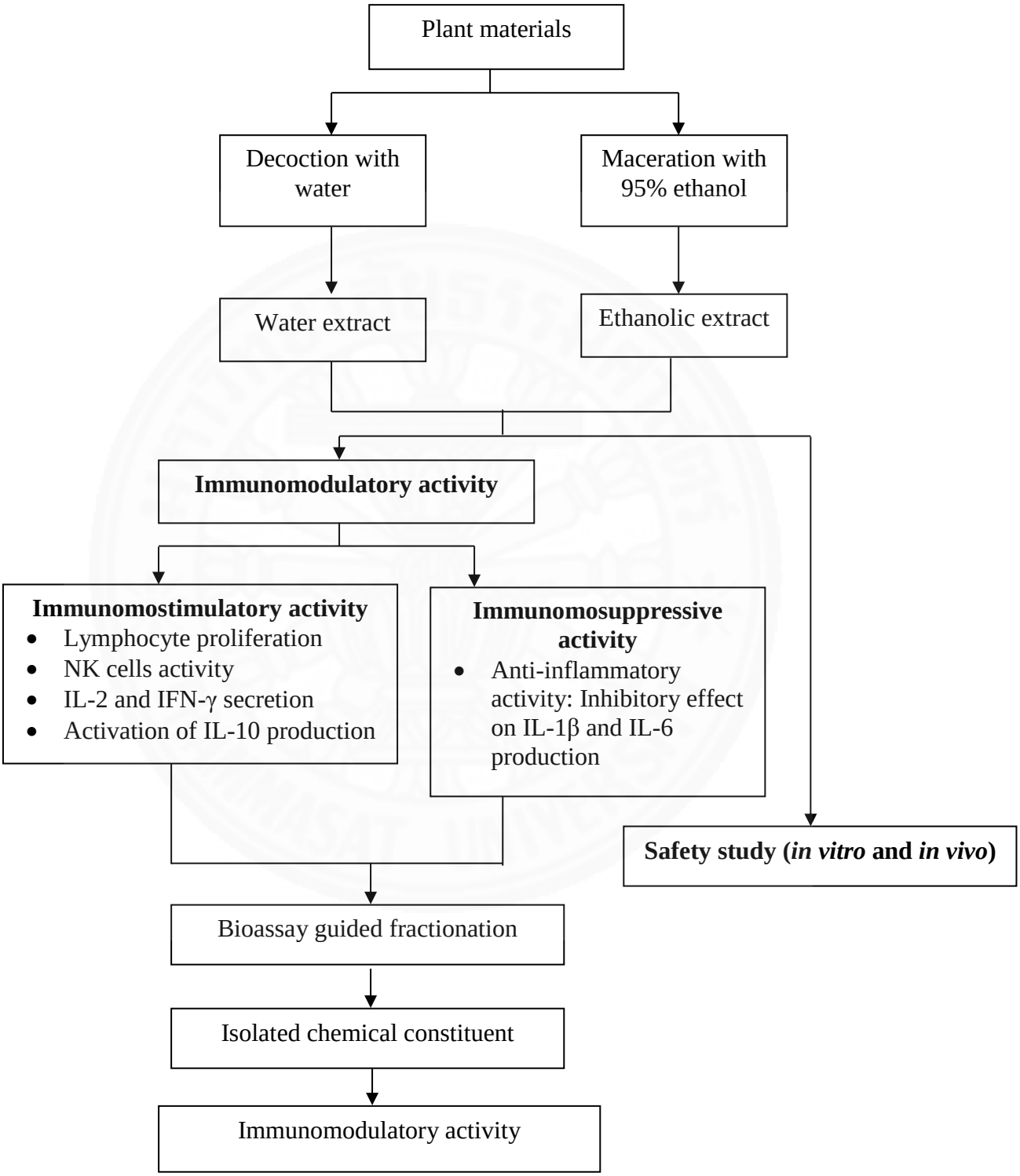
1.2.1.1 To study natural killer cell activity of ethanolic and aqueous extracts of Hua-Khao-Yen, Benjakul and their pure compounds.

1.2.1.2 To study lymphocyte proliferation of ethanolic and aqueous extracts of Hua-Khao-Yen, Benjakul and their pure compounds.

1.2.1.3 To study the effect of IL-2 and IFN-γ secretion of ethanolic and aqueous extracts of Hua-Khao-Yen, Benjakul and their pure compounds.

1.2.1.4 To study the effect of ethanolic and aqueous extracts of Hua-Khao-Yen, Benjakul and their pure compounds on inflammatory cytokine using RAW264.7 cell line.

1.3 Conceptual framework of thesis



CHAPTER 2
REVIEW OF LITERATURE

2.1 Immune system

The immune system is a protective system against invading pathogen, non-self-components and cancer cells. Early response to pathogens is called innate immunity and the late reaction is called adaptive immunity. Innate immunity is the first line of defense against microbes which consists of physical barriers, such as the skin and epithelial protection of internal organs and the cells that bear innate immune receptors, such as macrophages, dendritic cells, neutrophils, mast cells and natural killer cells. Adaptive immunity is a specific immune system and needs more response time than innate immunity. The adaptive immune system involves the antigen-specific receptors expressed on the surfaces of T and B cells (Chaplin, 2006).

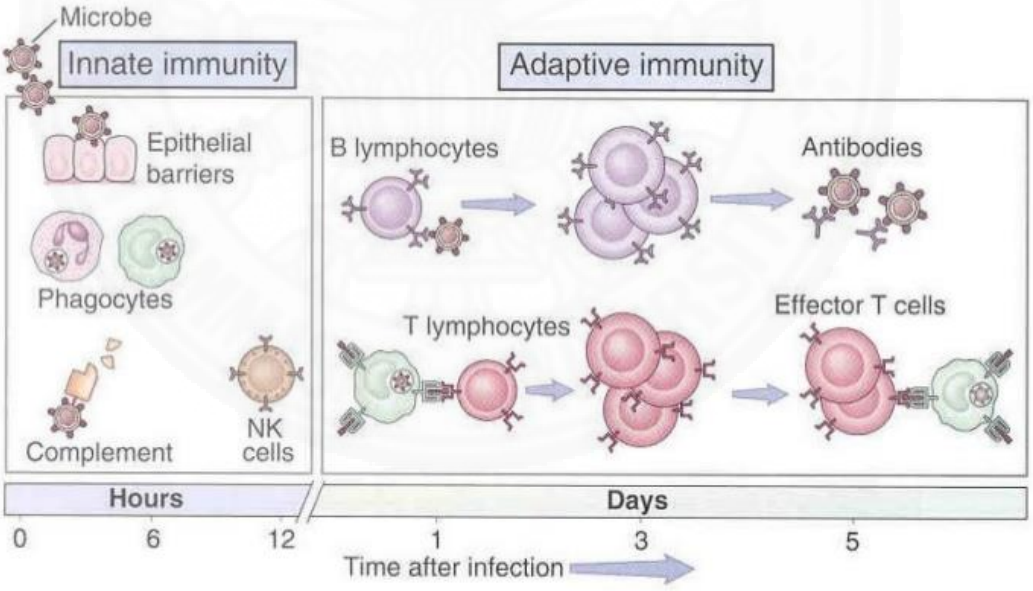


Figure 2.1 Integration of innate and adaptive immune system (Abbas et al., 2010).

The mechanism of innate immunity is for effector cells (neutrophils, mononuclear phagocytes and natural killer cells) to attack microbes that have branched epithelial barriers and have entered into tissues or the circulation system. Macrophages and neutrophils have surface receptors that have evolved to recognize

and bind common constituents of many pathogen surfaces. The response of macrophage induces the secretion of cytokines and other chemical mediators. The cytokines have a range of effects that are collectively known as inflammation (Abbas et al., 2010; Janeway & Travers, 1996). The four cardinal signs of inflammation are redness, heat, swelling and pain. The begining of inflammatory process can be called acute inflammation. The first stage of acute inflammation is an increase in the diameter of blood vessels. The engorged capillaries are responsible for tissue redness and an increase in tissue temperature. Then, the capillary increases its permeability. The fluid that accumulates has higher protein content than fluid normally secreted from the vasculature. Collection of exudate leads to tissue swelling. Phagocytes influx into the tissue and begin to ingest bacteria. If the pathogen cannot be eliminated, acute inflammation may progress to chronic inflammation. Chronic inflammation is the cause of tissue damage and is accompanied by simultaneous attempts at healing and repair. The extent and time course of chronic inflammation is variable and depends on the balance between the causing agent and the attempts of the body to remove it (Goldsby, Kindt, Osborne, & Kuby, 2003).

However, phagocytes cannot always eliminate infectious organisms and there are many pathogens that they cannot recognize. The adaptive immunity process is called into action against pathogens that are able to evade innate immunity. The adaptive immune system is divided into two parts including humoral immunity and cell mediated immunity. Humoral immunity is the interaction of B cells with antigen and differentiated into antibody-secreting plasma cells. The function of antibody is to neutralize antigen or to facilitate antigen elimination (Goldsby et al., 2003). Antibodies are specialized and different types of antibodies may activate different effector mechanisms. Cell mediated immunity or cellular immunity is mediated by T cells. The major roles of T cells are divided into two parts, namely lyses cells expressing specific antigens and release cytokines, which resist intracellular pathogens such as viruses, bacteria and parasites inaccessible to antibodies (Zabriskie, 2009).

2.1.1 Immune system to cancer

In the case of cancer, tumor cells express antigens recognizable by the immune system. NK cells are a line of defense against host cells that are stressed and

cancerous. The surface receptors of NK cells receive signals from the environment and determine their response to foreign or malignant cells. NK cells kill MHC class I-deficient cells. NK cells activity is controlled by positive and negative signals. MHC class I molecules are recognized by inhibitory receptors and blocks activation signals. The response of NK cells is producing effector molecules, which can both directly inhibit tumor growth and convey important information to the rest of the immune system (Moretta, Biassoni, Bottino, Mingari, & Moretta, 2000; Smyth, Hayakawa, Takeda, & Yagita, 2002). The helper T, or CD4+ T cells, is the important cell to regulator of partically all immune system functions. These cells produce protein mediators called lymphokines that act on other cells of the immune system and on bone marrow. The most important lymphokines secreted by the helper T cells consist of interleukin, granulocyte-monocyte colony stimulating factor (GM-CSF), and interferon (IFN)-γ. Lymphokines are produced by CD4+ T cells that regulate macrophage response. Macrophages are decreased the migration by lymphokines after they have been activated and accumulated at the site. These lymphokines also activate more productive phagocytosis, so they can destroy rapidly increasing numbers of toxins. The T-helper cell increases its ability by producing lymphokines, especially IL-2. This lymphokine improves the helper cell ability as well as the entire immune system's response to pathogens. Like cytotoxic T cells, the helper T cells also activate via MHC peptide complexes via class II MHC. Helper T cells increase the immune response by producing cytokines that activate either a cytotoxic T cell response (Th1 helper T cells) or an antibody response (Th2 helper T cells). These cytokines can activate B cells to generate antibodies or enhance cytotoxic T cell production. The role of the helper T cell depends on the antigen type it recognizes and the type of immune response required. The immune disorder involved with human cancer consist of changes within the immune system including cytokine unbalance of Th1/Th2 origin (Lauerova et al., 2002). Furthermore, chronic inflammation may lead to tumor initiation.

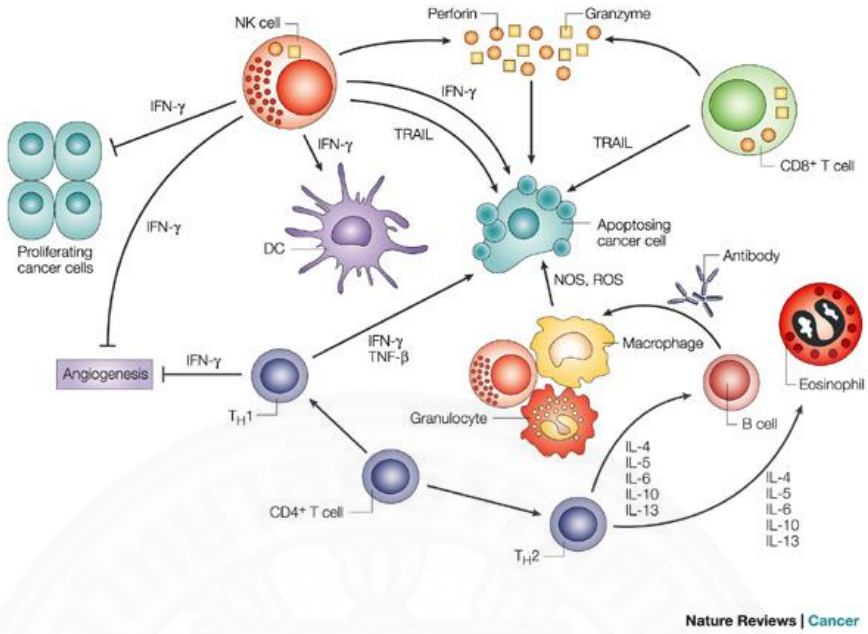


Figure 2.2 A coordinated cellular and humoral reaction mediates tumor destruction (Dranoff, 2004).

2.1.1.1 Natural killer cells

Natural killer (NK) cells are lymphocytes of the innate immune system. NKcells are generated from hematopoietic stem cells that develop in bone marrow, lymph nodes and liver (Mandal & Viswanathan, 2015). NK cells are classified into two subsets which depend on cell immunophenotyping namely $CD56^{bright}$ and $CD56^{dim}$. The $CD56^{bright}$ is 10% of NK cell population in circulating blood which are involved with cytokines production and immunoregulatory function. The $CD56^{dim}$ is about 90% of NK cell population and it plays the role in natural and Ab-mediated cell cytotoxicity (Chan et al., 2007; Mandal & Viswanathan, 2015). NK cells are activated via ligands on the cells such as the stress-induced self ligands recognized by NKG2D and Toll-like receptor ligands. Futhermore, some molecules on the target can inhibit or stimulate NK cell activity. NK cells are activated by MHC class-I deficient cells, where as NK cells express MHC class-I specific receptors and lose inhibitory signals when interaction cells that lack MHC class-I (Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008).

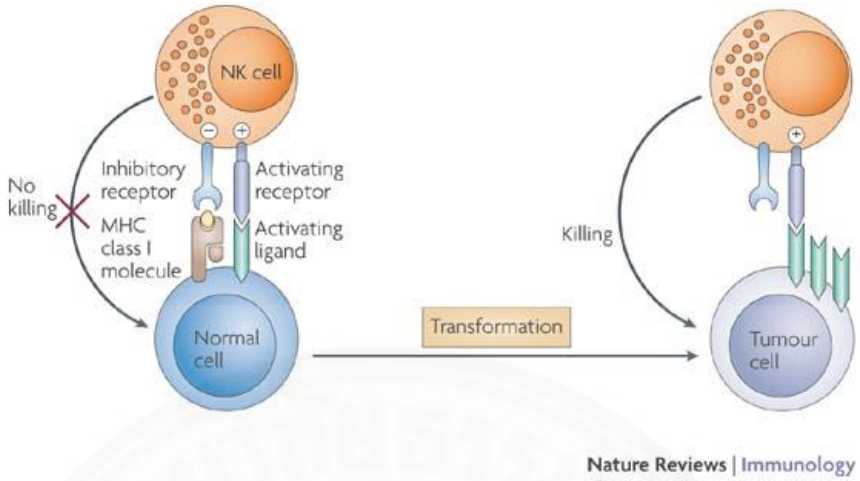


Figure 2.3 Missing self-recognition of tumor cells (Ljunggren & Malmberg, 2007).

When NK cells are activated, the cytolytic granules are released by exocytosis and induce the apoptosis of target cells. The cytolytic granules contain the serine protease granzymes A and B, the membrane-disrupting protein perforin, the antimicrobial lytic molecular granulysin. Perforin enters the target cell and releases lytic complexes from endocytic vehicles of the target cells. Granzyme that is carried by serglycin is facilitated into the target cell. Then, cells are induced to apoptosis via caspase-dependent apoptotic pathways (Uhrberg, 2005).

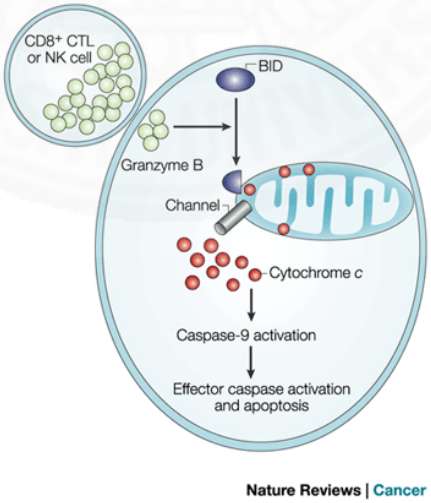


Figure 2.4 NK cell induced cell death (Andrews, Scalzo, Yokoyama, Smyth, & Degli-Esposti, 2003).

2.1.1.2 T cells

Cytotoxic T cells can kill tumor cells or malignant cells that express peptide from mutant cellular proteins and presented in association with MHC class I. Antigen presenting cells such as dendritic cells ingest peptide of tumor antigen and express to MHC class I molecule for recognition by Cytotoxic T cells. Moreover, the antigen presenting cells express tumor peptide to MHC class II molecules which activate CD4+ helper T cells. CD4+ helper T cells may secrete cytokines such as TNF-γ and IFN-γ that increase tumor cells class I MHC expression and sensitivity to lysis by cytotoxic T cells (Abbas et al., 2010).

2.1.1.3 Macrophage

Macrophages are cells in the innate immune response. They can be broadly divided to the classically-activated type 1 macrophages (M1) and the alternatively-activated type 2 macrophages (M2). The activation of M1 macrophage leads to destroy microbes, eliminate tumor cells, present antigen to T cells and produce pro-inflammatory cytokines. Moreover, M1 macrophages promote a strong immune response by IL-12, nitric oxide synthase and reactive oxygen species. The M2 polarization is promoted by IL-4, IL-10, IL-13, transforming growth factor-beta and prostaglandin E_2 (Quatromoni & Eruslanov, 2012). Some clinical studies show the role of macrophages in tumorigenesis promotion. It is reported that there is strong correlation between high macrophage density and the increasing survival in pancreatic cancer (Qian & Pollard, 2010). The M2 macrophages promote tumor development because it can boost tissue remodeling and wound healing such as digestion of extracellular matrix with matrix metalloproteinase, promotion of angiogenesis via vascular endothelial growth factor production and debris scavenging (Quatromoni & Eruslanov, 2012).

2.1.1.4 Cytokines in immune response to tumor

Cytokines are protein molecules that are involved in communications and interactions between cells. They produce immune cells in response to pathogen or tumor antigens. Some cytokines show multiple effects on the same target such as inducing or inhibiting the synthesis and effect of other cytokines (Khan, 2008). Cytokines directly activate immune cells at the tumor site and stimulate tumor

recognition by cytotoxic effector cells. In recent clinical study, cytokines have been used to stimulate immune response against cancer (S. Lee & Margolin, 2011).

(1) Interferon-γ (IFN-γ)

IFN-γ or type II interferon is produced by T helper cells, cytotoxic T cells and NK cells. IFN-γ enhance the expression of MHC-class II antigens to induce CD4+ T cell response. IFN-γ stimulates the expression of MHC class I proteins to promote the development of CD8+ T cells response and enhance the immunogenicity of the tumor. Moreover, IFN-γ is involved in Th1 cells development. Thus, it stimulates the IL-12 synthesis in antigen-presenting cells to facilitate Th1 production. Then, IFN-γ prevents Th2 cells development by inhibiting the IL-4 production. Furthermore, the activity of IFN-γ on macrophages is activation of the tumoricidal capacity of these cells (Dranoff, 2004; Mire-Sluis & Thorpe, 1998; Stark, Kerr, Williams, Silverman, & Schreiber, 1998) .

(2) Interleukin-2 (IL-2)

IL-2 is the important growth factor for T cells. IL-2 stimulates T helper cells proliferation and cytokine release from these cells. Moreover, IL-2 plays a role in the differentiation of CD4+ T cells into various functions of T cells (Rosenberg, 2014). IL-2 also increases the NK cell function and activates lymphokine to produce lymphokine-activated killer cells (LAK). LAK increase the potency of the ability of immune response to kill tumor cells (Khan, 2008). IL-2 was used in early-stage clinical trials for enhancing NK cells and CD8+ T cell function and increasing vascular permeability in cancer patients.

2.1.2 Inflammation and cancer

The inflammatory response defends the host from microbial infection and mediates tissue repair and regeneration, which may occur due to infectious or non-infectious tissue damage. During the inflammatory response, leukocyte recruitment occurs through improved expression of cellular adhesion molecules and chemo-attraction is involved in many soluble factors. Cytokines are one of the soluble factors of the inflammatory process which relate synergistic as well as antagonistic interactions and show both negative and positive regulatory effects on various target cells (Feghali & Wright, 1997). For acute inflammation, many systemic responses are due to the combined action of three pro-inflammatory cytokines, namely IL-1, TNF-α

and IL-6. IL-1 and TNF- α can activate fever by incresing prostaglandin E_2 production by the vascular endothelium of the hypothalamus and induce acute phase response (Feghali & Wright, 1997). Besides pro-inflammatory cytokines, IL-10 is anti-inflammatory cytokine which has an important role to limit the excessive inflammatory reaction (Opal & De Palo, 2000). However, if inflammation resolution is dysregulated, cellular response develops to the pattern of chronic inflammation.

Chronic inflammation is characterized by sustained tissue damage, damage-induced cellular proliferation, and tissue repair (Cordon-Cardo & Prives, 1999). The chronic inflammation microenvironment is predominated by macrophages (Coussens & Werb, 2002). Macrophages produce reactive oxygen and nitrogen species to against infection. However, in a setting of continuous tissue damage and cellular proliferation, the persistence of these infection-fighting agents is deleterious. They may produce mutagenic agents which lead to DNA mutation and cause mutations in proliferating epithelial and stroma cells (Maeda & Akaike, 1998). Macrophages and T cells may generate tumor necrosis factor-alpha (TNF-α) and macrophage migration inhibitory factor to exacerbate DNA damage (Polland, 2004). Tumor-associated macrophages (TAMs) contribute to tumor development through several mechanisms. Moreover, they release interleukin (IL)-10 and prostaglandin E_2, which suppress antitumor response (Elgert, Alleva, & Mullins, 1998). Furthermore, TAMs may activate TNF-α and iNOS, the role of which links inflammation to cancer. TNF has been found to be required in chemical carcinogen-elicited skin carcinogenesis (C. Huang, Li, Ma, & Dong, 1999) and also is a major inducer for nuclear factor-κB (NF-κB) activation, which shows anti-apoptotic activity (Philip, Rowley, & H., 2004).

2.2 Immunomodulator

Immunomodulators are substances which stimulate, suppress and modulate the immune system. In clinical practice, immunomodulators are divided into three categories: immunosuppressants, immunostimulants and immunoadjuvants (Enshasy, 2010).

2.2.1 Immunosuppressants

Immunosuppressants are substances which inhibit the immune system. They can be used in organ transplantation to control pathological immune response and to treat autoimmune disorders, hypersensitivity immune reaction and anti-microbials (Enshasy, 2010). An example of an immunosuppressant is cyclosporine which is used as immunosuppressant glucocorticoids to interfere in the activation and proliferation of CD8+ T cells (Fireman, DiMartini, Armstrong, & Cozza, 2004).

2.2.2 Immunostimulators

Immunostimulators are agents that stimulate the immune system. There has been recent interest in immunostimulants to boost the immune system. They enhance the ability of immune response against allergy, infection, cancer and auto-immunity (Enshasy, 2010). An example of an immunostimulant is isoprinosine which induces cytokines production and increases lymphocyte proliferation (Patil, Jaydeokar, & Bandawane, 2012). Another example of an immunostimulant is polysaccharides from mushroom. The mushroom polysaccharides can stimulate effector cells such as macrophages, T cells, NK cells and cytokine secretion. Futhermore, the mushroom polysaccharides show less toxicity and side effects when compared to other immunostimulants (Enshasy, 2010).

2.2.3 Immunoadjuvants

Immunoadjuvants are substances used to enhance the immunogenicity of antigen which is required for improvement of vaccines and drugs efficacy. They can be used as a specific immune stimulator effect (Enshasy, 2010; Rey-Ladino, Ross, Cripps, McManus, & Quinn, 2011). For example, saponin from *Quillaja saponaria* has been used to induce antibody response against cancer antigens in experimental cancer vaccines (Ragupathi et al., 2008). The extract from *Astragalus membranaceus* has been found to induce antibody responses to cancer antigens Globo H and GD3 following vaccination of mice (Ragupathi et al., 2010).

2.3 Medicinal plants for treatment of cancer

Currently, treatment for cancer is chemotherapy, radiotherapy and surgery. However, the effectiveness of chemotherapy is often limited by toxic effects on other

non-target tissues. Consequently, complementary and alternative therapies such as herbal therapies are increasingly used (Vickers, 2004). Use of plants for therapy is not new, indeed plants have been considered a valuable source of bioactive compounds for treatment of many conditions, including cancer, in almost all cultures and communities for many years (Desai et al., 2008). Moreover, many plants can stimulate, suppress or modulate any part of the immune system that is involved cancer treatment, including both innate and adaptive immune response (Kumar, Kumar, Rupesh, & Nitin, 2011).

In Thailand, Hua-Khao-Yen and Benjakul are Thai traditional preparations for treatment of cancer. In Thai traditional medicine, herbal drugs locally known as Hua-Khao-Yen, especially Hua-Khao-Yen-Nua and Hua-Khao-Yen-Tai, have long been used as ingredients in many Thai preparations, including those used in treatments of inflammation, lymphopathy, cancers, veneral diseases, leprosy and dermopathy (Boonyaratanakornkit & Chantarateptawan, 1993). Benjakul is consisted of five plants, namely *Piper chaba* Hunter, *Piper interruptum* Opiz, *Piper sarmentosum* Roxb., *Plumbago indica* Linn. and *Zingiber officinale* Roscoe. and is used for health balance and cancer treatment (Itharat et al., 1998).

2.3.1 *Dioscorea membranacea* Pierre or Hua-Khao-Yen-Tai

2.3.1.1 Description of *Dioscorea membranacea* Pierre

The rhizome is dark brown with white flesh. It is a wide running, probably even to 2 meters. The stem is slightly ridge and unbranched. Leaves are deeply tri-fid above a cordate base with the short acuminate 9 nerved, two primary nerves reaching the forerunner tip along with the midrib and the second pair reaching the tips of the latural lobes. The length of petioles is 1/2-2/3 of the blade. Male flowers have flowers and small sub sessile cymes. Moreover it has sepals 1 mm long and long-ovate. Stamens, similar the filaments insert just below the sepals 0.3 mm long. The anther is small and introse. Female flowers are on downwardly-directed spike-like racemes. Outer sepals are obovate, inner ones are lanceolate, and the inner are a little shorter than the outer. Style is short. Capsules are apart, about 1-2 cm (Burkill, 1951).

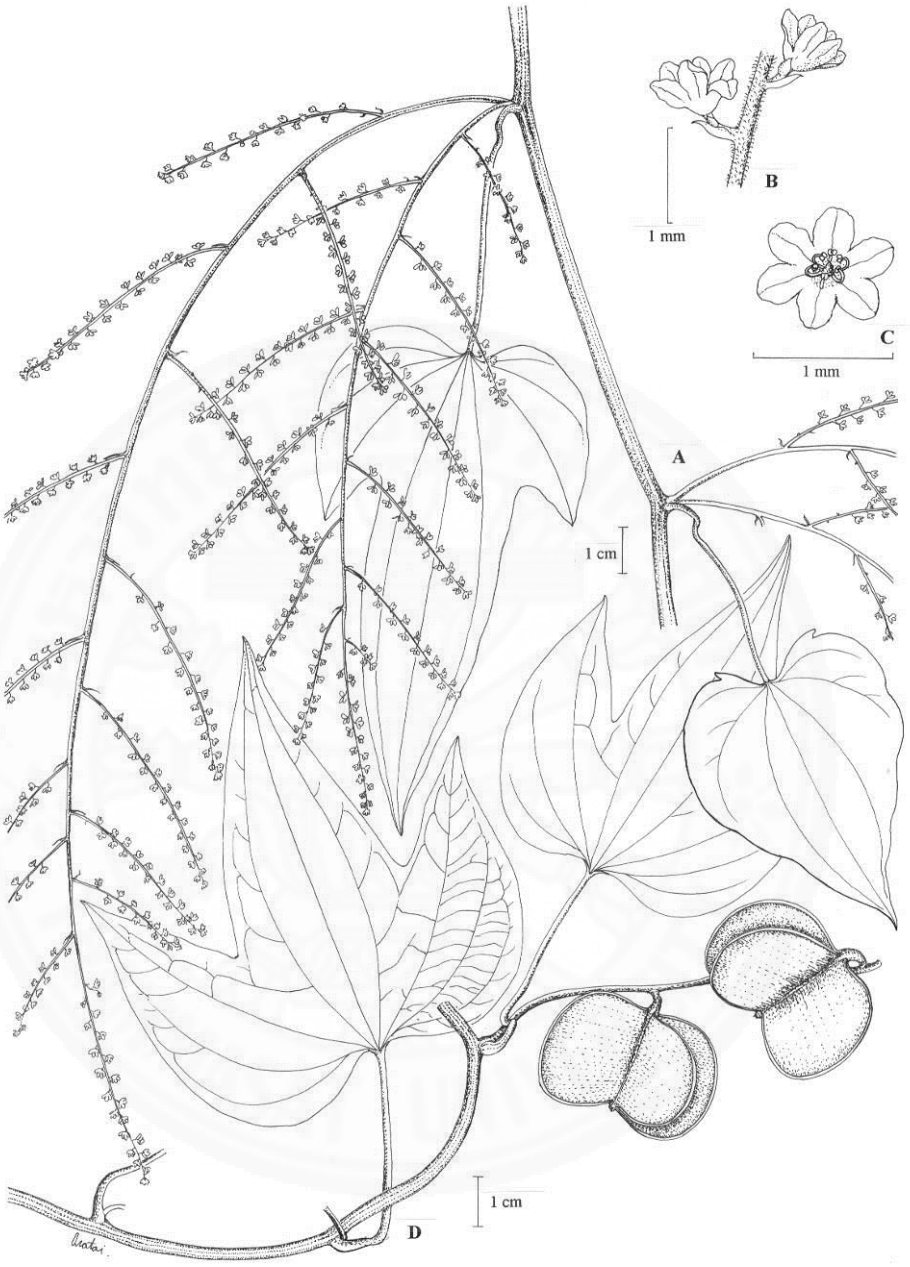
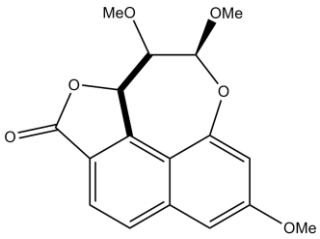


Figure 2.5 *Dioscorea membranacea* Pierre.; A and B: inflorescence; C: flower; D: stem (Boonyaratanakornkit & Chantarateptawan, 1993)

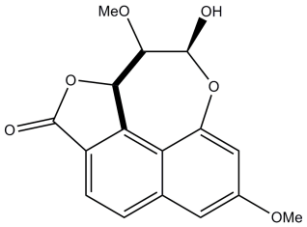
2.3.1.2 Chemical composition of *Dioscorea membranacea* Pierre

Table 2.1 Chemical composition of *Dioscorea membranacea* Pierre

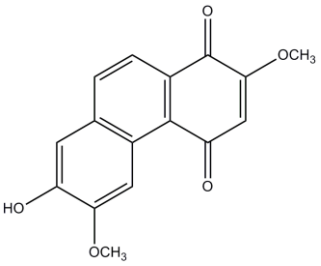
Part of used	Chemical composition	References
Rhizome	• Dioscorealide A	(Itharat, Kaewpradub, Plubrukarn, Chuchom, & Houghton, 2005; Itharat et al., 2003)
	• Dioscorealide B	(Itharat et al., 2005; Itharat et al., 2003)
	• Dioscoreanone	(Itharat et al., 2005; Itharat et al., 2003)
	• β-sitosterol	(Itharat et al., 2005)
	• Stigmasterol	(Itharat et al., 2005)
	• β-D-Sitosterol glucoside	(Itharat et al., 2005)
	• Diosgenyl 3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside	(Itharat et al., 2005)
	• Diosgenyl 3-O-β-D-rhamnopyranosyl (1→3)-β-D-glucopyranoside	(Itharat et al., 2005)
	• Diosgenin	(Itharat et al., 2005)
	• 5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene	(Itharat, Thongdeeying, & Ruangnoo, 2014)
	• 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene	(Itharat et al., 2014)
	• 2,5,6-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene	(Itharat et al., 2014)



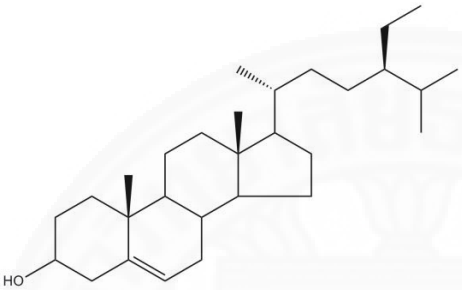
Dioscorealide A



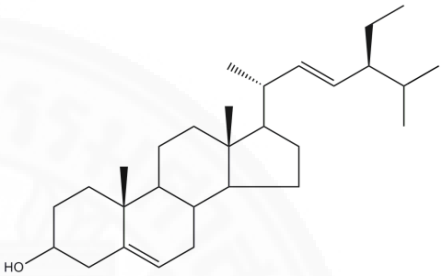
Dioscorealide B



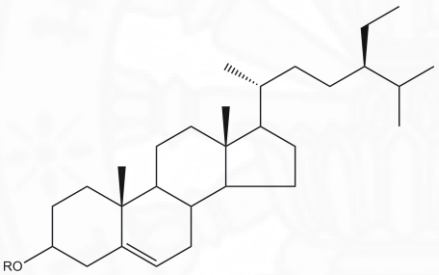
Dioscoreanone



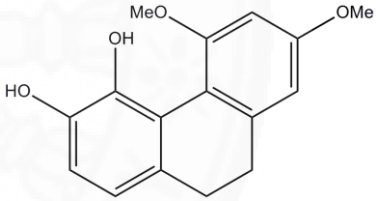
β-sitosterol



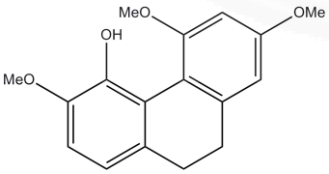
Stigmasterol



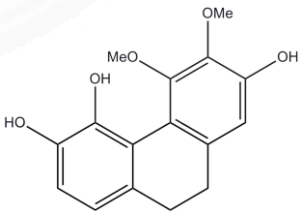
β-D-Sitosterol glucoside



5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene

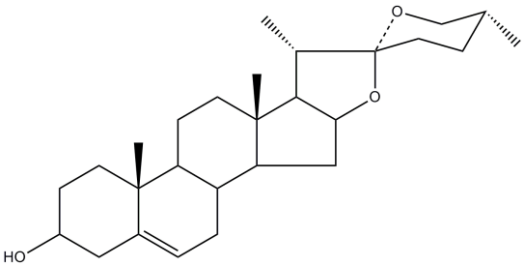


5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene

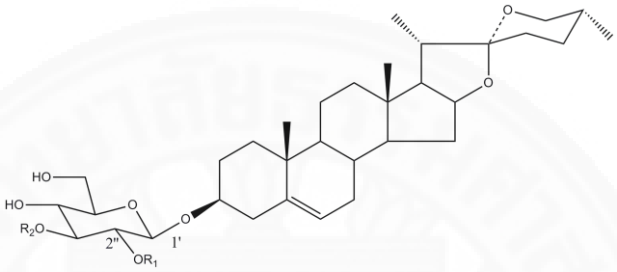


2,5,6-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene

Figure 2.6 Structure of chemical compounds in *Dioscorea membranacea* Pierre



Diosgenin



Diosgenyl 3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside (R_1=Fructose, R_2=H)

Diosgenyl 3-O-β-D-rhamnopyranosyl (1→3)-β-D-glucopyranoside (R_1=H, R_2=Glucose)

Figure 2.6 Structure of chemical compounds in *Dioscorea membranacea* Pierre (Cont.)

2.3.1.3 Biological activity of *Dioscorea membranacea* Pierre

The literature of biological activity of *D. membranacea* Pierre and its pure compounds are shown in the Table 2.2 and 2.3.

Table 2.2 Biological activities of *Dioscorea membranacea* Pierre.

Part used	Activities	Results	References
Rhizomes	Anti-allergy activity	• The ethanolic extract inhibited β-hexosaminidase release in RBL-2H3 cells with IC_{50} value of 37.5 μg/ml	(Tewtrakul & Itharat, 2006)
Rhizomes	Cytotoxic activity	• The water extract active against breast, colon, and lung cancer cell lines with IC_{50} value of 5.5, 15.6 and 16.3 μg/ml, respectively.	(Itharat et al., 2004)
		• The ethanolic extract showed anti-cancer activity on lung, colon and breast cancer cell lines (IC_{50} = 6.2, 16.7, 12.0 μg/ml, respectively).	(Itharat et al., 2004)
		• The ethanolic extract exhibited cytotoxic activity against lung cancer cell line CORL-23 and prostate cancer cell line PC3 with IC_{50} value of 4.63 and 7.55 μg/ml, respectively.	(Saetung et al., 2005)

Table 2.2 Biological activities of *Dioscorea membranacea* Pierre (Cont.)

Part used	Activities	Results	References
Rhizomes	Cytotoxic activity	• The ethanolic extract active against LS-174T and SW480 colon cancer cell lines with IC_{50} values of 45.97 and 62.83 μg/ml, respectively.	(Jaiaree, Ruangnoo, Thongdeeying, & Itharat, 2013)
Rhizomes	Anti-HIV activity	• The ethanolic extract against HIV-1 protease with IC_{50} value of 48 μg/ml.	(Tewtrakul, Itharat, & Rattanasuwan, 2006)
Rhizomes	Anti-inflammatory activity	• Ethanolic and water extracts exhibited inhibitory activities against LPS induced NO production in RAW264.7 cell lines with IC_{50} value of 23.6 and 57.8 μg/ml, respectively.	(Tewtrakul & Itharat, 2007)
Rhizomes	Anti-inflammatory activity	• The ethanolic extract (1,600 mg/kg) significantly decreased a paw edema induced by carrageenan in rats. The ethanolic and water extracts showed no effect on writhing test and yeast-induced fever.	(Reanmongkol, Itharat, & Bouking, 2007)

Table 2.2 Biological activities of *Dioscorea membranacea* Pierre (Cont.)

Part used	Activities	Results	References
Rhizomes	Anti-malarial activity	• The ethanolic extract showed good to moderate activities against K1 and 3D7 *P. falciparum* clone (IC_{50} = 5.1, 6.2 respectively).	(Thiengsusuk, Chaijaroenkul, & Na-Bangchang, 2013)

Table 2.3 Biological activities of pure compounds from *Dioscorea membranacea* Pierre

Activities	Compounds	Results	References
Anti-allergy activity	Dioscorealide A	• Dioscorealide A showed inhibitory activity against β-hexosaminidase, TNF-α and IL-4 release from RBL-2H3 cells with IC_{50} value of 27.9, 33.1 and 36.2 μM, respectively.	(Tewtrakul & Itharat, 2006)
	Dioscorealide B	• Dioscorealide B inhibited β-hexosaminidase, TNF-α and IL-4 release from RBL-2H3 cells (IC_{50} = 5.7, 22.0 and 73.6 μM, respectively).	(Tewtrakul & Itharat, 2006)
	Dioscoreanone	• Dioscoreanone inhibited LPS-induced β-hexosaminidase, TNF-α and IL-4 release from RBL-2H3 cells with IC_{50} values of 7.7, 8.1 and 6.0 μM, respectively.	(Tewtrakul & Itharat, 2006)
	Diosgenin	• Diosgenin exhibited inhibitory activity against β-hexosaminidase release from RBL-2H3 cells with IC_{50} value of 29.9 μM.	(Tewtrakul & Itharat, 2006)
Cytotoxic activity	Dioscorealide A	• Dioscorealide A is less effective against breast cancer cell line, MCF-7, with IC_{50} value of 27.4 μg/ml.	(Itharat et al., 2003)

Table 2.3 Biological activities of pure compounds from *Dioscorea membranacea* Pierre (Cont.)

Activities	Compounds	Results	References
Cytotoxic activity	Dioscorealide B	• Dioscorealide B active against large cell lung carcinoma COR-L23, colon adenocarcinoma LS-174T, breast adenocarcinoma MCF-7 (IC_{50}=1.59, 5.26,0.92 μg/ml, respectively).	(Itharat et al., 2003)
Cytotoxic activity	Dioscorealide B	• Dioscorealide B active against breast cancer cell lines MCF-7 and MDA-MB 468 (IC_{50}=2.76, 9.93 μM, respectively). Furthermore, dioscorealide B induced apoptosis in MCF-7 via activation of caspase-9 and caspase-7.	(Saekoo, Dechsukum, Graidist, & Itharat, 2010)
		• Dioscorealide B active against three types of human colon cancer cell lines (Caco-2, LS-174T, SW-480) with IC_{50} values of 10.26, 2.23 and 14.53 μM, respectively.	(Jaiaree et al., 2013)
	Dioscoreanone	• Dioscoreanone has cytotoxic activity against large cell lung carcinoma COR-L23, breast adenocarcinoma MCF-7, colon adenocarcinoma LS-174T (IC_{50}= 2.89, 3.76 and 9.96 μg/ml, respectively).	(Itharat et al., 2003)

Table 2.3 Biological activities of pure compounds from *Dioscorea membranacea* Pierre (Cont.)

Activities	Compounds	Results	References
Cytotoxic activity	Dioscoreanone	• Dioscoreanone showed high cytotoxic activity on human colon cancer cell lines Caco-2, LS-174T and SW-480 (IC_{50}=8.66, 2.56 and 4.98 μM, respectively).	(Jaiaree et al., 2013)
	5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene	•5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene exhibited cytotoxicity against prostate, lung, cervical, breast and liver cancer cell lines (IC_{50}= 19.04, 14.89, 82.62, 17.49 and 69.89 μM, respectively).	(Itharat et al., 2014)
	5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene	• 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene showed high cytotoxic activity on prostate, lung, cervical, breast and liver cancer cell lines with IC_{50} value of 23.54, 45.54, 127.28, 82.77 and 90.74 μM, respectively.	(Itharat et al., 2014)
	2,5,6-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene	•2,5,6-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene active against prostate, lung, breast and liver cancer cell lines (IC_{50}=51.89, 81.59, 31.47 and 107.07 μM, respectively). It showed cytotoxicity against normal cell lines MRC-5 with IC_{50} value of 37.94 μM.	(Itharat et al., 2014)

Table 2.3 Biological activities of pure compounds from *Dioscorea membranacea* Pierre (Cont.)

Activities	Compounds	Results	References
Cytotoxic activity	Diosgenin	• Diosgenin (10-25 μM) inhibited the HER2-overexpressing human breast cancer AU565 cells which decreased the expression of FAS in these cells.	(Chiang, Way, Tsai, & Lin, 2007)
	Diosgenin	• Diosgenin induced HepG2 apoptosis at concentration of 40 μM. It activated Bax expression and decreased Bid and Bcl-2 expression.	(Kim, Jeon, Lee, Woo, & Mun, 2012)
Anti-inflammatory activity	Diosgenin	• Diosgenin (0.1-10 μM) inhibited NO production, iNOS protein, IL-1 and IL-6 production. It reduced CK2, JNK, NF-κB and AP-1 activition.	(Jung et al., 2010)
	Diosgenin	• Diosgenin suppressed the NO and PGE_2 production in IL-1β stimulated OA chondrocytes. Moreover, it inhibited MMP-3, MMP-13 and iNOS expression.	(Wang et al., 2015)
	Dioscorealide B	• Dioscorealide B decreased NO production (IC_{50}=2.85μM) and mRNA expression of iNOS, IL-1β, IL-6, IL-10 (concentration of 6 μM).	(Hiransai et al., 2010)

Table 2.3 Biological activities of pure compounds from *Dioscorea membranacea* Pierre (Cont.)

Activities	**Compounds**	**Results**	**References**
Anti-inflammatory activity	Dioscoreanone	• Dioscoreanone inhibited NO production with IC_{50} value of 2.5 μM. Moreover, 6 μM dioscoreanone reduced mRNA expression of iNOS, IL-1β and IL-6.	(Itharat & Hiransai, 2012)
	•Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside •Dioscoreanone •Dioscorealide B	• Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside, dioscoreanone and dioscorealide B inhibited NO production from RAW 264.7 with IC_{50} value of 3.5, 9.8 and 24.9 μg/ml, respectively.	(Tewtrakul & Itharat, 2007)
Immunomodulatory activity	Diosgenin	• Diosgenin enhanced antigen-specific IgG2a and IFN-γ expression when was feed to BALB/c mice for 34 days.	(Jan, Wey, Kuan, Liao, & Wu, 2007)

2.3.2 *Smilax corbularia* Kunth or Hua-Khao-Yen-Nua

2.3.2.1 Description of *Smilax corbularia* Kunth

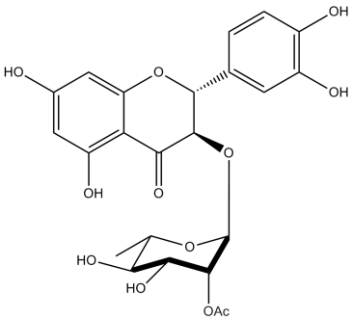
Smilax corbularia Kunth is a small climbing herb with woody stem and dense branches. Its branches are straight with internodes 3-10 cm long. Leaves are variable in shape and thickness. The blades are elliptic, cuneate, round or shallowly cordate at the base with a coriaceous, acuminate tip at the apex, and about 3-10 cm long by 1.5-5 cm wide. They are fresh-green and shiny on the upper surface, and strongly glaucous and more or less white-powdery on the lower side. Petioles are short, about 7-15 mm long and the tendrils develop only on sterile branches and stems. Flowering branches are 5-20 cm long and upper leaves reduced to bracts. The umbels with peduncles are 5-12 mm long. The staminate umbels have 10 to 40 flowers and the pistillate umbels have 8 to 20 flowers. The stamens have reddish perianth with free petals. There are 6 nearly sessile stamens with the elliptic anther, 1.3 mm long. The pistillate has a greenish to yellowish perianth, 1.5-2 mm long, with oblique petals. The ovary is ellipsoid, contracted at the apex, 2 mm long, and 1.5 mm wide, capped with 3-lobed stigma. There are 3 needle-like staminodes, 1.25 mm long. Berries are globose, purplish-black, 6-8 mm across with 1 to 3 seeds (Koyama, 1975).

Figure 2.7 *Smilax corbularia* Kunth; 1.branch; 2.fruit; 3.rhizome
(Boonyaratanakornkit & Chantarateptawan, 1993)

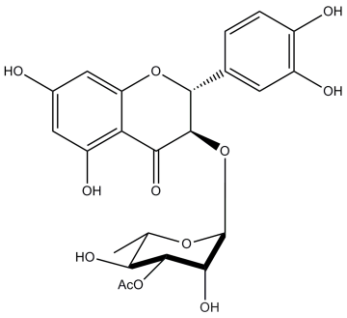
2.3.2.2 Chemical composition of *Smilax corbularia* Kunth

Table 2.4 Chemical composition of *Smilax corbularia* Kunth

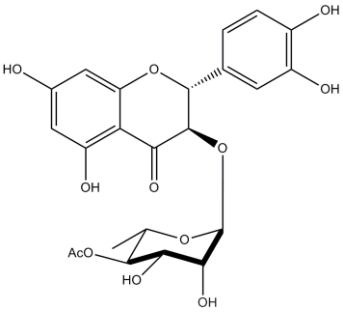
Part of used	Chemical composition	References
Rhizome	• 2’’-Acetyl astilbin	(Wungsintaweekul, Umehara, Miyase, & Noguchi, 2011)
	• 3’’-Acetyl astilbin	(Wungsintaweekul et al., 2011)
	• 4’’-Acetyl astilbin	(Wungsintaweekul et al., 2011)
	• 3’’-Acetyl engeletin	(Wungsintaweekul et al., 2011)
	• 4’’-Acetyl isoastilbin	(Wungsintaweekul et al., 2011)
	• Corbulain Ia	(Wungsintaweekul et al., 2011)
	• Corbulain Ib	(Wungsintaweekul et al., 2011)
	• Gnetumontanin E	(Wungsintaweekul et al., 2011)
	• Gnetumontanin F	(Wungsintaweekul et al., 2011)
	• Gnetumontanin G	(Wungsintaweekul et al., 2011)
	• 5,7,3’,4’-Tetrahydroxy-3-phenylcoumarin	(Wungsintaweekul et al., 2011)
	• Engeletin	(Chainakul, Supap, Tambunchong, & Picha, 2003; Ruangnoo, Jaiaree, et al., 2012)
	• Astilbin	(Chainakul et al., 2003; Ruangnoo, Jaiaree, et al., 2012)
	• Quercetin	(Ruangnoo, Jaiaree, et al., 2012)
	• Resveratrol	(Chainakul et al., 2003)
	• Stigmasteryl-3-O-β-D-glucopyranoside	(Chainakul et al., 2003)
	• β-sitosteryl-3-O-β-D-glucopyranoside	(Chainakul et al., 2003)



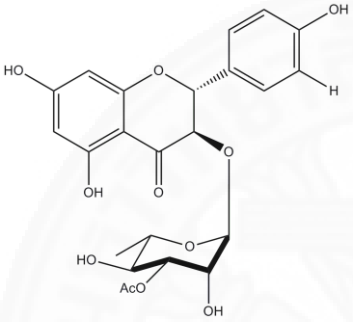
2’’-Acetyl astilbin



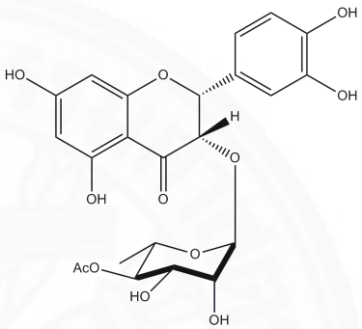
3’’-Acetyl astilbin



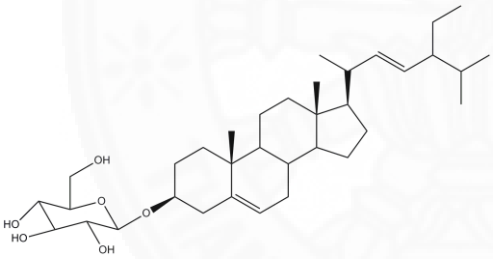
4’’-Acetyl astilbin



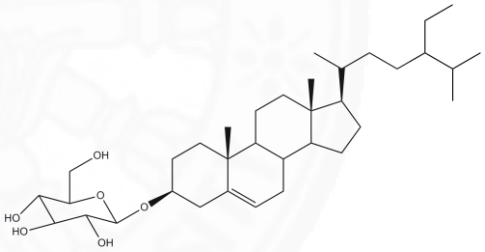
3’’-Acetyl engeletin



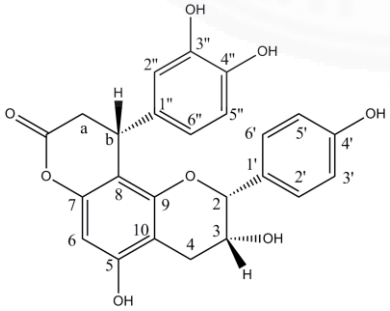
4’’-Acetyl isoastilbin



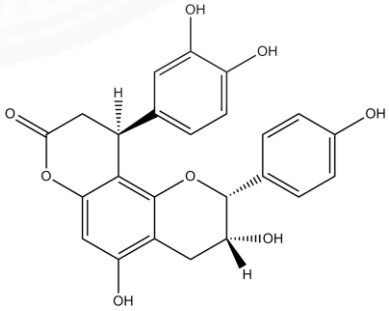
Stigmasteryl-3-O-β-D-glucopyranoside



β-sitosteryl-3-O-β-D-glucopyranoside

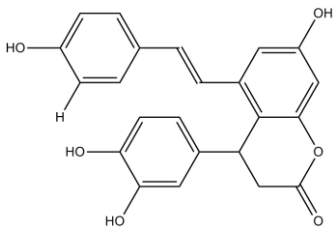


Corbulain Ia

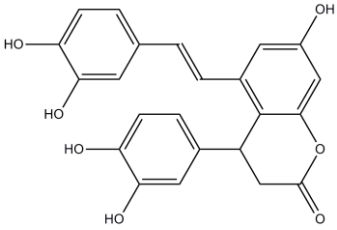


Corbulain Ib

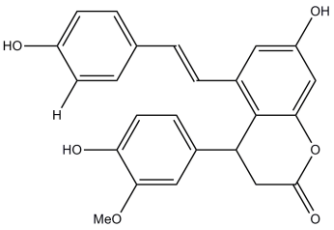
Figure 2.8 Structure of chemical compounds in *Smilax corbularia* Kunth



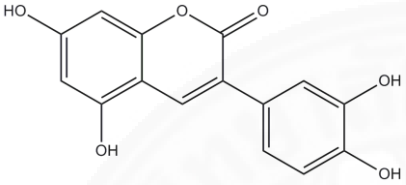
Gnetumontanin E



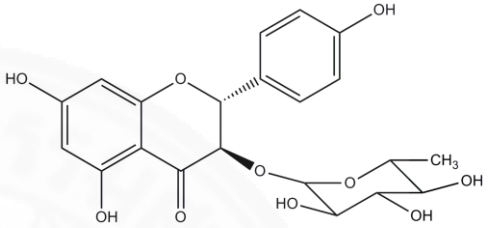
Gnetumontanin F



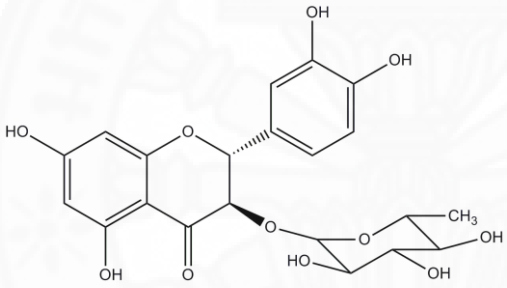
Gnetumontanin G



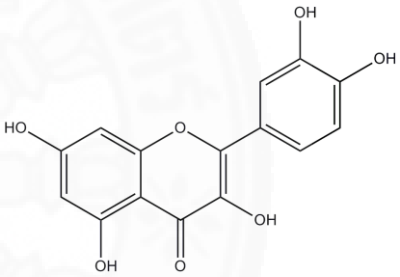
5,7,3’,4’-Tetrahydroxy-3-phenylcoumarin



Engeletin



Astilbin



Quercetin

Figure 2.8 Structure of chemical compounds in *Smilax corbularia* Kunth (Cont.)

2.3.2.3 Biological activity of *Smilax corbularia* Kunth

Previous report of biological activity of *S. corbularia* Kunth is shown in Table 2.5 and 2.6.

Table 2.5 Biological activities of *S. corbularia* Kunth

Part used	Activities	Results	References
Rhizomes	Anti–HIV activity	• The ethanolic and water extracts showed inhibitory activity against HIV-1 integrase activity with IC_{50} value of 1.9 and 5.4 μg/ml.	(Tewtrakul et al., 2006)
	Anti-inflammatory activity	• Ethanolic extract showed inhibitory activity on TNF-α and NO production with IC_{50} value of 61.97 and 83.90 μg/ml, respectively.	(Ruangnoo, Jaiaree, et al., 2012)
	Anti-inflammatory activity	• The ethanolic extract showed anti-inflammatory activity that significantly suppressed the paw edema induced by carragenin in rats but the water extract showed no effect.	(Reanmongkol et al., 2007a)
	Anti-estrogenic effect	• The flavononol rhamnosides suppressed estradiol on induced human breast cancer cell proliferation at 1 μM.	(Wungsintaweekul et al., 2011)

Table 2.6 Biological activities of pure compounds from *S. corbularia* Kunth

Activities	**Compounds**	**Results**	**References**
Cytotoxic activity	• Resveratol • Engeletin • Astilbin • Mixture of stigmasteryl-3-O-β-D-glucopyranoside and β-sitosteryl-3-O-β-D-glucopyranoside	• The ED_{50} values against P388 leukemic cell of resveratrol, engeletin, astilbin and mixture of stigmasteryl-3-O-β-D-glucopyranoside and β-sitosteryl-3-O-β-D-glucopyranoside were 3.5, over 100, 75 and over 100 μg/ml, respectively.	(Chainakul et al., 2003)
	Resveratol	• Resveratol exhibited cytotoxicity against MCF-7 cell with ED_{50} value of 9.4 μg/ml.	(Chainakul et al., 2003))
	Quercetin	• Quercetin significantly increased NKG2D ligands expression in cancer cell (K562, SNU1 and SNU-C4).	(J. H. Bae et al., 2010)
	Astilbin	• Astilbin significantly induced the apoptosis in Jurkat cells at concentration of 1 μg/ml.	(Yan & Xu, 2001)

Table 2.6 Biological activities of pure compounds from *S. corbularia* Kunth (Cont.)

Activities	**Compounds**	**Results**	**References**
Anti-allergy activity	Quercetin	• Quercetin (8 and 16 mg/kg/day) significantly decreased IL-4 and IL-5 production in BALB/c mice.	(Park et al., 2009)
Anti-inflammatory activity	Quercetin	• Quercetin had high inhibitory effect on NO production, TNF-α and PGE_2 release from RAW 264.7 cell line with IC_{50} value of 11.2, 1.25 and 19.9 µg/ml, respectively.	(Ruangnoo, Jaiaree, et al., 2012)
	Quercetin	• Quercetin (10-80 µM) dose-dependently inhibited IL-6 production by LPS-stimulated neutrophils.	(Liu et al., 2005)
	Quercetin	• Quercetin at concentration of 0.1 and 0.2 mM inhibited iNOS mRNA and decrease NO release in the macrophage cell line RAW 264.7	(Wadsworth & Koop, 1999)
	Quercetin	• TNF-γ production in vitro was decreased in blood sample by quercetin, but not in vivo in the blood of healthy volunteers.	(Boots et al., 2008)
	Quercetin	• Quercetin (10 µM) reduced NO production, iNOS mRNA and protein expression in BV-2 microglial cells	(Kao et al., 2010)

Table 2.6 Biological activities of pure compounds from *S. corbularia* Kunth (Cont.)

Activities	Compounds	Results	References
Anti-inflammatory activity	• Engeletin • Astilbin	• Engeletin and astilbin showed inhibitory effect on PGE_2 production with IC_{50} value of 14.4 and 19.6 μg/ml, respectively.	(Ruangnoo, Jaiaree, et al., 2012)
	• Astilbin • Engeletin	• Astilbin and engeletin (50 μM) showed inhibitory effect on IL-1β, IL-6, TNF-α, IL-10, MCP-1 and COX-2 mRNA expression in mouse J774A.1 macrophage cells.	(H. Huang et al., 2011)
	Astilbin	• Astilbin reduced the cell adhesion and T-lymphocyte activation to secrete TNF-α.	(Yi, Lu, Fang, Wang, & Xu, 2008)
	Astilbin	• Astilbin (25 and 50 mg/kg) increased IL-10 and TGF-β level in serum of DSS-treated mice and reduced IL-1β level.	(Ding et al., 2014)
	Resveratrol	• Resveratrol (0.05 and 0.1 mM) reduced nitrite release from LPS-stimulated RAW 264.7 cell lines. It increased TNF-α mRNA and cytokine release.	(Wadsworth & Koop, 1999)

Table 2.6 Biological activities of pure compounds from *S. corbularia* Kunth (Cont.)

Activities	Compounds	Results	References
Immunomodulatory activity	Resveratrol	• Resveratol inhibited T cell activation and IL-2, IFN-γ, IL-4 and IL-5 production from Th1 and Th2.	(Zou et al., 2013)
	Quercetin	• Quercetin (5, 25 and 50 μM) increased IFN-γ gene expression and IFN-γ production. On the other hand, it (0.5 – 50 μM) decreased IL-4 gene expression and the level of IL-4 production from PBMC.	(Nair et al., 2002)
	Quercetin	• Quercetin inhibited dendritic cells activation which reduced cytokines and chemokines secretion (IL-1β, IL-1α, IL-6 and IL-10) from dendritic cells.	(R. Y. Huang et al., 2010)
	Quercetin	• Quercetin decreased IFN-γ production from Th cells in mice. Moreover, quercetin reduced IL-2 and blocked IL-2-mediated gene transcription of IL-2Rα.	(Yu et al., 2008)

2.3.3 Benjakul preparation

Benjakul is a Thai traditional preparation which is composed of *Piper chaba* Hunter, *Piper interruptum* Opiz, *Piper sarmentosum* Roxb., *Plumbago indica* Linn. and *Zingiber officinale* Roscoe. It used to balance health and as an adaptogenic drug for cancer treatment. From in depth interviews with Thai traditional doctors, Benjakul was given to cancer patients for 2-3 weeks before other drugs. The reason is that it can modulate patient's immune system (Itharat et al., 1998). Benjakul showed no acute and chronic toxicity in rats (Chauvaltthamrong, Auttavich, Raksaman, & Jantapen, 1996; Itharat et al., 2011). A Benjakul safety study on normal volunteer showed that volunteers had no severe adverse event and clinical signs showed no significant change. The range of laboratory test results were within normal values (Amorndoljai, Kietinun, & Somparn, 2011).

2.3.3.1 Description of plants composing Benjakul

(1) *Piper chaba* Hunt.

Piper chaba Hunt. or *Piper retrofractum* Vahl. or *Piper longum* Linn is monoecious plant and climber. Its stem is stout, node dilated and rooting. It has petiole 0.8-1.2 cm long. Leaf is blade coriaceous that is glabrous, entire, oblong and ovate. Leaf is 4-6 cm wide and 10-17 cm long. Apex of leaf is acuminate and has base cordate, rounded and veins one or two pairs basal. The others arise alternately from midrib. Spike is straight up and is 3-5.5 cm long and 0.5-0.7 cm in diameter. Penduncle is 1-1.2 cm long that has bract or bicular, stamens 2 and stigmas 3. Fruiting is spike stout and conico-cylindric. It is 3.5-6 cm long. Drupe is globose and embedded on rachis. Moreover, it is red when ripe. Flowering all year round (Chaveerach, Mokkamul, Sudmoon, & Tanee, 2006).

(2) *Piper sarmentosum* Roxb.

P. sarmentosum Roxb. is monoecious plant. It is a small shrub, 30 cm tall and sometimes climber. Petiole is 1-2.5 cm long. Leaf blade is thin to thick and chartaceous or papery. Leaf is light to dark green and broadly ovate to elliptic. It is 4.5-6 cm wide and 7.5-9.5 cm long. Leaves are on epiphytic branches base and deeply, equally cordate with rounded lobes. Leaves are on free branch base cuneate to subtruncate all basal. Spike with male and female flowers together that is straight up

and cylindrical, 1-1.5 cm long and 0.3-0.5 cm in diameter. Bract is rounded which has stamen 1 and stigmas 3-4. Female spike is white cylindrical. Fruiting spike is 1-2 cm long and 0.5-1 cm in diameter. Flowering all year round or many in rainy season (Chaveerach et al., 2006).

(3) *Piper interruptum* Opiz.

Piper interruptum Opiz. leaf blades are large and usually less than 18 cm long. It is membranous to chartaceous-subcoriaceous and minor veins only weakly. Base of blade without a unilateral lobule, venation palmate to subpinnate, at least the finer venation above coplanar or hardly prominent and only irregularly so. Fruitlets free or concrescent with one another or on rachis. Peduncle of female inflorescence is much shorter than spike, fruiting spike is cylindrical and 2 cm or longer. Leaf blades are glabrous or pubescent. Fruitlets are 3-6 mm diameter. Bracts of female are spiked sessile. Vegetative parts are glabrous. Fruitlets are 2-3 mm diameter, not stalked and subglobose to ellipsoid. Leaf blades are chartaceous, seldom more than 10 x 4 cm, drying greyish and usually with conspicuous white glands. Bracts of male and female are spikes, sessile, elongate along rachis and adnate to it except marginally (Gardner, 2006).

(4) *Plumbago indica* L.

Plumbago indica L. or *Plumbago rosea* L. is widely used as medicinal plant. Its is a small bush up to 2 m tall, stem erect, climbing, and simple or branched from the base, sometime rooting at the nodes. Leaves alternate is simple and entire. Leaves have no stipules and auricles. They have short petiole and blade narrowly ovate to elliptical-ovate, 5-15 cm x 2-8 cm, base rounded to obtuse, apex acute, papery. Inflorescence is an elongated spike or raceme and many-flowered. It is 10-30 cm long and glabrous. Bracts ovate are 2-3 mm long and apex acuminate. Peduncle is 2-10 cm long. Flowers are bisexual, regular and 5-merous. Pedicel is 0-1 mm. long. Calyx tubular is 8-9 mm long, glandular and red. Corolla tube is 2.5-4.5 cm long. Lobes obovate are 1.5-3 cm in diameter, apex rounded. Futhermore, it is purple to red, ovary superior and ellipsoid-ovoid. It has 1-celled, filiform style and 5 stigma lobes (Schmelzer & Gurib-Fakim, 2008).

(5) *Zingiber officinale* Rosc.

Rhizome is fully pale yellow within or with a red external layer. Leafy stems are 50 cm tall and 5 mm diameter. They have glabrous but base of leaf blade have short. Leaf blades is dark green, narrow to slender tip, 17-18 cm long, ligule broad, thin glabrous, to 5 mm tall, slightly bilobed. Scape slender, to 12 cm in length, the upper sheaths with or without short leafy tips, inflorescence about 4.5 cm long and 15 mm diameter, bracts about 2.5 by 1.8 cm, green with pale sub marginal band and narrow clear margin, margins incurved, lower bracts with slender white tip. Bracteoles as long as bract, calyx with ovary 12 mm long, corolla tube 2.5 cm long, lobes yellowish, dorsal lobe is flattened and 18 by 8 mm, curving over the anther and narrowed to the tip, laterals narrower. Lip nearly circular, approximately 12 mm long, and wide, dull purple with cream blotches and base, side lobes about 6 by 4 mm, free almost to the base, colored at mid lobe. The anther cream is 9 mm long. Appendage dark purple, curved, 7 mm long (Ravindran & Babu, 2004).

2.3.3.2 Chemical composition of Benjakul preparation and its plant components

(1) Chemical composition of Benjakul preparation

Table 2.7 Chemical composition of Benjakul preparation

Chemical composition	References
• Piperine	(Sakpakdeejaroen, 2009; Sakpakdeejaroen & Itharat, 2009)
• Plumbagin	(Sakpakdeejaroen, 2009; Sakpakdeejaroen & Itharat, 2009)
• 6-Gingerol	(Sakpakdeejaroen, 2009; Sakpakdeejaroen & Itharat, 2009)
• Myristicin	(Rattarom, Sakpakdeejaroen, Hansakul, & Itharat, 2014)
• 6-shogaol	(Rattarom et al., 2014)
• Methyl piperate	(Rattarom et al., 2014)

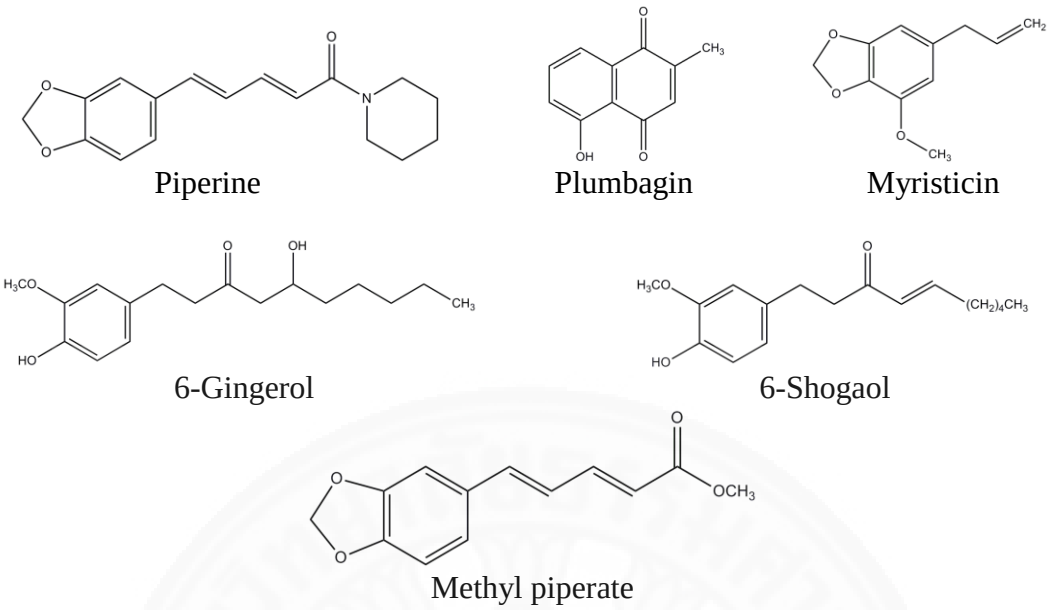


Figure 2.9 Structure of chemical compounds in Benjakul preparation

(2) Chemical composition of *P. chaba* Hunt.

Table 2.8 Chemical composition of *P. chaba* Hunt.

Part used	Chemical composition	References
Fruit	• Piperonal	(Matsuda et al., 2008)
	• Methyl piperate	(Matsuda et al., 2008)
	• Piperchabamide A	(Matsuda et al., 2008)
	• Piperanine	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Piperine	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Pipernonaline	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Piperroleine B	(Matsuda et al., 2008)
	• Piperchabamide B	(Matsuda et al., 2008)
	• Piperundecalidine	(Matsuda et al., 2008)
	• Piperchabamide C	(Matsuda et al., 2008)
	• Dihydropiperlonuminine	(Matsuda et al., 2008)
	• Piperlonguminine	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Piperchabamide E	(Matsuda et al., 2008)
	• Retrofractamide C	(Matsuda et al., 2008)
	• Retrofractamide A	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Piperchabamide D	(Matsuda et al., 2008)
	• Retrofractamide B	(Matsuda et al., 2008; Yamaguchi et al., 2014)
	• Guineensine	(Matsuda et al., 2008)
	• Brachystamide B	(Matsuda et al., 2008)
	• N-Isobutyl-(2E,4E)-decadienamide	(Matsuda et al., 2008)

Table 2.8 Chemical composition of *P. chaba* Hunt. (Cont.)

Part used	Chemical composition	References
Fruit	• N-Isobutyl-(2E,4E)-dodecadienamide	(Matsuda et al., 2008)
	• N-Isobutyl-(2E,4E)-octadecadienamide	(Matsuda et al., 2008)
	• N-Isobutyl-(2E,4E,14Z)-eicosatrienamide	(Matsuda et al., 2008)
Root	• E-piplatine	(Jyothi et al., 2009)
	• Z-piplatine	(Jyothi et al., 2009)
	• Chabamide	(Rama Subba Rao et al., 2011)
	• Chabamide F	(Rama Subba Rao et al., 2011; Rama Subba Rao et al., 2009)
	• Chabamide G	(Rama Subba Rao et al., 2011; Rama Subba Rao et al., 2009)
	• Piperine	(Rama Subba Rao et al., 2011)
	• Trichostachine	(Rama Subba Rao et al., 2011)
	• Pellitorine	(Rama Subba Rao et al., 2011)
	• Piplartine	(Rama Subba Rao et al., 2011)
	• 4,5-dihydropiperlongmine	(Rama Subba Rao et al., 2011)
	• Guineensine	(Rama Subba Rao et al., 2011)
	• Brachystamide B	(Rama Subba Rao et al., 2011)
	• Sesamin	(Rama Subba Rao et al., 2011)
	• Chabamide H	(Rama Subba Rao et al., 2011)
	• Chabamide I	(Rama Subba Rao et al., 2011)
	• Chabamide J	(Rama Subba Rao et al., 2011)
	• Chabamide K	(Rama Subba Rao et al., 2011)

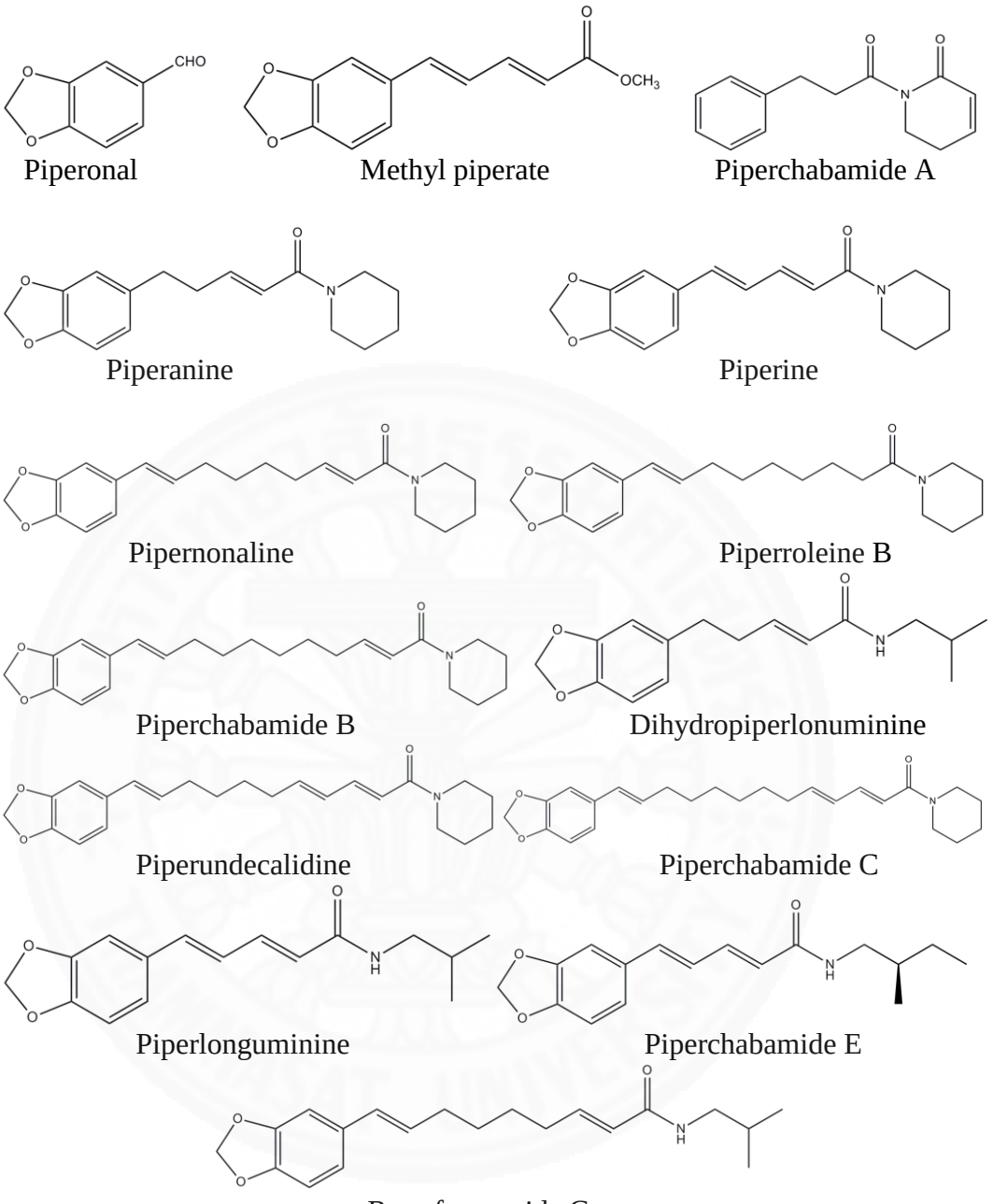


Figure 2.10 Structure of chemical compounds in *P. chaba* Hunt.

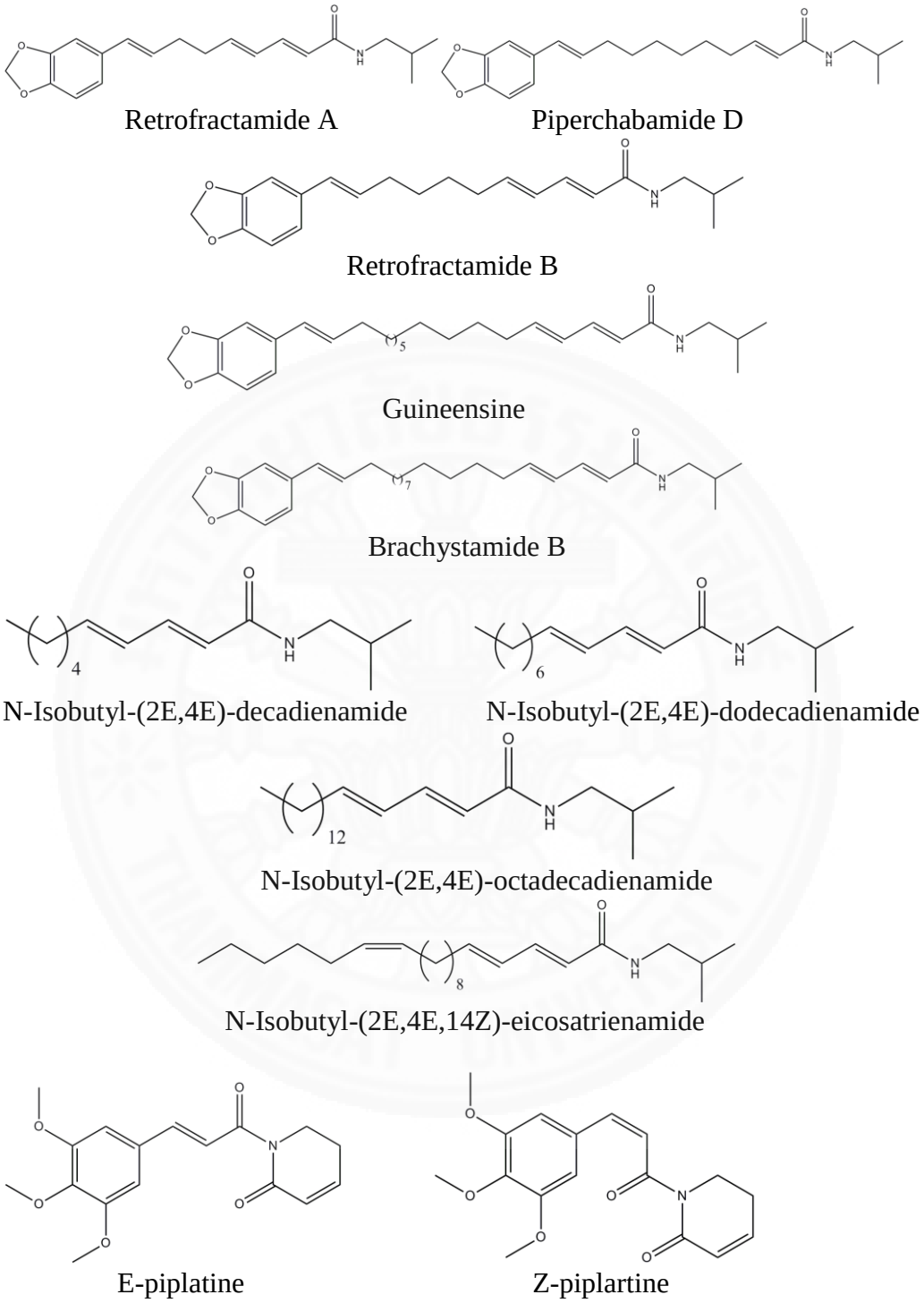


Figure 2.10 Structure of chemical compounds in *P. chaba* Hunt. (Cont.)

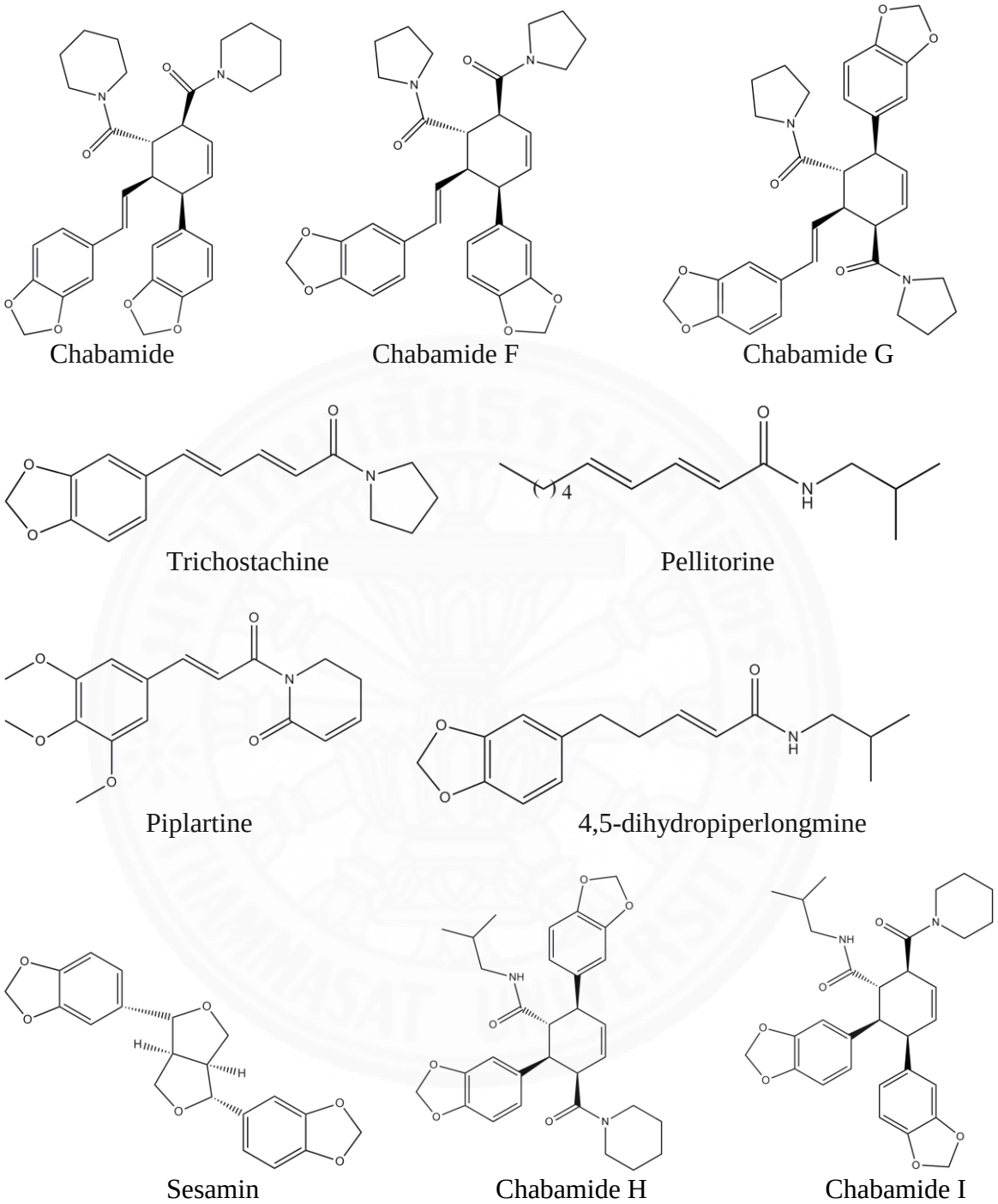
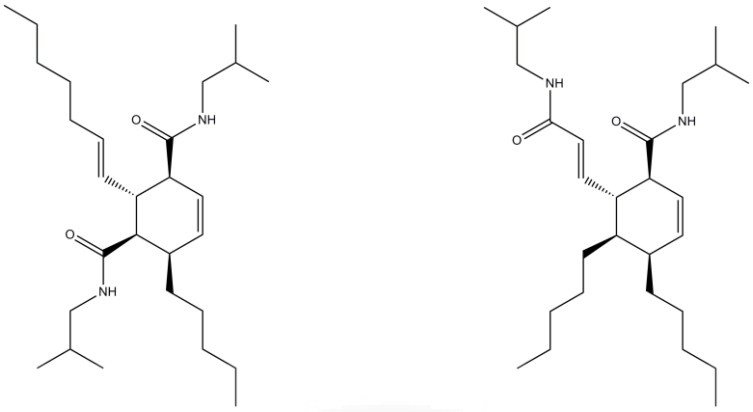


Figure 2.10 Structure of chemical compounds in *P. chaba* Hunt. (Cont.)



Chabamide J Chabamide K

Figure 2.10 Structure of chemical compounds in *P. chaba* Hunt. (Cont.)

(3) Chemical composition of *P. sarmentosum* Roxb.

Table 2.9 Chemical composition of *P. sarmentosum* Roxb.

Part used	Chemical composition	References
Fruit	• Pellitorine	(Rukachaisirikul et al., 2004)
	• Guineensine	(Rukachaisirikul et al., 2004)
	• Brachystamide B	(Rukachaisirikul et al., 2004)
	• Sarmentine	(Rukachaisirikul et al., 2004)
	• Brachyamide B	(Rukachaisirikul et al., 2004)
	• 1-piperettyl pyrrolidine	(Rukachaisirikul et al., 2004)
	• 3',4',5'-trimethoxycinnamoyl pyrrolidine	(Rukachaisirikul et al., 2004)
	• Sarmentosine	(Rukachaisirikul et al., 2004; Strunz & Finlay, 1995)
	• Asarinin	(Rukachaisirikul et al., 2004)
	• Sesamin	(Rukachaisirikul et al., 2004)
	• 1-(3,4-methylenedioxypheny)-1E-tetradecene	(Rukachaisirikul et al., 2004)
	• Methyl piperate	(Rukachaisirikul et al., 2004)
	• Mixture of β-sitosterol and stigmasterol	(Rukachaisirikul et al., 2004)
Leaf	• Chaplupyrrolidones A	(Damsud, Adisakwattana, & Phuwapraisirisan, 2013)
	• Chaplupyrrolidones B	(Damsud et al., 2013)
	• Deacetylsamentamide B	(Damsud et al., 2013)

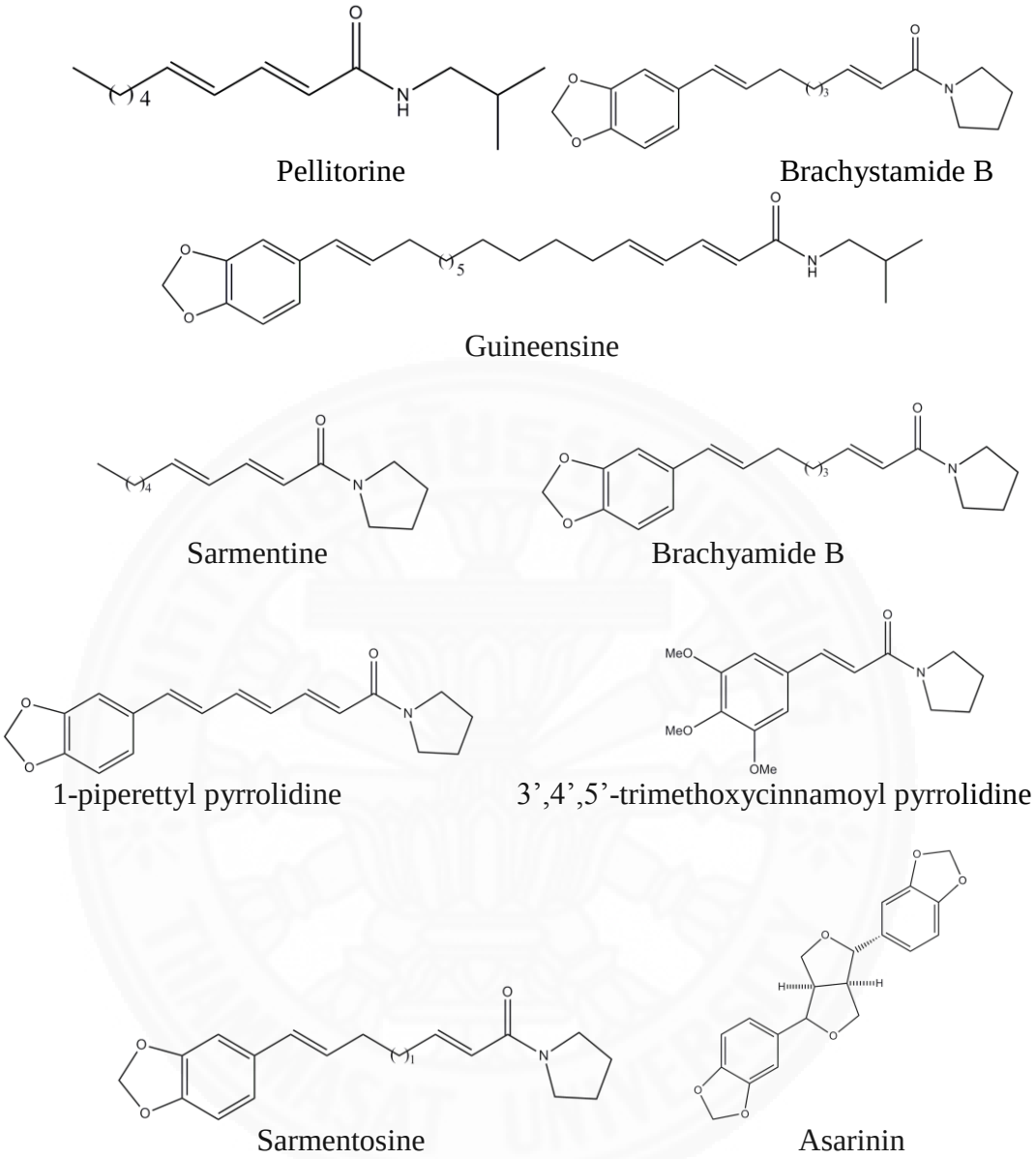
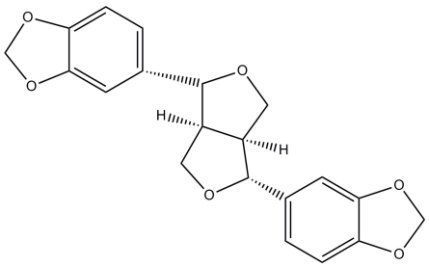
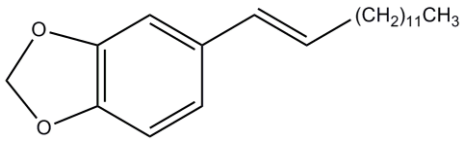


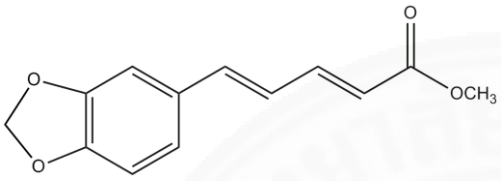
Figure 2.11 Structure of chemical compounds in *P. sarmentosum* Roxb.



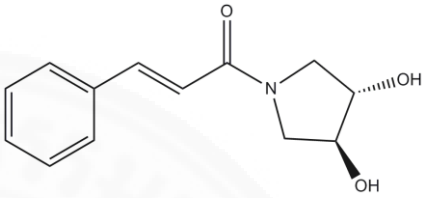
Sesamin



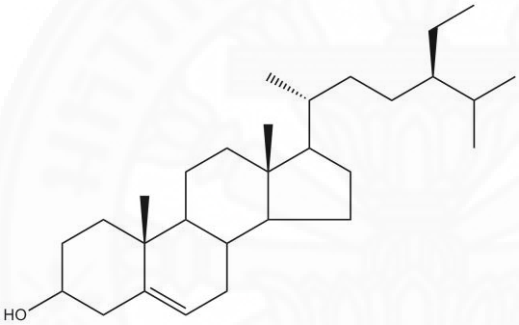
1-(3,4-methylenedioxypheny)-1E-tetradecene



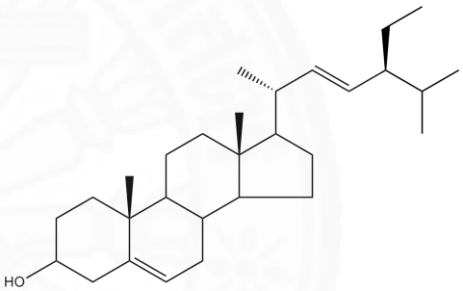
Methyl piperate



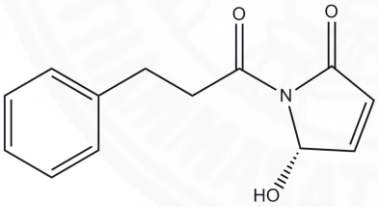
Deacetylsamentamide B



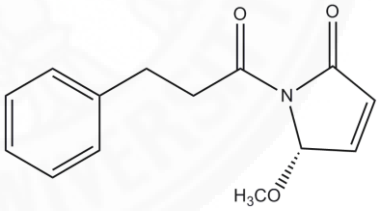
β-sitosterol



Stigmasterol



Chaplupyrrolidones A



Chaplupyrrolidones B

Figure 2.11 Structure of chemical compounds in *P. sarmentosum* Roxb. (Cont.)

(4) Chemical composition of *P. interruptum* Opiz.

Table 2.10 Chemical composition of *P. interruptum* Opiz.

Part used	Chemical composition	References
Stem	• Crotepoxide	(Thebpatiphat, RPengprecha, & Ternai, 1998)
	• Eupomatene	(Thebpatiphat et al., 1998)
	• Pipercallosine	(Thebpatiphat et al., 1998)

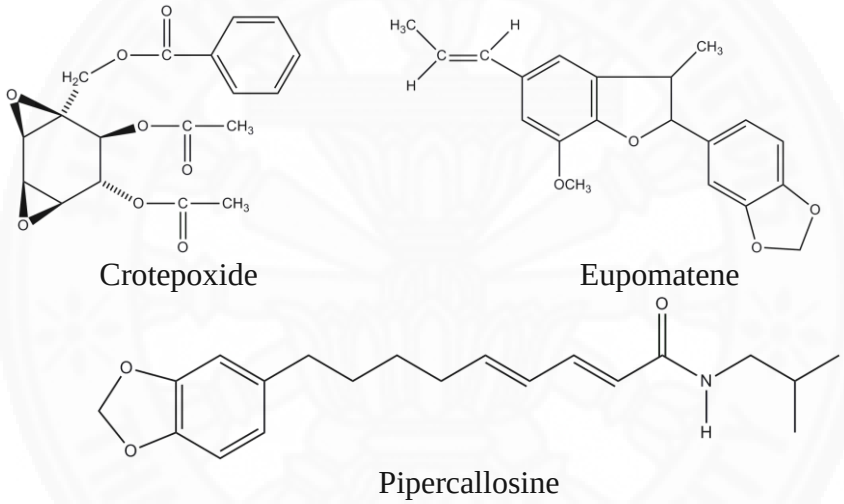
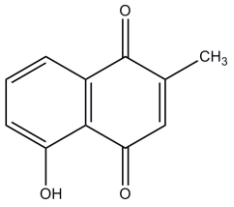


Figure 2.12 Structure of chemical compounds in *P. interruptum* Opiz.

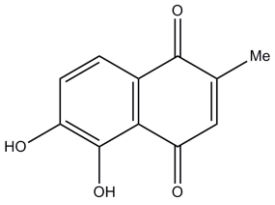
(5) Chemical composition of *P. indica* Linn

Table 2.11 Chemical composition of *P. indica* Linn

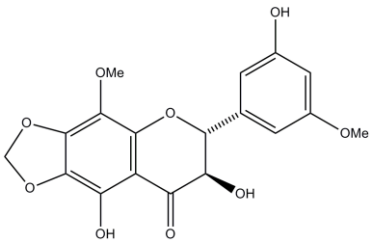
Part used	Chemical composition	References
Root	• Plumbagin	(Dinda & Chel, 1992)
	• 6-Hydroxyplumbagin	(Dinda & Chel, 1992)
	• Plumbaginol	(Dinda, Chel, & Achari, 1994)
	• Myricetin-3',3',5',7-tetra methyl ether	(Ariyanathan, Saraswathy, Rajamanickam, & Connolly, 2010)
	• Ampelopsin-3',4',5',7-tetra methyl ether	(Ariyanathan et al., 2010)
	• Plumbagic acid	(Ariyanathan et al., 2010)
	• Roseanoic acid	(Ariyanathan et al., 2010)



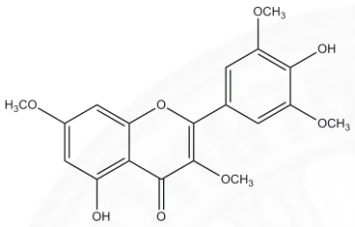
Plumbagin



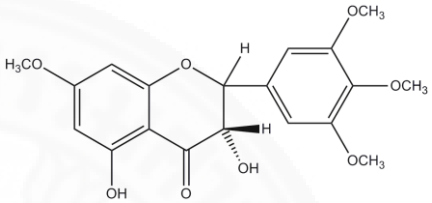
6-Hydroxyplumbagin



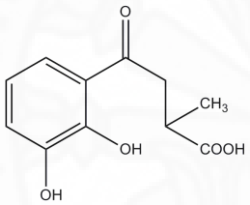
Plumbaginol



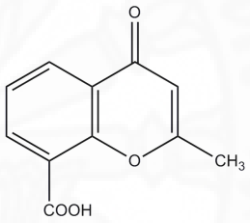
Myricetin-3’,3’,5’,7-tetra methyl ether



Ampelopsin-3’,4’,5’,7-tetra methyl ether



Plumbagic acid



Roseanoic acid

Figure 2.13 Structure of chemical compounds in *P. indica* Linn

(6) Chemical composition of *Z officinale* Rosc.

Table 2.12 Chemical composition of *Z. officinale* Rosc.

Part used	Chemical composition	References
Rhizome	[6]-Paradol	(Jolad et al., 2004)
	[7]-Paradol	(Jolad et al., 2004)
	[8]-Paradol	(Jolad et al., 2004)
	[9]-Paradol	(Jolad et al., 2004)
	[10]-Paradol	(Jolad et al., 2004)
	[11]-Paradol	(Jolad et al., 2004)
	[13]-Paradol	(Jolad et al., 2004)
	Methyl [6]-paradol	(Jolad et al., 2004)
	[4]-Gingerol	(Jolad et al., 2004)
	[6]-Gingerol	(Jolad et al., 2004)
	[7]-Gingerol	(Jolad et al., 2004)
	[8]-Gingerol	(Jolad et al., 2004)
	[10]-Gingerol	(Jolad et al., 2004)
	Methyl [4]-gingerol	(Jolad et al., 2004)
	Methyl [6]-gingerol	(Jolad et al., 2004)
	[4]-Shogaol	(Jolad et al., 2004)
	[6]-Shogaol	(Jolad et al., 2004)
	[8]-Shogaol	(Jolad et al., 2004)
	[10]- Shogaol	(Jolad et al., 2004)
	[12]- Shogaol	(Jolad et al., 2004)
	Methyl[6]-shogaol	(Jolad et al., 2004)
	Methyl[8]-shogaol	(Jolad et al., 2004)
	Acetoxy-[4]-gingerol	(Jolad et al., 2004)
	Acetoxy-[6]-gingerol	(Jolad et al., 2004)
	Acetoxy-[8]-gingerol	(Jolad et al., 2004)
	Acetoxy-[10]-gingerol	(Jolad et al., 2004)

Table 2.12 Chemical composition of *Z. officinale* Rosc. (Cont.)

Part used	Chemical composition	References
Rhizome	Methyl acetoxy-[6]-gingerol	(Jolad et al., 2004)
	1-Dehydro-[3]-gingerdione	(Charles, Garg, & Kumar, 2000; Jolad et al., 2004)
	1-Dehydro-[6]-gingerdione	(Jolad et al., 2004)
	1-Dehydro-[8]-gingerdione	(Jolad et al., 2004)
	1-Dehydro-[10]-gingerdione	(Jolad et al., 2004)
	[4]-Gingerdiol	(Jolad et al., 2004)
	[6]-Gingerdiol	(Jolad et al., 2004; Kikuzaki, Tsai, & Nakatani, 1992)
	[8]-Gingerdiol	(Jolad et al., 2004)
	[10]-Gingerdiol	(Jolad et al., 2004)
	5-Acetoxy-[4]-gingerdiol	(Jolad et al., 2004)
	5-Acetoxy-[6]-gingerdiol	(Jolad et al., 2004)
	5-Acetoxy-[7]-gingerdiol	(Jolad et al., 2004)
	Methyl 5-acetoxy-[4]-gingerdiol	(Jolad et al., 2004)
	Methyl 5-acetoxy-[6]-gingerdiol	(Jolad et al., 2004)
		(Jolad et al., 2004)
	Diacetoxy-[4]-gingerdiol	(Jolad et al., 2004)
	Diacetoxy-[6]-gingerdiol	(Jolad et al., 2004)
	Methyl diacetoxy-[4]-gingerdiol	(Jolad et al., 2004)
	Methyl diacetoxy-[6]-gingerdiol	(Jolad et al., 2004)
	Methyl diacetoxy-[10]-gingerdiol	(Jolad et al., 2004)
	3-Dihydro-[6]-demethoxyshogaol	(Jolad et al., 2004)

Table 2.12 Chemical composition of *Z. officinale* Rosc. (Cont.)

Part used	Chemical composition	References
Rhizome	5-Methoxy-[6]-gingerol	(Jolad et al., 2004)
	1,7-bis-(4'-Hydroxy-3'-methoxyphenyl)-4-heptene-3-one	
	1,7-bis-(4'-Hydroxy-3'-methoxyphenyl)-3,5-heptadione	(Jolad et al., 2004)
	1-Dehydro-3-dihydro-[10]-ginger-dione	(Jolad et al., 2004)
	6-Dihydroparadol	(Jolad et al., 2004)
	Acetoxy-6-dihydroparadol	(Jolad et al., 2004)
	1-(4'-Hydroxy-3'-methoxyphenyl)-7-octen-3-one	(Jolad et al., 2004)
	1-(4'-Hydroxy-3'-methoxyphenyl)-7-decen-3-one	(Jolad et al., 2004)
	1-(4'-Hydroxy-3'-methoxyphenyl)-7-dodecen-3-one	(Jolad et al., 2004)
	[4]-Isogingerol	(Jolad et al., 2004)

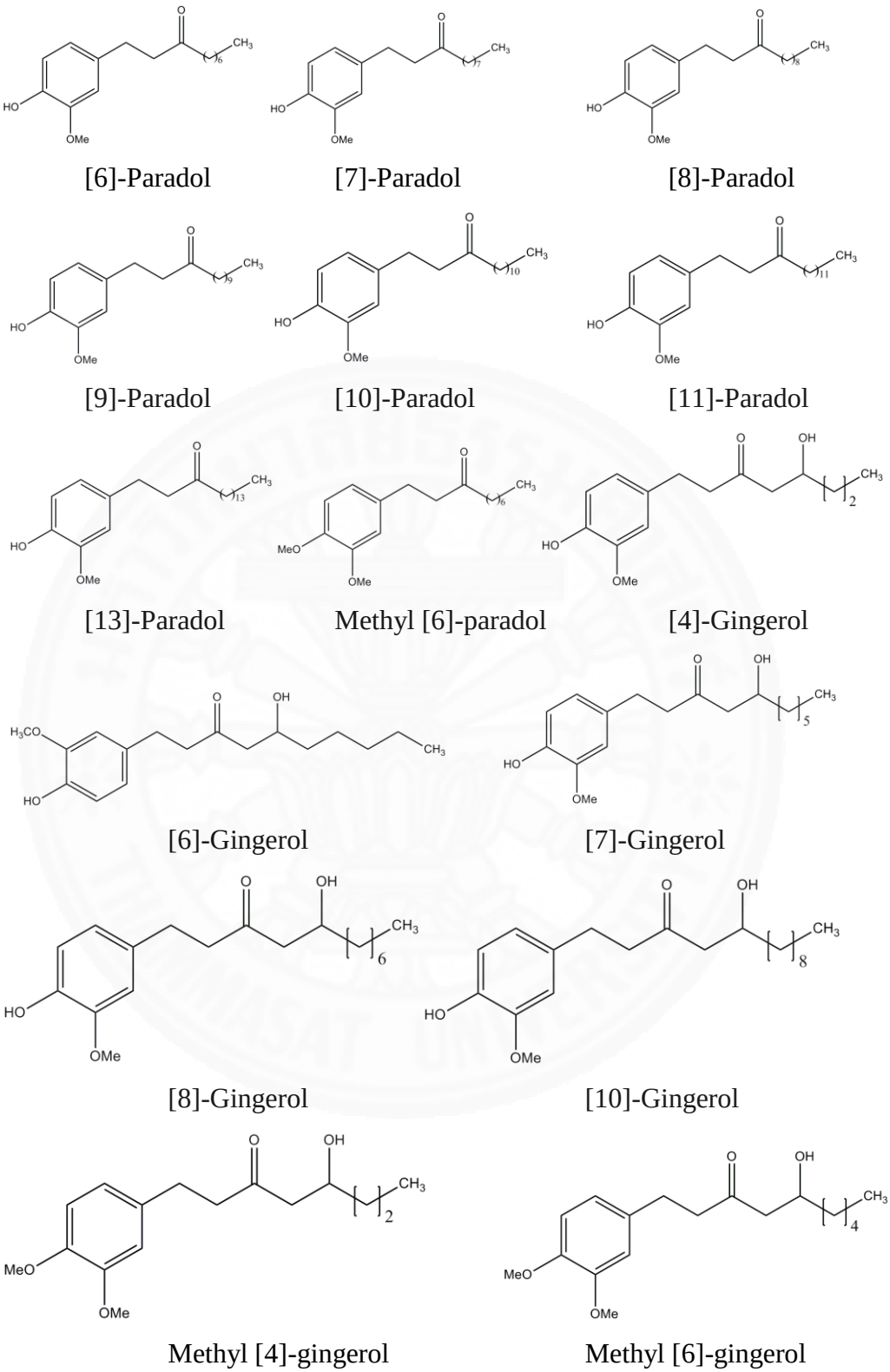


Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.**

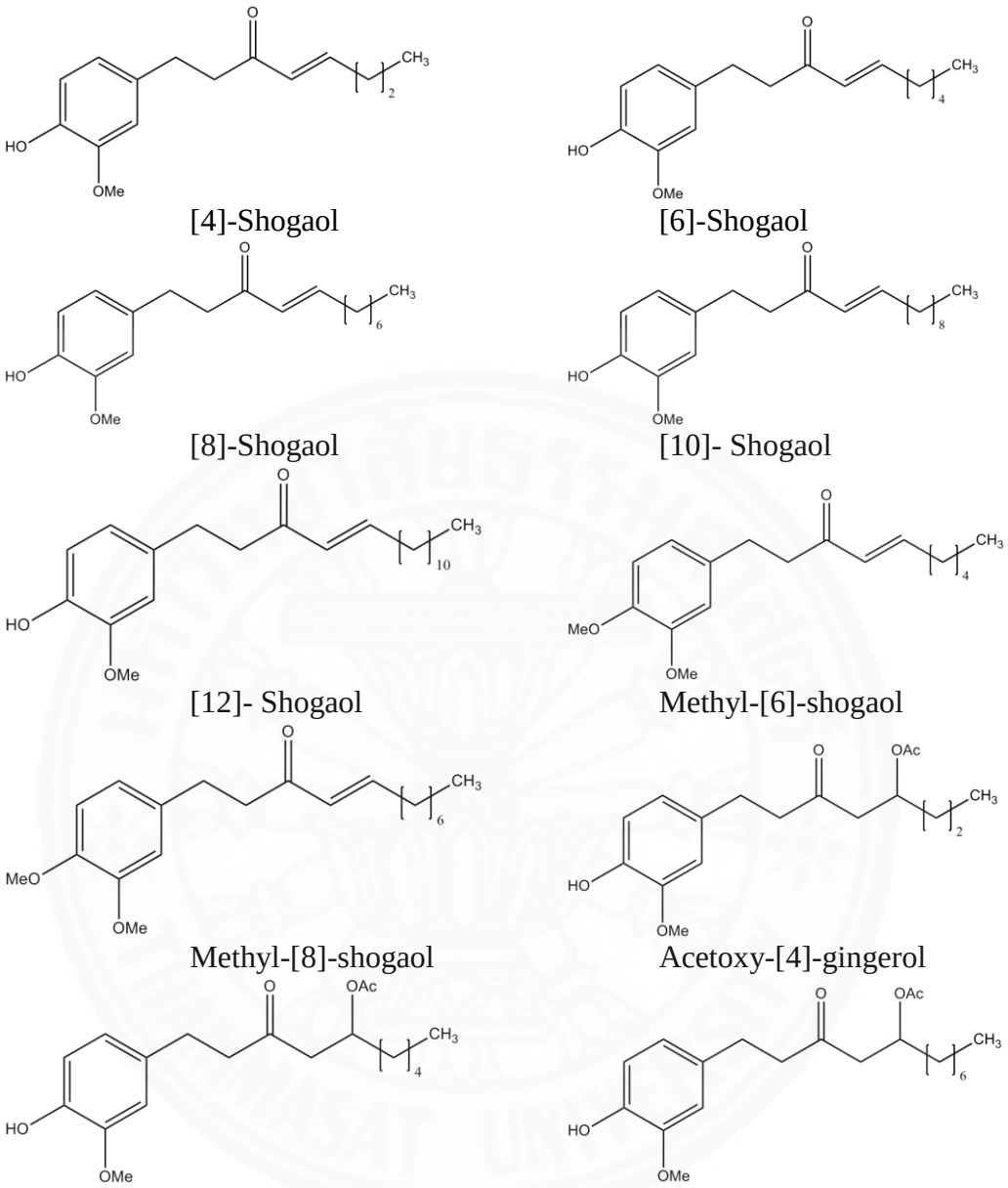
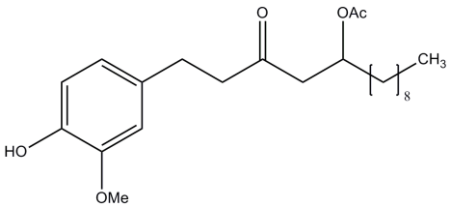
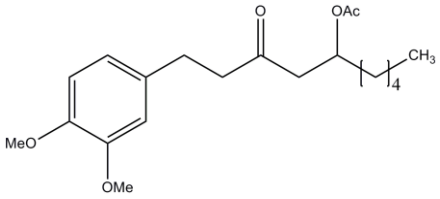


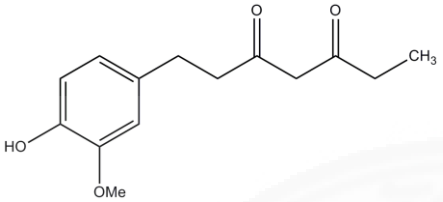
Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.** (Cont.)



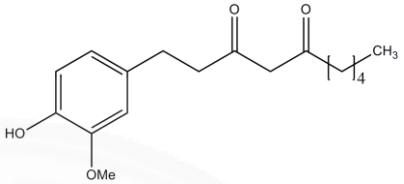
Acetoxy-[10]-gingerol



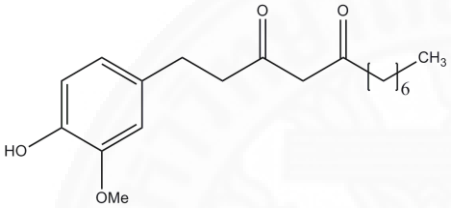
Methyl acetoxy-[6]-gingerol



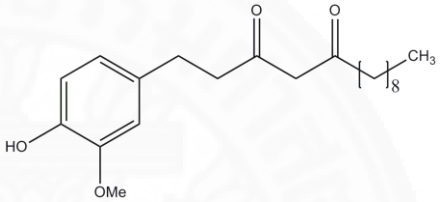
1-Dehydro-[3]-gingerdione



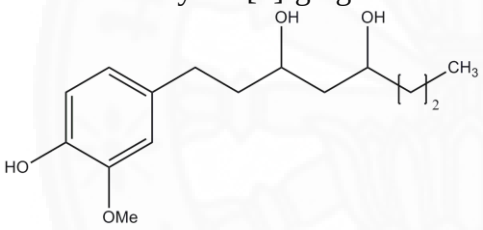
1-Dehydro-[6]-gingerdione



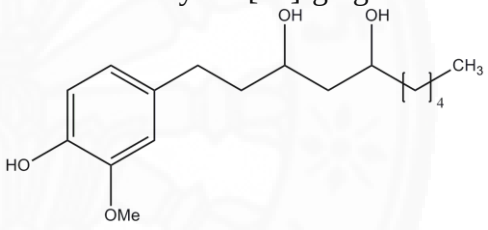
1-Dehydro-[8]-gingerdione



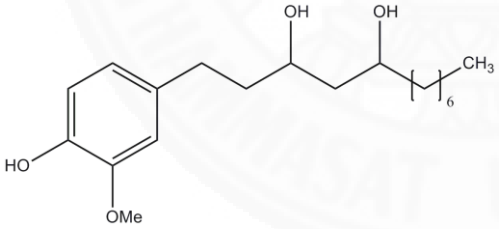
1-Dehydro-[10]-gingerdione



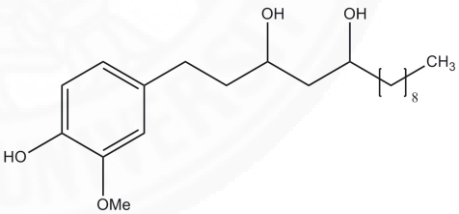
[4]-Gingerdiol



[6]-Gingerdiol

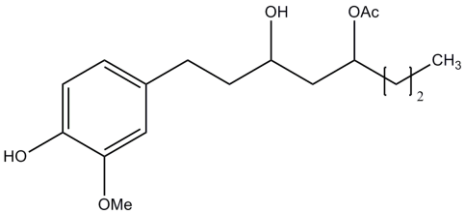


[8]-Gingerdiol

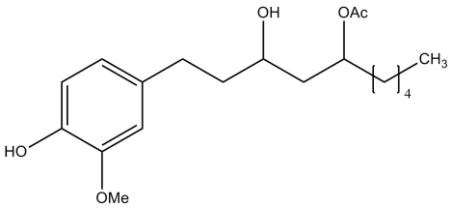


[10]-Gingerdiol

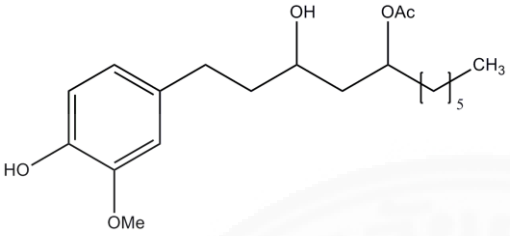
Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.** (Cont.)



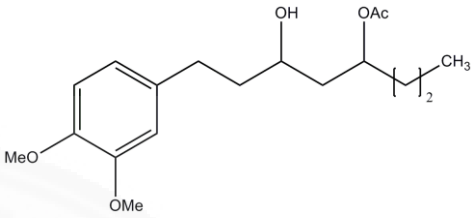
5-Acetoxy-[4]-gingerdiol



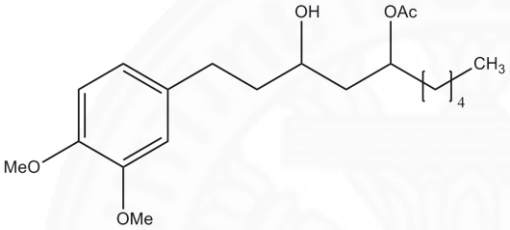
5-Acetoxy-[6]-gingerdiol



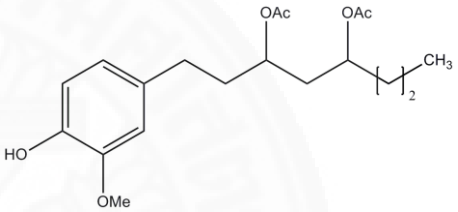
5-Acetoxy-[7]-gingerdiol



Methyl 5-acetoxy-[4]-gingerdiol



Methyl 5-acetoxy-[6]-gingerdiol



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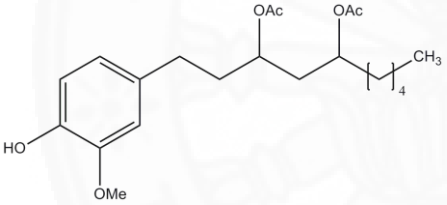
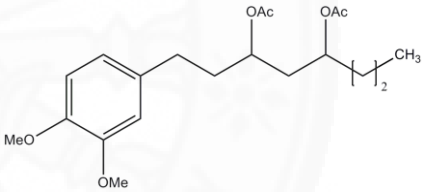


Diacetoxy-[6]-gingerdiol



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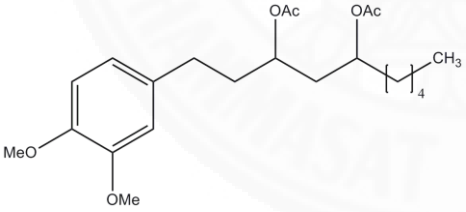

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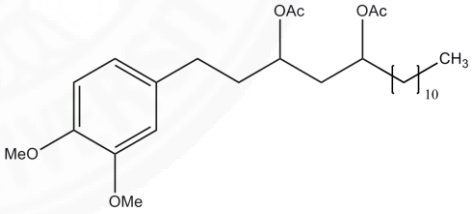
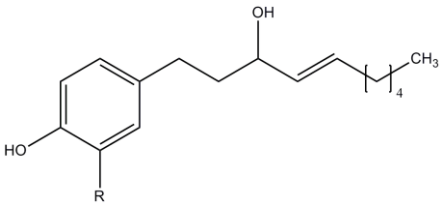
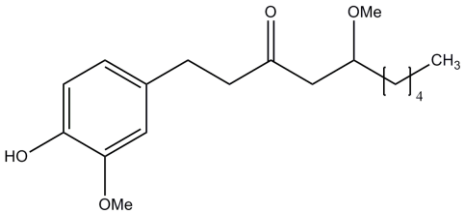


Methyl diacetoxy-[10]-gingerdiol

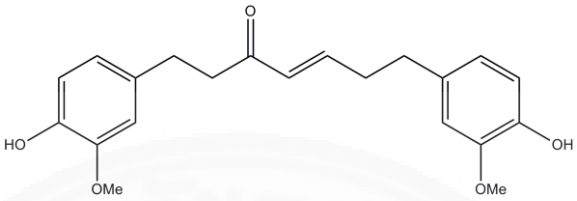
Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.** (Cont.)



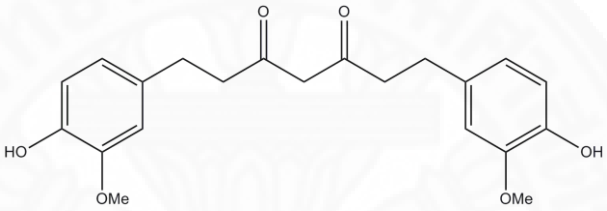
3-Dihydro-[6]-demethoxyshogaol



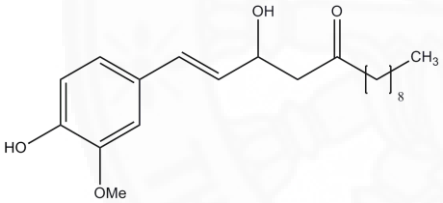
5-Methoxy-[6]-gingerol



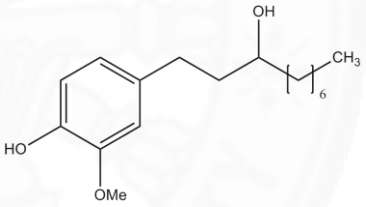
1,7-bis-(4’-Hydroxy-3’-methoxyphenyl)-4-heptene-3-one



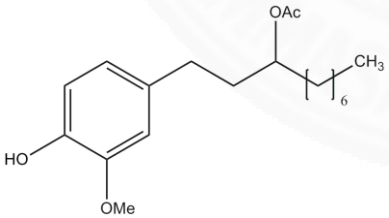
1,7-bis-(4’-Hydroxy-3’-methoxyphenyl)-3,5-heptadione



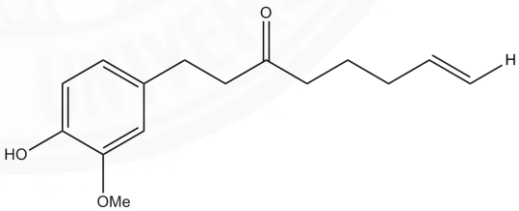
1-Dehydro-3-dihydro-[10]-ginger-dione



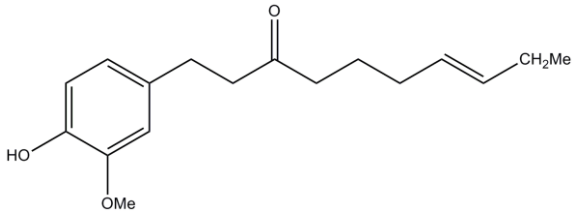
6-Dihydroparadol



Acetoxy-6-dihydroparadol

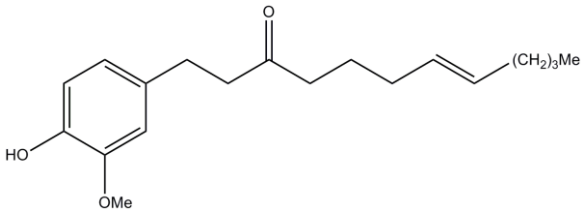


1-(4’-Hydroxy-3’-methoxyphenyl)-7-octen-3-one

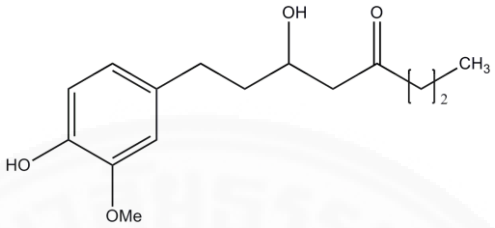


1-(4’-Hydroxy-3’-methoxyphenyl)-7-decen-3-one

Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.** (Cont.)



1-(4’-Hydroxy-3’-methoxyphenyl)-7-dodecen-3-one



[4]-Isogingerol

Figure 2.14 Structure of chemical compounds in *Z. officinale* Rosc**.** (Cont.)

2.3.3.3 Biological activity of Benjakul preparation and its pure compounds

Table 2.13 Biological activities of Benjakul preparation

Activities	Results	References
Cytotoxic activity	The ethanolic extract showed cytotoxic activity against breast cancer cell line (MCF-7) and lung cancer cell line (COR-L23) with IC_{50} value of 33.20 and 19.8 μg/ml, respectively. On the other hand, the water extract had no cytotoxic effect on breast cancer cell line.	(Ruangnoo, Itharat, et al., 2012; Sakpakdeejaroen & Itharat, 2009)
Cytotoxic activity	The ethanolic extract showed cytotoxic activity on lung cancer cell line (NCI-HI 688) with IC_{50} value of 36.15 μg/ml.	(Rattarom et al., 2014)
Genotoxic activity	The ethanolic extract showed significantly increased sister chromatid exchange level with concentration of 100, 200 and 400 μg/ml.	(Rattavalachai, Thitiorul, Tanuchit, Sakpakdeejaroen, & Itharat, 2012)
Anti-inflammatory activity	The ethanolic extract at the dose of 1 to 100 μg/ml showed significant anti-inflammatory activity against pro-inflammatory cytokine releases (TNF-α, IL-1β and IL-6) on intestinal epithelial cell line.	(Burodom & Itharat, 2013)

Table 2.13 Biological activities of Benjakul preparation (Cont.)

Activities	Results	References
Anti-malarial activity	The Benjakul extract was active against K1 and 3D7 *P. falciparum* clones with IC_{50} value of 32.1 and 11.3 μg/ml, respectively.	(Thiengsusuk et al., 2013)

Table 2.14 Biological activities of pure compounds from Benjakul preparation

Activities	Compounds	Results	References
Cytotoxic activity	• Piperine • 6-gingerol	• Piperine and 6-gingerol had activity against breast cancer cell line with IC_{50} value of 35.72 and 33.33 μM, respectively.	(Sakpakdeejaroen & Itharat, 2009)
	Plumbagin	• Plumbagin showed high cytotoxic activity against COR-L23, HepG2, Hela and MRC-5 with IC_{50} value of 2.55, 2.61, 4.61 and 11.54 μM, respectively.	(Ruangnoo, Itharat, et al., 2012)
		• Plumbagin induced apoptosis in lung cancer cell line and down-regulated G2/M regulatory proteins.	(Gomathinayagam et al., 2008)
Immunomodulatory activity	Piperine	• Piperine inhibited the production of IL-2 and IFN-γ production in the PBMCs.	(Chuchawankul, Khorana, & Poovorawan, 2012)
	Piperine	• Piperine (1.14 mg/dose/animal) increased white blood cells count in Balb/c mice	(Sunila & Kuttan, 2004)
	Piperine	• Piperine (100 μM) suppressed T lymphocyte proliferation and inhibited IFN-γ, IL-2, IL-4 and IL-17A.	(Doucette, Rodgers, Liwski, & Hoskin, 2015)

Table 2.14 Biological activities of pure compounds from Benjakul preparation (Cont.)

Activities	Compounds	Results	References
Immunomodulatory activity	6-gingerol	• 6-gingerol inhibited T-lymphocyte proliferation at concentration of 100 μM. 6-gingerol (25-50 μM) significantly inhibited IFN-γ production but it had no effect on IL-4 and IL-2 in T lymphocytes.	(Bernard, Furlong, Power Coombs, & Hoskin, 2015)
	Plumbagin	• Plumbagin (5 μM) inhibited the Con A-induced proliferation of lymphocytes. Plumbagin (50 nM) significantly inhibited IL-2, IL-4, IL-6 and IFN-γ production in T cells.	(Checker, Sharma, Sandur, Khanam, & Poduval, 2009)
Anti-inflammatory activity	Piperine	• Piperine inhibited TNF-α which did not inhibit IL-1β and IL-6 in peritoneal macrophage.	(G. S. Bae et al., 2010)
		• Piperine inhibited IL-6-induced Stat3 activation with IC_{50} value of 47.40 μM.	(S. W. Lee et al., 2010)
	6-shogaol	• 6-shogaol (0.1-100 μM) significantly inhibited IL-6, IL-8 and TNF-α secretion in HMC-1 cells.	(Sohn, Han, Lee, Cho, & Jung, 2013)

Table 2.14 Biological activities of pure compounds from Benjakul preparation (Cont.)

Activities	Compounds	Results	References
Anti-inflammatory activity	Myristicin	• Myristicin showed anti-inflammatory activity which inhibited IL-6, IL-10 and NO production of RAW 264.7 cells.	(J. Y. Lee & Park, 2011)

2.3.3.4 Biological activity of plants composing Benjakul preparation

Table 2.15 Biological activities of plants composition of Benjakul preparation

Scientific name	Activities	Results	References
P. chaba Hunt.	Anti-inflammatory activity	• *P. chaba* extract decreased both transudative and granuloma weights in rats.	(Sireeratawong et al., 2012)
		• The chloroform extract of *P. longum* reduced the neutrophil adhesion to endothelial monolayer and significantly inhibited TNF-α-induced expression of cell adhesion molecule-mediated interactions. Moreover, it inhibited NF-κB expression in endothelial cells.	(Singh et al., 2008)
		• The methanolic extract of *P. chaba* showed mild to moderate anti-inflammatory effect in rats at concentration of 125 and 250 mg/kg.	(Begum, Begum, Uddin, Haider, & Barman, 2012)

Table 2.15 Biological activities of plants composition of Benjakul preparation (Cont.)

Scientific name	Activities	Results	References
P. chaba Hunt.	Anti-inflammatory activity	• The EtOAc extract of *P. longum* at 40 mg/kg decreased CD4+ and CD8+ T cells and cytokine production in sensitized Balb/C mice.	(Devan, Bani, Suri, Satti, & Qazi, 2007)
		• The ethanolic extract of *P. longum* (10 mg/dose/mice) downregulated IL-1β, IL-6 and TNF-γ level of angiogenesis-induced mice after 9 days of tumor induction.	(Sunila & Kuttan, 2006)
	Immunomodulatory activity	• The ethanolic extract of *P. longum* (10 mg/dose/animal) increased the total white blood cells in Balb/c mice.	(Sunila & Kuttan, 2004)
		• The ethanolic extract of *P. longum* (10 mg/dose/mice) induced IL-2 production in mice on day 9 after tumor induction when compared to the control group.	(Sunila & Kuttan, 2006)

Table 2.15 Biological activities of plants composition of Benjakul preparation (Cont.)

Scientific name	Activities	Results	References
P. sarmentosum Roxb.	Anti-inflammatory activity	The water extract of *P. sarmentosum* showed anti-nociceptive activity and anti-inflammatory on acute inflammation in carrageenan-induced paw edema rat.	(Zakaria, Patahuddin, Mohamad, Israf, & Sulaiman, 2010)
P. interruptum Opiz.	Anti-inflammatory activity	The ethanolic extract of *P. interruptum* inhibited ethyl phenylpropiolate-induced ear edema and carrageenan-induced hind paw edema in rats.	(Sireeratawong et al., 2012)
P. indica Linn.	Immunomodulatory activity	The methanolic extract of *P. indica* (4 mg/ml) activated T-lymphocyte proliferation.	(Saraphanchotiwitthaya, Ingkaninan, & Sripalakit, 2007)
Z. officinale Rosc.	Immunomodulatory activity	The volatile oil (0.001-10 ng/ml) significantly inhibited T lymphocyte proliferation and decreased the number of T helper cells. Moreover, the volatile oil of ginger inhibited IL-1α secretion in mice peritoneal macrophages.	(Zhou, Deng, & Xie, 2006)

Table 2.15 Biological activities of plants composition of Benjakul preparation (Cont.)

Scientific name	Activities	Results	References
Z. officinale Rosc.	Immunomodulatory activity	*Z. officinale* extract enhanced macrophage, splenocyte and CD4+ CD8+ ratio. In addition, it increased the secretion of IL-1β and IL-3.	(X. Du et al., 2010)
	Anti-inflammatory activity	The methanol extract of *Z. officinale* inhibited IL-1β release from PBMCs with IC_{50} value of 3.17 μg/ml.	(Salim, Kumolosasi, & Jantan, 2014)
		Dried *Z. officinale* significantly reduced interferon-γ and interleukin-6 in the serum of ICR mice.	(Choi, Kim, Hong, Kim, & Yang, 2013)
		Ginger (2 gram/day) significantly decreased the TNF-α level in diabetes patients compared with placebo group (TNF-α level = 15.3 and 19.6 pg/ml, respectively).	(Mahluji, Ostadrahimi, Mobasseri, Ebrahimzade Attari, & Payahoo, 2013)
		The ethanolic extract of ginger (100 mg/kg body weight) showed significantly decreased NF-κB and TNF-γ expression in rats.	(Habib et al., 2008)

Table 2.15 Biological activities of plants composition of Benjakul preparation (Cont.)

Scientific name	Activities	Results	References
Z. officinale Rosc.	Anti-inflammatory activity	The extract (50 μg/ml) inhibited IL-1β production in human whole blood.	(Nievergelt, Marazzi, Schoop, Altmann, & Gertsch, 2011)
		Rhizome powder (200 mg/kg) modulated IL-6, IL-10 and IL-4 production in arthritic rats when compared with arthritic control rats.	(Ramadan, Al-Kahtani, & El-Sayed, 2011)

2.4 Contact hypersensitivity

Contact hypersensitivity is the delayed type hypersensitivity that response to epicutaneous application of chemical reagent, cosmetics, fragrances and natural product. Contact hypersensitivity is consisted of two phase including, sensitization phase and elicitation phase. Contact hypersensitivity involves a low molecular weight compounds termed haptens. The initial phase of contact hypersensitivity, sensitization phase, haptens are recognized by Langerhans cells, and then the mature Langerhans cells migrate from epidermis to the skin-draining lymph nodes. Langerhans cells present antigenic peptides to T cells. The reexposure of same hapten lead to the elicitation phase. Haptens activate T cells that recruit to the site of contact. CD4+ T cells play the important role in the elicitation phase of contact hypersensitivity. CD4+ T cells recognize antigen-peptide via MHC-II and produce various cytokines such as IL-4, IFN-γ and TNF-α. Furthermore, hapten also activates keratinocytes and induces pro-inflammatory cytokines TNF-α, IL-1 and GM-CSF. Contact hypersensitivity show cardinal signs such as redness, swelling, itching and blistering (Watanabe & Unger & Tuvel & Wang & Sauder, 2002). The previous report showed that contact hypersensitivity related with TRPV1 and TRPA1 channel in the plasma membrane of sensory nerves (Shiba et al., 2009).

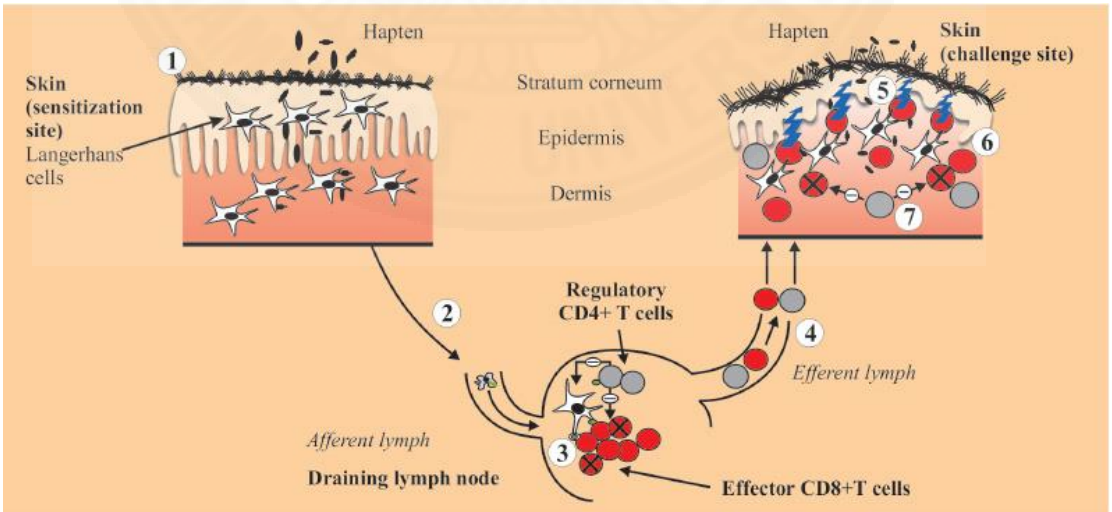


Figure 2.15 Contact hypersensitivity reactions (Hennino et al., 2005)

TRP channels are the family of ion channels which consists of six transmembrane domains. The TRP channel show important role in sensation such as, taste, vision, hearing, thermosensation. TRP are divided into seven subfamilies namely, TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML (Venkatachalam & Montell, 2007). The transient receptor potential vanilloid 1 and ankyrin 1 (TRPV1 and TRPA1) channels related the pain and neurogenic inflammation. TRPV1 channel is activated by capsaicin from chilli peppers, heat over 43°C and pH less than 5.9. TRPV1 channel is confirmed the effect on noxious heat sensation. TRPA1 also activate neurogenic inflammation (E.S. Fernandes & M.A. Fernandes & Keeble, 2012). Normally, TRPA1 is activated by mustard oil and cold temperature. TRPV1 and TRPA1 related to the adjuvant effect during contact hypersensitivity.

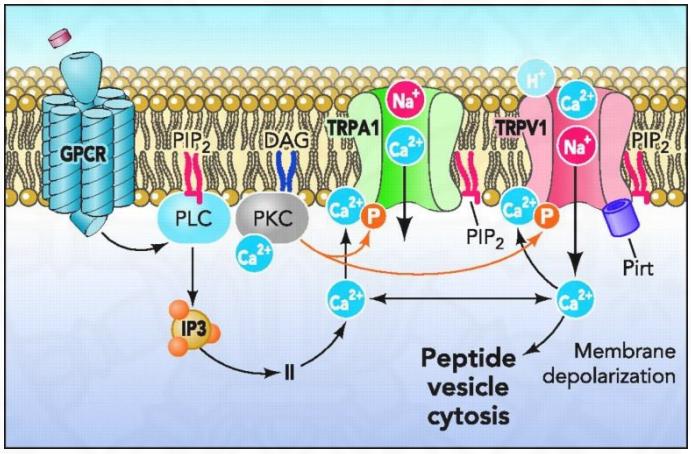


Figure 2.16 Structure of TRPV1 and TRPA1 channel (Bessac & Jordt, 2008)

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chemicals and materials

Table 3.1 The source of chemicals and materials

Chemicals and materials	Sources
• RPMI-1640 medium • Fetal bovine serum • Penicillin/Streptomycin • Trypsin-EDTA • HEPES • L-glutamine	Invitrogen Life Technologies Inc. (Carlsbad, CA, USA)
• Phosphate buffer saline	Amresco (Parkway Solon, Ohio, USA)
• 96-well microplates	Corning Inc. (New York, USA)
• 6-well plate	Corning Inc. (New York, USA)
• Lipopolysaccharide (LPS)	Sigma-Aldrich (St. Louis, MO, USA)
• 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	Sigma-Aldrich (St. Louis, MO, USA)
• Isoprep	Robbins Scientific Corporation (Sunnyvale, CA, USA)
• Sodium heparin blood collection tubes	Becton Dickinson (Franklin Lakes, NJ, USA)
• $Na_2{}^{51}CrO_4$ • ^{3}H-thymidine	PerkinElmer (Waltham, Massachusetts, USA)

Table 3.1 The source of chemicals and materials (Cont.)

Chemicals and materials	Sources
• Phytohaemaglutinin A (PHA)	Sigma-Aldrich (St. Louis, MO, USA)
• IL-1β ELISA Kit • IL-6 Mouse ELISA Kit • IL-10 ELISA Kit	Abcam (Cambridge, UK)
• Human IL-2 Plantinum ELISA Kit • Human IFN-γ Plantinum ELISA 96 test	Affymetrix, (Santa Clara, CA, USA)
• A dial thickness gauge	Mitutoyo (Kanagawa, Japan)
• 2,2--azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) • Hydrogen peroxide • Polyoxyethylene (20) sorbitan monolaurate • Kanamycin sulfate	Wako Pure Chemical Industries, Ltd. (Osaka, Japan)
• Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, MO, USA)
• Dulbecco's modified Eagle's medium (DMEM)	Nissui Pharmaceuticals (Tokyo, Japan)
• FITC	Dojindo Laboratories (Kumamoto, Japan)
• Pentobarbital	Kyoritsu Seiyaku Corporation (Tokyo, Japan)
• Phycoerythrin (PE)-conjugated hamster anti-CD11c • Monoclonal antibodies (mAb) (clone HL3; immunoglobulin G1 (IgG1)) • PE-conjugated hamster IgG1 isotype control (clone G235-2356)	BD Biosciences (San Jose, CA, U.S.A.)

Table 3.1 The source of chemicals and materials (Cont.)

Chemicals and materials	Sources
• Purified rat anti-mouse interferon-γ (IFN-γ) mAb (clone AN-18) • Biotin-conjugated rat anti-mouse IFN-γ mAb (clone R4-6A2) • Recombinant mouse IFN-γ	BioLegend (San Diego, CA, U.S.A.)
• Purified rat anti-mouse interleukin-4 (IL-4) mAb (clone 11B11) • Biotin-conjugated rat anti-mouse IL-4 mAb (clone BVD6-24G2) • Recombinant mouse IL-4	eBioscience (San Diego, CA, U.S.A.)
• Horseradish peroxidase (HRP)–avidin	Zymed Laboratories Inc. (South San Francisco, CA, U.S.A.)

3.2 Plant Materials

Plant materials were collected by Thai folk medicine practitioners. Plant identity was confirmed by comparison with authentic voucher specimens that are kept in the herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla, Thailand. The herbarium voucher specimen number is showed in Table 3.2

Table 3.2 Summarized data of plant materials

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Plants	Thai name	Source	Part of used	Voucher specimen number
Piper chaba Hunt.	Deeplee	Khaosaming, Chanthaburi	Fruit	SKP 146160301
Piper sarmentosum Roxb.	Chaplu	Chombueng, Ratchaburi	Root	SKP 146161901
Piper interruptum Opiz.	Sakhan	Phupan, Sakhonnakhon	Stem	SKP 146160901
Plumbago indica Linn.	Chettamun-phloeng-daeng	Thanlingchan, Bangkok	Root	SKP 148160901
Zingiber officinale Rosc.	Khing	Khaokho, Phetchabun	Rhizome	SKP 206261501
Smilax corbularia Kunth	Hua-Khao-Yen-Neua	Mae-Taeng, Chiangmai	Rhizome	SKP A179190315
Dioscorea membranacea Pierre	Hua-Khao-Yen-Tai	Patue, Chumporn	Rhizome	SKP A062041305

3.3 Preparation of plant extracts and Benjakul preparation

Each plant was cleaned, cut into small pieces, dried in oven at 50°C for 48 hours and ground to powder. These materials were divided into 2 parts; the first part was extracted by maceration and the second part was extracted by decoction. For Benjakul preparation, it is composed of 5 plants; *P. chaba* Hunt., *P. sarmentosum* Roxb., *P. interruptum* Opiz., *P. indica* Linn., *Z. officinale* Rosc. Thus, five plants were mixed in equal proportion and divided into 2 parts (for maceration and decoction). For maceration, dried plant powder was macerated with 95% ethanol for 3 days. After maceration, the solvent phase was filtered through Whatman No.1 paper. The plant residue was macerated and filtrated 2 times. The solvent phase was evaporated to dryness using a lyophilizer. For decoction, dried plant powder was boiled in water for 30 minutes, filtered through Whatman No. 1 and dehydrated by a lyophilizer. The ethanolic and water extract were weighed and stored at -20°C until required for use in the studies.

3.4 Bioassay-guided fractionation

The ethanolic extracts of *D. membranacea*, *S. corbularia* and Benjakul were dissolved in methanol and mixed with 120 g silica. The extract was separated to five fractions by vacuum liquid chromatography (VLC). The solvent polarity was ordered increase: hexane 1000 ml. hexane:chloroform (1:1) 2000 ml, chloroform 2000 ml, chloroform:methanol (1:1) 1000 ml and methanol 1000 ml. Solvents were evaporated by using rotary evaporator. Each fraction was tested for NK cells activity and selected for isolation of main pure compound.

3.5 Study of immunomodulatory effect of Hua-Khao-Yen and Benjkul on peripheral blood mononuclear cells (PBMCs)

3.5.1 Subjects

Six males and six females were enrolled in the study. Their ages ranged from 20-45 years old. They had not been infected with hepatitis B, C virus and HIV-1. They had not taken dietary supplements or nutraceutical products. Informed consent was obtained from each donor after the objective of this study had been completely

explained. The studies of Hua-Khao-Yen and Benjakul project were approved by the ethical review committees of Faculty of Medicine, Thammasat university under numbers 119/2555 and 120/2555.

3.5.2 Isolation of peripheral blood mononuclear cells

Approximately 20 ml of blood was collected from each donor into sodium heparin blood collection tubes. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood using Ficoll-Hypaque density gradient (Boyum, 1966). Blood samples were carefully and slowly overlayed to Ficoll-Hypaque isoprep in equal proportion. Whole bloods with isoprep were centrifuged at 380 x g (or 2,000 rpm) for 20 minutes at 4°C. PBMCs were in white band at interface which were collected and washed twice with RPMI-1640 medium. After washing, PBMCs were adjusted to $2x10^6$ cells/ml in complete RPMI (RPMI-1640 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin).

3.5.3 Cell viability by MTT assay

This assay was perfomed by the method of Mosmann (1983) with some modification. 100 µl PBMCs (2×10^6 cells/ml) were plated in 96 well-flat bottomed plates with 100 µl of plant extracts in various concentration and incubated at 37°C, 5% CO_2 for 18 hrs (for NK cells activity) and 72 hrs (for lymphocyte proliferation). After incubation, 100 µl of supernatant was discarded. 10 µl of MTT solution (5 mg/ml) was added into each well and incubated at 37°C, 5% CO_2 for 4 hours. The MTT formazan produced in wells containing living cells appeared as black, fuzzy crystals on the bottom of the well. After 4 hrs incubation, the medium was removed, and 100 µl of dimethylsulphoxide (DMSO) was added to dissolve the formazan in the cells. The optical density of formazan solution was measured with a microplate reader at 570 nm.

The percentage of survival was calculated according to the formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of sample} \times 100}{\text{OD of control}}$$

3.5.4 Evaluation of natural killer cells activity (NK cells activity)

Natural killer cells activity was performed by chromium release assay which used PBMCs as effector cells and K562 as target cells. The protocol was based by the method of Sriwanthana and Chavalittumrong (2001) with some modifications.

3.5.4.1 Cell and cultures

K562 (ATCC CCL-243), human erythroleukemic cell lines, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 medium with 10 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, and incubated at 37°C in an atmosphere containing 5% CO_2. The K562 cell lines were sub-cultured every three days.

3.5.4.2 Stimulation of PBMCs by the ethanolic and water extracts

PBMCs ($2x10^6$ cells/ml) were incubated in the absence or presence of the extracts at the final concentrations of 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml at 37°C, 5% CO_2 for 18-24 hours. After incubation period, PBMCs were washed with 1 ml of complete RPMI at 210 x g (or 1500 RPM) for 10 minutes and the supernatant removed. The cell pellet was re-suspended with 500 µl of complete RPMI.

3.5.4.3 Chromium-labelled target cells

The K562 (ATCC CCL-243), human erythroleukemic cell lines, were purchased from the American Type Culture Collection. The cells were used as target cells for evaluated natural killer cells (NK cells) activity. The K562 cell lines are cultured in complete RPMI medium and incubated at 37°C in 5% CO_2 atmosphere and subcultured every three days. K562 cells ($2x10^6$ cells/ml) were labelled with 100 µCi of $Na_2{}^{51}CrO_4$ for 90 minutes. After incubation time, target cells were washed three times with 10 ml of complete RPMI and adjusted to $2x10^4$ cells/ml.

3.5.4.4 Effect of the extracts or pure compounds on NK cells activity

The entire experiment was repeated on three further occasions. The effector cells to target cell ratio used 90:1, 30:1, 10:1 and 3:1, respectively. After incubation, 100 µl/well was transferred into microtiter tubes and counted in a gamma counter.

The percentage of cytolysis was calculated according to the formula:

$$\% \text{ cytolysis} = \frac{(\text{Experiment release} - \text{Spontaneous release})}{(\text{Maximal release} - \text{Spontaneous release})} \times 100$$

Spontaneous release was measured by incubation of target cells with media alone, while maximal release was measured by incubation of target cells with 5% Triton X-100. NK cells activity on cytotoxicity was expressed as a lytic unit (LU)/10^7 PBMCs. One LU was defined as the number of effector cells required for 20% specific lysis of 1×10^4 target cells.

3.5.5 Lymphocyte proliferation assay

Lymphocyte proliferation was determined via ^{3}H-thymidine uptake assay (Sriwanthana & Chavalittumrong, 2001). 100 µl PBMCs (2×10^6 cells/ml) were cultured in 96 well microtiter plates with the extracts or pure compounds. The final concentrations of these samples were 1 ng/ ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml and 100 µg/ml, and 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, 1 µg/ml and 10 µg/ml, respectively. Incubation was at 37°C with 5% CO_2 for 72 hours. After incubation, 20 µl of ^{3}H-thymidine (25 µCi/ml) was added into each 96 well plates and incubated at 37°C with 5% CO_2 for 18 hours. ^{3}H-thymidine uptake was measured by a liquid scintillation counting using Top-count Microplate Scintillation & Luminescence Counter. The degree of activation was expressed as the stimulation index. In any experiment, assays were carried out in triplicate for each concentration, and the entire experiment repeated on three further occasions. The stimulating index was calculated according to the formula:

$$\text{Stimulating index} = \frac{^3\text{H}-\text{thymidine uptake of sample with extract}}{^3\text{H}-\text{thymidine uptake of sample without extract}}$$

3.5.6 Effect of the extracts or pure compounds on IL-2 and IFN-γ secretion from PBMCs

This experiment was performed by the previous reported (Sriwanthana & Chavalittumrong, 2001; Sodsai, 2006) with some modification. 1 ml of PBMCs ($2x10^6$cells/ml) were incubated with the various concentrations of extracts of pure compounds and incubated with or without mitogen, PHA at 37°C with 5% CO_2 for 24 hours. After the incubation period, the supernatant was collected into 15 ml Fallcon-

tube and centrifuged at 210 x g, at 4°C for 10 minutes. IL-2 and IFN-γ production was determined by ELISA from the supernatant.

The strip wells were coated by immobilized IL-2 or IFN-γ antibody. 50 µl of IL-2 or IFN-γ standard was added into appropriate wells. For sample wells, 50 µl of sample diluent and supernatant was added into sample wells. Then, 50 µl of Biotinylated IL-2 or IFN-γ detection antibody was added to each well. ELISA plate was covered with cover a sheet and incubated for 2 hours at room temperature with gentle shaking. The solution was discarded and the plate washed 6 times with 300 µl of wash buffer. HRP-Streptavidin solution (100 µl) was added to each well and incubated for 1 hour at room temperature with gentle shaking. Discard solution and washed 6 times with wash buffer. 100 µl of TMB one-step substrate reagent was added to each well and incubated at room temperature in the dark with gently shaking. For IL-2 detection, ELISA plate was incubated for 30 minutes. For IFN-γ detection, ELISA plate was incubated for 10 minutes. Then, 100 µl of stop solution was added to each well and read at 450 nm immediately.

3.6 Effect of the extracts or pure compounds on inflammatory and anti-inflammatory cytokines production from RAW264.7 cells

3.6.1 Cell line and culture

RAW264.7 cell line, murine macrophage, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were sub-cultured every four days.

3.6.2 Evaluation of IL-1β, IL-6 and IL-10 production

Cytokine productions were determined using a method modified from the previous report (Shin et al., 2013; Razali, Ismail, Abidin, Suriza, &Shuib, 2014). RAW264.7 cells were seeded in 6-well plates ($2x10^6$ cells/well). Cells were incubated at 37°C, 5% CO_2 for 24 hours. After incubation, the medium was removed and replaced with fresh medium containing various concentrations of plant extracts or pure compounds or prednisolone as a positive control and 10 ng/ml of lipopolysaccharide (LPS). The cells were incubated at 37°C, 5% CO_2 for 24 hours. For evaluation of IL-10 production, the cells were incubated at 37°C, 5% CO_2 for 48

hours. Supernatant from each well was collected and IL-1β, IL-6 and IL-10 production detected by enzyme-linked immunosorbent assay (ELISA). ELISA kits were used according to specific manufacturer's instructions for each cytokine. Supernatants were added into the wells and incubated at room temperature for 2.5 hours. The wells were washed 4 times with wash buffer. After washing, 100 µl of the detection antibody (biotinylated-IL-1β or biotinylated-IL-6 or biotinylated-IL-10) was added into the wells and incubated at room temperature for 1 hour. An excess detection antibody was removed and washed 4 times with wash buffer. A Streptavidin-HRP conjugate was added into each well and incubated for 45 minutes at room temperature. After incubation, the excess streptavidin-HRP was removed and washed 4 times with wash buffer. TMB substrate solution (100 µl/well) was added into each wells and incubated for 30 minutes in the dark at room temperature. After incubation, 50 µl of the stop solution was added into the wells. The optical density of these wells was measured for colored product at 450 nm.

3.7 Effect of three *Piper* species and piperine on contact hypersensitivity.

3.7.1 Cell lines and culture

The HEK cells stably expressing human TRPV1 and the CHO cells stably expressing human TRPA1 were established with T-Rex system as described previously (Okumura et al., 2010). The cells were provided by Professor Tatsuo Watanabe (Laboratory of Food Chemistry, School of Food and Nutritional Sciences, University of Shizuoka).TRPV1-expressing HEK cells were cultured in Ham's F-12 medium/DMEM (1:1) with 10% FBS, 10 mM HEPES, 60 µg/ml kanamycin sulfate, 200 µg/ml zeocin and 5 µg/ml blasticidin. Cells were sub-cultured every 3 days.

3.7.2 Calcium imaging assay

TRPV1-expressing HEK cells or T-REx HEK cells or TRPA1-expressing CHO cells or T-Rex CHO cells (4×10^5 cells/well) were seeded into 96-well black plates coated with poly-L-lysine and incubated with 1µg/ml of tetracycline for 24 hrs at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, cells were replaced with 3 µM Fluo-4-AM in loading buffer with 250mM probenecid for 60-90 minutes at 37°C. Cells were washed with 100 µl loading buffer and then 180 µl

loading buffer was added into each well. The intracellular Ca^{2+} concentration was evaluated by Flex Station II (Molecular Devices, Sunnyvale, CA). After start of measurement, the sample was added into each well at 30 seconds and 5 μM ionomycin was added at 150 seconds to measure the maximum level of calcium. Each sample was dissolved in DMSO and added to the loading buffer. The final concentration of DMSO did not exceed 0.1%.The cells were treated with various concentrations of positive control (capsaicin for TRPV1 and AITC for TRPA1), piperine or the ethanolic extract of *Piper* species. Inhibitory activities of TRPV1 antagonist were performed by adding AMG9810 at various concentrations (30nM, 300 nM and 3μM) to piperine and *Piper* extract. Inhibitory activities of TRPA1 antagonist were investigated by adding HC030031 at various concentrations (0 μM, 1 μM and 10 μM) to piperine and *Piper* extract. The results of each sample are expressed as the percentage response to 5 μM ionomycin. Dose-response curves were plotted with Prism 5.0 software (Graph Pad Software, CA, USA).

3.7.3 Effects of *Piper* species and piperine during sensitization with hapten fluorescein isothiocyanate

Experiments were performed as described previously with some modifications (Imai, Kondo, Iizuka, Maruyama, & Kurohane, 2006). Mouse forelimbs were shaved for 2 days before sensitization. The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium. Mice were sensitized on days 0 and 7 with 160 μl of 0.5%FITC dissolved in acetone containing a test sample. On day 14, the baseline ear thickness at 0 hr was measured with a dial thickness gauge. After measurement, mice were challenged by painting 20 μl of 0.5% FITC in 10%DBP-acetone on the right auricle, while 20 μl of 10% DBP-acetone was applied on the left auricle as a control. Mouse ear thickness was measured at 24, 48 and 72 hrs after challenge. In some experiments, mice without FITC-sensitization were challenged with FITC to serve as a negative control.

$$\text{Ear swelling at X hrs} = (ER_X - EL_X) - (ER_0 - EL_0)$$

ER_X = Ear thickness of the right ear at X hrs

EL_X = Ear thickness of the left ear at X hrs

ER_0 = Ear thickness of the right ear at 0 hrs

EL_0 = Ear thickness of the left ear at 0 hrs

Statistical analysis was performed using ANOVA and Tukey's multiple comparison tests. Statistical significance was indicated at $p \leq 0.05$.

3.7.4 The trafficking of FITC-presenting dendritic cells

This assay was done according to the previous report (Imai et al., 2006) with some modification. Mouse forelimbs were shaved for 2 days before sensitization. The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium. Mice were sensitized with 160 µl of 0.5%FITC dissolved in acetone containing a test sample. 24 hours after sensitization, the mice were euthanized and collected lymph nodes. Cell suspensions of lymph nodes were prepared by teasing with 21 Guage needles. The cell suspensions were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN_3. After washing, cells were adjusted to $2x10^6$ cells/100 µl and incubated with 2 µg/ml of PE-conjugated anti-CD11c mAb or PE-isotype control mAb for 15 minutes at 4°C in a dark box. After incubation, cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN_3. Then, 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN_3 was added to the cells and filtered through a 70 µm cell strainer. A total of 5×10^5 cells were examined by a flow cytometer using gates for forward and side scatter to collect signals of cell-associated fluorescence. Data was analyzed by FACSDiva software.

3.7.5 Cytokine production from lymph node cells

The cytokine production was determined according to the previously report (Kobayashi, Kurohane, & Imai, 2012) with some modification. Mouse forelimbs were shaved for 2 days before sensitization. The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium. Mice were sensitized on days 0 and 7 with 160 µl of 0.5% FITC dissolved in acetone containing a test sample. 24 hours after the second sensitization, mice were euthanized and lymph nodes collected. Cell suspensions of lymph nodes were prepared by teasing with 21 Guage needles. The cells suspensions were washed twice with RPMI 1640 medium. After washing, cells were adjusted to 2.5×10^6 cells/ml in RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum, 30 µg/ml kanamycin. Cells were placed in 96-well plate and incubated at 37°C, 5% CO_2. After incubation, the supernatants were collected at 48 and 72 hrs.

IL-4 and IFN-γ production were detected by ELISA technique on supernatants. A monoclonal antibody specific for IL-4 or IFN-γ (0.5 μg/ml of purified rat anti-mouse IL-4 or 1 μg/ml of purified rat anti-mouse IFN-γ) was coated onto strip wells of ELISA plate and incubated for 18 hours at 4°C. The wells were washed with PBS three times. After washing, non-specific binding site was blocked with 1% BSA in PBS (200 μl/well) for 2 hours at room temperature. The plate was washed 3 times with 0.1% tween 20 in PBS. After washing, 100 μl of supernatants or standard recombinant protein was added into the wells and incubated for 1 hour at room temperature. The excess protein was discarded and washed 3 times with 0.1% tween 20 in PBS. The detection antibody (1 μg/ml of biotin-rat anti-mouse IL-4 or biotin-rat anti-mouse IFN-γ) was added to the wells for detected specific protein and incubated for 1 hour at room temperature. The ELISA plate was washed for 4 times with 0.1% tween 20 in PBS. The avidin-HRP (0.1 μg/ml, 100 μl/well) was added to the wells to detected biotinylated antibodies binding, and was incubated for 1 hour at room temperature. The plate was washed 5 times with 0.1% tween 20 in PBS. Then, 100 μl of 1 mM ABTS dissolved in 0.1 M citrate buffer containing H_2O_2 was added into each well and incubated at room temperature for 10-30 min. The optical density of the color product of these wells was measured at 405 nm.

3.7.6 Statistical analysis

The results were analyzed using one-way ANOVA followed by Dunnett's test with Graphpad Prism 5.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preparation of Plants extract and Benjakul extracts

Each plant was extracted by maceration and decoction. The percentage of yield of each plant is shown in Table 4.1.

Table 4.1 The percentage of yield of extracts

Sample	Extract	Code	%Yield of extract
S. corbularia	Ethanol	SCE	15.16
	Water	SCW	8.25
D. membranacea	Ethanol	DME	4.25
	Water	DMW	24.9
Benjakul	Ethanol	BJE	11.40
	Water	BJW	15.80
P. chaba	Ethanol	PCE	6.30
	Water	PCW	17.19
P. sarmentosum	Ethanol	PSE	6.21
	Water	PSW	15.54
P. interruptum	Ethanol	PIE	2.47
	Water	PIW	11.93
P. indica	Ethanol	PLE	15.56
	Water	PLW	29.88
Z. officinale	Ethanol	ZOE	12.59
	Water	ZOW	16.90

4.2 Immunomodulatory effect of Hua-Khao-Yen and Benjakul extracts on PBMCs

4.2.1 Cytotoxic activity of Hua-Khao-Yen and Benjakul extracts on PBMCs

PBMCs were isolated from from healthy donor's blood and tested for cytotoxic activity of the ethanolic and water extract of *S. corbularia* (Hua-Khao-Yen-Nua), *D. membranacea* (Hua-Khao-Yen-Tai) and Benjakul at 24 and 72 hours as showed in the Table below.

PBMCs were incubated with the ethanolic and water extract of *S. corbularia* at concentration of 0.01, 0.1, 1, 10 and 100 μg/ml for 24 and 72 hours. PBMCs showed over 70% survival at all concentrations (Table 4.2). These results show that the ethanolic and water extract of *S. corbularia* had no toxicity on PBMCs.

Table 4.2 The percentage of survival of PBMCs when incubated with *S. corbularia* extracts at 24 and 72 hours (n=6)

Concentration (μg/ml)	**% Survival (mean ± SEM)**			
	Ethanolic extract		**Water extract**	
	24 hours	**72 hours**	**24 hours**	**72 hours**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	102.53 ± 6.60	94.34 ± 3.11	101.53 ± 5.11	95.53 ± 3.35
0.1	95.11 ± 4.65	92.34 ± 4.31	101.35 ± 6.73	93.87 ± 4.68
1	100.44 ± 5.77	93.40 ± 3.37	95.03 ± 3.86	91.47 ± 4.68
10	103.36 ± 7.09	98.43 ± 5.39	99.02 ± 3.79	95.45 ± 4.76
100	128.11 ± 9.49*	103.84 ± 6.42	111.05 ± 3.35*	112.01 ± 2.98*
IC_{50} (μg/ml)	>100	>100	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

PBMCs were incubated with the ethanolic and water extract of *D. membranacea* at concentrations of 0.01, 0.1, 1, 10 and 100 µg/ml for 24 and 72 hours. From Table 4-3, PBMCs showed over 70% survival at all concentrations of *D. membranacea* extracts at 24 hours (Table 4.3). However, the ethanolic extract at 100 µg/ml and the water extract at 10 and 100 µg/ml showed toxic effect on PBMCs at 72 hours after incubation period.

Table 4.3 The percentage of survival of PBMCs when incubated with *D. membranacea* extracts at 24 and 72 hours (n=6)

Concentration (µg/ml)	**% Survival (mean ± SEM)**			
	Ethanolic extract		**Water extract**	
	24 hours	**72 hours**	**24 hours**	**72 hours**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	97.24 ± 3.65	89.98 ± 3.42*	101.19 ± 3.42	90.90 ± 3.06*
0.1	92.86 ± 2.40*	91.29 ± 3.98	93.39 ± 3.98	75.68 ± 4.84*
1	85.34 ± 3.59*	82.87 ± 5.05*	92.62 ± 5.17*	71.89 ± 4.86*
10	96.54 ± 3.70	104.08 ± 5.17	84.62 ± 5.17*	60.26 ± 4.78*
100	77.34 ± 11.87	55.85 ± 11.56*	79.83 ± 11.56*	49.40 ± 8.24*
IC_{50} (µg/ml)	>100	86.30 ± 6.49	>100	97.22 ± 2.22

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

For Benjakul preparation, the ethanolic and water extracts showed no toxic effect on PBMCs at all of concentrations. PBMCs exhibited over 70% survival at 24 and 72 hours after incubation as showed in Table 4.4.

Table 4.4 The percentage of survival of PBMCs when incubated with Benjakul extracts at 24 and 72 hours (n=6)

Concentration (µg/ml)	% Survival (mean ± SEM)			
	Ethanolic extract		Water extract	
	24 hours	72 hours	24 hours	72 hours
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	105.14 ± 5.75	94.08 ± 4.11	99.58 ± 5.32	89.03 ± 3.84
0.1	102.84 ± 4.25	95.76 ± 4.71	98.81 ± 3.48	89.99 ± 6.52*
1	97.93 ± 4.82	94.81 ± 6.34	92.60 ± 4.17	92.82 ± 2.65*
10	100.46 ± 4.88	96.53 ± 6.84	92.75 ± 2.28*	90.28 ± 1.95*
100	102.09 ± 5.77	93.30 ± 18.22	97.85 ± 3.18	94.38 ± 4.08
IC_{50} (µg/ml)	>100	>100	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

The extracts of five component plants in Benjakul preparation were investigated for their cytotoxic effect on PBMCs at 24 hours and 72 hours. The ethanolic extract of *P. indica* showed a toxicity effect on PBMCs at concentration of 100 µg/ml after incubation for 24 hours (Table 4.5). The ethanolic extract of *P. chaba* (100 µg/ml), *P. indica* (10 and 100 µg/ml) and *Z. officinale* (100 µg/ml) had cytotoxic effect on PBMCs at 72 hours after incubation as shown in Table 4.6. However, the water extracts of the five plants had no toxic effect on PBMCs at 24 and 72 hours (Table 4.7 and 4.8).

Table 4.5 The percentage of survival of PBMCs when incubated with the ethanolic extract of component plants in Benjakul preparation at 24 hours (n=6)

Concentration (µg/ml)	**% Survival (mean ± SEM)**				
	PCE	**PSE**	**PIE**	**PLE**	**ZOE**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	107.10 ± 4.75	92.36 ± 3.05	92.16 ± 2.29*	92.91 ± 2.62	108.60 ± 9.91
0.1	103.61 ± 5.25	95.10 ± 5.25	92.86 ± 1.51*	92.19 ± 2.78*	96.14 ± 3.74
1	103.04 ± 3.70	90.95 ± 3.56*	94.26 ± 2.18*	94.15 ± 2.37*	97.65 ± 3.88
10	107.55 ± 4.71	88.33 ± 4.48	98.21 ± 1.63	86.54 ± 5.76	101.60 ± 2.77
100	70.81 ± 5.69*	91.15 ± 5.55	131.16 ± 5.73*	20.20 ± 1.92*	107.24 ± 8.92
IC_{50} (µg/ml)	>100	>100	>100	84.15 ± 1.79	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.6 The percentage of survival of PBMCs when incubated with the ethanolic extract of component plants in Benjakul preparation at 72 hours (n=6)

Concentration (µg/ml)	**Survival (mean ± SEM)**				
	PCE	**PSE**	**PIE**	**PLE**	**ZOE**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	89.93 ± 6.16	87.03 ± 6.52	91.24 ± 3.91	92.91 ± 4.96	92.50 ± 3.52
0.1	90.61 ± 4.19	84.99 ± 5.95	89.78 ± 3.44*	90.87 ± 4.07	87.70 ± 3.96*
1	88.30 ± 5.17	84.69 ± 7.81	88.20 ± 2.74*	90.68 ± 3.63	88.89 ± 3.87*
10	96.03 ± 7.06	87.50 ± 6.75	90.70 ± 1.26*	69.22 ± 12.78	97.34 ± 4.05
100	23.36 ± 2.29*	86.54 ± 3.83*	84.87 ± 12.63	19.93 ± 0.92*	66.45 ± 11.46*
IC_{50} (µg/ml)	94.21 ± 2.14	>100	>100	72.41 ± 12.35	96.16 ± 2.43

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.7 The percentage of survival of PBMCs when incubated with the water extracts of component plants in Benjakul preparation at 24 hours (n=6)

Concentration (µg/ml)	**% Survival (mean ± SEM)**				
	PCW	**PSW**	**PIW**	**PLW**	**ZOW**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	96.62 ± 4.22	93.65 ± 2.47	90.88 ± 2.32*	95.69 ± 2.87	92.06 ± 2.95*
0.1	103.95 ± 7.56	93.31 ± 2.17*	92.05 ± 1.24*	96.21 ± 2.57	94.46 ± 2.61
1	95.37 ± 1.66*	95.95 ± 2.41	93.25 ± 1.56*	96.82 ± 2.32	93.44 ± 2.06*
10	99.46 ± 4.98	100.56 ± 3.39	96.28 ± 1.42*	100.57 ± 2.05	93.84 ± 2.71
100	102.01 ± 3.21	111.04 ± 5.56	104.52 ± 3.59	113.13 ± 3.47*	98.77 ± 3.65
IC_{50} (µg/ml)	>100	>100	>100	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.8 The percentage of survival of PBMCs when incubated with the water extracts of component plants in Benjakul preparation at 72 hours (n=6)

Concentration (µg/ml)	**% Survival (mean ± SEM)**				
	PCW	**PSW**	**PIW**	**PLW**	**ZOW**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	85.37 ± 4.91*	91.58 ± 3.44	92.56 ± 3.74	94.23 ± 6.10	86.90 ± 4.13*
0.1	83.22 ± 5.93*	88.91 ± 1.79*	91.52 ± 3.74	96.77 ± 6.67	88.18 ± 4.57*
1	86.01 ± 6.86	92.01 ± 2.75*	94.20 ± 5.41	97.03 ± 6.32	87.04 ± 2.67*
10	87.97 ± 6.75	94.46 ± 1.81*	96.49 ± 5.03	107.18 ± 6.81	89.47 ± 2.59*
100	100.80 ± 6.50	108.02 ± 2.52*	103.13 ± 5.44	122.26 ± 6.34*	97.11 ± 2.07
IC_{50} (µg/ml)	>100	>100	>100	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

4.2.2 Effect of Hua-Khao-Yen and Benjakul extracts on NK cells activity

NK cell activity was determined by chromium release assay using PBMCs as effector cells and K562 as target cells. PBMCs was incubated with the ethanolic and water extract of Hua-Khao-Yen and Benjakul at concentration of 0.01, 0.1, 1, 10 and 100 μg/ml for 24 hours.

From Table 4.9, the ethanolic extract of *S. corbularia* showed significantly increased NK cell activity at concentration of 0.01 μg/ml. The ethanolic extract stimulated NK cell activity about 1.2 times when compared with negative control (Figure 4.1). However, NK cell activity was decreased by the ethanolic extract of *S. corbularia* at high concentration (100 μg/ml) as shown in Table 4.9 and Figure 4.1. The water extract of *S. corbularia* showed no significant effect on NK cell activity. But, it tends to increased NK cells activity at high concentration (Table 4.9 and Figure 4.1).

Table 4.9 The lytic units/10^7 PBMCs of *S. corbularia* extracts (n=12)

Concentration (μg/ml)	**Lytic unit/10^7 PBMCs (mean ± SEM)**	
	SCE	**SCW**
Negative control	122.09 ± 26.15	158.86 ± 37.88
0.01	147.78 ± 28.34*	160.96 ± 38.51
0.1	130.19 ± 37.52	170.96 ± 37.10
1	142.13 ± 39.31	153.93 ± 41.81
10	171.30 ± 52.62	187.46 ± 53.72
100	50.00 ± 0.00*	183.23 ± 52.31
Positive control (PHA 15 μg/ml)	736.80 ± 26.15*	640.37 ± 95.09*

*Indicating a significant effect on NK cells activity with p-value < 0.05 when compared with negative control

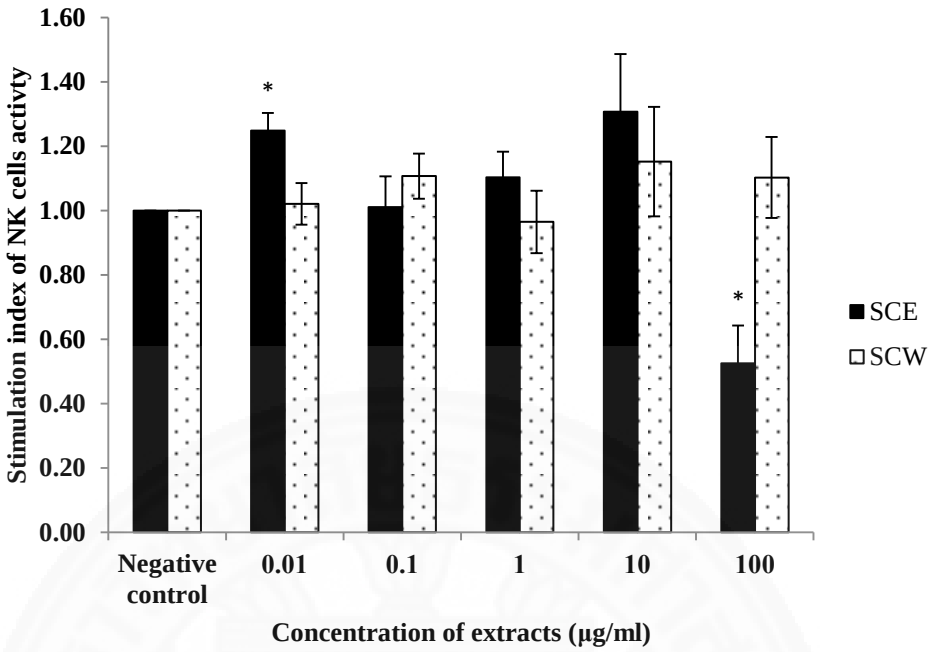

Figure 4.1 NK cell activities of the ethanolic and water extracts of *S. corbularia* were determined by chromium release assay. These results are shown as stimulation index of NK cell activity (mean ± SEM). *Indicating a significant effect of NK cells activity with p-value < 0.05 when compared with negative control.

For the extracts of Hua-Khao-Yen-Tai or *D. membranacea*, the ethanolic extract at concentration of 0.1 µg/ml and the water extract at all concentrations showed significantly increased NK cells activity. On the other hand, the ethanolic extract at concentration of 100 µg/ml showed significantly decreased NK cell activity as shown in Table 4.10 and Figure 4.2.

For Benjakul extracts, the ethanolic extract had no significant effect on NK cell activity but it tended to increase NK cell activity at concentration of 1 µg/ml and reduced NK cell activity at high concentration (100 µg/ml) as shown in Table 4.11 and Figure 4.3. However, the water extract of Benjakul showed significantly increased NK cell activity at concentration of 100 µg/ml (Table 4.12 and Figure 4.4). For plant component extracts, the ethanolic extract of *P. interruptum* (0.1 µg/ml) and *Z. officinale* (0.1 and 1 µg/ml) had significantly stimulated NK cell activity. However, all of the ethanolic extracts of plant components tended to decrease NK cell activity at concentration of 100 µg/ml (Table 4.11 and Figure 4.3). The water extracts of *P.*

sarmentosum, *P. indica* and *Z. officinale* showed a significant effect on NK cell activity as shown in Table 4.12 and Figure 4.4.

Table 4.10 The lytic unit/10^7 PBMCs of *D. membranacea* extracts (n=12)

Concentration	**Lytic unit/10^7 PBMCs (mean ± SEM)**	
(µg/ml)	**DME**	**DMW**
Negative control	283.75 ± 43.98	244.67 ± 65.81
0.01	386.39 ± 56.93	317.99 ± 77.36*
0.1	400.69 ± 60.19*	300.21 ± 74.96*
1	409.58 ± 74.03	373.20 ± 90.93*
10	383.92 ± 72.30	361.35 ± 89.60*
100	46.60 ± 3.40*	284.40 ± 73.61*
Positive control (PHA 15 µg/ml)	1159.34 ± 144.10*	1085.03 ± 194.60*

*Indicating a significant effect of NK cell activity with p-value < 0.05 when compared with negative control

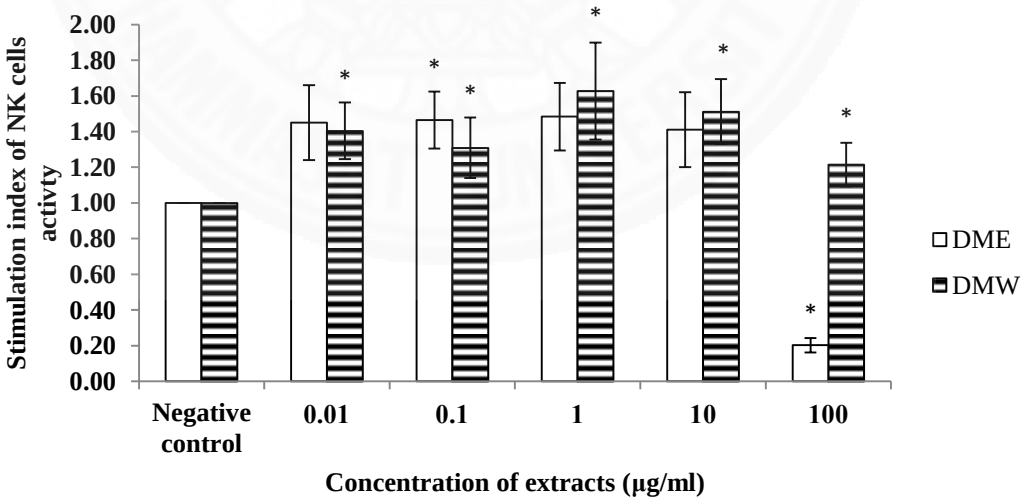

Figure 4.2 NK cells activity of the ethanolic and water extracts of *D. membrabacea* were determined by chromium release assay. These results are shown as stimulation index of NK cell activity (mean ± SEM). *Indicating a significant effect of NK cell activity with p-value < 0.05 when compared with negative control.

Table 4.11 The lytic units/10^7 PBMCs of the ethanolic extracts of Benjakul and component plants in Benjakul preparation (n=12)

Concentration (µg/ml)	Lytic units/10^7 PBMCs (mean ± SEM)					
	BJE	**PCE**	**PSE**	**PIE**	**PLE**	**ZOE**
Negative control	114.67 ± 10.19	171.86 ± 47.73	149.18 ± 34.98	343.03 ± 82.14	310.85 ± 119.30	260.95 ± 65.84
0.01	128.35 ± 16.60	148.17 ± 37.71	168.83 ± 41.84	355.84 ± 77.83	294.35 ± 85.28	247.43 ± 47.57
0.1	119.70 ± 13.30	173.86 ± 48.24	148.03 ± 33.12	416.34 ± 108.42*	439.83 ± 211.26	327.87 ± 65.33*
1	134.23 ± 20.38	178.37 ± 51.01	173.47 ± 40.74	414.93 ± 113.00	350.85 ± 134.91	357.07 ± 69.46*
10	94.24 ± 13.94	147.55 ± 39.77	94.44 ± 29.96	269.11 ± 80.94	56.48 ± 5.45	250.43 ± 48.34
100	50.00 ± 0.00	50.00 ± 0.00*	50.00 ± 0.00	77.90 ± 27.90*	50.00 ± 0.00*	50.00 ± 0.00*
Positive control	738.90 ± 74.17*	543.88 ± 79.48*	733.34 ± 120.28*	803.36 ± 176.22*	647.07 ± 148.43*	815.41 ± 143.98*

*Indicating a significant effect of NK cell activity with p-value < 0.05 when compared with negative control

Table 4.12 The lytic units/10^7 PBMCs of the water extracts of Benjakul and component plants in Benjakul preparation (n=12)

Concentration (µg/ml)	Lytic units/10^7 PBMCs (mean ± SEM)					
	BJW	**PCW**	**PSW**	**PIW**	**PLW**	**ZOW**
Negative control	134.19 ± 26.24	183.50 ± 41.26	173.07 ± 47.00	198.56 ± 61.29	234.12 ± 50.98	311.94 ± 73.12
0.01	137.89 ± 25.38	142.71 ± 27.86	235.51 ± 71.74	215.60 ± 70.31	277.15 ± 63.84	359.12 ± 97.50
0.1	130.70 ± 26.07	166.15 ± 33.34	183.03 ± 64.34	203.91 ± 60.12	253.80 ± 54.55	358.41 ± 93.04
1	147.18 ± 30.26	169.51 ± 45.73	230.41 ± 79.63	210.95 ± 72.49	321.21 ± 76.19*	433.69 ± 140.63
10	182.72 ± 49.02	200.88 ± 53.80	279.28 ± 130.93	236.27 ± 87.45	295.76 ± 74.19	458.90 ± 124.49*
100	215.00 ± 52.53*	196.34 ± 37.73	230.93 ± 70.25*	231.15 ± 70.78	376.73 ± 82.51*	684.15 ± 228.06*
Positive control	619.41 ± 78.29*	650.32 ± 67.70*	697.17 ± 99.52*	643.29 ± 78.43*	820.42 ± 111.89*	925.34 ± 205.52*

*Indicating a significant effect of NK cells activity with p-value < 0.05 when compared with negative control

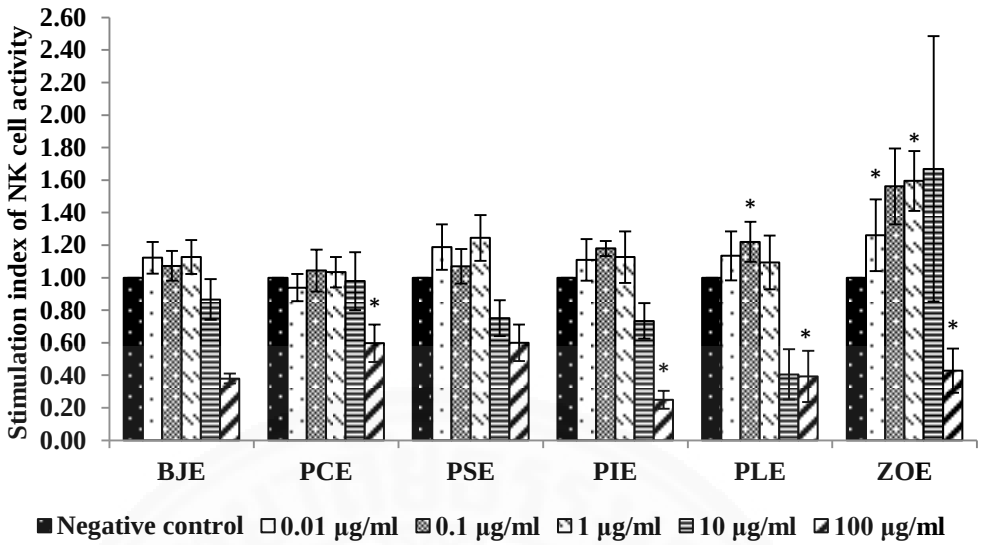

Figure 4.3 NK cell activity of the ethanolic extracts of Benjakul and component plants in Benjakul preparation was determined by chromium release assay. These results are shown as stimulation index of NK cell activity. *Indicating a significant effect of NK cell activity with p-value < 0.05 when compared with negative control.

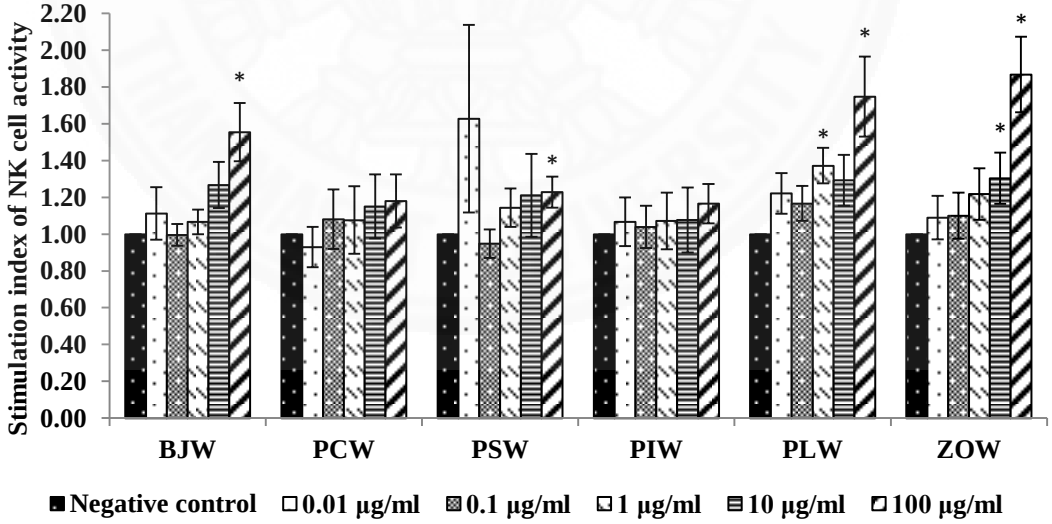

Figure 4.4 NK cell activity of the water extracts of Benjakul and component plants in Benjakul preparation was determined by chromium release assay. These results are shown as stimulation index of NK cell activity. * Indicating a significant effect of NK cell activity with p-value < 0.05 when compared with negative control.

4.2.3 Effect of Hua-Khao-Yen and Benjakul extracts on lymphocyte proliferation

The ethanolic and water extracts of Hua-Khao-Yen and Benjakul were investigated for lymphocyte proliferation by ^{3}H-thymidine uptake assay. PBMCs were incubated with the ethanolic and water extracts for 72 hours.

The ethanolic extract of *S. corbularia* showed significantly increased lymphocyte proliferation at concentration of 0.1 μg/ml (Table 4.13 and Figure 4.5). On the other hand, the water extract of *S. corbularia* showed no observable effect on lymphocyte proliferation but it tends to stimulate lymphocyte proliferation at high concentration (10-100 μg/ml).

From Table 4.14 and Figure 4.6, the ethanolic extract of *D. membranacea* significantly decreased lymphocyte proliferation at concentration of 10 and 100 μg/ml. The water extract showed significantly increased lymphocyte proliferation at concentration 0.01-10 μg/ml.

The ethanolic extract of Benjakul and component plants had significantly decreased lymphocyte proliferation at high concentration (10-100 μg/ml) as shown in Table 4.15 and Figure 4.7. The water extracts of Benjakul, *P. chaba*, *P. sarmentosum*, *P. interruptum* and *Z. officinale* showed significantly stimulated lymphocyte proliferation in a dose-dependent manner. On the other hand, the water extract of *P. indica* had no significant effect on lymphocyte proliferation (Table 4.16 and Figure 4.8).

Table 4.13 The stimulation index of lymphocyte proliferation of *S. corbularia* extracts (n=12)

Concentration (µg/ml)	Stimulation index of lymphocyte proliferation (mean ± SEM)	
	SCE	SCW
Negative control	1.00 ± 0.00	1.00 ± 0.00
0.01	1.16 ± 0.16	1.09 ± 0.09
0.1	1.40 ± 0.17*	0.95 ± 0.18
1	1.15 ± 0.11	0.99 ± 0.07
10	1.01 ± 0.17	1.13 ± 0.12
100	0.94 ± 0.13	1.19 ± 0.18
Positive control (PHA 15 µg/ml)	90.90 ± 20.44*	77.08 ± 11.82*

*Indicating significant lymphocyte proliferation activity with p-value < 0.05 when compared with negative control

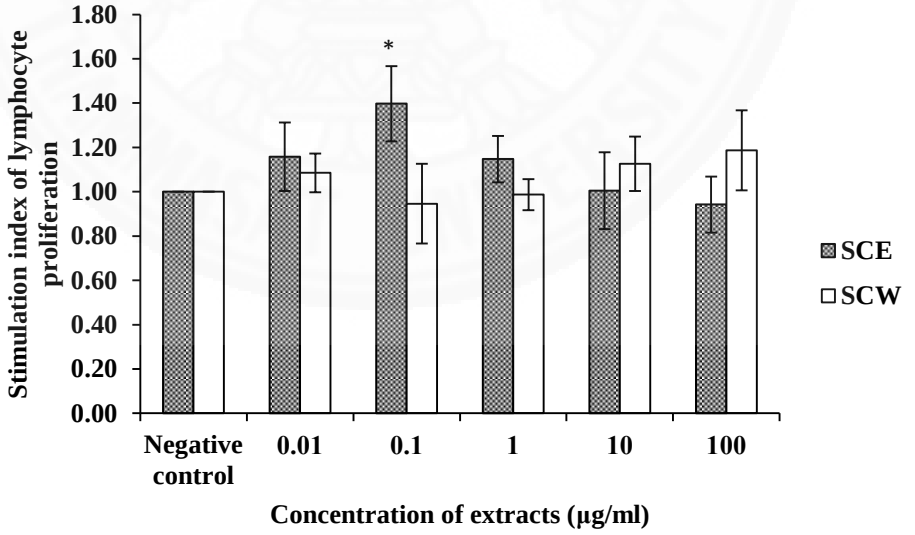

Figure 4.5 Stimulation index of lymphocyte proliferation of *S. corbularia* extracts were determined by ^{3}H-thymidine uptake assay. These results are shown as mean ± SEM. *Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control.

Table 4.14 The stimulation index of lymphocyte proliferation of *D. membranacea* extracts (n=12)

Concentration (µg/ml)	Stimulation index of lymphocyte proliferation (mean ± SEM)	
	DME	**DMW**
Negative control	1.00 ± 0.00	1.00 ± 0.00
0.01	0.90 ± 0.11	1.30 ± 0.11*
0.1	0.86 ± 0.08	1.38 ± 0.09*
1	0.75 ± 0.10	1.57 ± 0.25*
10	0.34 ± 0.05*	1.53 ± 0.10*
100	0.13 ± 0.02*	1.34 ± 0.14
Positive control (PHA 15 µg/ml)	85.94 ± 15.43*	189.76 ± 62.17*

*Indicating significant lymphocyte proliferation activity with p-value < 0.05 when compared with negative control

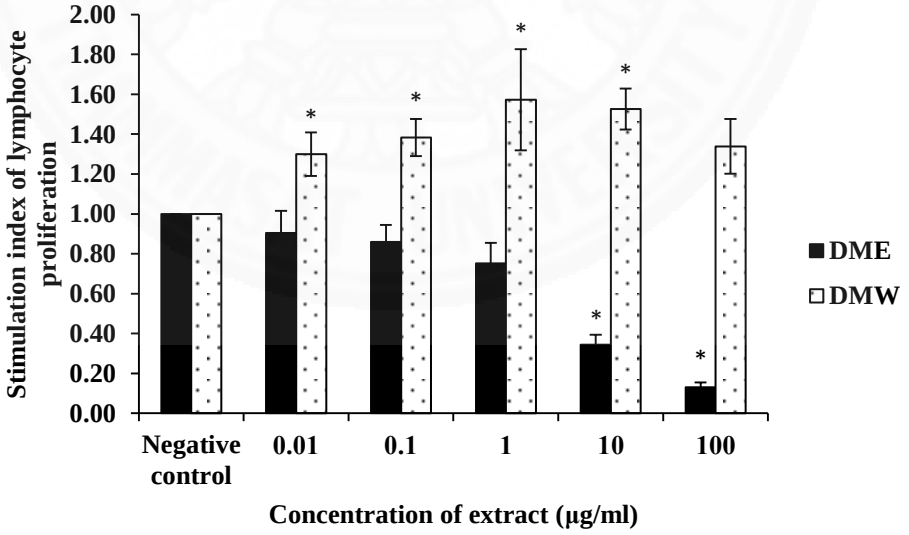

Figure 4.6 Stimulation index of lymphocyte proliferation of *D. membranacea* extracts were determined by ^{3}H-thymidine uptake assay. These results are shown as mean ± SEM. *Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control.

Table 4.15 The stimulating index of lymphocyte proliferation of the ethanolic extracts of Benjakul and component plants in Benjakul preparation (n=12)

Concentration	**Stimulation index of lymphocyte proliferation (mean ± SEM)**					
(µg/ml)	**BJE**	**PCE**	**PSE**	**PIE**	**PLE**	**ZOE**
Negative control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.01	1.29 ± 0.12	1.04 ± 0.08	1.03 ± 0.17	1.06 ± 0.08	1.14 ± 0.27	1.05 ± 0.17
0.1	0.98 ± 0.11	1.01 ± 0.10	0.89 ± 0.05	0.83 ± 0.12	0.80 ± 0.22	1.28 ± 0.12
1	1.10 ± 0.09	1.07 ± 0.10	1.02 ± 0.14	0.77 ± 0.09*	0.97 ± 0.17	1.34 ± 0.16
10	0.69 ± 0.11*	0.82 ± 0.16	0.54 ± 0.05*	0.34 ± 0.10*	0.23 ± 0.16*	0.62 ± 0.14
100	0.22 ± 0.06*	0.11 ± 0.04*	0.08 ± 0.03*	0.09 ± 0.03*	0.13 ± 0.08*	0.19 ± 0.12*
Positive control	120.41 ± 15.84*	100.09 ± 40.13*	95.33 ± 29.50*	90.37 ± 24.85*	88.38 ± 35.04*	109.70 ± 28.40*

*Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control

Table 4.16 The stimulating index of lymphocyte proliferation of the water extracts of Benjakul and plants component in Benjakul preparation (n=12)

Concentration	**Stimulation index of lymphocyte proliferation (mean ± SEM)**					
(µg/ml)	**BJW**	**PCW**	**PSW**	**PIW**	**PLW**	**ZOW**
Negative control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.01	1.68 ± 0.09*	1.27 ± 0.11*	1.28 ± 0.12*	1.28 ± 0.13*	0.90 ± 0.10	1.34 ± 0.22*
0.1	1.47 ± 0.09*	1.21 ± 0.10	1.08 ± 0.08*	1.28 ± 0.09*	1.05 ± 0.15	1.50 ± 0.10*
1	2.07 ± 0.27*	1.34 ± 0.21*	1.36 ± 0.21*	1.43 ± 0.20*	0.89 ± 0.06	2.22 ± 0.38*
10	1.78 ± 0.40*	1.56 ± 0.10*	1.61 ± 0.13*	1.60 ± 0.14*	0.86 ± 0.10	1.89 ± 0.44*
100	2.72 ± 0.30*	1.48 ± 0.12*	1.71 ± 0.30*	1.57 ± 0.22*	1.00 ± 0.12	1.94 ± 0.28*
Positive control	327.37 ± 49.56*	174.19 ± 65.74*	193.47 ± 59.58*	189.80 ± 59.63*	89.40 ± 23.89*	233.88 ± 86.87*

*Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control

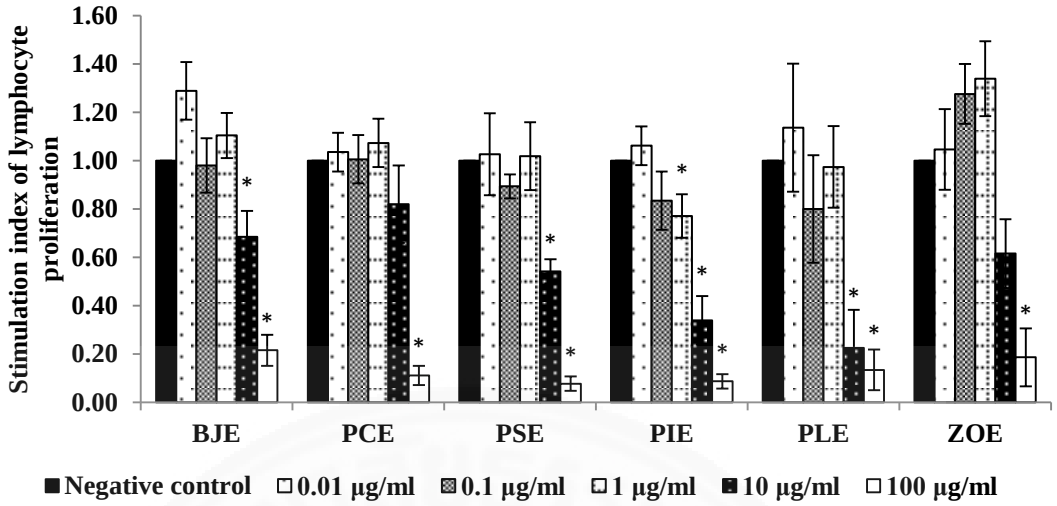

Figure 4.7 Stimulation index of lymphocyte proliferation of the ethanolic extracts of Benjakul and component plants were determined by ^{3}H-thymidine uptake assay. These results are shown as mean ± SEM. *Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control.

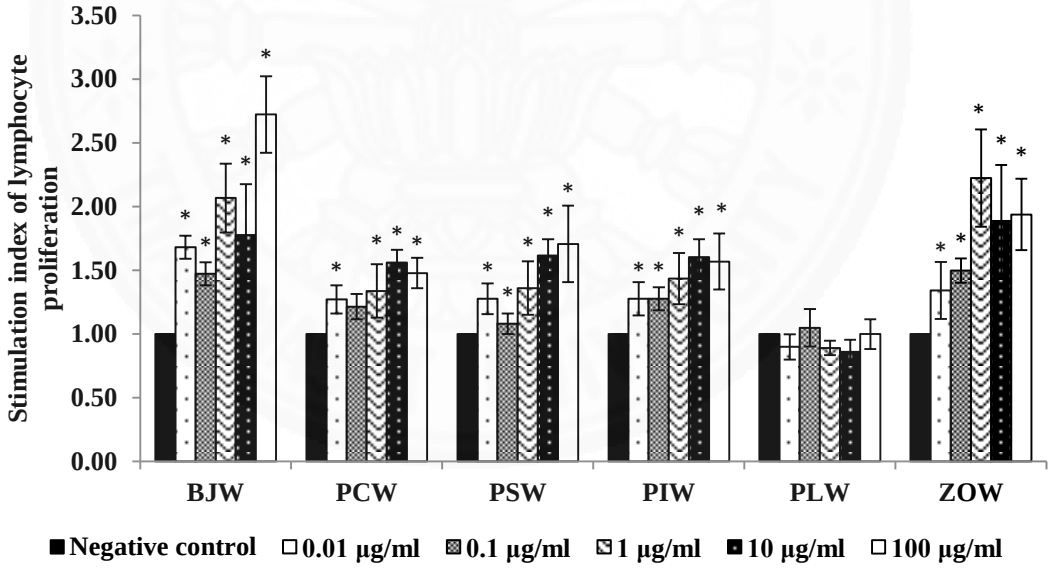

Figure 4.8 Stimulation index of lymphocyte proliferation of the water extract of Benjakul and component plants were determined by ^{3}H-thymidine uptake assay. These results are shown as mean ± SEM. *Indicating a significant effect of lymphocyte proliferation with p-value < 0.05 when compared with negative control.

4.2.4 Effect of Hua-Khao-Yen and Benjakul on IL-2 and IFN-γ production from PHA-stimulated PBMCs

PBMCs were treated with various concentrations of Hua-Khao-Yen and Benjakul extracts with or without PHA 5 μg/ml for 24 hours. After incubation, IL-2 production was detected by ELISA technique.

From Table 4.17 and Figure 4.9, the ethanolic extract and water extract of *S. corbularia* showed no effect on activation of IL-2 production in the absence of PHA condition. However, IL-2 production was significantly reduced in a dose-dependent manner with the ethanolic extract of *S. corbularia* in the presence of PHA condition. The water extract showed significant effect on IL-2 production in the presence of PHA that decreased IL-2 secretion at high concentration.

Table 4.17 Effect of the ethanolic and water extracts of *S. corbularia* on IL-2 production and the percentage of IL-2 production from PHA-stimulating PBMCs (n=4)

Concentration (µg/ml)	Ethanolic extract				Water extract			
	Without PHA 5 µg/ml		With PHA 5 µg/ml		Without PHA 5 µg/ml		With PHA 5 µg/ml	
	IL-2 production (pg/ml)	% IL-2 activation	IL-2 production (pg/ml)	% IL-2 activation	IL-2 production (pg/ml)	% IL-2 activation	IL-2 production (pg/ml)	% IL-2 activation
Negative control	61.72 ± 13.18	0.00 ± 0.00	1085.47 ± 281.61	100.00 ± 0.00	75.80 ± 39.09	0.00 ± 0.00	955.00 ± 302.75	100.00 ± 0.00
0.1	72.75 ± 3.97	0.91 ± 1.97	900.23 ± 187.25	84.08 ± 11.46	68.49 ± 12.13	1.79 ± 0.74	977.29 ± 353.71	96.78 ± 7.96
1	69.40 ± 4.78	0.64 ± 1.79	862.17 ± 215.61	77.82 ± 8.37	58.25 ± 9.17	1.37 ± 0.46	911.35 ± 370.47	85.72 ± 9.09
10	64.44 ± 2.66	-0.52 ± 2.29	815.33 ± 197.76	73.54 ± 6.61	66.95 ± 12.39	1.48 ± 0.51	799.27 ± 272.14	78.28 ± 5.15
100	62.45 ± 4.58	-0.37 ± 1.65	86.27 ± 9.74	1.98 ± 2.11	54.88 ± 11.62	1.09 ± 0.56	456.11 ± 167.63	34.22 ± 9.91

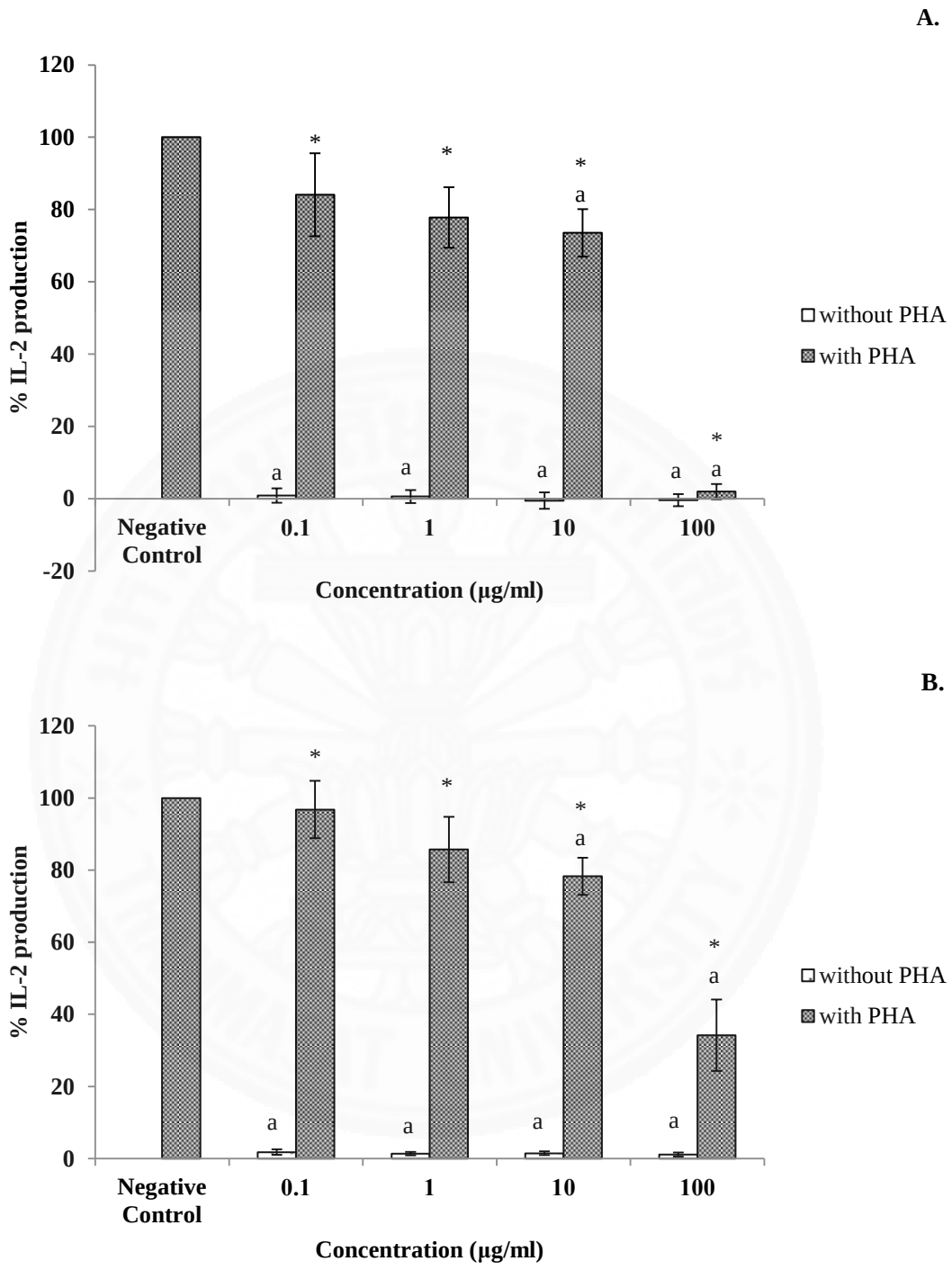

Figure 4.9 Effect of the ethanolic extract (A.) and the water extract (B.) of *S. corbularia* on IL-2 production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA); [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

The water extracts of *D. membranacea* had a significant effect on IL-2 production that induced IL-2 production at low concentration (0.1-1 μg/ml). However, the ethanolic and water extracts showed inhibitory effect on IL-2 production from PHA-activated PBMCs at high concentration (10-100 μg/ml), as shown in Table 4.18 and Figure 4.10.

Table 4.18 Effect of the ethanolic and water extracts of *D. membranacea* on IL-2 production and the percentage of IL-2 production from PHA-stimulating PBMCs (n=4)

Concentration (μg/ml)	Ethanolic extract				Water extract			
	Without PHA 5 μg/ml		With PHA 5 μg/ml		Without PHA 5 μg/ml		With PHA 5 μg/ml	
	IL-2 production (pg/ml)	% IL-2 production	IL-2 production (pg/ml)	% IL-2 production	IL-2 production (pg/ml)	% IL-2 production	IL-2 production (pg/ml)	% IL-2 production
Negative control	101.30 ± 32.86	0.00 ± 0.00	747.26 ± 163.58	100.00 ± 0.00	80.80 ± 37.88	0.00 ± 0.00	1069.91 ± 419.97	100.00 ± 0.00
0.1	74.28 ± 3.12	4.17 ± 1.53	728.09 ± 196.71	90.72 ± 9.94	60.71 ± 2.47	1.52 ± 0.79	1036.52 ± 380.75	107.11 ± 10.27
1	76.10 ± 8.72	3.91 ± 0.71	654.19 ± 190.17	77.70 ± 12.45	102.14 ± 47.90	32.89 ± 32.04	1032.30 ± 387.26	104.46 ± 11.96
10	115.49 ± 49.89	13.11 ± 10.46	491.90 ± 112.25	57.24 ± 6.04	62.14 ± 4.36	2.79 ± 2.03	611.66 ± 197.44	65.18 ± 13.54
100	73.58 ± 2.97	3.62 ± 0.97	81.69 ± 6.52	2.66 ± 1.83	80.71 ± 20.02	14.67 ± 13.82	106.64 ± 22.85	17.11 ± 14.51

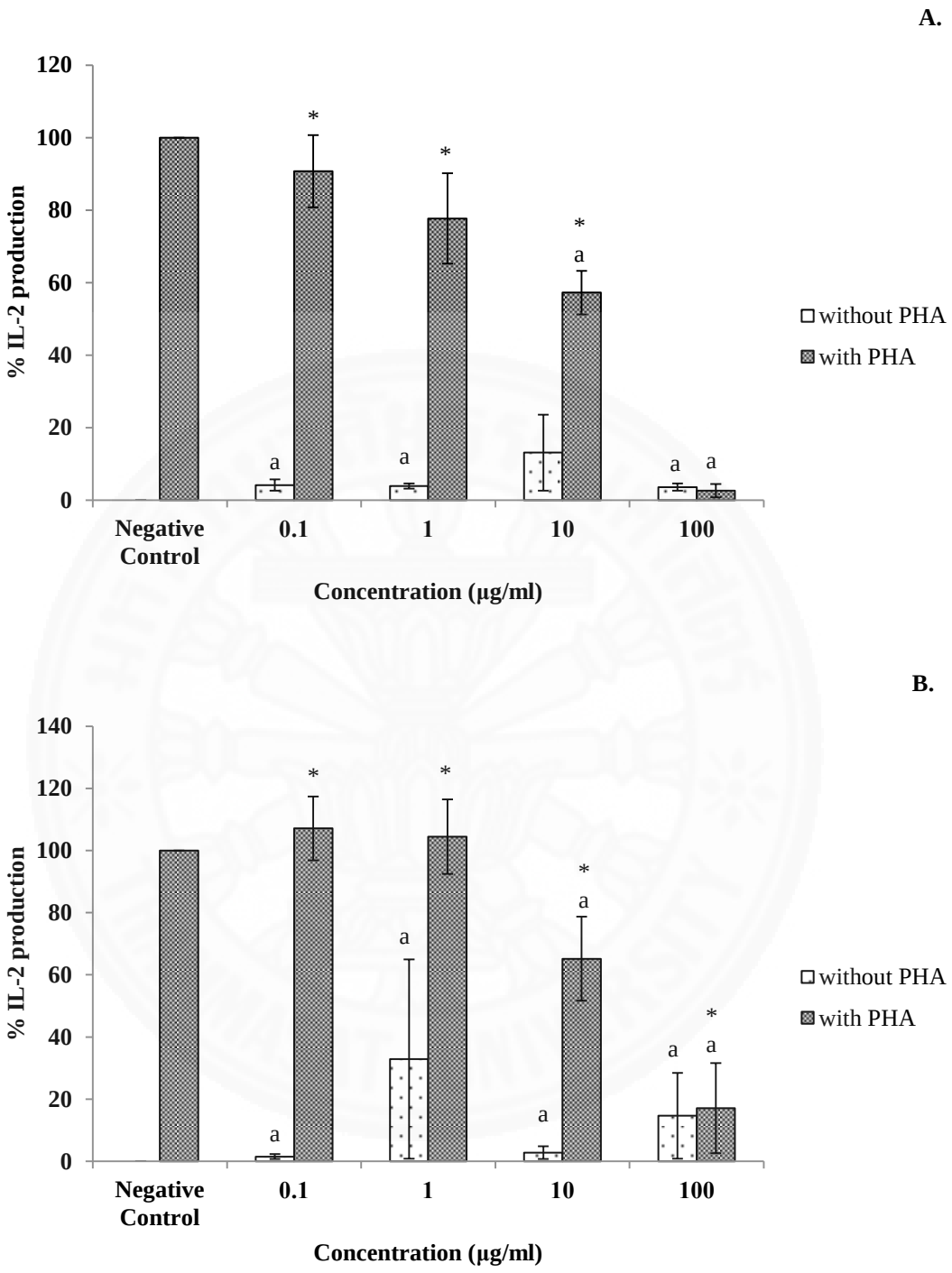

Figure 4.10 Effect of the ethanolic extract (A.) and the water extract (B.) of *D. membranacea* on IL-2 production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA); [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

The ethanolic extract of Benjakul up-regulated IL-2 at low concentration (0.1-1 μg/ml) but it reduced IL-2 production at high concentration (100 μg/ml). The water extract of Benjakul showed significantly increased IL-2 production at high concentration and slightly decreased IL-2 production at concentration 100 μg/ml, as shown in Table 4.19 and Figure 4.11.

Table 4.19 Effect of the ethanolic and water extracts of Benjakul on IL-2 production and the percentage of IL-2 production from PHA-stimulating PBMCs (n=4)

Concentration (μg/ml)	**Ethanolic extract**				**Water extract**			
	Without PHA 5 μg/ml		**With PHA 5 μg/ml**		**Without PHA 5 μg/ml**		**With PHA 5 μg/ml**	
	IL-2 production (pg/ml)	**% IL-2 production**	**IL-2 production (pg/ml)**	**% IL-2 production**	**IL-2 production (pg/ml)**	**% IL-2 production**	**IL-2 production (pg/ml)**	**% IL-2 production**
Negative control	41.44 ± 11.83	0.00 ± 0.00	836.82 ± 253.19	100.00 ± 0.00	31.78 ± 4.50	0.00 ± 0.00	786.16 ± 276.92	100.00 ± 0.00
0.1	51.48 ± 12.86	3.87 ± 2.14	1075.88 ± 321.80	125.29 ± 10.97	53.19 ± 10.67	4.25 ± 3.01	907.41 ± 320.12	115.19 ± 6.44
1	42.73 ± 17.40	4.82 ± 4.53	1014.48 ± 328.38	116.44 ± 7.20	51.24 ± 12.96	4.26 ± 3.34	918.03 ± 302.62	122.47 ± 5.39
10	41.00 ± 17.10	4.51 ± 4.27	761.55 ± 256.14	87.90 ± 3.56	52.59 ± 13.15	3.35 ± 1.80	908.97 ± 340.24	114.81 ± 4.35
100	56.60 ± 19.81	8.02 ± 6.67	94.08 ± 25.39	5.64 ± 1.27	49.03 ± 11.70	3.15 ± 2.07	783.34 ± 257.73	102.01 ± 2.97

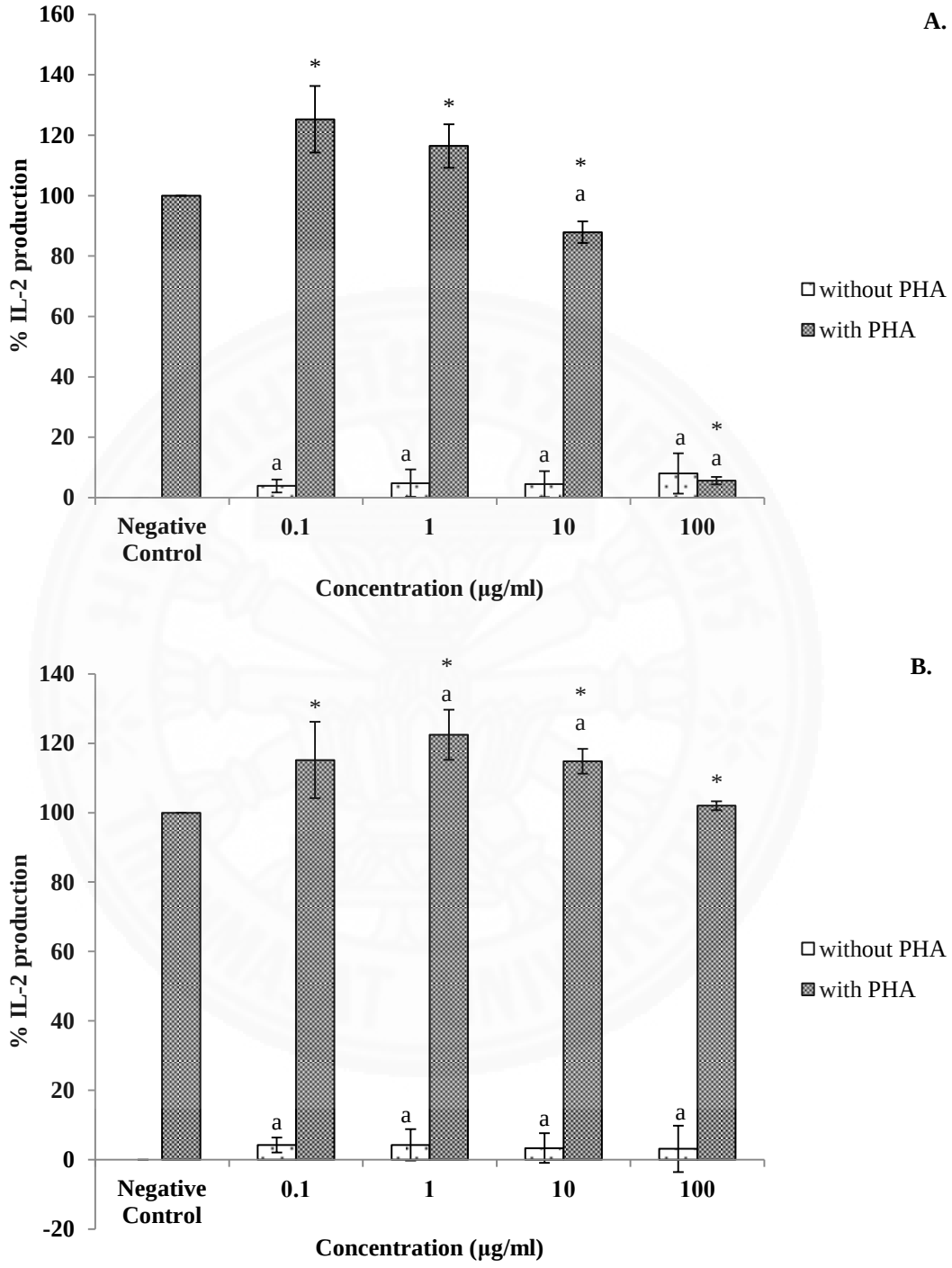

Figure 4.11 Effect of the ethanolic extract (A.) and the water extract (B.) of Benjakul on IL-2 production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA) ; [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

The ethanolic extract of *S. corbularia* significantly increased IFN-γ production at concentration of 1 and 10 μg/ml in the presence of PHA condition. In the absence of PHA, the ethanolic and water extracts showed no significant effect on IFN-γ production but they tended to increase IFN-γ production at concentration of 100 μg/ml. The water extract leaded to stimulate IFN-γ production but not significantly (Table 4.20 and Figure 4.12).

Table 4.20 Effect of the ethanolic and water extracts of *S. corbularia* on IFN-γ production and the percentage of IFN-γ production from PHA-stimulating PBMCs (n=4)

Concentration (μg/ml)	**Ethanolic extract**				**Water extract**			
	Without PHA 5 μg/ml		**With PHA 5 μg/ml**		**Without PHA 5 μg/ml**		**With PHA 5 μg/ml**	
	IFN-γ production (pg/ml)	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**
Negative control	< 0.99	0.00 ± 0.00	174.80 ± 65.24	100.00 ± 0.00	< 0.99	0.00 ± 0.00	115.18 ± 54.05	100.00 ± 0.00
0.1	< 0.99	0.00 ± 0.00	194.82 ± 61.20	123.84 ± 12.20	< 0.99	0.00 ± 0.00	301.89 ± 132.87	345.67 ± 164.38
1	< 0.99	0.00 ± 0.00	194.32 ± 56.37	127.87 ± 22.35	< 0.99	0.00 ± 0.00	287.06 ± 134.80	325.27 ± 166.54
10	< 0.99	0.00 ± 0.00	196.77 ± 54.06	132.63 ± 27.71	< 0.99	0.00 ± 0.00	423.37 ± 218.47	461.16 ± 269.02
100	21.61 ± 10.01	11.59 ± 7.50	171.45 ± 82.40	132.08 ± 84.58	22.24 ± 14.99	20.22 ± 14.81	391.67 ± 191.28	462.06 ± 236.92

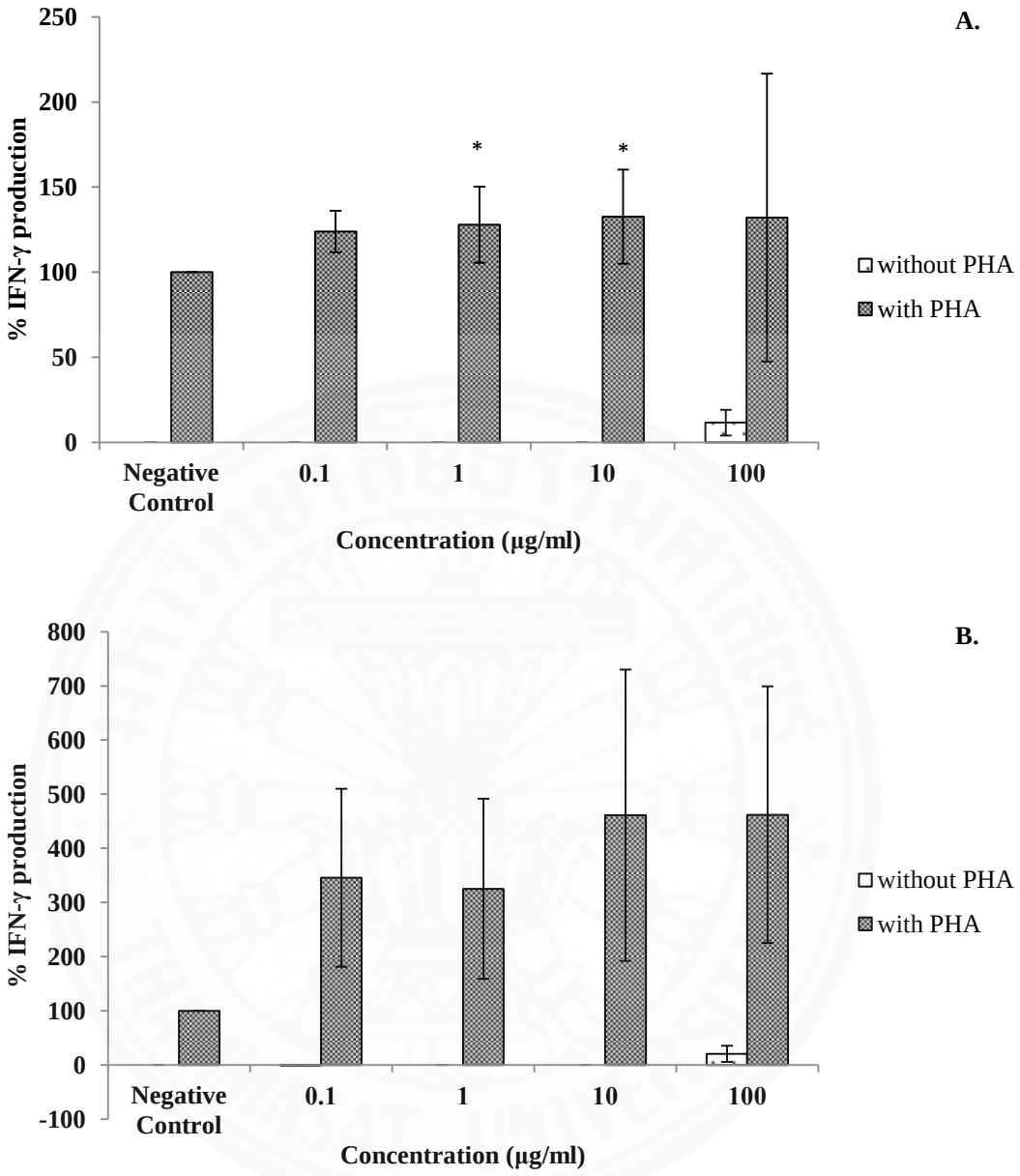

Figure 4.12 Effect of the ethanolic extract (A.) and the water extract (B.) of *S. corbularia* on IFN-γ production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA); [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

The ethanolic extract of *D. membranacea* showed significant effect on IFN-γ production from PHA-stimulated PBMCs. However, the ethanolic extract of *D. membranacea* at low concentration (0.1-10 μg/ml) increased IFN-γ production but it decreased IFN-γ production at concentration of 100 μg/ml (Table 4.21 and Figure 4.13). The water extract of *D. membranacea* significantly increased IFN-γ production at concentration of 0.1 μg/ml and significantly decreased IFN-γ production at concentration of 100 μg/ml, as shown in Table 4.21 and Figure 4.13.

Table 4.21 Effect of the ethanolic and water extracts of *D. membranacea* on IFN-γ production and the percentage of IFN-γ production from PHA-stimulating PBMCs (n=4)

Concentration (μg/ml)	**Ethanolic extract**				**Water extract**			
	Without PHA 5 μg/ml		**With PHA 5 μg/ml**		**Without PHA 5 μg/ml**		**With PHA 5 μg/ml**	
	IFN-γ production (pg/ml)	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**
Negative control	< 0.99	0.00 ± 0.00	169.83 ± 95.03	100 ± 0.00	< 0.99	0.00 ± 0.00	480.26 ± 183.36	100.00 ± 0.00
0.1	< 0.99	0.00 ± 0.00	169.32 ± 80.04	117.96 ± 33.10	0.12 ± 0.75	0.00 ± 0.00	559.98 ± 180.01	135.57 ± 18.24
1	0.43 ± 0.84	0.54 + 0.51	184.12 ± 89.32	116.11 ± 23.69	5.44 ± 1.29	0.74 ± 0.34	519.26 ± 137.49	168.54 ± 66.48
10	< 0.99	0.00 ± 0.00	181.17 ± 101.10	106.25 ± 17.64	1.44 ± 1.55	0.21 ± 0.14	81.95 ± 21.45	25.56 ± 9.40
100	0.64 ± 1.16	0.46 + 0.25	< 0.99	0.00 ± 0.00	0.70 ± 1.06	0.08 ± 0.03	4.34 ± 1.78	1.33 ± 0.85

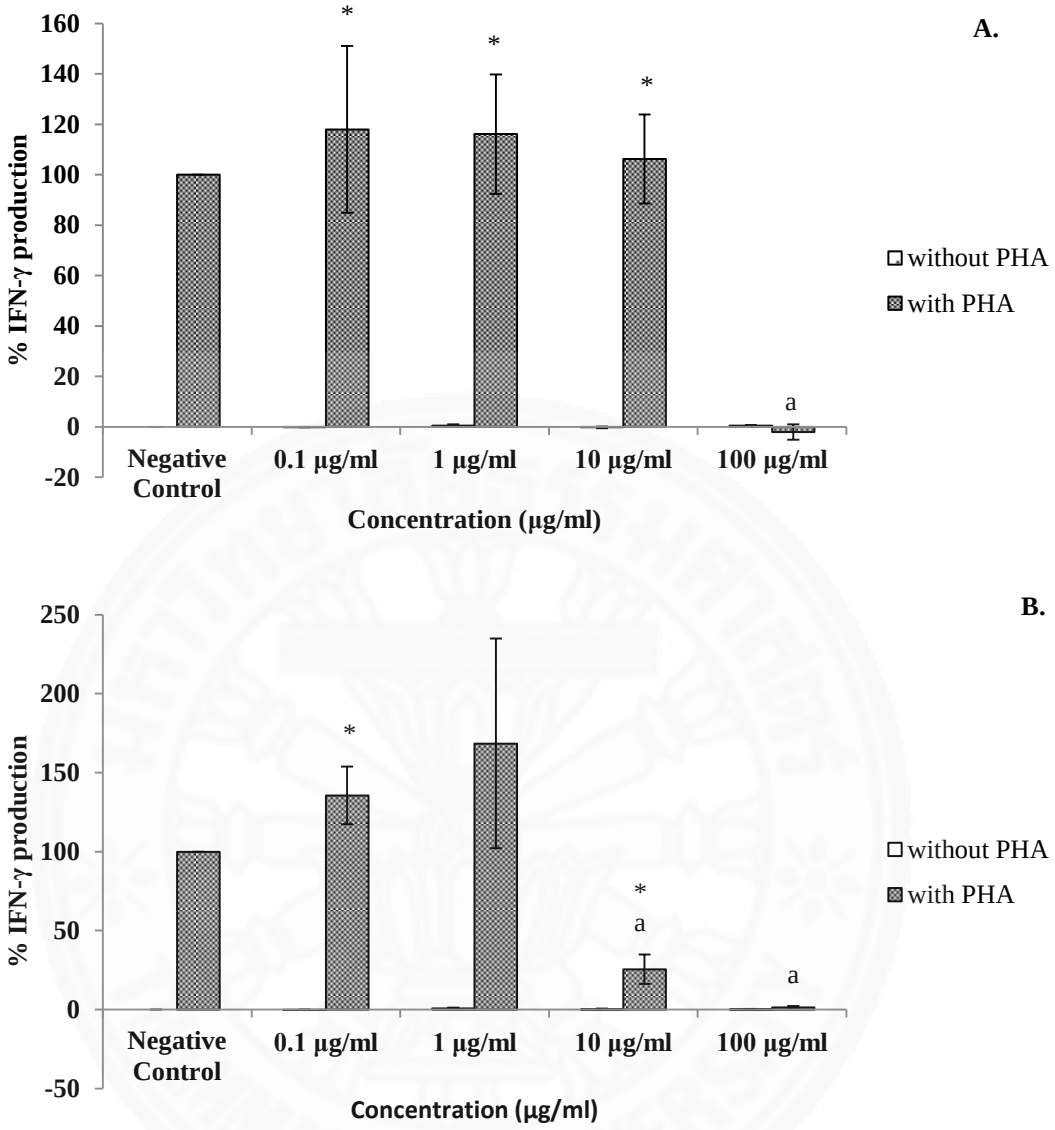

Figure 4.13 Effect of the ethanolic extract (A.) and the water extract (B.) of *D. membranacea* on IFN-γ production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA); [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

The water extract of Benjakul significantly stimulated IFN-γ production at concentration of 0.1-100 μg/ml. The ethanolic extract increased IFN-γ production at low concentration (0.1-10 μg/ml) and decreased IFN-γ production at high concentration (100 μg/ml) (Table 4.22 and Figure 4.14).

Table 4.22 Effect of the ethanolic and water extracts of Benjakul on IFN-γ production and the percentage of IFN-γ production from PHA-stimulated PBMCs (n=4)

Concentration (μg/ml)	**Ethanolic extract**				**Water extract**			
	Without PHA 5 μg/ml		**With PHA 5 μg/ml**		**Without PHA 5 μg/ml**		**With PHA 5 μg/ml**	
	IFN-γ production (pg/ml)	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**	**IFN-γ production (pg/ml)**	**% IFN-γ production**
Negative control	< 0.99	0.00 ± 0.00	259.05 ± 87.55	100.00 ± 0.00	< 0.99	0.00 ± 0.00	169.67 ± 75.97	100.00 ± 0.00
0.1	< 0.99	0.00 ± 0.00	282.72 ± 90.32	110.37 ± 10.98	< 0.99	0.00 ± 0.00	206.48 ± 82.00	125.56 ± 13.44
1	< 0.99	0.00 ± 0.00	284.58 ± 91.37	113.71 ± 5.94	9.28 ± 4.76	5.19 ± 2.28	260.01 ± 80.06	188.42 ± 41.67
10	< 0.99	0.00 ± 0.00	289.69 ± 97.27	109.53 ± 6.35	16.52 ± 11.09	8.01 ± 2.50	253.29 ± 84.07	174.48 ± 30.60
100	< 0.99	0.00 ± 0.00	62.74 ± 33.17	18.63 ± 7.17	19.88 ± 13.54	9.00 ± 2.58	242.93 ± 81.11	167.64 ± 24.62

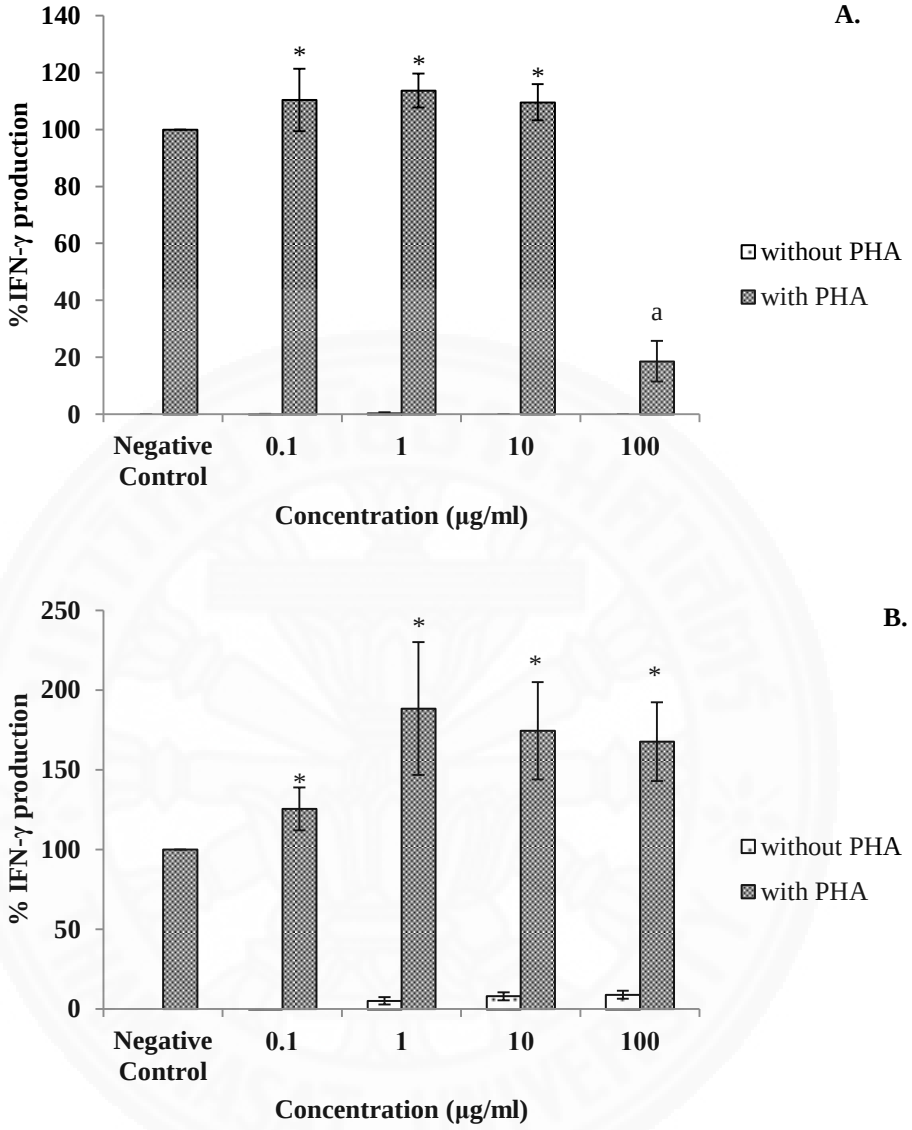

Figure 4.14 Effect of the ethanolic extract (A.) and the water extract (B.) of Benjakul preparation on IFN-γ production from PHA-stimulated PBMCs. Data are expressed as mean ± SEM, *p-value < 0.05 when compared with negative control (without PHA); [a] p-value < 0.05 when compared with negative control (with PHA 5 µg/ml).

4.3 Effect of Hua-Khao-Yen and Benjakul on cytokines production from LPS-stimulated RAW264.7 cell lines

The ethanolic and water extracts of Hua-Khao-Yen and Benjakul were investigated for cytotoxicity on RAW264.7 cell lines by MTT assay. RAW264.7 cell lines were incubated with various concentrations of extracts for 24 and 48 hours to observe cytotoxic effect of crude extracts, as shown in Table 4.23 to 4.25.

After that, RAW264.7 cells were incubated with these extracts at 24 hours to detect the effect of extracts on IL-1β and IL-6 production and 48 hours to detect the effect of extracts on IL-10 production from RAW264.7 cells. The IL-1β, IL-6 and IL-10 production were detected by ELISA technique. From Table 4.26, the ethanolic extracts of *D. membranacea* and Benjakul preparation showed less inhibitory effect on IL-6 production from RAW264.7 cells than prednisolone with IC_{50} value of 46.82, 51.83 and 0.1 μg/ml, respectively. Moreover, the ethanolic extract of *D. membranacea* and Benjakul also showed inhibitory effect on IL-1β production but their values were less than prednisolone with IC_{50} value of 0.90, 62.93 and 0.19 μg/ml, respectively (Table 4.27). All water extracts and the ethanolic extract of *S. corbularia* had no effect on IL-1β and IL-6 inhibition.

From Figure 4.15, the ethanolic extracts of *D. membranacea* up-regulated IL-10 production from RAW264.7 cells. The ethanolic extract showed the highest potency on IL-10 production at concentration of 30 μg/ml. Futhermore, the ethanolic extract of *D. membranacea* showed significant stimulation of IL-10 production at concentration of 10 μg/ml. The other samples had no stimulatory effect on IL-10 production. This extract also show higher up-regulated IL-10 production than prednisolone at the same concentration (10 μg/ml).

Table 4.23 The percentage of survival of RAW264.7 cells when incubated with *S. corbularia* extracts at 24 and 48 hours (n=3)

Concentration (µg/ml)	**% Survival (mean ± SEM)**			
	Ethanolic extract		**Water extract**	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
100	117.59 ± 4.84	99.33 ± 1.64	83.63 ± 5.50	99.23 ± 16.99
IC_{50} (µg/ml)	>100	>100	>100	>100

Table 4.24 The percentage of survival of RAW264.7 cells when incubated with *D. membranacea* extracts at 24 and 48 hours (n=3)

Concentration (µg/ml)	**% Survival (mean ± SEM)**			
	Ethanolic extract		**Water extract**	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 0.00	100.00 ± 0.00
1	95.73 ± 7.27	112.25 ± 9.23	85.97 ± 7.79	104.93 ± 12.80
10	71.61 ± 10.58	96.07 ± 23.70	72.25 ± 8.35	74.72 ± 13.81
30	75.73 ± 1.50	126.71 ± 16.98	61.68 ± 5.73	Not tested
50	86.10 ± 6.09	136.60 ± 12.17	61.87 ± 6.50	Not tested
100	48.73 ± 3.48	Not tested	57.00 ± 3.64	Not tested
IC_{50} (µg/ml)	68.42 ± 21.21	>50	>30	>30

Table 4.25 The percentage of survival of RAW264.7 cells when incubated with Benjakul extracts at 24 and 48 hours (n=3)

Concentration (µg/ml)	**% Survival (mean ± SEM)**			
	Ethanolic extract		**Water extract**	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1	100.59 ± 6.13	75.31 ± 10.83	85.54 ± 2.70	108.65 ± 2.00
10	88.17 ± 12.09	80.65 ± 15.77	77.84 ± 10.42	76.14 ± 4.92
30	78.96 ± 9.42	83.06 ± 15.09	47.42 ± 11.35	Not tested
50	81.21 ± 13.65	59.66 ± 6.67	51.58 ± 7.97	Not tested
100	79.23 ± 5.38	47.27 ± 5.20	52.48 ± 8.58	Not tested
IC_{50} (µg/ml)	>100	51.67 ± 26.49	>100	>10

Table 4.26 The percentage of inhibition and IC_{50} of Hua-Khao-Yen and Benjakul extracts on IL-6 production from LPS-stimulated RAW264.7 cell lines (n=2)

Extracts	%Inhibition of various concentrations of extracts							IC_{50} (µg/ml)
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
SCE	NT	NT	NT	NT	NT	NT	-64.10 ± 13.12	>100
SCW	NT	NT	NT	NT	NT	NT	-20.11 ± 0.34	>100
DME	NT	NT	-18.10 ± 3.62	16.73 ± 0.23	26.86 ± 1.20	56.47 ± 1.47	NT	46.82 ± 0.80
DMW	NT	NT	NT	-9.09 ± 4.45	NT	NT	NT	>10
BJE	NT	NT	13.02 ± 5.27	7.10 ± 3.24	NT	48.46 ± 4.37	67.35 ± 2.02	51.83 ± 3.98
BJW	NT	NT	NT	4.87 ± 7.92	NT	NT	NT	>10
Prednisolone	19.07 ± 1.35	51.48 ± 3.09	58.47 ± 1.04	84.00 ± 1.29	NT	NT	NT	0.10 ± 0.01

These results are expressed as mean ± SEM, NT means not tested

Table 4.27 The percentage of inhibition and IC_{50} of Hua-Khao-Yen and Benjakul extracts on IL-1β production from LPS-stimulated RAW264.7 cell lines (n=2)

Extracts	%Inhibition of various concentrations of extracts							IC_{50} (µg/ml)
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
SCE	NT	NT	NT	NT	NT	NT	27.36 ± 4.64	>100
SCW	NT	NT	NT	NT	NT	NT	42.32 ± 3.68	>100
DME	NT	-2.55± 6.55	55.14± 2.86	48.73± 3.27	NT	61.55 ± 2.45	NT	0.90± 0.05
DMW	NT	NT	NT	37.04± 7.41	NT	NT	NT	>10
BJE	NT	NT	-15.36 ±7.36	6.00± 6.00	44.45± 3.54	NT	65.82± 2.18	62.93± 7.66
BJW	NT	NT	NT	8.13 ± 5.86	NT	NT	NT	>10
Prednisolone	45.76± 4.25	82.91± 1.09	82.91± 1.09	106.41± 0.41	NT	NT	NT	0.19± 0.09

These results are expressed as mean ± SEM, NT means not tested

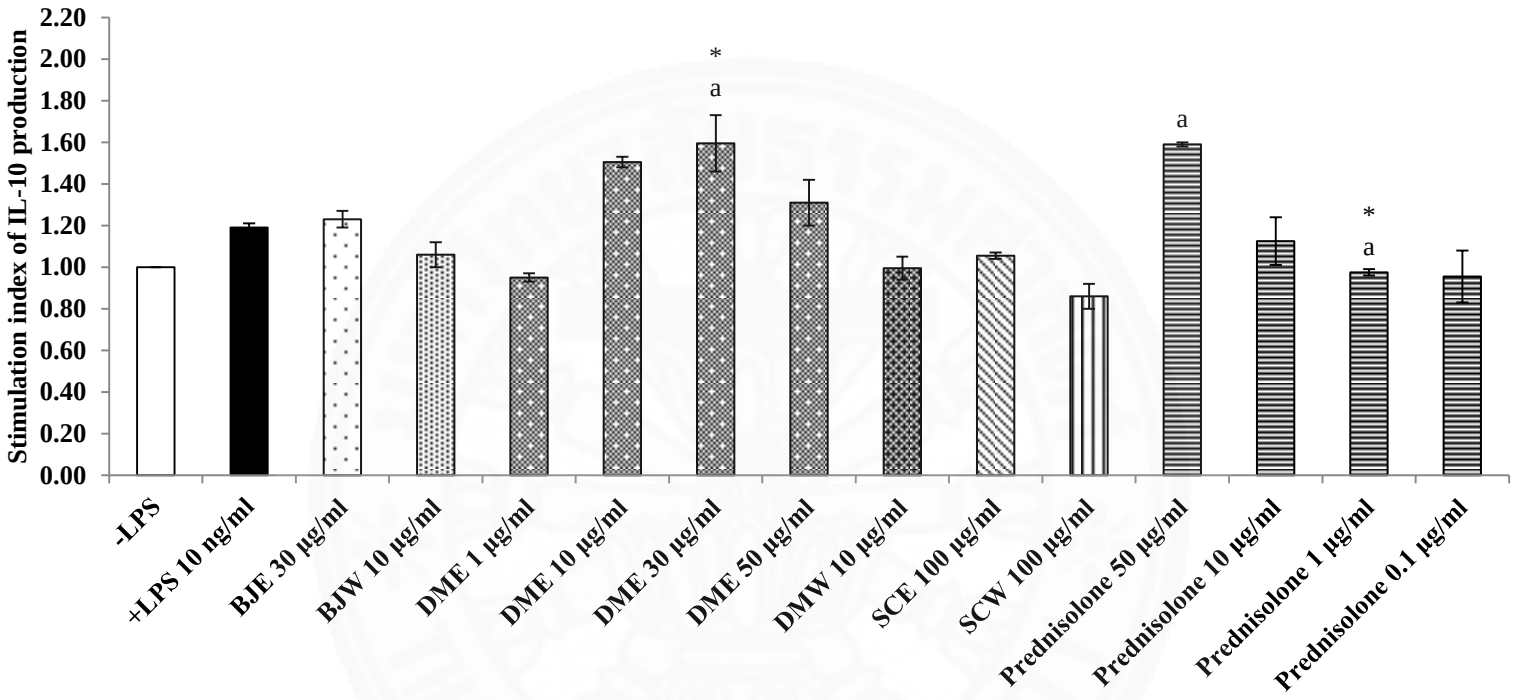

Figure 4.15 Effect of Hua-Khao-Yen and Benjakul extracts on IL-10 production from LPS-stimulated RAW264.7 cell lines (n=2). *Indicating a significant effect on IL-10 production with p-value < 0.05 when compared with the absence of LPS; a Indicating a significant effect on IL-10 production with p-value < 0.05 when compared with the presence of LPS.

4.4 Bioasaay-guided fractionation of the ethanolic extract of Hua-Khao-Yen and Benjakul preparation

The ethanolic extract of Hua-Khao-Yen and Benjakul were eluted by five solvent system such as hexane, hexane:chloroform, chloroform, chloroform:methanol and methanol, we got five fractions. The percentages of yield of fraction were showed in Table 4.28.

However, the hexane and hexane:chloroform fractions from Hua-Khao-Yen showed low yield. From previous reports, these two fractions have no effect on biological activity (Ruangnoo, 2012). Thus, the present study focused on chloroform, chloroform:methanol and methanol fraction of Hua-Khao-Yen. For Benjakul extract, all fractions have been reported for cytotoxic activity (Rattarom, 2013). All fractions were investigated for NK cell activity and then the active fraction was selected isolation of pure compounds.

Table 4.28 The percentage of yield and codes of five fractions from the ethanolic extract of Hua-Khao-Yen and Benjakul

Solvent	*S. corbularia*		*D. membranacea*		Benjakul	
	Code	% Yield	Code	% Yield	Code	% Yield
Hexane	F.SCE1	0.02%	F.DME1	0.23%	F.BJE1	0.55%
Hexane : Chloroform (1:1)	F.SCE2	0.01%	F.DME2	0.56%	F. BJE2	0.92%
Chloroform	F.SCE3	0.16%	F.DME3	14.21%	F. BJE3	25.99%
Chloroform : Methanol (1:1)	F.SCE4	45.69%	F.DME4	8.29%	F. BJE4	34.15%
Methanol	F.SCE5	30.13%	F.DME5	46.92%	F. BJE5	28.35%

From Table 4.29 to Table 4.31, the results show that F.DME4 and F.BJE3 had toxic effect on PBMCs at concentration of 100 μg/ml. However, other fractions showed no toxic effect on PBMCs.

Table 4.29 The percentage of survival of PBMCs when incubated with three fractions from the ethanolic extract of *S. corbularia* at exposure time as 24 hours (n=6)

Concentration of fractions (µg/ml)	**% Survival (mean ± SEM)**		
	F.SCE3	**F.SCE4**	**F.SCE5**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	84.33 ± 3.63*	89.45 ± 3.12*	100.15 ± 20.56
1	92.61 ± 2.51*	95.47 ± 5.47	98.20 ± 20.50
10	89.96 ± 4.33	94.50 ± 6.62	89.04 ± 6.71
100	92.94 ± 4.18	130.42 ± 8.42*	115.40 ± 3.38*
IC_{50} (µg/ml)	>100	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.30 The percentage of survival of PBMCs when incubated with three fractions from the ethanolic extract of *D. membranacea* at exposure time as 24 hours (n=6)

Concentration of fractions (µg/ml)	**% Survival (mean ± SEM)**		
	F.DME3	**F.DME4**	**F.DME5**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	87.53 ± 3.05*	83.49 ± 3.47*	81.86 ± 4.13*
1	88.22 ± 2.62*	84.83 ± 3.85*	83.02 ± 4.53*
10	94.52 ± 2.72	88.98 ± 3.07*	88.58 ± 5.35
100	55.99 ± 2.03*	125.66 ± 9.33*	108.97 ± 6.62
IC_{50} (µg/ml)	83.98 ± 16.02	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.31 The percentage of survival of PBMCs when incubated with all fractions from the ethanolic extract of Benjakul at exposure time as 24 hours (n=6)

Concentration of fractions (µg/ml)	**% Survival (mean ± SEM)**				
	F.BJE1	**F.BJE2**	**F.BJE3**	**F.BJE4**	**F.BJE5**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	88.95 ± 9.19	82.73 ± 2.60*	88.24 ± 13.64	87.57 ± 3.64*	82.69 ± 8.44*
1	92.04 ± 12.89	83.48 ± 4.90*	91.17 ± 8.90	84.59 ± 3.37*	80.28 ± 9.08*
10	92.85 ± 11.46	89.61 ± 5.81	87.53 ± 12.65	86.94 ± 3.73*	79.52 ± 10.49
100	102.90 ± 18.95	85.41 ± 3.12*	19.08 ± 5.39*	93.06 ± 5.76	83.67 ± 11.17
IC_{50} (µg/ml)	>100	>100	78.76 ± 12.92	>100	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

The fractions were investigated using NK cells activity by chromium release assay. From Table 4.32 and Figure 4.16, the F.SCE4 fraction from the ethanolic extract of *S. corbularia* showed a significant slightly decrease on NK cell activity at concentration of 1 µg/ml. Moreover, the F.SCE5 fraction significantly decreased NK cell activity at high concentration (100 µg/ml). However, previous reports showed that the chloroform:MeOH fraction had high potentcy to inhibit cancer cell lines. Thus, the chloroform:MeOH fraction F.SCE4 was chosen to isolate pure compounds.

Table 4.32 The lytic units/10^7 PBMCs of fractions from the ethanolic extract of *S. corbularia* (n=6)

Concentration (µg/ml)	Lytic units/10^7 PBMCs (mean ± SEM)		
	F.SCE3	F.SCE4	F.SCE5
Negative control	132.12 ± 37.68	142.15 ± 32.36	142.15 ± 32.36
0.1	85.83 ± 15.23	85.68 ± 32.94	98.01 ± 31.95
1	125.45 ± 4635	106.62 ± 26.85*	91.81 ± 26.68
10	86.57 ± 35.19	99.72 ± 35.52	81.80 ± 25.66
100	102.73 ± 38.83	98.36 ± 29.72	50.00 ± 0.00*
Positive control (PHA 15 µg/ml)	273.90 ± 106.10	283.81 ± 87.20	283.81 ± 87.20

*Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control

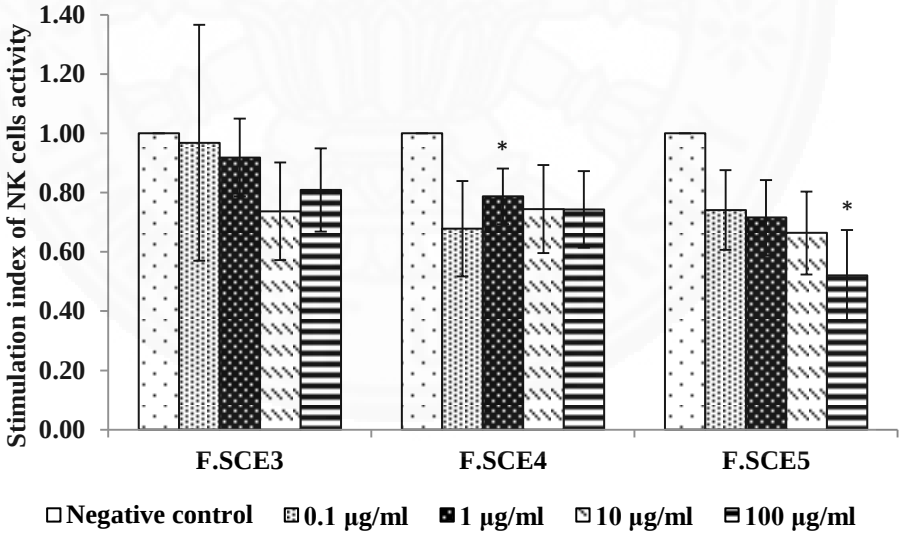

Figure 4.16 NK cell activity of three fractions from the ethanolic extract of *S. corbularia*. These fractions were subjected to chromium release assay. These results are shown as stimulation index of NK cell activity (mean ± SEM). * Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control.

From Table 4.33 and Figure 4.17, all of fractions tended to increase NK cell activity but they had no significant effect. However, FDME3 and FDME4 showed increasing NK activity. Thus, these fraction was isolated the active compound from *D. membranacea* and the method followed by the previous report (Ruangnoo, 2012).

Table 4.33 The lytic units/10^7 PBMCs of fractions from the ethanolic extract of *D. membranacea* (n=6)

Concentration	**Lytic units/10^7 PBMCs (mean ± SEM)**		
(µg/ml)	**F.DME3**	**F.DME4**	**F.DME5**
Negative control	146.70 ± 40.59	146.70 ± 40.59	146.70 ± 40.59
0.1	127.80 ± 34.91	173.35 ± 39.08	142.27 ± 25.42
1	137.06 ± 39.11	129.18 ± 39.84	115.30 ± 41.81
10	175.40 ± 64.87	104.35 ± 16.50	83.64 ± 22.04
100	50.00 ± 0.00	50.93 ± 0.93	58.33 ± 8.33
Positive control (PHA 15 µg/ml)	277.75 ± 71.41*	277.75 ± 71.41*	277.75 ± 71.41*

*Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control

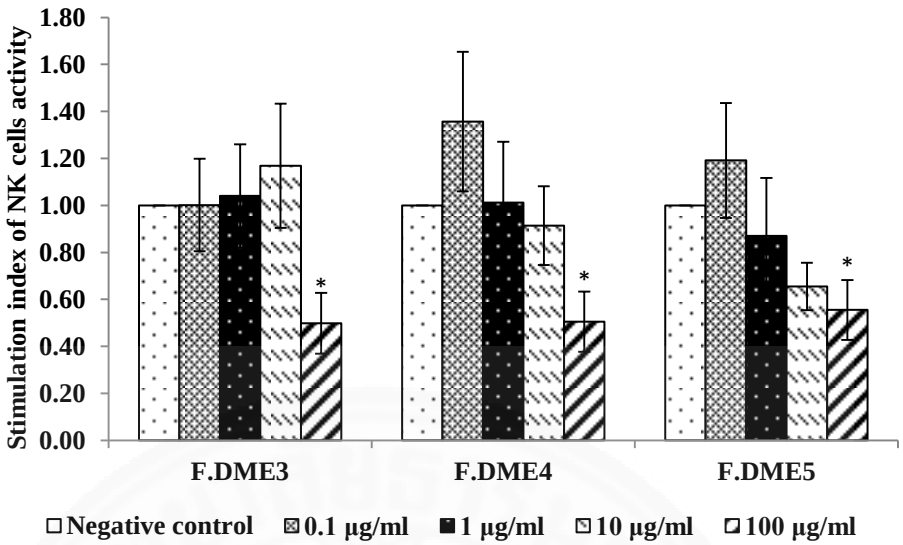

Figure 4.17 NK cell activity of three fractions from the ethanolic extract of *D. membranacea*. These fractions were subjected to chromium release assay. These results are shown as stimulation index of NK cell activity (mean ± SEM).
* Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control.

For Benjakul extract, the chloroform:methanol fraction (F.BJE4) showed strong effect on NK cell activity. Moreover, the F.BJE2 increased NK cell activity at 1 µg/ml. The F.BJE1, F.BJE2, F.BJE3 and BJE 4 reduced NK cell activity at high concentration (100 µg/ml).

Table 4.34 The lytic units/10^7 PBMCs of fractions from the ethanolic extract of Benjakul (n=6)

Concentration (µg/ml)	Lytic units/10^7 PBMCs (mean ± SEM)				
	F.BJE1	**F.BJE2**	**F.BJE3**	**F.BJE4**	**F.BJE5**
Negative control	202.81 ± 66.82	194.24 ± 57.12	220.09 ± 61.39	220.09 ± 61.39	220.09 ± 61.39
0.1	260.21 ± 91.58	145.10 ± 30.50	183.96 ± 40.72	372.47 ± 176.12	240.17 ± 118.83
1	172.83 ± 54.63	249.58 ± 104.10	185.13 ± 60.71	278.42 ± 140.03	235.45 ± 101.87
10	156.65 ± 60.23	189.63 ± 71.79	208.78 ± 73.69	248.41 ± 133.40	178.64 ± 71.52
100	75.00 ± 25.00*	50.00 ± 0.00*	50.00 ± 0.00*	54.69 ± 4.69*	208.82 ± 49.99
Positive control (PHA 15 µg/ml)	321.65 ± 92.71	274.29 ± 87.75	232.35 ± 62.44	232.35 ± 62.44	232.35 ± 62.44

*Indicating a significant effect on NK cells activity with p-value ≤ 0.05 when compared with negative control

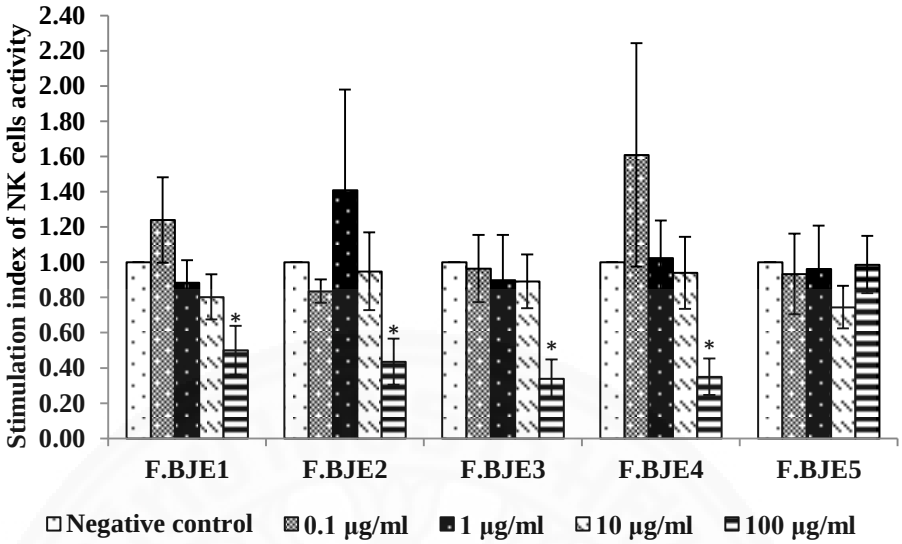

Figure 4.18 NK cell activities of five fractions from the ethanolic extract of Benjakul preparation. These fractions were subjected to chromium release assay. These results are shown as stimulation index of NK cell activity (mean ± SEM).
* Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control.

4.5 Isolation of chemical constituents from the ethanolic extract of Hua-Khao-Yen and Benjakul

The chemical constituents from the ethanolic extract of Hua-Khao-Yen and Benjakul were isolated by column chromatography using isocratic solvents. Pure compounds were isolated and detected by application of the general spraying reagent anisaldehyde in sulphuric acid. These compounds could be detected by UV at 254 nm wavelenght.

4.5.1 Isolated pure compounds from the ethanolic extract of *S. corbularia*

4.5.1.1 Astilbin

Astilbin is a white crystalline solid. The ^{1}H NMR 9(500 MHz in CDCl3) analysis and ^{13}C-NMR (125 MHz in CDCl3) of this compound was compared with authentic sample(Q. Du, Li, & Jerz, 2005)**.** The structure of this compound is shown in Figure 4.19.

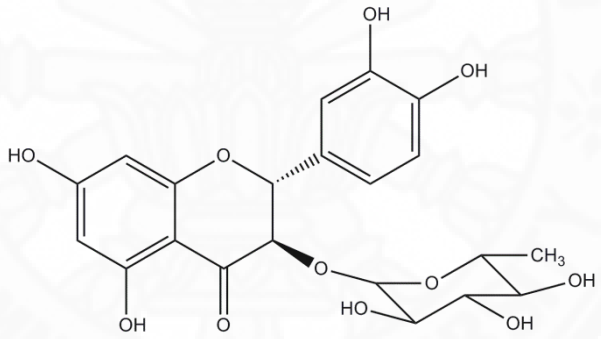

Figure 4.19 The chemical structure of astilbin

4.5.1.2 Quercetin

Quercetin is a yellow crystalline solid. This compound was compared with authentic sample of quercetin (Merck) by thin layer chromatography technique using 3 solvent systems and it gave identical behavior. The ^{1}H NMR spectrum was compared with previous report (Prieto, Braca, Morelli, Barker, & Schaffiner, 2004). The chemical structure of quercetin is shown in Figure 4.20.

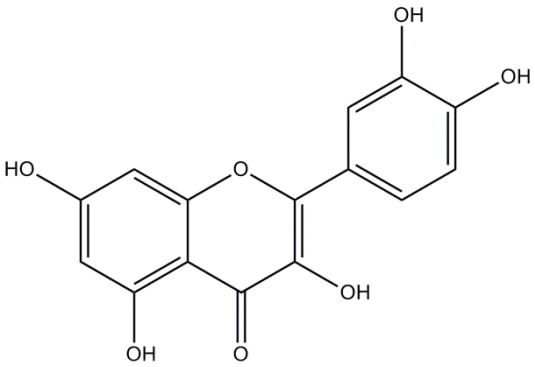

Figure 4.20 The chemical structure of quercetin

4.5.2 Isolated pure compounds from the ethanolic extract of *D. membranacea*

4.5.2.1 Dioscorealide B

This compound were identified by TLC technique using 3 solvent systems and compared with dioscorealide B standard. The ^{1}H NMR 9(500 MHz in CDCl3) analysis and ^{13}C-NMR (125 MHz in CDCl3) of this compound was compared with the previous 1H-NMR data of dioscorealide B (Itharat et al., 2003). The chemical structure of dioscorealide B is shown in Figure 4.21.

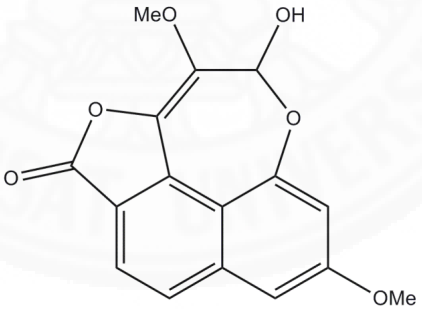

Figure 4.21 The chemical structure of dioscorealide B

4.5.2.2 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydro phenanthrene

This compound is a white solid. The ^{1}H-NMR and ^{13}C-NMR of this compound was compared with previous report (Rajaraman, Batta, & Rangaswami, 1975). The chemical structure of this compound is shown in Figure 4.22.

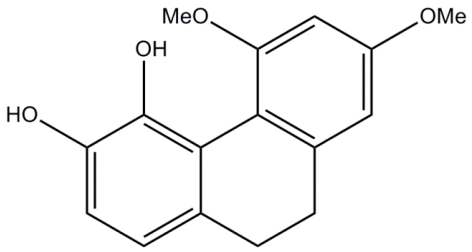

Figure 4.22 The chemical structure of 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene

4.5.3 Isolated pure compounds from the ethanolic extract of Benjakul preparation

Piperine was isolated from the ethanolic extract of Benjakul preparation. It is a yellowish clear crystal. This compound was compared with authentic sample of piperine (Merck) by thin layer chromatography technique using 3 solvent systems and it gave identical behavior. The chemical structure of piperine is shown in Figure 4.23.

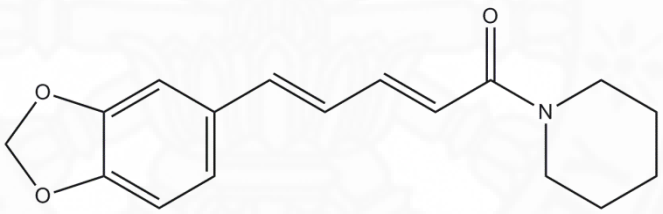

Figure 4.23 The chemical structure of piperine

4.5.4 Effect of chemical constituents from the ethanolic extract of Hua-Khao-Yen and Benjakul on immunomodulatory activity

4.5.4.1 Effect of pure compounds on NK cells activity

All pure compounds were investigated for their cytotoxic effect on PBMCs by MTT assay. The results showed that these compounds have no toxic effect on PBMCs at all concentrations as shown in Table 4.35. After that, the pure compounds were examined for NK cell activity. From Table 4.36 and Figure 4.24, quercetin and astilbin, isolated compounds from the ethanolic extract of *S. corbularia*, tend to increase NK cells activity at concentration of 1 μg/ml and 100 μg/ml, respectively. Dioscorelide B which was isolated from the ethanolic extract of *D. membranacea* stimulated NK cell activity at low concentration (0.01-0.1 μg/ml) but it had no significant effect. Another compound from the ethanolic extract of *D. membranacea*, 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene, slightly increased NK cell activity at concentration of 0.01 μg/ml. However, dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene decreased NK cells activity at high concentration (10-100 μg/ml). The isolated compound from Benjakul extract, piperine slightly increased NK cell activity at concentration of 1 μg/ml but it decreased NK cells activity at concentration of 100 μg/ml (Table 4.36 and Figure 4.24).

Table 4.35 The percentage of survival of PBMCs when incubated with pure compounds from the ethanolic extract of Hua-Khao-Yen and Benjakul extracts at 24 hours (n=6)

Concentration of pure compounds (µg/ml)	**% Survival (mean ± SEM)**				
	Quercetin	**Astilbin**	**Dioscorealide B**	**2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene**	**Piperine**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	92.64 ± 1.58	96.29 ± 1.81	93.95 ± 2.48	92.96 ± 1.78*	94.27 ± 2.89
0.1	98.31 ± 6.40*	93.65 ± 2.31*	90.69 ± 2.21*	93.18 ± 2.14*	93.90 ± 3.11
1	94.65 ± 3.67	93.51 ± 2.71	89.22 ± 2.45*	94.38 ± 2.69	90.94 ± 3.38*
10	103.96 ± 3.84	102.92 ± 2.92	85.78 ± 2.58*	97.01 ± 5.26	96.23 ± 3.71
100	171.53 ± 19.90*	152.03 ± 7.55*	80.78 ± 2.05*	33.52 ± 2.19*	112.49 ± 9.54
IC_{50} (µg/ml)	>100	>100	>100	60.19 ± 15.91	>100

*Indicating a significant effect of the percentage of survival with p-value < 0.05 when compared with negative control

Table 4.36 The lytic units/10^7 PBMCs of pure compounds from Hua-Khao-Yen and Benjakul extracts (n=12)

Concentration of pure compounds (µg/ml)	**Lytic units/10^7 PBMCs (mean ± SEM)**				
	Quercetin	**Astilbin**	**Dioscorealide B**	**2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene**	**Piperine**
Negative control	342.22 ± 55.26	241.32 ± 59.90	356.25 ± 92.91	241.32 ± 59.90	251.01 ± 64.25
0.01	367.98 ± 60.11	223.40 ± 57.55	392.65 ± 93.07	196.11 ± 45.43	204.16 ± 51.02
0.1	323.46 ± 57.56	234.88 ± 67.37	335.48 ± 73.87	214.02 ± 50.89	225.50 ± 55.47
1	382.03 ± 62.38	201.12 ± 52.34	340.79 ± 80.23	249.52 ± 77.66	253.66 ± 65.84
10	380.04 ± 61.75	228.04 ± 64.48	320.75 ± 79.71	100.01 ± 25.33*	204.77 ± 54.78
100	334.60 ± 56.73	272.46 ± 77.17	193.37 ± 49.18*	Toxic	135.46 ± 36.45*
Positive control (PHA 5 µg/ml)	537.93 ± 44.23*	428.06 ± 86.81*	688.64 ± 99.95*	428.06 ± 86.81*	447.70 ± 91.93*

*Indicating a significant effect on NK cell activity with p-value < 0.05 when compared with negative control.

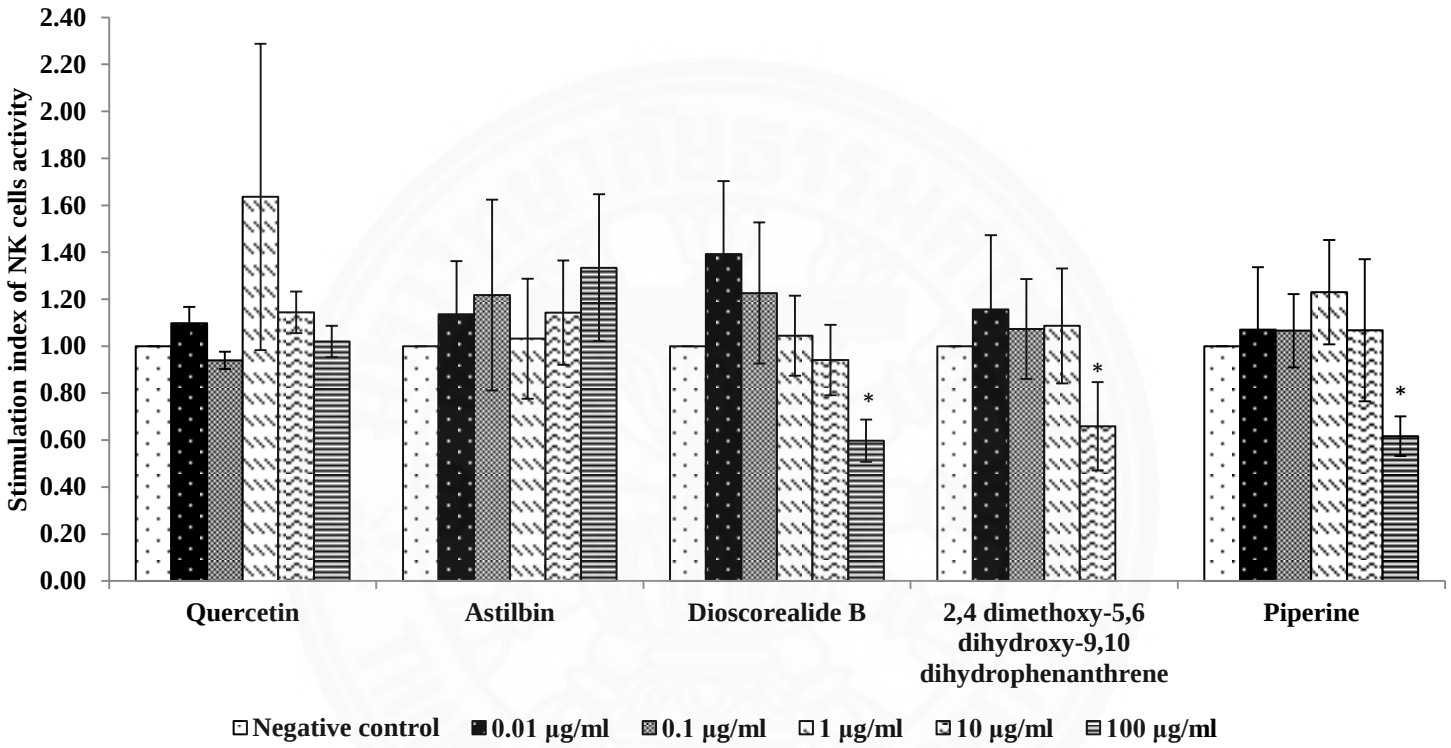

Figure 4.24 NK cells activity of pure compounds was determined by chromium release assay. These results are shown as stimulation index of NK cells activity (mean ± SEM). *Indicating a significant effect on NK cells activity with p-value < 0.05 when compared with negative control

4.5.4.2 Effect of pure compounds on lymphocyte proliferation

Quercetin and astilbin were investigated for cytotoxic effect on PBMCs at 72 hours. The results showed that quercetin had no toxic effect on PBMCs but dioscorealide B showed toxic effect on PBMCs at concentration of 100 μg/ml, as shown in Table 4.37.

After that, quercetin and dioscorealide B were examined for lymphocyte proliferation activity as shown in Table 4.38 and Figure 4.25. Quercetin increased lymphocyte proliferation at low concentration (0.01-0.1 μg/ml) but it decreased lymphocyte proliferation at 100 μg/ml. Dioscorealide B had no effect on lymphocyte activation. Moreover, dioscorealide B significantly decreased lymphocyte proliferation at concentration of 1 and 10 μg/ml (Table 4.38 and Figure 4.25).

Table 4.37 The percentage of survival of PBMCs when incubated with various concentrations of quercetin and dioscorealide B at 72 hours (n=6)

Concentration of pure compounds (μg/ml)	**% Survival of PBMCs (mean ± SEM)**	
	Quercetin	**Dioscorealide B**
Negative control	100.00 ± 0.00	100.00 ± 0.00
0.01	93.29 ± 6.58	88.34 ± 3.42
0.1	86.05 ± 5.96	86.75 ± 3.15
1	85.31 ± 6.03	86.36 ± 2.80
10	92.83 ± 5.97	72.84 ± 2.58
100	99.67 ± 4.25	56.75 ± 6.29
IC_{50} (μg/ml)	>100	85.89 + 3.78

Table 4.38 Stimulation index of lymphocyte proliferation of quercetin and dioscorealide B (n=12)

Concentration (µg/ml)	Stimulation index (mean ± SEM)	
	Quercetin	**Dioscorealide B**
Negative control	1.00 ± 0.00	1.00 ± 0.00
0.01	1.08 ± 0.09	1.08 ± 0.13
0.1	1.11 ± 0.09	1.00 ± 0.09
1	1.04 ± 0.08	0.97 ± 0.18*
10	0.96 ± 0.10	0.33 ± 0.15*
100	0.63 ± 0.07*	Toxic
Positive control (PHA 15 µg/ml)	158.93 ± 25.99*	117.09 ± 15.18*

*Indicating a significant effect on lymphocyte proliferation with p-value < 0.05 when compared with negative control

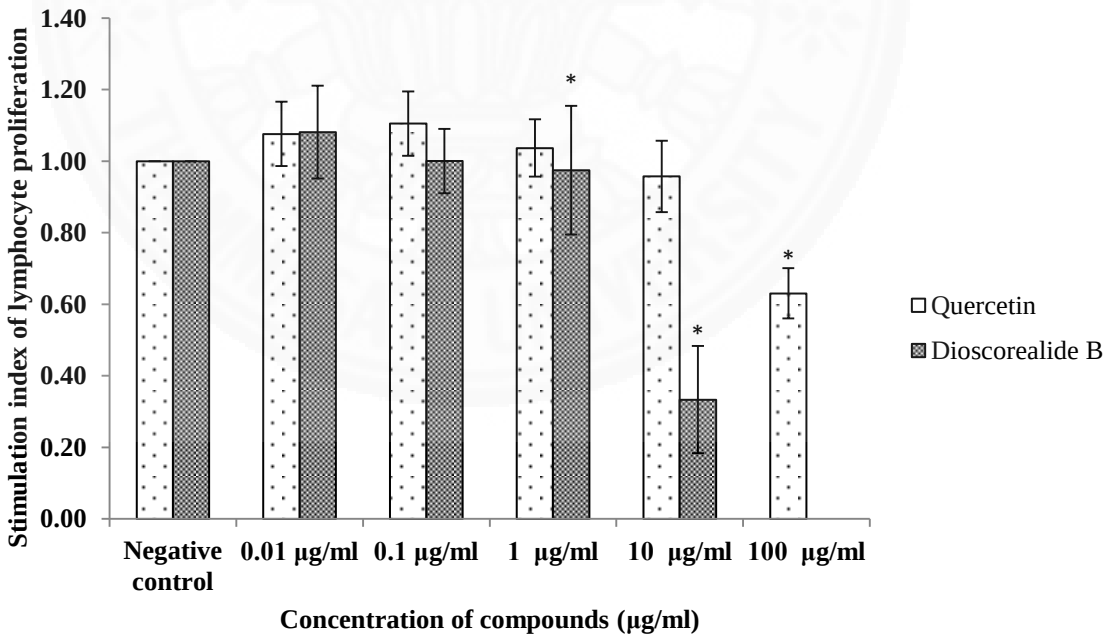

Figure 4.25 Stimulation index of lymphocyte proliferation of quercetin and dioscorealide B were determined by ^{3}H-thymidine uptake assay. These results are shown as mean ± SEM. *Indicating a significant effect on lymphocyte proliferation with p-value < 0.05 when compared with negative control.

4.5.4.3 Effect of pure compounds from Hua-Khao-Yen and Benjakul on cytokine secretion from LPS-stimulated RAW264.7 cell lines

From the results in Table 4.26, the ethanolic extracts of *D. membranacea* and Bejakul preparation inhibited IL-6 production. Thus, their isolated compounds were investigated for their cytotoxic effect on RAW264.7 cells (Table 4.39) and inhibitory effect on IL-6 production. Dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene, which were isolated from *D. membranacea*, showed inhibitory effects on IL-6 production with IC_{50} value of 8.59 and 4.38 μg/ml, respectively (Table 4.40). Moreover, 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene could inhibit IL-1β production with IC_{50} value of 9.30 μg/ml (Table 4.41). The isolated compound from Benjakul extract, piperine, decreased IL-6 production from RAW264.7 cells with IC_{50} value of 16.71 μg/ml. Dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene showed no toxic effect on RAW264.7 cells at 48 hours after incubation (Table 4.42). However, these two pure compounds could not stimulate anti-inflammatory cytokine, IL-10, although, dioscorealide B significantly decreased IL-10 production at 30 μg/ml (Figure.4.26).

Table 4.39 The percentage of survival of RAW264.7 cells when incubated with pure compounds at 24 hours (n=3)

Concentration (μg/ml)	**% Survival (mean ± SEM)**		
	Dioscorealide B	**2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene**	**Piperine**
Negative control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	NT	85.22 ± 6.50	NT
0.1	92.94 ± 4.79	83.44 ± 10.60	100.51 ± 6.31
1	94.14 ± 8.06	77.19 ± 3.29	76.58 ± 7.70
10	91.42 ± 6.00	71.96 ± 11.70	76.23 ± 13.75
30	76.74 ± 13.11	67.19 ± 4.87	NT
100	59.12 ± 3.32	44.98 ± 2.15	77.73 ± 0.43

These results are express as mean ± SEM, NT means not tested

Table 4.40 The percentage inhibition and IC_{50} of pure compounds from *D. membranacea* extracts on IL-6 production from LPS-stimulated RAW264.7 cell lines (n=2)

Compounds	%Inhibition of various concentrations of extracts						IC_{50} (µg/ml)
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	100 µg/ml	
Dioscorealide B	NT	1.47 ± 6.41	-3.33 ± 5.76	65.43 ± 2.98	77.96 ± 2.76	NT	8.59 ± 2.70
2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene	12.15 ± 3.11	0.44 ± 8.84	4.81 ± 4.46	80.06 ± 3.18	NT	NT	4.38 ± 2.96
Piperine	NT	-4.39 ± 4.48	-16.90 ± 4.48	7.43 ± 0.39	NT	75.59 ± 2.00	16.71 ± 3.57
Prednisolone	19.07 ± 1.35	51.48 ± 3.09	58.47 ± 1.04	84.00 ± 1.29	NT	NT	0.10 ± 0.01

These results are expressed as mean ± SEM, NT means not tested

Table 4.41 The percentage of inhibition and IC_{50} of 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene on IL-1β production from LPS-stimulated RAW264.7 cell lines (n=2)

Compounds	%Inhibition of various concentrations of extracts				IC_{50}
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	(µg/ml)
2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene	-4.1± 6.4	1.72± 6.27	-15.36 ± 7.36	72.23 ± 1.77	9.30 ± 0.1
Prednisolone	45.76± 4.25	82.91± 1.09	82.91± 1.09	106.41± 0.41	0.19± 0.09

These results are express as mean ± SEM

Table 4.42 The percentage of survival of RAW264.7 cells when incubated with pure compounds at 48 hours (n=3)

Concentration (µg/ml)	Dioscorealide B	2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene
Negative control	100.00 ± 0.00	100.00 ± 0.00
10	Not tested	74.42 ± 10.60
30	72.00 ± 6.82	Not tested

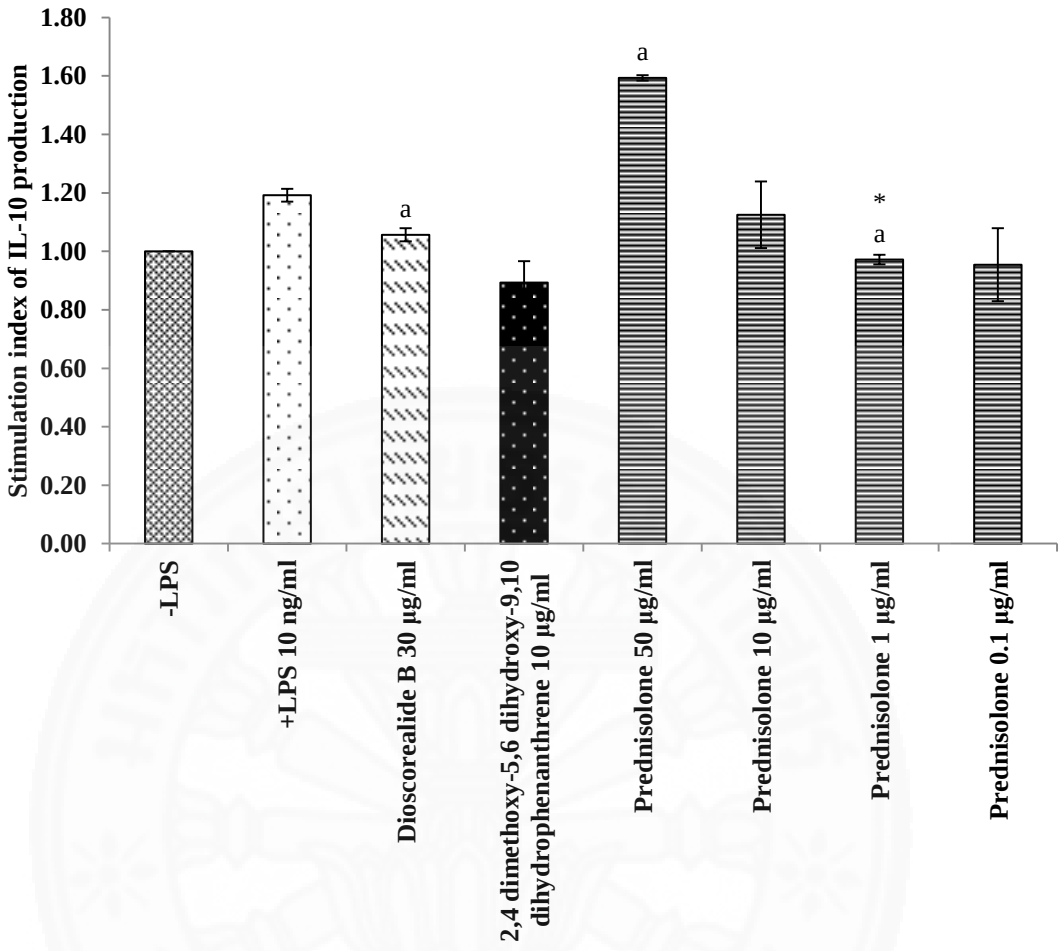

Figure 4.26 Effect of pure compounds from the ethanolic extract of *D. membranacea* on IL-10 production by LPS-stimulated RAW264.7. *Indicating a significant effect on IL-10 production with p-value < 0.05 when compared with the presence of LPS; [a]Indicating a significant effect on IL-10 production with p-value < 0.05 when compared with the presence of LPS.

4.6 The overall results of immunostimulatory and immunosuppressible effect of Hua-Khao-Yen and Benjakul

All results can be summarized in Table 4.43.

Table 4.43 The overall results of immunodulatory effects of Hua-Khao-Yen and Benjakul extracts and their isolated compounds

Sample	NK cell activity	Lymphocyte proliferation	IL-2 production	IFN-γ production	IL-10 production	IL-1β production (IC_{50})	IL-6 production (IC_{50})
BJE	0.01-1 μg/ml	0.01 μg/ml	0.1-1 μg/ml*	0.1-10 μg/ml*	NS	62.93 μg/ml	51.83 μg/ml
BJW	100 μg/ml*	0.01-100 μg/ml*	0.1-10 μg/ml*	0.1-100 μg/ml*	NS	>10 μg/ml	>10 μg/ml
Piperine	NS	NT	NT	NT	NT	NT	16.71 μg/ml
SCE	0.01 μg/ml*	0.1 μg/ml*	NS	1-10 μg/ml*	NS	>100 μg/ml	>100 μg/ml
SCW	0.01-100 μg/ml	10-100 μg/ml	NS	0.1-100 μg/ml	NS	>100 μg/ml	>100 μg/ml
Quercetin	0.01-10 μg/ml	0.1 μg/ml	NT	NT	NT	NI	NI
Astilbin	100 μg/ml	NT	NT	NT	NT	NI	NI

*Indicated significant effect, NS = Not significant stimulation, NT = Not tested, NI = No inhibitory effect

Table 4.43 The overall results of immunodulatory effects of Hua-Khao-Yen and Benjakul extracts and their isolated compounds. (Cont.)

Sample	NK cell activity	Lymphocyte proliferation	IL-2 production	IFN-γ production	IL-10 production	IL-1β production	IL-6 production
DME	0.1 μg/ml*	NS	NS	0.1-10 μg/ml*	10 μg/ml*	0.90 μg/ml	46.82 μg/ml
DMW	0.01-100 μg/ml*	0.01-10 μg/ml*	0.1-10 μg/ml*	0.1 μg/ml*	NS	>10 μg/ml	>10 μg/ml
Dioscorealide B	0.01 μg/ml	NS	NT	NT	NS	NT	8.59 μg/ml
2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene	NS	NT	NT	NT	NS	9.30 μg/ml	4.38μg/ml

*Indicated significant effect, NS = Not significant stimulation, NT = Not tested, NI = No inhibitory effect

4.7 Effect of three *Piper* species and piperine on contact hypersensitivity.

The ethanolic extract of *P. chaba*, *P. interruptum* and *P. sarmentosum* were investigated for adjuvant effect and immune response on skin sensitization. Firstly, the ethanolic extracts of *Piper* species were studied for TRPV1 and TRPA1 activation. Then, the extracts were investigated for their stimulatory effect on ear swelling, trafficking dendritic cell activation and cytokine production in mice.

4.7.1 The activation effect of *Piper* species on TRPV1 and TRPA1 receptor

From the previous report, the ethanolic extract of *P. chaba*, *P. interruptum* and *P. sarmentosum* contained piperine with the percentage of piperine content of 19.41, 11.99 and 0.99%, respectively as showed in Table 4.44 (Itharat et al., 2011). From Table 4.45 and Figure 4.27, the ethanolic extract of *P. chaba* and *P. interruptum* showed strong effect of TRPV1 activation with EC_{50} value of 0.65 and 1.42 μg/ml. Piperine also activated TRPV1 receptor with EC_{50} value of 0.31 μg/ml. The ethanolic extract of *P. sarmentosum* slightly increased TRPV1 activation that showed a stimulatory effect on normal cells (TRex-HEK cells).

Table 4.44 The percentage of piperine content in the ethanolic extract of *Piper* species (Itharat et al., 2011)

Ethanolic extract	Piperine content (mg/g of extract)	Percentage of piperine
P. chaba	194.10	19.41%
P. interruptum	119.89	11.99%
P. sarmentosum	0.90	0.09%

Table 4-45 The EC_{50} value of capsaicin, piperine and the ethanolic extract of *Piper* species on TRPV1 activation

Sample	**EC_{50} value**
Capsaicin	0.15 μg/ml (6 nM)
Piperine	0.31 μg/ml (1.08 μM)
PCE	0.65 μg/ml
PIE	1.42 μg/ml
PSE	> 60 μg/ml

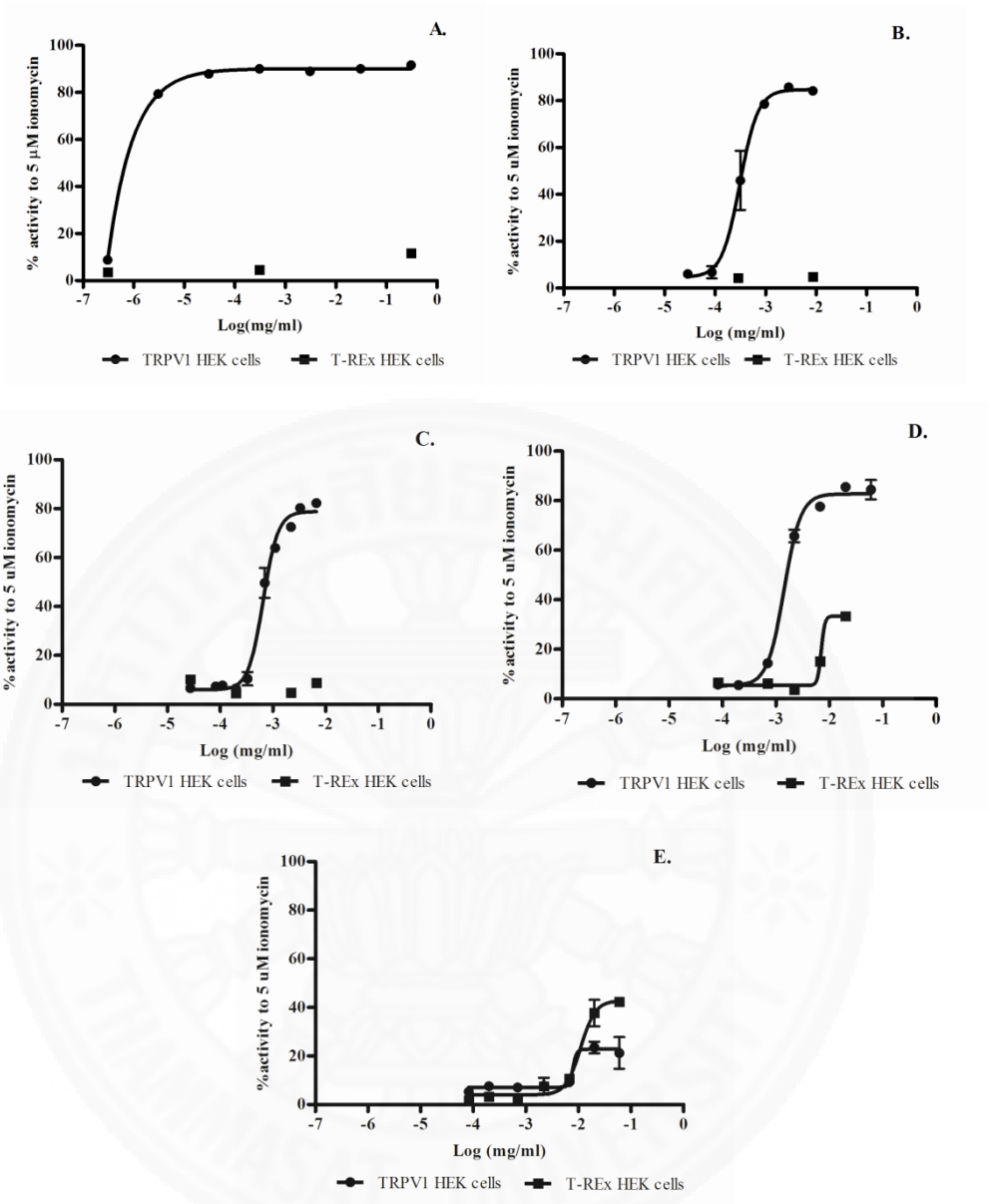

Figure 4.27 Calcium influx responses into TRPV1-expressing HEK cells(circles) or T-REx HEK cells (squares). The cells were treated with various concentrations of capsaicin (A.), piperine (B.) or the ethanolic extract of *P. chaba* (C.) or the ethanolic extract of *P. interruptum* (D.) or the ethanolic extract of *P. sarmentosum* (E.). The values represent the percent response relative to that treated with 5 µM ionomycin. Data are mean ± SEM (n = 2).

For the comparison of the effect of *Piper* extracts and piperine, the concentration of the ethanolic extract of three *Piper* species were plotted in a sigmoid curve based on the piperine content in the extract, as showed in Figure 4.28. The graph line of *P. chaba* extract and *P. interrutum* extract showed similar stimulation effect on the graph line of piperine. However, the *P. sarmentosum* extract showed the low activity on TRPV1 receptor (Figure 4.28). After that, the ethanolic extract of *P. chaba, P. interruptum* and piperine performed the specific effect on TRPV1 receptor. AMG9810 is TRPV1 antagonist that inhibited TRPV1 activation. The results in Figure 4.29 show that the effects of piperine, *P. chaba* and *P. interruptum* extract were decreased by AMG9810.

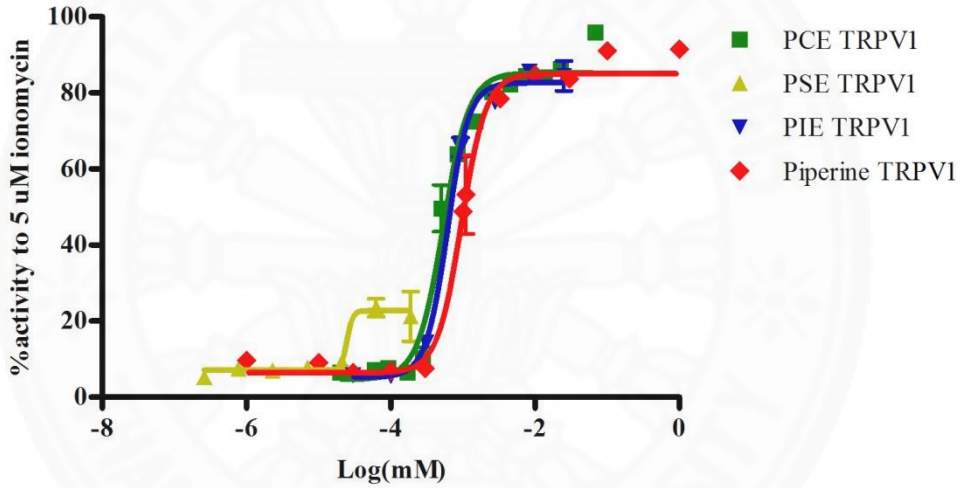

Figure 4.28 TRPV1-expressing HEK cells were activated by the ethanolic extract of *P. chaba* (green) or *P. sarmentosum* (yellow) or *P. interruptum* (blue) or pure piperine (red). For *Piper* species extract, data are plotted based on the piperine content (abscissa) in the extract. Data are mean ± SEM (n = 2)

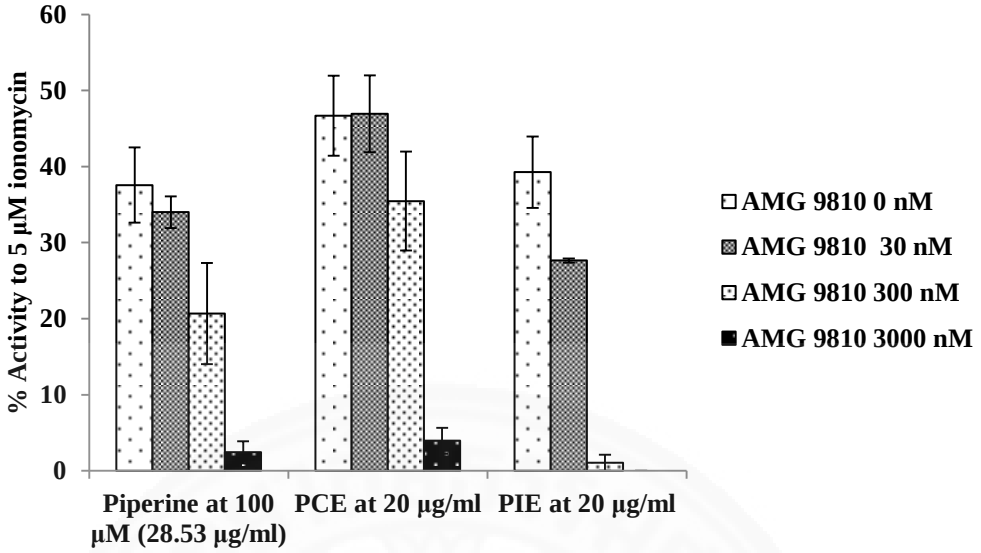

Figure 4.29 Inhibition of calcium influx by TRPV1 antagonist AMG 9810. Calcium influx responses (relative to the response to 5 µM ionomycin) are shown in the presence of various concentrations of AMG 9810 or in its absence. Concentrations of piperine, PCE and PIE were fixed as indicated in the abscissa. Data are mean ± SEM (n=2).

Then, the ethanolic extract of *P. chaba*, *P. interruptum* and piperine were investigated for their effect on TRPA1 activation. The ethanolic extract of *P. chaba* and *P. sarmentosum* could stimulate TRPA1 receptor with EC_{50} value of 9.22 and 6.83 µg/ml, respectively (Table 4.46 and Figure 4.30). The EC_{50} of *P. chaba* and *P. interruptum* on TRPA1 activation were converted to the concentration of piperine content in the extracts. The results showed that the EC_{50} value of piperine content in *P. chaba* and *P. interruptum* extract were 6.27 and 2.78 µM of piperine, respectively. The EC_{50} value of TRPA1 activation showed that the ethanolic extract of *P. chaba* and *P. interruptum* had stronger effect on TRPA1 activation than piperine, as showed in Figure 4.31. The *Piper* species extracts and piperine were then investigated for their specific effect on TRPA1 activation. HC030031 was used as TRPA1 antagonist. From Figure 4.32, the effect of the extracts and piperine were inhibited by HC030031.

Table 4.46 The EC_{50} values of Allyl isothiocyanate, piperine, the ethanolic extracts of *Piper* species on TRPA1 activation

Sample	EC_{50} value
Allyl isothiocyanate (AITC)	0.07 μg/ml (0.68 μM)
Piperine	0.42 μg/ml (14.86 μM)
P. chaba extract	9.22 μg/ml
P. interruptum extract	6.83 μg/ml

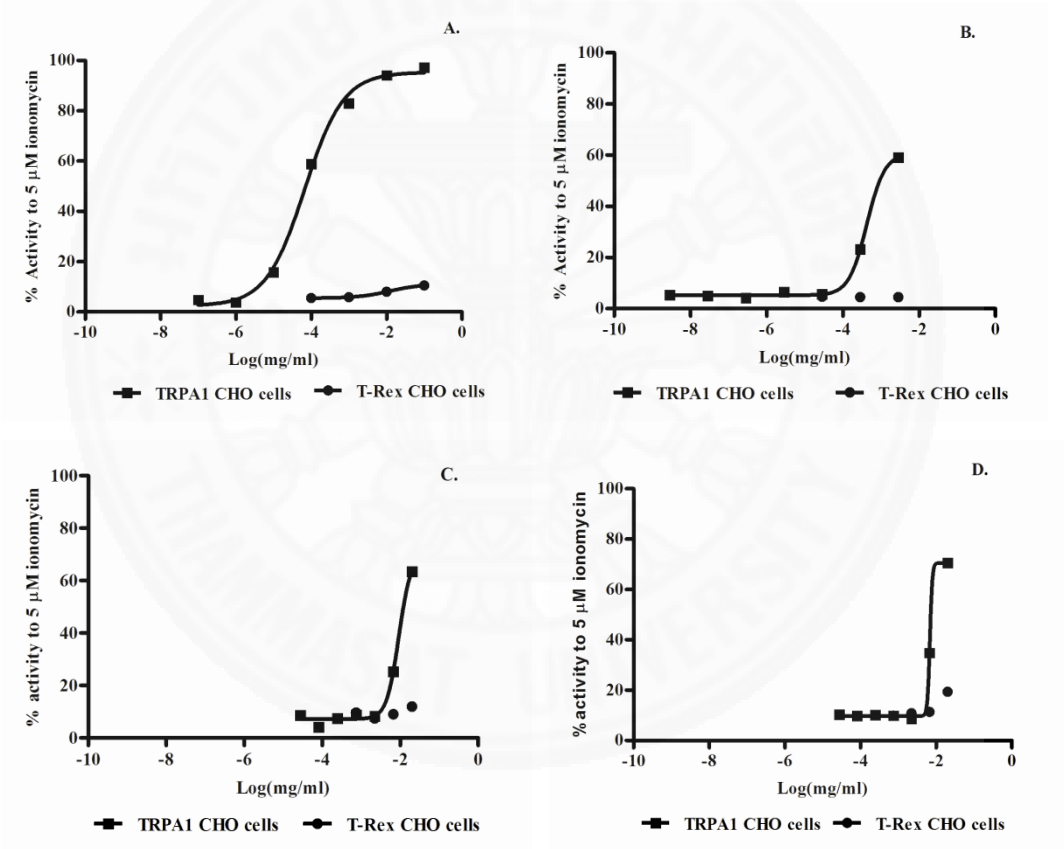

Figure 4.30 Calcium influx responses into TRPA1-expressing CHO cells(squares) or T-REx CHO cells (circles). The cells were treated with various concentrations of AITC (A.), piperine (B.) or the ethanolic extract of *P. chaba* extract (C.) or the ethanolic extract of *P. interruptum* (D.). The values represent the percent response relative to that treated with 5 μM ionomycin. Data are mean ± SEM (n = 2).

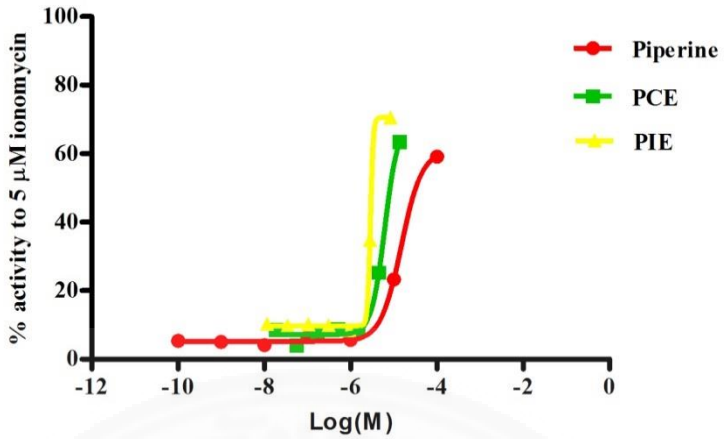

Figure 4.31 TRPA1-expressing CHO cells were activated by the ethanolic extract of *P. chaba* (green) or *P. interruptum* (yellow) or pure piperine (red). For *Piper* species extract, data are plotted based on the piperine content (abscissa) in the extract. Data are mean ± SEM (n = 2)

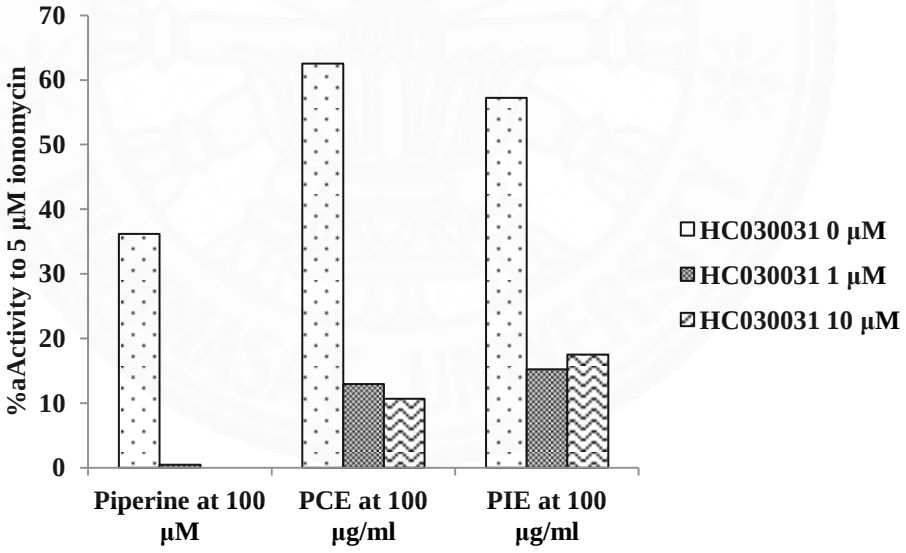

Figure 4.32 Inhibition of calcium influx by TRPA1 antagonist HC030031. Calcium influx responses (relative to the response to 5 µM ionomycin) are shown in the presence of two concentrations of HC030031 or in its absence. Concentrations of piperine, PCE and PIE were fixed as indicated in the abscissa.

4.7.2 Effect of the ethanolic extracts of *Piper* species and piperine sensitization to FITC by mouse ear swelling test

From section 4.7.1, piperine, the ethanolic extracts of *P. chaba* and *P. interruptum* showed stimulatory effect on TRPV1 and TRPA1 receptor. For this part, the ethanolic extracts of *P. chaba* and *P. interruptum* and piperine were investigated for their the adjuvant effects by mouse ear swelling test.

The ethanolic extract of *P. chaba* significantly increased the ear swelling in mice at concentration of 1% and 5% w/v. The ear swelling effect was maximum with *P. chaba* extract at 24 hours after sensitization. Then, the ear swelling effect was reduced at 48 and 72 hours (Figure 4.33). Moreover, the adjuvant effect of 1% *P. chaba* extract was similar to the adjuvant effect of 5% *P. chaba* extract. These results suggest that the adjuvant effect of *P. chaba* extract reached saturation at 1%. Piperine also showed stimulatory effect on mice ear swelling at 1% and 5% w/v. 5% w/v of piperine showed stronger effect than 1% w/v of piperine, as shown in Figure 4.35. However, the ethanolic extract of *P. interruptum* had no significant effect on mouse ear swelling, as shown in Figure 4.34.

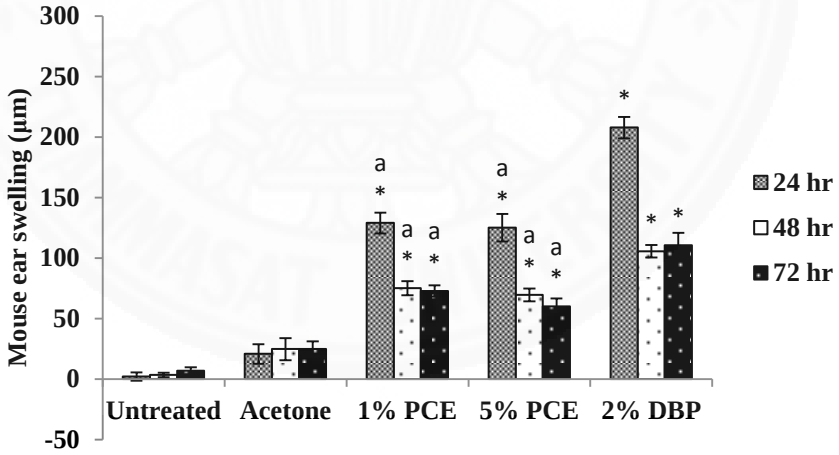

Figure 4.33 Effect of the ethanolic extract of *P. chaba* (PCE) on the sensitization to FITC by means of mouse ear-swelling test (n=5). BALB/c mice were sensitized with FITC in acetone, or FITC in 1% PCE extract, 5% PCE or 2% DBP. Mouse ear-swelling was measured at 24, 48 and 72 hours after challenge.* $p < 0.05$ when compared with acetone group; [a]$p < 0.05$ when compared with 2% DBP; [b]$p < 0.05$ when compared with untreated group.

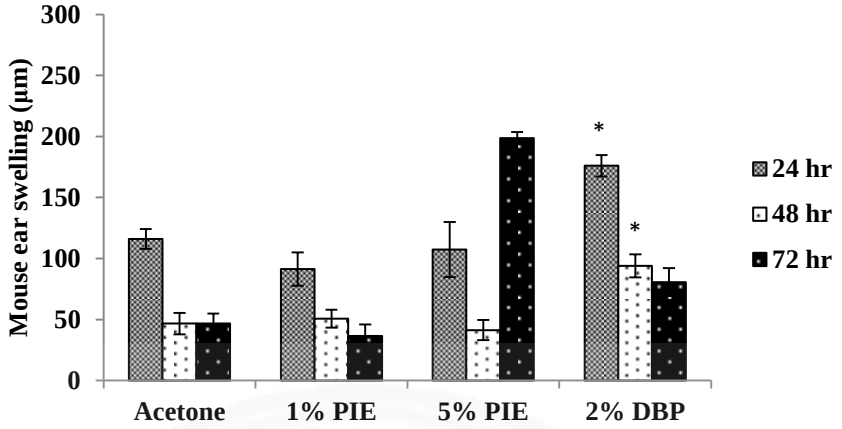

Figure 4.34 Effect of the ethanolic extract of *P. interruptum* (PIE) on the sensitization to FITC by means of mouse ear-swelling test (n=6). BALB/c mice were sensitized with FITC in acetone or FITC in 1% PIE, 5% PIE or 2% DBP. Mouse ear-swelling was measured at 24, 48 and 72 hours after challenge.* $p < 0.05$ when compared with acetone group; [a] $p < 0.05$ when compared with 2% DBP condition.

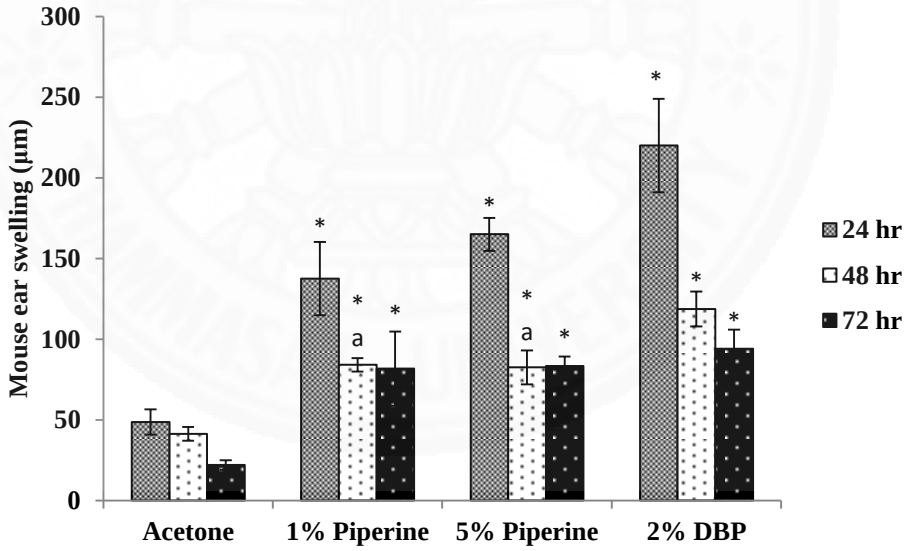

Figure 4.35 Effect of piperine on the sensitization to FITC by means of mouse ear-swelling test (n=5). BALB/c mice were sensitized with 0.5% FITC in acetone alone, or in acetone containing 1% piperine, 5% piperine or 2% DBP. Mouse ear-swelling was measured at 24, 48 and 72 hours after challenge with 0.5% FITC dissolved in 10% DBP in acetone.*$p < 0.05$ when compared with acetone group;[a] $p < 0.05$ when compared with 2% DBP condition.

4.7.3 Effect of the ethanolic extracts of *P. chaba* and piperine on trafficking of FITC-presenting dendritic cells

The ethanolic extract of *P. chaba* could activate TRPV1 and TRPA1 receptors. It also showed adjuvant effect in mice. Consequently, this extract was investigated for the ability to facilitate skin to lymph node trafficking of antigen presenting cells. Four mice were sensitized with 0.5% FITC in acetone alone or 1% *P. chaba* extract or 1% or 5% piperine or 2% DBP as a positive control. FITC positive dendritic cells were detected at 24 hours after sensitization by flow cytometry. The extract or hapten FITC is transported to lymph nodes cells to drain them by antigen-presenting cells. Dendritic cells play the important role as antigen-presenting cells in the immune system. The present study focused on FITC positive dendritic cells. Dendritic cells were detected and discriminated from other cells by the expression of cell surface marker (CD11c). Cells expressed CD11c and FITC in a quarter 2 (Figure 4.36) were detected and analysed.

The ethanolic extract of *P. chaba* significantly increased FITC positive dendritic cells at 1% w/v, as showed in Figure 4.37. On the other hand, piperine had no significant effect on draining lymph node cells at 1% and 0.2% w/v. The results showed that piperine content in *P. chaba* extract was not an active compound of *P. chaba* to induce dendritic cell trafficking.

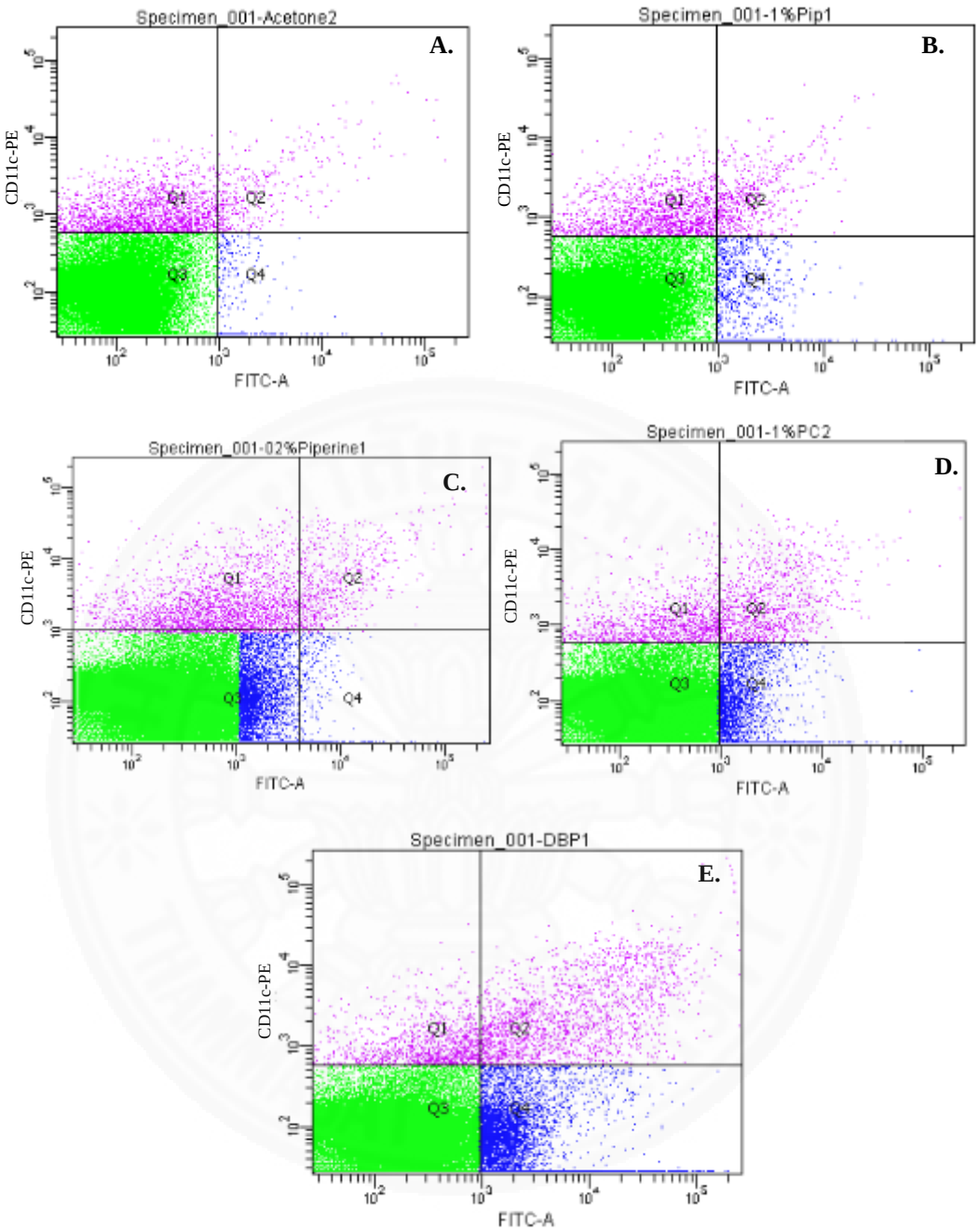

Figure 4.36 Flow cytometric dot plots of FITC positive dendritic cells from FITC sensitized-mice. Mice were sensitized by 0.5%FITC dissolved in acetone (A.), 1% piperine (B.), 0.2% piperine (C.), 1% PCE (D.) and 2% DBP (E.) on forelimb skin for 24 hours.

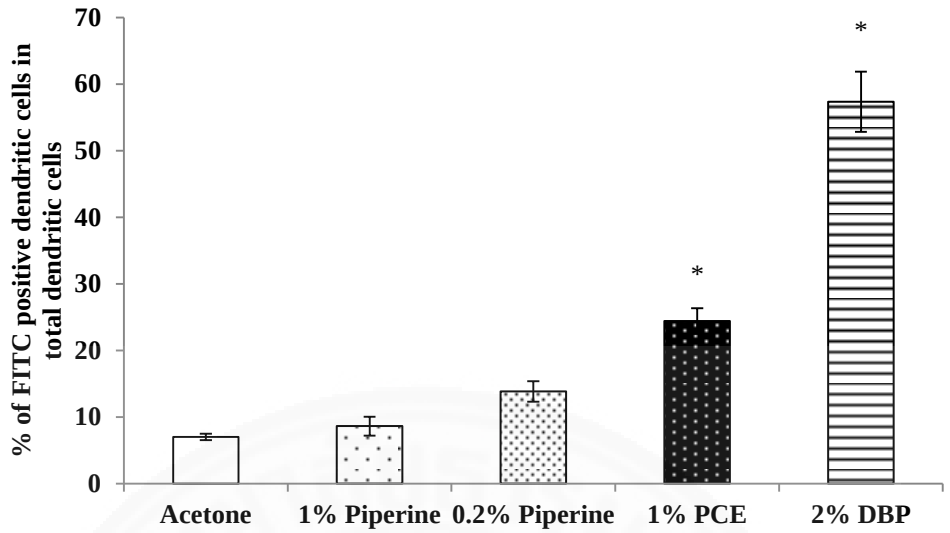

Figure 4.37 Effect of the ethanolic extract of *P. chaba* (PCE) and piperine on the stimulation to FITC positive dendritic cells (n=4). BALB/c mice were sensitized with 0.5% FITC in acetone alone, or with acetone containing 1% piperine, 0.2% piperine, 1% PCE or 2% DBP. FITC positive dendritic cells were measured at 24 hours after sensitization. *$p < 0.05$ when compared with acetone group. Statistical significance was analysed by one-way ANOVA, followed by Dunnett's test.

4.7.4 Effect of the ethanolic extracts of *P. chaba* and piperine on cytokines production by draining lymph nodes of FITC sensitized mice

From section 4.7.3, *P.chaba* extract showed potent effect on dendritic cell trafficking. Thus, this extract was investigated for the effect on cytokine production by draining lymph node cells. Eventhough piperine could not activate dendritic cells trafficking, it was investigated for its effect on cytokine production.

From Figure 4.38, the ethanolic extract of *P. chaba* significantly activated IL-4 and IFN-γ production by draining lymph node cells. The *P. chaba* extract showed the highest level of IL-4 at 48 hours and slightly decreased IL-4 production at 72 hours. Moreover, *P. chaba* extract significantly stimulated IFN-γ production at 72 hours. Piperine showed no effect on IL-4 and IFN-γ production by draining lymph node cells.

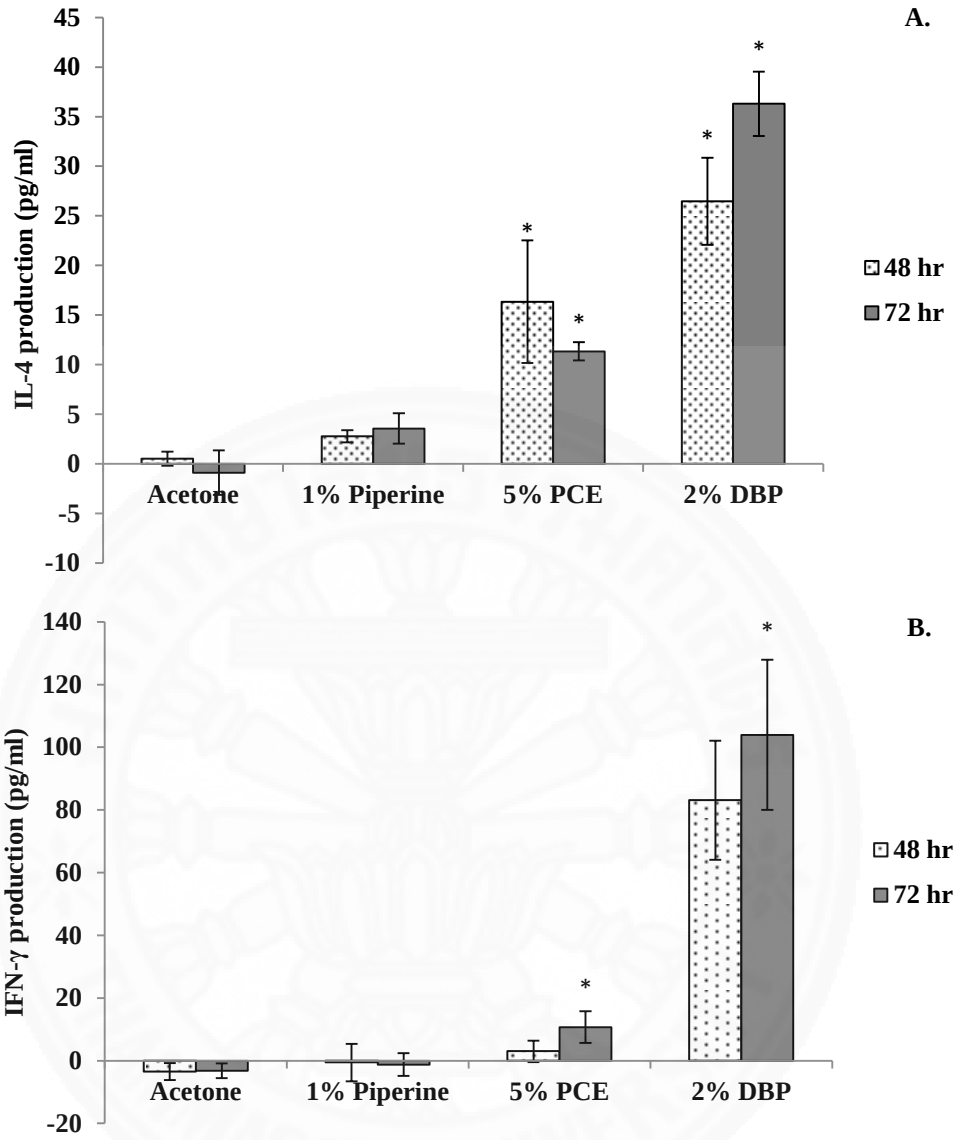

Figure 4.38 Effect of the ethanolic extract of *P. chaba* (PCE) and piperine on cytokine production by lymph nodes of FITC-sensitized mice. The lymph node cells were cultured for 48 and 72 hours and then the IL-4 (A.) and IFN-γ (B.) production were detected by ELISA. The data are shown by mean $\pm$ SEM (n=4). *$p < 0.05$ when compared with acetone group. Statistical significance was analysed by one-way ANOVA, followed by Dunnett's test.

4.8 Discussion

Hua-Khao-Yen-Nua or *S. corbularia*, Hua-Khao-Yen-Tai or *D. membranacea* and Benjakul preparation have long been used to treat cancer patients (Itharat et al., 1998; Vimolkhunakorn, 1979). The cytotoxic activity of them and their compounds have been reported (Itharat et al., 2004; Itharat et al., 2003; Jaiaree, Itharat, & Kumapava, 2010; Rattarom et al., 2014; Ruangnoo, 2012; Ruangnoo, Itharat, et al., 2012). However, there is no report on their immunomodulatory activity. Thus, the aims of this study focused on immunomodulatory activity.

Firstly, the crude extracts of *S. corbularia*, *D. membranacea* and Benjakul were tested for NK cells activity. NK cells are important cells of innate immunity to defend against viral infections and tumor cells in humans (Abbas et al., 2010). Abnormal cells that lack of MHC class I activate NK cells activity. After activation, NK cells secrete granzyme and perforin from cytolytic granule to kill tumor cells (Uhrberg, 2005). The ethanolic and water extracts of *S. corbularia*, *D. membranacea* and Benjakul were investigated for increase of NK cell activity. The ethanolic extract of *S. corbularia* (0.01 μg/ml) and *D. membranacea* (0.1 μg/ml) significantly increased NK cell activity. This result suggests that low concentration of Hua-Khao-Yen activated NK cells more than high concentration. Moreover, the water extract (0.01-100 μg/ml) of *D. membranacea* also showed a significant increase in NK cell activity. This result supports using its water extract by Thai folk doctors as an ingredient in many cancer remedies in Thai traditional medicine especially cancer preparation of Arokayasala , Kumpramong Temple which prepares a cancer remedy as water extract (Poonthananiwatkul, Lim, Howard, Pibanpaknitee, & Williamson, 2015). The water extract of Benjakul preparation showed a significant effect on NK cell activity at concentration of 100 μg/ml. Even though the ethanolic extract of Benjakul had no significant effect, it tended to stimulate NK cell activity at low concentration (0.01-1 μg/ml). This result is a good point of Benjakul because the ethanolic extract of Benjakul showed high cytotoxic activity against many cancer cells especially lung cancer with IC_{50} values as 19.8 μg/ml (Ruangnoo, Itharat, et al., 2012) so lower concentration showed both cytotoxic and NK cell stimulation effects.

Quercetin, an isolated compound from *S. corbularia*, increased NK cell activity at low concentration (0.01-10 μg/ml). Quercetin had been reported as

enhancing natural killer group 2D (NK G2D) ligands in tumor cells. The increasing of NK G2D ligands increased the susceptibility of tumor cells to NK cell-mediated cytotoxicity (Bae et al., 2010). The clinical study of quercetin has shown that quercetin had no significant effect on NK cell activity in 74 female volunteers with supplementation of 500 mg/d and 1,000 mg/d of quercetin for 12 weeks (Heinz, Henson, Nieman, Austin, & Jin, 2010). Another isolated compound from *S. corbularia*, astilbin, showed activation effect on NK cells at high concentration (100 μg/ml) and no toxic effect on PBMCs. Astibin is a main component from the ethanolic extract of *S. corbularia* (Ruangnoo, 2012) so it is a main ingredient for activation of NK cells in this extract. However, this result is the first report on NK cells for *S. corbularia* and astilbin as its main ingredient. The results of isolated compounds from *D. membranacea* showed that dioscorealide B stimulated NK cells activity at low concentration (0.01 μg/ml) and decreased NK cell activity at high concentration (100 μg/ml). This is good point of dioscorealide B because it showed high cytotoxic activity against many types of cancer cell and lower cytotoxicity against normal cells (Itharat, 2010; Ruangnoo, 2012). It also showed high immunomodulatory effect on activation of NK cells. This compound should be promoted to be anticancer drug in the future. 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene had no effect on NK cell activation. Piperine as an isolated compound and a main compound from the ethanolic extract of Benjakul (Itharat & Sakpakdeejaroen, 2010) slightly increased NK cell activity at low concentration (1 μg/ml) and significantly decreased NK cell activity at high concentration. This study agrees with Sunila (2014) who showed that piperine (1.14 mg/dose/animal) enhanced NK cell activity with maximum level of 40.3% in tumor bearing control animals at day 5 after drug administration.

From the past clinical studies, the lymphocyte proliferation of cancer patients was reduced and less responsive to the mitogen compared to healthy donors (Hannigan, Johnson, Collins, & Moriarty, 1984; Melichar et al., 1996). Thus, the second part of this study was regarding lymphocyte proliferation. The crude extracts of *S. corbularia*, *D. membranacea* and Benjakul were investigated for their effects on lymphocyte proliferation in healthy donor's blood. The ethanolic extract of *S. corbularia* significantly stimulated lymphocyte proliferation at concentration of 0.1

μg/ml. However, the water extract of *S. corbularia* had no effect on lymphocyte proliferation. The effect of *S. corbularia* on lymphocyte proliferation has not yet been reported. However, other plants in Smilacaceae family have shown effects on lymphocyte proliferation. For example, the aqueous extract of *S. glaba* (400 mg/kg) significantly stimulated T lymphocytes from adjuvant arthritis in rats 25 days after drug administration (Jiang & Xu, 2003). Quercetin, an isolated compound from the ethanolic extract of *S. corbularia*, showed no significant stimulating of lymphocyte proliferation but it significantly decreased lymphocyte proliferation at high concentration (100 μg/ml). Lugli (2009) showed that quercetin inhibited T cell proliferation and activation. Moreover, quercetin inhibited leukemic cell lines than on PBMC. The ethanolic extract of *D. membranacea* significantly reduced lymphocyte proliferation at high concentration but its water extract showed a significant effect at concentration of 0.01-10 μg/ml. This is a good effect of the water extract of *Diocorea membranacea* which showed high cytotoxic effect against breast cancer (IC_{50}=5.5 μg/ml) (Itharat et al., 2004), it also activated lymphocyte proliferation at the same concentration as cytotoxic activity. This extract should be promoted to treat breast cancer patients. Dioscorealide B, isolated from *D. membranacea* showed significantly inhibited lymphocyte proliferation similar to the effect of its ethanolic extract. This result agrees with the previous work which found that, the ethanolic extract of some *Dioscorea* species reduced the number of lymphocyte proliferation of mice after treatment dose of 10.04 g/kg (Makiya, Djati, Rifa, & Widodo, 2015). For Benjakul extracts, the ethanolic extract significantly decreased lymphocyte proliferation at high concentration (10-100 μg/ml). Moreover, the ethanolic extracts of component plants in Benjakul preparation showed effects similar to the ethanolic extract of Benjakul. On the other hand, the water extract of Benjakul and plants component, except the water extract of *P. indica*, significantly stimulated lymphocyte proliferation at all concentrations (0.01-100 μg/ml). Some extracts showed toxic effect on PBMCs, so the stimulation index of lymphocyte proliferation was also reduced. However, some extracts had no toxic effect on PBMCs but the stimulation index of lymphocyte proliferation was a low value such as the ethanolic extract of Benjakul, *P. sarmentosum*, *P. interruptum* and *Z. officinale*. Piperine, an isolate compound from Benjakul and *Piper* species, has been reported for its effect on mouse lymphocyte

activation. Piperine inhibited T lymphocyte proliferation at concentration of 25-100 μM without effects on cell viability. T lymphocyte was blocked in the cell cycle progression at G0/G1 phase by reduction of the cell cycle regulatory protein including G1-associated cyclin D3, CDK4, and CDK6 (Doucette et al., 2015). Moreover, the water extract of Benjakul at 100 μg/ml showed the highest potency in lymphocyte proliferation when compared with the water extract of its plant ingredients. The water extract of Benjakul may show synergistic effect on lymphocyte proliferation.

For cytokine secretion effect, it is well established that IL-2 enhances NK cell activity and improves perforin binding to tumor cells. The presence of IL-2 activates lymphocyte to lymphokine-activated killer (LAK) cells (Lehmann, Zeis, & Uharek, 2001; Whiteside, 2001). IFN-γ is a cytokine which is involved with cytotoxic and anti-tumor mechanism in adaptive immune response. IFN-γ induced T-cell migration to tumor sites (Nakajima et al., 2001). The present study showed that the ethanolic extract of *S. corbularia* significantly decreased IL-2 production from PHA-stimulated PBMCs in a dose-dependent manner. However, IFN-γ production was slightly increased at 0.1, 1 and 10 μg/ml of the ethanolic extract of *S. corbularia*. The water extract of *S. corbularia* had no effect on IL-2 and IFN-γ production but it tended to increased IFN-γ production at all concentrations (0.1-100 μg/ml). From the previous work, the aqueous extract of *Smilax* species induced IL-2 production from splenocytes from adjuvant arthritis rats (Jiang & Xu, 2003). The chemical constituents that are contained in the ethanolic extract of *S. cobularia*, may affect IL-2 and IFN-γ production. Nair et al. (2002) reported that quecetin significantly increased the gene expression and production of IFN-γ in PBMCs from normal subjects. Yu et al. (2008) described that quercetin (20-40 μM) inhibited IL-2 and IFN-γ production in arthritic rats. Moreover, astilbin (25-100 μg/ml) also decreased IL-2 and IFN-γ secretion from mice lymphocyte (Song et al., 2010). However, the ethanolic extract of *S. corbularia* in this study increased IFN-γ production. It may contain some chemical compounds which stimulate IFN-γ production. The water extract of *D. membranacea* showed significant effect on IFN-γ but it had no effect on IL-2 production. The ethanolic extract of *D. membranacea* also showed no stimulatory effect on IL-2 and IFN-γ production. Moreover, both extracts of *D. membranacea* decreased IL-2 and IFN-γ production at high concentration (100 μg/ml). The methanolic extract of *Dioscorea*

species has been reported as having dose-dependent stimulation of IL-2 and IFN-γ production from murine splenocytes (Dey & Chaudhuri, 2014). The ethanolic extract of Benjakul had no significant effect on IL-2 and IFN-γ production from PHA-stimulated PBMCs. However, the same extract tended to increase IL-2 and IFN-γ but this extract also tended to reduce cytokine production at high concentration. The water extract of Benjakul significantly increased IFN-γ production at concentration of 1 μg/ml but it had no significant effect on IL-2 production. Piperine is reported to inhibit IL-2 and IFN-γ production in PBMCs (Chuchawankul et al., 2012). The cause of ethanolic extract of Benjakul inhibiting IL-2 and IFN-γ production may cause from high yield of piperine.

Although the immune system can detect and kill tumor cells, some immune responses lead to stimulation of cancer. Inflammation is an immune response to pathogens and damaged cells. The inflammatory process is the cause of DNA damage and leads to tumor development, especially chronic inflammation (Meira et al., 2008; Rakoff-Nahoum, 2006). Acute inflammation is the primary state of the inflammatory response. Many cytokines are released to respond to the inflammation process. IL-1β and IL-6 are primarily involved in the pro-inflammatory process. IL-1β and IL-6 stimulate neutrophil-activated chemokines. Neutrophils produce reactive oxygen species and carcinogens. Thus, it has been proposed that IL-1β and IL-6 inhibition can protect chronic inflammation development and reduce tumor growth (Dinarello, 2006; McLoughlin et al., 2004). Moreover, IL-10 anti-inflammatory cytokines play the role of inflammatory cytokine inhibition and tumor-associated inflammation (Oft, 2014). The present study investigated the effect of these extracts on inflammatory and anti-inflammatory cytokines. The ethanolic and water extract of *S. corbularia* showed no inhibitory effect on IL-6 and IL-1β. Moreover, *S. corbularia* extracts could not activate IL-10 production. The ethnolic extract of *S. corbularia* was reported for inhibition of PGE2, TNF-α and NO produciton by LPS-stimulated RAW264.7 cells (Ruangnoo, Jaiaree, et al., 2012). The ethanolic extract of *D. membranacea* and isolated compounds dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene inhibited IL-6 production with IC_{50} value of 46.82, 8.59 and 4.38 μg/ml, respectively. The ethanolic extract of *D. membranacea* and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene also inhibited IL-1β

production (IC_{50} value = 0.9 and 9.3 μg/ml, respectively). Some phenanthrene showed inhibitory activity on nitric oxide production and phosphorelation and degradation of IκBα in LPS-activated RAW264.7 cells (Lin et al., 2013; Chen et al., 2016). 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol, phenanthrene, were reported that inhibited signal generated by TLR4 activation and down-regulated NF-κB (Datla et al., 2010). Moreover, the ethanolic extract of *D. membranacea* activated IL-10 production at 10-50 μg/ml. However, the water extract of *D. membranacea* had no effect on IL-6 and IL-10 production. The ethanolic and water extracts of *D. membranacea* showed inhibitory effects on NO production from RAW264.7 cells with IC_{50} value of 23.6 and 57.8 μg/ml, respectively (Tewtrakul & Itharat, 2007). However, these compounds could not stimulate IL-10 production. Dioscorealide B was reported for anti-inflammatory activity in RAW264.7 by inhibition of NO production and TNF-α with IC_{50} value of 24.9 and 93.2 μM, respectively (Tewtrakul & Itharat, 2007). Dioscorealide B significantly decreased iNOS, Il-1β and IL-6 mRNA expression. Moreover, dioscorealide B inhibited IκBα phosphorylation and interfered with NF-κB transcription factor. Dioscorealide B also reduced ERK/MAPK pathway and lead to inhibition of IL-10 mRNA expression (Hiransai et al., 2010). The ethanolic extract of Benjakul preparation and isolated compound, piperine, showed inhibitory effect on IL-6 production from LPS-stimulated RAW264.7 cells with IC_{50} of 51.83 and 16.71 μg/ml, respectively. Piperine is reported that decreased TNF-α, IL-1β and IL-6 but increased IL-10 production (Zhai et al., 2016). The research work of Ying et al. (2013) showed that piperine inhibited LPS-mediated activation of NF-κB by inhibition of IκB proteins and the translocations of p65 subunit of NF-κB from cytosol to the nucleus.

Benjakul consists of five plants, namely *P. chaba, P. sarmentosum, P. interruptum, P. indica* and *Z. officinale*. The clinical study report of Benjakul showed the side effect of Benjakul tablet that some volunteers had a burning stomach (Amorndoljai &Kietinun &Somparn, 2011). This preparation contains three *Piper* species so the main chemical constitiuent in Benjakul is piperine (Rattarom, 2013). Piperine is some kind of alkaloid that leaves a burning and hot sensation after taste. Benjakul also leaves a hot and peppery after taste. The cause of burning and peppery sensation is the activation of TRPV1 and TRPA1 receptors that are involved with

sensory nerve cells (Fernandes, Fernandes, & Keeble, 2012) and contact hypersensitivity (Shiba et al., 2009). Piperine has been reported as activating TRPV1 and TRPA1 receptors (Okumura et al., 2010). However, the effect of Benjakul and three *Piper* species has not ever been reported regarding TRPV1 and TRPA1 receptors. The present study focused on the effect of three *Piper* species activation of TRPV1 and TRPA1 receptors compared with piperine and contact hypersensitivity. The results showed that the ethanolic extract of *P. chaba* and *P. interruptum* strongly activated to TRP channel (TRPV1 and TRPA1) in similar way to piperine effect. However, the ethanolic extract of *P. sarmentosum* could not activate the TRP channel. The low piperine content in *P. sarmentosum* (Itharat et al., 2011), may be the reason. Previous studies showed TRPV1 and TRPA1 activation is involved in the adjuvant effect (Shiba et al., 2009). Here, *P. chaba* and *P. interruptum* extracts were investigated for their adjuvant effect. The results showed that *P. chaba* had on adjuvant effect which induced mice ear swelling at 24 hours after sensitization. However, *P.interruptum* could not activate mice ear swelling. The ethanolic extract of *P. chaba* also activated the trafficking dendritic cells and cytokines production; IL-4 and IFN-γ. IFN-γ and IL-4 promote T helper type 1 and T helper type 2 responses (Dittmer et al., 2001). The *P. chaba* effect is different from the piperine effect because piperine could not activate dendritic cell trafficking and cytokine production in mice. Okumura et al. (2010) reported that the other compounds of *P. chaba* such as brachystamide B, dehydropipernonaline, fragaramide, guineensine, methyl piperate, isopiperine, isochavicine, piperonal, piperlonguminine and retrofractamide) could activate the TRPV1 channel. These compounds in *P. chaba* extract may show a synergistic effect on TRPV1 activation. It is suggested these compounds may activate adjuvant effect and activate immune response in mice. An adjuvant is a substance which increases the immune response. Adjuvant substances are used in combination with specific antigens that stimulate more immune response than the antigens alone (Vogel, 1995). Natural compounds such as saponin have the ability of adjuvant effect which activates antibody production in the immune system (Rajput et al., 2007). The present study showed that *P. chaba* extract exhibited adjuvant effects in contact hypersensitivity which the mechanism of this action are this extract increased dendritic cells trafficking and activated cytokines production. However, piperine also

showed TRP channel activity and adjuvant effect but unknown mechanism. Chemical constituents in Benjakul are piperine, plumbagin, gingerol and shogaol (Rattarom, 2013). Theses compounds were reported that activated TRPV1 and TRPA1 channel (Vriens &Nilius &Vennekens, 2008; Morera et al., 2012). Thus, Benjakul may also activate TRPV1, TRPA1 and show adjuvant effect on contact hypersensitivity. Futhermore, Yu and Lu (2014) reported that the hapten increased personerlized chemoimmunotherapy. Thus, Benjakul may show adjuvant effect for hapten and improve ability of cancer drug.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Hua-Khao-Yen and Benjakul preparation have long been used for treatment of cancer patients. In Thailand, five species of Hua-Khao-Yen has been found but there are only two species which are widely used for cancer treatment namely Hua-Khao-Yen-Nua or *Smilax corbularia* and Hua-Khao-Yen-Tai or *Dioscorea membranacea*. Benjakul is a Thai traditional preparation used to treat cancer patients and most folk doctors gave Benjakul to cancer patients receiving other cancer drugs. Benjakul is composed of five plants, namely the fruits of *Piper chaba,* the stems of *Piper interruptum*, the stems of *Piper sarmentosum* Roxb., the roots of *Plumbago indica* Linn. and the rhizomes *Zingiber officinale*. The immunomodulatory activities of *S. corbularia, D. membranacea* and Benjakul preparation have not been reported. Thus, the present study focused on immunomodulatory effects of *S. corbularia, D. membranacea* and Benjakul preparation in cancer treatment. Immunomodulatory assays were performed on PBMCs and RAW264.7 macrophage cells. *S. corbularia, D. membranacea,* Benjakul preparation and component plants in Benjakul preparation were extracted by decoction with water and maceration with 95% EtOH to obtain water and ethanolic extracts respectively. These samples were investigated for cytotoxic effects on immunomodulatory cell such as PBMCs, NK cells activity, lymphocyte proliferation, IL-2 and IFN-γ production from PHA-stimulated PBMCs. The cytotoxic effect on RAW264.7 cells, IL-6 and IL-10 production from LPS-stimulated RAW264.7 cells which indicated inflammation, were also studied. Finally, the isolated compounds from *S. corbularia, D. membranacea* and Benjakul preparation were also investigated for immunomodulatory activity.

The ethanolic and water extracts of *S. corbularia* had no toxic effect on PBMC at 24 and 72 hours after incubation. The ethanolic extract of *S. corbularia* showed significant effect on NK cell activity and lymphocyte proliferation at low concentration (0.01 and 0.1 μg/ml) and decreased NK cells activity and lymphocyte proliferation at high concentration (100 μg/ml). IL-2 production from PHA-stimulated PBMCs was reduced in dose-response manner (0.1-100 μg/ml) by the ethanolic

extract of *S. corbularia*. The ethanolic extract of *S. corbularia* significantly increased IFN-γ production (1-10 μg/ml). The ethanolic extract of *S. corbularia* was divided into five fractions. Quercetin and astilbin are chemical constituents which were isolated from the ethanolic extract of *S. corbularia*. Quercetin (0.01-10 μg/ml) and astilbin (100 μg/ml) tended to increase NK cells activity. However, quercetin significantly reduced lymphocyte proliferation at high concentration (10-100 μg/ml).

The ethanolic extract (100 μg/ml) and water extract (10 and 100 μg/ml) of *D. membranacea* showed toxic effects on PBMCs at 72 hours after incubation. The ethanolic extract significantly stimulated NK cell activity (0.1 μg/ml) and inhibited lymphocyte proliferation (0.1-10 μg/ml). Moreover, the ethanolic extract of *D. membranacea* showed inhibitory effects on IL-2 and IFN-γ from PHA-stimulated PBMCs at concentration of 100 μg/ml. IL-6 production from LPS-induced RAW264.7 cells was inhibited by the ethanolic extract of *D. membranacea* (IC_{50} = 46.82 μg/ml). IL-10 production was significantly stimulated by the ethanolic extract of *D. membranacea* at 10 μg/ml. Additionally, the water extract of *D. membranacea* showed significant effect on NK cells and lymphocyte proliferation. However, the water extract showed stimulatory effect on IL-2, IFN-γ but it showed no effect on inflammatory cytokines. Thus, the ethanolic extract of *D. membranacea* was chosen for isolation of pure compounds. Two pure componds were isolated from *D. membranacea*, including: dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene. Dioscorealide B tended to increase NK cells activity at 0.01 μg/ml but it significantly decreased lymphocyte proliferation at 0.1 μg/ml. Dioscorealide B and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene showed inhibitory activity on IL-6 production by LPS-stimulated RAW264.7 cells with IC_{50} value of 8.59 and 4.38 μg/ml, respectively. However, two isolated compounds from *D. membranacea* had no stimulatory effect on IL-10 production.

The ethanolic and water extracts of Benjakul had no toxic effect on PBMCs. The water extract of Benjakul preparation showed a significant effect on NK cell activity at concentration of 100 μg/ml. The water extract also significantly stimulated lymphocyte proliferation at 10 and 100 μg/ml. The water extract significantly increased IL-2 and IFN-γ production at high concentration. However, the water extract had no significant effect on IL-6, IL-1β and IL-10 production. For the

ethanolic extract of Benjakul preparation, it tended to stimulate NK cells activity and lymphocyte proliferation at low concentration (0.01-1 μg/ml). Futhermore, it showed significantly increased IL-2 and IFN-γ production. However, the ethanolic extract showed inhibitory effect on IL-1β and IL-6 production in RAW264.7 cells (IC_{50} = 62.93 and 51.83 μg/ml). The ethanolic extract of Benjakul preparation was used to isolated pure compounds such as piperine. Eventhough piperine had no significant effect on NK cells, it slightly increased NK cell activity at low concentration. Moreover, piperine showed inhibitory effect on IL-6 production with IC_{50} value of 16.71 μg/ml. The component plants in Benjakul, *P. chaba* and *P. interruptum*, showed TRPV1 and TRPA1 activation. The ethanolic extract of *P. chaba* showed adjuvant effect and activated dendritic cell trafficking and cytokine in mice. For the ethanolic extract of *P. interruptum*, it had no adjuvant effect.

From the present study, the ethanolic extract of *D. membranacea* showed highest potency for immunomodulatory activity because it showed significant effect on NK cells, lymphocyte proliferation. Moreover, it showed an inhibitory effect on inflammatory cytokine and no toxic effect on PBMCs. However, it should be used at low concentration to avoid toxicity effects. *S. corbularia* extract showed significant effect on NK cells and lymphocyte proliferation but it had no effect on anti-inflammatory activity. The ethanolic extract of *S. corbularia* should be used for immunomodulatory treatment. The ethanolic extract of Benjakul preparation showed low potency to NK cells and lymphocyte proliferation. But it showed anti-inflammatory activity. Moreover, component plants showed TRPV1 and TRPA1 activation and contact hypersensitivity reaction. However, Benjakul may be developed to anti-inflammatory agent but it should be concerned about side effect of this preparation because some components plant in Benjakul preparation showed contact hypersensitivity and induced sensory neuron. Benjakul should be investigated as an adjuvant and for TRP channel activation to confirm its side effect.

Further study of the immunomodulatory activities of *S.corbularia*, *D.membranacea* and Benjakul should be performed in animals to confirm this activity. Moreover, *D. membranacea* and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene should be performed in anti-inflammatory mechanism such as the activation of p38, JNK, and ERK1/2 MAPK pathway. In addition these data can

provide basic knowledge and can use to develop as an immunomodulatory medicine for cancer patients treatment.

REFERENCES

Books and Book Articles

Abbas, A. K., Litchman, A. H., & Pillai, S. (2010). *Cellular and Molecular Immunology* (6 th ed.). Philadelphia: W.B. Saunders Co.

Ancient Medicine Association. (1962). *Tamra ya silajaruek nai Wat Phra Chetuphonwimonmankalaram*. Bangkok: Mahamakut Buddhist University.

Ancient Medicine Association. (1978). *Pramuan supphakun ya Thai 1*. Bangkok: School of Wat Phra Chetuphon.

Burkill, I. H. (1951). Dioscoreaceae *Flora Malesiana* (Vol. 1).

Goldsby, R. A., Kindt, T. K., Osborne, B. A., & Kuby, J. (2003). *Immunology* (5th ed.). New York: W.H. Freeman and Company.

Itharat, A. (2002). *Study on bioactivity of five Thai medicinal plants called Hua-Khao-Yen.* (Doctoral dissertation), King's Colleage, University of London, London, UK.

Itharat, A., Sinchangchai, P., & Ratanasuwan, P. (1998). *Wisdom of Southern Thai traditional doctors.* Songkhla: Prince of Songkla University.

Itharat, A. (2002). *Study on bioactivity of five Thai medicinal plants called Hua-Khao-Yen.* (Doctor of philosophy in Pharmacognosy), King's Colleage, University of London, London, UK.

Itharat, A., Chunlaratthanabhorn, S., Vannasiri, S., Tappayuthpijarn, P., Sriyakul, K., & Kietinun, S. (2011). *Development of Benjakul extract as health products for cancer patients*. Pathumthani, Thailand: Faculty of Medicine, Thammasat university.

Khan, M. M. (2008). *Immunopharmacology*. New York, US: Springer.

Koyama, T. (1975). *Flora of Thailand* (Vol. 2). Bangkok: Applied Scientific Research Cooperation of Thailand.

Mutita. (1989). *Tamra ya jaruek nai wat Pho 1*. Bangkok.

Pongbunrod, S. (1976). *Mai Thiet Mueang Thai*. Bangkok: Kasembanakit.

Price, P., Sikora, K., & Illidge, T. (2008). *Treatment of Cancer* (Fifth Edtion ed.). USA: CRC Press.

Rattarom, R. (2013). *Regulatory of apoptosis on lung cancer cells through caspase-dependent mechanism by Benjakul extract and its ingredients.* (Doctor of philosophy in Medical Sciences), Thammasat University.

Ravindran, P. N., & Babu, K. N. (2004). *Ginger: The Genus Zingiber*. New York, USA: CRC Press.

Rosenbaum, T., & Simon, S. A. (2007). TRPV1 Receptors and Signal Transduction. In W. B. Liedtke & S. Heller (Eds.), TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades. Boca Raton (FL).

Ruangnoo, S. (2012). *Stability and Formulation Development of Hua-khao-yen Extracts as Anticancer Drug*: Faculty of Medicine, Thammasat University.

Sakpakdeejaroen, I. (2009). *Study on Cytotoxicity and Chemical Fingerprint of Ethanolic Extract of Benjakul Preparation*: Faculty of Medicine, Thammasat University.

Schmelzer, G. H., & Gurib-Fakim, A. (2008). *Medicinal Plants 1*. Wageningen, Netherlands: PROTA Foundation.

Sodsai, A. (2006). Study of immunomodulatory effect of Curcuma comosa Roxb. (Doctor of Philosophy in Toxicology), Mahidol University.

Sunila, E. S. (2014). *Anticancer antimetastatic and immunomodulatory activity of medicinal plants piper longum and thuja occidentalis.* (Doctor of Philosophy in immunology), University of Calicut.

Tungtrongjit, K. (1978). *Pramuan Suphakun Ya Thai* (Vol. 2). Bangkok.

Vimolkhunakorn, P. (1979). *Medicinal plant drug formular*. Nonthaburi: Wat Phikul Ngen.

Zabriskie, J. B. (2009). *Essential Clinical Immunology*. New York: Cambridge University Press.

Articles

Agarwal, S. S., & Singh, V. K. (1999). Immunomodulators: a review ofstudies on Indian medicinal plants and synthetic peptides Part II: synthetic peptides. *Proceedings of the Indian National Science Academy B65, 6,* 377-392.

Amorndoljai, P., Kietinun, S., & Somparn, N. (2011). Study on safety of Benjakul recipies extract tablets in normal volunteers. *Thammasat Medical Journal, 11*(2), 195-202.

Andrews, D. M., Scalzo, A. A., Yokoyama, W. M., Smyth, M. J., & Degli-Esposti, M. A. (2003). Functional interactions between dendritic cells and NK cells during viral infection. *Nature Immunology, 4*(2), 175-181.

Aravindaram, K., & Yang, N. S. (2010). Anti-inflammatory plant natural products for cancer therapy. *Planta Medica, 76*(11), 1103-1117.

Ariyanathan, S., Saraswathy, A., Rajamanickam, G. V., & Connolly, J. D. (2010). Polyphenols from the roots of *Plumbago rosea*. *Indian Journal of Chemistry, 49B*, 386-389.

Bae, G. S., Kim, M. S., Jung, W. S., Seo, S. W., Yun, S. W., Kim, S. G., Park R. K., Kim, E.C., Song, H. J., & Park, S. J. (2010). Inhibition of lipopolysaccharide-induced inflammatory responses by piperine. *European Journal of Pharmacology, 642*(1-3), 154-162.

Bae, J. H., Kim, J. Y., Kim, M. J., Chang, S. H., Park, Y. S., Son, C. H., Park, S.J., Chung, J. S., Lee, E. Y., Kim, S. H., & Kang, C. D. (2010). Quercetin enhances susceptibility to NK cell-mediated lysis of tumor cells through induction of NKG2D ligands and suppression of HSP70. *Journal of Immunotherapy, 33*(4), 391-401.

Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *Lancet, 357*(9255), 539-545.

Becknell, B., & Caligiuri, M. A. (2005). Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Advances in Immunology, 86*, 209-239.

Begum, F., Begum, Z. A., Uddin, M. R., Haider, A. H. M. Z., & Barman, R. C. (2012). Effects of methanol extract of Piper chaba stem bark on acute inflammation in rats. *Faridpur Medical College Journal, 7*(1), 26-28.

Bernard, M., Furlong, S. J., Power Coombs, M. R., & Hoskin, D. W. (2015). Differential Inhibition of T Lymphocyte Proliferation and Cytokine Synthesis by [6]-Gingerol, [8]-Gingerol, and [10]-Gingerol. *Phytotherapy Research, 29*(11), 1707-1713.

Bessac, B.F., Jordt, S.E. (2008). Breathing TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology*, 23(6), 360-370.

Boonyaratanakornkit, L., & Chantarateptawan, V. (1993). Identification and specification of Khaao-Yen-Neua, Khaao-Yen-Tai. *Thai Journal of Pharmaceutical Sciences, 17*, 79-90.

Boots, A. W., Wilms, L. C., Swennen, E. L., Kleinjans, J. C., Bast, A., & Haenen, G. R. (2008). In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition, 24*(7-8), 703-710.

Boyum, A. (1966). Separation of leucocytes from blood and bone marrow. *Scandinavian Journal of Clinical and Laboratory Investigation, 21*, 97.

Burodom, A., & Itharat, A. (2013). Inflammatory suppressive effect of Benjakul, a Thai traditional medicine on intestinal epithelial cell line. *Journal of Medicinal Plants Research, 7*(44), 3286-3291.

Chainakul, S., Supap, C., Tambunchong, C., & Picha, P. (2003). Study on chemical constituents and cytotoxicity of substances isolated from *Smilax corbularia* Kunth. on leukemic (P388) and breast carcinoa (MCF-7) cells. *Srinakharinwirot Science Journal, 19*, 108-114.

Chan, A., Hong, D. L., Atzberger, A., Kollnberger, S., Filer, A. D., Buckley, C. D., . . . Bowness, P. (2007). CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *The Journal of Immunology 179*(1), 89-94.

Chaplin, D. D. (2006). 1. Overview of the human immune response. *Journal of Allergy and Clinical Immunology, 117*(2 Suppl Mini-Primer), S430-435.

Charles, R., Garg, S. N., & Kumar, S. (2000). New gingerdione from the rhizomes of *Zingiber officinale*. *Fitoterapia, 71*, 716-718.

Chauvaltthamrong, P., Auttavich, A., Raksaman, P., & Jantapen, P. (1996). Subchronic toxicity of Benjakul traditional medicine. *Thai Journal of Pharmaceutical Sciences, 20*(1), 39-51.

Chaveerach, A., Mokkamul, P., Sudmoon, R., & Tanee, T. (2006). Ethanobotany of the genus *Piper* (Piperaceae) in Thailand. *Ethanobotany reseach and applications, 4*, 223-231.

Checker, R., Sharma, D., Sandur, S. K., Khanam, S., & Poduval, T. B. (2009). Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes. *International Immunopharmacology*, *9*(7-8), 949-958.

Chen, L., Shen, X., Hu, B., Lin, Y., Igbe, I., Zhang, C., Zhang, G., Yuan, X., & Wang, F. (2016). Nitric oxide production inhibition and mechanism of phenanthrene analogs in lipopolysaccharide-stimulated RAW264.7 macrophages. *Bioorganic & Medicinal Chemistry Letters*, *26*(10), 2521-2525.

Chiang, C. T., Way, T. D., Tsai, S. J., & Lin, J. K. (2007). Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. *FEBS Letters, 581*(30), 5735-5742.

Choi, Y. Y., Kim, M. H., Hong, J., Kim, S. H., & Yang, W. M. (2013). Dried Ginger (*Zingiber officinalis*) Inhibits Inflammation in a Lipopolysaccharide-Induced Mouse Model. *Evidence-Based Complementary and Alternative Medicine, 2013*, 914563.

Chuchawankul, S., Khorana, N., & Poovorawan, Y. (2012). Piperine inhibits cytokine production by human peripheral blood mononuclear cells. *Genetics and molecular research, 11*(1), 617-627.

Cordon-Cardo, C., & Prives, C. (1999). Commentary: at the crossroads of inflammation and tumorigenesis. *The Journal of Experimental Medicine, 190*, 1367-1370.

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420*(6917), 860-867.

Damsud, T., Adisakwattana, S., & Phuwapraisirisan, P. (2013). Three new phenylpropanoyl amides from the leaves of *Piper sarmentosum* and their a-glucosidase inhibitory activities. *Phytochemistry Letters*, *6*, 350-354.

Datla, P., Kalluri, M.D., Basha, K., Bellary, A., Kshirsagar, R., Kanekar, Y., Upadhyay, S., Singh, S., & Rajagopal, V. (2010). 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol, from Eulophia ochreata, inhibits inflammatory signalling mediated by Toll-like receptors. *British Journal of Pharmacology*, *160*(5), 1158-1170.

Desai, A. G., Qazi, G. N., Ganju, R. K., El-Tamer, M., Singh, J., Saxena, A. K., Bedi, Y. S., Taneja, S. C., & Bhat, H. K. (2008). Medicinal plants and cancer chemoprevention. *Current Drug Metabolism, 9*(7), 581-591.

Devan, P., Bani, S., Suri, K. A., Satti, N. K., & Qazi, G. N. (2007). Immunomodulation exhibited by piperinic acid through suppression of proinflammatory cytokines. *International Immunopharmacology, 7*(7), 889-899.

Dey, P., & Chaudhuri, T. K. (2014). In vitro modulation of TH1 and TH2 cytokine expression by edible tuber of *Dioscorea alata* and study of correlation patterns of the cytokine expression. *Food Science and Human Wellness, 3*, 1-8.

Dinarello, C. A. (2006). The paradox of pro-inflammatory cytokines in cancer. *Cancer and Metastasis Reviews, 25*(3), 307-313.

Dinda, B., & Chel, G. (1992). 6-Hydroxyplumbagin, a naphthoquinone from *Plumbago indica*. *Phytochemistry, 31*(10), 3652-3653.

Dinda, B., Chel, G., & Achari, B. (1994). A dihydroflavonol from *Plumbago indica*. *Phytochemistry, 35*(4), 1083-1084.

Ding, Y., Liang, Y., Deng, B., Qiao, A., Wu, K., Xiao, W., & Gong, W. (2014). Induction of TGF-beta and IL-10 production in dendritic cells using astilbin to inhibit dextran sulfate sodium-induced colitis. *Biochemical and Biophysical Research Communications, 446*(2), 529-534.

Dittmer, U., Peterson, K. E., Messer, R., Stromnes, I. M., Race, B., & Hasenkrug, K. J. (2001). Role of interleukin-4 (IL-4), IL-12, and gamma interferon in primary and vaccine-primed immune responses to Friend retrovirus infection. *Journal of Virology, 75*(2), 654-660.

Doucette, C. D., Rodgers, G., Liwski, R. S., & Hoskin, D. W. (2015). Piperine From Black Pepper Inhibits Activation-Induced Proliferation and Effector Function of T Lymphocytes. *Journal of Cellular Biochemistry, 116*(11), 2577-2588.

Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer, 4*(1), 11-22.

Du, Q., Li, L., & Jerz, G. (2005). Purification of astilbin and isoastilbin in the extract of *Smilax glabra* rhizome by high-speed counter-current chromatography. *Journal of Chromatography A, 1077*(1), 98-101.

Du, X., Pan, H., Zhang, C., Zhang, H., Liu, H., Chen, Z., & Zeng, X. (2010). *Zingiber officinale* extract modulates g-rays-induced immunosuppression in mice. *Journal of Medicinal Plants Research, 4*(16), 1647-1655.

Dzivenu, O. K., & O'Donnell-Tormey, J. (2003). *Cancer and the immune system: The vital connection*. New York: Cancer research institute.

Elgert, K., Alleva, D., & Mullins, D. (1998). Tumor-induced immune dysfunction: the macrophage connection. *Journal of Leukocyte Biology, 64*, 275-290.

Enshasy, H. E. (2010). Immunomodulators. In M. Hofrichter (Ed.), *Industrial Applications* (2nd ed., pp. 165-194). Heidelberg, Berlin: Springer-Verlag.

Feghali, C. A., & Wright, T. M. (1997). Cytokines in acute and chronic inflammation. *Frontiers in Bioscience, 2*, 12-26.

Fernandes, E. S., Fernandes, M. A., & Keeble, J. E. (2012). The functions of TRPA1 and TRPV1: moving away from sensory nerves. *British Journal of Pharmacology, 166*(2), 510-521.

Fireman, M., DiMartini, A. F., Armstrong, S. C., & Cozza, K. L. (2004). Immunosuppressants. *Psychosomatics, 45*(4), 354-360.

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research & Therapy, 8 Suppl 2*, S3.

Gardner, R. O. (2006). *Piper* (Piperaceae) in the Philippine islands: the climbing species. *BLUMEA, 51*, 569-586.

Gaspari, A.A., Katz, S.I. (2006). Contact hypersensitivity. *Current Protocols in Immunology* 113, 4.2.1-4.2.7.

Gatti, L. L., Burbano, R. R., de Assumpcao, P. P., Smith Mde, A., & Payao, S. L. (2004). Interleukin-1beta polymorphisms, *Helicobacter pylori* infection in individuals from Northern Brazil with gastric adenocarcinoma. *Clinical and Experimental Medicine, 4*(2), 93-98.

Gomathinayagam, R., Sowmyalakshmi, S., Mardhatillah, F., Kumar, R., Akbarsha, M. A., & Damodaran, C. (2008). Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells. *Anticancer Research, 28*(2A), 785-792.

Granucci, F., Vizzardelli, C., Pavelka, N., Feau, S., Persico, M., Virzi, E., Rescigno, M., Moro, G., & Ricciardi-Castagnoli, P. (2001). Inducible IL-2 production by

dendritic cells revealed by global gene expression analysis. *Nature Immunology, 2*(9), 882-888.

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell, 140*(6), 883-899.

Habib, S. H., Makpol, S., Abdul Hamid, N. A., Das, S., Ngah, W. Z., & Yusof, Y. A. (2008). Ginger extract (*Zingiber officinale*) has anti-cancer and anti-inflammatory effects on ethionine-induced hepatoma rats. *Clinics (Sao Paulo), 63*(6), 807-813.

Hannigan, B., Johnson, A. H., Collins, P. B., & Moriarty, M. (1984). Lymphocyte lactate dehydrogenase isoenzymes in association with depressed mitogen responsiveness. *Cell Biology International Reports, 8*(10), 857-865.

Heinz, S. A., Henson, D. A., Nieman, D. C., Austin, M. D., & Jin, F. (2010). A 12-week supplementation with quercetin does not affect natural killer cell activity, granulocyte oxidative burst activity or granulocyte phagocytosis in female human subjects. *British Journal of Nutrition, 104*(6), 849-857.

Hennino, A., Vocanson, M., Chavagnac, C., Saint-Mezard, P., Dubois, B., Kaiserlian, D., Nicolas, J.F. (2005). Update on the pathophysiology with special emphasis on CD8 effector T cells and CD4 regulatory T cells. *Anais Brasileiros de Dermatologia*, 80(4), 335-347.

Hiransai, P., Ratanachaiyavong, S., Itharat, A., Graidist, P., Ruengrairatanaroj, P., & Purintrapiban, J. (2010). Dioscorealide B suppresses LPS-induced nitric oxide production and inflammatory cytokine expression in RAW 264.7 macrophages: The inhibition of NF-kappaB and ERK1/2 activation. *Journal of Cellular Biochemistry, 109*(5), 1057-1063.

Huang, C., Li, J., Ma, W. Y., & Dong, Z. (1999). JNK activation is required for JB6 cell transformation induced by tumor necrosis factor-alpha but not by 12-O-tetradecanoylphorbol-13-acetate. *Journal of Biological Chemistry, 274*(42), 29672-29676.

Huang, C. F., Lin, S. S., Liao, P. H., Young, S. C., & Yang, C. C. (2008). The immunopharmaceutical effects and mechanisms of herb medicine. *Cellular & Molecular Immunology, 5*(1), 23-31.

Huang, H., Cheng, Z., Shi, H., Xin, W., Wang, T. T., & Yu, L. L. (2011). Isolation and characterization of two flavonoids, engeletin and astilbin, from the leaves of Engelhardia roxburghiana and their potential anti-inflammatory properties. *Journal of Agricultural and Food Chemistry, 59*(9), 4562-4569.

Huang, R. Y., Yu, Y. L., Cheng, W. C., OuYang, C. N., Fu, E., & Chu, C. L. (2010). Immunosuppressive effect of quercetin on dendritic cell activation and function. *Journal of Immunology, 184*(12), 6815-6821.

Hussain, S. P., Hofseth, L. J., & Harris, C. C. (2003). Radical causes of cancer. *Nature Reviews Cancer, 3*(4), 276-285.

Imai, Y., Kondo, A., Iizuka, H., Maruyama, T., & Kurohane, K. (2006). Effects of phthalate esters on the sensitization phase of contact hypersensitivity induced by fluorescein isothiocyanate. *Clinical & Experimental Allergy, 36*(11), 1462-1468.

Itharat, A. (2010). Cytotoxic effect and its mechanism of dioscorealide B from Dioscorea membranacea against breast cancer cells. *Journal of the medical association of thailand, 93*(Suppl 7), 277-282.

Itharat, A., Chunlaratthanabhorn, S., Vannasiri, S., Tappayuthpijarn, P., Sriyakul, K., & Kietinun, S. (2011). *Development of Benjakul extract as health products for cancer patients*. Pathumthani, Thailand: Faculty of Medicine, Thammasat university.

Itharat, A., & Hiransai, P. (2012). Dioscoreanone suppresses LPS-induced nitric oxide production and inflammatory cytokine expression in RAW 264.7 macrophages by NF-kappaB and ERK1/2 signaling transduction. *Journal of Cellular Biochemistry, 113*(11), 3427-3435.

Itharat, A., Houghton, P. J., Eno-Amooquaye, E., Burke, P. J., Sampson, J. H., & Raman, A. (2004). In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of Ethnopharmacology, 90*(1), 33-38.

Itharat, A., Kaewpradub, N., Plubrukarn, A., Chuchom, T., & Houghton, P. J. (2005). *A cytotoxic steroid saponin from Dioscorea membranacea*. Paper presented at the The 53rd Annual Meeting of the Society of Medicinal Plants Research and Joint Congress with the Italian Society of Phytochemistry, Florence, Italy. August 21-23, 2005.

Itharat, A., Plubrukarn, A., Kongsaeree, P., Bui, T., Keawpradub, N., & Houghton, P. J. (2003). Dioscorealides and dioscoreanone, novel cytotoxic naphthofuranoxepins, and 1,4-phenanthraquinone from Dioscorea membranacea Pierre. *Organic Letters, 5*(16), 2879-2882.

Itharat, A., & Sakpakdeejaroen, I. (2010). Determination of cytotoxic compounds of Thai traditional medicine called Benjakul using HPLC. *Journal of the medical association of thailand, 93 Suppl 7,* S198-203.

Itharat, A., Thongdeeying, P., & Ruangnoo, S. (2014). Isolation and characterization of a new cytotoxic dihydrophenanthrene from *Dioscorea membranacea* rhizomes and its activity against five human cancer cell lines. *Journal of Ethnopharmacology, 156*, 130-134.

Jaiaree, N., Itharat, A., & Kumapava, K. (2010). Cytotoxic saponin against lung cancer cells from *Dioscorea birmanica* Prain & Burkill. *Journal of the medical association of thailand, 93 Suppl 7,* S192-197.

Jaiaree, N., Ruangnoo, S., Thongdeeying, P., & Itharat, A. (2013). Cytotoxic compounds from *Dioscorea membranacea* against three types of colon cancer cells. *Journal of Health Research, 27*(1), 7-11.

Jan, T. R., Wey, S. P., Kuan, C. C., Liao, M. H., & Wu, H. Y. (2007). Diosgenin, a steroidal sapogenin, enhances antigen-specific IgG2a and interferon-gamma expression in ovalbumin-sensitized BALB/c mice. *Planta Medica, 73*(5), 421-426.

Janeway, C. A., & Travers, P. (1996). *Immunobiology: the immune system in health and disease*. U.S.A.: Garland Publishing.

Jiang, J., & Xu, Q. (2003). Immunomodulatory activity of the aqueous extract from rhizome of *Smilax glabra* in the later phase of adjuvant-induced arthritis in rats. *Journal of Ethnopharmacology, 85*(1), 53-59.

Jolad, S. D., Lantz, R. C., Solyom, A. M., Chen, G. J., Bates, R. B., & Timmermann, B. N. (2004). Fresh organically grown ginger (*Zingiber officinale*): composition and effects on LPS-induced PGE2 production. *Phytochemistry, 65*(13), 1937-1954.

Jung, D. H., Park, H. J., Byun, H. E., Park, Y. M., Kim, T. W., Kim, B. O., Um, S. H., & Pyo, S. (2010). Diosgenin inhibits macrophage-derived inflammatory

mediators through downregulation of CK2, JNK, NF-kappaB and AP-1 activation. *International Immunopharmacology, 10*(9), 1047-1054.

Jyothi, D., Vanathi, P., Mangala Gowri, P., Rama Subba Rao, V., Madhusudana Rao, J., & Sreedhar, A. S. (2009). Diferuloylmethane augments the cytotoxic effects of piplartine isolated from *Piper chaba*. *Toxicology in Vitro, 23*(6), 1085-1091.

Kao, T. K., Ou, Y. C., Raung, S. L., Lai, C. Y., Liao, S. L., & Chen, C. J. (2010). Inhibition of nitric oxide production by quercetin in endotoxin/cytokine-stimulated microglia. *Life Sciences, 86*(9-10), 315-321.

Kikuzaki, H., Tsai, S., & Nakatani, N. (1992). Gingerdiol related compounds from the rhizomes of Zingiber officinale. *Phytochemistry, 31*(5), 1783-1786.

Kim, D. S., Jeon, B. K., Lee, Y. E., Woo, W. H., & Mun, Y. J. (2012). Diosgenin Induces Apoptosis in HepG2 Cells through Generation of Reactive Oxygen Species and Mitochondrial Pathway. *Evidence-Based Complementary and Alternative Medicine, 2012*, 981675.

Kobayashi, C., Kurohane, K., & Imai, Y. (2012). High dose dietary pyridoxine induces T-helper type 1 polarization and decreases contact hypersensitivity response to fluorescein isothiocyanate in mice. *Biological and Pharmaceutical Bulletin, 35*(4), 532-538.

Kronke, M., Leonard, W. J., Depper, J. M., Arya, S. K., Wong-Staal, F., Gallo, R. C., Waldmann, T. A., & Greene, W. C. (1984). Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proceedings of the National Academy of Sciences, 81*(16), 5214-5218.

Kumar, S. V., Kumar, S. P., Rupesh, D., & Nitin, K. (2011). Immunomodulatory effects of some traditional medicinal plants. *Journal of Chemical and Pharmaceutical Research, 3*(1), 675-684.

Lauerova, L., Dusek, L., Simickova, M. K., I., Vagundova, M., Zaloudik, J., & Kovarik, J. (2002). Malignant melanoma associates with Th1/Th2 imbalance that coincides with disease progression and immunotherapy response. *Neoplasma, 49*(3), 159-166.

Lee, J. Y., & Park, W. (2011). Anti-inflammatory effect of myristicin on RAW 264.7 macrophages stimulated with polyinosinic-polycytidylic acid. *Molecules, 16*(8), 7132-7142.

Lee, S., & Margolin, K. (2011). Cytokines in Cancer Immunotherapy. *Cancers, 3*, 3856-3893.

Lee, S. W., Kim, M. S., Park, M. H., Park, S. J., Lee, W. S., Chang, J. S., & Rho, M. C. (2010). Alkamides from *Piper longum* and *Piper nigrum* as Inhibitors of IL-6 action. *Bulletin of the Korean Chemical Society., 31*(4), 921-924.

Lehmann, C., Zeis, M., & Uharek, L. (2001). Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. *British Journal of Haematology, 114*(3), 660-665.

Lin, Y., Wang, F., Yang, L.J., Chun, Z., Bao, J.K., & Zhang, G.L. (2013). Anti-inflammatory phenanthrene derivatives from stems of *Dendrobium denneanum*. *Phytochemistry, 95*, 242-251.

Liu, J., Li, X., Yue, Y., Li, J., He, T., & He, Y. (2005). The inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils. *Cellular & Molecular Immunology, 2*(6), 455-460.

Ljunggren, H. G., & Malmberg, K. J. (2007). Prospects for the use of NK cells in immunotherapy of human cancer. *Nature Reviews Immunology, 7*(5), 329-339.

Lugli, E., Ferraresi, R., Roat, E., Troiano, L., Pinti, M., Nasi, M., Nemes, E., Bertoncelli, L., Gibellini, L., Salomoni, P., Cooper, E. L., & Cossarizza, A. (2009). Quercetin inhibits lymphocyte activation and proliferation without inducing apoptosis in peripheral mononuclear cells. *Leukemia Research, 33*(1), 140-150.

Maeda, H., & Akaike, H. (1998). Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry (Moscow), 63*(7), 854-865.

Mahluji, S., Ostadrahimi, A., Mobasseri, M., Ebrahimzade Attari, V., & Payahoo, L. (2013). Anti-inflammatory effects of zingiber officinale in type 2 diabetic patients. *Advanced Pharmaceutical Bulletin, 3*(2), 273-276.

Makiya, S., Djati, M., Rifa, M., & Widodo, I. (2015). *Lymphocyte proliferation on hypersensitivity of Balb/C mice after given ethanol extract tuber of Dioscorea*

alata L. Paper presented at the The 3rd International Conference on Biological Science 2013.

Malek, T. R. (2008). The biology of interleukin-2. *Annu Rev Immunol, 26*, 453-479.

Mandal, A., & Viswanathan, C. (2015). Natural killer cells: In health and disease. *Hematology/Oncology and Stem Cell Therapy, 8*(2), 47-55.

Matsuda, H., Ninomiya, K., Morikawa, T., Yasuda, D., Yamaguchi, I., & Yoshikawa, M. (2008). Protective effects of amide constituents from the fruit of *Piper chaba* on D-galactosamine/TNF-alpha-induced cell death in mouse hepatocytes. *Bioorganic & Medicinal Chemistry Letters, 18*(6), 2038-2042.

McLoughlin, R. M., Hurst, S. M., Nowell, M. A., Harris, D. A., Horiuchi, S., Morgan, L. W., Wilkinson, T. S., Yamamoto, N., Topley, N., & Jones, S. A. (2004). Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms. *The Journal of Immunology, 172*(9), 5676-5683.

McNamara, F.N., Randall, A., Gunthorpe, M.J. (2005). Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *British Journal of Pharmacology*, 144, 781-790.

Meira, L. B., Bugni, J. M., Green, S. L., Lee, C. W., Pang, B., Borenshtein, D., Rickman, B. H., Rogers, A. B., Moroski-Erkul, C. A., McFaline, J. L., Schauer, D. B., Dedon, P. C., Fox, J. G., & Samson, L. D. (2008). DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. *ournal of Clinical Investigation, 118*(7), 2516-2525.

Melichar, B., Jandik, P., Krejsek, J., Solichova, D., Drahosova, M., Skopec, F., Mergancova, J., & Voboril, Z. (1996). Mitogen-induced lymphocyte proliferation and systemic immune activation in cancer patients. *Tumori, 82*(3), 218-220.

Mire-Sluis, A. R., & Thorpe, R. (1998). *Cytokines*. California, USA: Academic Press.

Morera, E., De Petrocellis, L., Morera, L., Moriello, A. S., Nalli, M., Di Marzo, V., & Ortar, G. (2012). Synthesis and biological evaluation of [6]-gingerol analogues as transient receptor potential channel TRPV1 and TRPA1 modulators. *Bioorganic & Medicinal Chemistry Letters*, 22(4), 1674-1677.

Moretta, A., Biassoni, R., Bottino, C., Mingari, M. C., & Moretta, L. (2000). Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Viewpoint Immunology Today, 21*(5), 228-234.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2), 55-63.

Nair, M. P., Kandaswami, C., Mahajan, S., Chadha, K. C., Chawda, R., Nair, H., Kumar, N., Nair, R. E., & Schwartz, S. A. (2002). The flavonoid, quercetin, differentially regulates Th-1 (IFNgamma) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochimica et Biophysica Acta, 1593*(1), 29-36.

Nakajima, C., Uekusa, Y., Iwasaki, M., Yamaguchi, N., Mukai, T., Gao, P., Tomura, M., Ono, S., Tsujimura, T., Fujiwara, H., & Hamaoka, T. (2001). A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Research, 61*(8), 3399-3405.

Nievergelt, A., Marazzi, J., Schoop, R., Altmann, K. H., & Gertsch, J. (2011). Ginger phenylpropanoids inhibit IL-1beta and prostanoid secretion and disrupt arachidonate-phospholipid remodeling by targeting phospholipases A2. *Journal of Immunology, 187*(8), 4140-4150.

Oft, M. (2014). IL-10: master switch from tumor-promoting inflammation to antitumor immunity. *Cancer Immunology Research, 2*(3), 194-199.

Okumura, Y., Narukawa, M., Iwasaki, Y., Ishikawa, A., Matsuda, H., Yoshikawa, M., & Watanabe, T. (2010). Activation of TRPV1 and TRPA1 by black pepper components. *Bioscience, Biotechnology, and Biochemistry, 74*(5), 1068-1072.

Opal, S. M., & De Palo, V. A. (2000). Anti-inflammatory cytokines. *Chest Journal, 117*(4), 1162-1172.

Park, H. J., Lee, C. M., Jung, I. D., Lee, J. S., Jeong, Y. I., Chang, J. H., Chun, S. H., Kim, M. J., Choi, I. W., Ahn, S. C., Shin, Y. K., Yeom, S. R., & Park, Y. M. (2009). Quercetin regulates Th1/Th2 balance in a murine model of asthma. *nternational Immunopharmacology, 9*(3), 261-267.

Patil, U. S., Jaydeokar, A. V., & Bandawane, D. D. (2012). Immunomodulators: a phamacological review. *Int J Pharm Pharm Sci, 4*(Suppl 1), 30-36.

Philip, M., Rowley, D. A., & H., S. (2004). Inflammation as a tumor promoter in cancer induction. *Seminars in Cancer Biology, 14*(433), 439.

Polland, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasi. *Nature Reviews Cancer, 4*(1), 71-78.

Poonthananiwatkul, B., Lim, R. H., Howard, R. L., Pibanpaknitee, P., & Williamson, E. M. (2015). Traditional medicine use by cancer patients in Thailand. *Journal of Ethnopharmacology, 168*, 100-107.

Prieto, J. M., Braca, A., Morelli, I., Barker, A., & Schaffiner, U. (2004). A new acylated quercetin glycoside from Ranunculus lanuginosus. *Fitoterapia, 75*(6), 533-538.

Qian, B. Z., & Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell, 141*(1), 39-51.

Quatromoni, J. G., & Eruslanov, E. (2012). Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *American Journal of Translational Research, 4*(4), 376-389.

Ragupathi, G., Damani, P., Deng, K., Adams, M. M., Hang, J., George, C., Livingston, P. O., & Gin, D. Y. (2010). Preclinical evaluation of the synthetic adjuvant SQS-21 and its constituent isomeric saponins. *Vaccine, 28*(26), 4260-4267.

Ragupathi, G., Yeung, K. S., Leung, P. C., Lee, M., Lau, C. B., Vickers, A., Hood, C., Deng, G., Cheung, N. K., Cassileth, B., & Livingston, P. (2008). Evaluation of widely consumed botanicals as immunological adjuvants. *Vaccine, 26*(37), 4860-4865.

Rajaraman, K., Batta, A. K., & Rangaswami, S. (1975). Structures of three new steroidal sapogenins from *Dioscorea prazeri*. *Journal of the Chemical Society 1*(16), 1560-1609.

Rajput, Z. I., Hu, S. H., Xiao, C. W., & Arijo, A. G. (2007). Adjuvant effects of saponins on animal immune responses. *Journal of Zhejiang University SCIENCE B, 8*(3), 153-161.

Rakoff-Nahoum, S. (2006). Why cancer and inflammation? *Yale Journal of Biology and Medicine, 79*(3-4), 123-130.

Rama Subba Rao, V., Suresh, G., Suresh Babu, K., Satyanarayana Raju, S., Vishnu vardhan, M. V. P. S., Ramakrishna, S., & Madhusudana Rao, J. (2011). Novel dimeric amide alkaloids from Piper chaba Hunter: isolation, cytotoxic activity, and their biomimetic synthesis. *Tetrahedron, 67*, 1885-1892.

Rama Subba Rao, V., Suresh Kumar, G., Sarma, V. U. M., Satyanarayana Raju, S., Hari Babu, K., Suresh Babu, K., Hari Babu, T., Rekha, K., & Madhusudana Rao, J. (2009). Chabamides F and G, two novel dimeric alkaloids from the roots of *Piper chaba* hunter. *Tetrahedron, 50*, 2774-2777.

Ramadan, G., Al-Kahtani, M. A., & El-Sayed, W. M. (2011). Anti-inflammatory and anti-oxidant properties of *Curcuma longa* (turmeric) versus *Zingiber officinale* (ginger) rhizomes in rat adjuvant-induced arthritis. *Inflammation, 34*(4), 291-301.

Rattarom, R., Sakpakdeejaroen, I., Hansakul, P., & Itharat, A. (2014). Cytotoxic activity against small cell lung cancer cell line and chromatographic fingerprinting of six isolated compounds from the ethanolic extract of Benjakul. *Journal of the medical association of thailand, 97*(8), 70-75.

Rattavalachai, T., Thitiorul, S., Tanuchit, S., Sakpakdeejaroen, I., & Itharat, A. (2012). In vitro cytotoxic effects of ethanolic extract of Pipkutbenjakul on human lymphocytes. *Thammasat Medical Journal, 12*(2), 262-268.

Razali, F. N., Ismail, A., Abidin, N. Z., Shuib, A. S. (2014). Stimulatory effects of plysaccharide fraction from Solanum nigrum on RAW 264.7 murine macrophage cells. *PLoS One*, 9(10), 1-8.

Reanmongkol, W., Itharat, A., & Bouking, P. (2007a). Evaluation of the anti-inflammatory, antinociceptive and antipyretic activities of the extracts from *Smilax corbularia* Kunth rhizomes in mice and rats (in vivo). *Songklanakarin Journal of Science and Technology, 29*(Suppl.1), 59-67.

Reanmongkol, W., Itharat, A., & Bouking, P. (2007b). Investigation of the anti-inflammatory, analgesic and antipyretic activities of the extracts from the rhizome of *Dioscorea membranacea* Pierre in experimental animals. *Songklanakarin Journal of Science and Technology*, *29*(Supp.1), 49-57.

Rey-Ladino, J., Ross, A. G., Cripps, A. W., McManus, D. P., & Quinn, R. (2011). Natural products and the search for novel vaccine adjuvants. *Vaccine, 29*(38), 6464-6471.

Rosenberg, S. A. (2014). IL-2: the first effective immunotherapy for human cancer. *Journal of Immunology, 192*(12), 5451-5458.

Ruangnoo, S., Itharat, A., Sakpakdeejaroen, I., Rattarom, R., Tappayutpijam, P., & Pawa, K. K. (2012). In vitro cytotoxic activity of Benjakul herbal preparation and its active compounds against human lung, cervical and liver cancer cells. *Journal of the medical association of thailand, 95 Suppl 1*, S127-134.

Ruangnoo, S., Jaiaree, N., Makchuchit, S., Panthong, S., Thongdeeying, P., & Itharat, A. (2012). An in vitro inhibitory effect on RAW 264.7 cells by anti-inflammatory compounds from *Smilax corbularia* Kunth. *Asian Pacific Journal of Allergy and Immunology, 30*(4), 268-274.

Rukachaisirikul, T., Siriwattanakit, P., Sukcharoenphol, K., Wongvein, C., Ruttanaweang, P., Wongwattanavuch, P., & Suksamrarn, A. (2004). Chemical constituents and bioactivity of *Piper sarmentosum*. *Journal of Ethnopharmacology, 93*(2-3), 173-176.

Saekoo, J., Dechsukum, C., Graidist, P., & Itharat, A. (2010). Cytotoxic effect and its mechanism of dioscorealide B from *Dioscorea membranacea* against breast cancer cells. *Journal of the medical association of thailand, 93*(Suppl.7), S277-S282.

Saetung, A., Itharat, A., Dechsukum, C., Wattanapiromsakul, C., Keawpradub, N., & Ratanasuwan, P. (2005). Cytotoxicity activity of Thai medicinal plants. *Songklanakarin Journal of Science and Technology, 27*(Suppl.2), 469-478.

Sakpakdeejaroen, I., & Itharat, A. (2009). Cytotoxic compounds against breast adenocarcinoma cells (MCF-7) from Pikutbenjakul. *Journal of Health Research, 23*(2), 71-76.

Salim, E., Kumolosasi, E., & Jantan, I. (2014). Inhibitory effect of selected medicinal plants on the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated human peripheral blood mononuclear cells. *Journal of Natural Medicines, 68*(3), 647-653.

Saraphanchotiwitthaya, A., Ingkaninan, K., & Sripalakit, P. (2007). Immunomodulating activity of Thai rejuvenating plants. *Naresuan University Journal 15*(3), 149-157.

Schmelzer, G. H., & Gurib-Fakim, A. (2008). *Medicinal Plants 1*. Wageningen, Netherlands: PROTA Foundation.

Shiba, T., Maruyama, T., Kurohane, K., Iwasaki, Y., Watanabe, T., & Imai, Y. (2009). TRPA1 and TRPV1 activation is a novel adjuvant effect mechanism in contact hypersensitivity. *Journal of Neuroimmunology, 207*(1-2), 66-74.

Shin, J.H., Ryu, J. H., Kang, M. J., Hwang, C.R., Han, J. Kang, D. (2013). Short-term heating reduces the anti-inflammatory effects of fresh raw garlic extracts on the LPS-induced production of NO and pro-inflammatory cytokines by downregulating allicin activity in RAW 264.7 macrophages. *Food and Chemical Toxicology*, 58, 545-551.

Singh, N., Kumar, S., Singh, P., GRaj, H. G., Prasad, A. K., Parmar, V. S., & Ghosh, B. (2008). *Piper longum* Linn. Extract inhibits TNF-a-induced expression of cell adhesion molecules by inhibiting NF-jB activation and microsomal lipid peroxidation. *Phytomedicine, 15*, 284-291.

Sireeratawong, S., Itharat, A., Lerdvuthisopon, N., Piyabhan, P., Khonsung, P., Boonraeng, S., & Jaijoy, K. (2012). Anti-Inflammatory, Analgesic, and Antipyretic Activities of the Ethanol Extract of *Piper interruptum* Opiz. and Piper chaba Linn. *ISRN Pharmacology, 2012*, 480265.

Smyth, M. J., Hayakawa, Y., Takeda, K., & Yagita, H. (2002). New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature Reviews Cancer, 2*(11), 850-861.

Sohn, Y., Han, N. Y., Lee, M. J., Cho, H. J., & Jung, H. S. (2013). [6]-Shogaol inhibits the production of proinflammatory cytokines via regulation of NF-kappaB and phosphorylation of JNK in HMC-1 cells. *Immunopharmacol Immunotoxicol, 35*(4), 462-470.

Song, S. H., Shen, X. Y., Ding, G. S., Liu, F., Wang, Z. M., & Fu, Z. R. (2010). Effects of astilbin on maturation and immunologic function of mouse bone marrow-derived dendritic cells. *Journal of Chinese integrative medicine, 8*(2), 145-151.

Spelman, K., Burns, J., Nichols, D., Winters, N., Ottersberg, S., & Tenborg, M. (2006). Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Alternative Medicine Review, 11*(2), 128-150.

Sriwanthana, B., & Chavalittumrong, P. (2001). In vitro effect of *Derris scandens* on normal lymphocyte proliferation and its activities on natural killer cells in normals and HIV-1 infected patients. *Journal of Ethnopharmacology, 76*(1), 125-129.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., & Schreiber, R. D. (1998). How cells respond to interferons. *Annual Review of Biochemistry, 67*, 227-264.

Strunz, G. M., & Finlay, H. (1995). Synthesis of sarmentosine, an amide alkaloid from *Piper sarmentosum*. *Phytochemistry, 39*(3), 731-733.

Sunila, E. S., & Kuttan, G. (2004). Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. *Journal of Ethnopharmacology, 90*(2-3), 339-346.

Sunila, E. S., & Kuttan, G. (2006). *Piper longum* inhibits VEGF and proinflammatory cytokines and tumor-induced angiogenesis in C57BL/6 mice. *International Immunopharmacology, 6*(5), 733-741.

Tewtrakul, S., & Itharat, A. (2006). Anti-allergic substances from the rhizomes of *Dioscorea membranacea*. *Bioorganic & Medicinal Chemistry Letters, 14*(24), 8707-8711.

Tewtrakul, S., & Itharat, A. (2007). Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. *Journal of Ethnopharmacology, 109*(3), 412-416.

Tewtrakul, S., Itharat, A., & Rattanasuwan, P. (2006). Anti-HIV-1 protease- and HIV-1 integrase activities of Thai medicinal plants known as Hua-Khao-Yen. *Journal of Ethnopharmacology, 105*(1-2), 312-315.

Thebpatiphat, S., RPengprecha, S., & Ternai, B. (1998). Some constituents of the stems of *Piper interruptum* Opiz. *Journal of The Science Society of Thailand, 14*, 225-231.

Thiengsusuk, A., Chaijaroenkul, W., & Na-Bangchang, K. (2013). Antimalarial activities of medicinal plants and herbal formulations used in Thai traditional medicine. *Parasitology Research, 112*(4), 1475-1481.

TraditionalLannaThaiMedicine. (1982). *The palm leaf test studies program*. Chiangmai, Thailand: A Joint Project between the National Museum of Ethnology Osaka Japan and Social Research Institute Chiang Mai University.

Uchida, K., Tominaga, Y., Haba, T., Katayama, A., Matsuoka, S., Goto, N., Ueki, T., Kimata, T., Takeda, A., Morozumi, K., Takagi, H., & Nakao, A. (2004). Clinical success of Neoral absorption profile. *Transplantation Proceedings, 36*(2 Suppl), 461S-464S.

Uhrberg, M. (2005). The CD107 mobilization assay: viable isolation and immunotherapeutic potential of tumor-cytolytic NK cells. *Leukemia, 19*(5), 707-709.

Venkatachalam, K., Montell, C. (2007). TRP Channels. *Annual Review of Biochemistry*, 76, 387-417.

Vickers, A. (2004). Alternative cancer cures:'unproven'or'disproven'? *Cancer Journal for Clinicians, 54*(2), 110-118.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology, 9*(5), 503-510.

Vogel, F. R. (1995). Immunologic adjuvants for modern vaccine formulations. *Annals of the New York Academy of Sciences, 754*, 153-160.

Vriens, J., Nilius, B., & Vennekens, R. (2008). Herbal compounds and toxins modulating TRP channels. *Current Neuropharmacology*, 6(1), 79-96.

Wadsworth, T. L., & Koop, D. R. (1999). Effects of the wine polyphenolics quercetin and resveratrol on Pro-inflammatory cytokine expression in RAW 264.7 macrophage. *Biochemical Pharmacology, 57*, 941-949.

Wang, L., Ma, T., Zheng, Y., Lv, S., Li, Y., & Liu, S. (2015). Diosgenin inhibits IL-1beta-induced expression of inflammatory mediators in human osteoarthritis chondrocytes. *International Journal of Clinical and Experimental Pathology, 8*(5), 4830-4836.

Watanabe, H., Unger, M., Tuvel, B., Wang, B., Sauder, D.N. (2002). Contact hypersensitivity: The mechanism of immune response and T cell balance. *J Interferon Journal of Interferon & Cytokine Research*, 22, 407-412.

Whiteside, T. L. (2001). Isolation of human NK cells and generation of LAK activity. *Current Protocols in Immunology, Chapter 7*, Unit 7 7.

Wungsintaweekul, B., Umehara, K., Miyase, T., & Noguchi, H. (2011). Estrogenic and anti-estrogenic compounds from the Thai medicinal plant, *Smilax corbularia* (Smilacaceae). *Phytochemistry, 72*(6), 495-502.

Yamaguchi, I., Matsuda, H., Zhang, H., Hamao, M., Yamashita, C., Kogami, Y., Kon'I, H., Yoshikawa, M. (2014). Adipogenic effects of piperlonguminine in 3T3-L1 cells and plasma concentrations of several amide constituents from *Piper chaba* extracts after treatment of mice. *Journal of Natural Medicines, 68*(1), 74-82.

Yan, R., & Xu, Q. (2001). Astilbin selectively facilitates the apoptosis of interleukin-2-dependent phytohemagglutinin-activated Jurkat cells. *Pharmacological Research, 44*(2), 135-139.

Yi, H. W., Lu, X. M., Fang, F., Wang, J., & Xu, Q. (2008). Astilbin inhibits the adhesion of T lymphocytes via decreasing TNF-alpha and its associated MMP-9 activity and CD44 expression. *International Immunopharmacology, 8*(10), 1467-1474.

Ying, X., Yu, K., Chen, X., Chen, H., Hong, J., Cheng, S., & Peng, L. (2013). Piperine inhibits LPS induced expression of inflammatory mediators in RAW 264.7 cells. *Cellular Immunology, 285*(1-2), 49-54.

Yu, B., & Lu, Y. (2014). Hapten-enhanced personalized chemoimmunotherapy by intratumoral drug delivery in advanced pancreatic cancer. *Journal of Clinical Oncology, 32*(suppl 3), 333.

Yu, E. S., Min, H. J., An, S. Y., Won, H. Y., Hong, J. H., & Hwang, E. S. (2008). Regulatory mechanisms of IL-2 and IFNgamma suppression by quercetin in T helper cells. *Biochem Pharmacol, 76*(1), 70-78.

Zaidi, M. R., & Merlino, G. (2011). The two faces of interferon-gamma in cancer. *Clinical Cancer Research, 17*(19), 6118-6124.

Zakaria, Z. A., Patahuddin, H., Mohamad, A. S., Israf, D. A., & Sulaiman, M. R. (2010). In vivo anti-nociceptive and anti-inflammatory activities of the aqueous extract of the leaves of *Piper sarmentosum*. *Journal of Ethnopharmacology, 128*(1), 42-48.

Zhai, W., Zhang, Z., Xu, N., Guo, Y., Qiu, C., Li, C., Deng, G., Guo, M. (2016). Piperine plays an anti-inflammatory role in *Staphylococcus aureus* endometritis by inhibiting activation of NF-κB and MAPK Pathways in mice. *Evidence-Based Complementary and Alternative Medicine*, *2016*, 1-10.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International Anesthesiology Clinics, 45*(2), 27-37.

Zhou, H. L., Deng, Y. M., & Xie, Q. M. (2006). The modulatory effects of the volatile oil of ginger on the cellular immune response in vitro and in vivo in mice. *Journal of Ethnopharmacology, 105*(1-2), 301-305.

Zou, T., Yang, Y., Xia, F., Huang, A., Gao, X., Fang, D., Xiong, S., & Zhang, J. (2013). Resveratrol Inhibits CD4+ T cell activation by enhancing the expression and activity of Sirt1. *PLoS One, 8*(9), e75139.

Other Materials

Bureau of Drug Control, T. (2012). National list of essential medicines. Retrieved from http://drug.fda.moph.go.th:81/nlem.in.th/medicine/herbal/list

Office of the Permanent Secretary for Public Health, M. o. P. H. (2011). Death rates per 100,000 population by leading cause of death and sex, whole kingdom : 2004-2011. Retrieved from http://service.nso.go.th/nso/web/statseries/statseries09.html

APPENDICES

APPENDIX A
CHEMICAL REAGENTS AND EQUIPMENTS FOR CELL CULTURE

1. RPMI 1640 medium

RPMI 1640 powder medium	10.4	g
Sodium bicarbonate	2.0	g

Adjust to pH 7.2-7.4 with 10% NaOH or 10% HCl.

Bring to 1,000 ml with sterile deionized water.

2. MTT solution 5 mg/ml

MTT powder	1	g

Adjust volume to 200 ml with sterile PBS and keep at 4°C (protect from light)

3. Dulbeco's modified eagle medium (DMEM)

DMEM powder medium	13.4	g
Sodium bicarbonate	3.7	g

Adjust to pH 7.2-7.4 with 10% NaOH or 10% HCl.

Bring to 1,000 ml with sterile deionized water.

4. Loading buffer

10X HANK's balanced salt solution	100	ml
1 M HEPES	20	ml
25 mM $CaCl_2$	40	ml
10% BSA	10	ml

Adjust volume to 1,000 ml with Milli Q water.

5. Phosphate buffer saline

PBS	1	tablet

Bring to 100 ml with deionized water

6. 96-well black plates coated with poly-L-lysine

Poly-L-lysine 40 µl was added to each well of 96-well black plate and incubated at room temperature for 20 minutes. Then, poly-L-lysine was removed from wells. The plate was washed once time with 200 µl of sterile Milli Q water. Finally, dry the plate in the larminar flow for 20 minutes. The plate was keep at 4°C and protected from the light.

APPENDIX B

CHEMICAL REAGENTS FOR ELISA

1. IL-1β, IL-6 and IL-10 ELISA Kit

1.1 1X Assay Diluent B

5X Assay Diluent B	15	ml

Adjust volume to 75 ml with sterile DI water. Keep at 4°C.

1.2 1X Wash buffer concentrate

20X wash buffer concentrate	25	ml

Adjust volume to 500 ml with sterile DI water. Mix gently to avoid foaming. Keep at 4°C.

1.3 1X Biotinylated Detection Antibody

Biotinylated Detection Antibody	1	vial
1X Assay Diluent B	100	µl

Keep at -20°C for 2 months. The detection antibody concentrate must be diluted 80-fold with 1X Assay Diluent B before use in Assay Procedure.

1.4 1X HRP-Streptavidin Solution

Spin the 200X HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use.HRP-Streptavidin concentrate must be diluted 200-fold with 1X Assay Diluent B prior to use in the Assay Procedure.

2. IL-2 and IFN-γ ELISA Kit

2.1 1X Wash buffer

20X Wash buffer concentrate	50	ml

Bring to final volume of 1,000 ml with sterile deionized water. Mix gently to avoid foaming. Keep at 2-25 °C for 30 days.

2.2 1X Assay buffer

20X Assay buffer concentrate 5 ml

Bring to final volume of 100 ml with sterile deionized water. Mix gently to avoid foaming. Keep at 2-25 °C for 30 days.

2.3 Biotin-Conjugate solution

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) before use. Biotin-Conjugate should be used within 30 minutes after dilution.

2.4 Streptavidin-HRP

Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) before use. Streptavidin-HRP should be used within 30 minutes after dilution.

APPENDIX C
RADIONUCLIDE DECAY TABLE

1. Chromium-51 decay table

Half-life = 27.7 days

Days	0	1	2	3	4	5	6	7	8	9
0	1	0.9753	0.9512	0.9277	0.9048	0.8824	0.8606	0.8394	0.8186	0.7984
10	0.7787	0.7594	0.7407	0.7224	0.7045	0.6871	0.6701	0.6536	0.6374	0.6217
10	0.6063	0.5913	0.5767	0.5625	0.5486	0.535	0.5218	0.5089	0.4963	0.4841
30	0.4721	0.4604	0.4491	0.438	0.4272	0.4166	0.4063	0.3963	0.3865	0.3769
40	0.3676	0.3585	0.3497	0.341	0.3326	0.3244	0.3164	0.3086	0.3009	0.2935
50	0.2862	0.2792	0.2723	0.2655	0.259	0.2526	0.2463	0.2403	0.2343	0.2285
60	0.2229	0.2174	0.212	0.2068	0.2017	0.1967	0.1918	0.1871	0.1825	0.178

2. ^{3}H-Thymidine decay table

Half-life = 12.28 years

Years \ Months	0	1	2	3	4	5	6	7	8	9	10	11
0	1.000	0.995	0.991	0.986	0.981	0.977	0.972	0.968	0.963	0.959	0.954	0.950
1	0.945	0.941	0.936	0.932	0.928	0.923	0.919	0.915	0.910	0.906	0.902	0.898
2	0.893	0.889	0.885	0.881	0.877	0.873	0.869	0.865	0.860	0.856	0.852	0.848
3	0.844	0.841	0.837	0.833	0.829	0.825	0.821	0.817	0.813	0.810	0.806	0.802
4	0.798	0.794	0.791	0.787	0.783	0.780	0.776	0.772	0.769	0.765	0.762	0.758
5	0.754	0.751	0.747	0.744	0.740	0.737	0.733	0.730	0.727	0.723	0.720	0.716
6	0.713	0.710	0.706	0.703	0.700	0.697	0.693	0.690	0.687	0.684	0.680	0.677
7	0.674	0.671	0.668	0.665	0.661	0.658	0.655	0.652	0.649	0.646	0.643	0.640
8	0.637	0.634	0.631	0.628	0.625	0.622	0.619	0.616	0.614	0.611	0.608	0.605
9	0.602	0.599	0.597	0.594	0.591	0.588	0.585	0.583	0.580	0.577	0.575	0.572
10	0.569	0.567	0.564	0.561	0.559	0.556	0.553	0.551	0.548	0.546	0.543	0.541
11	0.538	0.535	0.533	0.530	0.528	0.526	0.523	0.521	0.518	0.516	0.513	0.511
12	0.509	0.506	0.504	0.501	0.499	0.497	0.494	0.492	0.490	0.487	0.485	0.483

BIOGRAPHY

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Publications

Panthong, S., Ruangnoo, S., Thongdeeying, P., Sriwanthana, B., Itharat, A. (2014). Immunomodulatory activity of *Dioscorea membranacea* Pierre rhizomes and of its main active constituent Dioscorealide B. BMC Complement Altern Med. 14(403), 1-7.(Ph.D. publication)

Panthong, S., Itharat, A. (2014). Effect of *Piper chaba* Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz on natural killer cells activity and lymphocyte proliferation. J Med Assoc. 97(Suppl. 8), 133-139. (Ph.D. publication)

Panthong, S., Imai, Y., Matsuoka T., Suzuki, W., Watanabe, T., Terada, Y., Kurohane, K., Sekiguchi, K., Ogawa, E., Endo, Y., Itharat, A. (2016). The role of *Piper chaba* Hunt. and its pure compound, piperine, on TRPV1 activation and adjuvant effect. Phytomedicine. (In press). (Ph.D. publication)

Uttama, S., Itharat, A., Rattarom, R., Makchuchit, S., **Panthong, S**., Sakpakdeejaroen I. (2014). Biological activities and chemical content of Sung Yod Rice Bran Oil extracted by expression and soxhlet extraction methods. J Med Assoc. 97(Suppl. 8), 125-132.

Lairungruang, K., Itharat, A., **Panthong, S.** (2014). Antimicrobial activity of extracts from Thai traditional remedy called Kabpi for oral and throat infection and its plant components. J Med Assoc. 97(Suppl. 8), 108-115.

Panthong, S., Itharat, A., Reuangnoo, S., Thongdeeying, P., Sriwanthana, B. (2013). Immunomodulatory activity of *Dioscorea membranacea* Pierre and its compound. Planta Med. 79, p. 1267.

Jaiaree, N., Itharat, A., Suthon, P., **Panthong, S**. (2013). Anti-inflammatory, anti-bacterial and antioxidant activities of Thai medicinal plants for diarrheal treatment. Planta Med. 79, p. 1259.

Ruangnoo, S., Jaiaree, N., Makchuchit, S., **Panthong, S**., Thongdeeying, P., Itharat, A. (2012). An in vitro inhibitory effect on RAW 264.7 cells by anti-inflammatory compounds from *Smilax corbularia* Kunth. Asian Pac J Allergy Immunol. 30, 268-274.

Chunthorng-Orn, J., **Panthong, S**., Itharat, A. (2012). Antimicrobial, antioxidation activities and total phenolic content of Thai medicinal plants used to treat HIV patients. J Med Assoc. 95(Suppl. 1), 154-158.

Prajuabjinda, O., **Panthong, S**., Itharat, A. (2012). Antimicrobial activities of Thai medicinal preparation of Khampramong temple used for cancer treatment and its plant components. J Med Assoc. 95(Suppl. 1), 159-165.

Presentations

Panthong, S., Itharat, A. 2014. Study on natural killer cell activity of Three *Piper* species. (p.74). Book of program and abstracts of 18th World Congress on Clinical Nutrition, 1-3 December 2014 Ubon Ratchathani. Ubon Rachathani: Ubn Ratchathani Univercity.

Panthong, S., Itharat, A., Sakpakdeejaroen, I., Sriwanthana, B. 2014. Effect of *Piper interruptum* Opiz. on lymphocyte proliferation and natural killer cell activity. (p.73). Book of abstracts of 1st Joint ACS AGFD-ACS ICSCI Symposium on Agriculture and Food Chemistry, 4-5 March 2014 Montier Riverside Hotel Bangkok. Bangkok.

Panthong, S., Itharat, A., Sriwanthana, B., Ruangnoo, S. 2012. Study on lymphocyte proliferation of Hua-Khao-Yen. (p.58). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012 Thammasat University. Bangkok: Thammasat Press.

Pukdeekumjorn, P., Ruangnoo, S., **Panthong, S**., Itharat, A. 2012. In vitro antioxidant, anti-inflammatory and antimicrobial activities of *Cinnamomum poreectum* wood (Thep-Ta-Ro). (p.59). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012 Thammasat University. Bangkok: Thammasat Press.

Thongdeeying, P., Makchuchit, S., **Panthong, S**., Ruangnoo, S., Itharat, A. 2012. Inhibitory effect on LPS-induced PGE_2 release in RAW264.7 cells of compounds from *Dioscorea membranacea*. (p.54). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012 Thammasat University. Bangkok: Thammasat Press.

Suthon, S., Jaiaree, N., **Panthong, S**., Itharat, A. 2012. Comparison of antibacterial acitivity of the extracts from Thatbunjob and *Terminalla chebula* Retz.(p.45). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012 Thammasat University. Bangkok: Thammasat Press.

Panthong, S., Itharat, A., Ruangnoo, S., Sriwanthana, B. 2012. In vitro immunomodulatory activity of *Dioscorea membranacea*. (p.283). Abstract and proceeding of Natural products Research and development Center, 28-30 November 2012 Chiangmai Thailand. Chiangmai: Sangreeya Publishing Co.

Thongdeeying, P., Makchuchit, S., **Panthong, S**., Ruangnoo, S., Itharat, A. 2012. Inhibitory effect on LPS-induced PGE2 release in RAW264.7 cells of

Dioscorea membranacea extract and its ingredient. (p.440). Abstract and proceeding of Natural products Research and development Center, 28-30 November 2012 Chiangmai Thailand. Chiangmai: Sangreeya Publishing Co.

Suthon, P., Jaiaree, N., **Panthong, S**., Itharat, A. 2012. Antioxidant activity and total phenolic content of eleven Thai medicinal plants for diarrheal treatment.(p.287). Abstract and proceeding of Natural products Research and development Center, 28-30 November 2012 Chiangmai Thailand. Chiangmai: Sangreeya Publishing Co.

Awards

The 1st runner up award of oral presentation, "Immunomodulatory activity of Thai medicinal plants in adaptogenic remedy". TUMED Conference Practical Points for Best Practitioners, 11-12 July 2013, Thailand Science park Convention Center, Pathumthani, Thailand.

The winner award of oral presentation, "Immunomodulatory effect of Smilax corbularia and *Dioscorea membranacea* extracts". TUMED Conference Misconception in everyday practice, 14-16 October 2015, Faculty of Medicine, Thammasat University, Pathumthani, Thailand.