ANTI-ALLERGIC AND ANTI-INFLAMMATORY ACTIVITIES OF PRASAPROHYAI AND BENJAKUL REMEDIES USED FOR ALLERGIC RHINITIS

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MISS SUNITA MAKCHUCHIT

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
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ABSTRACT

Prasaprohyai and Benjakul remedies are Thai polyherbal formulation, which have been indicated in the list of herbal medicinal products of the national list of essential medicines of Thailand 2013. In Thai traditional medicine group, Prasaphohyai is used for treatments of fever, cold and allergy. It consists of nineteen plants namely rhizomes of *Kaempferia galanga* L., which the main component in 20 proportions and other components in equal proportions as each 1 proportion namely fruits of *Amomum testaceum* Ridl., fruits of *Anethum graveolens* L., roots of *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., roots of *Angelica sinensis* (Oliv.) Diels, all parts of *Artemisia annua* L., rhizomes of *Atractylodes lancea* (Thunb.) DC., fruits of *Cuminum cyminum* L., stems of *Dracaena loureiroi* Gagnep., fruits of *Foeniculum vulgare* Mill., seeds of *Lepidium sativum* L., rhizomes of *Ligusticum sinense* Oliv., flowers of *Mammea siamensis* (Miq.) T. Anders., flowers of *Mesua ferrea* L., flowers of *Mimusops elengi* L., stems, arils and seeds of *Myristica fragrans* Houtt., pollens of *Nelumbo nucifera* Gaertn., seeds of *Nigella sativa* L., and flowers of *Syzygium aromaticum* (L.) Merr. & L. M. Perry. Many of biological activities

of Prasaprohyai have also been reported including anti-allergy, anti-inflammation, antibacterial, anticancer, etc. However, it has not been reported on *in vivo* anti-allergy. In part of Benjakul, it has long been used for balanced health, controlled abnormal of element in the body, reliefed of flatulence, carminative and adaptogenic drug before using other drugs. Benjakul consists of five plants in equal proportions namely stems of *Piper interruptum* Opiz., fruits of *Piper longum* L., roots of *Piper sarmentosum* Roxb., roots of *Plumbago indica* L., and rhizomes of *Zingiber officinale* Roscoe. Benjakul has been reported on adaptogenicity, antimalarial, cytotoxicity, etc. But it has not been reported on *in vitro* and *in vivo* anti-allergy and *in vitro* anti-inflammation. Thus, the objectives of present study aimed to evaluate Benjakul the *in vitro* anti-allergic activity using antigen-induced β-hexosaminidase release in RBL-2H3 cells, *in vivo* anti-allergic activity using OVA-induced allergic rhinitis reaction in BALB/c mice, and *in vitro* anti-inflammatory activity using lipopolysaccharide (LPS)-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in RAW 264.7 cells. For Prasaprohyai, it was focused only *in vivo* anti-allergic effect. The isolation of pure compounds from *Atractylodes lancea* which showed to be active plant ingredient was investigated by using vacuum liquid chromatography (VLC) and medium pressure liquid chromatography (MPLC) techniques. The stability of crude extract and capsule products from these remedies were also determined.

Benjakul ethanolic extract exhibited the most potent β-hexosaminidase release with IC_{50} value of 12.69 μg/ml and showed potent inhibitory activity on NO production with IC_{50} value of 16.60 μg/ml, but not active on the release of TNF-α. Moreover, *Z. officinale* and *P. indica* ethanolic extracts, plant components of Benjakul, represented the high inhibition on β-hexosaminidase release (IC_{50} = 12.93 and 13.31 μg/ml, respectively) and also possessed the high potent effect on NO production (IC_{50} = 13.44 and 14.06 μg/ml, respectively) and the inhibitory effect on NO production of these plants showed higher activity than Benjakul ethanolic extract. The ethanolic extract of *P. interruptum* exhibited the potential inhibiton on TNF-α release (IC_{50} = 21.84 μg/ml). In addition, 6-shogaol and plumbagin, which are isolated

compounds from Benjakul ethanolic extract, showed the strongest activities on both β-hexosaminidase release (IC_{50} = 0.28 and 4.03 μg/ml, respectively) and NO production (IC_{50} = 0.92 and 0.002 μg/ml, respectively) but only 6-shogaol exhibited the potent effect on TNF-α relese (IC_{50} = 9.01 μg/ml).

BALB/c mice were sensitized with ovalbumin (OVA) mixed with alum intraperitoneally and then treated with three doses of both Prasaprohyai and Benjakul and challenged intranasally with OVA. Prasaprohyai (100 mg/kg) treatment group reduced the number of grades of mucus production in nasal tissues. It also suppresses OVA-specific IgE and IgG1 serum levels and increased OVA-specific IgG2a level. Moreover, IL-4, IL-5 and IL-13 mRNA expressions in the nasal tissues of allergic mice were inhibited by treatment with Prasaprohyai ethanolic extract (100 mg/kg). The expressions of Th1 response (IL-12p35) and Treg response (IL-10) were slightly increased. However, the expressions of IFN-γ, IL-12p40 and TGF-β were unchanged in the nasal mucosa. As for Benjakul, serum OVA-specific IgE and IgG1 levels, the number of grades of inflammatory cells and mast cells of allergic mice lessened after treated with Benjakul ethanolic extract (37.5 mg/kg). In addition, Benjakul (37.5 mg/kg) treatment group also showed down-regulation of IL-5 and IL-13, which are Th2 cytokines and slightly up-regulated IL-10 mRNA expression. However, the expressions of IFN-γ, IL-12p35 and IL-12p40 were down-regulated and the TGF-β mRNA level was unchanged in nasal tissues.

Three compounds including taraxerol acetate, isolated from *Atractylodes lancea*, atractylodin TS and β-eudesmol, authentic pure compounds from *A. lancea*, showed no effect on LPS-incuded NO production in RAW 264.7 cells. Atractylodin TS showed strong inhibitory activity on antigen-activated β-hexosaminidase release in RBL-2H3 cells (IC_{50} = 0.07 μg/ml) while β-eudesmol exhibited weak effect on this study (IC_{50} = 86.21 μg/ml).

The stability study after keeping under accelerated conditions (40°C, 75% RH for 6 months) of Prasaprohyai ethanolic extracts was unaltered in β-hexosaminidase release and NO production inhibition. While, Benjakul ethanolic

extract was unaltered in NO production but it altered β-hexosaminidase release at days 120, 150 and 180.

Three formulations consist of one formulation of Prasaprohyai (P3) and two formulations of Benjakul (B1 and B4) were prepared in solid dosage form as capsule containing 10% w/w of extract, Avecel® PH-101, Avecel® PH-102, lactose, Aerosil®, magnesium stearate and talcum having a total weight of 500 mg per capsule. Moreover, the weight variation and disintegration time of these capsules were performed in accordance with the BP 2013 standards. The stability test under accelerated conditions (40ºC, 75% RH for 6 months) of P3, B1 and B4 formulations were unchanged in the moisture content and NO production inhibition. Additionally, B1 and B4 formulations were unchanged in inhibiting β-hexosaminidase release but P3 formulation was changed in β-hexosaminidase release at days 150 and 180. The P3 formulation showed significantly increased on the release of β-hexosaminidase in RBL-2H3 cells when compared with day 0.

These results can be concluded that Benjakul ethanolic extract showed the high effects on *in vitro* anti-allergic activirty which inhibited the release of β-hexosaminidase and on *in vitro* anti-inflammatory activity which inhibited the NO production. Moreover, Benjakul and Prasaprohyai ethanolic extracts might be considered a potential therapeutic agent in treating allergic rhinitis. Benjakul ethanolic extract could be stored for one year at room temperature, due to the amount of plumbagin which rapidly changed. Therefore, B1 and B4 formulations could be stored for two years at room temperature without loss of activity. In addition, Prasaprohyai ethanolic extract could be stored for two years at room temperature; while P3 formulation could be stored for 1.5 years at room temperature without loss of activity. Thus, these results may provide some sciencetific support for the use of Benjakul and Prasaprohyai for treatment of allergy-related diseases and inflammation-related diseases.

Keywords: Allergic rhinitis, Anti-allergic activity, Anti-inflammatory activity, Benjakul, Cytokine, β-hexosaminidase, Mouse model, Nitric oxide, Prasaprohyai, Stability test, Thai traditional medicine

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Miss Sunita Makchuchit

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
°C	Degree(s) Celsius
%	Percent
>	More than
<	Less than
=	Equal
/	*Per*
μg	Microgram(s)
μg/ml	Microgram per milliliter
μl	Microliter(s)
μM	Micromolar
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
Anti-DNP IgE	Monoclonal anti-dinitrophenyl antibody
AR	Allergic rhinitis
BJK	Benjakul remedy
BSA	Bovine serum albumin
$CaCl_2.2H_2O$	Calcium chloride dihydrate
cDNA	Complementary DNA
$CHCl_3$	Chloroform
CO_2	Carbondioxide
CPM	Chlorpheniramine maleate
DMSO	Dimethylsulphoxide
DNP-BSA	Dinitrophenylated bovine serum albumin
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
g	Gram(s)

Symbols/Abbreviations	Terms
HCl	Hydrochloric acid
H & E	Hematoxylin and eosin
HPLC	High performance liquid chromatography
HRP	Houseradish peroxidase
IC_{50}	The half maximal inhibitory concentration
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
i.n.	Intranasal
i.p.	Intraperitoneal injection
KCl	Potassium chloride
LPS	Lipopolysaccharide
m	Milliliter(s)
M	Molar
MEM	Minimum essential medium
MeOH	Methanol
mg	Milligram(s)
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
mg/kg	Milligram per kilogram
mg/ml	Milligram per milliliter
ml	Milliliter(s)

Symbols/Abbreviations	Terms
mM	Millimolar
MPLC	Medium pressure liquid chromatography
mRNA	Messenger RNA
MT	Montelukast (sodium salt)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
ng/ml	Nanogram per milliliter
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance spectrum
NO	Nitric oxide
OD	Optical density
OVA	Ovalbumin
PAS	Periodic acid-Shchift
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% Tween-20
PCR	Polymerase chain reaction
pH	Power of hydrogen ion
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PNAG	4-Nitrophenyl N-acetyl-β-D-glucosaminide
P/S	Penicillin Streptomycin

Symbols/Abbreviations	Terms
PPY	Prasaprohyai remedy
qPCR	Quantitative real-time polymerase chain reaction
RAW 264.7	Murine macrophage cell line
RBL-2H3	Rat basophilic leukemia cell line
RH	Relative humidity
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
TGF-β	Transforming growth factor beta
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
Vis	Visible
VLC	Vacuum liquid chromatography
v/v	Volume by volume
w/w	Weight by weight

CHAPTER 1
INTRODUCTION

1.1 Background and rational

The allergy or type I hypersensitivity is the clinical manifestation of an unwanted immune response after repeated contact with normally harmless environmental substance that is known as an allergen, such as pollen, mold spores, animal dander, stinging insects, dust mites or foods (Garbo, Tessema, & Brown, 2013). Type I hypersensitivity also called immediate hypersensitivity is mediated mainly by the host specific IgE to an allergen that has gained entry into the host body may be by ingestion, inhalation, injection or direct contact (Hou, Xu, & Wang, 2011). The allergic response is evident within 30 minutes after encountering the allergen. When the host is first exposed to the allergen helper T cells secrete the cytokine IL-4, which in turn causes B cell to release IgE. This initial exposure is the sensitization period where usually no symptomatic reaction occurs. Memory cells are formed and no subsequent exposure to the allergen a more significant reaction can be elicited. IgE antibodies do not freely circulate, instead their tail portions attach to both mast cells and basophils, which produce and store inflammatory mediators. Mast cells tend to congregate at areas, which come in contact with environment. When a suitable allergen attaches to the IgE molecule it causes break of the mast cells or basophils and release of an abundance of inflammatory mediators (O'Connor & Nichol, 2015).

One clinical manifestation, allergic rhinitis (AR), is defined as inflammation of the nasal mucous membranes resulting from an IgE-mediated immunological reaction following exposure to allergen. The AR symptoms are sneezing, itching (nasal pruritus), nasal congestion or blockage (upper airway obstruction), runny nose (nasal discharge or rhinorrhea), and postnasal drip (Brozek et al., 2017; May & Dolen, 2017). Although allergic rhinitis is a major human health problem and leading cause of death in the world, it is a major cause of morbidity and decreased quality of life by

affecting sleep, school, work productivity, and social life (Lim & Leong, 2010; May & Dolen, 2017). The prevalence of allergic rhinitis is increasing worldwide, particularly in low and middle income countries. Over the last few decades, it is reported to affect 10% to 40% of the global population and its prevalence is increasing both in children and adults (Bousquet, Van Cauwenberge, & Khaltaev, 2004). The prevalence of AR is estimated to be approximately 12% to 30% in USA, 5.5% to 45.1% in Latin America, 23% to 30% in Europe, 7.2% to 54.1% in Africa, 7.4% to 45.2% in Middle East, 12% to 41.3% in Australia, and 12% to 30% in South East Asia (Gutiérrez-Cardona et al., 2017). In Thailand, the incidence of allergy increases every year, and is predicted to increase 300-400% within the next 20 years (Tungsukruthai, Nootim, Worakunphanich, & Tabtong, 2018). Recently, Thai people suffer from allergy approximately 25% of the population or 16-17 million people (Bunnag, Jareoncharsri, Tantilipikorn, Vichyanond, & Pawankar, 2009). Furthermore, the incidence of AR in Thailand is about 10% to 25% of the population, approximately 14% to 44% of Thai children and 20% of Thai adults (Bunjean et al., 2012).

The inflammation is a pervasive phenomenon that operates during severe perturbations of homeostasis, such as infection like bacteria, viruses or fungi, injury like scrapes or foreign objects, and exposure to contaminants like chemicals. It is triggered by innate immune receptors that recognize pathogens and damaged cells (Ashley, Weil, & Nelson, 2012). The major symptoms of inflammation are characterized by redness, heat, swelling, pain and loss of function. These symptoms are due to dilation of the blood vessels increases the flow of blood to the inflamed regions, whereas increased permeability of the vessel wall allows plasma constituents to enter the surrounding injured tissue, and circulating white blood cells also emigrate from the blood to the tissues, which clear microbes and debris and initiate reactions of the immune response (Furie, 2017; Iwalewa, McGaw, Naidoo, & Eloff, 2007).

Inflammation is divided into two categories: as an acute inflammation and a chronic inflammation. Acute inflammation is short-lasting process occurring from few minutes up to few days. The acute inflammatory may be regarded as the first line of defense against injury. It is characterized by change in the

microcirculation: exudation of fluid and emigration of leukocytes from blood vessels to the area of injury. While, chronic inflammation is an inflammatory response of prolonged duration, days to year, and is typified by influx of lymphocytes and macrophages with associated vascular proliferation and scarring. The tissues affected by chronic inflammation commonly show evidence of the pathologic processes, such as immune response, phagocytosis, necrosis, and repair (Chandrasoma & Taylor, 1998).

Nowadays, natural products continue to be extremely important as sources of medicinal agents and are believed to be highly important as source of new chemical substances with potential therapeutic effects. The use of plants as medicines has a long history in the treatment of many diseases worldwide. Plants especially those with ethanopharmacological uses have been the primary sources of medicine for early drug discovery (Veeresham, 2012).

In Thailand, Prasaprohyai remedy is a Thai traditional medicine commonly used for fever and cold treatment. Prasaprohyai consists of nineteen medicinal plants: fruit of *Amomum testaceum* Ridl., fruit of *Anethum graveolens* L., root of *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., root of *Angelica sinensis* (Oliv.) Diels, all parts of *Artemisia annua* L., rhizome of *Atractylodes lancea* (Thunb.) DC., fruit of *Cuminum cyminum* L., stem of *Dracaena loureiroi* Gagnep., fruit of *Foeniculum vulgare* Mill., rhizome of *Kaempferia galanga* L., seed of *Lepidium sativum* L., rhizome of *Ligusticum sinense* Oliv., flower of *Mammea siamensis* (Miq.) T. Anders., flower of *Mesua ferrea* L., flower of *Mimusops elengi* L., stem, aril and seed of *Myristica fragrans* Houtt., pollen of *Nelumbo nucifera* Gaertn., seed of *Nigella sativa* L., and flower of *Syzygium aromaticum* (L.) Merr. & L. M. Perry (National Drug Committee, 2013). The Prasaprohyai remedy has been studied on anti-allergic, anti-inflammatory and antioxidant activities (Makchuchit, 2010), antimalarial activity (Thiengsusuk, Chaijaroenkul, & Na-Bangchang, 2013), antimicrobial activity (Sattaponpan & Kondo, 2011), and cytotoxic activity (Mahavorasirikul, Viyanant, Chaijaroenkul, Itharat, & Na-Bangchang, 2010). In addition, it has been report on analgesic, anticancer, anti-inflammatory and antipyretic effects in animal model

(Plengsuriyakarn et al., 2012). However, there is no report on anti-allergic activity of Prasaprohyai in animal model.

According to the national list of essential medicine (National Drug Committee, 2013), Benjakul remedy is Thai traditional medicine usually used for controlling abnormality of elements in the body and balancing health. Benjakul composes of five plants: stem of *Piper interruptum* Opiz., fruit of *Piper longum* L., root of *Piper sarmentosum* Roxb., root of *Plumbago indica* L., and rhizome of *Zingiber officinale* Roscoe. The Benjakul remedy has been reported on biological activities, such as antimalarial activity (Thiengsusuk et al., 2013), anti-inflammatory effect (Burodom & Itharat, 2013), antimicrobial activity (Kondo, Sattaponpan, Phongpaichit, Srijan, & Itharat, 2010), genotoxic activity (Ratanavalachai, Thitiorul, Tanuchit, Sakpakdeejaroen, & Itharat, 2012), and cytotoxicity against many types of cancer cells (Mahavorasirikul et al., 2010; Rattarom, Sakpakdeejaroen, Hansakul, & Itharat, 2014; Rattarom, Sakpakdeejaroen, & Itharat, 2010; Sakpakdeejaroen & Itharat, 2009). Moreover, Benjakul has been reported in the clinical study. The Benjakul tablet did not change of clinical signs (phase I) (Amorndoljai, Kietinun, & Somparn, 2011). The Benjakul capsule showed clinical efficacy in relieving symptoms of primary osteoarthritis (OA) knee (Rachawat, Pinsornsak, Kanokkangsadal, & Itharat, 2017). However, there is no report about *in vitro* anti-inflammatory effect of Benjakul and anti-allergic activity of Benjakul both *in vitro* and *in vivo* model.

Therefore, the objectives of this study were to investigate the effects of Prasaprohyai and Benjakul remedies, Benjakul's plant components and Benjakul's pure compounds on anti-allergic and anti-inflammatory effects. Moreover, isolation of pure compounds of *Atractylodes lancea*, one of main active plant which showed high biological activities in Prasaprohyai remedy, was also tested on anti-allergy and anti-inflammation. In addition, formulations of capsule products from these remedies were produced. Finally, the stability studies of the formulations were also investigated. These investigations may provide some scientific support for the use of Prasaprohyai and Benjakul for the allergic and inflammatory treatment.

1.2 Objectives

1.2.1 Overall objective

Overall objective of this study was to investigate anti-allergic and anti-inflammatory activities of Prasaprohyai, Benjakul, Benjakul's plant ingredients and Benjakul's pure compounds by *in vitro* study and also tested anti-allergic effect by *in vivo* study. Additionally, formulations of the capsule products from Prasaprohyai and Benjakul for allergic rhinitis drug were also investigated.

1.2.2 Specific objectives

1.2.2.1 To investigate anti-allergic activity of ethanolic extract of Prasaprohyai, Benjakul, Benjakul's plant ingredients and Benjakul's pure compounds using RBL-2H3 cells.

1.2.2.2 To investigate anti-allergic activity of ethanolic extract of Prasaprohyai and Benjakul using BALB/c mice.

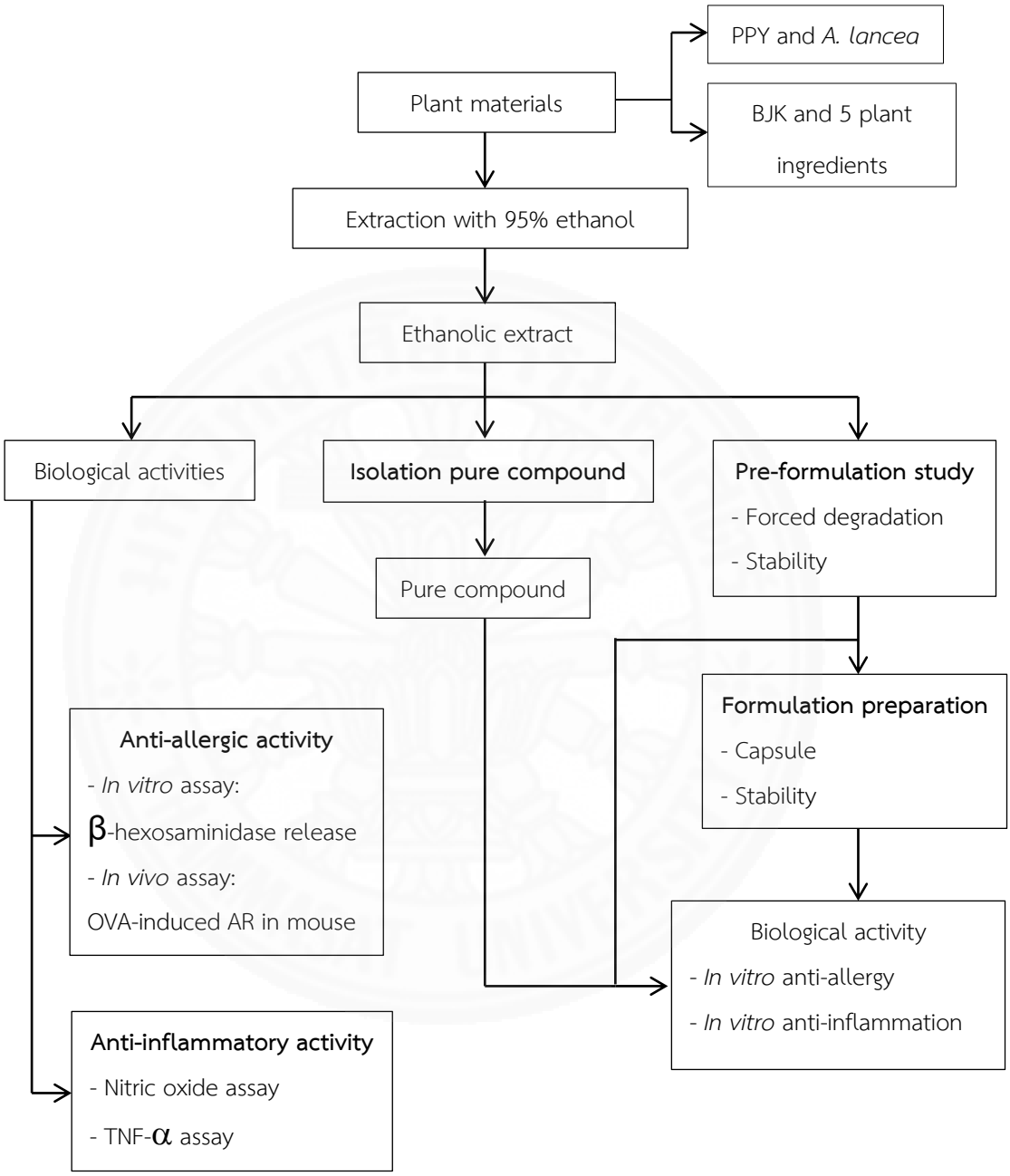
1.2.2.3 To investigate anti-inflammatory activity of ethanolic extract of Prasaprohyai, Benjakul, Benjakul's plant ingredients and Benjakul's pure compounds using RAW 264.7 cells.

1.2.2.4 To isolate and characterize pure compound from *Atractylodes lancea* and study anti-allergic and anti-inflammatory activities using cell line.

1.2.2.5 To prepare capsule formulations from ethanolic extract of Prasaprohyai and Benjakul.

1.2.2.6 To determine stability of the ethanolic extracts and capsule formulation of Prasaprohyai and Benjakul under accelerated storage conditions.

1.3 Conceptual framework



CHAPTER 2
REVIEW OF LITERATURE

2.1 Allergy

Allergy is a hypersensitive state acquired through exposure to a particular allergen, re-exposure to which produces a heightened capacity to react. Allergic reactions cover a broad range of clinical manifestations, from mild, delayed reactions developing as long as 48 hours after exposure to the antigen, to immediate and life-threatening reactions developing within seconds of exposure (Malamed, 2015).

Hypersensitivity diseases are classified according to the immune response and the effector mechanism responsible for cell and tissue injury (Table 2.1). This classification was originally developed by Philip Gell and Robin Coombs (Abbas, Lichtman, & Pillai, 2012). The most common form of allergy is IgE-mediated hypersensitivity. Sensitization can occur: following allergen exposure in the airways by inhalation, in the gastrointestinal tract by ingestion, in body fluids by insect sting or in the skin by physical contact. Regardless of the route of exposure, symptoms can manifest in one or more tissues; for example, in the eyes (conjunctivitis), nose (rhinitis), lungs (asthma), skin, either as a rash (urticarial), inflammation (atopic dermatitis), or swelling (angioedema), or in the whole body accompanies by a drop in blood pressure (anaphylaxis) (Figure 2.1) (Larsen, Broge, & Jacobi, 2016).

Table 2.1 Classification of immunologic diseases

Type of hypersensitivity	**Pathologic immune mechanisms**	**Mechanisms of tissue injury and disease**
Immediate hypersensitivity (type I)	IgE antibody	Mast cells and their mediators (vasoactive amines, lipid mediators, cytokines)
Antibody mediated (type II)	IgM, IgG antibodies against cell surface or extracellular matrix antigens	Opsonization and phagocytosis of cells Complement- and Fc receptor-mediated recruitment and activation of leukocytes (neutrophils, macrophages) Abnormalities in cellular functions, e.g., hormone receptor signaling
Immune complex mediated (type III)	Immune complexes of circulating antigens and IgM or IgG antibodies	Complement- and Fc receptor-mediated recruitment and activation of leukocytes
T cell mediated (type IV)	$CD4^+$ T cell (cytokine-mediated inflammation) $CD8^+$ CTLs (T cell-mediated cytolysis)	Recruitment and activation of leukocytes Direct target cell killing, cytokine-mediated inflammation

Note. From *Cellular and Molecular Immunology* (p. 408), by A. K. Abbas, A. H. Lichtmam, and S. Pillai, 2012, Philadelphia, PA: Saunders.

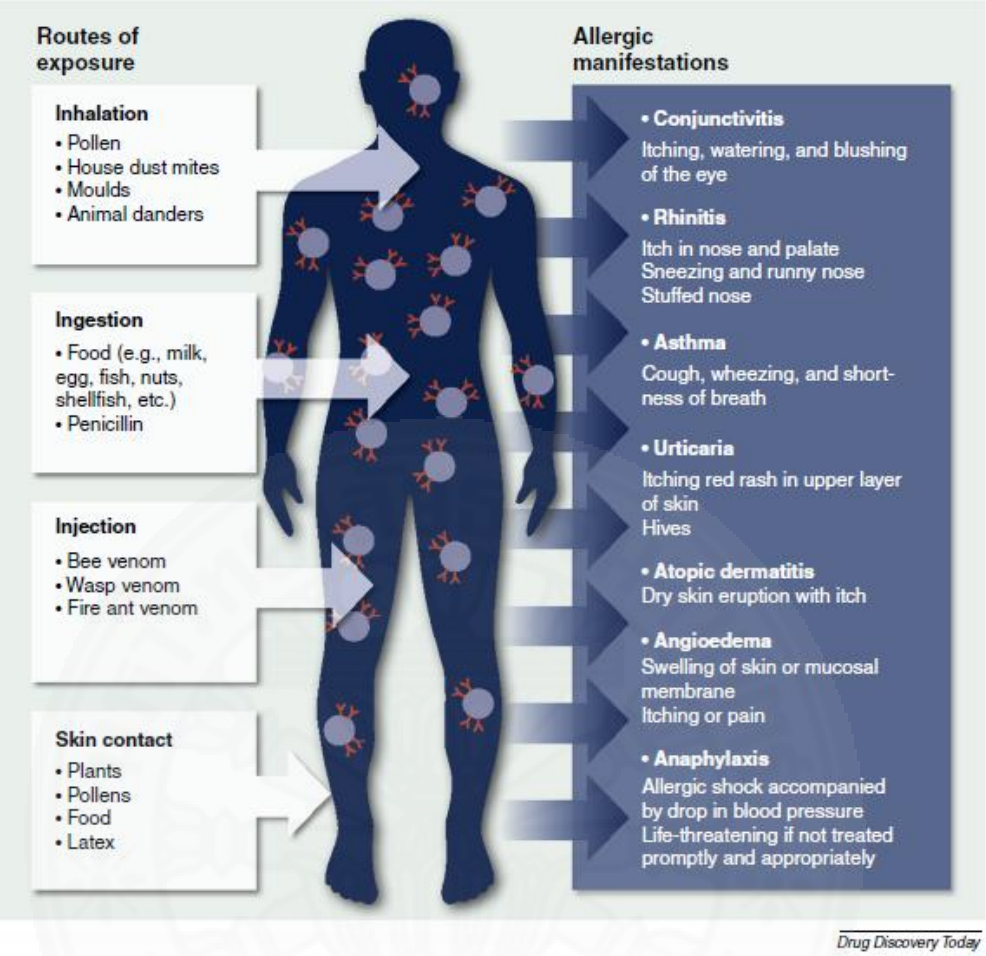


Figure 2.1 The route of exposure and allergic manifestations (Larsen et al., 2016)

2.1.1 Mechanisms of allergy

During sensitization to allergen, the sensitization is triggered when the new allergen is taken up by dendritic cells (DCs), which are professional antigen-presenting cells (APCs), or macrophages in the airway lumen or in the epithelium of the airway mucosa. Result in uptake of the allergen by DCs is into peptide fragments. The allergenic peptide obtained in the activated DC is presented *via* major histocompatibility complex (MHC) class II molecule on DC to $CD4^+$ T cell receptor (TCR) on naïve T cell. After antigen presented by DCs, naïve $CD4^+$ T cells can differentiate into the well-established Th1 and Th2 effector cells and more recently discovered Th9, Th17, Th22, Treg and Tfh effector cells based on micro-environmental stimuli to they are exposed in the presence of antigen. Allergic

diseases are primarily driven by Th2 cells, with IL-4, IL-5 and IL-13 as the major cytokines, and are opposed by the activity of Treg cells (Wambre, James, & Kwok, 2012). Th2 cells are involved in other components of the immediate hypersensitivity reaction in addition to promotion of switching to IgE. An ordered sequence of T-B cell interactions involving both cell-cell contact and secreted cytokines drives class switching. Antigenic peptides displayed on the B cell surface, bound to MHC II molecules, engage TCR of the Th cells off the same antigenic specificity (a cognate interaction) leading both to cytokine transcription, including IL-4 and to expression of CD40L (CD154), an activation molecule not present on resting Th cells. CD40L binding to CD40 (back on the presenting B cell), along with IL-4, drives germline transcription and activates the expression of components of the pathway of deletional class switching. CD40 activation also drives expression of B7 family costimulatory molecules, which engage receptors on the Th cell and amplify cytokine responses and proliferation (Oettgen & Broide, 2012). Allergen-specific IgE produced by plasmablasts and plasma cells enters the circulation and binds to Fc receptors on tissue mast cells, so that these cells are sensitized and poised to react to a subsequent encounter with the allergen. Circulating basophils are also capable of binding IgE (Figure 2.2A) (Abbas et al., 2012).

During re-exposure to the same allergen, IgE sensitizes mast cells and basophils by binding the high-affinity receptor or IgE (Fc**ε**RI), which is expressed at the surface of these cells. On crosslinking of the IgE-Fc**ε**RI (high-affinity receptor for IgE) complexes by allergen, mast cells and basophils degranulate, releasing vasoactive amines (such as histamine), lipid mediators (such as prostaglandin D, platelet-activating factor (PAF), and leukotriene C_4 (LTC_4), LTD_4 and LTE_4), chemokines (CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 2 (CCL2), CCL4 and CCL5) and other cytokines (such as IL-4, IL-5 and IL-13), all of which characterize the immediate phase of the allergic reaction (Figure 2.2B). IgE also binds Fc**ε**RI at the surface of dendritic cells (DCs) and monocytes, as well as the low-affinity receptor for IgE, Fc**ε**RII (also known as CD23), at the surface of B cells. These process increases the uptake of allergen by these antigen-presenting cells (APCs) and the subsequent

presentation of allergen-derived peptide to specific $CD4^+$ T cells, which drive the late phase of allergic reaction. Following migration to sites of allergen exposure under the influence of chemokines and other cytokines, allergen-specific T cells are reactivated and clonally expand. Local IgE-facilitated antigen presentation by DCs increases T cells activation. Local IgE production is seen in allergic rhinitis and asthma but not in allergic skin inflammation. Eosinophils are one of the main inflammatory cells in the lungs of asthmatic individuals but not in skin of those with atopic dermatitis. Th1 cells, which produce interferon-γ (IFN-γ) and tumor necrosis factor (TNF), contribute to the activation and apoptosis of keratinocytes (in the skin), bronchial epithelial cells and pulmonary smooth-muscle cells. Activation of mast cells and basophils, which release histamine, chemokines and other cytokines, also contributes to the late phase allergic reaction (Figure 2.2C) (Larché, Akdis, & Valenta, 2006).

2.1.2 Cytokines in allergy

Cytokines are secreted proteins with growth, differentiation, and activation functions that are critical in allergic intercellular communication networks, and they contribute to disease pathology through the recruitment and activation of pro-inflammatory leukocytes and in chronic disease to pro-fibrotic/remodeling events (Commins, Borish, & Steinke, 2010; Williams, Rahman, Hubeau, & Ma, 2012).

2.1.2.1 Th1 cytokines

Th1 cells secrete a Th1 profile of cytokines, after cognate stimulation of the naïve T cell by antigen presenting cells (APCs), such as dendritic cell and macrophage. Th1 cells form a natural counterbalance to Th2 cells driving protective cell-mediated immunity, and are induced on exposure to foreign agents. Th1 cytokines include IL-12 and interferon-γ, which inhibit Th2 responses (Chung, 2001).

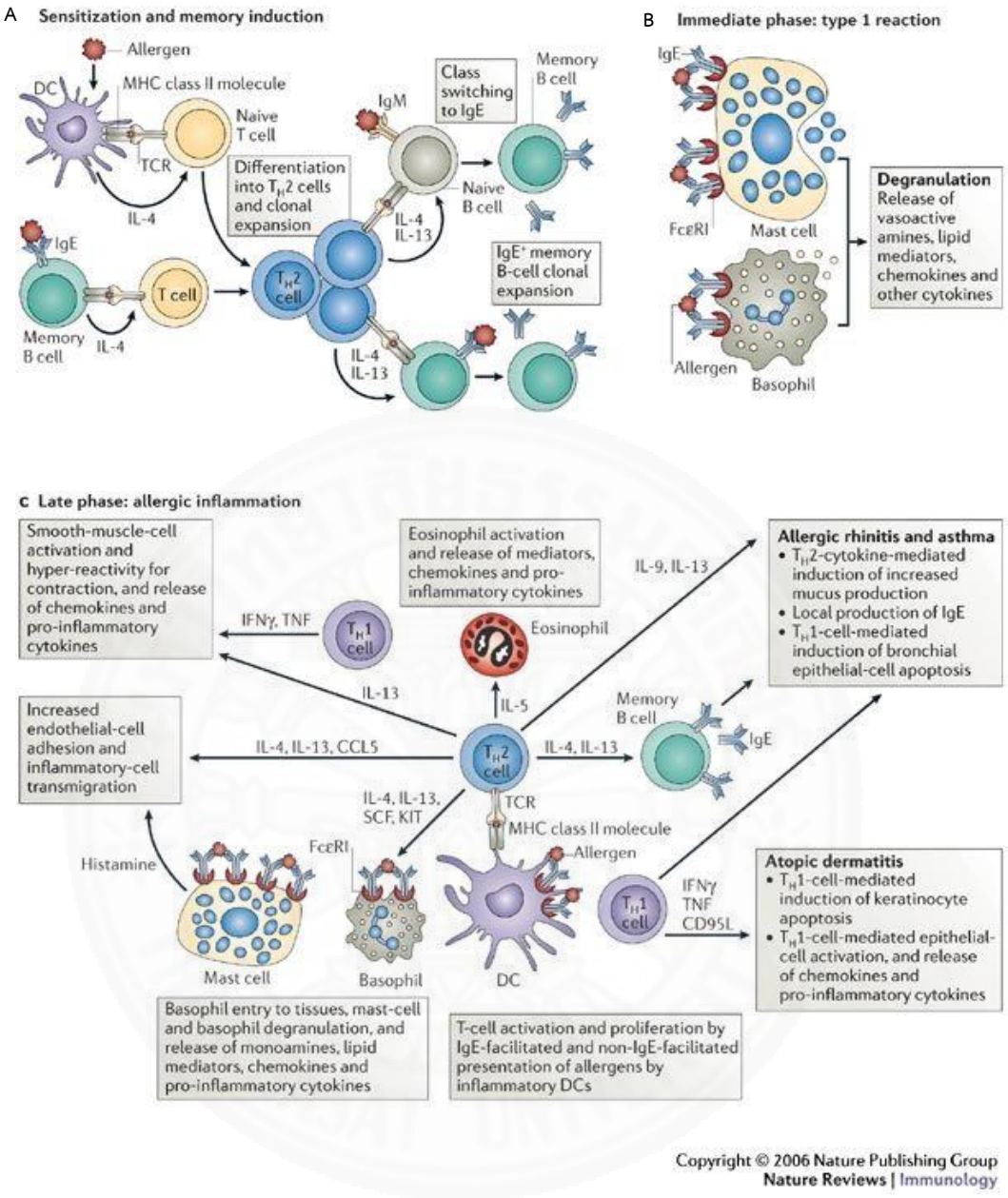


Figure 2.2 Mechanisms of allergic reactions. (A) Sensitization to allergens and development of specific B cell and T cell memory. (B) Type I hypersensitivity reaction or immediate phase of the allergic reaction. (C) Allergic inflammation or late phase of the allergic reaction (Larché et al., 2006)

(1) Interferon-γ (IFN-γ)

IFN-γ or type II interferon is the most important cytokine responsible for cell-mediated immunity. It is the signature cytokine produced by Th1 cells but is also derived from cytotoxic T cells and natural killer (NK) cells, and exerts inhibitory effects on Th2 cell differentiation. IFN-γ production by professional APCs, such as DCs and monocytes/macrophages, acting locally may be important in cell self-activation and activation of nearby cell. Moreover, IFN-γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptive immune response. In addition, IFN-γ is an inhibitor of Th2-mediated allergic inflammatory responses through its capacity to suppress many IL-4-mediated effects (Chung, 2001; Commins et al., 2010; Schroder, Hertzog, Ravasi, & Hume, 2004).

(2) Interleukin-12 (IL-12)

IL-12 is a heterodimer consisting of a larger (IL-12b, p40) subunit and a smaller (IL-12a, p35) subunit. IL-12 is naturally produced by APCs, such as DCs, monocytes/macrophages and B lymphocytes. It stimulates IFN-γ production and activates and induces proliferation, cytotoxicity, and cytokine production of NK cells. In addition, IL-12 regulates Th1 cells differentiation and suppresses the expansion of Th2 cells and inhibits IL-4 dependent IgE production (Chung, 2001; Commins et al., 2010).

2.1.2.2 Th2 cytokines

Th2 cells secrete a Th2 profile of cytokines, after cognate stimulation of the naïve T cell by antigen presenting cells (APCs), such as dendritic cell and macrophage. Some Th2 cytokines include IL-4, which is essential to IgE production, IL-5, which drives the terminal differentiation and IL-13, which promotes mucus secretion, airway hyperresponsiveness and tissue remodeling (Chung, 2001; Williams et al., 2012). Th2 cytokines play an important role in including B cells to switch class and express IgE, induce eosinophil proliferation in the bone marrow, and up-regulate adhesion molecules on blood vessels to promote tissue infiltration of

circulating inflammatory cells associated with allergic inflammation, such as eosinophils and basophils (Oettgen & Broide, 2012).

(1) Interleukin-4 (IL-4)

IL-4 is secreted mainly by activated T cells but also by basophils, NK T cells, eosinophils, and mast cells. In both eosinophils and basophils, IL-4 exists as a preformed, granule-associated peptide that can be rapidly released in allergic inflammatory responses. IL-4 is the major stimulus for the production of IgE antibodies and for the development of Th2 cells from naïve $CD4^+$ T cell. IL-4 stimulates B cell Ig heavy chain class switching to IgE isotype, development of Th2 cells and functions as an autocrine growth factor for differentiated Th2 cells. Furthermore, IL-4 together with IL-13 contributes to an alternative form of macrophage activation, stimulates the recruitment of leukocytes, and stimulates peristalsis in the gastrointestinal tract (Abbas et al., 2012; Commins et al., 2010).

(2) Interleukin-5 (IL-5)

IL-5 is the most important eosinophilopoietin. IL-5 is derived from Th2 lymphocytes, NK T cells, mast cells, eosinophils, and airway smooth muscle and epithelial cells, and is primarily responsible for the maturation and release of eosinophils in the bone marrow (Greenfeder, Umland, Cuss, Chapman, & Egan, 2011). IL-5 is a key factor in regulating the growth, differentiation, recruitment, activation, and survival of eosinophils by blocking apoptosis. Other activities of IL-5 include the development, metabolism, and function of basophils. In addition, IL-5 also stimulates the production of IgA antibodies (Abbas et al., 2012; Commins et al., 2010; Corren, 2012).

(3) Interleukin-13 (IL-13)

IL-13 is structurally and functionally similar to IL-4. IL-13 is produced mainly by the Th2 cells, but basophils, eosinophils, NK T cells and innate lymphoid cells may also secrete the cytokine. IL-13 together with IL-4, produces biologic effects associated with allergic inflammatory, and in defense against helminthes. IL-3 functions with IL-4 to induce alternative macrophage activation, which contributes to tissue repair and fibrosis. IL-13 increases mucus secretion from airway, an important component of allergic reactions including asthma, and gut

epithelium cells. Likewise, IL3 can activates B cells to switch to IgE and some IgG isotypes and recruit leukocytes, but not involved in Th2 differentiation (Abbas et al., 2012; Commins et al., 2010; Gour & Wills-Karp, 2015).

2.1.2.3 Treg cytokines

Regulatory T cells (Treg cells) are generated by self-antigen recognition in the thymus (also known as natural regulatory cells) and probably to a lesser extent by antigen recognition in peripheral lymphoid organs (also known as inducible or adaptive regulatory cells). Treg cells secrete a Treg profile of cytokines, such as IL-10 and transforming growth factor-β, and suppress immune response, thereby maintaining homeostasis and self-tolerance. Treg cells are able to inhibit the ability of APCs to stimulate T cells (Abbas et al., 2012; Kondelková et al., 2010).

(1) Interleukin-10 (IL-10)

IL-10 is a potent an anti-inflammatory cytokine that plays a central role in limiting host immune response to pathogens, like preventing damage to the host and maintaining normal tissue homeostasis. It is produced by activated macrophages and dendritic cells, regulatory T cells, Th1 and Th2 cells, B cells, neutrophils, NK cells and keratinocytes. IL-10 inhibits IFN-γ and IL-12 production by Th1 lymphocytes, and the production of IL-4 and IL-5 by Th2 cells. IL-10 inhibits the expression of costimulators and class II MHC molecules on dendritic cells and macrophages, and limits the production of pro-inflammatory cytokines (such as TNF-α, IL-1α and β, IL-6 and IL-8) and chemokines (such as MCP1, MCP5, IL-8 and MIP-2). Importantly, IL-10 regulates innate and adaptive Th1 and Th2 responses by limiting T cell activation and differentiation in the lymph nodes, as suppressing pro-inflammatory responses in tissues, leading to impaired pathogen control and/or reduced immunopathology (Chung, 2001; Couper, Blount, & Riley, 2008; Iyer & Cheng, 2012).

(2) Transforming growth factor-β (TGF-β)

TGF-β is synthesized and secreted by eosinophils, monocytes, activated macrophages and $CD4^+$ regulatory T cells. It has many important and quite driver roles in the immune system. TGF-β inhibits the

proliferation and effector functions of T cells and activation of macrophages. Moreover, it manages the differentiation of functionally distinct subsets of T cells, and stimulates IgA antibodies production by switching B cells to IgA isotype. Additionally, TGF-β promotes tissue repair after local immune and inflammatory reactions subside, thereby stimulating of fibrosis, inducing formation of the extracellular matrix and promoting wound healing and scar formation (Abbas et al., 2012; Commins et al., 2010).

2.1.4 Allergic rhinitis (AR)

Allergic rhinitis (AR) is a global health problem that causes major illness and disability worldwide and affects approximately 500 million people worldwide (Ozdoganoglu & Songu, 2012). Even though AR is not considered a life-threatening it contributes to reduced quality of life, lower work productivity, school learning performance, sleep problems and increasing medical costs (Pawankar, Mori, Ozu, & Kimura, 2011). AR is an inflammatory disease of the nasal mucosa mediated by an immunoglobulin E (IgE)-associated reaction resulting from inflammation of the airway mucosa with hypersensitivity. It is characterized by an inflammatory infiltrate made up of eosinophils, T cells, mast cells and basophils, which secrete several mediators, chemokines and cytokines, regulation of the local and systemic IgE synthesis, and communication with the immune system and the bone marrow (Pawankar et al., 2011). Furthermore, AR is also characterized by one or more of the following nasal symptoms: sneezing, nasal congestion, nasal itching (pruritus of the nose) and rhinorrhea (runny nose) (Deraz, 2010).

2.1.4.1 Pathophysiology of AR

The response in AR can also be further divided into two phases including immediate or early phase and late phase response. Early phase response that occurs immediately within seconds to minutes of exposure to an allergen and lasts for about 2 to 3 hours. One of the cardinal components of early phase response is the degranulation of the mast cells or basophils at the affected site. Therefore, the mediators are produced and stored in the granules of mast cells or basophils before degranulation include preformed mediators such as biogenic amines (histamine and serotonin), serglycin proteoglycans (heparin and chondroitin),

serine proteases (tryptases, chymases and carboxypeptidases) and other enzymes. In addition, mediators are synthesized and releases after activated mast cells or basophils include lipid-derived mediators such as prostaglandin D_2 and sulfidopeptidyl leukotriene (LT) C_4, LTD_4 and LTE_4 (Gentile & Skoner, 2010; Pawankar et al., 2011). These mediators cause bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production (Galli, Tsia, & Piliponsky, 2008). The other phase is late phase response that responses have many features in common with early phase responses. Late phase response which occurs 4 to 6 hours after antigen stimulation and lasts for about 18 to 24 hours. It is thought to reflect the actions of innate (such as basophils, eosinophils, neutrophils and monocytes) and adaptive (such as T lymphocytes) immune cells. Thus, the mediators are produced by these cells including leukotrienes, kinins, histamine, which result in the continuation of the symptoms and the development of the late phase. Moreover, the mast cells can releases a broad range of newly synthesized cytokines, chemokines and growth factor, such as TNF-α, LTB4, IL-8, IL-5, IL-10 and TGF-β, which have the potential to recruit other immune cells, to activate innate immune cells, and to affect many aspects of the biology of DCs, T cells and B cells (Galli et al., 2008; Gentile & Skoner, 2010; Pawankar et al., 2011).

2.1.4.2 Classification of AR

AR can be classified as seasonal or perennial results from timing and duration of allergen exposure, and the allergen pathogenesis. Seasonal allergic rhinitis (commonly called hay fever) is most frequently results from IgE-mediated sensitivity to pollen allergens (tree, grass, or weed) and outdoor mold spores. Typical symptoms during pollen exposure include the explosive onset of profuse, watery rhinorrhea, itching, and sneezing, along with frequent allergic symptoms of the eye. While, congestion also occurs but usually is not the most troubling symptom. Alternatively, perennial allergic rhinitis is most commonly caused by allergy to dust mites, cockroaches, indoor molds, and cat, dog, and other dander. It is defined as a disease that persists for longer than 9 months each year and produced two or more of the following symptoms: serous or seromucus

hypersecretion, nasal blockage caused by a swollen nasal mucosa, and sneezing paroxysms. In addition, nasal congestion and mucous production symptoms predominate in most patients, while sneezing, itching, and watery rhinorrhea may be minimal (Gentile & Skoner, 2010; Ozdoganoglu & Songu, 2012; Wheatley & Togias, 2015).

2.1.5 Management of allergy

The treatment of AR includes allergen avoidance, pharmacotherapy, immunotherapy, and complementary and alternative medicine. The mainly goal of treatment should be to control the symptoms and to improve the quality of life inflammatory processes as well as concomitantly decreases inflammation and comorbidities.

2.1.5.1 Allergen avoidance

Allergen exposure leads to the symptoms. In consequence, avoidance of allergic triggers is the primary treatment for allergy, as well as the primary preventive strategy, i.e. avoiding food triggers, avoiding outdoor sports in the springtime, banishing furred pets from the house, reducing dust mite in the house, etc. However, complete avoidance of allergens is difficult if not impossible. Sometimes a total change of environment might be of value (Gentile & Skoner, 2010; Scadding, Church, & Borish, 2012).

2.1.5.2 Pharmacotherapy (Barnes, 1999; Garbo et al., 2013; Gentile & Skoner, 2010; Scadding et al., 2012)

(1) Antihistamines

Antihistamines (nasal spray, oral medication) are a first-line medical therapy for allergy. These drugs work as an H_1-receptor antagonist, preventing histamine-induced reaction, such as vascular permeability, smooth muscle contraction, mucous production, and pruritus. Antihistamines have a good effect on early-phase reactions, but little effect on congestion. Nowadays, these drugs are classified into the older or first-generation antihistamines (e.g. chlorpheniramine, diphenhydramine and hydroxyzine) that cause sedation and the newer or second-generation antihistamines (i.e. loratadine, rupatadine, etc.) that cause less sedation than first-generation antihistamine. Antihistamines are the drug of choice for the

management of allergic rhinitis, allergic conjunctivitis, urticaria and atopic dermatitis, asthma, and anaphylaxis.

(2) Corticosteroids

Corticosteroids (intranasal, oral medication) are usually treated allergic rhinitis. However, it must be imported that steroids have potentially debilitating, unwanted effects when used systemically in a chronic fashion, incorrectly or inappropriately. New-generation inhaled corticosteroids for asthma (i.e. budesonide, fluticasone, propionate, etc.) have a high level of anti-inflammatory action with minimal side effects, as the swallowed fraction of drugs is largely removed by hepatic metabolism. After that, new soft steroids (e.g. ciclesonide) seem to be more promising, and corticosteroids that are inactivated in plasma are now in development. These drugs are treated asthma, allergic rhinitis, allergic conjunctivitis, and urticaria and atopic dermatitis.

(3) Decongestants

Decongestants (intranasal, oral medication) are α-adrenergic receptor agonists that cause vasoconstriction, reducing turbinate congestion and improving patency of the airway. However, these drugs (e.g. pseudoephedrine) have no effect on other symptoms such as rhinorrhea, pruritis, or sneezing.

(4) Leukotriene receptor antagonist (antileukotrienes)

Leukotriene receptor antagonists (antileukotrienes) have potent relaxing effects on the vascular smooth muscles of the nasal mucosa, enhancing effects on vascular permeability, and stimulating effects on eosinophil migration. Antileukotrienes are the drug for nasal blockage. These drugs (e.g. montelukast, pranlukast and zafirlukast) are treated asthma, allergic rhinitis, and urticaria and atopic dermatitis.

(5) Mast cell stabilizers

Mast cell stabilizers (intranasal, oral, ocular) work as block the release of mediators from mast cells. These drugs (i.e. cromolyn, azelastine, ketotifen, etc.) are used when other drugs (antihistamines, corticosteroids) are

ineffective or not well-tolerated. However, these drugs have minimal effects on congestion.

(6) Recombinant human monoclonal antibody to IgE (anti-IgE therapy)

Recombinant human monoclonal antibody to IgE (anti-IgE therapy) acts through binding circulating IgE molecules. This drug (omalizumab) is indicated for moderately persistent or severe asthma refractory to standard treatment.

2.1.5.3 Immunotherapy (Saporta, 2012; Scadding et al., 2012)

Immunotherapy is a long-term treatment modality that can modify the immunological response of the allergy sufferer so that the affected individual will stop reacting to involved allergens. It involves the administration of regular, gradually increasing amounts of allergen extracts, by injections (subcutaneous immunotherapy, SCIT) or sublingual drops (sublingual immunotherapy, SLIT).

(1) Subcutaneous immunotherapy (SCIT)

Subcutaneous immunotherapy (SCIT) refers to injectable vaccines commonly known as allergy shots. It involves repeated injection of the allergen at regular intervals and is effective in allergic rhinoconjunctivitis with clear-cut allergens such as pollen, animal dander, and mites.

(2) Sublingual immunotherapy (SLIT)

Sublingual immunotherapy (SLIT) refers to oral vaccines where the allergens are administered as drops to the sublingual area. Although, SLIT is a very old treatment modality it is also safe and easy to administration for patients.

2.1.5.4 Complementary and alternative medicine (Bielory, 2014)

Complementary and alternative medicine (CAM) therapies are frequently used to treat various allergic diseases such as allergic rhinitis, asthma, and atopic dermatitis. These therapies include traditional Chinese medicine (TCM), acupuncture, Ayurvedic medicine, a variety of herbal therapies, and several others.

(1) Traditional Chinese medicine (TCM)

Traditional Chinese medicine (TCM) includes herbal therapy, acupuncture, massage, and dietary therapy.

(2) Acupuncture

Acupuncture is a component of traditional Chinese medicine (TCM). This treatment involves inserting tiny needles into specific meridians or areas of the body for redistribution of Qi, the life energy.

(3) Ayurvedic medicine

Ayurvedic medicine is a medical tradition originating from India and derived from the teachings of ancient Hindu healers. Theses therapeutic interventions include yoga, breathing exercises, meditation, and herbal preparations.

(4) Herbal therapies

Most herbal preparations contain several components, each with potentially varying physiologic and pharmacologic properties. However, herbal therapies that differ by name may contain identical components and thus share similar clinical effects and adverse effects. Specific herbal agents are butterbur, cinnamon bark, Tinofend, etc.

2.2 Inflammation

Inflammation is a complex set of interactions among soluble factors and cells that can arise in any tissue in response to traumatic, infections, post-ischaemic, toxic or autoimmune injury (Nathan, 2002). At its basic level, it is a tissue-destroying process that involves the recruitment of blood-derived products, such as plasma proteins, fluid, and leukocytes, into perturbed tissue. This migration is facilitated by alterations in the local vasculature that lead to vasodilation, increased vascular permeability, and increased blood flow (Ashley et al., 2012). Inflammation may release or generate a diverse population of pro-inflammatory mediators include bradykinins, serotonin, histamines, prostaglandins, and nitric oxide. These substances contribute to the cardinal signs of inflammation that include tumor (swelling of the tissue), calor (elevated tissue temperature), rubor (blood color-like redness of

vascularized tissue at the inflammation site), dolor (intensive sensation of a noxious stimulus), and functio laesa (disturbance of function, i.e. impaired function of the organ affected). Notably, although the four cardinal signs including tumor, calor, rubor and dolor only apply to acute inflammation accompanying wounds and infections, functio laesa is the only universal sign that accompanies all inflammatory processes (Medzhitov, 2010; Stankov, 2012). Inflammation is divided into two basic types based on duration of inflammation as acute and chronic inflammation.

Acute inflammation is an immediate response to infection and injury characterized by the influx of granulocytes followed by phagocytosing mononuclear phagocytes (Motwani et al., 2017). It is of short duration, lasting for a few minutes, several hours, or few days and characterized by the exudation of fluid and plasma proteins and emigration of leukocytes (predominantly neutrophils) from blood into inflammatory site. Mechanisms of acute inflammation, featuring the initial intravascular events that lead to increased permeability changes, activation of endothelial cells (increased expression of adhesion molecules for neutrophils (PMNs)), adhesion of PMNs to the endothelial surfaces, and platelet activation (resulting in their aggregation and adhesion to one another as well as to endothelial surfaces). Platelets are often present in areas of fibrin deposition. Further, tissue responses (in extravascular compartment) feature edema, transmigration of PMNs, fibrin deposition, and hemorrhage if the structural integrity of the vascular barrier has been compromised (Ward, 2010). Moreover, acute inflammation is mediated by the release of a number of mediators such as vasoactive amines (histamine and serotonin), vasoactive peptides (bradykinin), and lipid mediators (prostaglandin (PG) and leukotriene (LT)) (Medzhitov, 2008).

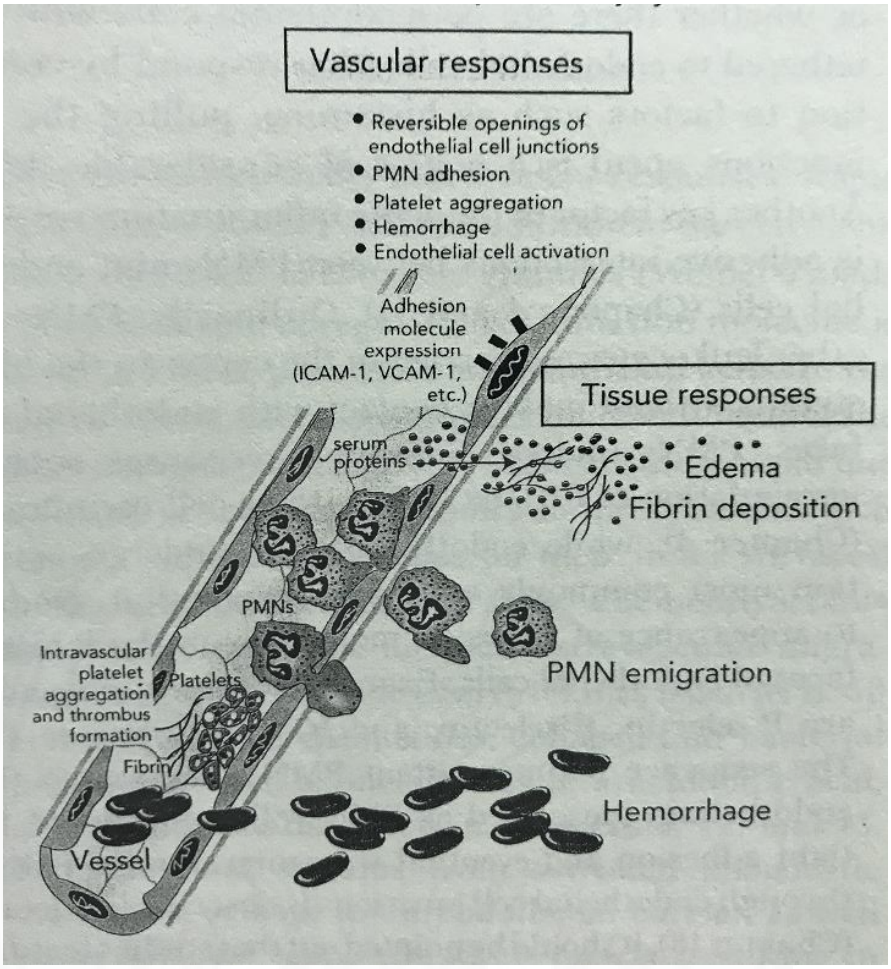


Figure 2.3 Mechanisms of acute inflammatory responses (Ward, 2010)

Chronic inflammation has many features of acute inflammation but it usually of low grade and persistent, resulting in response that lead to tissue degeneration (Franceschi & Campisi, 2014). It is of longer duration, lasting for several weeks or months to years and associated histologically by lymphocytes and macrophages, proliferation of blood vessels, fibrosis and necrosis. Mechanisms of chronic inflammatory responses are triggered by vascular responses that involve the appearance of adhesion molecules on endothelial cell surfaces that cause adhesion of lymphocytes and monocytes, and the eventual transmigration of these cells into the extravascular space. Activated endothelial cells express adhesion molecules like VCAM-1 that facilitate adhesion of lymphocytes and monocytes to endothelial surfaces, followed by their eventual transmigration. In the extracellular compartment,

lymphocytes and macrophages secrete factors that stimulate extracellular collagen formation and perpetuate the inflammatory response. Transmigrated monocytes “mature” into macrophages. Addition, plasma cells secrete various subclasses of antibodies (Ward, 2010). Moreover, chronic inflammation involves the release of a number of mediators that are not prominent in the acute response. There are inflammatory cytokines (tumor necrosis factor-alpha (TNF-α), interleukins (IL) 1, 2 and 3, interferon (IFN)) and lipid mediator (platelet-derived growth factor (PDGF)) (Medzhitov, 2008).

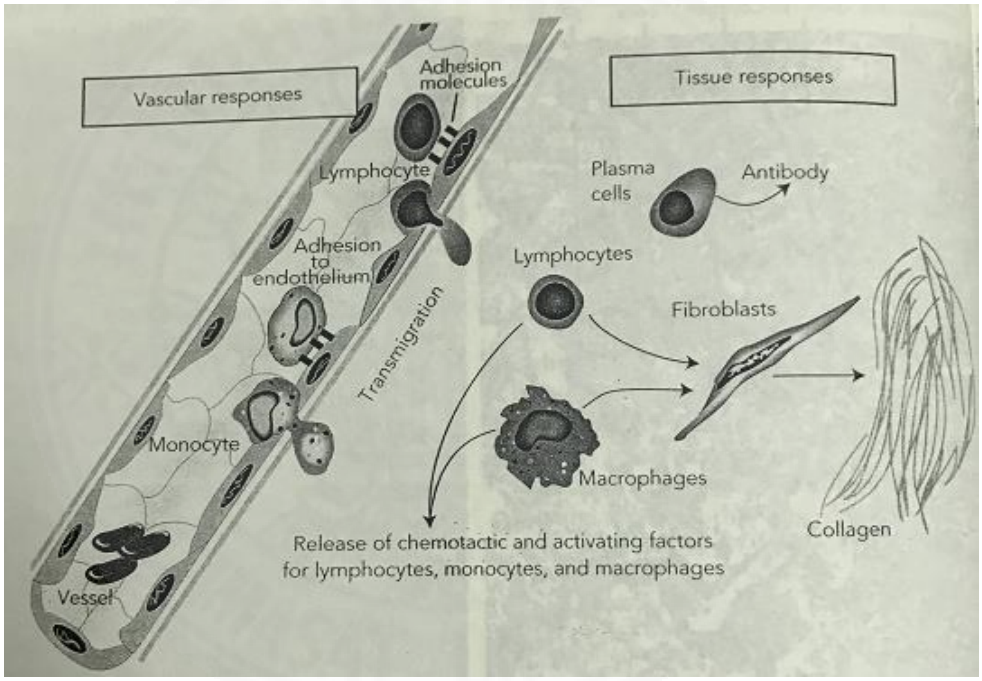


Figure 2.4 Mechanisms of chronic inflammatory responses (Ward, 2010)

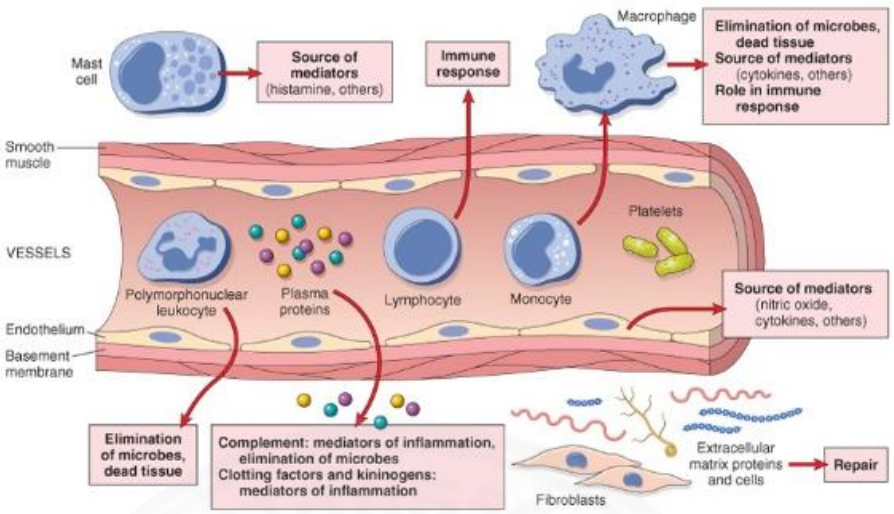


Figure 2.5 The components of acute and chronic inflammatory responses and their principal functions (Kumar, Abbas, & Aster, 2013)

Table 2.2 Actions of the principle mediators of inflammation

Mediator	Principal Sources	Actions
Cell-Derived		
Histamines	Mast cells, basophils, platelets	Vasodilation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasodilation, increased vascular permeability
Prostaglandins	Mast cells, leukocytes	Vasodilation, pain, fever
Leukotrienes	Mast cells, leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet-activating factor	Leukocytes, mast cells	Vasodilation, increased vascular permeability, leukocyte adhesion, chemotaxis, degranulation, oxidative burst
Reactive oxygen species	Leukocytes	Killing of microbes, tissue damage
Nitric oxide	Endothelium, macrophages	Vascular smooth muscle relaxation, killing of microbes
Cytokines (tumor necrosis factor [TNF], interleukin 1 [IL-1])	Macrophages, endothelial cells, mast cells	Local endothelial activation (expression of adhesion molecules), fever/pain/anorexia/hypotension, decreased vascular resistance (shock)
Chemokines	Leukocytes, activated macrophages	Chemotaxis, leukocyte activation
Plasma Protein-Derived		
Complement products (C5a, C3a, C4a)	Plasma (produced in liver)	Leukocyte chemotaxis and activation, vasodilation (mast cell stimulation)
Kinins	Plasma (produced in liver)	Increased vascular permeability, smooth muscle contraction, vasodilation, pain
Proteases activated during coagulation	Plasma (produced in liver)	Endothelial activation, leukocyte recruitment

Note. From *Robbins Basic Pathology* (p. 45), by V. Kumar, A. K. Abbas, and J. C. Aster, 2013, Philadelphia, PA: Saunders.

2.2.1 Nitric oxide (NO)

Nitric oxide (NO, formula •N=O) plays an important regulatory or modulatory role in a variety of inflammatory conditions. NO is a small, fairly short-lived molecules (with a half-life of 6 seconds) that is released from a variety of cells in response to homeostatic and pathologic stimuli (Blantz & Munger, 2002; Sharma, Al-Omran, & Parvathy, 2007). NO is the metabolic by-product of the conversion of amino acid L-arginine to L-citrulline with stoichiometric formation of NO, a gaseous free radical, by three isoforms of nitric oxide synthase (NOS). This conversion occurs in two steps: two-electron oxidation of L-arginine to N^{ω}-hydroxy-L-arginine followed by three-electron oxidation of N^{ω}-L-hydroxy arginine to NO and L-citrulline and also requires nicotinamide-adenine-dinucleotide phosphate (NADPH) and O_2 as co-substrates and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX (haem) as co-factor (Figure 2.6) (Förstermann & Sessa, 2012; Stuehr, Kwon, Nathan, & Griffith, 1991).

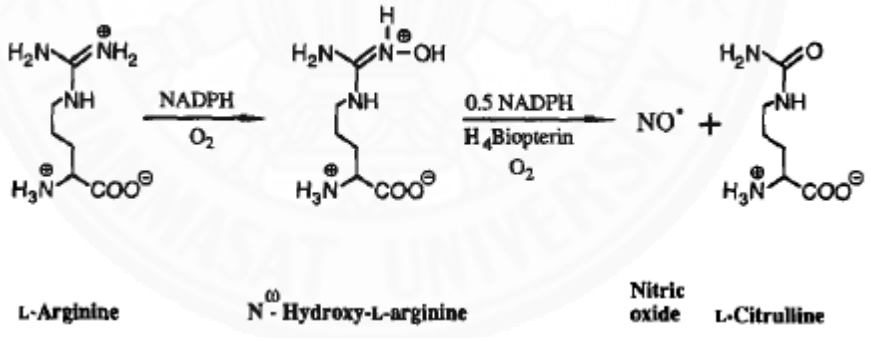


Figure 2.6 The reaction catalyzed by nitric oxide synthase (NOS) (Stuehr et al., 1991)

At present, there are three different NOS isoforms. The first isoform, neuronal NOS (nNOS or NOS I) is predominantly expressed in neurons in brain and peripheral nervous system. The second isoform, the endothelial NOS (eNOS or NOS III) is mainly expressed in endothelial cells. The third isoform, is the inducible NOS (iNOS or NOS II) is not expressed in resting cells, but is expressed only in response to certain inflammatory stimuli such as bacterial products (lipopolysaccharide, LPS),

cytokines (IL-1, TNF-α and IFN-γ), and lipid mediators (Korhonen, Lahti, Kankaanranta, & Moilanen, 2005; Laroux et al., 2001).

Both eNOS and nNOS produce NO in relatively low amounts whereas iNOS can synthesize prodigious amounts of NO for extended periods of time. Low concentrations of NO derived from eNOS and nNOS are beneficial that inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion and transmigrations. On the other hand, large amounts of NO produced by iNOS can be toxic, pro-inflammatory, damaging and contributes to the injury of host tissues (Guzik, Korbut, & Adamek-Guzik, 2003; Laroux et al., 2001).

2.2.2 Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) is a member of a growing family of peptide mediators comprising at least 19 cytokines, including lymphotoxin-α, Fas ligand, and CD40 ligand (Popa, Netea, Van Riel, Van Der Meer, & Stalenhoef, 2007). It is a pro-inflammatory cytokine that exerts both homeostatic and pathophysiological roles in the central nervous system (CNS). In addition, TNF-α plays crucial roles in the innate and adaptive immunity, cell proliferation, and apoptotic processes. The cytokine is produced by different kind of cells, including macrophages, monocytes, mast cells, NK cells, endothelial cells, neutrophils, T and B lymphocytes, smooth and cardiac muscle cells, astrocytes, adipocytes, and fibroblasts (Bradley, 2008; Olmos & Lladó, 2014).

2.3 Stability testing

Stability testing is required to provide evidence on the quality of a product, substance or material as it varies with environmental factors and the passage of time period for shelf-life and storage conditions. The stability is affected by physical factors such as temperature, humidity and light, and chemical factors such as hydrolysis, oxidation, isomerization and polymerization etc. These factors are the most vital factor which investigates whether a compound or a mixture of compounds can be developed into a pharmaceutical product. Moreover, stability

testing involves the investigating of quality and potency of a product at suitable time intervals is conducted for a period corresponding to the normal time that the product is likely to remain in stock or in use (Hussain, Ismail, Sadikun, & Ibrahim, 2009). Stability testing procedures have been categorized into four types including real-time stability testing, accelerated stability testing, retained sample stability testing and cyclic temperature stress testing (Bajaj, Singla, & Sakhuja, 2012). Degradation is slowly at room temperature and shelf-life may go up to several years. Since the period can be as long as two years, stability testing for this period will be time consuming and expensive. Therefore, accelerated stability testing is designed, which enables a rapid prediction of long term stability of a product (Hussain et al., 2009).

Accelerated stability testing is widely used for the prediction of storage stability and quality and for the estimation of shelf-life and safe storage temperature of labile products (Franks, 1994). It can be used to settle temporary age of the drug preparation. In the end of tested period, if the drug has the qualities following by its standard regulation, its shelf-life will be established two years. Besides the long term stability has to be tested couple with accelerated test for setting real shelf-life of the drug (International Conference on Harmonization, 2003).

Table 2.3 Storage conditions of stability testing of active substances and pharmaceutical products

Study	Storage condition	Minimum time period covered by data at submission
Long term*	25 ± 2ºC/60 ± 5% RH or 30 ± 2ºC/65 ± 5% RH	12 months
Intermediate**	30 ± 2ºC/65 ± 5% RH	6 months
Accelerated	40 ± 2ºC/75 ± 5% RH	6 months

Note. From *ICH Harmonized Tripartite Guideline: Stability Testing of New Drug Substances and Products Q1A (R2)* (p.3), by International Conference on Harmonization, 2003, Retrieved from http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf

* Long term stability studies are performed at 25 ± 2ºC/60 ± 5% RH or 30 ± 2ºC/65 ± 5% RH.

** If 30 ± 2ºC/65 ± 5% RH is the long term condition, there is no intermediate condition.

2.4 Prasaprohyai remedy

Prasaprohyai is a polyherbal Thai traditional remedy which is composed of *Kaempferia galanga* L. as the main component, *Amomum testaceum* Ridl., *Anethum graveolens* L., *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., *Angelica sinensis* (Oliv.) Diels, *Artemisia annua* L., *Atractylodes lancea* (Thunb.) DC., *Cuminum cyminum* L., *Dracaena loureiroi* Gagnep., *Foeniculum vulgare* Mill., *Lepidium sativum* L., *Ligusticum sinense* Oliv., *Mammea siamensis* (Miq.) T. Anders., *Mesua ferrea* L., *Mimusops elengi* L., *Myristica fragrans* Houtt., *Nelumbo nucifera* Gaertn., *Nigella sativa* L., and *Syzygium aromaticum* (L.) Merr. & L. M. Perry. It is used in treatments of fever and cold (National Drug Committee, 2013).

Figure 2.7 Plant components of Prasaprohyai remedy

(https://www.samunpri.com/traditionalmedicine/24-ยาประสะเปราะใหญ่/)

2.4.1 Biological activities of Prasaprohyai remedy

Previous reports of biological activities of Prasaprohyai remedy are shown in Table 2.4.

Table 2.4 Biological activities of Prasaprohyai remedy

Activities	Results	References
Analgesic	- The mice were treated with 4,000 mg/kg body weight of ethanolic extract exhibited a significant extendibility of the latency of analgesic response in hot plate test compared with the untreated control.	Plengsuriyakarn et al., 2012
Anti-allergic	- The ethanolic extract showed inhibitory effect on β-hexosaminidase release in RBL-2H3 cells with IC_{50} value of 16.59 μg/ml.	Makchuchit, 2010
Antibacterial	- The ethanolic extract inhibited the growth of gram-positive bacteria, i.e. *S. aureus* ATCC 25923, *S. aureus*, MRSA, and *S. pyogenes* with the MIC/MBC values from 1.5 to 2.5 mg/ml.	Sattaponpan & Kondo, 2011
	- The volatile oil inhibited the growth of both gram-positive and gram-negative bacterial, i.e. *S. aureus* ATCC 25923, *S. aureus*, MRSA, *S. pyogenes*, *E. coli* ATCC 25922, *E. coli* (extended-spectrum β-lactamase), *A. baumannii*, *A. baumannii* (multidrug resistant strain), *K. pneumonia*, and *K. pneumonia* (multidrug resistant strain) with the MIC/MBC values from 5 to > 10 mg/ml.	Sattaponpan & Kondo, 2011

Table 2.4 Biological activities of Prasaprohyai remedy (Cont.)

Activities	Results	References
Anticancer	- The ethanolic extract (5,000 mg/kg body weight) showed low activity against cholangiocarcinoma in nude mice on day 48.	Plengsuriyakarn et al., 2012
Anti-inflammatory	- The ethanolic extract showed inhibitory effect on LPS-induced NO production in RAW 264.7 cells with IC_{50} value of 18.40 μg/ml.	Makchuchit, 2010
	- The ethanolic extract at dose of 1,000, 2,000 and 4,000 mg/kg body weight reduced the paw edema volume induced by carrageenan in rats.	Plengsuriyakarn et al., 2012
Antimalarial	- The ethanolic extract exhibited good to moderate against 3D7 *P. falciparum* clone (IC_{50} = 9.1 μg/ml) and weak against K1 *P. falciparum* clone (IC_{50} = 15.7 μg/ml).	Thiengsusuk et al., 2013
Antioxidant	- The ethanolic extract showed moderate antioxidant activity using DPPH scavenging assay (EC_{50} = 42.98 μg/ml).	Makchuchit, 2010
Antipyretic	- The ethanolic extract at dose of 2,000 and 4,000 mg/kg body weight exhibited a significant antipyretic activity using Brewer's yeast-induced pyrexia in rats at 5 hours after administration and 3 up to 6 hours after observation time starting, respectively.	Plengsuriyakarn et al., 2012

Table 2.4 Biological activities of Prasaprohyai remedy (Cont.)

Activities	Results	References
Cytotoxic	- The ethanolic extract showed cytotoxicity against cholangiocarcinoma cell line (CL-6), human hepatocarcinoma cell line (HepG2), human laryngeal carcinoma cell line (Hep-2) and normal human epithelial cell (HRE) using MTT assay with IC_{50} values of 44.12, 125.07, 20.99 and 263.51 μg/ml, respectively.	Mahavorasirikul et al., 2010

2.5 *Atractylodes lancea* (Thunb.) DC.

Atractylodes lancea (Thunb.) DC. belongs to the Asteraceae (Compositae) family. It is known as Kot khamao in Thai and Atractylis in English and Cangzhu in Chinese. It is widely distributed in East Asia, especially in China (Huang et al., 2016). *A. lancea* is a perennial plant, up to 30 to 80 cm high, with thick rhizome. The rhizome is irregularly beaded or nodular-cylindrical, slightly curved, occasionally branched, 3 to 10 cm long, 1 to 2 cm in diameter. Externally greyish-brown, marked with wrinkles, horizontal curved striations and remains of fibrous roots, and stem scar or remains of stem base at apex. Texture compact, scattered with many reddish-brown oil spots, and white fine needle crystals appear after exposing for a long time (Zhengtao & Peishan, 2015).

Figure 2.8 The rhizomes of *A. lancea*

2.5.1 The chemical structures of *A. lancea*

The investigation on chemical structures of *A. lancea* is shown in Figure 2.9.

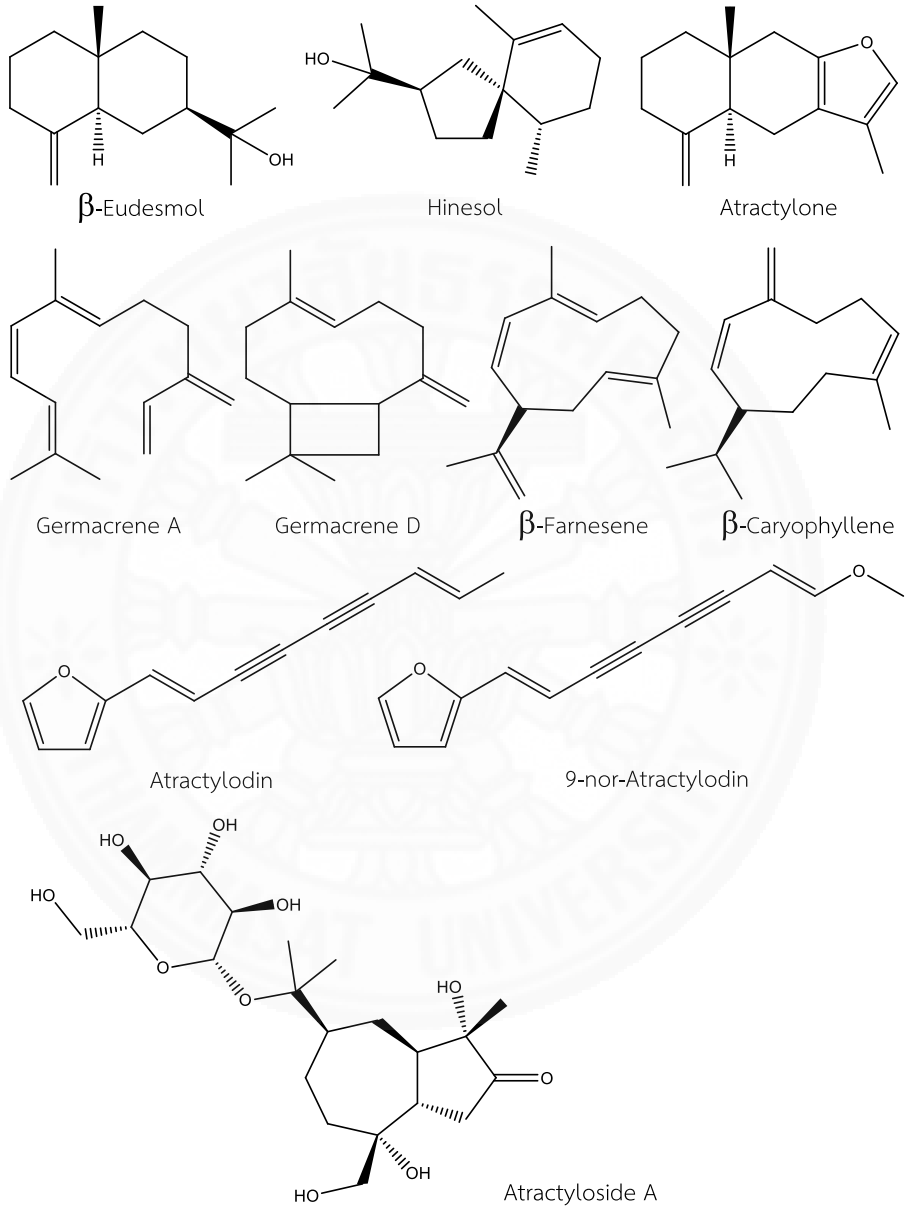
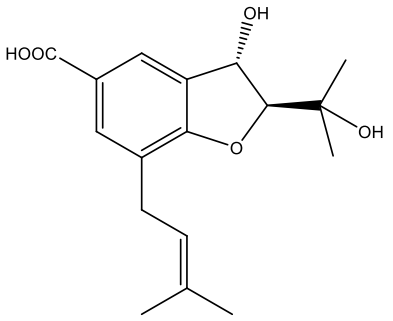
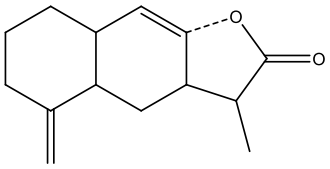


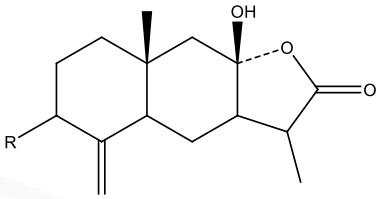
Figure 2.9 Chemical constituents of the rhizomes of *A. lancea* (Ahmed et al., 2016; Chen, Wu, Wang, & Gao, 2012; Duan, Wang, Qian, Su, & Tang, 2008; Koonrungsesomboon, Na-Bangchang, & Karbwang, 2014; Xu, Feng, Jiang, Yang, & Zhang, 2017; Yin et al., 2015)



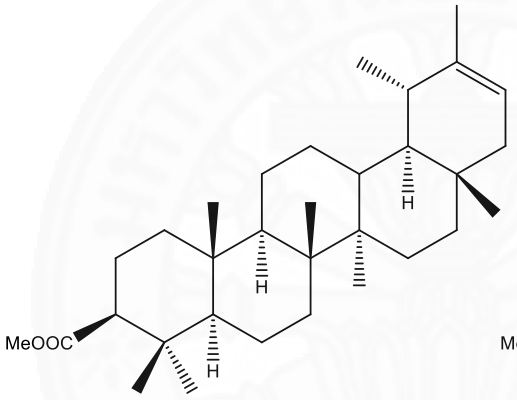
trans-2-Hydroxyisoxypropyl-3-hydroxy-7-isopentene-2,3-dihydrobenzofuran-5-carboxylic acid



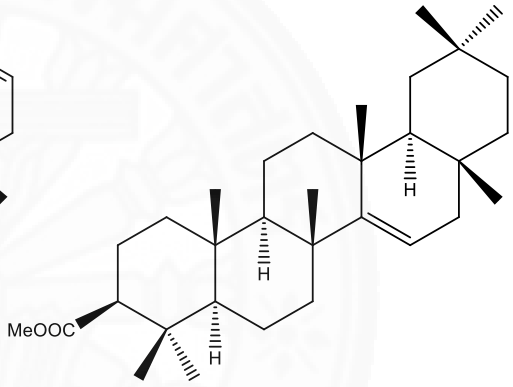
Atractylenolide Ⅱ



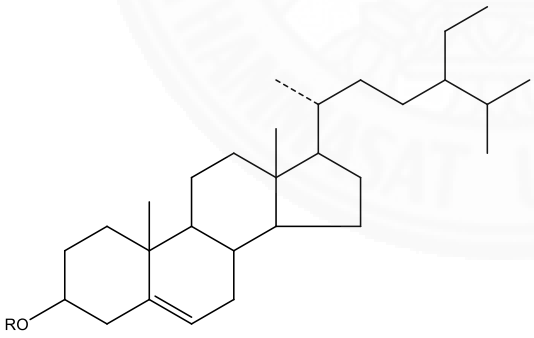
Atractylenolide Ⅲ R = H
Atractylenolide Ⅳ R = OCOMe



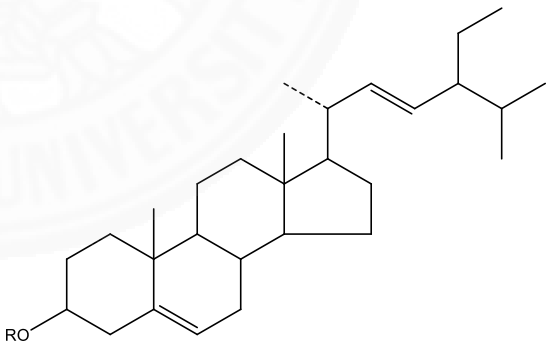
φ-Taraxasteryl acetate



Taraxerol acetate



β-Sitosterol R = H
Daucosterol R = glc



Stigmasterol R = H
Stigmasterol 3-O-β-D-glucopyranoside R = glc

Figure 2.9 Chemical constituents of the rhizomes of *A. lancea* (Ahmed et al., 2016; Chen, Wu, Wang, & Gao, 2012; Duan, Wang, Qian, Su, & Tang, 2008; Koonrungsesomboon, Na-Bangchang, & Karbwang, 2014; Xu, Feng, Jiang, Yang, & Zhang, 2017; Yin et al., 2015) (Cont.)

2.5.2 Biological activities of the rhizomes of *A. lancea*

Previous reports of biological activities of the rhizomes of *A. lancea* are shown in Table 2.5.

Table 2.5 Biological activities of the rhizomes of *A. lancea*

Activities	Results	References
Anti-allergic	- The ethanolic extract showed the strong anti-allergic activity on β-hexosaminidase release with the IC_{50} value of 13.60 μg/ml.	Makchuchit, 2010
Anti-inflammatory	- Atractylenolide I significantly reduced TNF-α and IL-6 levels in serum at concentration of 10, 20 and 40 mg/kg. Moreover, atractylenolide I decreased IL-1β at concentration of 20 and 40 mg/kg.	Wang et al., 2016
	- Atractylodin inhibited IL-6 production in PMA and A23187-induced HMC-1 cells with the IC_{50} value of 6.32 μM and showed inhibitory effect on TNF-α, CFS2, IL-4 and IL-6 mRNA expression in a dose-dependent manner (0.8, 4 and 20 μM).	Chae, Kim, & Chin, 2016
	- Atractylenolide I (5, 10 and 20 mg/kg body weight) decreased TNF-α, IL-6, IL-1β, IL-13 and MIF production in BALF of LPS-activated acute lung injury (ALI) mice in a dose-dependent manner, but it increased IL-10 production.	Zhang, Huang, & Zeng, 2015

Table 2.5 Biological activities of the rhizomes of *A. lancea* (Cont.)

Activities	Results	References
Anti-inflammatory	- The petroleum ether fraction (0.82 and 1.64 mg/kg body weight) decreased TNF-α, IL-6, IL-17, and IL-1β levels in the serum, collagen loss, and hind paw size in collagen-induced arthritis (CIA) in rats.	Liu et al., 2016
	- The ethanolic extract showed strong anti-inflammatory activity on NO production with the IC_{50} value of 9.70 μg/ml.	Makchuchit et al., 2010

2.6 Benjakul remedy

Benjakul is a Thai traditional remedy which is composed of *Piper interruptum* Opiz., *Piper longum* L., *Piper sarmentosum* Roxb., *Plumbago indica* L., and *Zingiber officinale* Roscoe. It is used for controlled abnormal of element in the body, balanced health and relieved flatulence (National Drug Committee, 2013).

2.6.1 General description of plants in Benjakul remedy

2.6.1.1 *Piper interruptum* Opiz. or *Piper ribesioides* Wall.

Piper interruptum Opiz. or *Piper ribesioides* Wall. is in the Piperaceae family. It is commonly known as Sa-khan in Thai and is found in the Northern and Northeastern parts of Thailand, Taiwan, Indonesia, and Philippines. Sa-khan is a climber dioecious. The stems are rigged, glabrous, 2 to 4.5 mm thick. The petiole is glabrous, sheathed at base only, 1 to 4 cm long; leaf blade ovate to long ovate, 4 to 7 cm wide, 6 to 13 cm long, membranous or papery, without evident glands, both surfaces glabrous, base rounded or shortly tapered, symmetric, apex acute or shortly acuminate; veins 5, all basal; reticulate veins abaxially prominent, lax. The inflorescences are spikes leaf-opposed; male spikes 1.5 to 3 mm wide, 11 to 27 cm long; peduncle as long as petioles, glabrous; bracts oblong, 3 to 4 mm long, adnate to rachis, margin free, apex or rounded, stamens 2; female spikes 7 to 17 cm long. The flowers are unevenly developed, sparse or interrupted in fruit; peduncle nearly as long as opposite leaves, glabrous; rachis and bracts as in male spikes. The ovary is distinct, ovoid, apex acute; stigmas 4 or 5. The fruits are ovoid or ovoid-globose and smooth (Flora of China, 1999).

Figure 2.10 The stems of *P. interruptum*

2.6.1.2 *Piper longum* L. or *Piper chaba* Hunt. or *Piper retrofractum* Vahl.

Piper longum L. or *Piper chaba* Hunt. or *Piper retrofractum* Vahl. is a medicinal plant that belongs to the Piperaceae family. It is commonly known as Dee-plee in Thai and Long pepper or Indian long pepper in English. *P. longum* is cultivated in China, India, Malaysia, Nepal, Sri Lanka, and Vietnam (Flora of China, 1999). Dee-plee is a climber, glabrous, rather fleshy, with the aid of adventitious roots, rarely creeping; stems much branched, stout, cylindrical, thickened above nodes. The leaves are simple, alternate, 2 to 13 cm wide, 3 to 20 cm long, lower ones ovate or lanceolate with cordate base, upper ones oblong-oval to oblong with obtuse, cordate or cuneate base, unequal, all tapering or acuminate, entire, glabrous, reticulate venation sunk above and raised beneath; petioles of lower leaves 1.5 to 3 cm long, of the upper ones 0.5 to 1.5 cm long; stipules 1 to 1.5 cm long, membranous, lanceolate, obtuse, enclosing the bud but soon falling off. The inflorescences are spikes, erect or patent; peduncles 0.7 to 2 cm long; bracts broadly oval-ovate, 1.5 to 2 mm long. The flowers are unisexual, bisexual; male spikes 2.5 to 8.5 cm long, stamens 2, rarely 3, very short; female spikes 1.7 to 3 cm long, stigmata 2 to 3, short, obtuse, persistent. The fruiting spikes are cylindro-conic; berries connate and adnate to stalk of bract, broadly rounded, bright red. The seeds are 2 to 2.5 mm in diameter (Department of Medical Sciences, 2000).

Figure 2.11 The fruits of *P. longum*

2.6.1.3 *Piper sarmentosum* Roxb.

Piper sarmentosum Roxb. belongs to the Piperaceae family and is known as Cha-phlu in Thai and Betel or Vietnamese pepper in English. It is widely distributed in Cambodia, China, Indonesia, Laos, Malaysia, Myanmar, Philippines, Thailand, and Vietnam (Hussain, Hashmi, Latif, Ismail, & Sadikun, 2012). *P. sarmentosum* is a glabrous, erect or creeping, growing up to 50 to 60 cm high, often stoloniferous, swollen node. The leaves are simple, alternate, and heart-shaped; suborbicular ovate, or ovate-oblong, 5 to 10 cm wide, 7 to 15 cm long, light to dark green; surface glabrous or short hairs; apex acute to shortly acuminate; base cordate to obliquely obtuse or rounded; margin entire, slightly undulate; veins palmately 5- to 7-nerved, prominent on lower surface; petioles 2 to 5 cm long. The inflorescence is in axillary spike, tubular, unisexual. Male spikes are white, 1.5 to 3 cm long; peduncle 0.5 to 1.5 cm long; stamens 2 to 3, filament very short; anthers subglobose. Female spikes are whitish, 2 to 5 cm long; peduncle 0.5 to 1.5 cm long; stigmas 3 to 4. The fruit drupe is obovoid, sweet tasting when ripe, turn black on maturity, dry, and have several rounded bulges. The seeds are small (Department of Medical Sciences, 2011; Rahman, Sijam, & Omar, 2016).

Figure 2.12 The roots of *P. sarmentosum*

2.6.1.4 *Plumbago indica* L. or *Plumbago rosea* L.

Plumbago indica L. or *Plumbago rosea* L. is a plant belonging to the Plumbaginaceae family and it is called Chettamun phloeng daeng in Thai and Indian leadwort or Rosy-flowered leadwort or Scarlet leadwort in English (Pooma & Suddee, 2014). It is widely distributed in tropical climate, especially in South Africa, Australia, South Asia, and Southeast Asia including Thailand (Sukkasem, Chatuphonprasert, Tatiya-aphiradee, & Jarukamjorn, 2016). *P. indica* is a perennial plant or small shrub, growing up to 0.8 to 1.5 m high; young shoots red. The stems are tubular with red nodes. The leaves are simple, alternate, broadly elliptic-ovate, 3 to 5 cm wide, 6 to 10 cm long. The inflorescence is in terminal or axillary, spiciform raceme; calyx gland-bearing. The flowers are red, bisexual and regular. The fruit is circumscissile capsule (Saralamp, Chuakul, Temsiririrkkul, & Clayton, 1996).

Figure 2.13 The roots of *P. indica*

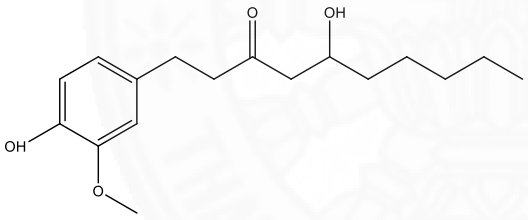
2.6.1.5 *Zingiber officinale* Roscoe

Zingiber officinal Roscoe is medicinal plant in the Zingiberaceae family. It is native to Southeast Asia and is cultivated in Africa, China, India, Jamaica, Haiti, and Nigeria. *Z. officinale* is commonly known as Khing in Thai and Ginger in English. It is an erect perennial plant with thick tuberous rhizomes (underground stems) from which the aerial stem grows up to 1 m high. The leaves are simple, alternate, lanceolate-oblong, smooth and pale green, 1.5 to 2 cm wide, 15 to 20 cm long. The flower stems shorter than the leaf stems and bearing a few flowers, each surrounded by a thin bract and situated in axils of large, greenish yellow obtuse bracts, which are closely arranged at end of flower stem forming collectively an ovate-oblong spike. Each flower shows a superior tubular calyx, split part way down one side; an orange yellow corolla composed of a tube divided above into 3 linear-oblong, blunt lobes; 6 staminodes in 2 rows, the outer row of 3 inserted at mouth of corolla; the posterior 2, small, horn-like; the anterior petaloid, purple and spotted and divided in to 3 rounded lobes; an inferior, 3-celled ovary with tufted stigma. The fruit is a capsule with small arillate seeds (Leung & Foster, 1996; Saralamp et al., 1996; World Health Organization, 1999).

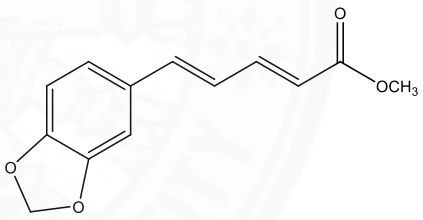
Figure 2.14 The rhizomes of *Z. officinale*

2.6.2 The chemical structures of some components of plants in Benjakul remedy

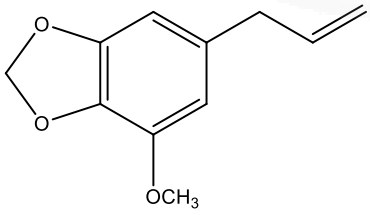
The investigations on chemical structures of plants in Benjakul remedy are shown in Figure 2.15 to 2.20.



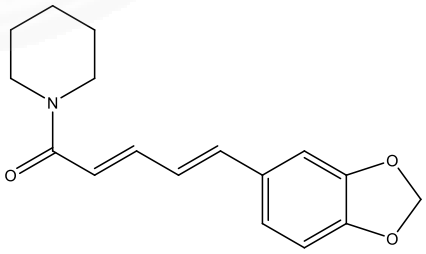
6-Gingerol



Methyl piperate

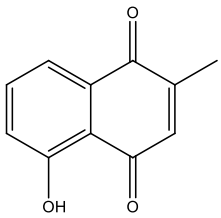


Myristicin

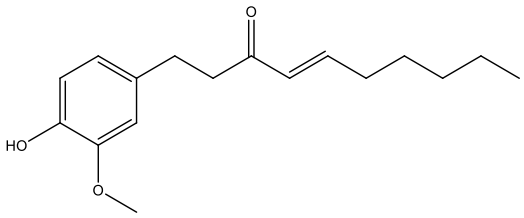


Piperine

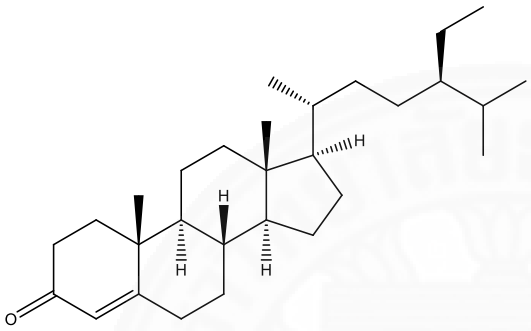
Figure 2.15 Chemical constituents of Benjakul remedy (Rattarom, 2013; Sakpakdeejaroen & Itharat, 2009)



Plumbagin



6-Shogaol



β-Sitostenone

Figure 2.15 Chemical constituents of Benjakul remedy (Rattarom, 2013; Sakpakdeejaroen & Itharat, 2009) (Cont.)

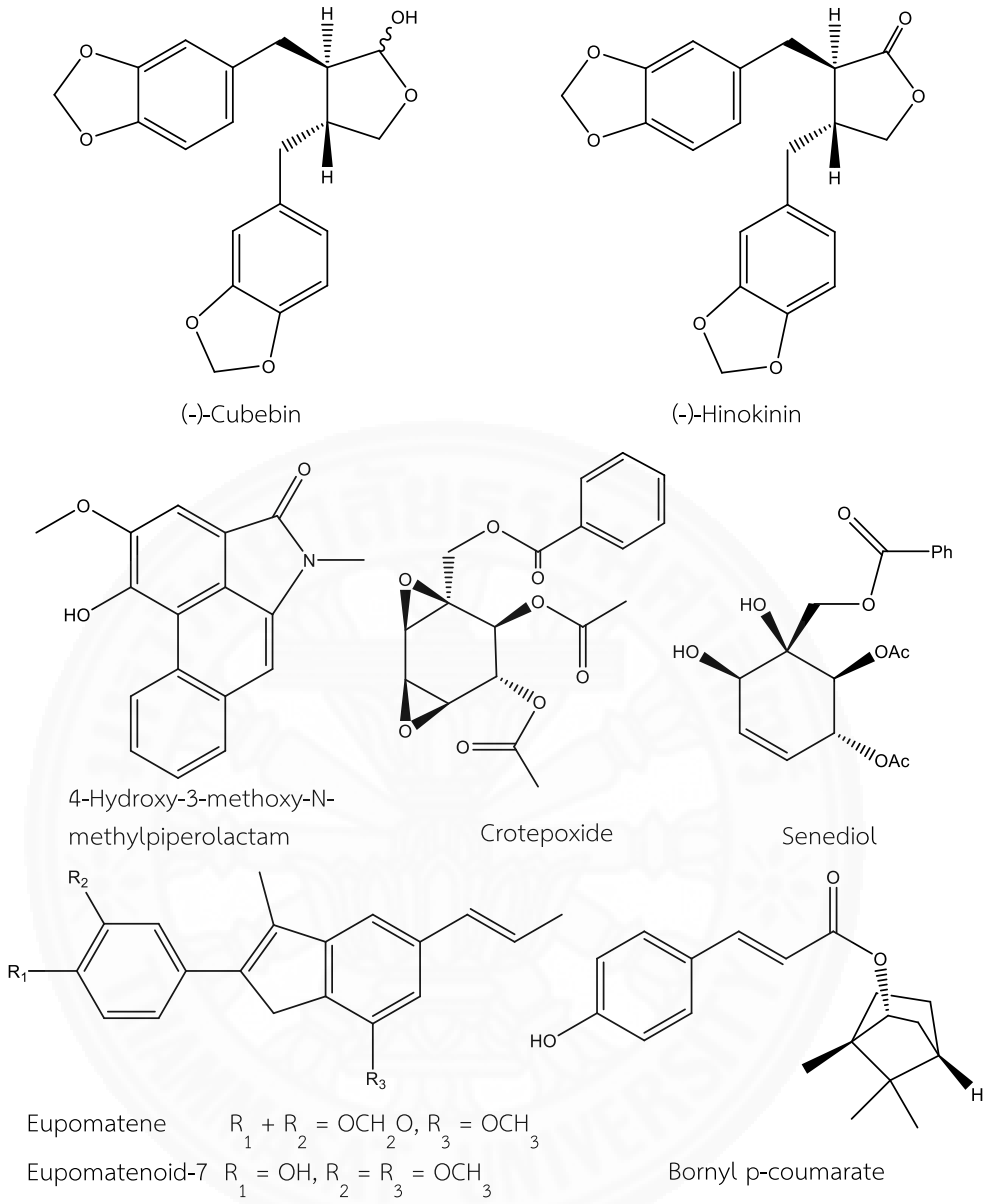


Figure 2.16 Chemical constituents of the stems of *P. interruptum* (Parmar et al., 1997; Thebpatiphat, Pengprecha, & Ternai, 1988)

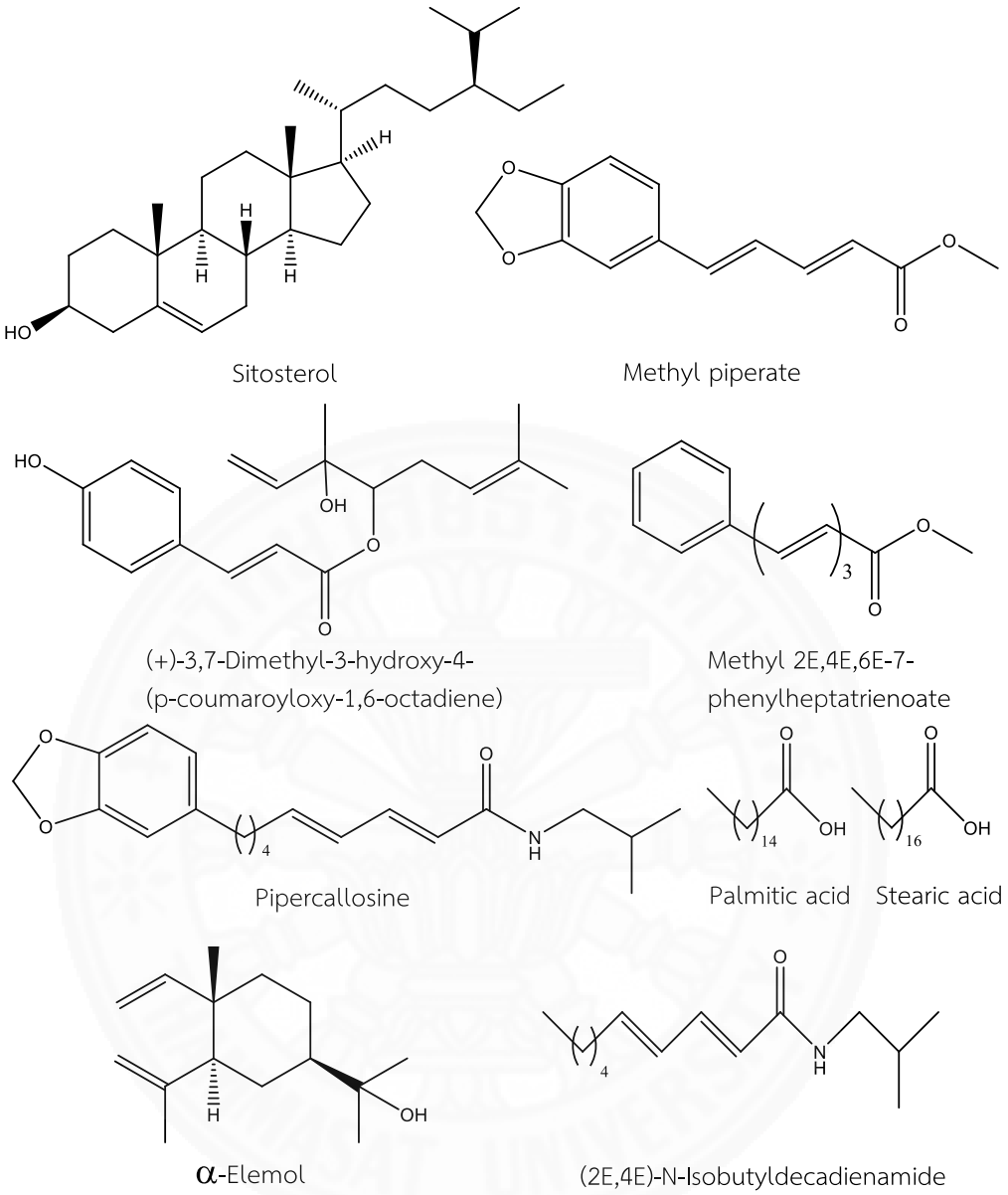


Figure 2.16 Chemical constituents of the stems of *P. interruptum* (Parmar et al., 1997; Thebpatiphat, Pengprecha, & Ternai, 1988) (Cont.)

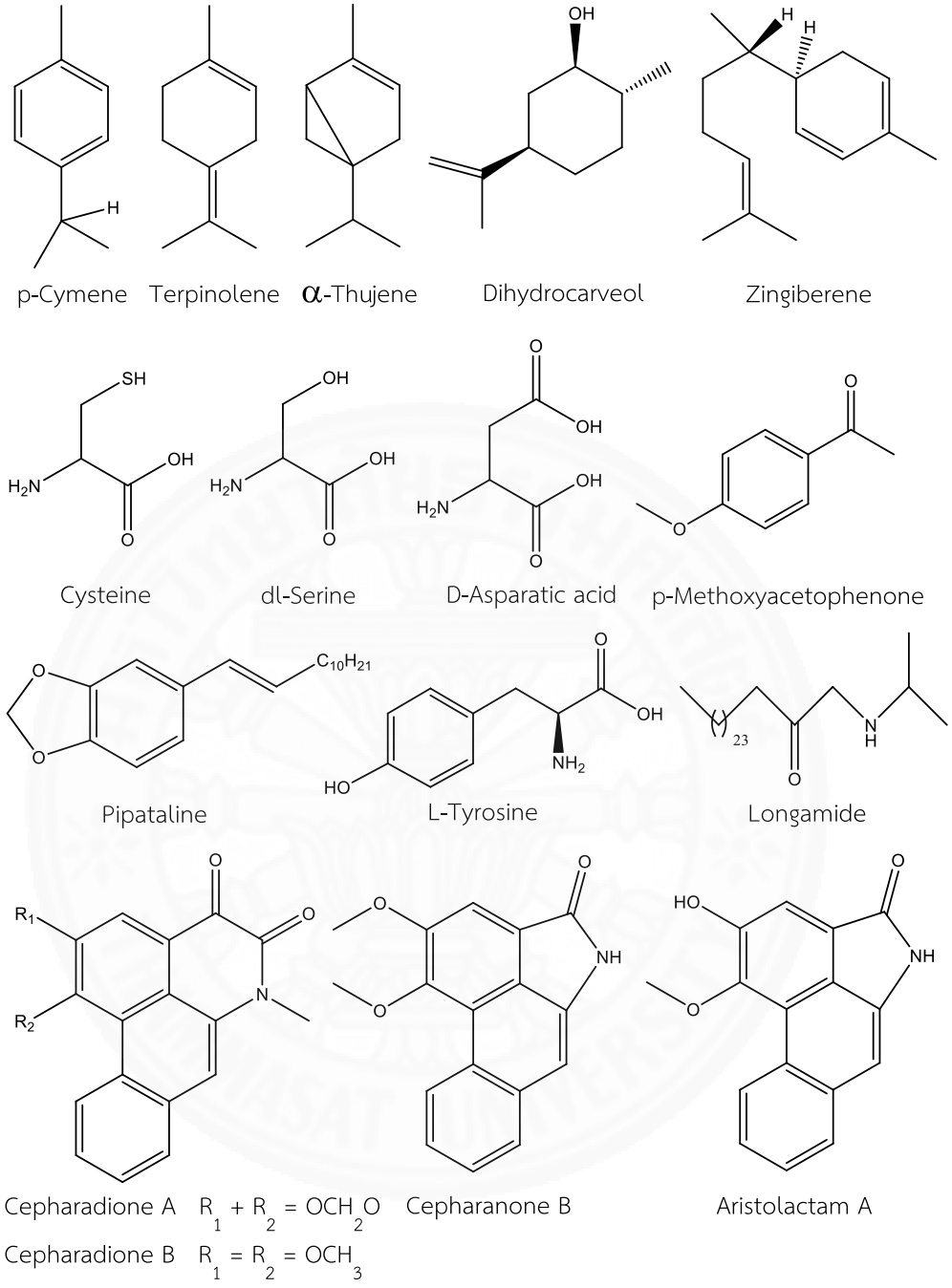


Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001)

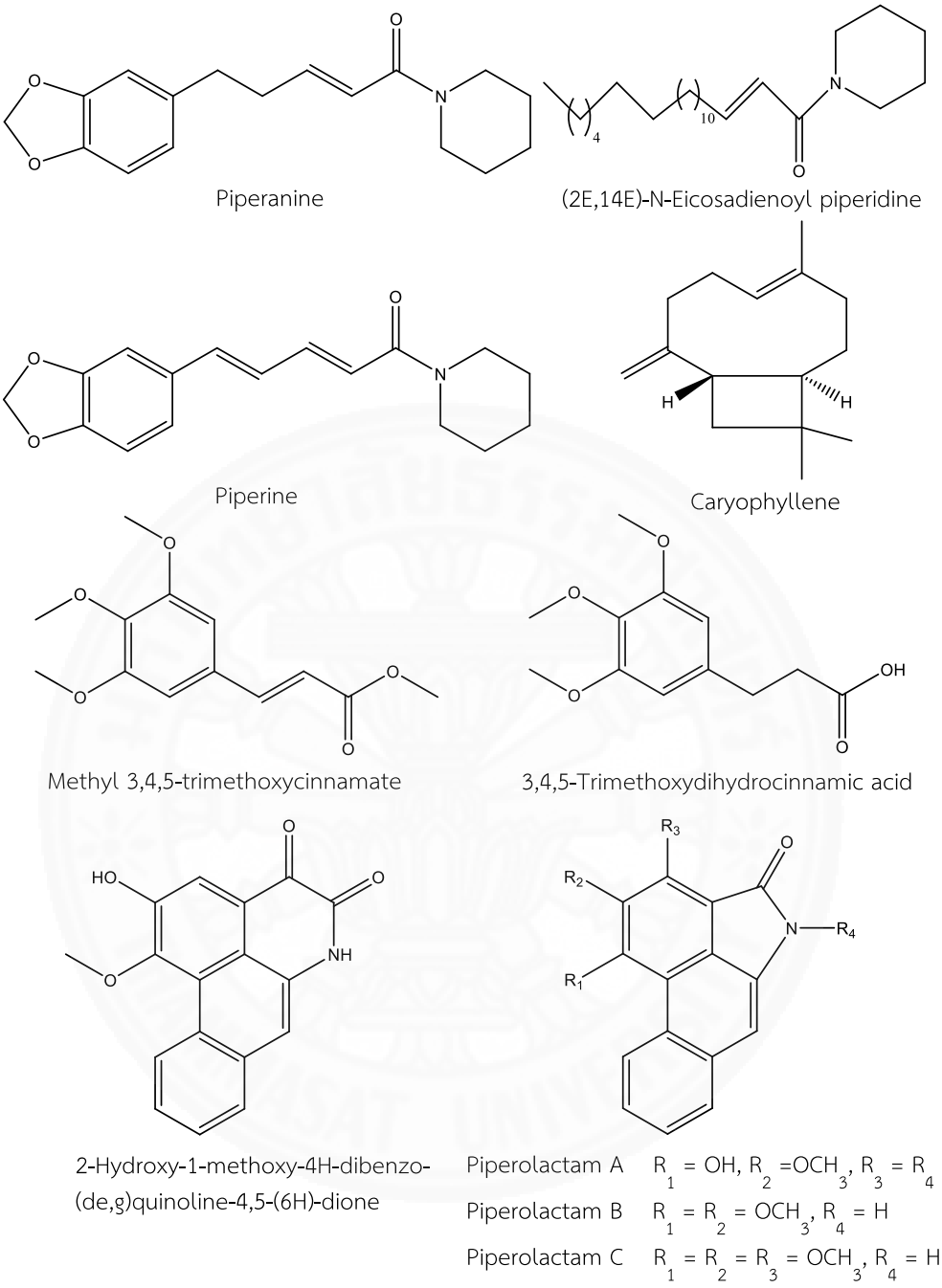


Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)

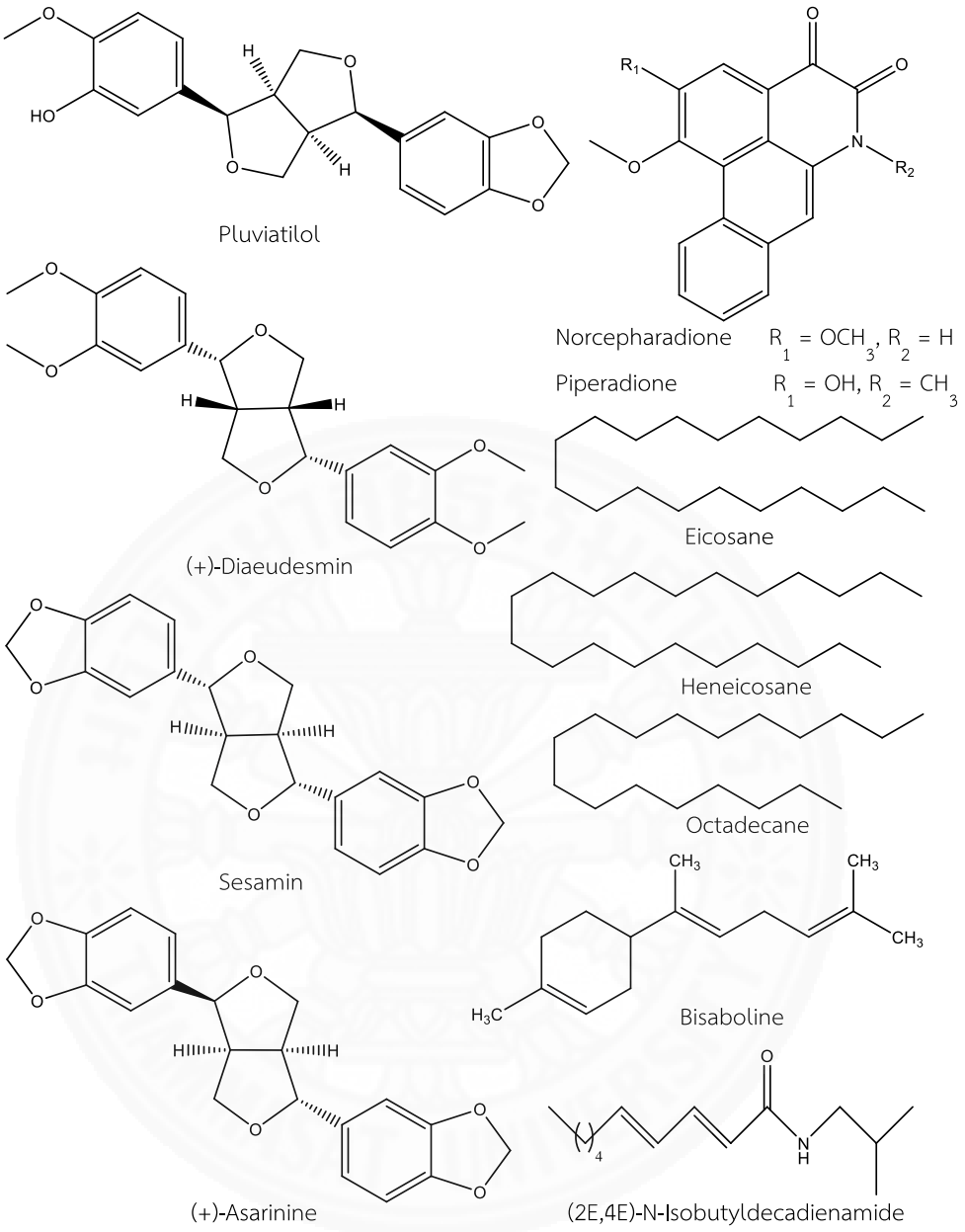


Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)

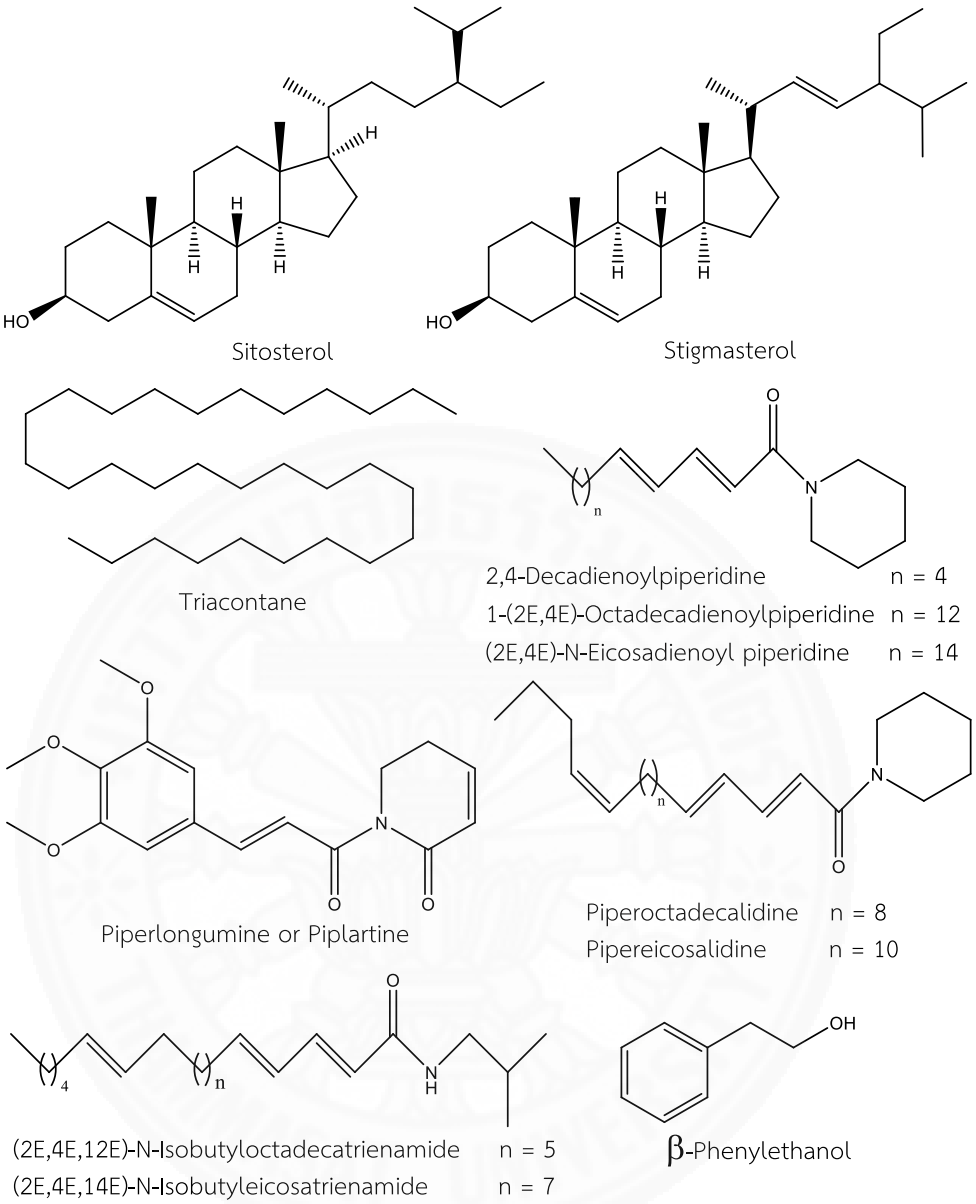


Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)

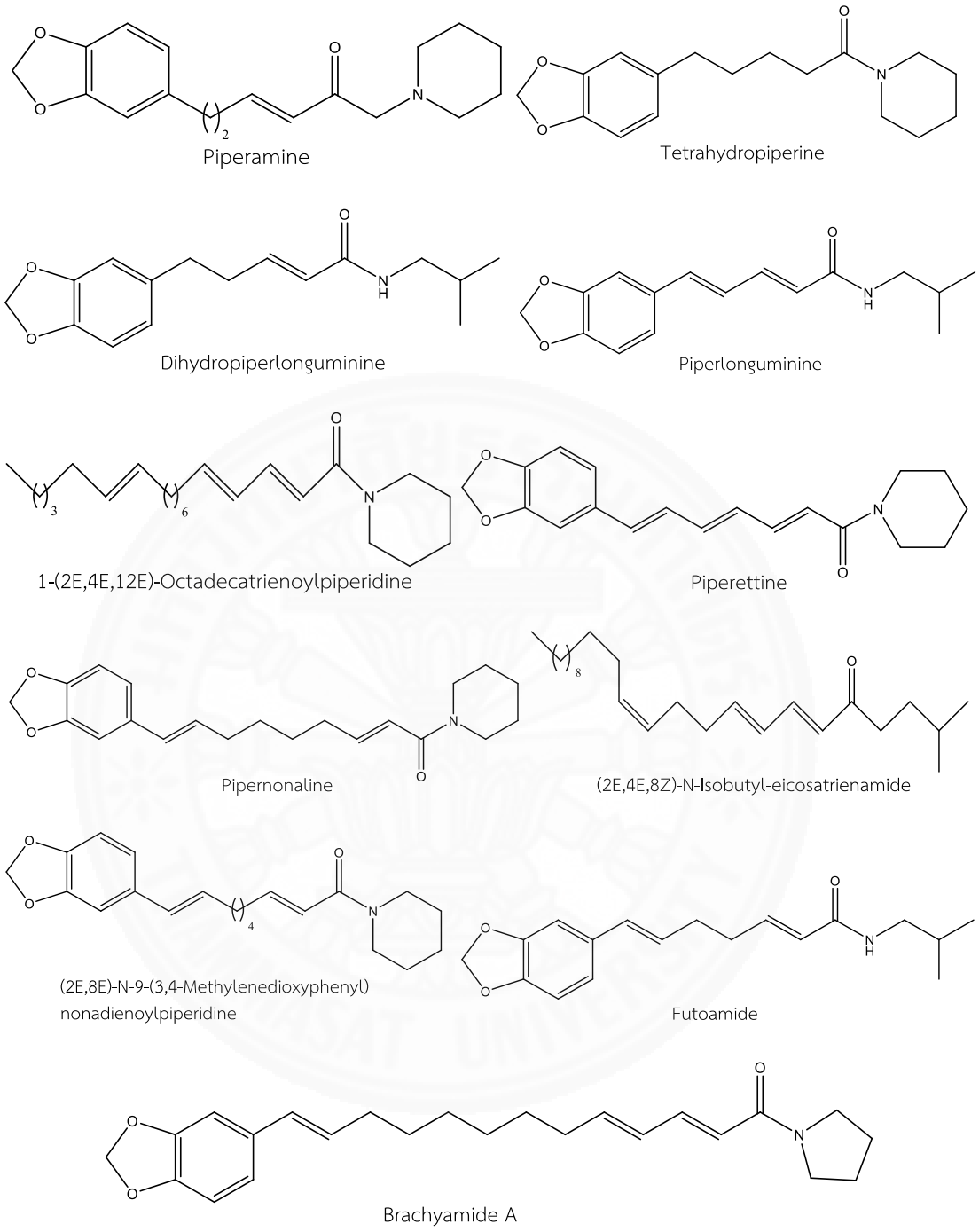


Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)

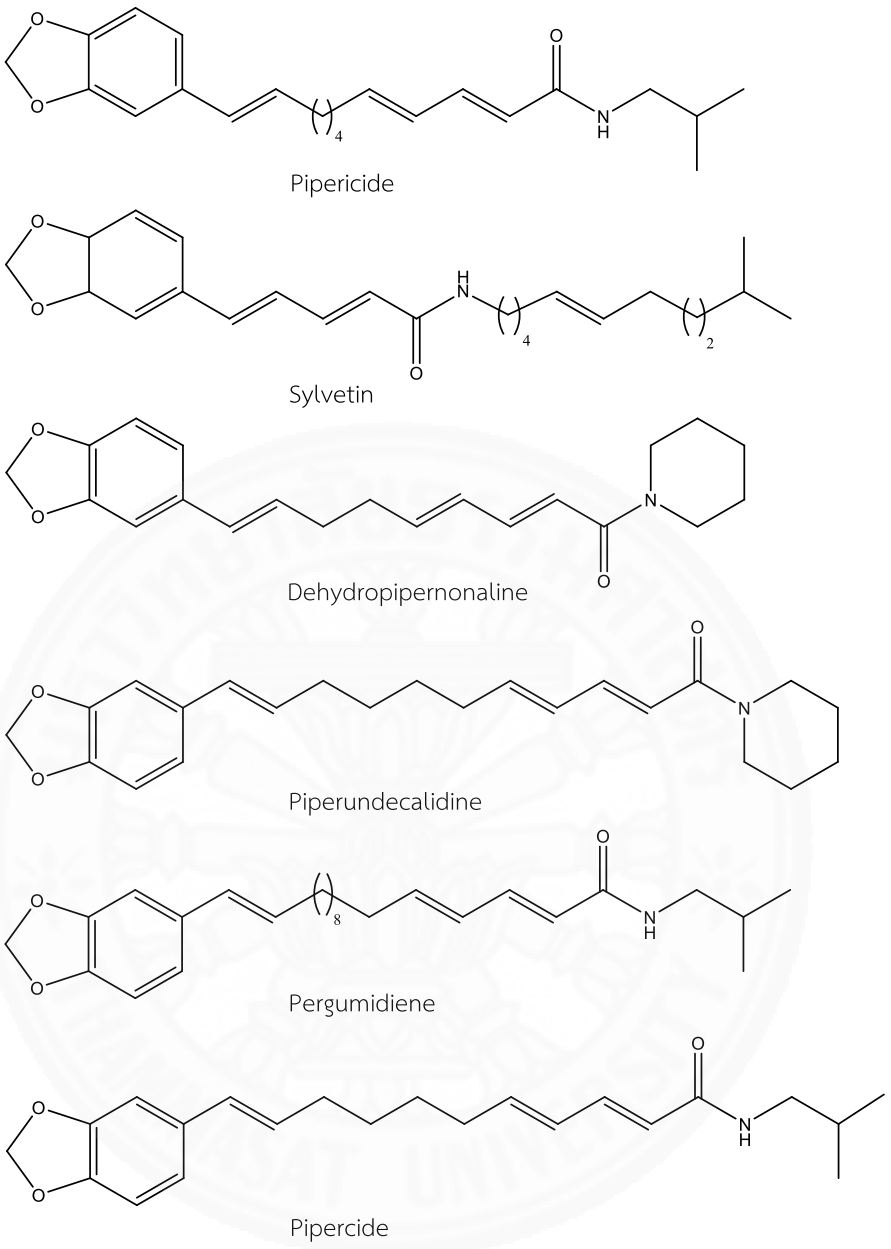
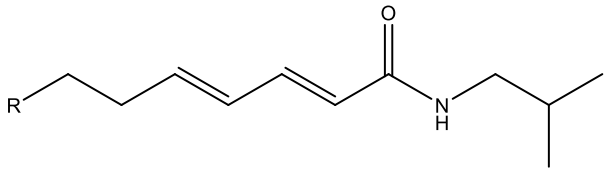
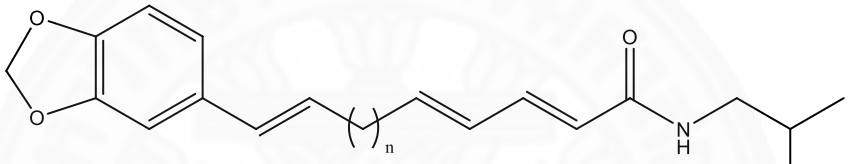


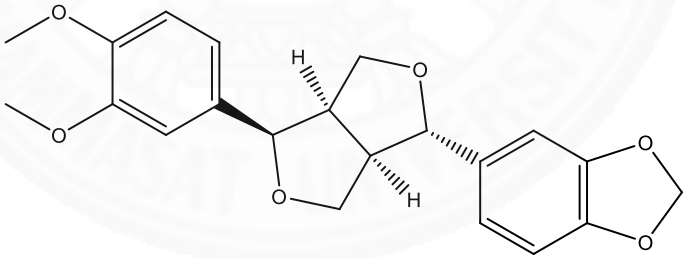
Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)



Pellitorine	R = C_3H_7
N-Isobutyl-2E,4E-dodecadienamide	R = C_5H_{11}
N-Isobutyl-2E,4E-tetradecadienamide	R = C_7H_{15}
N-Issobutyl-2E,4E-hexadecadienamide	R = C_9H_{19}
N-Isobutyl-2E,4E-octadecadienamide	R = $C_{11}H_{23}$
N-Isobutyl-2E,4E-eicosadienamide	R = $C_{13}H_{27}$



Retrofractamide A	n = 2
Retrofractamide D	n = 3
Retrofractamide B	n = 4
Guineensine	n = 6
Brachystamide B	n = 8



Fargesin

Figure 2.17 Chemical constituents of the fruits of *P. longum* (Dhanalakshmi, Umamaheswari, Balaji, Santhanalakshmi, & Kavimani, 2016; Parmar et al., 1997; Stôhr, Xiao, & Bauer, 2001) (Cont.)

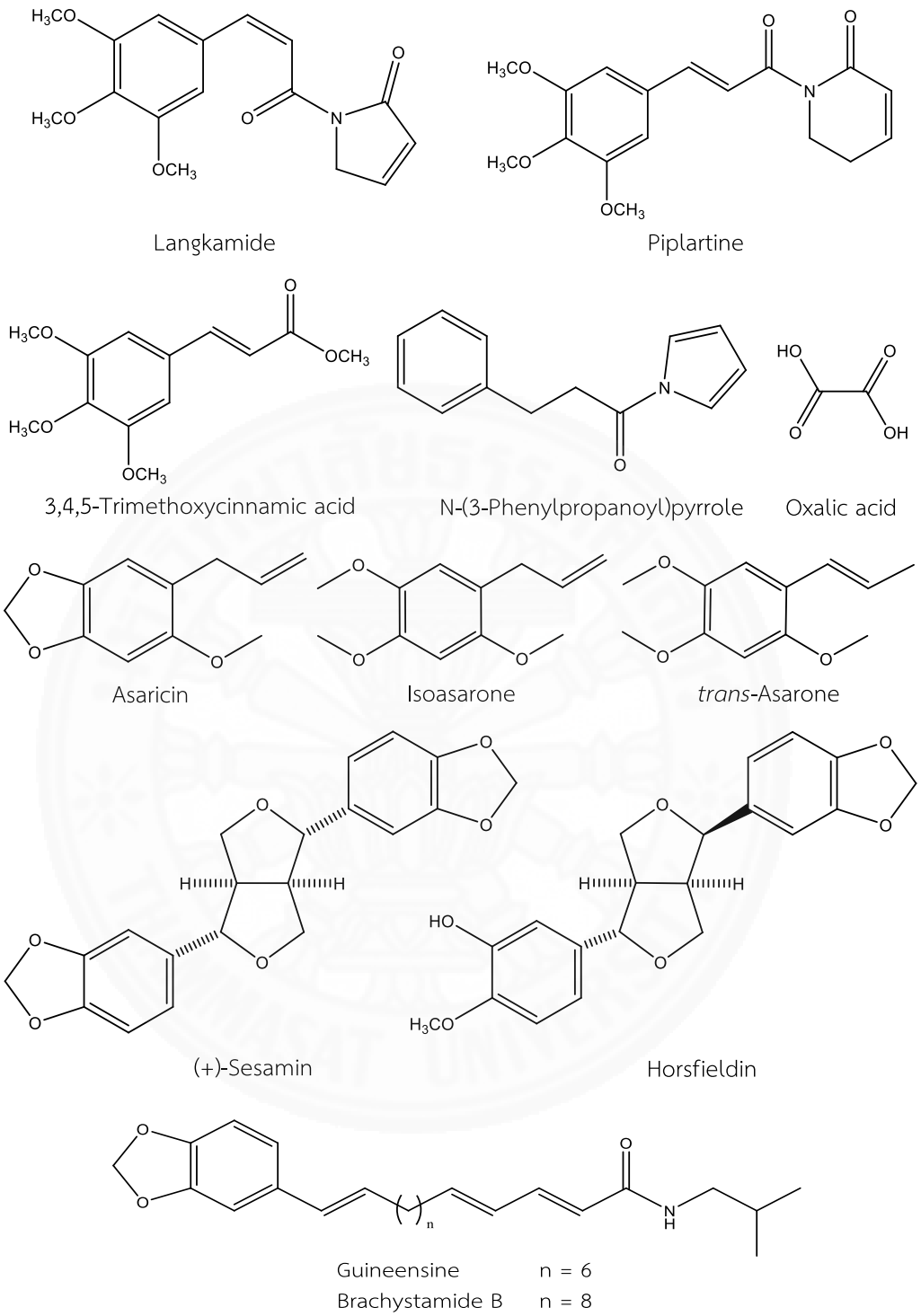


Figure 2.18 Chemical constituents of the roots of *P. sarmentosum* (Bokesch et al., 2011; Hematpoor et al., 2016; Parmar et al., 1997; Tuntiwachwuttikul, Phansa, Pootaeng-on, & Taylor, 2006)

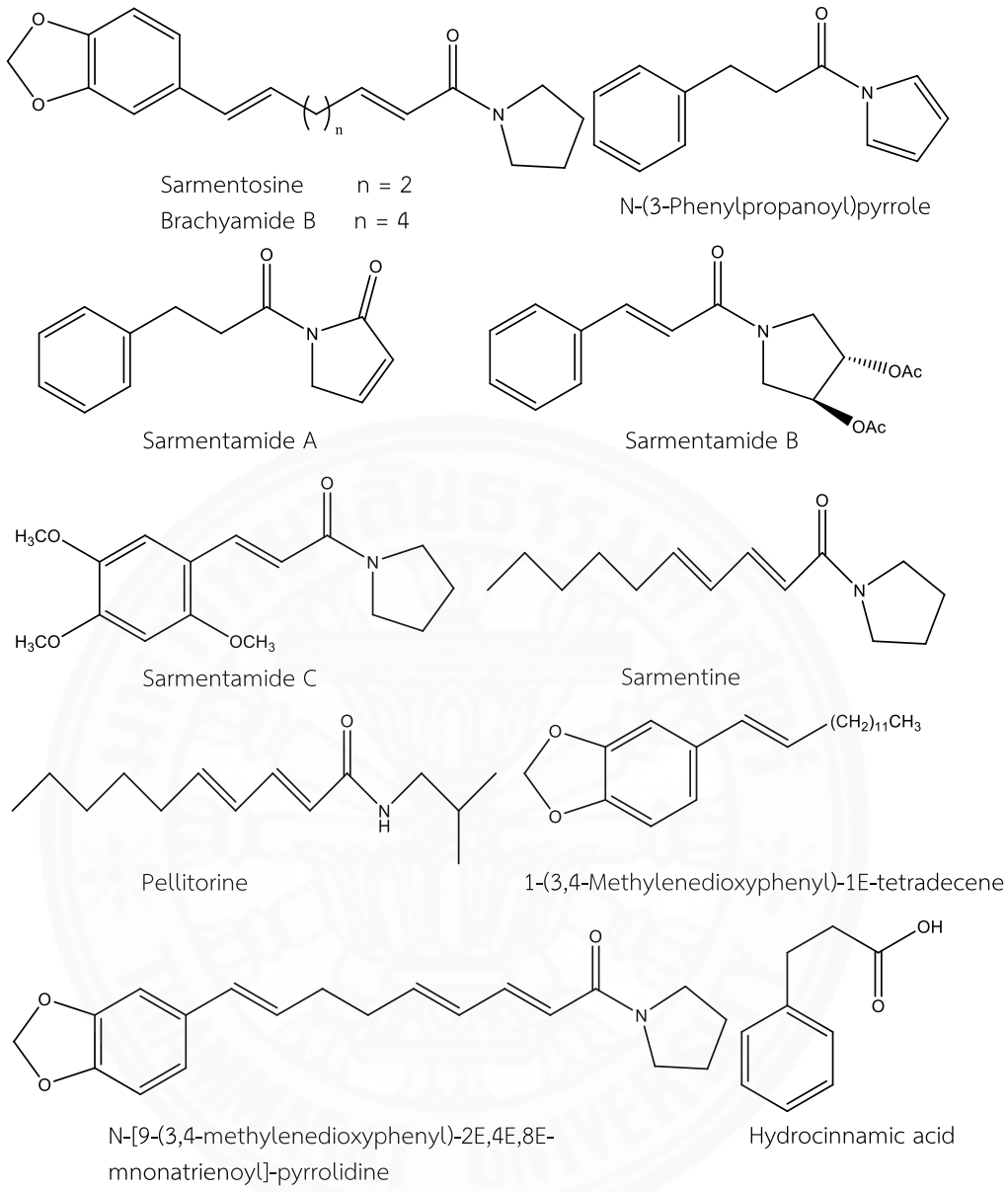
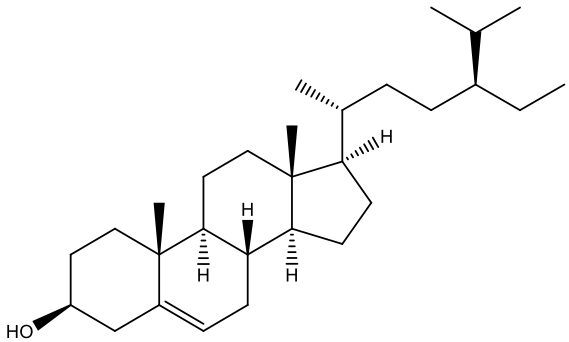
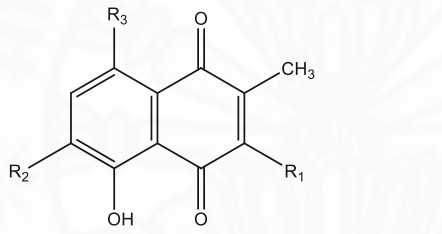


Figure 2.18 Chemical constituents of the roots of *P. sarmentosum* (Bokesch et al., 2011; Hematpoor et al., 2016; Parmar et al., 1997; Tuntiwachwuttikul, Phansa, Pootaeng-on, & Taylor, 2006) (Cont.)



Sitosterol

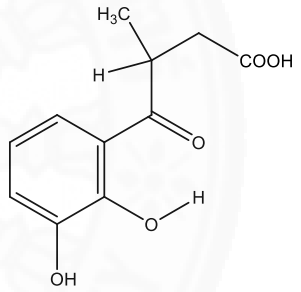
Figure 2.18 Chemical constituents of the roots of *P. sarmentosum* (Bokesch et al., 2011; Hematpoor et al., 2016; Parmar et al., 1997; Tuntiwachwuttikul, Phansa, Pootaeng-on, & Taylor, 2006) (Cont.)



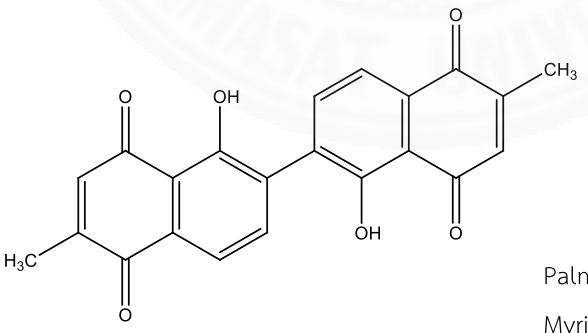
Plumbagin $R_1 = R_2 = R_3 = H$

3-Bromoplumbagin $R_1 = Br, R_2 = R_3 = H$

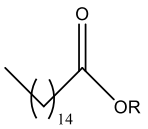
6,8-Dibromoplumbagin $R_1 = H, R_2 = R_3 = Br$



Plumbagic acid



Elliptinone



Palmitic acid R = H

Myricyl palmitate $R = CH_3(CH_2)_{29}$

Figure 2.19 Chemical constituents of the roots of *P. indica* (Dinda et al., 1999; Kaewbumrung & Panichayupakaranant, 2012; Sukkasem et al., 2016)

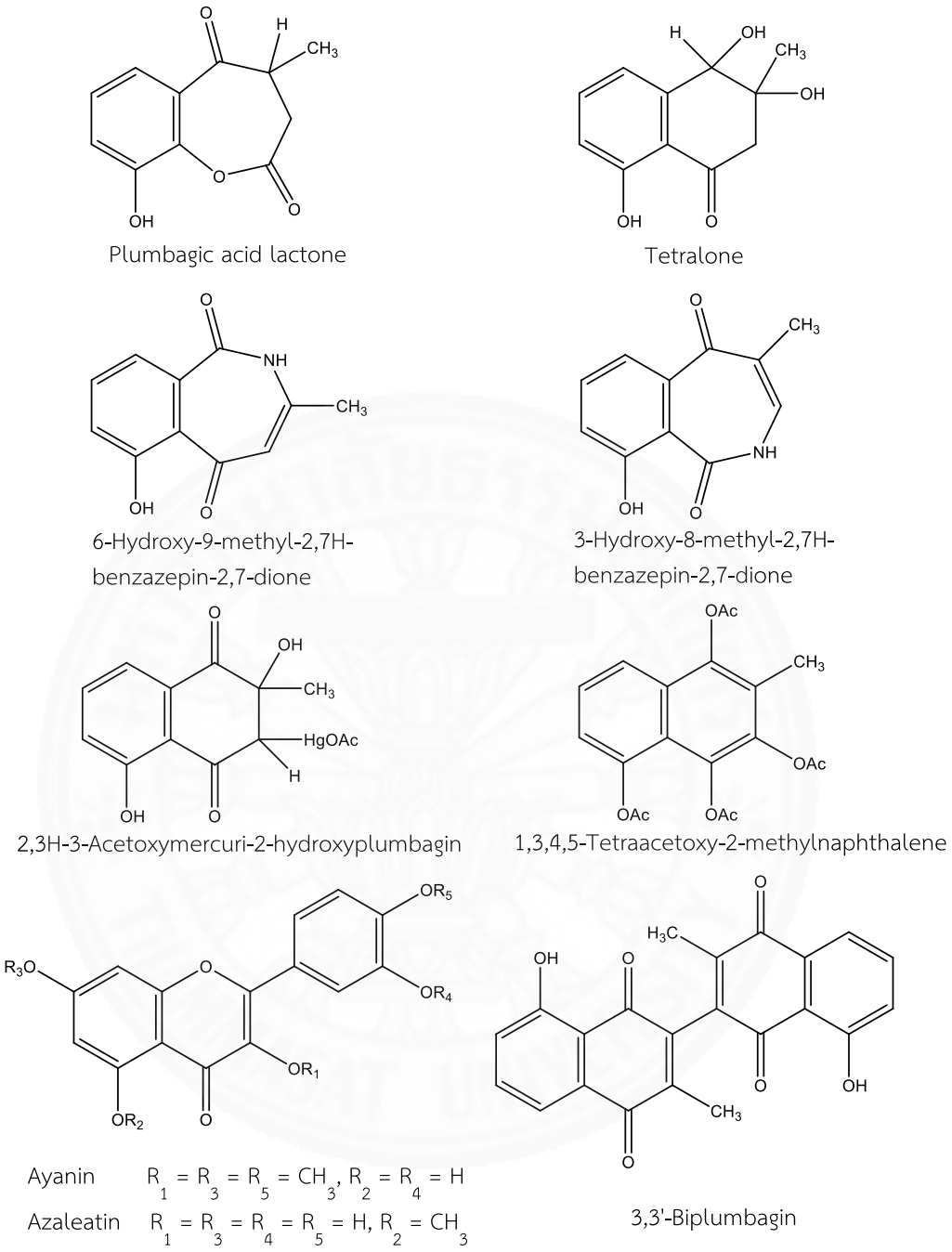


Figure 2.19 Chemical constituents of the roots of *P. indica* (Dinda et al., 1999; Kaewbumrung & Panichayupakaranant, 2012; Sukkasem et al., 2016) (Cont.)

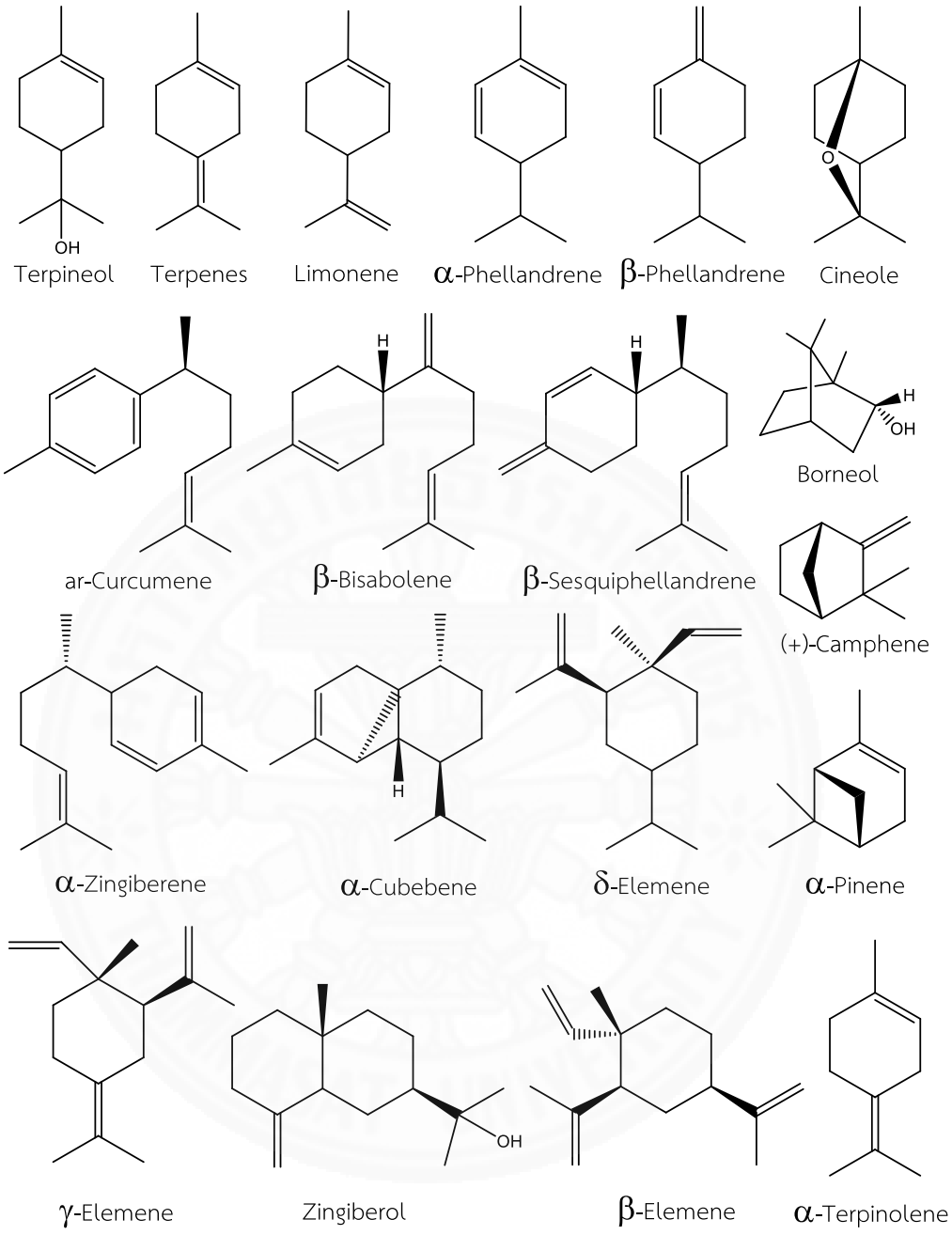


Figure 2.20 Chemical constituents of the rhizomes of *Z. officinale* (Ali, Blunden, Tanira, & Nemmar, 2008; Chen et al., 2009; Dhanik, Arya, & Nand, 2017; Feng et al., 2011; Gupta & Sharma, 2014; Sharma, Singh, & Ali, 2016)

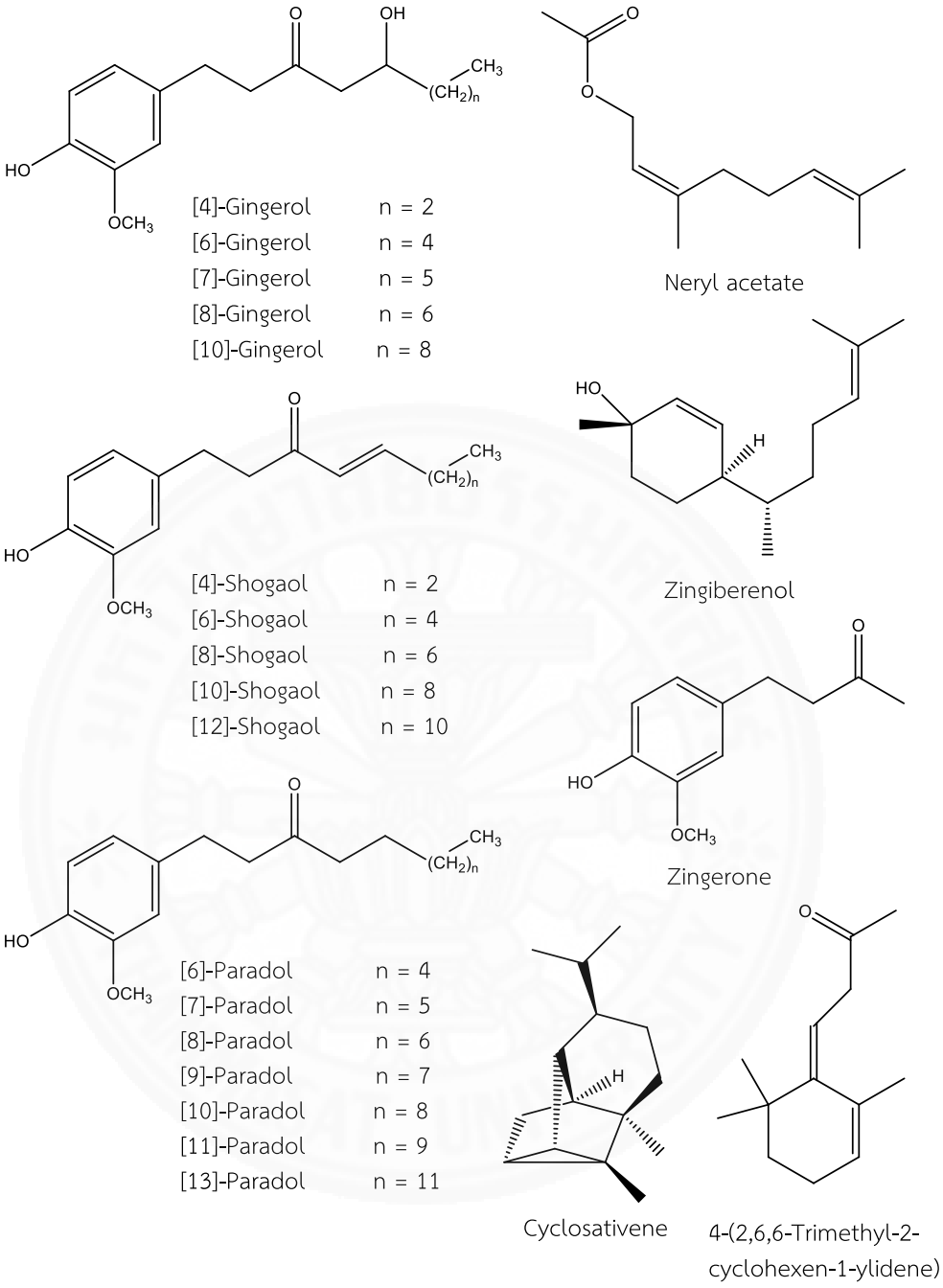


Figure 2.20 Chemical constituents of the rhizomes of *Z. officinale* (Ali, Blunden, Tanira, & Nemmar, 2008; Chen et al., 2009; Dhanik, Arya, & Nand, 2017; Feng et al., 2011; Gupta & Sharma, 2014; Sharma, Singh, & Ali, 2016) (Cont.)

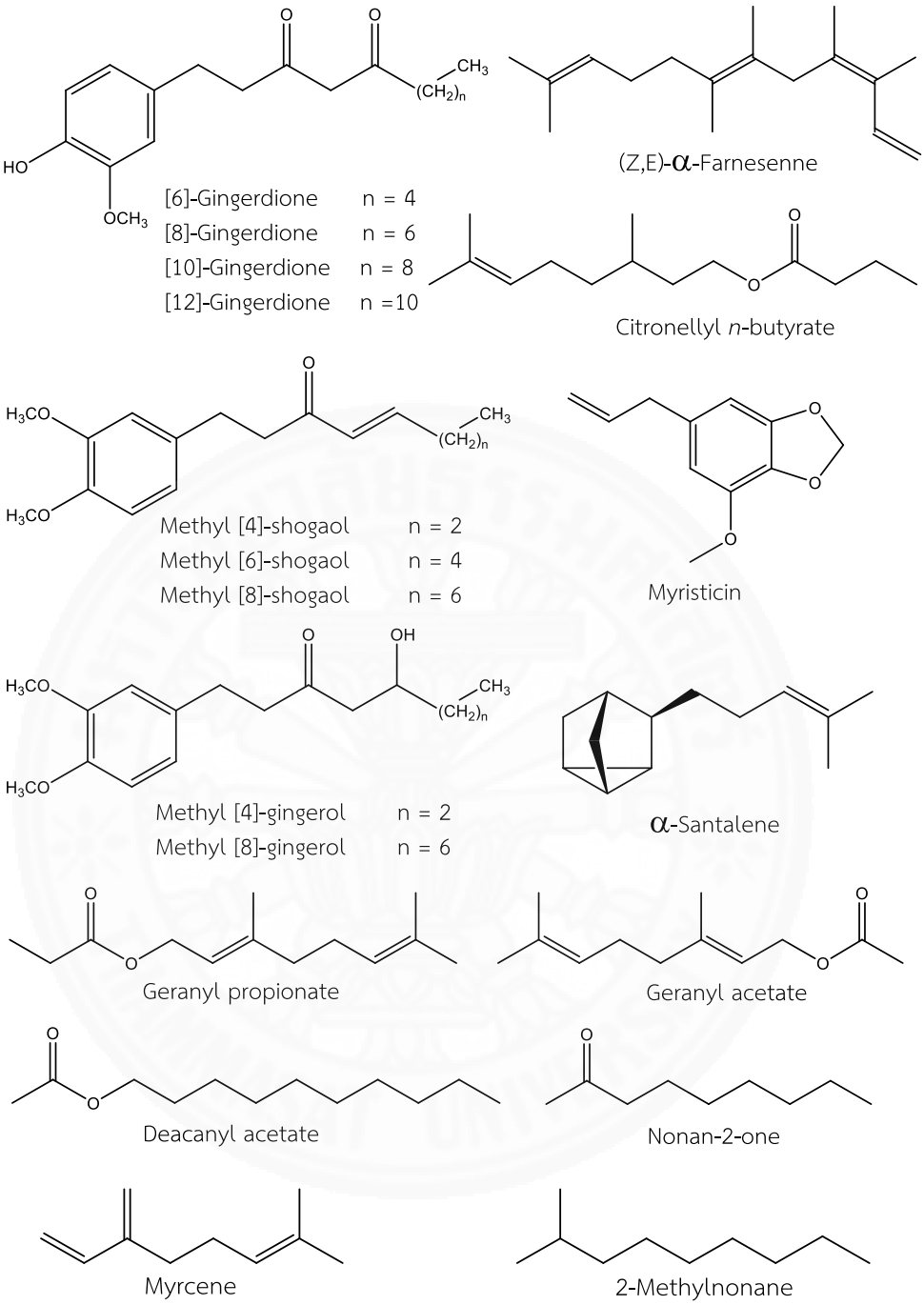


Figure 2.20 Chemical constituents of the rhizomes of *Z. officinale* (Ali, Blunden, Tanira, & Nemmar, 2008; Chen et al., 2009; Dhanik, Arya, & Nand, 2017; Feng et al., 2011; Gupta & Sharma, 2014; Sharma, Singh, & Ali, 2016) (Cont.)

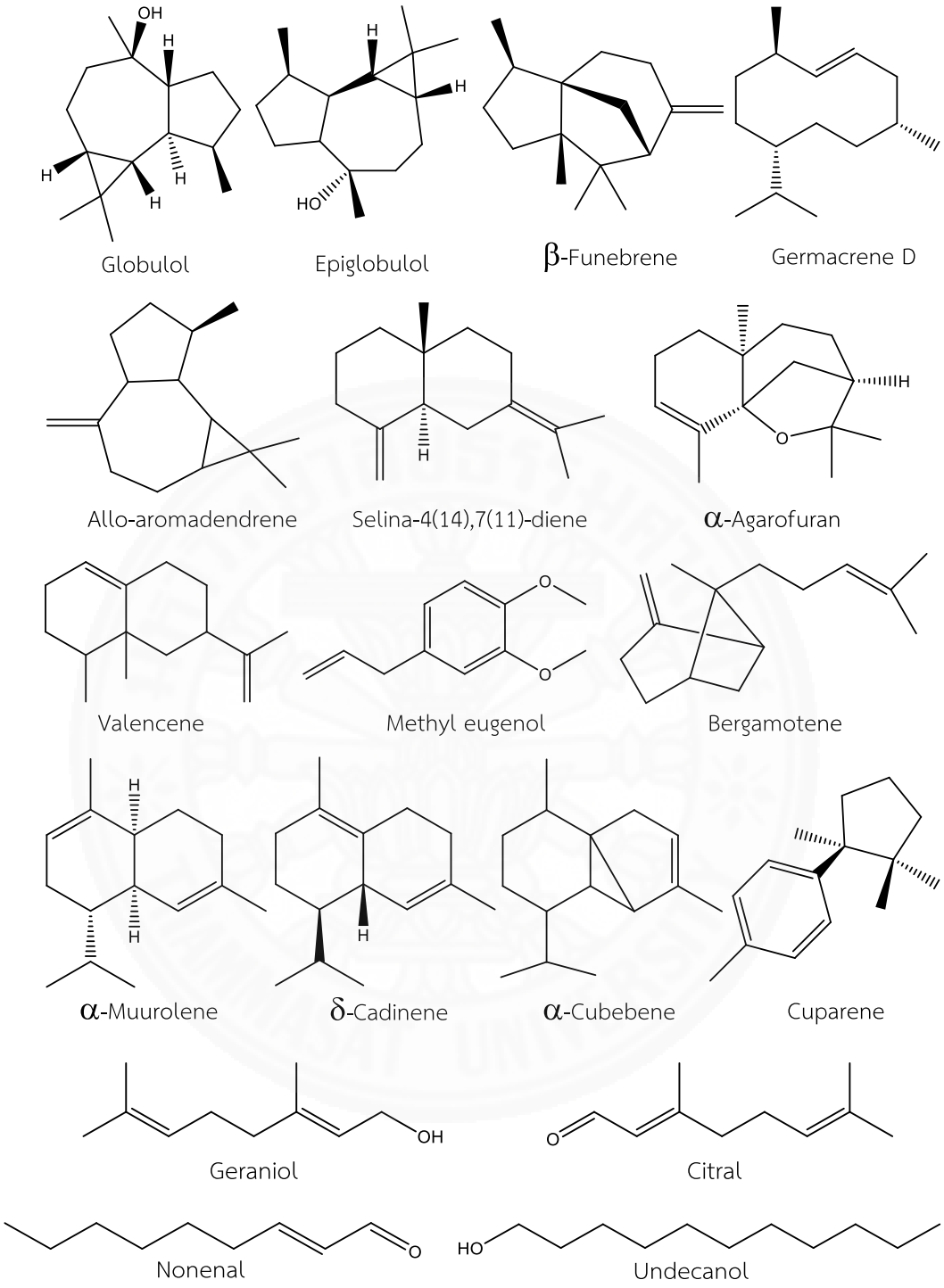


Figure 2.20 Chemical constituents of the rhizomes of *Z. officinale* (Ali, Blunden, Tanira, & Nemmar, 2008; Chen et al., 2009; Dhanik, Arya, & Nand, 2017; Feng et al., 2011; Gupta & Sharma, 2014; Sharma, Singh, & Ali, 2016) (Cont.)

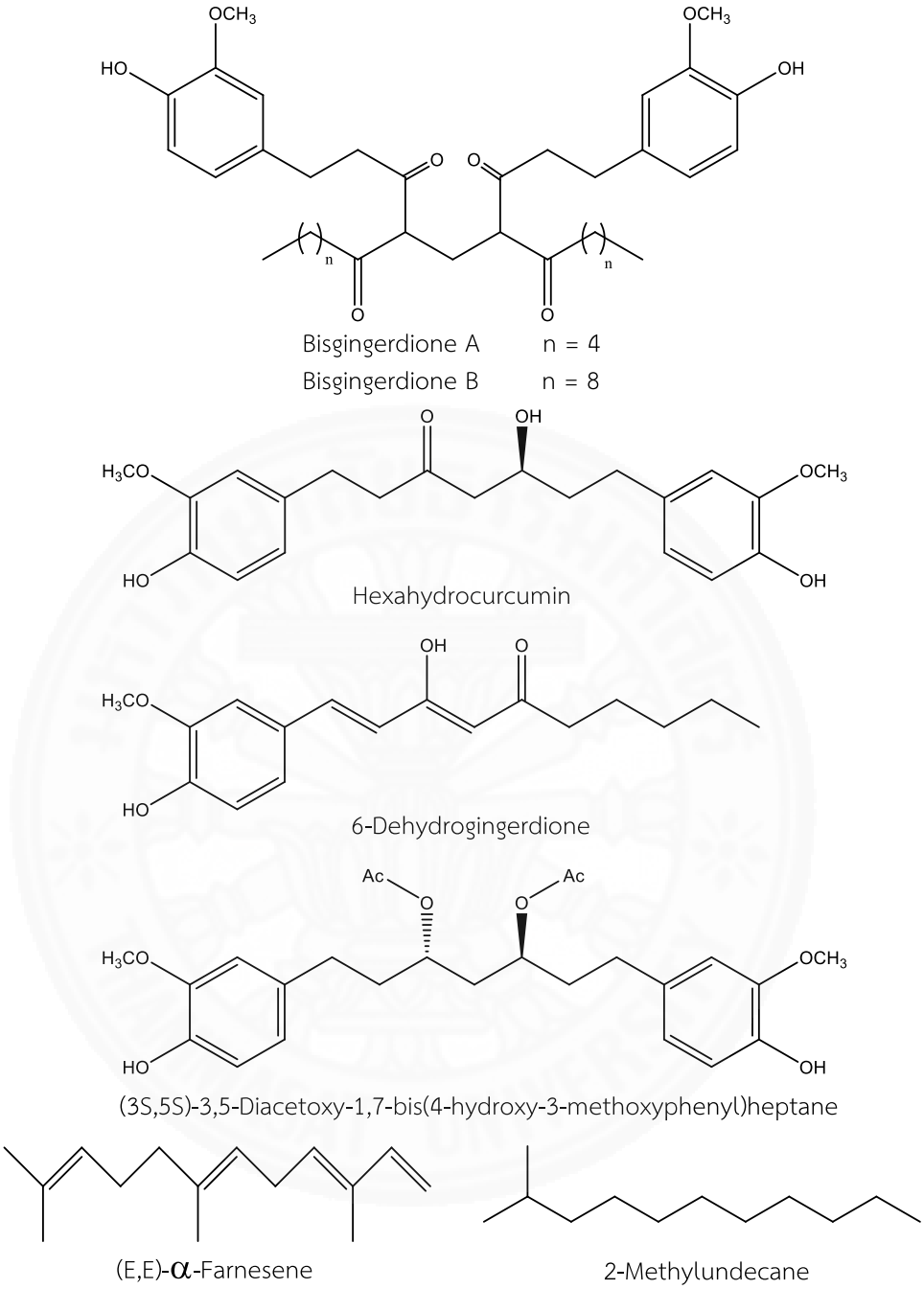


Figure 2.20 Chemical constituents of the rhizomes of *Z. officinale* (Ali, Blunden, Tanira, & Nemmar, 2008; Chen et al., 2009; Dhanik, Arya, & Nand, 2017; Feng et al., 2011; Gupta & Sharma, 2014; Sharma, Singh, & Ali, 2016) (Cont.)

2.6.3 Biological activities of Benjakul remedy and its components

Previous reports of biological activities of Benjakul remedy and its components are shown in Table 2.6 to 2.11.

Table 2.6 Biological activities of Benjakul remedy

Activities	Results	References
Adaptogenic	- The aqueous extract (41.3 and 413 mg/kg) showed improvement of pancreatic senescence by reduction of oxidative stress and islet cell proliferation, insulin signaling by transcription of IRS-2 gene and lipid deposition in the acinar cells in high-fat fed rats model.	Vongthoung, Kamchansuppasin, Temrangsee, Munkong, & Kaendee, 2016
Anti-inflammatory	- The ethanolic extract (1, 10 and 100 μg/ml) significantly reduced TNF-α, IL-1β and IL-6 production in LPS-induced colonic epithelial (Caco-2) cells at dose-dependently manner.	Burodom & Itharat, 2013
Antimalarial	- The ethanolic extract exhibited good to moderate antimalarial activity against K1 and 3D7 *P. falciparum* clones with the IC_{50} values of 3.7 and 10.5 μg/ml, respectively.	Thiengsusuk et al., 2013
Antimicrobial	- The ethanolic extract exposed antimicrobial activity against *Vibrios* with the MIC values between 2.5 to < 5 mg/ml, *Shigella* spp. with the MIC value more than 5 mg/ml and *S. aureus* with the MIC value of 5 mg/ml.	Kondo, Sattaponpan, Phongpaichit, Srijan, & Itharat, 2010

Table 2.6 Biological activities of Benjakul remedy (Cont.)

Activities	Results	References
Cytotoxic	- The ethanolic extract displayed no cytotoxicity against cholangiocarcinoma cell line (CL-6), human hepatocarcinoma cell line (HepG2), human laryngeal carcinoma cell line (Hep-2) using MTT assay.	Mahavorasirikul et al., 2010
	- The ethanolic extract showed moderated cytotoxicity against breast adenocarcinoma cell (MCF-7, IC_{50} = 33.20 μg/ml), human small cell lung cancer cell line (NCI-HI 688, IC_{50} = 36.15 μg/ml), human large cell lung carcinoma cell line (COR-L23, IC_{50} = 43.64 μg/ml), human small cell lung carcinoma cell line (A549, IC_{50} = 48.96 μg/ml), and normal human lung fibroblasts (MRC-5, IC_{50} = 60.83 μg/ml) using SRB assay.	Rattarom et al., 2010, 2014; Sakpakdeejaroen & Itharat, 2009
	- Piperine exhibited cytotoxicity against MCF-7 and COR-L23 cells with the IC_{50} values of 9.80 and 17.00 μg/ml, respectively.	Rattarom et al., 2010; Sakpakdeejaroen & Itharat, 2009
	- 6-Gingerol showed promising cytotoxic activity against MCF-7 and COR-L23 cells with the IC_{50} values of 10.18 and 15.19 μg/ml, respectively.	Rattarom et al., 2010; Sakpakdeejaroen & Itharat, 2009

Table 2.6 Biological activities of Benjakul remedy (Cont.)

Activities	Results	References
Cytotoxic	- Plumbagin showed strong activity against A549, NCI-HI 688, COR-L23, HepG2, Hela (cervical cancer cell line) and MRC-5 cancer cells (IC_{50} = 0.59 μg/ml, 1.41 μg/ml, 2.55 μM, 2.61 μM, 4.61 μM and 11.54 μM, respectively).	Rattarom et al., 2010, 2014; Ruangnoo et al., 2012
	- Shogaol displayed cytotoxic activity against NCI-HI 688 with the IC_{50} value of 6.45 μg/ml.	Rattarom et al., 2014
Genotoxic	- The ethanol extract exposed genotoxicity (at concentration of ≥ 100 μg/ml) and cytotoxicity (at concentration of ≥ 500 μg/ml) against human lymphocytes using sister chromatid exchange (SCE) assay.	Ratanavalachai et al., 2012

Table 2.7 Biological activities of the stems of *P. interruptum*

Activities	Results	References
Analgesic	- The ethanolic extract displayed significantly analgesic activity on early phase and late phase of formalin test in mice at the doses of 300, 600 and 1,200 mg/kg when compared with the control group.	Sireeratawong et al., 2012
Anti-inflammatory	- In acute inflammatory model, the ethanolic extract exposed significant inhibitory effect on ethyl phenylpropiolate (EPP)-induced rats ear edema at the dose of 1 mg/ear. Moreover, the ethanol extract (300, 600 and 1,200 mg/kg) significantly inhibited the paw edema at 1, 3 and 5 h after carrageenan injection.	Sireeratawong et al., 2012
	- In chronic inflammatory model, the ethanolic extract at the dose of 1,200 mg/kg showed the reduction of transudative weight, body weight gain and thymus weight by cotton pellet-induced granuloma formation in rats.	Sireeratawong et al., 2012
Antipyretic	- The ethanolic extract (300, 600 and 1,200 mg/kg) significantly diminished the rectal temperature on yeast-induced hyperthermia in rats.	Sireeratawong et al., 2012
Immunomodulatory	- The water extract (1 ng/ml – 100 μg/ml) increased lymphocyte proliferation, but did not stimulate natural killer (NK) cell activity.	Panthong & Itharat, 2014

Table 2.8 Biological activities of the fruits of *P. longum*

Activities	Results	References
Analgesic	- The ethanolic extract (300, 600 and 1,200 mg/kg) significantly reduced the licking time on both early phase and late phase of formalin test in mice.	Sireeratawong et al., 2012
Anti-allergic	- *In vivo* model, the petroleum ether, alcoholic, and aqueous extracts (50, 100 and 200 mg/kg) significantly decreased paw volume at 2 h on passive paw anaphylaxis in rats. Moreover, only the petroleum ether and aqueous extracts (200 mg/kg) significantly attenuated the number of leukocytes on milk-induced leukocytosis in mice when compared with the control group.	Kaushik, Rani, Kaushik, Sacher, & Yadav, 2012
Anti-asthmatic	- *In vitro* model, the petroleum ether, alcoholic, and aqueous extracts (100 μg/ml) significantly inhibited the histamine-induced contraction of isolated guinea-pig ileum preparation.	Kaushik et al., 2012
	- *In vivo* model, the petroleum ether, alcoholic, and aqueous extracts (50, 100 and 200 mg/kg) significantly increased the latent period of convulsion on histamine-induced bronchospasm in guinea-pigs and showed significant activity on haloperidol-induced catalepsy in mice when compared with the control group.	Kaushik et al., 2012

Table 2.8 Biological activities of the fruits of *P. longum* (Cont.)

Activities	Results	References
Anti-inflammatory	- In acute inflammatory model, the ethanol extract (1 mg/ear) significantly inhibited the EPP-induced ear edema formation in rats. Additionally, the ethanol extract (1,200 mg/kg) showed significant inhibitory effect on carrageenan-induced paw edema at 3 and 5 h after carrageenan injection.	Sireeratawong et al., 2012
	- In chronic inflammatory model, the ethanol extract (1,200 mg/kg) inhibited transudative weight, granuloma weight, body weight gain and thymus weight using cotton pellet-induced granuloma formation in rats.	Sireeratawong et al., 2012
	- The n-hexane extract exhibited inhibition of leukotriene (5-LOX, 22%) and prostaglandin (COX-1, 40%) activities.	Stöhr et al., 2001
	- The ethanol extract showed strong inhibitory effect on NO production in LPS-induced RAW 264.7 cells (IC_{50} = 9.98 μg/ml).	Anuthakoengkun & Itharat, 2014
	- The chloroform extract inhibited the TNF-α-induced expression of ICAM-1, VCAM-1 (at concentration of 17.5 μg/ml) and E-selectin (at concentration of 15 μg/ml) on the surface of endothelial cells.	Singh et al., 2008

Table 2.8 Biological activities of the fruits of *P. longum* (Cont.)

Activities	Results	References
Anti-inflammatory	- The EtOAc extract (40 mg/kg) reduced lymphocytes and cytokine levels, such as IL-2 and IFN-γ in sensitized BALB/c mice.	Devan, Bani, Suri, Santti, & Qazi, 2007
	- Piperine (100 mg/kg) significantly decreased the levels of TNF-α, IL-1β, and PGE_2 and increased the level of IL-10 in rats by collagen induced arthritis (CIA).	Umar et al., 2013
	-Piperine (5 and 10 mg/kg) suppressed the production of NO and TNF-α and also decreased FFA-induced TLR4 mediated inflammation.	Gupta, Motiwala, Dumore, Danao, & Ganjare, 2015
Antipyretic	- The ethanolic extract (600 and 1,200 mg/kg) showed significant antipyretic activity on yeast-induced hyperthermia in rats.	Sireeratawong et al., 2012
Immunomodulatory	- The water extract (1 ng/ml – 100 μg/ml) increased lymphocyte proliferation, but did not stimulate NK cell activity.	Panthong & Itharat, 2014
	- The alcoholic extract (10 mg/dose) and piperine (1.14 mg/dose) raised the total white blood cells in BALB/c mice.	Sunila & Kuttan, 2004

Table 2.9 Biological activities of the roots of *P. sarmentosum*

Activities	Results	References
Anti-inflammatory	- In acute inflammatory model, the ethanol extract (1 mg/ear) inhibited the EPP-induced ear edema formation in rats. Moreover, the ethanol extract (1,200 mg/kg) showed inhibitory effect on carrageenan-induced paw edema at 3 and 5 h after carrageenan injection.	Sireeratawong, Vannasiri, Sritiwong, Itharat, & Jaijoy, 2010
	- In chronic inflammatory model, the ethanol extract (1,200 mg/kg) inhibited transudative weight by cotton pellet-induced granuloma formation in rats.	Sireeratawong et al., 2010
Anti-nociceptive	- The ethanolic extract (300, 600 and 1,200 mg/kg) presented inhibitory effect on the formalin test in mice.	Sireeratawong et al., 2010
Antipyretic	- The ethanolic extract (300, 600 and 1,200 mg/kg) displayed significant antipyretic activity on yeast-induced hyperthermia in rats.	Sireeratawong et al., 2010
Immunomodulatory	- The water extract (1 ng/ml – 100 μg/ml) increased lymphocyte proliferation and increased NK cell activity at a concentration of 100 μg/ml.	Panthong & Itharat, 2014

Table 2.10 Biological activities of the roots of *P. indica*

Activities	Results	References
Anti-inflammatory	- The ethanol extract showed the potent inhibitory effect on NO secretion with the IC_{50} value of 9.74 μg/ml.	Anuthakoengkun & Itharat, 2014
Immunomodulatory	- The methanol extract (4 mg/ml) induced T-lymphocyte proliferation (the SI value of about 1.6).	Saraphanchotiwitthaya, Ingkaninan, & Sripalakit, 2007

Table 2.11 Biological activities of the rhizomes of *Z. officinale*

Activities	Results	References
Analgesic	- The ethanol extract (50-800 mg/kg) dose-dependently showed analgesic activities on electrical heat-induced pain and acetic acid-induced writhes in mice model.	Ojewole, 2006
	- 6-Gingerol (25 and 50 mg/kg) significantly inhibited acetic acid-induced writhing response and the late phase of formalin-induced pain in mice when compared with the control group.	Young et al., 2005
Anti-allergic	- The ethanol extract (500 mg/kg body weight) significantly reduced both mRNA levels and protein levels of IL-4 and IL-5 in BALF, also suppressed total serum IgE level in allergic asthma mouse model.	Khan, Shahzad, Asim, Imran, & Shabbir, 2015
	- The water extract (720 mg/kg body weight) inhibited both mRNA levels and protein levels of IL-4 and IL-5 in BALF, also decreased the total IgE level in serum.	Khan et al., 2015
	- Hexahydrocurcumin, 6-dehydrogingerdione, 10-gingerol, 6-shogaol, and 6-gingerol showed inhibitory effect on β-hexosaminidase release from RBL-2H3 cells.	Chen et al., 2009

Table 2.11 Biological activities of the rhizomes of *Z. officinale* (Cont.)

Activities	Results	References
Anti-allergic	- *In vitro* model, 6-shogaol (0.1-100 μM) dose-dependently exhibited anti-allergic activity on histamine release in compound 48/80-induced rat peritoneal mast cells (RPMCs).	Sohn, Han, Lee, Cho, & Jung, 2013
	- *In vivo* model, 6-shogaol (1 and 5 mg/kg) inhibited the IgE-mediated passive cutaneous anaphylaxis (PCA) in rats by 44.9% and 72.1%, respectively when compared with the control group.	Sohn et al., 2013
Anti-inflammatory	- 6-, 8- and 10-Gingerol inhibited both the PGE_2 production (IC_{50} < 0.1 μg/ml) and the COX-2 expression (30-55%) in LPS-stimulated U937 cells.	Lantz et al., 2007
	- 6-Shogaol had no inhibitory effects on PGE_2 production and COX-2 expression.	Lantz et al., 2007
	- 8-Paradol showed the potent inhibitory activity against COX-1 enzyme with the IC_{50} value of 4 μM.	Nurtjahja-Tjendraputra, Ammit, Roufogalis, Tran, & Duke, 2003
	- 10-Gingerol, 8-shogaol and 10-shogaol showed COX-2 inhibition, but not COX-1 inhibition (IC_{50} = 32.0, 17.5 and 7.5 μM, respectively).	Van Breemen, Tao, & Li, 2011

Table 2.11 Biological activities of the rhizomes of *Z. officinale* (Cont.)

Activities	Results	References
Anti-inflammatory	- The chloroform partition showed both COX-2 and COX-1 inhibition with IC_{50} values of 7.5 and 20.0 μg/ml, respectively.	Van Breemen et al., 2011
	- 6-Shogaol, 8-paradol and 10-gingerol displayed strong inhibition of COX-2 enzyme in IL-1β-activated A549 cells with the IC_{50} values of 2.1, 3.4 and 3.7 μM, respectively.	Tjendraputra, Tran, Liu-Brennan, Roufogalis, & Duke, 2001
	- The dichloromethane extract inhibited PGE_2 production in LPS-induced U937 cells with the IC_{50} value of 0.06 μg/ml. Moreover, it prevented joint inflammation and granuloma formation in rheumatoid arthritis rat model.	Funk, Frye, Oyarzo, & Timmermann, 2009
	- The ethanol extract (1 μl/ml) exposed the inhibitory effect on TNF-α, IL-12, IL-1β, RANTES, and MCP-1 production in LPS-activated murine peritoneal macrophages cells.	Tripathi, Bruch, & Kittur, 2008
	- The ethanol extract (50-800 mg/kg) presented anti-inflammatory activity on rats hind paw edema induced by fresh egg albumin in dose-dependent manner.	Ojewole, 2006

Table 2.11 Biological activities of the rhizomes of *Z. officinale* (Cont.)

Activities	Results	References
Anti-inflammatory	- 6-Gingerol (50 and 100 mg/kg) significantly showed anti-inflammatory activity against carrageenan-induced paw edema in rats.	Young et al., 2005
	- 6-Shogaol showed inhibitory effects on NO and PGE_2 secretion, and also suppressed iNOS and COX-2 in LPS-induced RAW 264.7 cells, while 6-gingerol showed mild inhibitory effects on NO and PGE_2 production, and iNOS expression in RAW 264.7 cells.	Pan et al., 2008

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chemicals, materials and instruments

The chemicals, materials and instruments used in the present studies are summarized in Table 3.1.

Table 3.1 The list of chemicals, materials and instruments used in the studies

Chemicals, materials and instruments	Sources
4-Nitrophenyl N-acetyl-β-D-glucosaminide (PNAG)	Sigma-Aldrich Inc., USA
[6]-Gingerol	Wako Pure Chemical Industries, Ltd., Japan
[6]-Shogaol	Wako Pure Chemical Industries, Ltd., Japan
24-Well plate flat bottom	Corning Inc.., USA
96-Well plate EIA/RIA flat bottom without lid	Corning Inc., USA
96-Well plate flat bottom with lid	Corning Inc., USA
96-Well plate flat bottom without lid	Corning Inc., USA
ABTS® Peroxidase substrate	KPL, USA
Albumin from bovine serum (BSA, code A8022)	Sigma-Aldrich Inc., USA
Albumin dinitrophenyl (DNP-BSA)	Sigma-Aldrich Inc., USA
Albumin from chicken egg white grade V (OVA)	Sigma-Aldrich Inc., USA
Anchored oligo dT primers	Thermo Fisher Scientific, USA
Atractylodin TS	Wako Pure Chemical Industries, Ltd., Japan
Biosafety cabinet	Labconco, USA
Bovine serum albumin (heat shock fraction) (BSA)	Sigma-Aldrich Inc., USA
Brilliant II SYBR® green QPCR master mix	Agilent Technologies, USA

Table 3.1 The list of chemicals, materials and instruments used in the studies (Cont.)

Chemicals, materials and instruments	Sources
Calcium chloride dihydrate	Merck, Germany
Cell culture flask, canted neck 25 and 75 cm^2	Corning Inc., USA
Centrifuge	Hettich, Germany
Centrifuge tube 15 and 50 ml	Corning Inc., USA
Chloroform (AR grade)	RCI Labscan, Thailand
Chlorpheniramine maleate (CPM)	Sigma-Aldrich Inc., USA
Citric acid monohydrate	Merck, Germany
CO_2 incubator	Thermo Fisher Scientific, USA
D-(+)-glucose	Sigma-Aldrich Inc., USA
Dimethylsulphoxide (DMSO) (AR grade)	RCI Labscan, Thailand
Disposable serological pipette 5, 10 and 25 ml	Corning Inc., USA
ELISA washer	BioTek, USA
β-Eudesmol	Wako Pure Chemical Industries, Ltd., Japan
Fetal bovine serum (FBS)	Life Technologies, USA
Gavage needle (No. 8 size 1.5”)	NLAC, Thailand
Gelatin type B from bovine skin	Sigma-Aldrich Inc., USA
Goat-anti-mouse IgG1-biotin	Southern Biotech, USA
Hematocytometer	Brand, Germany
Hexane (AR grade)	RCI Labscan, Thailand
High performance liquid chromatography (HPLC, 1200 series)	Agilent Technologies, USA
Hot air oven	Memmert, Germany
Hydrochloric acid (AR grade)	RCI Labscan, Thailand
Imject™ alum adjuvant	Thermo Fisher Scientific, USA
Inverted microscope	Nikon, Japan

Table 3.1 The list of chemicals, materials and instruments used in the studies (Cont.)

Chemicals, materials and instruments	Sources
Isopropanol (Propan-2-ol) (AR grade)	RCI Labscan, Thailand
iTaq™ universal SYBR® green supermix	Bio-Rad Laboratories, USA
Light microscope (model BX41)	Olympus, Japan
Lipopolysaccharide from *E. coli* O55:B5 (LPS)	Sigma-Aldrich Inc., USA
Magnesium chloride hexahydrate	Merck, Germany
Methanol (AR grade and HPLC grade)	RCI Labscan, Thailand
Micropipettes	Gilson, USA
Microplate reader	BioTek, USA
Micro tube	Axygen Inc., USA
Minimum essential medium (MEM)	Life Technologies, USA
Moisture analysis (model SMO-01)	BOECO, Germany
Monoclonal anti-dinitrophenyl antibody produced in mouse	Sigma-Aldrich Inc., USA
Montelukast (sodium salt)	Cayman Chemical, USA
Multichannel pipettor	Corning Inc., USA
Nanodrop	Thermo Fisher Scientific, USA
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma-Aldrich Inc., USA
Paraformaldehyde	Sigma-Aldrich Inc., USA
Penicillin Streptomycin (P/S)	Life Technologies, USA
pH meter	WTW Inolab, Germany
Phosphate buffered saline (PBS)	Amresco, USA
Phosphoric acid	Sigma-Aldrich Inc., USA
Piperine	Merck, Germany
PIPES sodium salt	Amresco, USA
Pipette controller	Brand, Germany
Plumbagin	Sigma-Aldrich Inc., USA
Polymerase chain reaction machine (PCR)	Eppendorf, USA

Table 3.1 The list of chemicals, materials and instruments used in the studies (Cont.)

Chemicals, materials and instruments	Sources
Potassium chloride	Merck, Germany
Prednisolone	Sigma-Aldrich Inc., USA
Quantikine mouse TNF-α ELISA kit	R&D Systems Inc., USA
Rat-anti-mouse IgE-biotin	Southern Biotech, USA
Rat-anti-mouse IgG2a-biotin	Southern Biotech, USA
Real-time PCR machine (model Mx3005P)	Agilent Technologies, USA
RevertAid first stand cDNA synthesis kit	Thermo Fisher Scientific, USA
RNA*later* RNA stabilization reagent	QIAGEN, Germany
RPMI medium 1640	Life Technologies, USA
Rotary evaporator	Buchi, Switzerland
SepacorControl preparative chromatography (MPLC) (model C-620)	Buchi, Switzerland
Sodium bicarbonate	BHD, England
Sodium carbonate	Merck, Germany
Sodium chloride	Merck, Germany
Sodium hydroxide	BHD, England
Stability machine (model KB 8400F)	Termaks, Norway
Streptavidin-horseradish peroxidase (HRP) conjugate	Southern Biotech, USA
Sucrose	Univar, Australia
Sulfanilamide	Sigma-Aldrich Inc., USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich Inc., USA
Total RNA mini kit (tissue)	Geneaid Biotech Ltd., Taiwan
Trisodium citrate dihydrate	Merck, Germany
Trypan blue stain (0.4%)	Life Technologies, USA
Trypsin-EDTA (0.5%)	Life Technologies, USA
Tween-20	Affymetrix, USA

Table 3.1 The list of chemicals, materials and instruments used in the studies (Cont.)

Chemicals, materials and instruments	Sources
Tween-80	Sigma-Aldrich Inc., USA
Vortex	Scientific Industries, USA
Water (HPLC grade)	RCI Labscan, Thailand
Water bath	Memmert, Germany

3.2 Plant materials

3.2.1 Prasaprohyai remedy

The plant materials were purchased from Australia, China, India, Indonesia and Thailand. The identification of plant materials were confirmed by comparison with authentic voucher specimens deposited at the Herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The data of plant materials in Prasaprohyai remedy are summarized in Table 3.2.

Table 3.2 The summary of data of plant materials in Prasaprohyai remedy

Plant species (Family)	Thai name	Source	Part used	Ratio	Voucher specimen number
Amomum testaceum Ridl. (Zingiberaceae)	Krawan	Chanthaburi, Thailand	Fruit	1	SKP 206 01 11 01
Anethum graveolens L. (Apiaceae)	Thian ta takkataen	India	Fruit	1	SKP 199 01 07 01
Angelica dahurica (Hoffm.) Benth. & Hook. F. ex Franch. & Sav. (Apiaceae)	Kot so	China	Root	1	SKP 199 01 04 01

Table 3.2 The summary of data of plant materials in Prasaprohyai remedy (Cont.)

Plant species (Family)	Thai name	Source	Part used	Ratio	Voucher specimen number
Angelica sinensis (Oliv.) Diels (Apiaceae)	Kot chiang	China	Root	1	SKP 199 01 09 01
Artemisia annua L. (Asteraceae)	Kot chula lampha	China	All parts	1	SKP 051 01 01 01
Atractylodes lancea (Thunb.) DC. (Asteraceae)	Kot khamao	China	Rhizome	1	SKP 051 01 12 01
Cuminum cyminum L. (Apiaceae)	Thian khao	India	Fruit	1	SKP 199 03 03 01
Dracaena loureiroi Gagnep. (Asparagaceae)	Chan daeng	Ratchaburi and Kanchanaburi, Thailand	Stem	1	SKP 065 04 12 01
Foeniculum vulgare Mill. (Apiaceae)	Thian khao plueak	India	Fruit	1	SKP 199 06 22 01
Kaempferia galanga L. (Zingiberaceae)	Proh hom	Ratchaburi and Kanchanaburi, Thailand	Rhizome	20	SKP 206 11 07 01
Lepidium sativum L. (Brassicaceae)	Thian daeng	India	Seed	1	SKP 057 12 19 01
Ligusticum sinense Oliv. (Apiaceae)	Kot hua bua	China	Rhizome	1	SKP 199 12 19 01
Mammea siamensis (Miq.) T. Anders. (Calophyllaceae)	Saraphi	Ratchaburi and Kanchanaburi, Thailand	Flower	1	SKP 083 13 19 01
Mesua ferrea L. (Calophyllaceae)	Bunnak	Ratchaburi and Kanchanaburi, Thailand	Flower	1	SKP 083 13 06 01
Mimusops elengi L. (Sapotaceae)	Phikun	Ratchaburi and Kanchanaburi, Thailand	Flower	1	SKP 171 13 05 01

Table 3.2 The summary of data of plant materials in Prasaprohyai remedy (Cont.)

Plant species (Family)	Thai name	Source	Part used	Ratio	Voucher specimen number
Myristica fragrans Houtt. (Myristicaceae)	Chan thet	Australia	Stem	1	SKP 121 13 06 01
Myristica fragrans Houtt. (Myristicaceae)	Mace	Suratthani, Thailand	Aril	1	SKP 121 13 06 01
Myristica fragrans Houtt. (Myristicaceae)	Nutmeg	Suratthani, Thailand	Seed	1	SKP 121 13 06 01
Nelumbo nucifera Gaertn. (Nelumbonaceae)	Bua luang	Ratchaburi and Kanchanaburi, Thailand	Pollen	1	SKP 125 14 14 01
Nigella sativa L. (Ranunculaceae)	Thian dam	India	Seed	1	SKP 160 14 19 01
Syzygium aromaticum (L.) Merr. & L. M. Perry (Myrtaceae)	Kan phlu	Indonesia	Flower	1	SKP 123 19 01 01

3.2.2 Benjakul remedy

The plant materials were collected from several provinces of Thailand. The identification of plant materials were confirmed by comparison with authentic voucher specimens deposited at the Herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The data of plant materials in Benjakul remedy are summarized in Table 3.3.

Table 3.3 The summary of data of plant materials in Benjakul remedy

Plant species (Family)	Thai name	Source	Part used	Ratio	Voucher specimen number
Piper interruptum Opiz. (Piperaceae)	Sa-khan	Sakhonnakhon	Stem	1	SKP 146 16 09 01
Piper longum L. (Piperaceae)	Dee-plee	Chanthaburi	Fruit	1	SKP 146 16 03 01
Piper sarmentosum Roxb. (Piperaceae)	Cha-phlu	Ratchaburi	Root	1	SKP 146 16 19 01
Plumbago indica L. (Plumbaginaceae)	Chettamun phloeng daeng	Bangkok	Root	1	SKP 148 16 09 01
Zingiber officinale Roscoe (Zingiberaceae)	Khing	Phetchabun	Rhizome	1	SKP 206 26 15 01

3.3 Preparation of plant extracts

Plant materials were cleaned, crushed thinly, oven-dried at 50°C for 24 hours, ground into powder with an electric-grinder and then the powdered plants were extracted with 95% ethanol that extraction method is similar to those practice by Thai traditional doctors.

3.3.1 Preparation of Prasaprohyai (PPY) extract

The powdered plants were mixed according to the proportion of combination (Table 3.1). The powdered PPY (300 g) was extracted with 95% ethanol (900 ml) at room temperature for 3 days and the extracted solution was filtered through Whatman No.1 paper. The residue from the filtration was extracted again twice using the same procedure. The total solvent extract (2,700 ml) was concentrated to dryness under reduced pressure (45°C) using a rotary film

evaporator. The concentrated extract was dried to constant weight and stored in air-tight glass container at -20ºC until used.

3.3.2 Preparation of Benjakul (BJK) extract

The powdered plants were mixed according to the proportion of combination (Table 3.2). The powdered BJK (1,000 g) and each of five plants were extracted with 95% ethanol (5,000 ml) at room temperature for 3 days and the extracted solution was filtered through Whatman No.1 paper. The residue from the filtration was extracted again twice using the same procedure. The total solvent extract (15,000 ml) was concentrated to dryness under reduced pressure (45ºC) using a rotary film evaporator. The concentrated extract was dried to constant weight and stored in air-tight glass container at -20ºC until used.

3.4 *In vitro* assay for anti-allergic activity

3.4.1 Cell culture

RBL-2H3 (ATCC® Catalog No. CRL-2256™) cells were cultured in minimum essential medium (MEM) with Earle's salts and L-glutamine, added 2.2 g sodium bicarbonate ($NaHCO_3$), supplemented with 15% heat-inactivated fetal bovine serum (FBS), 10,000 Units/ml penicillin and 10,000 μg/ml streptomycin. The cells were maintained in an incubator at 37°C in a humidified 5% CO_2/95% air atmosphere, with change of cultured medium three times every 7 days.

3.4.2 Determination of antigen-induced β-hexosaminidase release from RBL-2H3 cells

The degranulation of RBL-2H3 cells were determined by measuring β-hexosaminidase release. The inhibition effect of the extract on the release of β-hexosaminidase from RBL-2H3 cells was demonstrated by the following modified method (Tewtrakul, Subhadhirasakul, & Kummee, 2008). The RBL-2H3 cells were seeded at a density of 2×10^5 cells/well in 24-well plates and allowed to adhere for 2 hours at 37ºC in 5% CO_2. Then, the cells were sensitized with monoclonal anti-dinitrophenyl antibody (anti-DNP IgE) (0.45 μg/ml), and incubated at 37ºC in 5% CO_2

for 24 hours. The cells were washed with 400 μl of buffer A [buffer A; consisting of 119 mM NaCl, 5 mM KCl, 5.6 mM D-(+)-glucose, 0.4 mM $MgCl_2.6H_2O$, 1 mM $CaCl_2.2H_2O$, 25 mM PIPES, 0.1% BSA and 40 mM NaOH, pH 7.2], and then incubated in 160 μl of buffer A for an additional 10 min at 37ºC in 5% CO_2. After that, various concentrations of test samples (20 μl) were added to each well and incubated for 10 min, followed by addition of 20 μl of antigen (DNP-BSA, final concentration is 10 μg/ml) for 20 min at 37ºC in 5% CO_2 to stimulate the cells into producing allergic reactions. Aliquots (50 μl) of supernatant were transferred into 96-well plates and incubated with 50 μl of substrate (PNAG) in 0.1 M citric/citrate buffer (pH 4.5) at 37ºC in 5% CO_2 for 2 hours. The reaction was stopped by adding 200 μl of a stop solution (0.1 M $Na_2CO_3/NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in DMSO, and the solution was added to buffer A (final DMSO concentration was 0.1%).

The percentage of inhibitory effects on β-hexosaminidase release by the test samples was calculated by the following equation, and IC_{50} values were calculated from the Prism program.

$$\text{Inhibition (\%)} = [1 - (T - B - N)/(C - B - N)] \times 100$$

Where Control (C): DNP-BSA (+), test sample (-); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (-), test sample (+); Normal (N): DNP-BSA (-), test sample (-)

3.5 *In vivo* assay for anti-allergic activity

3.5.1 Preparation of Prasaprohyai extract, Benjakul extract and reference drug

For Prasaprohyai extract, the ethanolic extract of Prasaprohyai at dose 100, 200 and 400 mg/kg/body weight were dissolved in 5% Tween-80 for administration for mice.

For Benjakul extract, the ethanolic extract of Benjakul at dose 37.5, 75 and 150 mg/kg/body weight were dissolved in 10% Tween-80 for administration for mice.

For reference drug, the Montelukast at dose 10 mg/kg/body weight was dissolved in distilled water for administration for mice.

3.5.2 Preparation of mouse models of OVA-induced allergic rhinitis, sham mice, control mice and sample collection

For each experiment, thirty-six male BALB/c mice, aged 4-6 weeks were used in the experiment. The mice were purchased from the National Laboratory Animal Center, Mahidol University, Nakhonpathom, Thailand. The animals were maintained under standard conditions at a temperature of 23 ± 2ºC, 12 hours light/dark cycle and fed with laboratory chow and tap water throughout the experiment. All animals were acclimatized for at least one week prior to sensitization period. The mice were randomly divided into six groups (six mice per group) (i.e. sham mice, control mice and four groups of allergenized mice).

3.5.2.1 Sham mice

Each sham mouse was injected with PBS in alum (2:1 v/v) by intraperitoneal injection (i.p.) on day 0, 7 and 14. On days 21 to 27, each mouse was daily fed with 50 μl of PBS by oral gavage. After fed 3 hours, each mouse was challenged with 20 μl of PBS (10 μl per nostril) by intranasal (i.n.). On day 28, blood and tissues were collected from each mouse.

3.5.2.2 Control mice

Each control mouse was sensitized with 25 μg ovalbumin (OVA) in aluminum hydroxide (alum) (2:1 v/v) by intraperitoneal injection on days 0, 7 and 14. On days 21 to 27, each mouse was daily fed with 50 μl of solvent that dissolved the crude extract by oral gavage (see below). After 3 hours, each mouse was challenged with 20 μl of 500 μg of OVA (10 μl into each nostril) by intranasal. On day 28, blood and tissues were collected from each mouse.

- Prasaprohyai treatment: 5% Tween-80
- Benjakul treatment: 10%Tween-80

3.5.2.3 Allergenized mice

Allergic mice were prepared as described previously (Mo et al., 2013) with modification. The timeline used for allergen sensitization and treatment in mice is shown in Figure 3.1.

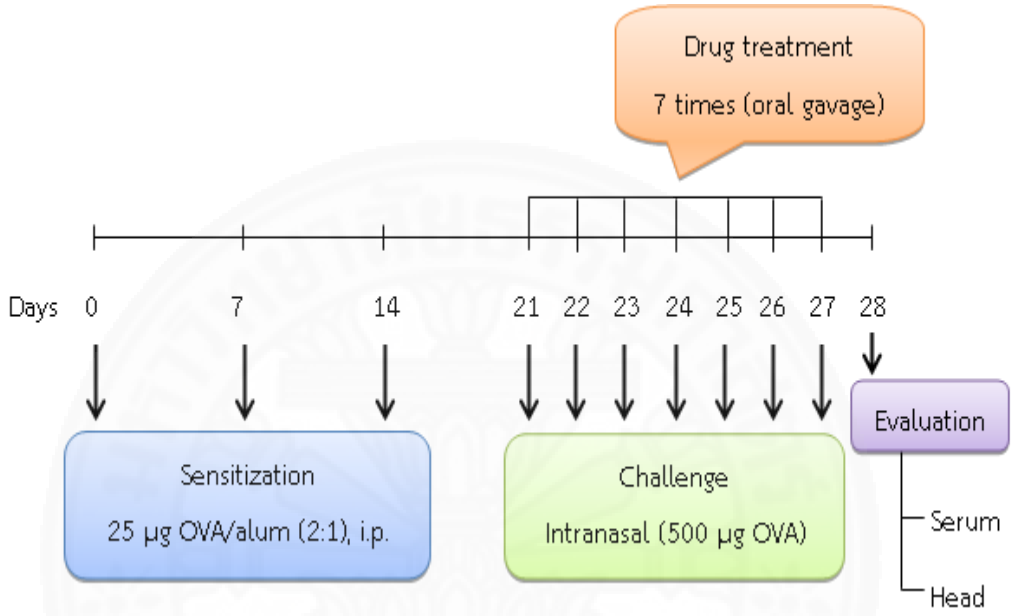


Figure 3.1 Sensitization and challenge protocol for the experimental mouse allergic rhinitis model

For each allergy model, each mouse was sensitized with 25 µg OVA in alum (2:1 v/v) by intraperitoneal injection on days 0, 7 and 14. On days 21 to 27, the mice were daily fed with various dose of the crude extract by oral gavage (see below). After fed 3 hours, each mouse was challenged with 20 µl of 500 µg of OVA (10 µl per nostril) by intranasal. On day 28, blood and tissues were collected from each mouse.

- Prasaprohyai treatment: 100, 200 and 400 mg/kg/body weight
- Benjakul treatment: 37.5, 75 and 150 mg/kg/body weight
- Montelukast treatment: 10 mg/kg/body weight

3.5.2.4 Mouse sample collection

The sham, control and allergenized mice were sacrificed and blood and tissues were collected one day after the last OVA challenge. Each mouse was bled individually and serum was collected separately for testing OVA-specific immunoglobulin E (IgE), immunoglobulin G1 (IgG1) and immunoglobulin G2a (IgG2a) levels by indirect ELISA. For allergic rhinitis model, each sacrificed mouse was decapitated and the head was cut longitudinally for studying histopathology and cytokine profiles by quantitative real-time PCR (qPCR).

3.5.3 Indirect ELISA for measuring the levels of OVA-specific IgE, IgG1 and IgG2a in the mouse serum

The levels of OVA-specific IgE, IgG1 and IgG2a in mouse serum were determined by indirect ELISA (Meechan et al., 2013). Each well of 96-well ELISA plate was coated with 1 μg of OVA in 100 μl of coating buffer (carbonate-bicarbonate buffer, pH 9.6) and incubated at 37ºC until dried. After that, the coated plate was washed with 300 μl of 0.05% PBST (5 times) and blocked with 200 μl of blocking solution (1% BSA in PBS) and then incubated at 37ºC in moist chamber for 1 hour and re-washed. One hundred μl of individual mouse serum (diluted 1:4 for specific IgE and 1:1,000 for specific IgG1 and IgG2a in diluent as well as 0.2% gelatin and 0.2% BSA in PBS) was added into antigen coated plate and then the coated plate was incubated at 37ºC for 1 hour and kept at 4ºC overnight. After incubating, the coated plate was washed again and added with 100 μl of primary antibody such as rat-anti-mouse IgE-biotin (diluted 1:1,000 in diluent), goat-anti-moue IgG1-biotin (diluted 1:10,000 in diluent) and rat-anti-mouse IgG2a-biotin (diluted 1:10,000 in diluent), and then incubated at 37ºC in moist chamber for 1 hour and washed with 0.05% PBST. The coated plate was added with 100 μl of streptavidin-horseradish peroxidase (HRP) conjugate (diluted 1:8,000 in diluent) and incubated at 37ºC in moist chamber for 1 hour and washed with 0.05% PBST. Then, the coated plate was added with 100 μl of ABTS substrate (peroxidase substrate) and kept in the dark at room temperature for 20 min for IgE determination and 5 min for IgG1 and IgG2a determination. The reaction was stopped by adding 100 μl of a stop solution (1% SDS). The optical

density was measured using a microplate reader at 405 nm ($OD_{405\ nm}$). Data are shown as means ± SEM of $OD_{405\ nm}$ of mice of the same treatment group.

3.5.4 Histopathological study of nasal tissues

The right side of each mouse head was immediately fixed in fixative (5% paraformaldehyde and 4% sucrose in PBS) and stored at 4ºC. Each of the fixed nasal tissue was embedded in paraffin, sectioned (5 μm thick). After that, each section was stained with hematoxylin and eosin (H & E) stains for inflammatory cells, toluidine blue stain for mast cells, and Periodic acid-Schiff (PAS) stain for mucus production. All tissue sections were counted under a light microscope (magnification ×400) with DP2-BSW software. The numbers of inflammatory cells which infiltrated into the epithelium were graded arbitrarily using a scale from 1 to 4. The numbers of mast cells were counted in 10 microscopic fields per section per mouse. PAS-stained mucus glands in the tissue were graded arbitrarily based on the intensity of the tissue color (magenta) using a scale from 1 to 3.

3.5.5 Cytokine gene expressions in nasal cavity tissues of sham, control and allergenized mice by quantitative real-time PCR (qPCR)

The cytokine gene expression in nasal cavity tissue of each mouse was determined using qPCR (Sookrung et al., 2008).

3.5.5.1 RNA extraction

The left side of each mouse head was immediately preserved in RNA*later* RNA stabilization reagent and stored at -80ºC. Soft tissues from the area of the tip of the nose to nose cavity were scratched and the total RNA was extracted by Total RNA mini kit (tissue) according to the manufacturer's protocol. The total RNA was quantified from the light absorption at 260 and 280 nm (A260/A280) using a NanoDrop. Complementary DNA (cDNA) was synthesized using RevertAid first stand cDNA synthesis kit and anchored oligo dT primers under conditions provided by the manufacturer. The cDNA was used as a template for quantification of housekeeping gene (β-actin) and cytokine gene expression (mRNA) which included Th2 cytokines (IL-4, IL-5 and IL-13), Th1 cytokines (IFN-γ, IL-12a (p35) and IL-12b (p40)) and regulatory T cell cytokines (IL-10 and TGF-β).

3.5.5.2 Nucleotide primers for amplification

The nucleotide primers for qPCR amplification of individual cytokine gene are listed in Table 3.4 (Tasaniyananda, Chaisri, Tungtrongchitr, Chaicumpa, & Sookrung, 2016). The cDNA of mRNA was amplified by polymerase chain reaction (PCR). The qPCR was carried out on 1 μl of cDNA and 100 nM of each PCR primer in SYBR Green PCR Master Mix following the manufacturer's protocol. The reaction was performed using Mx3005P QPCR System and MxPro QPCR software was used for data analysis. Thermal cycling conditions included an initial denaturation at 95ºC for 10 min, followed by 40 cycles of amplification at 95ºC for 30 sec, annealing at 60ºC for 1 min and extension at 72ºC for 30 sec. The levels of mRNA expression of each target gene were normalized to the mRNA of the housekeeping gene.

Table 3.4 The oligonucleotide primers used in qPCR for monitoring cytokine gene expression

Gene	Primer	Size of PCR product (bp)
Th2 cytokine		
IL-4	F: 5'-TCGGCATTTTGAACGAGGTC-3'	218
	R: 5'-GAAAAGCCCGAAAGAGTCTC-3'	
IL-5	F: 5'-ATGATCGTGCCTCTGTGCCTGGAGC-3'	242
	R: 5'-CTGTTTTTCCTGGAGTAAACTGGGG-3'	
IL-13	F: 5'-AGACCAGACTCCCCTGTGCA-3'	123
	R: 5'-TGGGTCCTGTAGATGGCATTG-3'	
Th1 cytokine		
IFN-γ	F: 5'-AACGCTACACACTGCATCTTGG-3'	237
	R: 5'-GACTTCAAAGAGTCTGAGG-3'	
IL-12a (p35)	F: 5'-CCACCCTTGCCCTCCTAAAC-3'	132
	R: 5'-GTTTTTCTCTGGCCGTCTTCA-3'	
IL-12b (p40)	F: 5'-GGAAGCACGGCAGCAGAATA-3'	180
	R: 5'-AACTTGAGGGAGAAGTAGGAATGG-3'	
Treg cytokine		
IL-10	F: 5'-CGGGAAGACAATAACTG-3'	191
	R: 5'-CATTTCCGATAAGGCTTGG-3'	
TGF-β	F: 5'-CAAGGGCTACCATGCCAACT-3'	84
	R: 5'-AGGGCCAGGACCTTGCTG-3'	
House keeping		
β-actin	F: 5'-GGCCAACCGTGAAAAGATGA-3'	251
	R: 5'-CACGCTCGGTCAGGATCTTC-3'	

3.6 *In vitro* assay for anti-inflammatory activity

3.6.1 Cell culture

RAW 264.7 (ATCC® Catalog No. TIB-71™) cells were cultured in RPMI medium 1640 with L-glutamine, added 2.0 g sodium bicarbonate ($NaHCO_3$), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10,000 Units/ml penicillin and 10,000 μg/ml streptomycin. The cells were maintained in an incubator at 37°C in a humidified 5% CO_2/95% air atmosphere, with change of cultured medium three times every 7 days.

3.6.2 Determination of lipopolysaccharide (LPS)-induced nitric oxide (NO) production from RAW 264.7 cells

The inhibitory effect of the extract on NO production from RAW 264.7 cells was determined by following modified method (Tewtrakul & Itharat, 2007). The RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 96-well plates and allowed to adhere for 24 hours at 37ºC in 5% CO_2. After that, the medium was replaced with fresh medium (100 μl/well) containing 10 ng/ml of LPS, together with test samples at various concentrations (100 μl/well), and incubated at 37°C in 5% CO_2 for 24 hours. Aliquots (100 μl) of supernatant were transferred into 96-well plates and added Griess reagent (100 μl) (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid). After aliquots supernatant, cell viability was determined using the MTT assay. Briefly, the MTT solution (10 μl, 5 mg/ml in PBS) was added to each well and incubated at 37ºC in 5% CO_2 for 2 hours. Then, the medium was removed and added isopropanol containing 0.04 M HCl to each well to dissolve the formazan production in the cells. The absorbance was measured with a microplate reader at 570 nm. The test sample was considered to be cytotoxic when the optical density of the sample group was less than 70% of that in the control group. The test sample was dissolved in DMSO, and the solution was added to RPMI medium 1640 (final DMSO concentration was 0.2%).

The percentage of inhibitory effect on NO production by the test samples was calculated by the following equation, and IC_{50} values were calculated from the Prism program.

$$\text{Inhibition (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

3.6.3 Determination of lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) release from RAW 264.7 cells

The inhibitory effect of the extract on TNF-α release from RAW 264.7 cells was determined with Quantikine mouse TNF-α ELISA kit according the manufacturer's protocol. The RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 96-well plates and allowed to adhere for 24 hours at 37°C in 5% CO_2. After that, the medium was replaced with RPMI medium 1640 (100 μl/well) containing 10 ng/ml of LPS, together with test samples at various concentrations (100 μl/well), and incubated at 37°C in 5% CO_2 for 24 hours. Then, the supernatant was transferred into 96-well ELISA plate and then TNF-α concentration was determined. The absorbance was measured with a microplate reader at 450 nm. The test sample was dissolved in DMSO, and the solution was added to RPMI medium 1640 (final DMSO concentration was 0.2%).

The percentage of inhibitory effect on TNF-α release by the test samples was calculated by the following equation, and IC_{50} values were calculated from the Prism program.

$$\text{Inhibition (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

3.7 Bioassay-guided fractionation and isolation of chemical constituents from the ethanolic extract of *A. lancea*

3.7.1 Bioassay-guided fractionation of the ethanolic extract of *A. lancea*

The ethanolic extract of *A. lancea* (50.08 g), main active plant which showed the potential activity of both anti-allergy and anti-inflammation by *in vitro* assay, was separated by vacuum liquid chromatography (VLC) on silica gel 60 and eluted with hexane, hexane: chloroform (1:1), chloroform, chloroform: methanol (1:1) and methanol in order of increasing polarity. Each fraction was concentrated to dryness under reduced pressure (45ºC) using a rotary film evaporator to obtain five primary fractions (F1-F5). The yields were no yield (F1), 1.90 g (F2), 8.44 g (F3), 30.44 g (F4) and 5.50 g (F5). The percentage of yield of each fraction as % w/w of initial weight of crude extract is shown in Figure 3.2. Thereafter, each fraction was tested for *in vitro* anti-allergic activity (see section 3.4) and selected for isolation of pure compound.

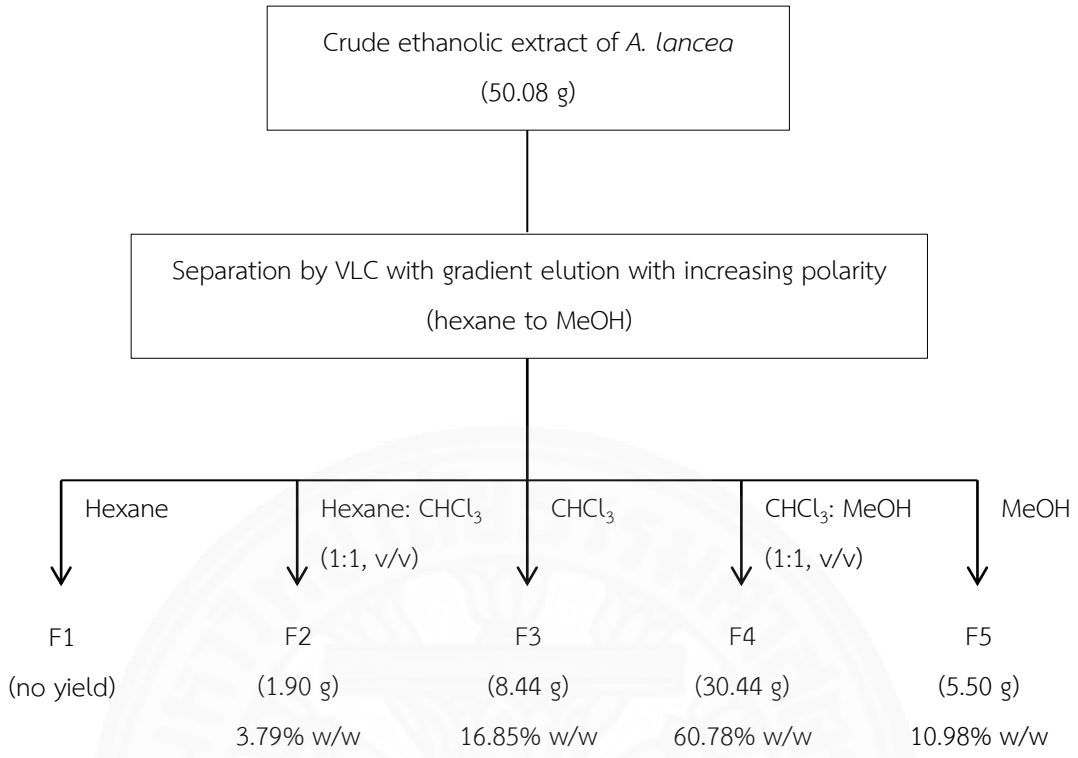


Figure 3.2 Flow diagram of the isolation procedure of the ethanolic extract of *A. lancea*

3.7.2 Isolation of chemical constituents from the ethanolic extract of *A. lancea*

The active fraction was isolated by medium pressure liquid chromatography (MPLC) with gradient solvents.

Fraction F3 (5.0027 g) was further purified by SepacorControl Preparative Chromatography (MPLC) packed with silica gel 60 (121.21 g) and eluted with hexane gradually enriched with $CHCl_3$ (30-90%) followed by $CHCl_3$ gradually enriched with MeOH (10-90%). Each fraction with similar TLC chromatogram characteristics and detection with acidic anisaldehyde spraying reagent was combined and concentrated to dryness under reduced pressure (45°C) using a rotary film evaporator to obtain twenty-four sub-fractions (Fr1-Fr24). After that, sub-fraction Fr8 was precipitated with MeOH to obtain FA1 compound. Purity of pure compound was verified by nuclear magnetic resonance spectrum (NMR). The percentage of yield

of FA1 compound as % w/w of initial weight of crude extract is shown in Figure 3.3. Thereafter, sub-fraction Fr8 was tested for *in vitro* anti-allergic (see section 3.4.2) and anti-inflammatory activities (see section 3.6.2).

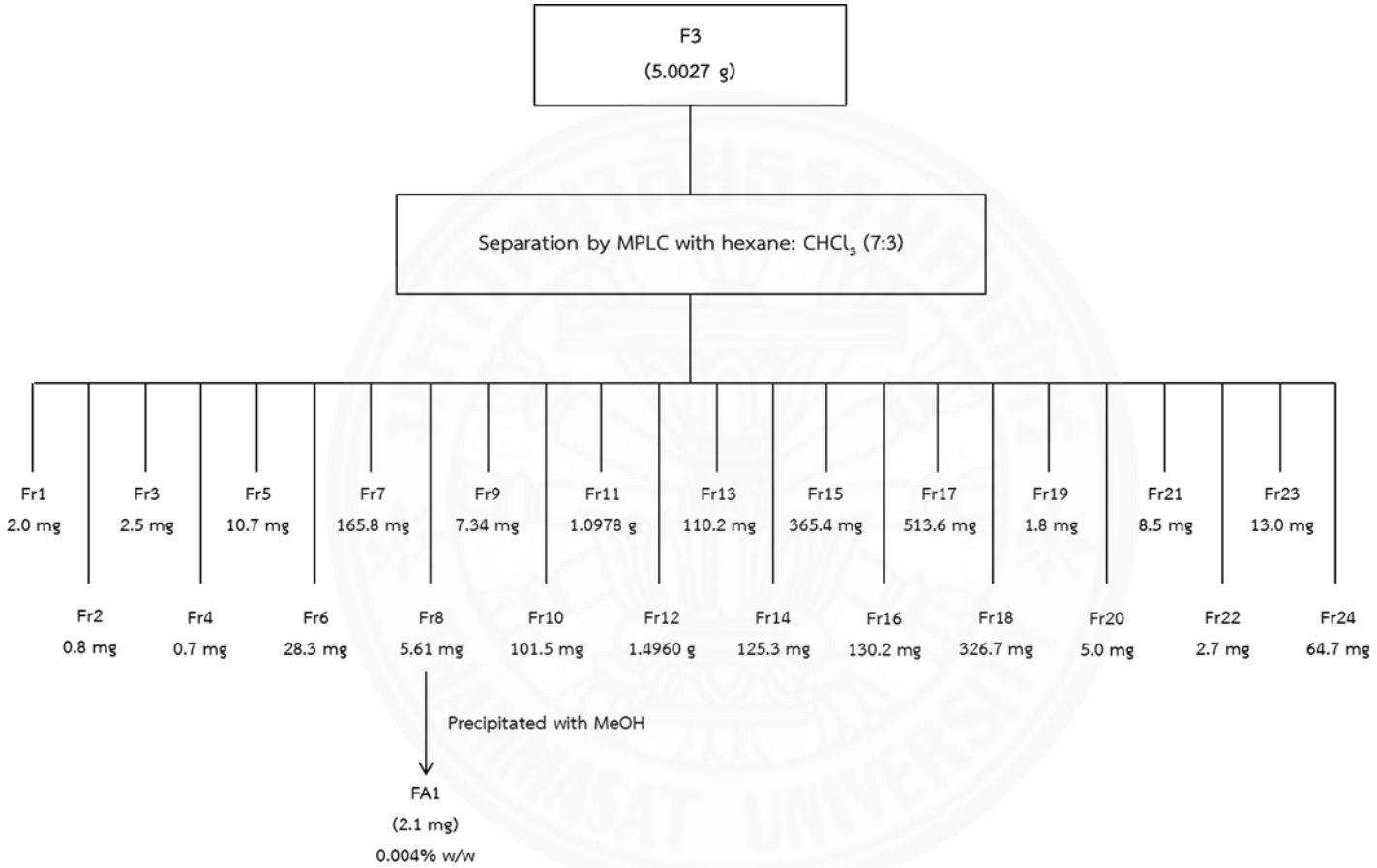


Figure 3.3 Flow diagram of the isolation procedure of FA1 from chloroform fraction (F3) of the ethanolic extract of *A. lancea*

3.8 Formulation development

3.8.1 Pre-formulation studies (International Conference on Harmonization, 2003)

Forced degradation studies (or stress testing) of Prasaproyai ethanolic extract and Benjakul ethanolic extract were investigated to stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation with heated at 80ºC for 3 hours. Thereafter, the anti-allergic and anti-inflammatory activities of each sample were tested and compared with normal condition.

3.8.1.1 Moisture hydrolysis

Each crude extract (50 mg) was weighed into test tube and added deionized water (3 drops). Each sample was heated at 80ºC for 3 hours and left at room temperature. The forced samples were determined for both of *in vitro* anti-allergic and anti-inflammatory activities.

3.8.1.2 Acid hydrolysis

Each crude extract (50 mg) was weighed into test tube. The samples were added 3N hydrochloric acid (3 drops). After that, each sample was heated at 80ºC for 3 hours, left at room temperature and then neutralized with sodium hydroxide solution. The forced samples were studied for both of *in vitro* anti-allergic and anti-inflammatory activities.

3.8.1.3 Alkaline hydrolysis

Each crude extract (50 mg) was weighed into test tube and added 3N sodium hydroxide (3 drops). Each sample was heated at 80ºC for 3 hours, left at room temperature and neutralized with hydrochloric acid solution. The forced samples were tested for both of *in vitro* anti-allergic and anti-inflammatory activities and comp.

3.8.1.4 Temperature degradation

Each crude extract (50 mg) was weighed into test tube, heated at 80ºC for 3 hours and left at room temperature. The forced samples were determined for both of *in vitro* anti-allergic and anti-inflammatory activities.

3.8.1.5 Oxidation

Each crude extract (50 mg) was weighed into test tube and added 30% hydrogen peroxide (3 drops). Then, the samples were heated at 80ºC for 3 hours and left at room temperature. The forced samples were determined for both of *in vitro* anti-allergic and anti-inflammatory activities.

3.8.1.6 Stability testing under accelerated condition

Both of the ethanolic extracts of Prasaprohyai and Benjakul were performed under accelerated condition (International Conference on Harmonization, 2003). The ethanolic extracts (10 mg) were packed into capped glass vials and stored under controlled temperature (40 ± 2ºC) and relative humidity (75 ± 5% RH) for 6 months. The samples were withdrawn at periods 0, 15, 30, 45, 60, 90, 120, 150 and 180 days. The biological activity characteristics at difference periods were determined using β-hexosaminidase and NO assays. Moreover, the changes of amount of active compounds in the Benjakul ethanolic extract were evaluated by HPLC method as described previously (Itharat & Sakpakdeejaroen, 2010) with modification.

3.8.2 Formulation of Prasaprohyai extract and Benjakul extract into solid dosage form (capsule)

The formulation of Prasaprohyai capsule and Benjakul capsule which composed of hydrophilic silicon dioxide as a adsorbent and anti-adherent (Aerosil®), microcrystalline cellulose as a adsorbent and glidant (Avicel® PH-101 and Avicel® PH-102), lactose as a filler, talcum as a glidant and magnesium stearate as a lubricant by total weight of 500 mg per one capsule were used in the capsule formulation. The composition of different formulation of Prasaprohyai capsule and Benjakul capsule is shown in Table 3.5.

Table 3.5 The composition of Prasaprohyai and Benjakul formulations

Ingredients	Formulations (mg)									
	Prasaprohyai capsule					Benjakul capsule				
	P1	P2.1	P2.2	P3	P4	B1	B2.1	B2.2	B3	B4
PPY	50	50	50	50	50	-	-	-	-	-
BJK	-	-	-	-	-	50	50	50	50	50
Avicel® PH-101	425	305	300	175	212.5	425	305	300	175	212.5
Avicel® PH-102	-	-	-	-	212.5	-	-	-	-	212.5
Lactose	-	125	125	250	-	-	125	125	250	-
Talcum	15	15	15	15	15	15	15	15	15	15
Magnesium stearate	5	5	5	5	5	5	5	5	5	5
Aerosil®	5	-	5	5	5	5	-	5	5	5
Total	**500**					**500**				

3.8.2.1 Uniformity of weight (Mass)

Randomly selected twenty capsules were accurately weighed in an analytical balance. The average weight was recorded and standard deviation was calculated. Each capsule must be in range 92.5-107.5% of average weight. Not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation and none deviates by more than twice that percentage (British Pharmacopoeia Commission, 2013).

3.8.2.2 Disintegration time

One capsule was placed in each of the six tubes and the basket rack was positioned in one liter of water as the disintegration medium. The apparatus was operated at 37 ± 2ºC for 30 min. Then, the basket was moved from the medium and observed the capsules. The results were noted as the time require for completely disintegration of the capsules. All of capsules must be disintegrated completely. If one or two capsules fail to disintegrate, repeat the test on 12

additional capsules. The requirements of the test are met if not less than 16 of the 18 capsules tested are disintegrated (British Pharmacopoeia Commission, 2013).

3.8.2.3 Stability testing

The capsules were investigated under accelerated conditions (International Conference on Harmonization, 2003). The capsules were packed in blister pack has backing of a lidding seal of aluminum foil and stored under controlled temperature (40 ± 2ºC) and relative humidity (70 ± 5% RH) for 6 months. The capsules were withdrawn at periods 0, 15, 30, 45, 60, 90, 120, 150 and 180 days. The physical and biological activity characteristic changes at difference periods were determined using moisture analysis for physical characteristic change and using β-hexosaminidase and NO assays for biological activity changes.

(1) Determination of moisture content (Department of Medical Sciences, 2000).

The sample was weighed about 2 g and spread to the dish. The sample was dried in moisture analyzer at 105ºC until reaches a constant weight. Each sample was tested in triplicate and the percentage of loss on drying was calculated by the following equation.

$$\text{Loss on drying (\%)} = [(W1 - W2) \times 100]/W1$$

Where W1: initial weight (g) of sample; W2: weight (g) of sample after drying

3.9 Statistical analysis

For *in vitro* model, the results were presented as mean ± standard error of the mean (SEM) of three individual experiments. The IC_{50} values were calculated using GraphPad Prism 5. Data were analyzed by one-way ANOVA followed by Dunnett's test. Significant levels were considered at *p*-value less than 0.05 ($p < 0.05$).

For *in vivo* model, the results were expressed as mean ± standard error of the mean (SEM). The results were analyzed using one-way ANOVA followed by the independent *t*-test for analyses of antibody levels and cytokine data. Percentage of grades of histopathological was compared by Chi-square test. $P < 0.05$ was considered statistically significant.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preparation of Prasaprohyai extract and Benjakul extract

Each plant of Prasaprohyai and Benjakul remedies was extracted by maceration with 95% ethanol. The percentage of yield of each plant of Prasaprohyai and Benjakul remedies is shown in Table 4.1 and Table 4.2, respectively.

For seven pure compounds of Benjakul ethanolic extract, methyl piperate, myristicin and β-sitostenone were established and kindly provided by Dr. Ruchilak Rattarom (Rattarom, 2013). Other pure compounds including 6-gingerol, 6-shogaol, piperine, and plumbagin were purchased from Wako Pure Chemical Industries, Merck and Sigma-Aldrich.

Table 4.1 The percentage of yield of Prasaprohyai and its component extracts

Plants	Thai name	%Yield (w/w)
A. testaceum	Krawan	2.42
A. graveolens	Thian ta takkataen	4.34
A. dahurica	Kot so	5.12
A. sinensis	Kot chiang	15.05
A. annua	Kot chula lampha	4.27
A. lancea	Kot khamao	16.89
C. cyminum	Thian khao	8.73
D. loureiroi	Chan daeng	17.87
F. vulgare	Thian khao plueak	6.69
K. galanga	Proh hom	6.39
L. sativum	Thian daeng	9.20

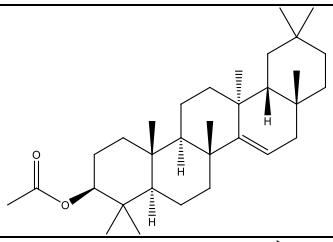
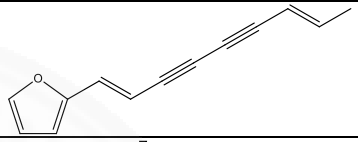
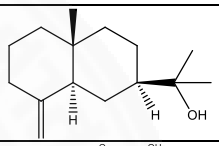
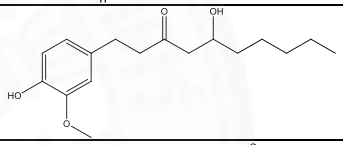
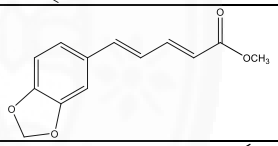
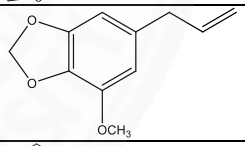
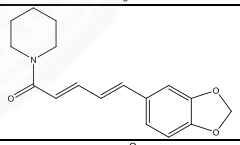
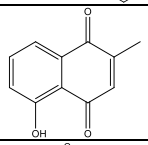
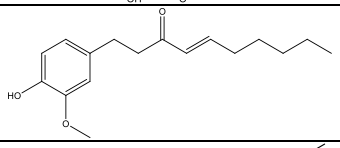
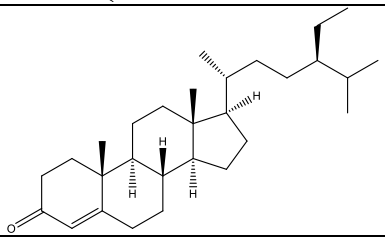
Table 4.1 The percentage of yield of Prasaprohyai and its component extracts (Cont.)

Plants	Thai name	%Yield (w/w)
L. sinense	Kot hua bua	12.19
M. siamensis	Saraphi	32.78
M. ferrea	Bunnak	23.17
M. elengi	Phikun	8.82
M. fragrans	Chan thet	7.07
M. fragrans	Mace	18.97
M. fragrans	Nutmeg	13.67
N. nucifera	Bua luang	10.59
N. sativa	Thian dam	32.29
S. aromaticum	Kan phlu	31.24
Prasaprohyai remedy		18.66

Table 4.2 The percentage of yield of Benjakul and its component extracts

Plants	Thai name	%Yield (w/w)
P. interruptum	Sa-khan	2.47
P. longum	Dee-plee	10.89
P. sarmentosum	Cha-phlu	6.21
P. indica	Chettamun phloeng daeng	10.61
Z. officinale	Khing	4.30
Benjakul remedy		11.10

Table 4.3 The summary of pure compound structures used in the studies

Plant/Remedy	Pure compounds	Chemical structures
A. lancea	Taraxerol acetate	
	Atractylodin TS	
	β-Eudesmol	
Benjakul	6-Gingerol	
	Methyl piperate	
	Myristicin	
	Piperine	
	Plumbagin	
	6-Shogaol	
	β-Sitostenone	

4.2 *In vitro* assay for anti-allergic activity

4.2.1 Determination of antigen-induced β-hexosaminidase release from RBL-2H3 cells

For Prasaprohyai remedy (PPY), the inhibitory effects of the ethanolic extracts of Prasaprohyai and its plant components on the release of β-hexosaminidase in RBL-2H3 cells were tested and reported in my previous thesis. The ethanolic extract of Prasaprohyai remedy showed the potent anti-allergic activity with the IC_{50} value of 16.59 μg/ml (Makchuchit, 2010).

For Benjakul remedy (BJK), the inhibition effects of the ethanolic extracts of Benjakul, five plant components, and seven pure compounds from the Benjakul ethanolic extract on antigen-induced β-hexosaminidase release from RBL-2H3 cells were determined and compared to the standard drug as well as chlorpheniramine. As the results are shown in Table 4.4 and Figure 4.1, the Benjakul ethanolic extract showed the greatest anti-allergic activity, followed by *Z. officinale*, *P. indica*, *P. interruptum*, *P. longum* and *P. sarmentosum* in a dose-dependently manner with the IC_{50} values of 12.69, 12.93, 13.31, 14.13, 15.82 and 21.73 μg/ml, respectively.

For pure compounds isolated from BJK, 6-shogaol presented the highest inhibitory effect (IC_{50} = 0.28 μg/ml), followed by plumbagin, methyl piperate, piperine, 6-gingerol and β-sitostenone (IC_{50} = 4.03, 12.44, 14.14, 18.30 and 24.27 μg/ml, respectively). Nevertheless, myristicin exhibited mild anti-allergic activity (IC_{50} = 80.80 μg/ml). These results indicated 6-shogaol and plumbagin had significantly decreased inhibitory effect on β-hexosaminidase release from RBL-2H3 cells when compared with chlorpheniramine (CPM) (IC_{50} = 17.98 μg/ml) which is a positive control drug.

Table 4.4 The percentage of inhibition and the IC_{50} value of the ethanolic extracts of Benjakul remedy, its plant components and its pure compounds on β-hexosaminidase release from RBL-2H3 cells (n = 3)

Crude extracts and pure compounds	%Inhibition at various concentrations of extracts (mean ± SEM, μg/ml)						IC_{50} ± SEM, μg/ml (μM for pure compound)
	0.01	0.1	1	10	50	100	
P. interruptum	-	-	-52.02 ± 26.66	25.99 ± 5.60	63.56 ± 4.76	93.85 ± 2.64	14.13 ± 0.92
P. longum	-	-	-40.76 ± 6.34	24.50 ± 5.78	65.11 ± 9.14	100.54 ± 3.69	15.82 ± 2.00
P. sarmentosum	-	-	-29.53 ± 6.61	18.78 ± 1.36	46.49 ± 1.77	81.05 ± 4.63	21.73 ± 2.14
P. indica	-	-	-55.37 ± 16.44	31.42 ± 5.21	85.25 ± 11.52	102.83 ± 1.91	13.31 ± 1.78
Z. officinale	-	-	-29.86 ± 18.01	35.19 ± 5.15	70.12 ± 3.23	84.14 ± 3.21	12.93 ± 1.28
Benjakul	-	-	-23.42 ± 5.12	37.37 ± 4.92	68.16 ± 9.61	89.65 ± 4.40	12.69 ± 1.25
6-Gingerol	-	-	-27.87 ± 19.81	27.15 ± 3.42	71.89 ± 4.12	93.57 ± 5.58	18.30 ± 3.38 (62.16 μM)
Methyl piperate	-	-	-4.85 ± 10.75	40.01 ± 4.75	77.87 ± 3.29	90.59 ± 0.81	12.44 ± 1.09 (53.57 μM)
Myristicin	-	-	-45.83 ± 7.43	0.34 ± 0.91	31.82 ± 4.90	75.29 ± 2.38	80.80 ± 2.80* (420.37 μM)
Piperine	-	-	-48.92 ± 23.94	30.52 ± 2.83	65.05 ± 9.22	80.52 ± 2.09	14.14 ± 1.46 (49.55 μM)
Plumbagin	-	-	15.65 ± 14.79	101.81 ± 0.97	102.58 ± 1.51	102.31 ± 0.79	4.03 ± 0.78* (21.42 μM)
6-Shogaol	-44.82 ± 16.45	9.92 ± 4.42	30.94 ± 5.99	68.23 ± 4.69	-	-	0.28 ± 0.11* (1.01 μM)
β-Sitostenone	-	-	-34.57 ± 1.64	11.99 ± 2.55	55.39 ± 4.02	77.49 ± 3.53	24.27 ± 2.29 (58.81 μM)
Chlorpheniramine	-	-	-38.89 ± 8.01	17.82 ± 1.01	68.35 ± 1.50	91.33 ± 1.22	17.98 ± 0.78 (46.00 μM)

(-) means not tested; * Significant difference at the *p*-value < 0.05 compared with chlorpheniramine (a positive control drug)

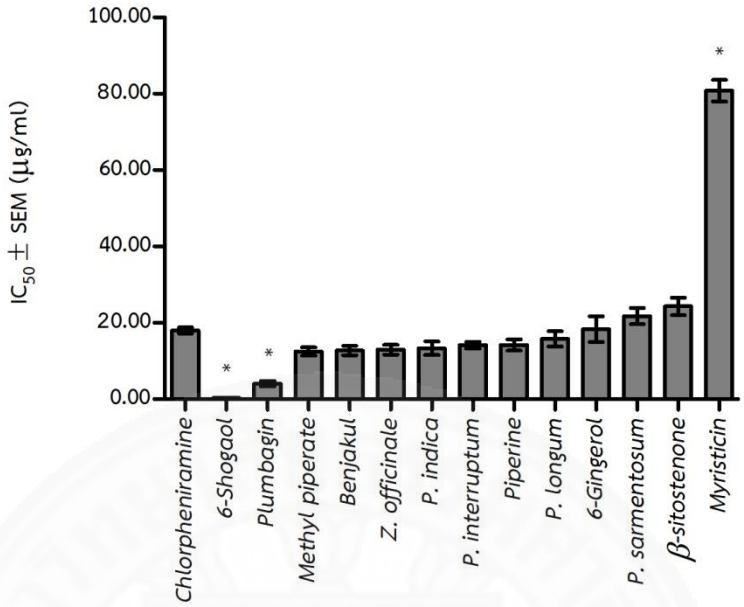


Figure 4.1 Effect of the ethanolic extracts of Benjakul, its plant components and its pure compounds on β-hexosaminidase release from RBL-2H3 cells. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with chlorpheniramine (CPM, a positive control drug).

4.3 *In vivo* assay for anti-allergic activity

4.3.1 Serum levels of OVA-specific immunoglobulins

4.3.1.1 Serum specific immunoglobulins in sham, control and OVA allergic mice after receiving Prasaprohyai remedy

Serum levels of OVA-specific IgE, IgG1 and IgG2a in sham, control and OVA allergic mice after receiving Prasaprohyai and challenged with OVA are shown in Table 4.5 and Figure 4.2.

(1) Serum specific IgE

The IgE levels from allergic mice of all treatment groups (all doses of PPY and MT) were lower than in the control group (not significant). In addition, the allergenized mice that were treatment by receiving PPY at concentrations of 200 and 400 mg/kg had significantly higher IgE levels than the allergic mice that received with montelukast, a positive control group ($p < 0.05$).

(2) Serum specific IgG1

The allergic mice that treated with PPY at concentrations of 100 and 200 mg/kg had significantly different in serum specific IgG1 levels ($p < 0.05$). Moreover, the IgG1 levels of allergic mice that received PPY at concentrations of 200 and 400 mg/kg had significantly different from the positive drug group, MT ($p < 0.05$).

(3) Serum specific IgG2a

The OVA allergic mice that treated with PPY at concentrations of 100, 200 and 400 mg/kg had a slight increase of IgG2a levels in serum but not significant when compared with the control group.

Table 4.5 The average number (mean) and standard error of the mean (SEM) of optical density (OD_{405nm}) of OVA-specific serum IgE, IgG1 and IgG2a of sham, control and allergenized mice after obtaining Prasaprohyai remedy

Mouse group (6 mice per group)	Mean ± SEM of OD_{405nm}		
	IgE	IgG1	IgG2a
Sham mice	0.215 ± 0.026	0.086 ± 0.008	0.086 ± 0.007
Control 5% Tween-80 mice	0.935 ± 0.117	1.444 ± 0.006	0.137 ± 0.015
PPY 100 mg/kg/bw mice	0.785 ± 0.064	1.392 ± 0.005*	0.155 ± 0.012
PPY 200 mg/kg/bw mice	0.882 ± 0.041#	1.516 ± 0.015*,#	0.145 ± 0.007
PPY 400 mg/kg/bw mice	0.880 ± 0.070#	1.478 ± 0.014#	0.150 ± 0.010
MT 10 mg/kg/bw mice	0.675 ± 0.040	1.420 ± 0.014	0.156 ± 0.016

* $p < 0.05$ when compared with control group; # $p < 0.05$ when compared with montelukast group (MT, a positive control drug)

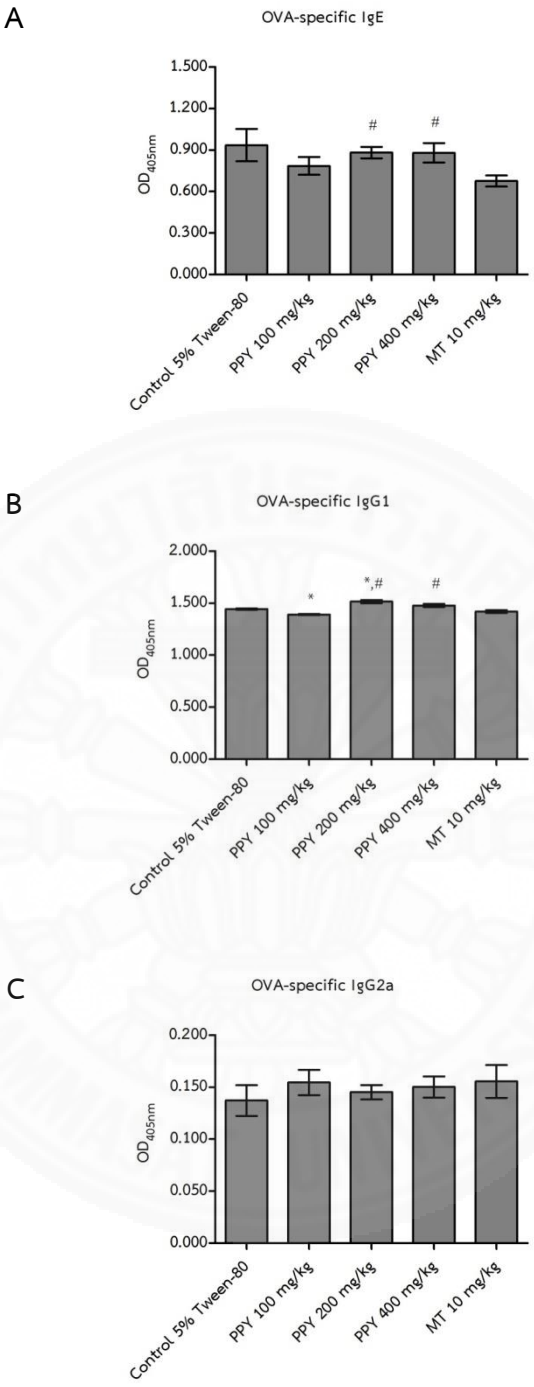


Figure 4.2 Mean and standard error of the mean (SEM) of indirect ELISA OD_{405nm} of OVA-specific serum IgE (A), IgG1 (B) and IgG2a (C) of OVA-allergic mice after receiving Prasaprohyai treatment. Data were analyzed by using independent *t*-test. * Significant difference ($p < 0.05$) when compared with control group; # $p < 0.05$ when compared with montelukast group (a positive control drug).

4.3.1.2 Serum specific immunoglobulins in sham, control and OVA allergic mice after receiving Benjakul remedy

Serum levels of OVA-specific IgE, IgG1 and IgG2a in sham, control and OVA allergic mice after receiving Benjakul and challenged with OVA are shown in Table 4.6 and Figure 4.3.

(1) Serum specific IgE

The allergic mice that were treatment by BJK at concentrations of 37.5 and 75 mg/kg had significantly decreased the serum levels of OVA-specific IgE in both the control (10% Tween-80) and the positive drug (montelukast) groups ($p < 0.05$).

(2) Serum specific IgG1

The allergenized mice that treated with Benjakul (BJK) at concentrations of 37.5, 75 and 150 mg/kg and montelukast (MT) at concentration of 10 mg/kg had slightly increased in serum IgG1 levels when compared to the control group (not significant difference).

(3) Serum specific IgG2a

The IgG2a levels of the allergic mice that received with BJK at concentrations of 37.5, 75 and 150 mg/kg and MT at concentration of 10 mg/kg-treatments were not significant difference when compared with the control group.

Table 4.6 The average number (mean) and standard error of the mean (SEM) of optical density (OD_{405nm}) of OVA-specific serum IgE, IgG1 and IgG2a of sham, control and allergenized mice after obtaining Benjakul remedy

Mouse group (6 mice per group)	Mean ± SEM of OD_{405nm}		
	IgE	IgG1	IgG2a
Sham mice	0.133 ± 0.006	0.067 ± 0.002	0.072 ± 0.001
Control 10% Tween-80 mice	0.575 ± 0.041	1.917 ± 0.042	0.119 ± 0.005
BJK 37.5 mg/kg/bw mice	0.386 ± 0.043$^{*,\#}$	1.959 ± 0.066	0.166 ± 0.020
BJK 75 mg/kg/bw mice	0.381 ± 0.052$^{*,\#}$	2.004 ± 0.031	0.141 ± 0.013
BJK 150 mg/kg/bw mice	0.798 ± 0.102	1.942 ± 0.053	0.121 ± 0.014
MT 10 mg/kg/bw mice	0.880 ± 0.152	1.952 ± 0.049	0.146 ± 0.013

* $p < 0.05$ when compared with control group; # $p < 0.05$ when compared with montelukast group (MT, a positive control drug)

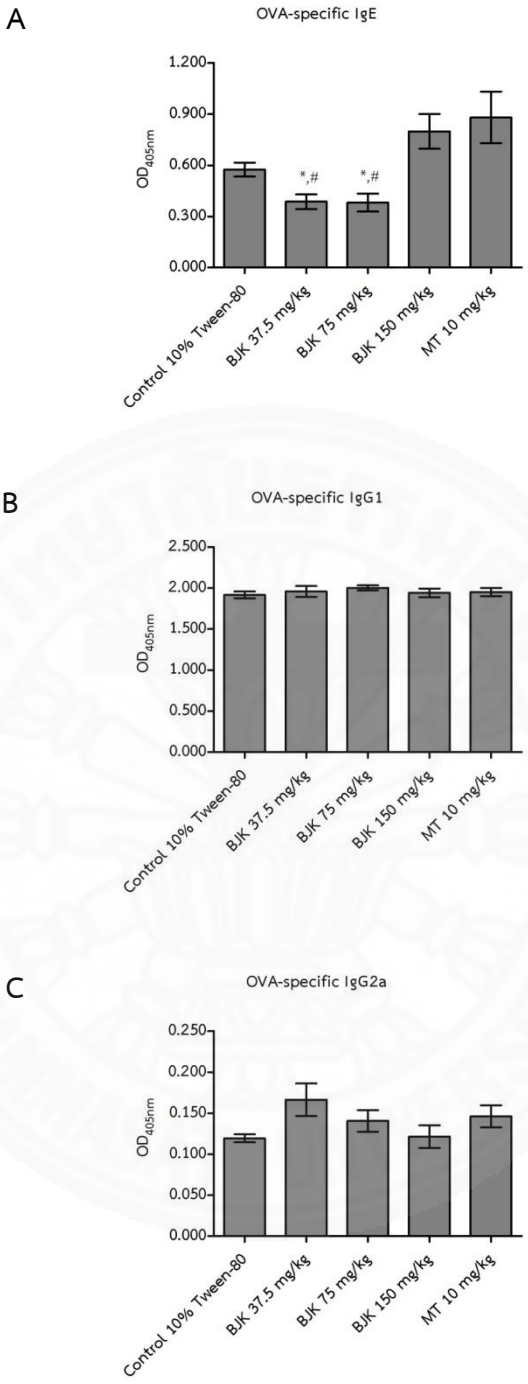


Figure 4.3 Mean and standard error of the mean (SEM) of indirect ELISA OD_{405nm} of OVA-specific serum IgE (A), IgG1 (B) and IgG2a (C) of OVA-allergic mice after receiving Benjakul treatment. Data were analyzed by using independent *t*-test. * Significant difference ($p < 0.05$) when compared with control group; # $p < 0.05$ when compared with montelukast group (a positive control drug).

4.3.2 Histological features of nasal tissues of allergic mice after receiving Prasaprohyai and Benjakul remedies

4.3.2.1 Grades of inflammatory cells

The grades of inflammatory cells in nasal mucosa stained with hematoxylin and eosin (H & E) dyes are shown in Figure 4.4.

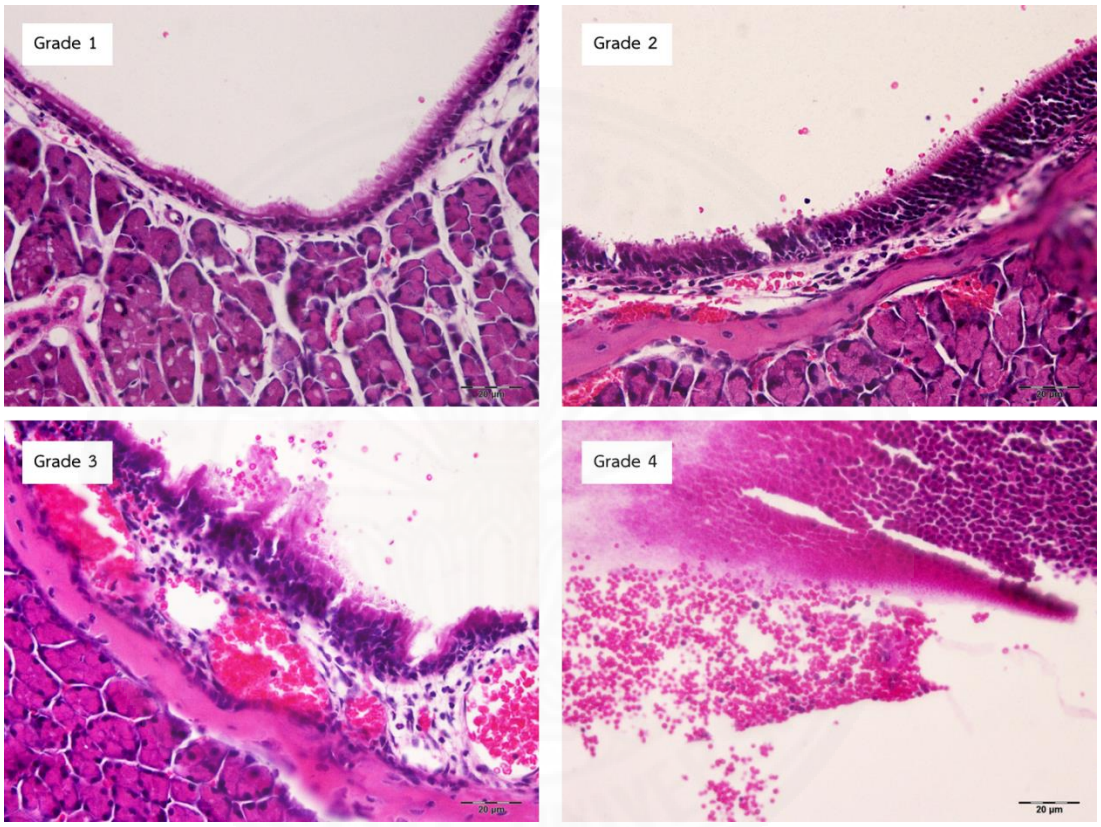


Figure 4.4 Grades of inflammatory cells in epithelia of nasal mucosa stained with H & E stain (original magnification ×400). Grade 1 means epithelial cells normal with some inflammatory cells (not more than 10). Grade 2 means some epithelial cells degenerate, moderate inflammatory cells (between 10 and 20). Grade 3 means some epithelial cells necrosis or detached from basal line (more than 20). Grade 4 means almost of epithelial cells necrosis with a lot of inflammatory cells accumulate in nasal cavity.

(1) Grades of inflammatory cells of allergic mice after receiving Prasaprohyai

The percentage of individual and average grades of inflammatory cells of allergic mice after treatment with Prasaprohyai and challenged with OVA in nasal mucosae along the epithelia are shown in Table 4.7.

As the results are shown in Table 4.7, the average grades of inflammatory cells of allergic mice after receiving PPT at concentrations of 100, 200 and 400 mg/kg body weight were close to control mice (not significant) (average grades = 2.17 ± 0.12, 2.42 ± 0.12, 2.13 ± 0.08 and 2.12 ± 0.20, respectively). In addition, there was no significant difference between the control group and the montelukast group, a positive drug (average grades = 2.20 ± 0.12 and 2.12 ± 0.20, respectively). However, the percentage of the grade 2-inflammatory cells of PPY at concentrations of 100, 200 and 400 mg/kg-treatment groups had significantly different when compared with control group ($p < 0.05$). And the percentage of the grade 3-inflammatory cells of MT at concentration of 10 mg/kg-treatment group had significantly different from control group ($p < 0.05$).

Table 4.7 The percentage of individual and average grades of the H & E stained inflammatory cells in epithelia of nasal tissues of control and allergic mice after receiving Prasaprohyai remedy (6 mice per group)

Group of mice (Total microscopic fields)	%Individual of inflammatory cells grades*				Mean ± SEM of inflammatory cells grades#
	Grade 1	Grade 2	Grade 3	Grade 4	
Control 5% Tween-80 (217)	26.73[a]	47.00[a]	17.05[a]	9.22[a]	2.12 ± 0.20[a]
PPY 100 mg/kg/bw (191)	31.41[a]	30.89[b]	26.70[a]	11.00[a]	2.17 ± 0.12[a]
PPY 200 mg/kg/bw (212)	23.11[a]	34.91[b]	26.89[a]	15.09[a]	2.42 ± 0.12[a]
PPY 400 mg/kg/bw (195)	34.36[a]	28.21[b]	28.21[a]	9.23[a]	2.13 ± 0.08[a]
MT 10 mg/kg/bw (231)	33.77[a]	27.27[a]	29.00[b]	9.96[a]	2.20 ± 0.12[a]

* Percentage of individual inflammatory cells grades was analyzed by Chi-square test

Mean of inflammatory cells grades was analyzed by independent *t*-test

Entries with different superscripts along vertical axis (a *versus* b) are statistically different ($p < 0.05$)

(2) Grades of inflammatory cells of allergic mice after receiving Benjakul

The percentage of individual and average grades of inflammatory cells of allergic mice after treatment with Benjakul and challenged with OVA in nasal mucosae along the epithelia are shown in Table 4.8.

As the results are shown in Table 4.8, there was no significant difference between control group and all experiments groups with means of 2.06 ± 0.08 (control), 1.77 ± 0.14 (BJK 37.5 mg/kg), 1.94 ± 0.08 (BJK 75 mg/kg), 1.96 ± 0.05 (BJK 150 mg/kg) and 1.84 ± 0.13 (MT 10 mg/kg). The percentage of all grades

(1-4) of inflammatory cells was no significant difference but statistically different only grade 3-inflammatory cells of allergic mice receiving BJK 75 mg/kg group which had significantly different when compared to control group ($p < 0.05$). Moreover, the allergic mice after receiving BJK at concentration of 37.5 mg/kg showed decreased the percentage of the grades 4-, 3- and 2-inflammatory cells and increased the percentage of the grade 1-inflammatory cells.

Table 4.8 The percentage of individual and average grades of the H & E stained inflammatory cells in epithelia of nasal tissues of control and allergic mice after receiving Benjakul remedy (6 mice per group)

Group of mice (Total microscopic fields)	%Individual of inflammatory cells grades*				Mean ± SEM of inflammatory cells grades#
	Grade 1	Grade 2	Grade 3	Grade 4	
Control 10% Tween-80 (316)	37.66[a]	25.63[a]	30.70[a]	6.01[a]	2.06 ± 0.08[a]
BJK 37.5 mg/kg/bw (275)	56.36[a]	13.82[a]	26.55[a]	3.27[a]	1.77 ± 0.14[a]
BJK 75 mg/kg/bw (159)	40.88[a]	33.33[a]	18.24[b]	7.55[a]	1.94 ± 0.08[a]
BJK 150 mg/kg/bw (178)	35.40[a]	38.20[a]	22.47[a]	3.93[a]	1.96 ± 0.05[a]
MT 10 mg/kg/bw (279)	54.84[a]	16.84[a]	20.43[a]	7.89[a]	1.84 ± 0.13[a]

* Percentage of individual inflammatory cells grades was analyzed by Chi-square test

Mean of inflammatory cells grades was analyzed by independent *t*-test

Entries with different superscripts along vertical axis (a *versus* b) are statistically different ($p < 0.05$)

4.3.2.2 Mast cells enumeration

The examples of mast cells in the right side of the head of mouse stained with toluidine blue dye are shown in Figure 4.5.

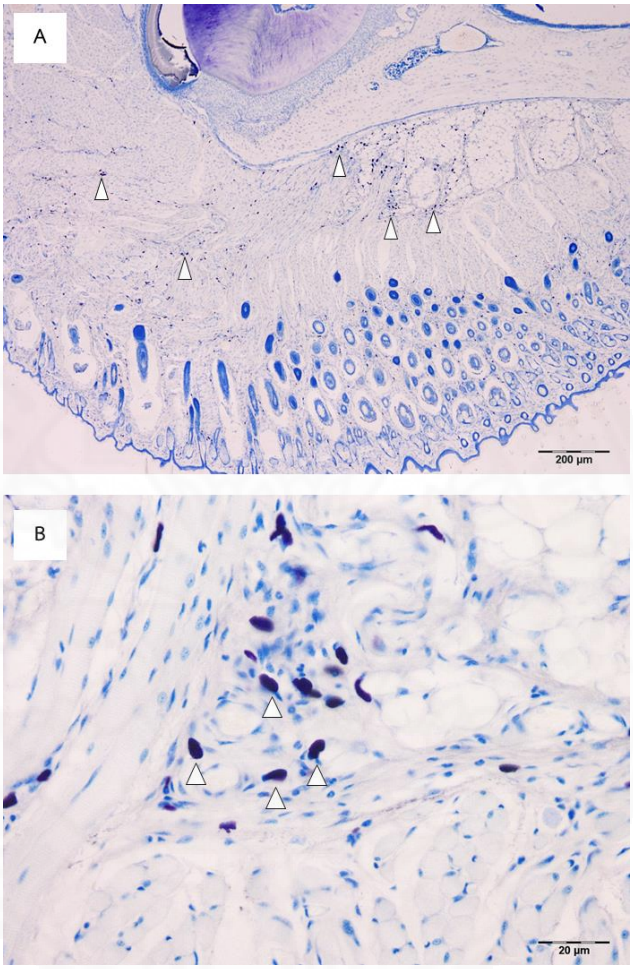


Figure 4.5 Mast cells stained with toluidine blue stain in the right side of the head of mouse are shown in low magnification (original magnification ×4) (A) and high magnification (original magnification ×400) (B). Mast cells were indicated by white arrow heads.

(1) Mast cells enumeration of allergic mice after receiving Prasaprohyai

The mean and standard error of the mean (SEM) of total mast cells in individual mouse groups after treatment with Prasaprohyai and challenged with OVA is shown in Table 4.9 and Figure 4.6.

From Table 4.9 and Figure 4.6, mast cell numbers in nasal tissues of allergic mice after treatment with PPY at concentrations of 100 and 200 mg/kg body weight were lower than the control group but not statistically significant.

Table 4.9 The average number (mean) and standard error of the mean (SEM) of enumerated mast cells in the right side of the head of control and allergic mice after receiving Prasaprohyai remedy

Mouse group (Total number of mice)	**Mean ± SEM of number of mast cells***
Control 5% Tween-80 (6)	244.83 ± 21.31
PPY 100 mg/kg/bw (6)	203.33 ± 13.60
PPY 200 mg/kg/bw (6)	191.00 ± 19.12
PPY 400 mg/kg/bw (6)	246.17 ± 23.69
MT 10 mg/kg/bw (6)	235.83 ± 18.87

* Mean ± SEM was calculated from the numbers of mast cells in 10 microscopic fields in the right side of the head of individual mice in each groups and analyzed by independent *t*-test

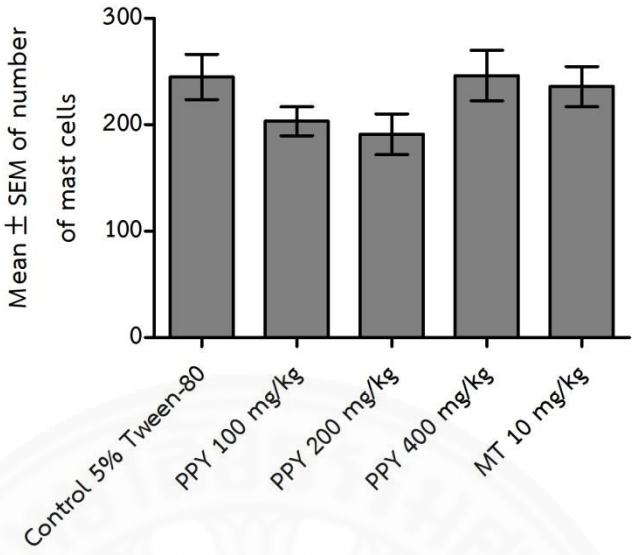


Figure 4.6 Mean and standard error of the mean (SEM) of numbers of mast cells stained with toluidine blue stain in the right side of the head of allergenized mice after treatment with Prasaprohyai.

(2) Mast cells enumeration of allergic mice after receiving Benjakul

The mean and standard error of the mean (SEM) of total mast cells in individual mouse groups after treatment with Benjakul and challenged with OVA is shown in Table 4.10 and Figure 4.7.

From Table 4.10 and Figure 4.7, the numbers of mast cells of allergenized mice after receiving BJK at concentrations of 37.5, 75 and 150 mg/kg body weight, and MT (a positive drug) at concentration of 10 mg/kg body weight were lower than the control group but not statistically significant.

Table 4.10 The average number (mean) and standard error of the mean (SEM) of enumerated mast cells in the right side of the head of control and allergic mice after receiving Benjakul remedy

Mouse group (Total number of mice)	Mean ± SEM of number of mast cells*
Control 10% Tween-80 (6)	272.00 ± 29.49
BJK 37.5 mg/kg/bw (6)	234.33 ± 14.26
BJK 75 mg/kg/bw (6)	244.17 ± 7.29
BJK 150 mg/kg/bw (6)	236.67 ± 18.29
MT 10 mg/kg/bw (6)	216.67 ± 8.34

* Mean ± SEM was calculated from the numbers of mast cells in 10 microscopic fields in the right side of the head of individual mice in each groups and analyzed by independent *t*-test

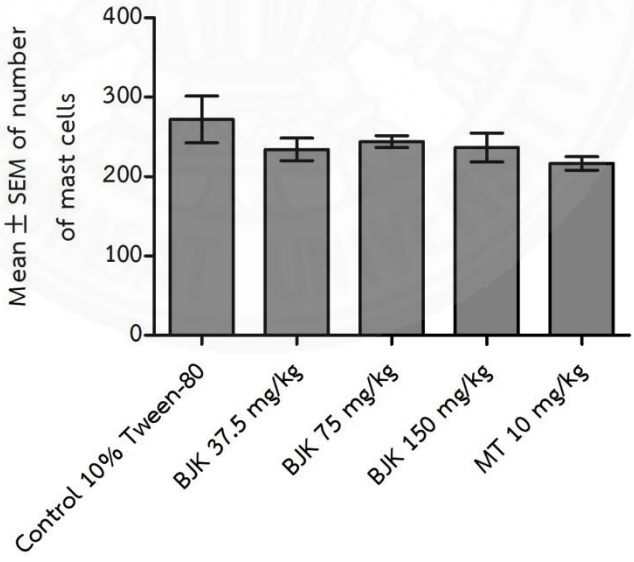


Figure 4.7 Mean and standard error of the mean (SEM) of numbers of mast cells stained with toluidine blue stain in the right side of the head of allergenized mice after treatment with Benjakul.

4.3.2.3 Grades of mucus production

The grades of color intensity of mucus glands in nasal tissues stained with Periodic acid-Schiff (PAS) dye are shown in Figure 4.8. The different color of mucus glands is shown in magenta color as the results of the reaction of PAS dye and glycogens in the mucus glands.

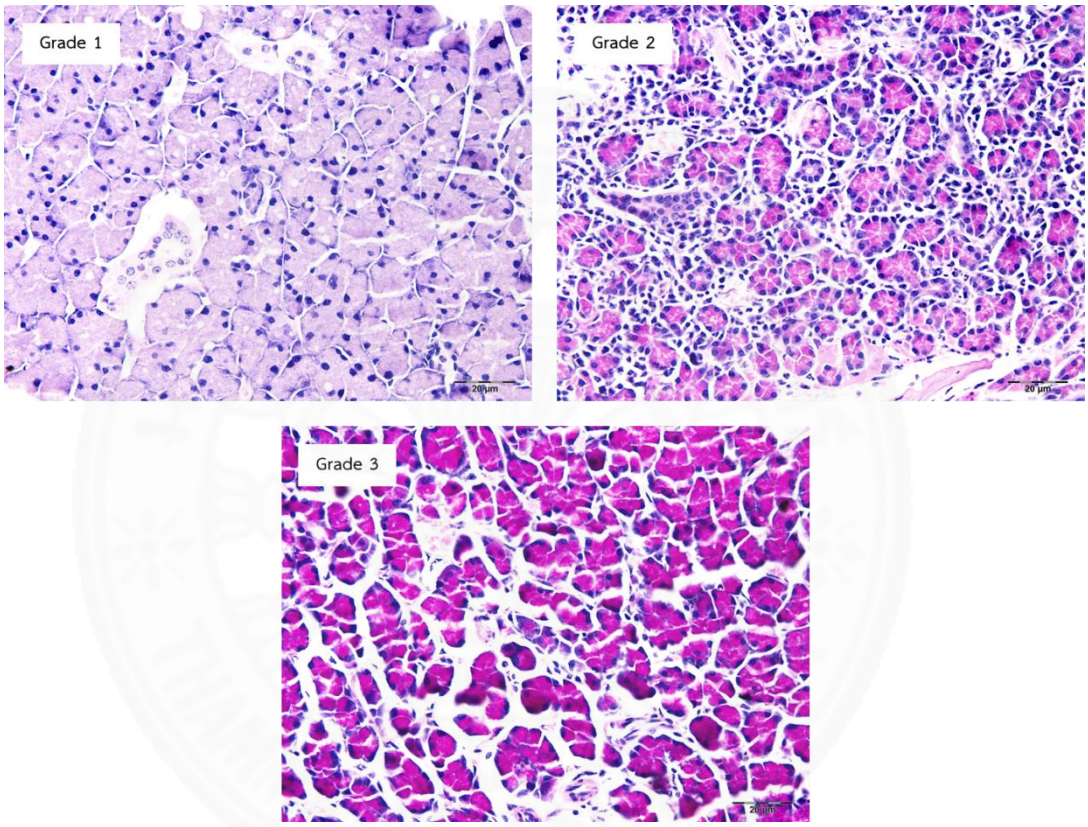


Figure 4.8 Grades of color intensity of mucus glands in the nasal tissues stained with PAS stain (original magnification ×400). Grades 1-3 were grades according to the color reaction illustrated and shown in magenta color.

(1) Grades of mucus glands of allergic mice after receiving Prasaprohyai

The percentage of individual and average grades of mucus glands of allergic mice after treatment with Prasaprohyai and challenged with OVA in nasal tissues are shown in Table 4.11.

The average grades of mucus glands of allergenized mice after receiving PPY at concentrations of 100, 200 and 400 mg/kg, and MT at concentration of 10 mg/kg had no significant effect on mucus production compared to the control mice with the average grades values of 1.47 ± 0.22 (control 5% Tween-80), 1.32 ± 0.26 (PPY 100 mg/kg), 1.25 ± 0.16 (PPY 200 mg/kg), 1.26 ± 0.16 (PPY 400 mg/kg) and 1.44 ± 0.28 (MT 10 mg/kg).

Prasaprohyai at concentration of 400 mg/kg and montelukast at concentration of 10 mg/kg treatments showed reduced in the grade 3-mucus gland and also increased the grade 1 and the grade 2 in allergic mice compared with control mice, but not significantly different. Furthermore, PPY at concentrations of 100 and 200 mg/kg displayed reduced in the grade 3 and increased only the grade 1 in allergenized mice compared to the control group (not significant).

Table 4.11 The percentage of individual and average grades of color intensity of PAS stained mucus glands in nasal tissues of control and allergic mice after receiving Prasaprohyai remedy (6 mice per group)

Group of mice	%Individual of mucus grades*			Mean ± SEM of
(Total microscopic fields)	Grade 1	Grade 2	Grade 3	mucus grades#
Control 5% Tween-80 (103)	71.85[a]	13.59[a]	14.56[a]	1.47 ± 0.22[a]
PPY 100 mg/kg/bw (62)	90.32[a]	3.23[a]	6.45[a]	1.32 ± 0.26[a]
PPY 200 mg/kg/bw (55)	83.64[a]	5.45[a]	10.91[a]	1.25 ± 0.16[a]
PPY 400 mg/kg/bw (60)	78.33[a]	15.00[a]	6.67[a]	1.26 ± 0.16[a]
MT 10 mg/kg/bw (64)	71.88[a]	21.87[a]	6.25[a]	1.44 ± 0.28[a]

* Percentage of individual mucus grades was analyzed by Chi-square test

Mean of mucus grades was analyzed by independent *t*-test

Entries with different superscripts along vertical axis (a *versus* b) are statistically different ($p < 0.05$)

(2) Grades of mucus glands of allergic mice after receiving Benjakul

The percentage of individual and average grades of mucus glands of allergic mice after treatment with Benjakul and challenged with OVA in nasal tissues are shown in Table 4.12.

The average grades of mucus glands of allergenized mice after treatment with BJK at concentrations of 37.5, 75 and 150 mg/kg body weight, and MT at concentration of 10 mg/kg body weight were close to control group but not significant difference (average grades = 2.17 ± 0.12, 2.24 ± 0.14, 2.35 ± 0.14, 2.21 ± 0.09 and 2.25 ± 0.15, respectively).

Allergic mice receiving BJK at concentrations of 37.5 and 75 mg/kg showed slightly decreased in the grade 3-mucus gland and slightly increased in the grade 2 when compared with control mice.

Table 4.12 The percentage of individual and average grades of color intensity of PAS stained mucus glands in nasal tissues of control and allergic mice after receiving Benjakul remedy (6 mice per group)

Group of mice (Total microscopic fields)	%Individual of mucus grades*			Mean ± SEM of mucus grades#
	Grade 1	Grade 2	Grade 3	
Control 10% Tween-80 (114)	24.56[a]	34.21[a]	41.23[a]	2.25 ± 0.15[a]
BJK 37.5 mg/kg/bw (143)	23.08[a]	38.46[a]	38.46[a]	2.17 ± 0.12[a]
BJK 75 mg/kg/bw (103)	24.28[a]	37.86[a]	37.86[a]	2.24 ± 0.14[a]
BJK 150 mg/kg/bw (72)	15.28[a]	40.28[a]	44.44[a]	2.35 ± 0.14[a]
MT 10 mg/kg/bw (93)	20.43[a]	36.56[a]	43.01[a]	2.21 ± 0.09[a]

* Percentage of individual mucus grades was analyzed by Chi-square test

Mean of mucus grades was analyzed by independent *t*-test

Entries with different superscripts along vertical axis (a *versus* b) are statistically different ($p < 0.05$)

4.3.3 Expressions of cytokine genes in nasal tissues of mice

4.3.3.1 Cytokine gene expressions in nasal tissues of OVA-allergic mice after treatment with Prasaprohyai remedy

The fold changes of cytokine gene expressions including IL-4, IL-5, IL-13, IFN-γ, IL-12a (p35), IL-12b (p40), TGF-β and IL-10 were investigated by the quantitative real-time PCR (qPCR) in nasal tissues of OVA-allergic mice after receiving Prasaprohyai remedy and challenged with OVA compared with the control group and a positive drug (montelukast, MT) are shown in Table 4.13 and Figure 4.9 to 4.15.

Table 4.13 The overall results of fold changes of cytokine gene expressions of OVA-allergic mice after receiving Prasaprohyai remedy and OVA challenge

Mouse group (6 mice/group)	Mean ± SEM of fold changes							
	IL-4	IL-5	IL-13	IFN-γ	IL-12a	IL-12b	TGF-β	IL-10
Control 5% Tween-80 mice	0.189 ± 0.068	1.623 ± 0.376	3.036 ± 0.886	6.576 ± 0.968	0.956 ± 0.590	4.461 ± 1.147	3.895 ± 0.493	1.048 ± 0.108
PPY 100 mg/kg/bw mice	0.063 ± 0.010	0.982 ± 0.084$^{\#}$	1.848 ± 0.440	3.537 ± 0.447*	1.677 ± 0.190	0.871 ± 0.147*	2.004 ± 0.103*	1.855 ± 0.348
PPY 200 mg/kg/bw mice	0.137 ± 0.013	3.101 ± 0.644	4.174 ± 1.121	1.590 ± 0.371$^{*,\#}$	2.560 ± 0.504	1.130 ± 0.185*	2.926 ± 0.446	1.820 ± 0.277*
PPY 400 mg/kg/bw mice	0.524 ± 0.240	2.072 ± 0.571	0.003 ± 0.001$^{*,\#}$	3.080 ± 0.844*	4.590 ± 1.544	4.160 ± 1.025	2.167 ± 0.231*	13.945 ± 5.672
MT 10 mg/kg/bw mice	0.131 ± 0.049	2.641 ± 0.502	3.465 ± 0.954	4.318 ± 1.051	2.938 ± 0.997	2.167 ± 0.885	3.042 ± 0.498	4.836 ± 1.546

* $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the montelukast group by independent *t*-test

(1) IL-4 gene expressions of Prasaprohyai treatment

The expressions of IL-4 (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.9.

The fold changes of level of IL-4 mRNA in OVA-allergic mice after receiving all doses of PPY and OVA-allergen challenge were not significantly different from the control group and the montelukast group, a positive control drug. Nevertheless, allergic mice after receiving PPY at concentrations of 100 and 200 mg/kg showed reduced on mRNA expression of IL-4 cytokine in nasal mucosa when compared with the control group.

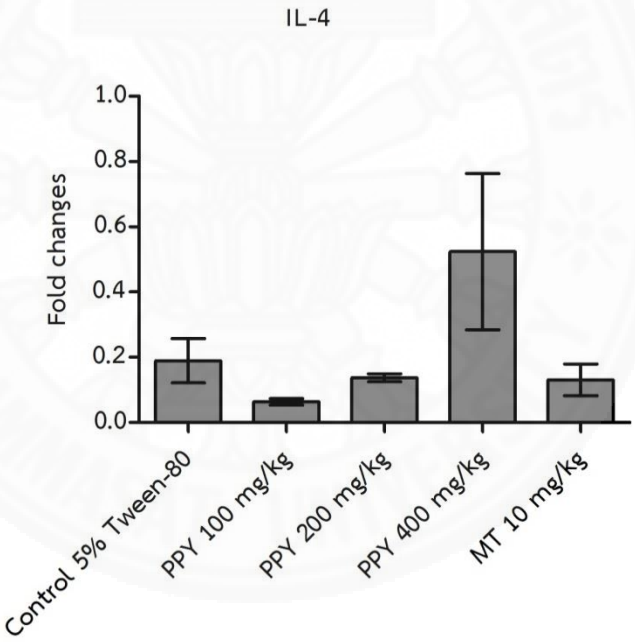


Figure 4.9 Fold changes of IL-4 gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

(2) IL-5 gene expressions of Prasaprohyai treatment

The expressions of IL-5 (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.10.

The expressions of IL-5 in allergenized mice after receiving all doses of PPY were not significant difference when compared with the control mice. However, the allergic mice after receiving PPY at concentration of 100 mg/kg revealed decreased on mRNA expression of IL-5 cytokine in nasal mucosa. Moreover, allergic mice after receiving PPY at concentration of 100 mg/kg also showed significantly reduced on mRNA expression of IL-5 cytokine in nasal mucosa when compared with the positive control group (MT group).

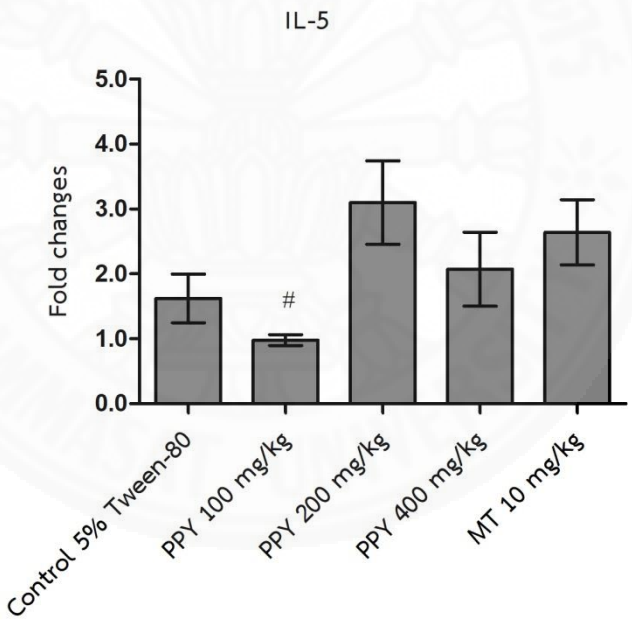


Figure 4.10 Fold changes of IL-5 gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

(3) IL-13 gene expressions of Prasaprohyai treatment

The expressions of IL-13 (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.11.

For IL-13, the allergic mice after receiving PPY at concentration of 100 mg/kg showed decreased on mRNA expression of IL-13 cytokine in nasal mucosa when compared with the control group. In addition, the level of IL-13 mRNA in allergic mice receiving PPY at concentration of 400 mg/kg body weight was significantly lower than allergic mice that received solvent control as the control group and montelukast as the positive control group ($p < 0.05$).

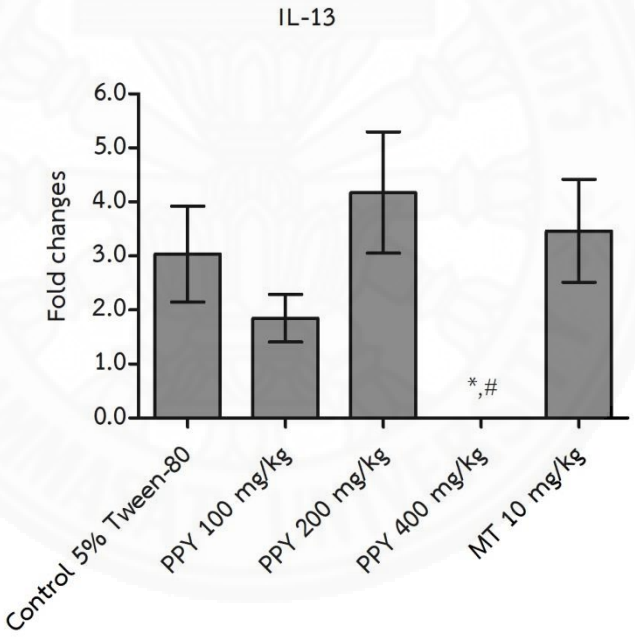


Figure 4.11 Fold changes of IL-13 gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

(4) IFN-γ gene expressions of Prasaprohyai treatment

The expressions of IFN-γ (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.12.

The expressions of IFN-γ cytokine in the allergic mice that received the PPY at concentrations of 100, 200 and 400 mg/kg were significantly lower than in the solvent control group ($p < 0.05$) but only the PPY at concentration of 200 mg/kg-treatment group was significantly lower than in the positive control group (MT 10 mg/kg) ($p < 0.05$).

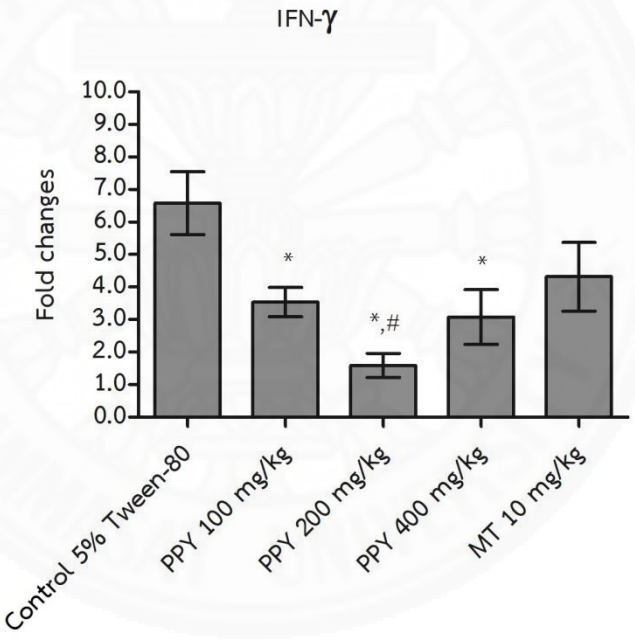


Figure 4.12 Fold changes of IFN-γ gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

(5) IL-12a (p35) and IL-12b (p40) gene expressions of Prasaprohyai treatment

The expressions of IL-12a (p35) and IL-12b (p40) (mRNAs) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.13.

For IL-12a (p35) and IL-12b (p40), the trend of IL-12b (p40) cytokine of allergic mice after treatment with PPY at concentrations of 100 and 200 mg/kg had significantly lower than that of the positive control group (MT mice) ($p < 0.05$), while the levels of IL-12a (p35) cytokine of PPY at concentrations of 100, 200 and 400 mg/kg-treatment groups showed increased but no significant difference from the solvent control group and the positive control group.

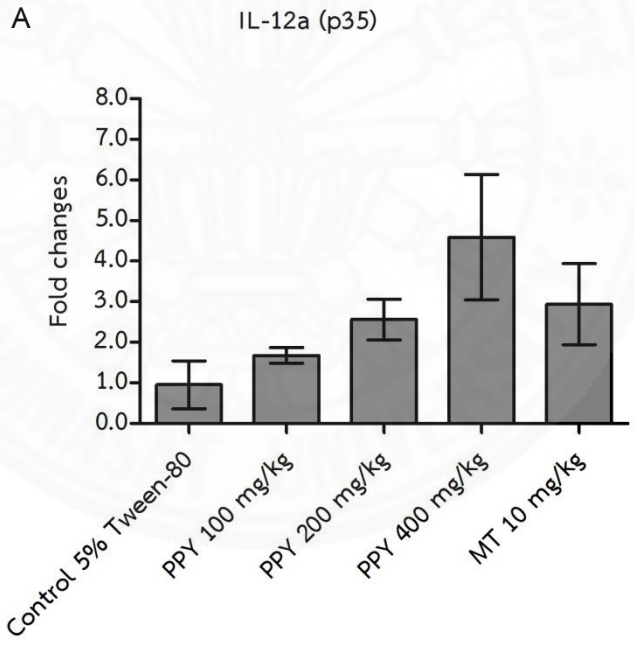


Figure 4.13 Fold changes of IL-12a (p35) (A) and IL-12b (p40) (B) gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

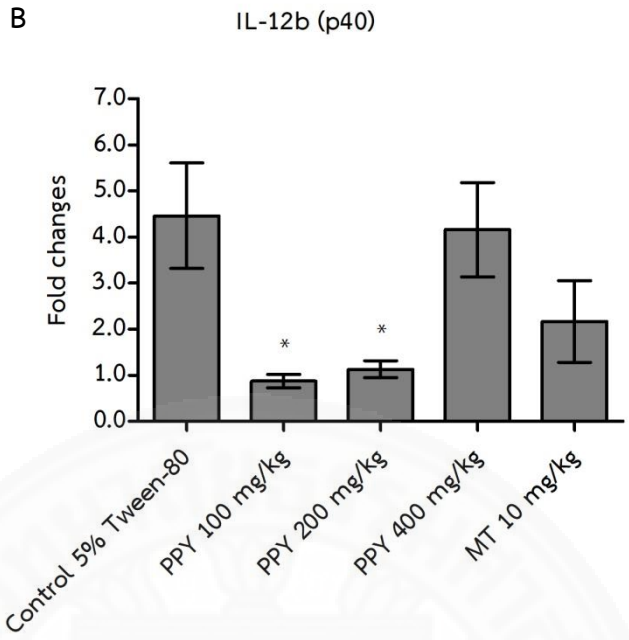


Figure 4.13 Fold changes of IL-12a (p35) (A) and IL-12b (p40) (B) gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug). (Cont.)

(6) TGF-β gene expressions of Prasaprohyai treatment

The expressions of TGF-β (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.14.

Both the mRNA of TGF-β expressions of PPY at concentrations of 100 and 400 mg/kg-treatment groups were significantly lower than that of the control group ($p < 0.05$).

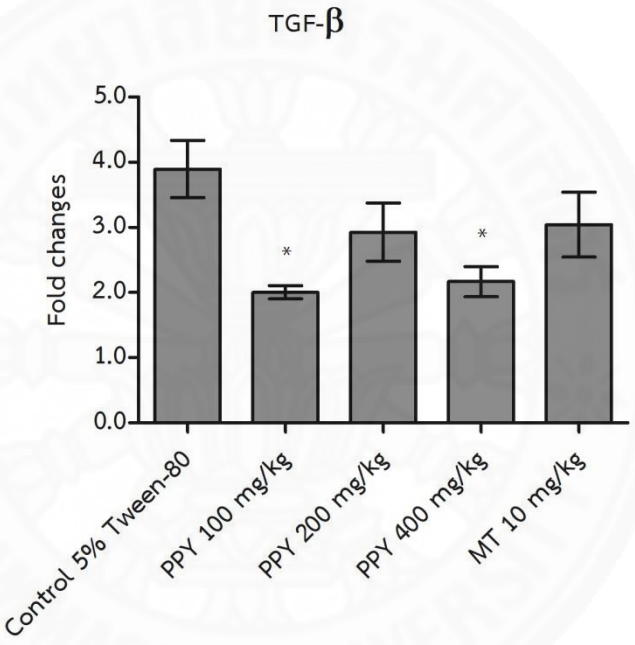


Figure 4.14 Fold changes of TGF-β gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

(7) IL-10 gene expressions of Prasaprohyai treatment

The expressions of IL-10 (mRNA) in allergic mice after treated with PPY (100, 200 and 400 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.15.

The mRNA expressions of the IL-10 gene in allergic mice after treated with PPY (100, 200 and 400 mg/kg) were increased when compared with the control group. Furthermore, the mRNA of IL-10 expression of PPY at concentration of 200 mg/kg-treatment group was significantly increased in the nasal mucosa when compared with the solvent control group ($p < 0.05$).

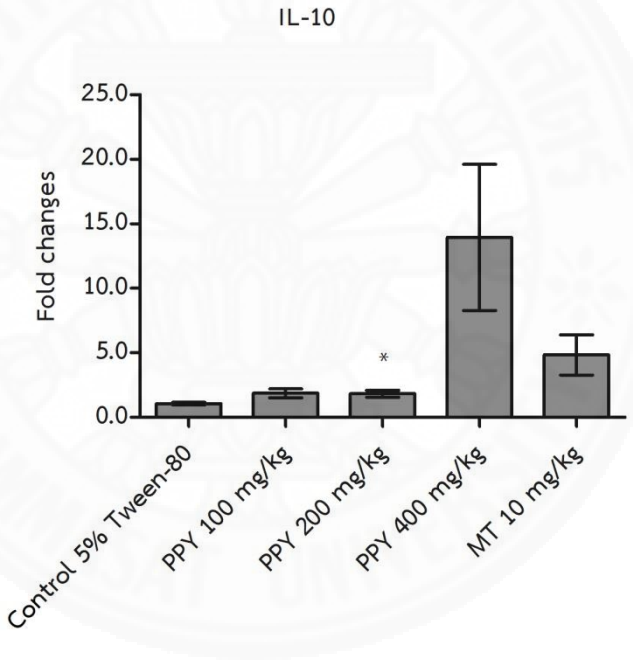


Figure 4.15 Fold changes of IL-10 gene expressions in nasal tissues of allergic mice after treatment with PPY and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared with control group; # $p < 0.05$ compared with montelukast group (a positive control drug).

4.3.3.2 Cytokine gene expressions in nasal tissues of OVA-allergic mice after treatment with Benjakul remedy

The fold changes of cytokine gene expressions including IL-4, IL-5, IL-13, IFN-γ, IL-12a (p35), IL-12b (p40), TGF-β and IL-10 were investigated by the quantitative real-time PCR (qPCR) in nasal tissues of OVA-allergic mice after treatment with Benjakul remedy and challenged with OVA compared with the control group and montelukast group, a positive drug, are shown in Table 4.14 and Figure 4.16 to 4.22.

Table 4.14 The overall results of fold changes of cytokine gene expressions of OVA-allergic mice after treatment with Benjakul remedy and OVA challenge

Mouse group (6 mice/group)	Mean ± SEM of fold changes							
	IL-4	IL-5	IL-13	IFN-γ	IL-12a	IL-12b	TGF-β	IL-10
Control 10% Tween-80 mice	1.633 ± 0.579	2.990 ± 0.436	1.877 ± 0.269#	2.212 ± 0.722	4.476 ± 1.496	2.024 ± 0.187	0.621 ± 0.110#	1.413 ± 0.365
BJK 37.5 mg/kg/bw mice	2.084 ± 1.037	2.345 ± 0.400	0.415 ± 0.149*	0.856 ± 0.721	2.137 ± 0.848	0.875 ± 0.225*	0.469 ± 0.149#	1.493 ± 0.402
BJK 75 mg/kg/bw mice	11.915 ± 4.107*	2.718 ± 0.957	0.265 ± 0.034*	1.108 ± 0.401	3.365 ± 0.997#	1.108 ± 0.254*	0.517 ± 0.113#	0.368 ± 0.082*,#
BJK 150 mg/kg/bw mice	2.513 ± 1.052	6.198 ± 0.394*	0.237 ± 0.025*	0.083 ± 0.223	1.977 ± 0.386	1.285 ± 0.153*	0.093 ± 0.018*	0.512 ± 0.047#
MT 10 mg/kg/bw mice	0.367 ± 0.068	4.149 ± 0.936	0.312 ± 0.106*	1.008 ± 0.237	1.035 ± 0.310	1.702 ± 0.467	11.960 ± 3.616*	1.141 ± 0.288

* $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the montelukast group by independent *t*-test

(1) IL-4 gene expressions of Benjakul treatment

The expressions of IL-4 (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.16.

The reductions of IL-4 gene expression were not detected in the allergenized mice treated with BJK at concentrations of 37.5, 75 and 150 mg/kg compared with the solvent control group.

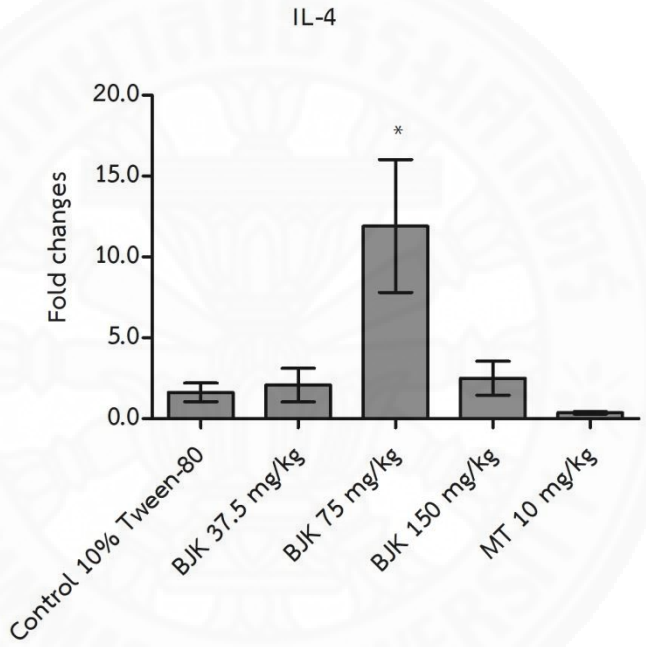


Figure 4.16 Fold changes of IL-4 gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

(2) IL-5 gene expressions of Benjakul treatment

The expressions of IL-5 (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.17.

The mRNA expression of IL-5 cytokine decreased in the nasal mucosa of the BJK at concentrations of 37.5 and 75 mg/kg treatment groups compared with the solvent control mice (not significant).

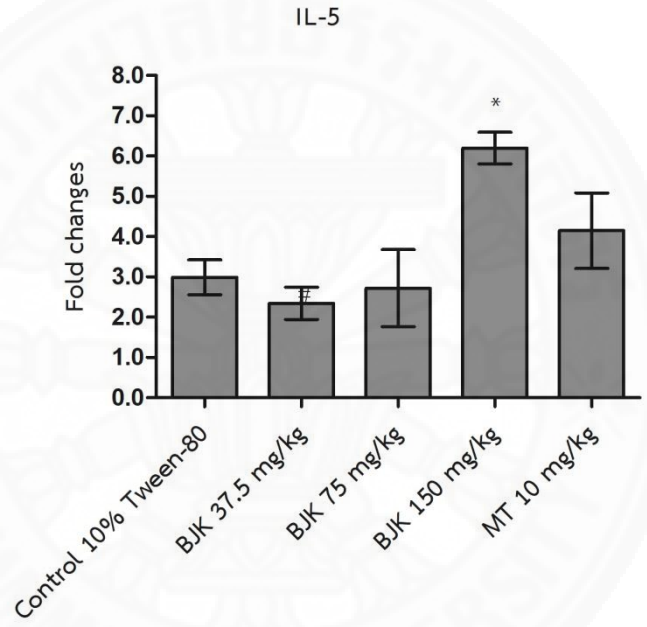


Figure 4.17 Fold changes of IL-5 gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

(3) IL-13 gene expressions of Benjakul treatment

The expressions of IL-13 (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.18.

Allergic mice that were treated with the BJK at concentrations of 37.5, 75 and 150 mg/kg and the MT at concentration of 10 mg/kg had significantly decreased on IL-13 gene expression in nasal tissues when compared with the control mice ($p < 0.05$).

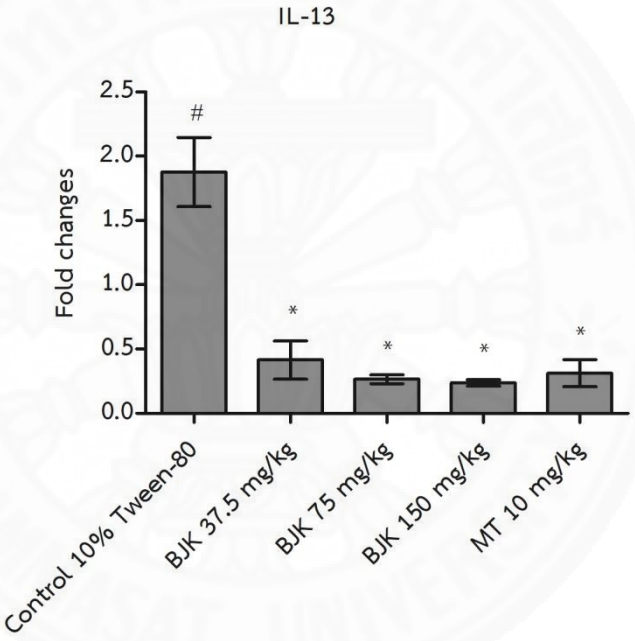


Figure 4.18 Fold changes of IL-13 gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

(4) IFN-γ gene expressions of Benjakul treatment

The expressions of IFN-γ (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.19.

The levels of IFN-γ gene of the allergic mice receiving BJK at concentrations of 37.5, 75 and 150 mg/kg were not increased when compared with the control group.

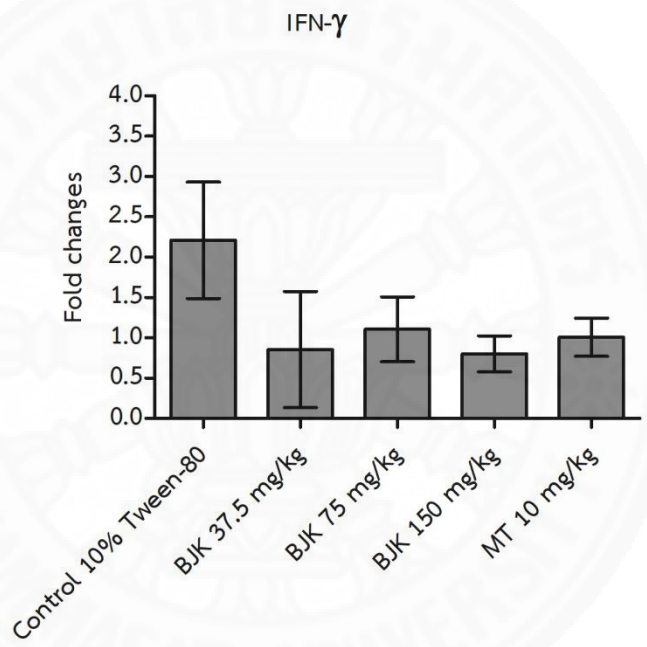


Figure 4.19 Fold changes of IFN-γ gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

(5) IL-12a (p35) and IL-12b (p40) gene expressions of Benjakul treatment

The expressions of IL-12a (p35) and IL-12b (p40) (mRNAs) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.20.

For IL-12a (p35) and IL-12b (p40), the levels of IL-12a (p35) and IL-12b (p40) genes in the allergenized mice receiving BJK at concentrations of 37.5, 75 and 150 mg/kg were not increased when compared to the solvent control group.

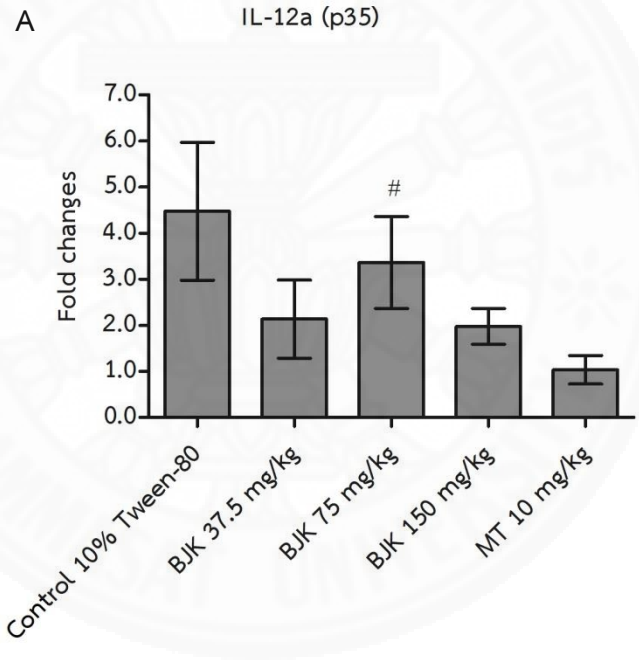


Figure 4.20 Fold changes of IL-12a (p35) (A) and IL-12b (p40) (B) gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

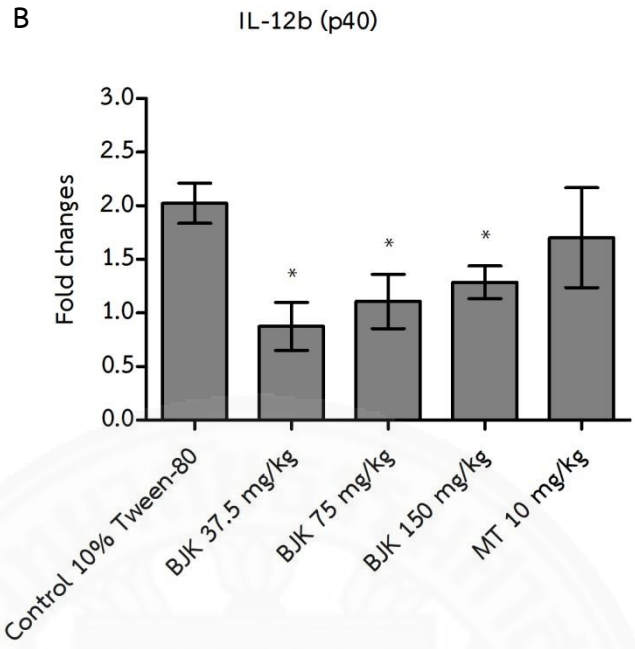


Figure 4.20 Fold changes of IL-12a (p35) (A) and IL-12b (p40) (B) genes expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug). (Cont.)

(6) TGF-β gene expressions of Benjakul treatment

The expressions of TGF-β (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.21.

The mRNA expressions of TGF-β were not increased in the allergic mice receiving BJK at concentrations of 37.5, 75 and 150 mg/kg when compared to the solvent control group.

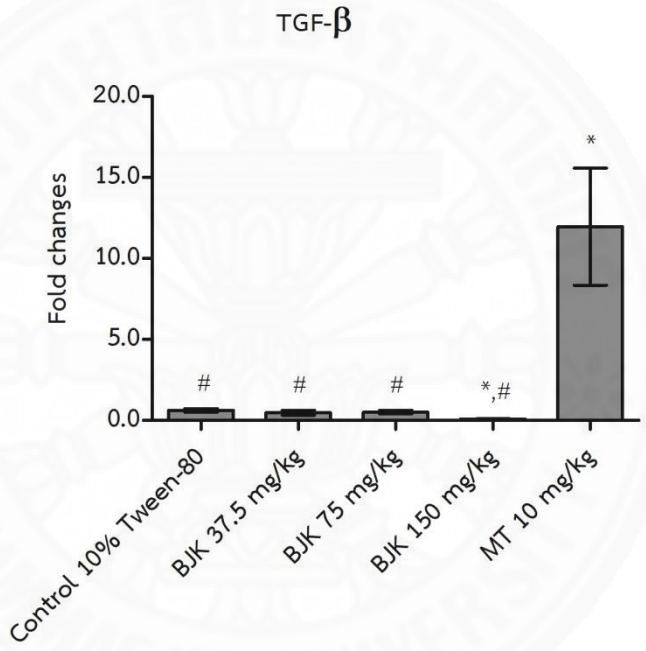


Figure 4.21 Fold changes of TGF-β gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

(7) IL-10 gene expressions of Benjakul treatment

The expressions of IL-10 (mRNA) in allergic mice after receiving BJK (37.5, 75 and 150 mg/kg) and MT (10 mg/kg) and challenged with OVA are shown in Figure 4.22.

The mRNA expression of IL-10 had slightly increased in the BJK 37.5 mg/kg treatment group when compared to the solvent control group (not significant).

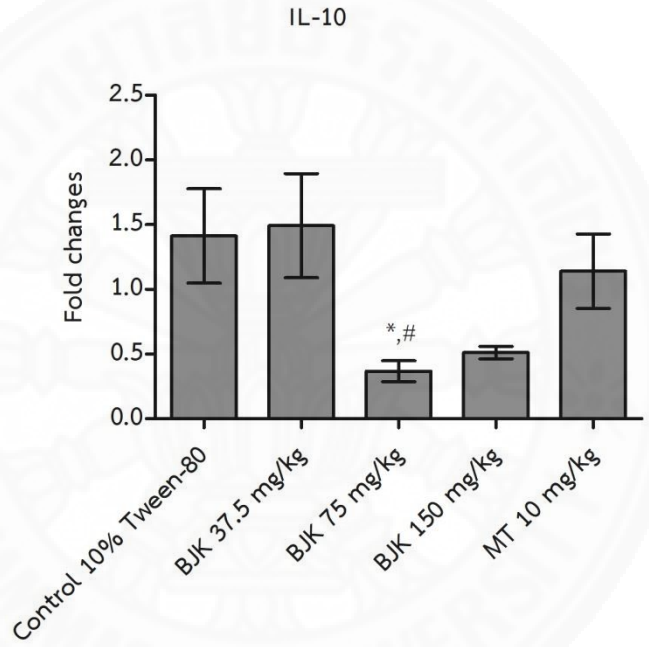


Figure 4.22 Fold changes of IL-10 gene expressions in nasal tissues of allergic mice after receiving BJK and MT and OVA-allergen challenge. Data were analyzed by independent *t*-test. * Significant difference ($p < 0.05$) compared to control group; # $p < 0.05$ compared to montelukast group (a positive control drug).

4.4 *In vitro* assay for anti-inflammatory activity

4.4.1 Determination of lipopolysaccharide (LPS)-induced nitric oxide (NO) production from RAW 264.7 cells

For Prasaprohyai remedy (PPY), the inhibition effects of the ethanolic extracts of Prasaprohyai and its individual plants on NO production in RAW 264.7 cells were determined and reported in my previous thesis. The ethanol extract of Prasaprohyai presented strong inhibitory effect on NO production with the IC_{50} value of 18.40 μg/ml (Makchuchit, 2010).

For Benjakul remedy (BJK), the inhibition effects of the ethanolic extracts of Benjakul, five plant components and seven pure compounds from the Benjakul ethanolic extract on lipopolysaccharide-stimulated NO production from RAW 264.7 cells were studied and compared with the standard drug as prednisolone. The results of this study are shown in Table 4.15 and Figure 4.23. The ethanolic extracts of *Z. officinale*, *P. indica*, Benjakul, *P. interruptum*, *P. longum* and *P. sarmentosum* exhibited significantly increased the levels of NO production when compared with the positive control (prednisolone, IC_{50} = 0.59 μg/ml) with the IC_{50} values of 13.44, 14.06, 16.60, 21.59, 22.28 and 22.42 μg/ml, respectively.

For pure compounds isolated from BJK, plumbagin showed the most potent inhibitory effect on NO production (IC_{50} = 0.002 μg/ml) and higher than prednisolone (IC_{50} = 0.59 μg/ml, a positive control), but not significantly different. And 6-shogaol also showed the great inhibitory activity on NO production (IC_{50} = 0.92 μg/ml) (not significant). Moreover, the results indicated that piperine possessed moderate activity (IC_{50} = 38.86 μg/ml), whereas 6-gingerol displayed mild activity (IC_{50} = 72.25 μg/ml). Both of piperine and 6-gingerol had significantly increased the inhibitory effect on NO production when compared to prednisolone, a positive control.

Table 4.15 The percentage of inhibition and the IC_{50} value of the ethanolic extracts of Benjakul remedy, its plant components and its pure compounds on nitric oxide (NO) production from RAW 264.7 cells (n = 3)

Crude extracts and pure compounds	%Inhibition at various concentrations of extracts (mean ± SEM, μg/ml)												IC_{50} ± SEM (μg/ml)
	0.00001	0.0001	0.001	0.01	0.1	1	10	15	20	30	50	100	
P. interruptum	-	-	-	-	-6.00±6.07	4.37±3.04	27.86±1.80	-	48.40±3.46	62.65±5.25	-	-	21.59±1.69*
P. longum	-	-	-	-	-4.30±1.65	3.77±1.33	27.81±1.69	-	48.79±5.94	63.67±8.93	-	-	22.28±2.79*
P. sarmentosum	-	-	-	-	-	-8.99±0.82	20.94±3.72	-	-	-	84.19±6.21	94.93±1.18	22.42±3.61*
P. indica	-	-	-	-	-3.49±4.33	0.82±3.50	30.85±15.47	-	78.87±7.32	92.09±1.31[a]	-	-	14.06±2.91*
Z. officinale	-	-	-	-	-7.14±1.18	-1.86±1.30	37.58±3.43	54.16±1.61	-	-	-	-	13.44±0.78*
Benjakul	-	-	-	-	-4.20±5.14	4.97±3.45	28.83±8.10	-	59.86±4.74	76.93±3.42	-	-	16.60±1.85*
6-Gingerol	-	-	-	-	-	-0.45±1.27	9.45±2.01	-	-	20.75±4.32	34.56±5.34	70.74±3.94	72.25±7.75* (245.42 μM)
Methyl piperate	-	-	-	-	-	-	0.88±2.64	-	-	-	-	-	> 10
Myristicin	-	-	-	-	-	-	-	-	-	-	-	35.89±10.83	> 100
Piperine	-	-	-	-	-	0.61±0.98	18.48±0.26	-	-	40.08±0.71	64.66±1.38	89.94±4.38[a]	38.89±0.79* (136.29 μM)
Plumbagin	8.45±2.94	25.65±18.94	51.10±12.69	77.08±7.66	88.57±3.20[a]	-	-	-	-	-	-	-	0.002±0.002 (0.01 μM)
6-Shogaol	-	-	-12.98±3.79	-8.01±2.25	-3.97±1.44	5.09±0.43	81.95±9.02	-	-	-	-	-	0.92±0.31 (3.33 μM)
β-Sitostenone	-	-	-	-	-	-	-	-	-	-	-	-1.09±0.55	> 100
Prednisolone	-	-	-	-	39.71±5.79	62.78±8.89	70.42±9.10	-	-	75.95±8.32	81.47±6.19	-	0.59±0.32 (1.64 μM)

(-) means not tested; [a] Cytotoxic effect was observed; * Significant difference at the *p*-value < 0.05 compared with prednisolone (a positive control drug)

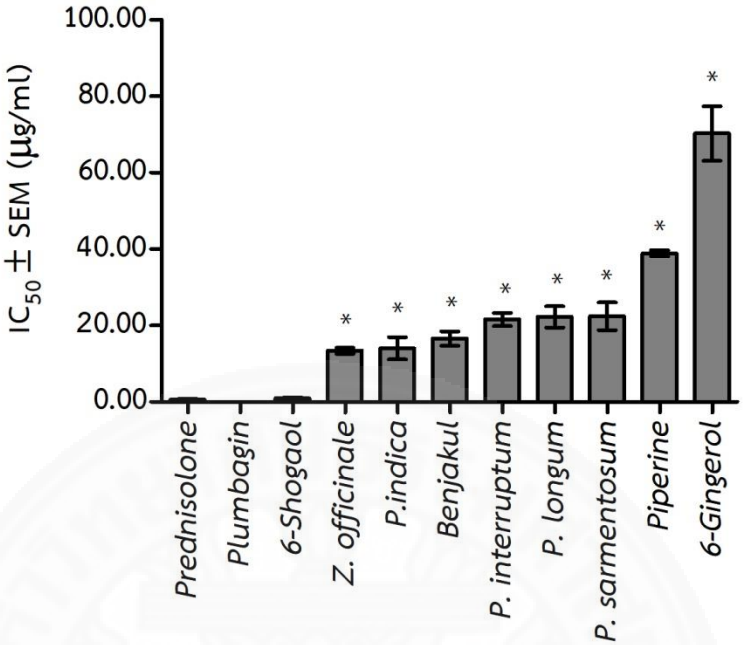


Figure 4.23 Effect of the ethanolic extracts of Benjakul, its plant components and its pure compounds on NO production from RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with prednisolone (a positive control drug).

4.4.2 Determination of lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) from RAW 264.7 cells

For Prasaprohyai remedy (PPY), the inhibition effects of the ethanolic extracts of Prasaprohyai and its individual plants on TNF-α production in RAW 264.7 cells were studied and reported in my previous thesis. The ethanol extract of Prasaprohyai had inhibition of TNF-α release *in vitro* assay with the IC_{50} value of 20.34 μg/ml (Makchuchit, 2010).

From Table 4.16 and Figure 4.24, the inhibition effects of the ethanolic extracts of Benjakul, five plant components and seven pure compounds from the Benjakul ethanolic extract on lipopolysaccharide-activated TNF-α release from RAW 264.7 cells were determined and compared with the standard drug as prednisolone. The results were found that Benjakul ethnolic extract was no effect on LPS-induced TNF-α release in RAW 264.7. In addition, 6-shogaol revealed the highest potent inhibitory effect on TNF-α release with the IC_{50} value of 9.16 μg/ml but lower than the positive control as prednisolone (IC_{50} = 5.77 μg/ml). Other extracts such as *P. interruptum*, *P. sarmentosum*, myristicin and 6-gingerol had anti-inflammatory activity *via* TNF-α method with IC_{50} values of 19.81, 34.33, 47.52 and 65.47 μg/ml, respectively. These results indicated that *P. sarmentosum*, myristicin and 6-gingerol had significantly increased the inhibitory effect on TNF-α release compared to prednisolone ($p < 0.05$).

Table 4.16 The percentage of inhibition and the IC_{50} value of the ethanolic extracts of Benjakul remedy, its plant components and its pure compounds on tumor necrosis factor-α (TNF-α) from RAW 264.7 cells (n = 3)

Crude extracts and pure compounds	%Inhibition at various concentrations of extracts (mean ± SEM, μg/ml)									IC_{50} ± SEM, μg/ml (μM for pure compound)
	0.01	0.1	1	10	15	20	30	50	100	
P. interruptum	-	-	15.94±1.39	34.68±3.29	-	46.98±3.86	57.84±1.55	-	-	21.84±3.26
P. longum	-	-	-	-	-	-	47.01±0.36	-	-	NA
P. sarmentosum	-	-	25.65±1.99	37.22±2.51	-	-	-	55.02±2.04	81.90±0.89	30.31±8.07*
P. indica	-	-	-	-	-	-0.31±1.35	-	-	-	NA
Z. officinale	-	-	-	-	29.77±1.04	-	-	-	-	NA
Benjakul	-	-	-	-	-	-	44.26±2.12	-	-	NA
6-Gingerol	-	-	-30.95±15.70	-9.35±15.55	-	-	-	38.04±2.14	72.98±1.54	67.83±2.45* (230.41 μM)
Methyl piperate	-	-	-	-	-	-	-	-	33.11±1.29	> 100
Myristicin	-	-	-22.08±13.10	-6.20±9.09	-	-	-	52.31±1.30	75.54±0.88	47.81±1.11* (248.74 μM)
Piperine	-	-	-	-	-	-	-	21.77±4.68	18.11±0.30[a]	NA
Plumbagin	35.31±1.67	70.91±0.98[a]	-	-	-	-	-	-	-	NA
6-Shogaol	-39.40±13.65	-29.67±8.79	-14.43±5.98	68.26±1.09	-	-	-	-	-	9.01±0.31 (32.60 μM)
β-Sitostenone	-	-	-	-	-	-	-	-	4.42±0.87	> 100
Prednisolone	-	22.06±5.86	28.50±3.27	56.16±3.78	-	-	-	80.86±1.76	-	6.72±2.51 (18.64 μM)

(-) means not tested; [a] Cytotoxic effect was observed; NA means not active; * Significant difference at the *p*-value < 0.05 compared with prednisolone (a positive control drug)

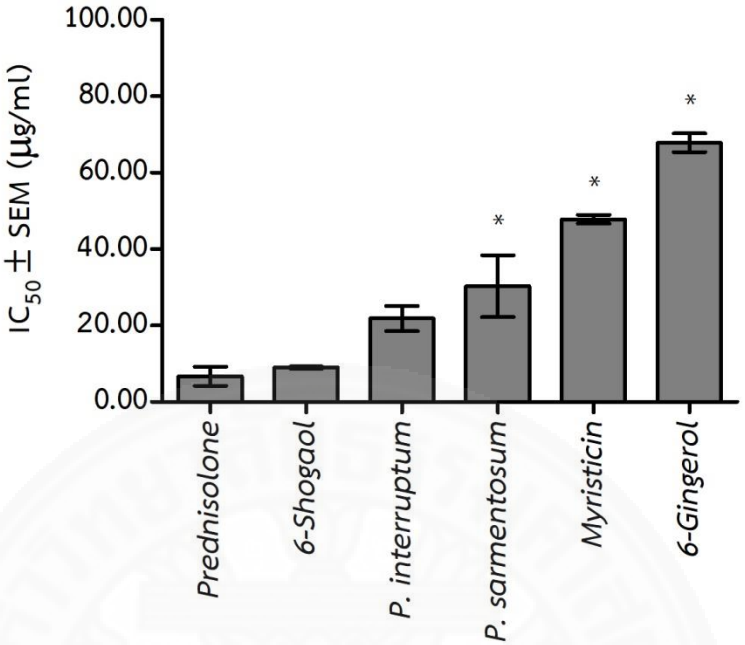


Figure 4.24 Effect of the ethanolic extracts of Benjakul, its plant components and its pure compounds on TNF-α release from RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with prednisolone (a positive control drug).

4.5 Bioassay-guided fractionation and isolation of chemical constituents from the ethanolic extract of *A. lancea*

The ethanolic extract of *A. lancea* was fractionated by VLC and eluted with increasing polarity solvents from hexane to MeOH. Therefore, these fractions were determined for *in vitro* anti-allergic activity on β-hexosaminidase release from RBL-2H3 cells and selected for isolation of pure compound. Out of the five fractions obtained (F1-F5), fraction F3 was found to present the highest potent anti-allergic activity with the IC_{50} value of 19.56 ± 3.07 μg/ml followed by fraction F4 and fraction F5 with the IC_{50} values of 25.80 ± 9.24 and 42.19 ± 8.47 μg/ml, respectively (Table 4.17). Therefore, fraction F3 was further purified with MPLC using gradient solvents as hexane: $CHCl_3$ (7:3, 5:5, 3:7 and 1:9) followed by MeOH: $CHCl_3$ (1:9, 3:7, 5:5, 7:3 and 9:1). Twenty-four sub-fractions were collected by combining elutes according to similar TLC chromatogram and detection with acidic anisaldehyde spraying reagent by UV at 254 nm.

Bioassay-guided fractionation and isolation of the ethanolic extract of *A. lancea* led to the isolation of FA1 compound. The structure of the FA1 compound was identified as taraxerol acetate with various spectroscopic methods such as 1D-NMR (^{1}H NMR (400 MHz in $CDCl_3$), ^{13}C-NMR (100 MHz in $CDCl_3$), DEPT) and 2D-NMR (COSY, HMBC, HMQC) (see Appendix C) as well as comparison with the previous reported (Duan et al., 2008). These results showed that FA1 compound as taraxerol acetate (%yield = 0.004%) is a white crystalline solid and the structure of this compound is shown in Figure 4.25.

After purified compound, the pure compound was investigated for anti-allergic and anti-inflammatory effects *in vitro* assays. From Table 4.18, the results showed that taraxerol acetate had no inhibition on β-hexosaminidase release from RBL-2H3 cells (IC_{50} > 100 μg/ml) and it had toxic effect on RAW 264.7 cells. Moreover, two authentic pure compounds as atractylodin TS and β-eudesmol, which were previously isolated from *A. lancea* were chose (Duan et al., 2008). Atractylodin TS and β-eudesmol were also tested in these assays. The result found that

atractylodin TS showed the most potent inhibitory effect on β-hexosaminidase release with the IC_{50} value of 0.07 ± 0.01 μg/ml. However, it showed no anti-inflammatory activity by nitric oxide assay because of the toxicity in RAW 264.7 cells. For β-eudesmol, it presented mild anti-allergic activity with the IC_{50} value of 86.21 ± 0.27 μg/ml and showed toxic effect on RAW 264.7 cells at concentrations of 50 and 100 μg/ml.

Table 4.17 The percentage of yield and the IC_{50} value of five fractions isolated from the ethanolic extract of *A. lancea* on β-hexosaminidase release from RBL-2H3 cells (n = 3)

Crude extract and its fractions	%Yield (w/w)	IC_{50} ± SEM (μg/ml)
A. lancea ethanolic extract	16.89	13.65 ± 1.32
F1 (hexane)	no yield	-
F2 (hexane: $CHCl_3$, 1:1)	3.79	> 100
F3 ($CHCl_3$)	16.85	19.56 ± 3.07
F4 ($CHCl_3$: MeOH, 1:1)	60.78	25.80 ± 9.24
F5 (MeOH)	10.98	42.19 ± 8.47

(-) means not tested

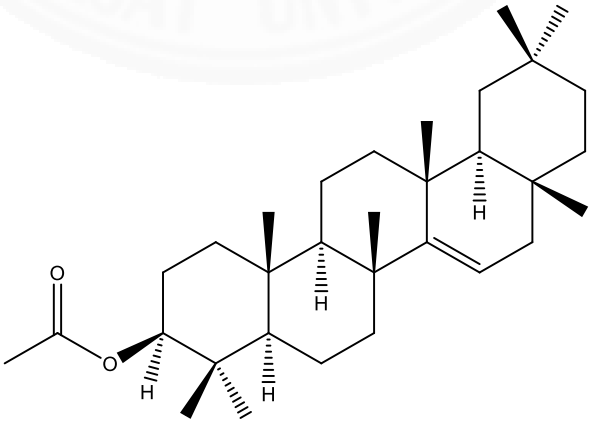


Figure 4.25 The chemical structure of taraxerol acetate

Table 4.18 The IC_{50} value of pure compounds isolated from *A. lancea* on β-hexosaminidase release from RBL-2H3 cells (n = 2) and on NO production from RAW 264.7 cells (n = 3)

Pure compounds	%Inhibition at various concentrations of pure compounds (mean ± SEM, μg/ml)								IC_{50} ± SEM, μg/ml
	0.0001	0.001	0.01	0.1	1	10	50	100	
β-Hexosaminidase assay									
Taraxerol acetate	-	-	-	-	-44.15±8.11	-9.49±4.54	14.96±1.91	22.01±5.15	> 100
Atractylodin TS	-0.47±0.69	-0.97±0.28	3.58±0.72	63.98±2.04	-	-	-	-	0.07±0.01 (0.38 μM)
β-Eudesmol	-	-	-	-	-3.95±1.71	0.04±0.55	23.92±1.05	60.33±1.31	86.21±0.27 (387.68 μM)
Nitric oxide assay									
Taraxerol acetate	-	-	-	-	-27.40±11.20	-27.20±9.99[a]	-13.28±5.15[a]	3.23±3.42[a]	Toxic
Atractylodin TS	-13.20±10.23	-14.68±13.61	4.47±13.59	97.90±0.33[a]	98.59±0.23[a]	-	-	-	Toxic
β-Eudesmol	-	-	-	-	-27.12±6.19	-10.37±7.24	88.74±0.27[a]	96.52±0.46[a]	Toxic

(-) means not tested; [a] Cytotoxicity was observed

4.6 Formulation development

4.6.1 Pre-formulation studies

4.6.1.1 Forced degradation study or stress test

Prasaprohyai ethanolic extract and Benjakul ethanolic extract were investigated to stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation with heated at 80ºC for 3 hours. After that, these extracts were tested anti-allergic effect on β-hexosaminidase from RBL-2H3 cells and anti-inflammatory effect on NO production from RAW 264.7 cells and compared with normal condition.

As the results are shown in Table 4.19 and Figure 4.26, the Prasaprohyai extracts after exposure to moisture, acid, alkaline, temperature and oxidation conditions had significant effect on β-hexosaminidase release when compared with normal condition ($p < 0.05$). These results indicated that the Prasaprohyai extract displayed unstable allergic activity under stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation. In addition, the Prasaprohyai extract showed significant effect on NO production after exposure to moisture, acid, temperature and oxidation conditions, whereas after exposure to alkaline hydrolysis had no significant difference. Thus, the Prasaprohyai extract showed stable NO activity under alkaline hydrolysis.

As the results are shown in Table 4.20 and Figure 4.27, the Benjakul ethanolic extract after exposure to moisture, acid, alkaline, temperature and oxidation conditions had no significant effect on β-hexosaminidase release when compared with normal condition. These results indicated that the Benjakul extract exhibited the stability under moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation. Moreover, only the Benjakul ethanolic extract after exposure to alkaline hydrolysis showed significant effect on NO production. These results indicated that the Benjakul extract showed stable activity under moisture, acid, temperature and oxidation conditions but unstable activity under alkaline condition for NO production.

Table 4.19 The percentage of inhibition and the IC_{50} value of Prasaprohyai ethanolic extract after stress tested on β-hexosaminidase release from RBL-2H3 cells and nitric oxide production from RAW 264.7 cells (n = 3)

Conditions	%Inhibition at various concentrations of Prasaprohyai extract (mean ± SEM, μg/ml)					IC_{50} ± SEM (μg/ml)
	1	10	30	50	100	
β-Hexosaminidase assay						
Normal	-12.86 ± 5.68	42.16 ± 4.22	-	63.53 ± 1.17	81.74 ± 2.37	11.71 ± 0.94
Moisture hydrolysis	-27.97 ± 2.68	5.53 ± 2.89	-	34.41 ± 4.30	67.19 ± 4.03	81.45 ± 5.01*
Acid hydrolysis	-9.01 ± 3.65	11.04 ± 2.41	-	37.26 ± 3.79	67.45 ± 1.07	75.75 ± 3.65*
Alkaline hydrolysis	-18.59 ± 7.33	3.65 ± 4.99	-	30.77 ± 1.28	51.00 ± 0.71	98.26 ± 1.34*
Temperature degradation	-12.40 ± 15.69	16.53 ± 1.76	-	45.25 ± 3.37	62.64 ± 2.10	61.59 ± 6.50*
Oxidation	-21.84 ± 4.82	0.07 ± 2.29	-	31.45 ± 2.26	59.11 ± 2.85	86.25 ± 4.56*
Nitric oxide assay						
Normal	-7.92 ± 6.22	5.08 ± 2.11	31.96 ± 1.98	50.21 ± 1.52	89.61 ± 5.20	49.95 ± 1.89
Moisture hydrolysis	-9.21 ± 1.03	-4.23 ± 1.74	9.97 ± 3.76	24.70 ± 4.59	54.30 ± 3.63	91.38 ± 7.45*
Acid hydrolysis	-7.20 ± 2.04	1.16 ± 0.73	16.68 ± 2.34	30.57 ± 4.16	60.56 ± 3.24	80.88 ± 6.95*
Alkaline hydrolysis	-7.75 ± 1.43	4.70 ± 1.76	31.63 ± 0.51	59.10 ± 3.79	91.19 ± 2.05	43.49 ± 2.19
Temperature degradation	-12.46 ± 4.70	-8.41 ± 3.69	-3.57 ± 2.80	3.42 ± 1.72	18.01 ± 1.86	> 100
Oxidation	-15.73 ± 2.91	-1.48 ± 3.73	13.42 ± 3.69	37.04 ± 4.09	90.66 ± 3.55[a]	Toxic

(-) means not tested; [a] Cytotoxicity was observed; * Significant difference at the level $p < 0.05$ compared with normal condition

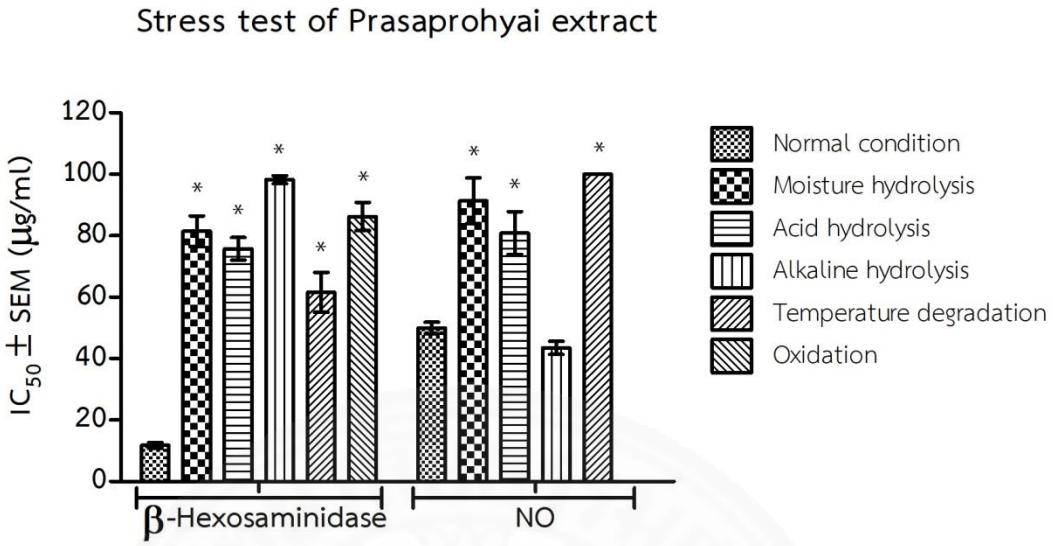


Figure 4.26 Effects of Prasaprohyai extracts after forced degradation tested on β-hexosaminidase release from RBL-2H3 cells and NO production from RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with normal condition.

Table 4.20 The percentage of inhibition and the IC_{50} value of Benjakul ethanolic extract after stress tested on β-hexosaminidase release from RBL-2H3 cells and nitric oxide production from RAW 264.7 cells (n = 3)

Conditions	%Inhibition at various concentrations of Benjakul extract (mean ± SEM, μg/ml)						IC_{50} ± SEM (μg/ml)
	1	10	20	30	50	100	
β-Hexosaminidase assay							
Normal	-10.67 ± 7.82	24.03 ± 0.64	-	-	53.94 ± 0.38	74.97 ± 0.93	22.86 ± 3.21
Moisture hydrolysis	-17.79 ± 1.90	11.11 ± 2.97	-	-	57.44 ± 1.94	93.48 ± 0.73	35.49 ± 5.95
Acid hydrolysis	-16.80 ± 8.34	23.05 ± 1.60	-	-	63.91 ± 4.91	93.52 ± 1.85	19.92 ± 1.04
Alkaline hydrolysis	-16.86 ± 11.41	26.71 ± 4.56	-	-	64.10 ± 7.65	92.23 ± 1.63	18.46 ± 2.93
Temperature degradation	-27.94 ± 6.73	20.59 ± 1.80	-	-	58.41 ± 9.93	88.29 ± 4.17	18.95 ± 2.46
Oxidation	-27.77 ± 6.22	17.93 ± 3.11	-	-	59.74 ± 0.90	95.09 ± 0.32	22.21 ± 4.22
Nitric oxide assay							
Normal	3.33 ± 1.82	34.52 ± 10.00	57.62 ± 5.54	81.26 ± 3.29	-	-	15.77 ± 3.63
Moisture hydrolysis	-1.92 ± 4.88	22.06 ± 3.17	-	74.93 ± 10.40	94.73 ± 2.16	96.60 ± 0.24[a]	20.62 ± 2.56
Acid hydrolysis	-8.63 ± 3.96	24.64 ± 9.10	-	77.29 ± 10.18	95.00 ± 2.04	95.70 ± 0.50[a]	18.94 ± 4.00
Alkaline hydrolysis	-11.39 ± 1.15	5.22 ± 3.18	-	24.36 ± 7.62	49.53 ± 7.94	84.24 ± 5.12	50.94 ± 6.59*
Temperature degradation	0.28 ± 1.64	34.27 ± 3.14	61.91 ± 7.99	81.86 ± 7.34	96.30 ± 1.49[a]	95.79 ± 1.25[a]	16.34 ± 2.89
Oxidation	-3.34 ± 2.89	25.86 ± 3.97	-	78.85 ± 4.55	96.39 ± 0.79	97.53 ± 0.23[a]	18.06 ± 2.01

(-) means not tested; [a] Cytotoxicity was observed; * Significant difference at the level $p < 0.05$ compared with normal condition

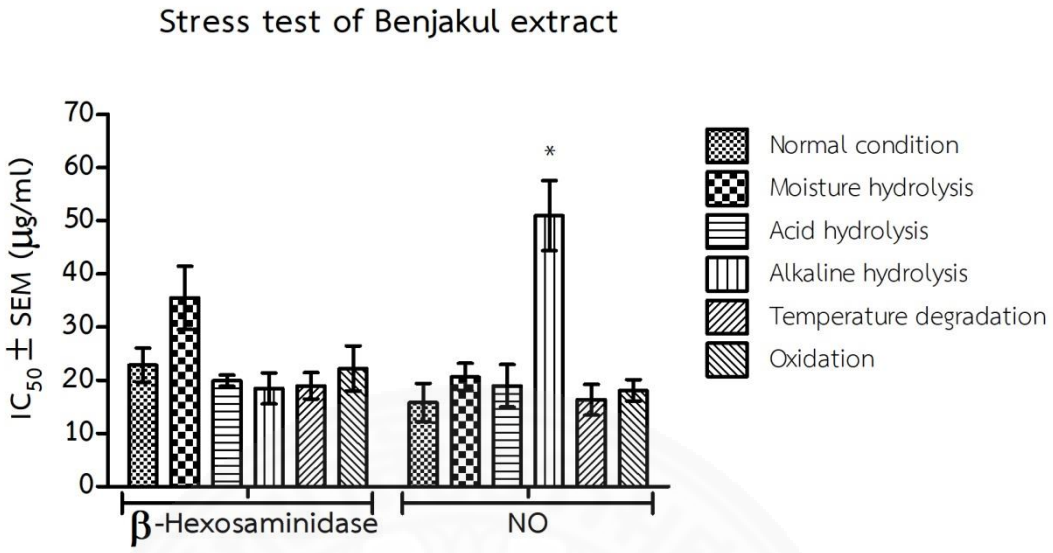


Figure 4.27 Effects of Benjakul extracts after forced degradation tested on β-hexosaminidase release from RBL-2H3 cells and NO production from RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with normal condition.

4.6.1.2 Stability testing under accelerated condition

(1) Prasaprohyai ethanolic extract

The crude ethanolic extract of Prasaprohyai was investigated under accelerated condition at 40 ± 2°C and 75 ± 5% RH for 6 months. After 6 months, the biological activity characteristics as inhibitory effects on β-hexosaminidase release and NO production of Prasaprohyai ethanolic extracts in various storage periods (0, 15, 30, 45, 60, 90, 120, 150 and 180 days) were tested.

From Table 4.21 and Figure 4.28, these results indicated that the inhibitory activities of Prasaprohyai ethanolic extract after keeping under accelerated conditions on both the release of β-hexosaminidase and the production of nitric oxide had no change after keeping under accelerated conditions when compared with day 0.

Table 4.21 The IC_{50} value of the stability of Prasaprohyai ethanolic extract on β-hexosaminidase release and NO production under accelerated conditions at 40ºC and 75% RH for 6 months at the various storage times (n = 3)

Periods	IC_{50} ± SEM (μg/ml)	
	β-Hexosaminidase release	**NO production**
Day 0	11.71 ± 0.94	49.95 ± 1.89
Day 15	17.23 ± 1.26	50.97 ± 5.46
Day 30	11.44 ± 1.89	49.46 ± 4.62
Day 45	12.09 ± 2.51	47.76 ± 4.15
Day 60	14.98 ± 1.30	47.32 ± 2.68
Day 90	13.70 ± 1.17	45.35 ± 4.32
Day 120	22.09 ± 6.67	44.17 ± 3.44
Day 150	14.33 ± 2.94	48.01 ± 6.03
Day 180	14.35 ± 1.94	46.60 ± 8.10

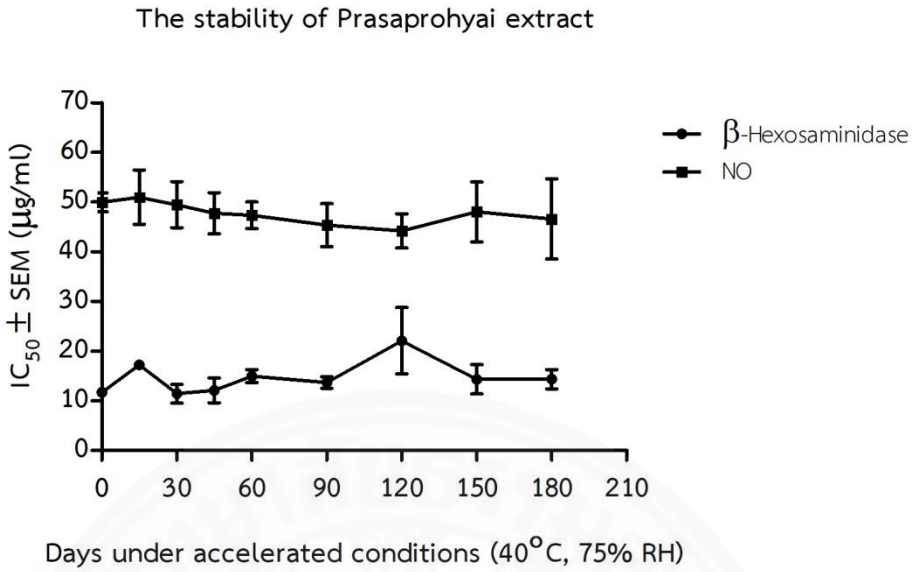


Figure 4.28 The stability of Prasaprohyai ethanolic extract on β-hexosaminidase release and NO production under accelerated conditions at 40ºC and 75% RH for 6 months at the various storage times. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3).

(2) Benjakul ethanolic extract

The crude ethanolic extract of Benjakul was investigated under accelerated condition at 40 ± 2ºC and 75 ± 5% RH for 6 months. After 6 months, the biological activity characteristics as inhibitory effects on β-hexosaminidase release and NO production of Benjakul ethanolic extracts in various storage time periods (0, 15, 30, 45, 60, 90, 120, 150 and 180 days) were determined (Table 4.22 and Figure 4.29). In particular, the changes of amount of piperine, myristicin, 6-shogaol and plumbagin, active compounds, in the Benjakul ethanolic extract were evaluated by HPLC method (Table 4.23 and 4.24 and Figure 4.30 to 4.32).

From Table 4.22 and Figure 4.29, these results indicated that crude ethanolic extract of Benjakul after keeping under accelerated conditions at days 120, 150 and 180 had significant change on anti-allergic activity *via*

β-hexosaminidase release when compared with day 0 ($p < 0.05$). At the same time, these extracts exhibited no significant change on anti-inflammatory activity *via* nitric oxide production when compared with day 0.

From Table 4.24 and Figure 4.32, the results displayed that the percentage of content of some pure compounds, such as piperine, myristicine, 6-shogaol and plumbagin in Benjakul ethanolic extract after keeping under accelerated conditions were significantly different with day 0 ($p < 0.05$). Especially, the amount of plumbagin in Benjakul ethanolic extract showed rapidly reduced change at day 90, 120, 150 and 180.

Table 4.22 The IC_{50} value of the stability of Benjakul ethanolic extract on β-hexosaminidase release and NO production under accelerated conditions at 40ºC and 75% RH for 6 months at the various storage times (n = 3)

Periods	IC_{50} ± SEM (μg/ml)	
	β-Hexosaminidase release	NO production
Day 0	20.20 ± 3.24	15.77 ± 3.63
Day15	16.96 ± 2.81	25.83 ± 1.27
Day 30	15.15 ± 7.77	18.68 ± 1.93
Day 45	11.59 ± 0.53	22.03 ± 2.94
Day 60	10.31 ± 0.23	20.69 ± 1.82
Day 90	14.23 ± 1.46	23.93 ± 1.92
Day 120	75.63 ± 7.30*	21.32 ± 3.30
Day 150	90.41 ± 4.92*	20.66 ± 2.34
Day 180	76.95 ± 3.97*	22.25 ± 2.53

* Significant difference at the *p*-value < 0.05 compared with day 0

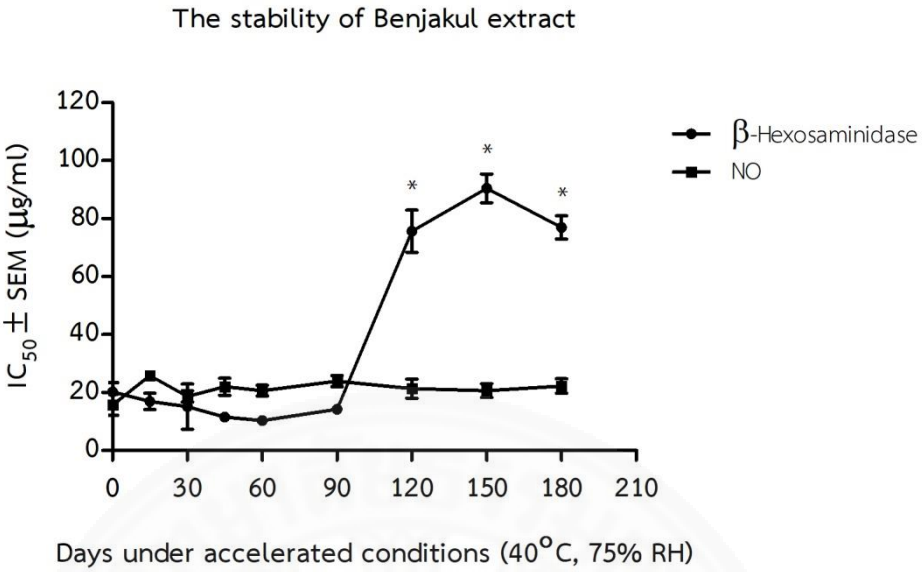


Figure 4.29 The stability of Benjakul ethanolic extract on β-hexosaminidase release and NO production under accelerated conditions at 40ºC and 75% RH for 6 months at the various storage times. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are expressed as the IC_{50} ± SEM values (µg/ml) (n = 3). * Significant difference ($p < 0.05$) compared with day 0.

Table 4.23 HPLC conditions for analysis of piperine, myristicin, 6-shogaol and plumbagin in the Benjakul ethanolic extract

Operating parameters	Conditions
Stationary phase	Phenomenex Luna 5 μ C18(2) 100A (250 × 4.6 mm, 5 μm)
Mobile phase	water: acetonitrile with gradient elution as follows: 0 min, 65:35; 30 min, 50:50; 50 min, 5:95; 65 min, 5:95; 65.10 min, 65:35; 70 min, 65:35
Flow rate	1.0 ml/min
Wavelength	UV 256 nm for piperine and myristicin UV 215 nm for 6-shogaol Vis 415 nm for plumbagin
Injection volume	20 μl

The peak of piperine, myristicin, 6-shogaol and plumbagin in HPLC chromatogram was separated from other compounds in the Benjakul ethanolic extract by using chromatographic condition that described above. The results of HPLC chromatogram of mixture standards including piperine, myristicin, 6-shogaol and plumbagin are shown in Figure 4.30. From Figure 4.32, the results showed that piperine is a major compound from the Benjakul ethanolic extract, which retention time was 27.71 min.

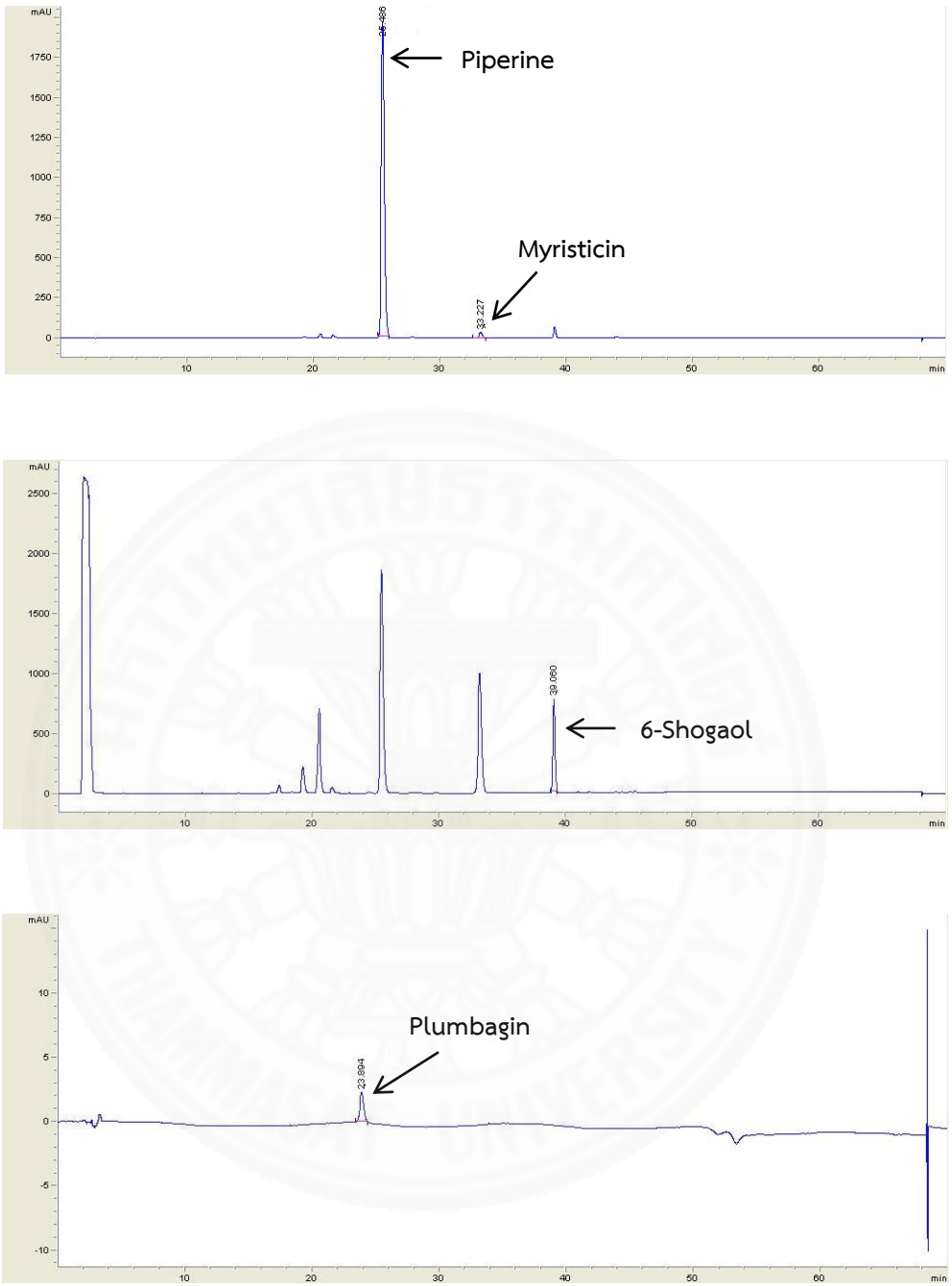


Figure 4.30 HPLC chromatogram obtained from the mixture of piperine, myristicin, 6-shogaol and plumbagin standards; chromatogram of piperine and myristicin standards at UV 256 nm (A), chromatogram of 6-shogaol standard at UV 215 nm (B) and chromatogram of plumbagin standard at Vis 415 nm (C).

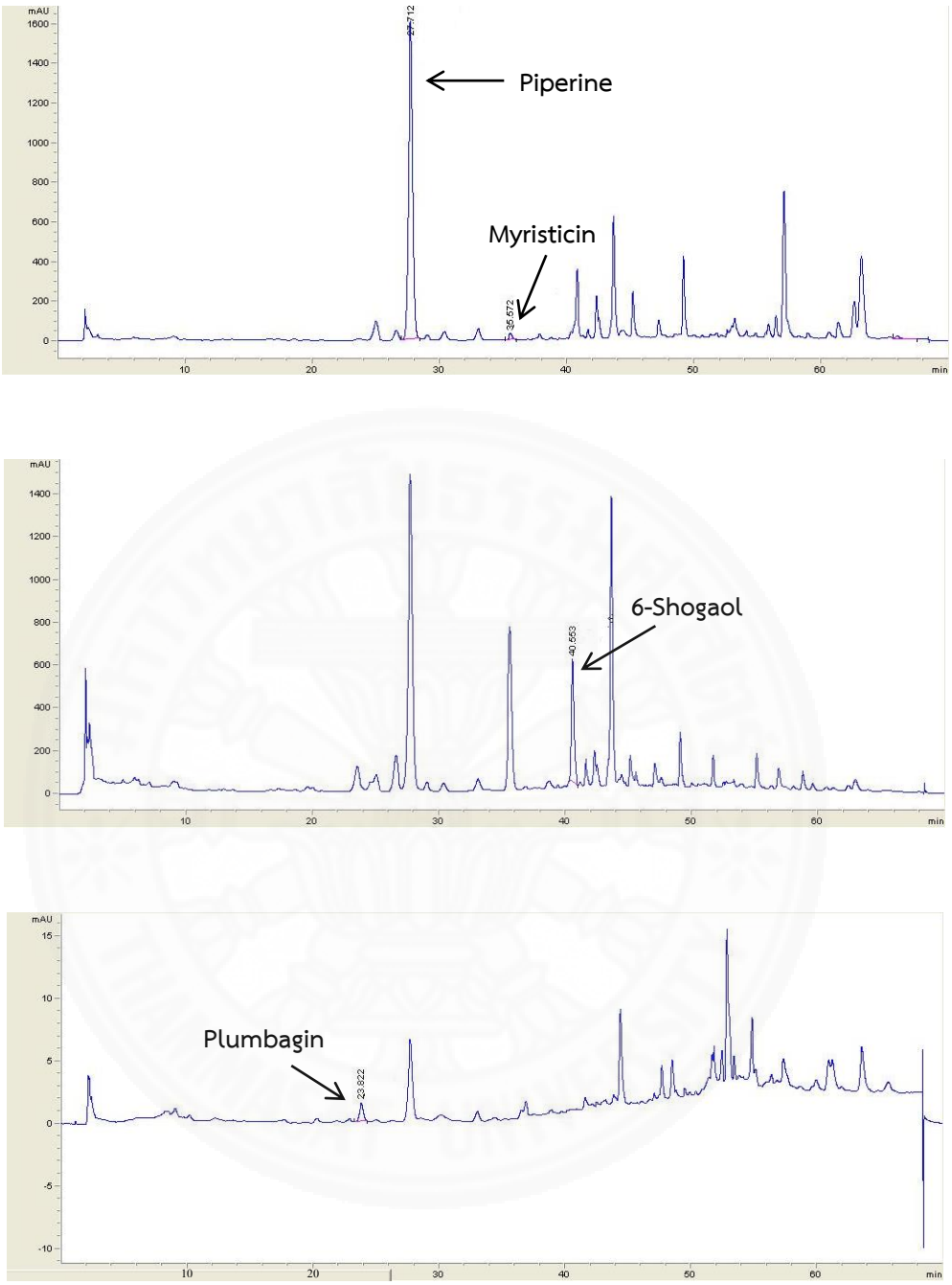


Figure 4.31 HPLC chromatogram obtained from the ethanolic extract of Benjakul; chromatogram of piperine and myristicin at UV 256 nm (A), chromatogram of 6-shogaol at UV 215 nm (B) and chromatogram of plumbagin at Vis 415 nm (C).

Table 4.24 Piperine, myristicin, 6-shogaol and plumbagin contents in the Benjakul ethanolic extract after accelerated storage conditions (40°C, 75% RH) for 6 months at the various storage times (n = 2)

Periods	Piperine[a]		Myristicin[b]		6-Shogaol[c]		Plumbagin[d]	
	Content (mg/g)	% Content	Content (mg/g)	% Content	Content (mg/g)	% Content	Content (mg/g)	% Content
Day 0	170.00 ± 0.23	100.00 ± 0.00	12.18 ± 0.53	100.00 ± 0.00	16.68 ± 0.75	100.00 ± 0.00	0.32 ± 0.00	100.00 ± 0.00
Day 15	162.92 ± 2.97	95.84 ± 1.90	12.73 ± 0.43	104.82 ± 8.05	16.28 ± 0.77	97.55 ± 0.21	0.22 ± 0.01*	67.69 ± 1.53*
Day 30	242.56 ± 5.43*	142.69 ± 3.39*	18.91 ± 0.25*	155.59 ± 8.77*	23.90 ± 0.79*	143.31 ± 1.71*	0.52 ± 0.02*	162.22 ± 4.80*
Day 45	215.46 ± 6.38*	126.75 ± 3.93*	16.03 ± 1.06*	132.17 ± 14.45*	21.95 ± 0.87*	131.58 ± 0.70*	0.27 ± 0.01	85.28 ± 3.54
Day 60	237.63 ± 16.65*	139.80 ± 9.99*	18.40 ± 0.95*	151.66 ± 14.35*	24.84 ± 1.45*	148.76 ± 1.99*	0.24 ± 0.02*	74.75 ± 6.69*
Day 90	133.74 ± 0.12	78.67 ± 0.18	10.05 ± 0.25	82.68 ± 5.62	14.19 ± 0.28	85.16 ± 2.16	0.05 ± 0.00*	14.40 ± 0.22*
Day 120	228.57 ± 15.38*	134.47 ± 9.23*	17.56 ± 1.43*	144.91 ± 17.98*	25.05 ± 1.54*	150.01 ± 2.46*	0.06 ± 0.01*	19.25 ± 4.02*
Day 150	161.32 ± 1.90	94.90 ± 1.25	12.75 ± 0.01	104.82 ± 4.60	17.67 ± 0.24	106.04 ± 3.31	0.02 ± 0.00*	6.82 ± 0.63*
Day 180	195.43 ± 4.26	114.97 ± 2.66	15.39 ± 0.19	126.59 ± 7.04	22.77 ± 0.29*	136.65 ± 4.38*	0.02 ± 0.01*	6.77 ± 1.95*

[a] All data were calculated as the standard linear equation of piperine and detection at UV 256 nm (see Appendix D)

[b] All data were calculated as the standard linear equation of myristicin and detection at UV 256 nm (see Appendix D)

[c] All data were calculated as the standard linear equation of 6-shogaol and detection at UV 215 nm (see Appendix D)

[d] All data were calculated as the standard linear equation of plumbagin and detection at Vis 415 nm (see Appendix D)

* Significant difference ($p < 0.05$) compared with day 0

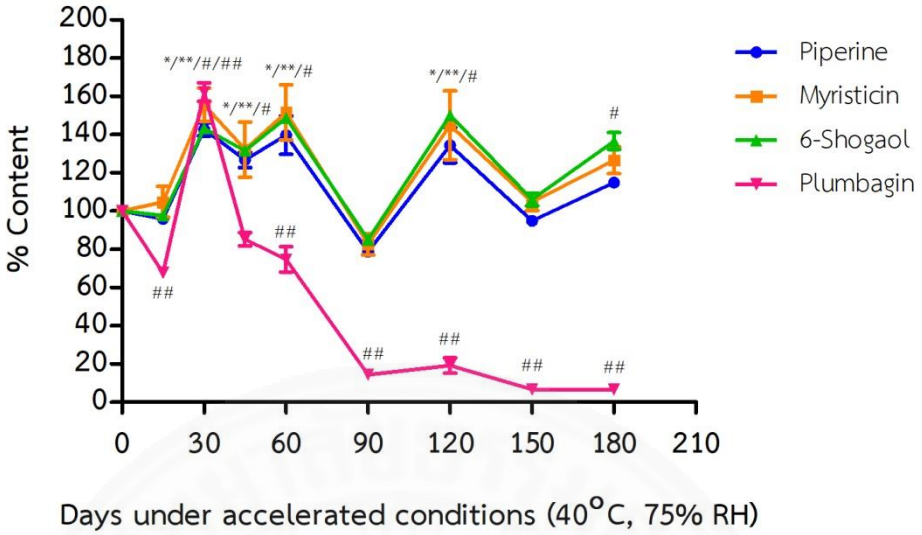


Figure 4.32 The stability of piperine, myristicin, 6-shogaol and plumbagin (% content) in the Benjakul ethanolic extract under accelerated storage conditions (40ºC, 75% RH) for 6 months. *, **, #, ## means significant difference ($p < 0.05$) for piperine, myristicin, 6-shogaol and plumbagin, respectively when compared with day 0 (n = 2).

4.6.2 Formulation of Prasaprohyai extract and Benjakul extract into capsule

The formulations of Prasaprohyai and Benjakul capsules which composed of hydrophilic silicon dioxide as a adsorbent and anti-adherent (Aerosil®), microcrystalline cellulose as a adsorbent and glidant (Avicel® PH-101 and Avicel® PH-102), lactose as a filler, talcum as a glidant and magnesium stearate as a lubricant were used in the capsule formulation. In this study, ten formulations consist of five formulations of Prasaprohyai and five formulations of Benjakul were prepared in solid dosage form as capsule containing 10% w/w of extract, Avecel® PH-101, Avecel® PH-102, lactose, Aerosil®, magnesium stearate and talcum by total weight of 500 mg per capsule (see section 3.8.2 and Table 3.5).

4.6.2.1 Uniformity of weight (Mass)

Each of 20 capsules of formulations was determined by analytical balance.

For Prasaprohyai capsule formulations, the P3 capsule formulation had weight variation not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation (Percentage deviation = 7.5) in BP 2013 (British Pharmacopoeia Commission, 2013) as shown in Table 4.25.

For Benjakul capsule formulations, the B1 and B4 capsule formulations had weight variation not more than specification in BP 2013 which stated that weight of individually 20 capsules not more than 7.5% difference from average weight of capsule as shown in Table 4.26.

4.6.2.2 Disintegration time

One capsule was placed in each of the six tubes and the basket rack was positioned in one liter of water as the disintegration medium. The apparatus was operated at 37 ± 2ºC for 30 min. These results found that all of Prasaprohyai capsule formulations and all of Benjakul capsule formulations had disintegration time within 30 minutes for complete disintegration of capsule.

Table 4.25 The weight variation and the percentage deviation of Prasaprohyai capsule formulations

No.	P1		P2.1		P2.2	
	Weight (mg)	%Deviation	Weight (mg)	%Deviation	Weight (mg)	%Deviation
1	401.6	0.2	271.2	29.0	382.4	2.1
2	395.0	1.9	401.0	-5.0	356.0	8.9
3	393.9	2.2	419.3	-9.8	360.9	7.6
4	411.8	-2.3	379.9	0.5	431.6	-10.5
5	445.8	-10.7	440.5	-15.3	404.9	-3.6
6	348.3	13.5	421.9	-10.4	406.1	-3.9
7	470.7	-16.9	448.8	-17.5	398.9	-2.1
8	399.0	0.9	255.7	33.1	418.5	-7.1
9	362.8	9.9	371.9	2.6	382.9	2.0
10	303.1	24.7	454.5	-19.0	334.4	14.4
11	435.8	-8.2	397.5	-4.1	411.4	-5.3
12	416.2	-3.4	352.8	7.6	418.9	-7.2
13	367.0	8.8	456.8	-19.6	353.4	9.5
14	400.1	0.6	313.6	17.9	433.9	-11.1
15	439.8	-9.2	396.2	-3.7	342.2	12.4
16	367.6	8.7	459.4	-20.3	466.7	-19.5
17	412.1	-2.4	330.7	13.4	382.4	2.1
18	440.7	-9.5	340.0	11.0	358.2	8.3
19	387.1	3.8	263.5	31.0	359.4	8.0
20	453.9	-12.7	465.2	-21.8	410.3	-5.0
Mean	**402.6**	**-**	**382.0**	**-**	**390.7**	**-**

Table 4.25 The weight variation and the percentage deviation of Prasaprohyai capsule formulations (Cont.)

No.	P3		P4	
	Weight (mg)	%Deviation	Weight (mg)	%Deviation
1	448.2	3.7	378.2	12.7
2	443.3	4.7	405.7	6.4
3	449.9	3.3	473.8	-9.3
4	457.6	1.7	444.2	-2.5
5	479.1	-2.9	412.4	4.8
6	462.6	0.6	474.7	-9.6
7	486.8	-4.6	396.3	8.5
8	462.7	0.6	451.9	-4.3
9	469.8	-0.9	386.5	10.8
10	469.5	-0.9	387.0	10.7
11	467.1	-0.4	368.5	15.0
12	461.9	0.8	479.2	-10.6
13	492.6	-5.8	439.1	-1.3
14	499.2	-7.3	484.2	-11.7
15	441.0	5.2	484.2	-11.7
16	469.8	-0.9	468.9	-8.2
17	429.1	7.8	460.9	-6.4
18	459.7	1.2	337.4	22.1
19	462.5	0.6	469.4	-8.3
20	496.0	-6.6	463.1	-6.9
Mean	**465.4**	**-**	**433.3**	**-**

Table 4.26 The weight variation and the percentage deviation of Benjakul capsule formulations

No.	B1		B2.1		B2.2	
	Weight (mg)	%Deviation	Weight (mg)	%Deviation	Weight (mg)	%Deviation
1	446.0	-4.7	467.6	-4.3	420.8	5.9
2	425.2	0.1	403.4	10.0	393.4	12.0
3	450.8	-5.9	530.6	-18.4	407.0	9.0
4	456.5	-7.2	454.6	-1.4	417.3	6.7
5	407.7	4.3	435.6	2.8	427.5	4.4
6	415.0	2.5	446.1	0.5	465.5	-4.1
7	412.2	3.2	459.0	-2.4	470.2	-5.2
8	445.8	-4.7	494.5	-10.3	468.3	-4.7
9	428.9	-0.7	414.3	7.6	421.8	5.7
10	435.9	-2.4	376.0	16.1	430.3	3.8
11	403.8	5.2	520.9	-16.2	465.4	-4.1
12	404.3	5.0	426.4	4.9	493.4	-10.4
13	437.4	-2.7	420.1	6.3	404.1	9.6
14	420.6	1.2	494.7	-10.4	442.2	1.1
15	418.0	1.8	452.0	-0.8	459.7	-2.8
16	427.1	-0.3	351.8	21.5	475.0	-6.2
17	442.7	-4.0	473.7	-5.7	506.6	-13.3
18	415.6	2.4	487.9	-8.8	488.6	-9.3
19	409.8	3.8	451.8	-0.8	430.4	3.7
20	411.9	3.3	404.2	9.8	455.3	-1.8
Mean	**425.8**	**-**	**448.3**	**-**	**447.1**	**-**

Table 4.26 The weight variation and the percentage deviation of Benjakul capsule formulations (Cont.)

No.	B3		B4	
	Weight (mg)	%Deviation	Weight (mg)	%Deviation
1	478.3	-5.2	401.1	2.6
2	468.8	-3.1	429.7	-4.4
3	460.2	-1.2	404.0	1.9
4	474.9	-4.4	391.5	4.9
5	471.1	-3.6	416.7	-1.2
6	455.9	-0.2	414.1	-0.6
7	399.0	12.3	429.4	-4.3
8	443.5	2.5	414.2	-0.6
9	465.0	-2.2	405.1	1.6
10	474.4	-4.3	408.2	0.9
11	482.2	-6.0	418.8	-1.7
12	423.2	6.9	410.6	0.3
13	470.9	-3.5	413.0	-0.3
14	410.8	9.7	392.5	4.7
15	457.8	-0.7	432.9	-5.1
16	477.0	-4.9	403.8	1.9
17	437.8	3.7	394.8	4.1
18	479.9	-5.5	430.9	-4.7
19	447.2	1.7	398.6	3.2
20	418.8	7.9	424.0	-3.0
Mean	**454.8**	**-**	**411.7**	**-**

From these results, the **P3** formulation which contained 35% w/w Avecel® PH-101, 50% w/w lactose, 1% w/w Aerosil®, 1% w/w magnesium stearate, and 3% w/w talcum and the **B1** formulation which contained 85% w/w Avecel® PH-101, 1% w/w Aerosil®, 1% w/w magnesium stearate, and 3% w/w talcum and the last one, the **B4** formulation which contained 42.5% w/w Avecel® PH-101, 42.5% w/w Avecel® PH-102, 1% w/w Aerosil®, 1% w/w magnesium stearate, and 3% w/w talcum revealed an optimum capsule formulations. As the optimum capsule formulations both of Prasaprohyai and Benjakul showed good weight variation and disintegration time of the capsules in accordance with the BP 2013 requirements. In consequence, the stability studies of the P3, B1 and B4 capsule formulations were performed according to ICH 2003 on stability testing of new drug substances and products (International Conference on Harmonization, 2003).

4.6.2.3 Stability study

(1) Moisture content of the P3, B1 and B4-capsule formulations

The physical characteristic changes presented as moisture content of the P3, B1 and B4-capsule formulations were tested under accelerated storage conditions at 40 ± 2ºC and 75 ± 5% RH for 6 months.

As the results are shown in Table 4.27 and Figure 4.33, the Prasaprohyai (P3)-capsule formulation and the Benjakul (B1 and B4)-capsule formulations were not change on high moisture content when compared with day 0.

Table 4.27 The percentage of loss on drying of the P3, B1 and B4-capsule formulations under accelerated storage conditions at 40ºC and 75% RH for 6 months (n = 3)

Periods	% Loss on drying (mean ± SEM)		
	P3	**B1**	**B4**
Day 0	6.14 ± 1.41	5.57 ± 0.55	5.44 ± 0.84
Day 15	4.95 ± 0.22	5.22 ± 0.48	5.20 ± 0.24
Day 30	5.43 ± 0.28	5.24 ± 0.27	5.10 ± 0.62
Day 45	6.37 ± 1.23	6.28 ± 0.63	5.44 ± 0.79
Day 60	6.00 ± 0.36	4.87 ± 0.60	7.32 ± 1.32
Day 90	4.56 ± 0.23	4.72 ± 0.52	4.21 ± 1.24
Day 120	6.71 ± 0.61	4.67 ± 0.68	4.96 ± 0.34
Day 150	5.51 ± 0.64	5.19 ± 0.36	4.09 ± 0.35
Day 180	4.53 ± 1.03	4.73 ± 0.19	5.36 ± 0.33

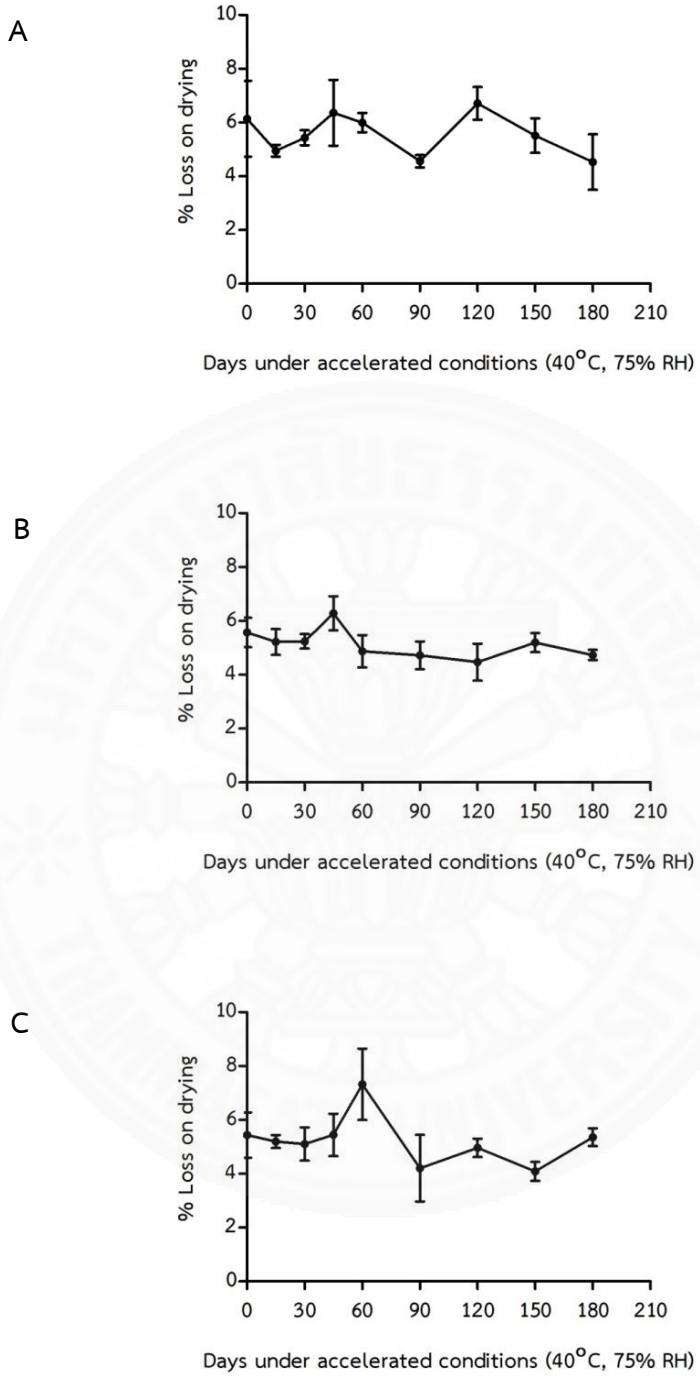


Figure 4.33 The percentage of loss on drying of the P3 (A), B1 (B) and B4 (C)-capsule formulations under accelerated storage conditions (40ºC, 75% RH) at the various storage times (n = 3).

(2) Anti-allergic activity change of the P3, B1 and B4-capsule formulations

The biological activity characteristic changes presented as anti-allergic activity of the P3, B1 and B4-capsule formulations were studied under accelerated storage conditions at 40 ± 2ºC and 75 ± 5% RH for 6 months.

From Table 4.28 and Figure 4.34, the results revealed that the P3-capsule formulation from Prasaprohyai extract at day 150 and day 180 showed significantly increased on the release of β-hexosaminidase in RBL-2H3 cells when compared with day 0 ($p < 0.05$). On the other hand, the B1 and B4-capsule formulations from Benjakul extract at different storage times showed no effect on the release of β-hexosaminidase.

Table 4.28 The IC_{50} values of the P3, B1 and B4-capsule formulations under accelerated storage conditions at 40ºC and 75% RH for 6 months on the release of β-hexosaminidase from RBL-2H3 cells (n = 2)

Periods	IC_{50} ± SEM (μg/ml)		
	P3	B1	B4
Day 0	15.14 ± 4.57	34.07 ± 19.94	21.23 ± 3.55
Day 15	9.79 ± 0.34	21.56 ± 9.48	31.88 ± 18.87
Day 30	11.27 ± 0.16	27.41 ± 13.09	18.48 ± 4.08
Day 45	17.75 ± 2.46	44.36 ± 21.46	20.68 ± 5.69
Day 60	15.45 ± 0.51	21.51 ± 6.89	23.62 ± 10.85
Day 90	18.24 ± 4.57	18.71 ± 0.23	14.95 ± 4.00
Day 120	17.67 ± 3.59	19.34 ± 7.02	23.15 ± 8.60
Day 150	32.72 ± 2.42*	23.84 ± 10.16	13.99 ± 0.20
Day 180	33.72 ± 6.93*	22.38 ± 0.64	9.79 ± 1.17

* Significant difference ($p < 0.05$) when compared with day 0

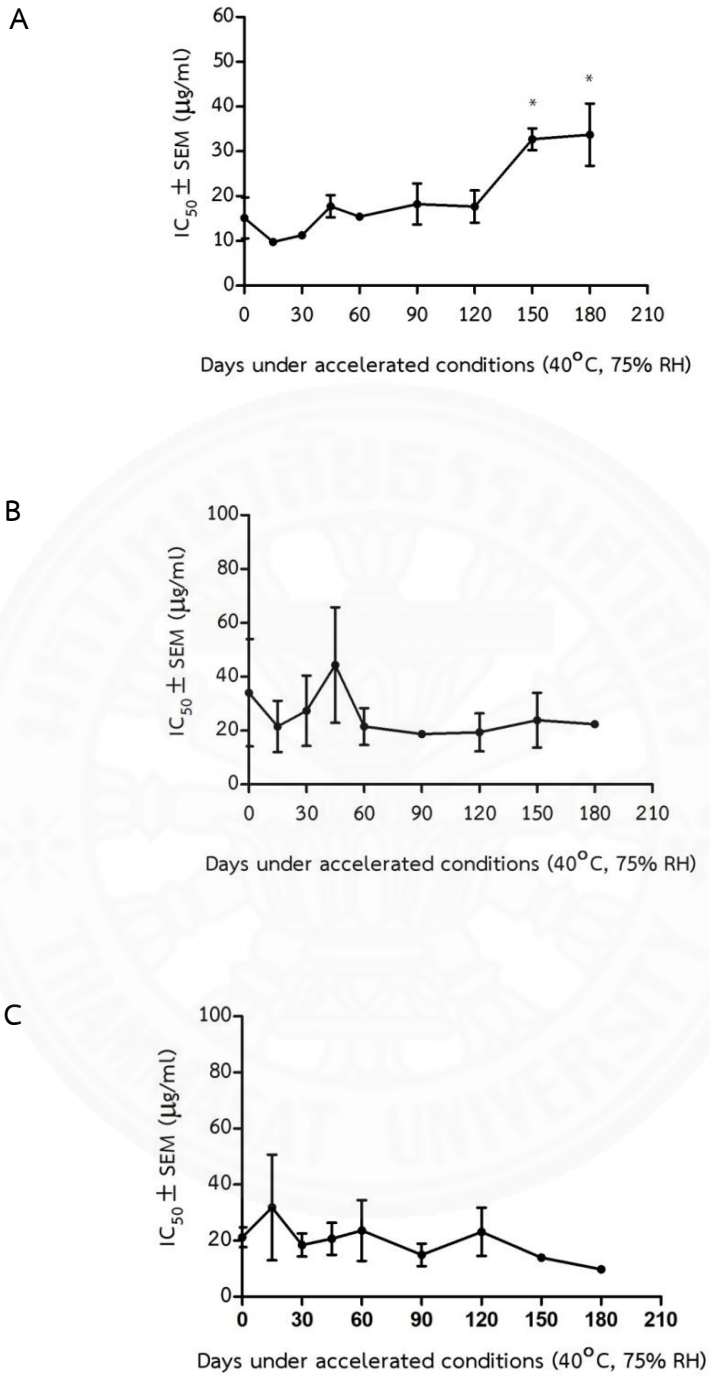


Figure 4.34 The stability of anti-allergic activity on β-hexosaminidase release of the P3 (A), B1 (B) and B4 (C)-capsule formulations under accelerated storage conditions (40°C, 75% RH) at the various storage times (n = 2).

(3) Anti-inflammatory activity change of the P3, B1 and B4-capsule formulations

The biological activity characteristic changes exhibited as anti-inflammatory effect on NO production of the P3, B1 and B4-capsule formulations were tested under accelerated storage conditions at 40 ± 2ºC and 75 ± 5% RH for 6 months.

The results showed that the anti-inflammatory effect on NO production of Prasaprohyai (P3) and Benjakul (B1 and B4)-capsule formulations at different storage times were not significantly different when compared with day 0.

Table 4.29 The IC_{50} values of the P3, B1 and B4-capsule formulations under accelerated storage conditions at 40ºC and 75% RH for 6 months on the release of nitric oxide from RAW 264.7 cells (n = 3)

Periods	IC_{50} ± SEM (μg/ml)		
	P3	B1	B4
Day 0	41.22 ± 6.97	65.13 ± 7.81	48.70 ± 2.56
Day 15	47.98 ± 2.54	51.27 ± 6.39	58.14 ± 3.11
Day 30	50.66 ± 1.64	56.54 ± 6.66	55.29 ± 2.99
Day 45	56.86 ± 6.21	55.78 ± 4.45	54.11 ± 0.64
Day 60	54.33 ± 4.51	52.55 ± 3.89	55.11 ± 2.58
Day 90	49.55 ± 4.68	44.72 ± 3.11	55.89 ± 2.60
Day 120	53.83 ± 5.77	47.56 ± 4.17	52.60 ± 1.55
Day 150	47.99 ± 6.33	45.81 ± 2.47	57.41 ± 2.26
Day 180	52.85 ± 8.12	49.28 ± 6.57	51.25 ± 2.32

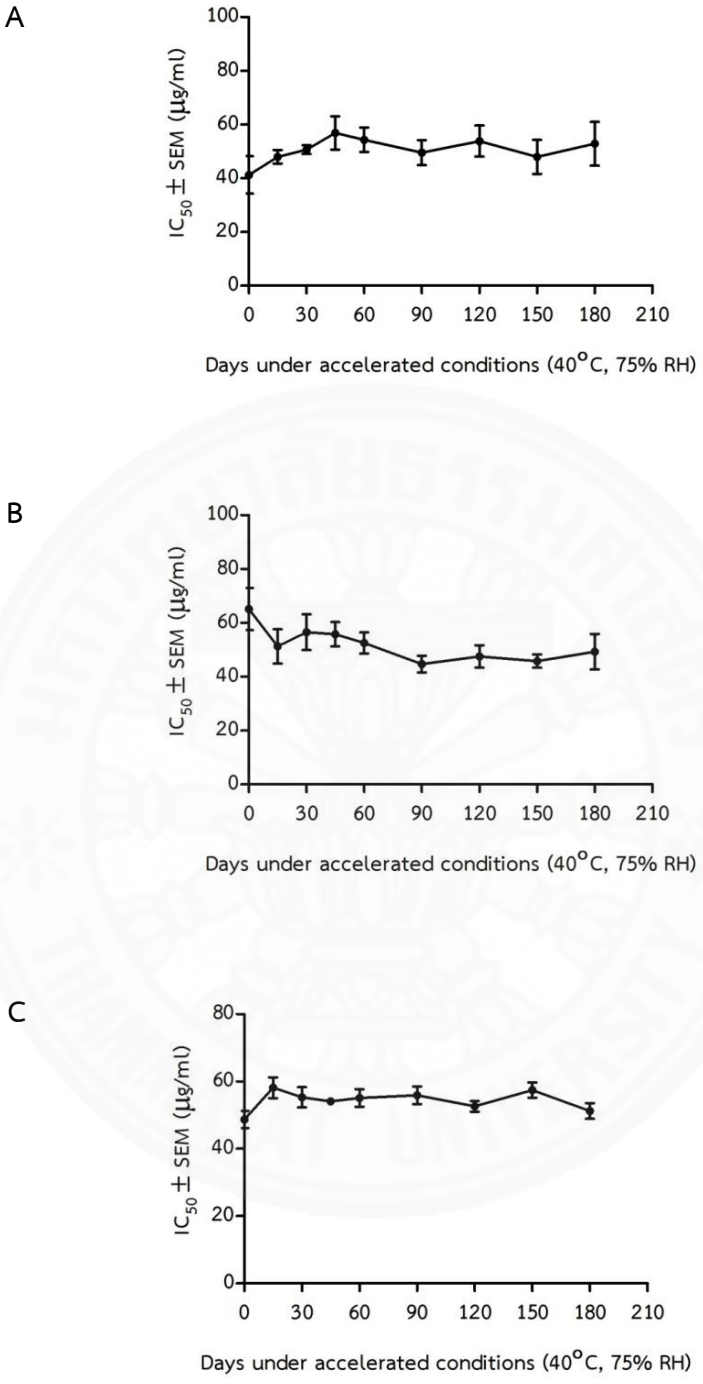


Figure 4.35 The stability of anti-inflammatory activity on NO production of the P3 (A), B1 (B) and B4 (C)-capsule formulations under accelerated storage conditions (40ºC, 75% RH) at the various storage times (n = 3).

4.7 Discussion

Prasaprohyai and Benjakul remedies are polyherbal medicine remedy derived from Thai traditional knowledge of folk doctors. For Prasaprohyai remedy, it has long been used to treat patients with fever and cold (National Drug Committee, 2013). Several studies have been done on Prasaprohyai remedy to find some specific potentials, i.e. anti-allergic activity (Makchuchit, 2010), anti-inflammatory activity (Makchuchit, 2010; Plengsuriyakarn et al., 2012), antimalarial activity (Thiengsusuk et al., 2013), etc. However, there has been no report on Prasaprohyai of the *in vivo* anti-allergic activity.

For Benjakul remedy, it has long been used for balanced health in patients and healthy people (Itharat & Sakpakdeejaroen, 2010; National Drug Committee, 2013). In addition, Benjakul remedy have been reported on antimalarial (Thiengsusuk et al., 2013), anti-inflammation (Burodom & Itharat, 2013), antimicrobial (Kondo et al., 2010), genotoxicity (Ratanavalachai et al., 2012) and cytotoxicity (Mahavorasirikul et al., 2010; Rattarom et al., 2010, 2014; Sakpakdeejaroen & Itharat, 2009). However, *in vitro* and *in vivo* anti-allergic and *in vitro* anti-inflammatory effects of Benjakul, its components and its pure compounds have not been reported.

Thus, the present study aimed to focus on both *in vitro* and *in vivo* anti-allergic and *in vitro* anti-inflammatory activities of Benjakul remedy and also focused on *in vivo* anti-allergic activity of Prasaprohyai remedy.

4.7.1 β-Hexosaminidase release assay in RBL-2H3 cells

Mast cells and basophils are granule-containing secretory cells in the immune system. They are important effector cells in both early- and late-phase reaction of allergy as well as type I hypersensitivity. Activation of mast cells and basophils can be initiated by allergen cross-linking of FcεRI, a high-affinity IgE receptor, and then these cells degranulate to release various preformed mediators like β-hexosaminidase, histamine and serotonin (Abramson, Licht, & Pecht, 2006). Therefore, the release of β-hexosaminidase from activated RBL-2H3 cells is commonly measured as an indicator of degranulation. This assay is a convenient tool

for monitoring the capacity of potential drugs to block mast cells and basophils activation and degranulation (Passante, Ehrhardt, Sheridan, & Frankish, 2009; Passante & Frankish, 2009).

Benjakul is adaptogenic remedy in Thailand and also it is used before changing weather such as change from winter to summer, etc. The weather changing can make people be sick than usual, especially allergy. Furthermore, Benjakul remedy was proven about allergic assay and it showed the greatest anti-allergic activity (IC_{50} = 12.69 μg/ml) and it has higher anti-allergic activity than each plant and chlorpheniramine (IC_{50} = 17.98 μg/ml) as positive control. This result indicated that it is the effect of combined synergistic effect of five plants which are constituents of this remedy. Additionally, five plant extracts as constituent of Benjakul remedy also showed higher anti-allergic effect than chlorpheniramine except *P. sarmentosum*. This discovery is the first report on anti-allergic effect of Benjakul ethanolic extract. Some individual plant components of Benjakul remedy such as *P. interruptum*, *P. longum*, *P. sarmentosum* and *P. indica* are also the first report.

Z. officinale was the only plant in Benjakul remedy reported on anti-allergic effect. Its ethanolic extract inhibited β-hexosaminidase release from RBL-2H3 cells with the IC_{50} value of 40.3 μg/ml (Tewtrakul & Subhadhirasakul, 2007). However, *Z. officinale* ethanolic extract showed stronger anti-allergic activity with the IC_{50} value of 12.93 μg/ml better than in the previous study (Tewtrakul & Subhadhirasakul, 2007). It depends on several factors including seasons of plant collection, geographical place of collection and the method of preparation of the extracts. Previous report, 2% ginger diet suppressed allergic effect on ovalbumin (OVA)-induced allergic rhinitis in animal model (Kawamoto et al., 2016). Moreover, both ethanol extract (500 mg/kg) and water extract (700 mg/kg) of ginger rhizomes showed inhibitory effect on Th2-mediated immune responses such as IL-4 and IL-5 cytokines in OVA-induced allergic asthma in mouse model (Khan et al., 2015). In addition, the aqueous extract of ginger at concentration of 360 mg/kg inhibited Th2-mediated immune responses in allergen-induced airway inflammation (Ahui et al.,

2008). Thus, ginger has the highest anti-allergy compared with each individual plant of Benjakul, and 6-shogaol which is an ingredient in ginger also showed the highest anti-allegic activity. These all results support that Benjakul remedy can be used as alternative drug for anti-allergy. However, some factors can affect the amount of active compound. Thus, the adequate of quality of herbal should be concerned. Although, 6-gingerol, 6-shogaol, piperine and β-sitostenone, pure compounds isolated from Benjakul, had ever been reported on anti-allergic ability (Chen et al., 2009; Huang et al., 2014; Wu, Chan, Shi, & Jhong, 2017) but plumbagin, methyl piperate and myristicin were reported for first time for inhibitory activity on β-hexosaminidase release. Both 6-gingerol and 6-shogaol, two major constituents of ginger, exhibited the potent inhibitory activity against β-hexosaminidase release in RBL-2H3 cells (Chen et al., 2009). In another study, 6-gingerol has been inhibited both OVA-induced allergic rhinitis (Kawamoto et al., 2016) and OVA-induced airway inflammation (Ahui et al., 2008). In addition, piperine that isolated from *F. piperis* showed inhibitory effect on the expression of cytokines such as IL-4, IL-13, and TNF-α, and suppressed histamine and β-hexosaminidase release from RBL-2H3 cells (Huang et al., 2014). Recent study showed that β-sitostenone isolated from *C. militaris* inhibited β-hexosaminidase secretion from activated mast cells (Wu et al., 2017). However, Benjakul's pure compounds as well as 6-shogaol, plumbagin, methyl piperate and piperine elicited higher anti-allergic activity than chlorpheniramine. Thus, these compounds of Benjakul ethanolic extract have synergistic effect and promote the Benjakul to inhibit allergic reactions. Moreover, plumbagin and methyl piperate are first reported on high anti-allergic activity. These results suggested that 6-shogaol, plumbagin, piperine and methyl piperate should be used as markers for analysis and quality control of the Benjakul ethanolic extract.

Thereby, the result from Figure 4.1 showed that release of β-hexosaminidase from RBL-2H3 cells was significantly inhibited in a dose-dependent manner by treatment with plumbagin and 6-shogaol compared to a positive control (CPM). The reduction in β-hexosaminidase levels upon plumbagin and 6-shogaol

treatments indicate that plumbagin and 6-shogaol are capable of inhibiting the degranulation process of mast cells and basophils.

4.7.2 Effects of Prasaprohyai and Benjakul remedies in a mouse model of allergic rhinitis

Allergic rhinitis (AR) is an extremely global health problem that causes major illness and disability worldwide. AR is broadly defined as an inflammation of the nasal mucosa caused by IgE-mediated immune response to environmental allergens and is characterized by symptoms such as sneezing, pruritus, mucosal edema (Aswar, Shintre, Chepurwar, & Aswar, 2015). Therefore, it is necessary to establish an animal model to show the similar allergic symptoms for evaluation therapeutical effects of the test sample. In the literature, it has been reported that ovalbumin (OVA) sensitization and challenge in mice model induced symptoms of AR and led to an increase in inflammatory cell infiltration in epithelium and subepithelium of the nasal mucosa, increase the levels of histamine, IL-4, and antigen-specific IgE in serum (Rhee, 2013). OVA-induced AR in mice shows similar nasal allergic symptoms as observed in humans (Wang et al., 2007). Thus, the aim of this study was to evaluate anti-allergic effect of Prasaprohyai and Benjakul remedies using model of OVA-induced AR in mice.

4.7.2.1 Prasaprohyai extract treatment group

Immunoglobulin isotype switching and the induction of antibody production are complex processes driven by IL-4 or IL-13 to the promoter regions of IgE and IgG1 (a Th2 cytokine) and also induced by IFN-γ to the promoter region of IgG2a (a Th1 cytokine) (Ormstad, Groeng, Duffort, & Løvik, 2003). Furthermore, IgE and IgG1 levels represent Th2 activity and IgG2a also represent Th1 activity. In the present study, the levels of OVA-specific serum IgE, a pathogenic antibody isotype for allergy, in all doses of PPY (100, 200 and 400 mg/kg) treatment groups were decreased compared with the control group (not significant) but their levels were more than montelukast, a positive control group. Not only treatment of PPY (100 mg/kg) significantly reduced of the level of serum OVA-specific IgG1, a Th2-related immunoglobulin, when compared with control group but also reduced (not

significant) the level of IgG1 when compared with montelukast. In addition, PPY (100, 200 and 400 mg/kg) treatment groups increased the levels of serum OVA-specific IgG2a, a Th1-related immunoglobulin, when compared with control group but their levels were less than montelukast. These results indicate that PPY (100 mg/kg) treatment group suppressed Th2 response and enhanced Th1 response. That means a shift of Th2 to Th1 response by treatment occurs with PPY at concentration of 100 mg/kg.

The average histopathological grades of inflammatory cells, mast cells and mucus glands were not different in allergic mice after treatment with all doses of PPY (100, 200 and 400 mg/kg) compared with control group and montelukast group. Nevertheless, allergic mice that received PPY (100 and 400 mg/kg) down regulated (significant) the numbers of grade 2-inflammatory cells in the nasal cavity and increased the numbers of grade 1-inflammatory cells when compared with control group although not significant. In addition, allergic mice that received with PPY (100 and 200 mg/kg) reduced the mast cell number in nasal tissues compared with control group and montelukast group (not significant). Moreover, allergic mice that received PPY (100 and 200 mg/kg) also down regulated the numbers of grade 2 and 3-mucus glands in the nasal cavity and increased the numbers of grade-1 mucus glands at the same time compared with control group but not significant. These results indicate that PPY (100 and 200 mg/kg) can decreases the mast cells, which are dependent of IgE-mediated activation pathway by the synthesis of cytokines (Galli et al, 2008). Therefore, PPY (100 and 200 mg/kg) treatments caused decreasing of the mucus production in nasal tissues.

T helper type 2 (Th2) cells may play an important roles in allergic diseases, including allergic rhinitis and asthma. Th2 cells produce various effector Th2 cytokines, particularly IL-4, IL-5 and IL-13 (Chung, 2001; Williams et al., 2012). IL-4 is upstream cytokine that regulates allergic inflammation by promoting Th2 cells differentiation and IgE production. IL-5 is a cytokine that is highly specific for eosinophilic inflammation. IL-13 has many actions similar to those of IL-4 and also regulates IgE production but it does not regulate T cell differentiation to Th2 cells and T lymphocytes do not respond to IL-13 (Barnes, 2001). The IL-4, IL-5 and IL-13

mRNA levels in the nasal tissues of allergic mice treated with PPY (100 mg/kg) were down-regulated and they had reduced more than montelukast group but not significant difference. Additionally, allergic mice treated with PPY (200 mg/kg) inhibited IL-4 secretion and PPY (400 mg/kg) treatment decreased the production of IL-13. These findings suggest that PPY (100 mg/kg) inhibited the production of Th2 cytokines due to decreased serum OVA-specific IgE and IgG1 levels.

T helper type 1 (Th1) cells are recognized by their secretion of IFN-γ and IL-12. IFN-γ is a pro-inflammatory cytokine, which inhibits Th2 cytokines and promotes Th1 differentiation. IL-12 is a pro-inflammatory heterodimeric cytokine, composed of two covalently-linked subunits, IL-12p35 and IL-12p40, which is a potent simulator of T-cell functions, including proliferation, cytotoxicity and the release of cytokines. Moreover, IL-12 induces IFN-γ production and regulates T cell differentiation to Th1 cells (Hamza, Barnett, & Li, 2010; Lauw, Florquin, Speelman, Van Deventer, & Van Der Poll, 2001). In this study, only IL-12p35 mRNA expression, that was up-regulated in nasal tissues at all doses of PPY (100, 200 and 400 mg/kg) treatment groups. Apart from the production of IL-12p35, the expressions of IFN-γ and also IL-12p40 mRNA in nasal tissues of allergic mice after treatment with all doses of PPY (100, 200 and 400 mg/kg) decreased. The reason that IFN-γ and IL-12p40 profiles decreased is unclear, however, it might be due to the suppression of other cytokines. This needs to be clarified further.

Regulatory T helper cells or Treg (inducible Treg, iTreg) cells play an important role in immunological tolerance by producing immunosuppressive cytokines including IL-10 and TGF-β (Kawase et al., 2006). IL-10 is an inhibitory cytokine of inflammation which inhibits allergen-specific IgE production, down-regulates eosinophil activities and suppresses IL-5 production (Hawrylowicz & O'Garra, 2005). TGF-β plays important roles in multifunctional cytokine involved in cell growth and differentiation, inflammation and repair and host immunity (Clark & Coker, 1998; Jin, Wi, Choi, Hong, & Bae, 2014). In the present study, there was no increase in TGF-β mRNA expression in nasal tissues of allergic mice treated with all doses of PPY (100, 200 and 400 mg/kg). Whereas, the allergic mice received all doses

of PPY (100, 200 and 400 mg/kg) had increase in IL-10 mRNA, especially PPY (200 mg/kg) had a slightly significant rise in IL-10 compared with control group.

This finding is the first report on anti-allergic effects of Prasaprohyai ethanolic extract in OVA-induced allergic rhinitis mouse model. However, some individual plant components of Prasaprohyai remedy have been reported in animal model of allergy. The 70% ethanolic extract of *A. dahurica* (50 and 100 mg/kg) showed significant reduction of IL-4, IL-5 and TNF-α levels in BALF, and reduced inflammatory cells in mice model (Lee, Seo et al., 2011). In addition, the 80% ethanolic extract of *A. annua* (100, 200 and 400 mg/kg) diminished systemic anaphylactic shock, histamine release, scratching behavior and vascular permeability in compound 48/80-stimulated rat model (Deng, Liu, & Geng, 2016). Moreover, some active compounds from *A. annua*, including artesunate (Cheng et al., 2013) and dihydroartemisinin (Wei et al., 2013) inhibited *in vivo* anti-allergic activity. Recently, many extracts of *N. sativa* i.e. fixed oil (Abdel-Aziz et al., 2014), oil (Balaha, Tanaka, Yamashita, Rahman, & Inagaki, 2012) and hydro-ethanolic (Boskabady, Keyhanmanesh, Khameneh, Doostdar, & Khakzad, 2011) were reported that they showed inhibitory effect on animal experiments of allergic asthma. Thymoquinone an active compound from *N. sativa* reduced blood cytokines and changed the histopathological in lung lavage fluid in guinea pig model of asthma (Hossen et al., 2017). Additionally, thymoquinone also showed anti-allergic effect in allergic conjunctivitis mouse model (Hayat et al., 2011).

4.7.2.2 Benjakul extract treatment group

In this study, BJK (37.5 and 75 mg/kg) treatment groups significantly decreased serum OVA-specific IgE when compared to control group and montelukast group. On the other hand, all doses of BJK (37.5, 75 and 150 mg/kg) treatment groups were not difference in the level of OVA-specific IgG1 when compared with control group and montelukast group. The levels of OVA-specific serum IgG2 in all doses of BJK (37.5, 75 and 150 mg/kg) treatment groups were slightly increased when compared with the control group (not significant) but only BJK (37.5 mg/kg) treatment group increased the levels of OVA-specific serum IgG2a

better than montelukast, a positive control group. These results indicate that BJK at concentration of 37.5 mg/kg inhibits the allergic response, especially through the rise of OVA-specific IgG2a and the reduction of OVA-specific IgE.

The number of grades of inflammatory cells, mast cells and mucus glands were not significantly different in allergic mice after treatment with all of doses of BJK (37.5, 75 and 150 mg/kg) compared with control group and montelukast group. However, allergic mice that treated with BJK (37.5 mg/kg) showed reducing of the numbers of grade 2, 3 and 4-inflammatory cells infiltration into nasal tissues and increasing of the numbers of grade-1 inflammatory cells when compared with control group similar to montelukast group (not significant). Further, all doses of BJK (37.5, 75 and 150 mg/kg) treatments decreased amount of mast cells in nasal tissue sections as compared to control group but demonstrated high amount of mast cells as compared to montelukast group (not significant). These results imply that BJK (37.5 mg/kg) can reduce the migration of inflammatory cells such as eosinophils, neutrophils and lymphocytes to the epithelia of nasal tissues. These cells may activate Th2 cells and produce cytokines including IL-4, IL-5 and IL-13 during the allergic process (Vieira et al., 2018).

The IL-5 mRNA level in the nasal tissues of allergic mice treated with BJK (37.5 and 75 mg/kg) was reduced but not significant different when compared with control group. Moreover, all doses of BJK (37.5, 75 and 150 mg/kg) showed significant inhibition of IL-13 secretion in allergic mice compared to control group. These findings suggest that BJK (37.5 up to 75 mg/kg) inhibited the production of Th2 cytokines due to decreased serum OVA-specific IgE and IgG1 levels.

In this study, the IFN-γ and also IL-12 (p35 and p40) mRNA levels of allergic mice after received all doses of BJK (37.5, 75 and 150 mg/kg) were reduced. In the expected outcomes, the results may be increasing tendency of the IFN-γ and IL-12 (p35 and p40) expressions.

For Treg cytokines, the levels of TGF-β mRNA in nasal tissues of allergic mice treated with BJK (37.5 and 75 mg/kg) were at the same levels of control group. Further, the only slightly up-regulation was observed in the level of IL-

10 mRNA of allergic mice treated with BJK (37.5 mg/kg) compared with control group (not significant). This result might indicate the regulatory T cell generation of the allergic mice treated with BJK (37.5 mg/kg).

This is the first evidence to show anti-allergic effects of Benjakul ethanolic extract in OVA-induced allergic rhinitis mouse model. In previous report, there have reports of some individual plant constituents of Benjakul remedy in animal model of allergy. Initially, the petroleum ether, alcoholic and aqueous extracts of *P. longum* revealed anti-allergic and anti-asthmatic activities in various animal models (Kaushik et al., 2012). Addition, the ehanolic extract (500 mg/kg) and the aqueous extract (720 mg/kg) of *Z. officinale* reduced both mRNA levels and protein levels of IL-4 and IL-5 in BALF, also suppressed total serum IgE level in allergic asthma mouse model (Khan et al., 2015). Moreover, 6-Shogaol, a major compound of *Z. officinale*, inhibited the IgE-mediated passive cutaneous anaphylaxis (PCA) in rat at concentrations of 1 and 5 mg/kg (Sohn et al., 2013), suppressed Th2-mediated immune responses in mouse allergic asthma (Ahui et al., 2008), relieved allergic dermatitis-like skin lesions by inhibiting immune mediators in mouse model (Park, Oh, Lee, Lee, & Kim, 2016). Other active compound of *Z. officinale*, 6-gingerol inhibited the expression of Th2 and Th1 cytokines in OVA-induced spleen cells (Kawamoto et al., 2016).

4.7.3 Nitric oxide and tumor necrosis factor-α production assays in RAW 264.7 cells

Inflammatory reactions are protective biological processes carried out by endogenous mediators to eliminate harmful stimuli. The most common inflammatory process involves pro-inflammatory cytokines, chemokines, and inflammatory immune cells (Majdalawieh & Ro, 2010). Activation of macrophage is related to release pro-inflammatory mediators and cytokines, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukine-1β (IL-1β), and interleukin-6 (IL-6) by inflammatory cytokines and bacterial products. Therefore, overexpression of these mediators and cytokines that can leads to tissue injury, cell death, and multiple organ failure (Pan, Chiou, Tsai, &

Ho, 2011) and relate to many diseases including asthma, cancer, osteoarthritis, rheumatoid arthritis, and diabetes (Zhang et al., 2015).

Nitric oxide (NO) is a free radical messenger molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under normal physiological conditions, but on the other hand it is considered as a pro-inflammatory mediator that relates inflammation due to excess production in abnormal situations (Sharma et al., 2007). Further, activation of RAW 264.7 murine macrophage cells to induce NO and TNF-α production requires LPS. The Griess assay was used to determine NO production since the method is simplicity, inexpensive, and high sensitivity enough to detect the induced form of NO production (Sun, Zhang, Broderick, & Fein, 2003).

In the present study, the crude ethanolic extract of Benjakul, its individual plant components and its pure compounds were investigated for their anti-inflammatory effects using LPS-induced NO and TNF-α in RAW 264.7 cells. These results firstly found that myristicin showed the moderate inhibitory activity on TNF-α release. Apart from myristicin, 6-shogaol, plumbagin, piperine and 6-gingerol which were isolated from Benjakul ethanolic extract that compounds had been reported to have anti-inflammatory activity (Dugasani et al., 2010; Kumar et al., 2007; Wang et al., 2014). Moreover, these results also found that 6-shogaol showed mightily potent inhibitory effects on NO production and TNF-α release in LPS-induced RAW 264.7 murine macrophage cells. Interestingly, this discovery revealed the results as in the previous report that 6-shogaol displayed the inhibitory effect on NO production in RAW 264.7 cells (Dugasani et al., 2010; Imm, Zhang, Chan, Nitteranon, & Parkin, 2010). Previous report, 6-shogaol also demonstrated to cause inhibition of LPS-induced inflammation in cell-based assay, such as inhibition of iNOS and COX-2 genes (Li et al., 2012; Shim, Kim, Choi, Kwon, & Kwon, 2011). Therefore, 6-shogaol diminished the levels of inflammatory cytokines and inhibited the expression of COX-2 and iNOS in brain of MCAO-induced mice (Na, Song, Lee, Kim, & Kwon, 2016). In addition, plumbagin, a major pure compound of *P. zeylanica*, inhibited LPS-activated NO, TNF-α, PGE_2 and IL-6 production in RAW 264.7 cells (Checker et al., 2014). A recent

studies reported that piperine, a pure compound of *P. longum*, showed inhibitory effect on NF-κB activation in TNF-α induced endothelial cells (Kumar et al., 2007), inhibitory effect on COX-2 expression in PMA-induced RAW 264.7 cells (Kim, Han et al., 2012) and inhibitory effect on endotoxic shock in LPS-induced C57BL/6 mice (Bae et al., 2010). Additionally, 6-gingerol showed anti-inflammatory activities on *in vitro* (Dugasani et al., 2010; Imm et al., 2010) and *in vivo* (Young et al., 2005) models. Several studies have examined the use of 6-shogaol and plumbagin to suppress on allergic and inflammatory reactions (Checker, Sharma, Sandur, Khanam, & Poduval, 2009; Lantz et al., 2007; Pan et al., 2008; Shim, Kim, Choi, Kwon, & Kwon, 2011; Van Breemen et al., 2011; Wang et al., 2014; Young et al., 2005). In the present study, 6-shogaol and plumbagin showed strong inhibitory effects on allergy and inflammation. In addition, 6-shogaol and plumbagin are present in the Benjakul ethanolic extract. It also explained that compounds caused the Benjakul ethanolic extract showed anti-allergy and anti-inflammation. Moreover, the results indicated that Benjakul at concentrations between 10-20 μg/ml also exhibited inhibitory effects on β-hexosaminidase release and NO production but not TNF-α activity.

Recently study, Benjakul has been reported *in vitro* model (Burodom & Itharat, 2013) and in animal models of inflammation and analgesic (Sireeratawong, 2008). In additional, some plant components in Benjakul remedy have been reported both *in vitro* and *in vivo* studies of anti-inflammatory effect. For instance, *in vitro* studies, the *Z. officinale* methanolic extract presented strongly inhibitory effect on IL-1β release in human peripheral blood mononuclear cells (PBMCs) with the IC_{50} value of 3.17 μg/ml (Salim, Kumolosasi, & Jantan, 2014), COX-1 and COX-2 with the IC_{50} values of 20.0 and 7.5 μM, respectively (Van Breemen et al., 2011). The ethanolic extract of both *P. longum* fruits and *P. indica* roots displayed the potential inhibitory effect on NO production with IC_{50} values of 9.98 and 9.74 μg/ml, respectively (Anuthakoengkun & Itharat, 2014). Additional, *in vivo* studies, the *Z. officinale* aqueous extract also reduced the production of IFN-γ, IL-6, iNOS, COX-2 (Choi, Kim, Hong, Kim, & Yang, 2013), and prostaglandin E_2 (PGE_2) (Thomson et al., 2002). Recently study has reported that the ethanolic extract of *P. interruptum*

stems, *P. chaba* fruits (Sireeatawong et al., 2012), and *P. sarmentosum* roots (Sireeratawong et al., 2010) have presented anti-inflammation in animal model.

4.7.4 β-Hexosaminidase release and nitric oxide production of pure compounds from *A. lancea* using *in vitro* cell based assay

Taraxerol acetate (%yield = 0.004%) is a pentacyclic triterpene isolated from the rhizomes of *A. lancea* ethanolic extract. Triterpenes are also known to increase the inhibitory effects of anticancer drugs (Yamai et al., 2009) and also to benefit in Alzhiemer's and Parkinsonism (Ngo & Li, 2013). In this study, taraxerol acetate exhibited no appreciable anti-allergic activity *via* β-hexosaminidase release because it showed the IC_{50} value more than 100 μg/ml. Furthermore, taraxerol acetrate showed no anti-inflammatory activity *via* nitric oxide production because this compound also induced severe cytotoxicity in RAW 264.7 cells. However, there is no previous report of taraxerol acetate being anti-allergic and anti-inflammatory compound from *Atractylodes lancea.* Nevertheless, according the previous report, taraxerol acetate was isolated from *Artemisia roxburghiana* and studied for its effect in both *in vitro* and *in vivo* anti-inflammatory activity. For *in vitro* assay, taraxerol acetate showed considerable inhibitory activity against both COX-1 and COX-2 enzymes with IC_{50} values of 116.3 and 94.7 μM, respectively (Rehman et al., 2013). For *in vivo* assay, taraxerol acetate (60 mg/kg/bw) significantly decreased the edema induced by carrageenan in rats (Rahman, Ali et al., 2016). Additionally, taraxerol acetate, isolated from *A. roxburghiana*, significantly decreased the anti-pyretic activity in yeast induced pyrexia model which is dose-dependent (Rahman, Durrani, Ubaidullah, Ali, & Rahman, 2016). Taraxerol acetate was also reported to have potent anticancer activities both *in vitro* and *in vivo* models *via* the induction of apoptosis, autophagy, and cell cycle arrest and inhibition of cell migration (Hong et al., 2016). Besides, taraxerol acetate showed inhibitory effects on human gastric epithelial (AGC) cell growth through inducing G(2)/M arrest and apoptosis promotion (Tan, Shi, Ji, & Xie, 2011).

Atractylodin is a polyacetylenic compound isolated from the rhizomes of *A. lancea* (Nakai et al., 2003). Atractylodin TS, an authentic compound

isolated from *A. lancea*, demonstrated strong inhibitory effect on β-hexosaminidase release in RBL-2H3 cells with IC_{50} value of 0.07 μg/ml, but it showed no inhibitory effect on nitric oxide production in LPS-activated RAW 264.7 cells because this compound also induced severe cytotoxicity in RAW 264.7 cells. This result of anti-inflammation does not relate with the previous reports that presented anti-inflammatory effect of atractylodin *in vitro* and *in vivo* experiment. Atractylodin was reported that it inhibited the inflammatory mediator expression *via* suppressing regulated MAPKs and NPM-ALK signaling pathway in human mast cells (Chae et al., 2016). In addition, atractylodin diminished LPS-induced acute lung injury by suppression of inflammatory cytokines production in mouse model (Tang, Fan, Wang, & Bian, 2018) and also reduced jejunal inflammation in rat model (Yu et al., 2017). Atractylodin also showed anticancer activity *in vitro* model through inducing activity on cell cycle arrest at the G1 phase and induction of cell apoptosis in CCA cells (Kotawong, Chaijaroenkul, Muhamad, & Na-Bangchang, 2018).

β-Eudesmol is a sesquiterpene isolated from the rhizomes of *A. lancea* (Ouyang et al., 2012). β-Eudesmol, an authentic compound isolated from *A. lancea*, showed weak anti-allergic activity on β-hexosaminidase release in RBL-2H3 cells (IC_{50} = 82.21 μg/ml) and this compound revealed no anti-inflammatory effect on nitric oxide production in LPS-stimulated RAW 264.7 cells because this compound also induced severe cytotoxicity in RAW 264.7 cells. Results from previously anti-inflamtory studies have indicated that β-eudesmol (2 and 20 μM) showed regulation of IL-6 through regulation of p38 MAPK and NF-κB and suppressed the expression of RIP2 and the activation of caspase-1 (Seo et al., 2011). Another previous report, β-eudesmol exhibited the protective effect on NF-κB-mediated inflammatory signaling activities (Kim, 2018). β-Eudesmol showed anticancer activity *in vitro* study through cell cycle arrest promotion and cell apoptosis induction (Kotawong et al., 2018) and *in vivo* study in CCA-xenografted nude mice (Plengsuriyakarn, Karbwang, & Na-Bangchang, 2015).

These findings suggest that atractylodin could be considered a potential major active compound of *A. lancea* for allergy as a result of the inhibion

of β-hexosaminidase release in antigen-stumulated RBL-2H3 cells and can be marker in this plant because this plant is an ingredient in Prasaprhoyai and showed the highest anti-allergic activity in the previously report (Makchuchit, 2010).

4.7.5 Formulation development

For pre-formulation studies, forced degradation studies or stress testing are important for help to develop and demonstrate specificity of stability-indicating method and to determine the degradation pathways and degradation products of the active ingredients (Hotha, Reddy, Raju, & Ravindranath, 2013). Degradation products generated from forced degradation studies are potential degradation products but they assist in the developing stability indicating method (Blessy, Patel, Prajapati, & Agrawal, 2014). The results indicated that Prasaprohyai remedy showed unstable anti-allergic activity under stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation because the IC_{50} values of β-hexosaminidase release were increased and showed stable NO activity under alkaline hydrolysis. In contrast to Prasaprohyai, Benjakul remedy displayed stable anti-allergic effect under stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation and oxidation because the IC_{50} values of β-hexosaminidase release did not change and displayed unstable NO activity under alkaline hydrolysis. Because the *Piper* species in Benjakul remedy have an alkaloid compound such as piperine which can change the molecule in alkaline condition. For the stability testing under accelerated conditions, the results showed that Prasaprohyai ethanolic extract showed no change on both the release of β-hexosaminidase and the production of nitric oxide. For Benjakul ethanolic extract under accelerated conditions, the results found that Benjakul ethanolic extract showed no change on nitric oxide production, while it exhibited change on β-hexosaminidase release at days 120, 150 and 180. Furthermore, plumbagin in Benjakul ethanolic extract showed rapidly reduced level after keeping under accelerated condition at day 90, 120, 150 and 180, indicating that plumbagin was unstable. The results of stability testing found that stability of Benjakul ethanolic extract related with stability of Benjakul from previous report (Sakpakdeejaroen,

2009; Suthanurak, Sakpakdeejaroen, Rattarom, & Itharat, 2010). From the results presented here, it may be concluded that the anti-allergic effect of Benjakul was change where cause of change of this activity may be plumbagin.

For formulation studies, the results suggested that the suitable excipients of Prasaprohyai and Benjakul capsule formulations were hydrophilic silicon dioxide as a adsorbent and anti-adherent (Aerosil®), microcrystalline cellulose as a adsorbent and glidant (Avicel® PH-101 and Avicel® PH-102), lactose as a filler, talcum as a glidant and magnesium stearate as a lubricant were used in the capsule formulation. Capsules were adjusted weight to about 500 mg/kg per capsule. There were one formulation of Prasaprohyai (P3-capsule) and two formulations of Benjakul (B1- and B4-capsule), which revealed good weight variation and disintegration time of the capsules in accordance with the BP 2013 requirements (British Pharmacopoeia Commission, 2013). In addition, the stability of P3, B1 and B4-capsule formulations after keeping under accelerated condition revealed no change of moisture content and inhibitory effect on nitric oxide production. Meanwhile, P3-capsule formulation changed when tested the release of β-hexosaminidase at days 150 and 180. Consequently, it may be concludes that P3-capsule formulation of Prasaprohyai remedy could be stored for two years at room temperature without loss of the anti-inflammatory activity, but it could be kept for 1.5 years without loss of the anti-allergic activity. Whereas, B1- and B4-capsule formulations of Benjakul remedy could be stored for two years at room temperature without loss of both the anti-allergic and anti-inflammatory activities (International Conference on Harmonization, 2003).

Table 4.30 The summary of all results of Prasaprohyai remedy in the studies

Sample	Anti-allergy		Anti-inflammatory		Pre-formulation	Formulation	Stability under accelerated conditions	
	In vitro (IC_{50}, μg/ml)	*In vivo* (Dose, mg/kg)	NO (IC_{50}, μg/ml)	TNF-α (IC_{50}, μg/ml)			Extract	Capsule
Prasaprohyai (PPY)	11.71	100	49.95	20.34	Unstable (All conditions)	P3	β-HEX (Stable) NO (Stable)	β-HEX (Unstable at D150-D180) NO (Stable)
A. lancea (AtL)	13.60	-	9.70	24.35	-	-	-	-
Pure compounds (AtL)								
- Atractylodin	0.07	-	Toxic	-	-	-	-	-
- β-Eudesmol	86.21	-	Toxic	-	-	-	-	-
- Taraxerol acetate	> 100	-	Toxic	-	-	-	-	-

(-) means not tested

Table 4.31 The summary of all results of Benjakul remedy in the studies

Sample	Anti-allergy		Anti-inflammatory		Pre-formulation	Formulation	Stability under accelerated conditions	
	In vitro (IC_{50}, μg/ml)	*In vivo* (Dose, mg/kg)	NO (IC_{50}, μg/ml)	TNF-α (IC_{50}, μg/ml)			Extract	Capsule
Benjakul (BJK)	12.69	37.5	16.60	NA	Unstable (Alkaline condition)	B1 and B4	β-HEX (Unstable at D120-D180) NO (Stable)	β-HEX (Stable) NO (Stable)
Plant ingredients (BJK)								
- *P. interruptum*	14.13	-	21.59	21.84	-	-	-	-
- *P. longum*	15.82	-	22.28	NA	-	-	-	-
- *P. sarmentosum*	21.73	-	22.42	30.31	-	-	-	-
- *P. indica*	13.31	-	14.06	NA	-	-	-	-
- *Z. officinale*	12.93	-	13.44	NA	-	-	-	-
Pure compounds (BJK)								
- 6-Gingerol	18.30	-	72.25	67.83	-	-	-	-
- Methyl piperate	12.44	-	> 10	> 100	-	-	-	-
- Myristicin	80.80	-	> 100	47.48	-	-	-	-
- Piperine	14.14	-	38.89	NA	-	-	-	-
- Plumbagin	4.03	-	0.002	NA	-	-	-	-
- 6-Shogaol	0.28	-	0.92	9.01	-	-	-	-
- β-Sitostenone	24.27	-	> 100	> 100	-	-	-	-

NA means not active; (-) means not tested

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

In Thai traditional medicine, Prasaprohyai remedy has long been used for treatment of fever, common cold and allergy. Prasaprohyai remedy is composed of nineteen plants, namely the rhizomes of *Kaempferia galanga* L. as the main component, the fruits of *Amomum testaceum* Ridl., the fruits of *Anethum graveolens* L., the roots of *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., the roots of *Angelica sinensis* (Oliv.) Diels, all parts of *Artemisia annua* L., the rhizomes of *Atractylodes lancea* (Thunb.) DC., the fruits of *Cuminum cyminum* L., the stems of *Dracaena loureiroi* Gagnep., the fruits of *Foeniculum vulgare* Mill., the seeds of *Lepidium sativum* L., the rhizomes of *Ligusticum sinense* Oliv., the flowers of *Mammea siamensis* (Miq.) T. Anders., the flowers of *Mesua ferrea* L., the flowers of *Mimusops elengi* L., the stems, arils and seeds of *Myristica fragrans* Houtt., the pollens of *Nelumbo nucifera* Gaertn., the seeds of *Nigella sativa* L., and the flowers of *Syzygium aromaticum* (L.) Merr. & L. M. Perry. Another remedy is Benjakul that has long been used for supporting the body in achieving a state of balance or homeostasis, controlled abnormal of element in the body, relieved flatulence, and treated cancer patients by Thai folk doctors. Benjakul remedy is composed of five plants, including the stems of *Piper interruptum* Opiz., the fruits of *Piper longum* L., the roots of *Piper sarmentosum* Roxb., the roots of *Plumbago indica* L., and the rhizomes of *Zingiber officinale* Roscoe.

In vitro anti-allergic and anti-inflammatory activities of Benjkaul remedy and *in vivo* anti-allergic activity of Prasaprohyai and Benjakul remedies have not been reported. Therefore, the present study proposed on *in vitro* and *in vivo* anti-allergic and *in vitro* anti-inflammatory activities of Prasaprohyai remedy and Benjakul remedy and individual plant components of Benjkaul and pure compounds of Benjakul. Anti-allergic effects were investigated on RBL-2H3 cells (*in vitro* assay) and BALB/c mice (*in vivo* assay). Anti-inflammatory effects were tested on RAW 264.7 cells (*in vitro* assay). In addition, the isolated compounds from *Atractylodes lancea*, which marked the

high anti-allergic and anti-inflammatory effects, were also tested for *in vitro* anti-allergic and anti-inflammatory activities. Moreover, formulations of capsule from Prasaprohyai and Benjakul remedies were produced. Finally, the stabilities of the formulations were also determined.

In vitro anti-allergic and anti-inflammatory activities, the ethanolic extract of Benjakul showed the most potent effect on β-hexosaminidase release in RBL-2H3 cells. In addition, the ethanolic extract of Benjakul presented the potential effect on LPS-activated NO production in RAW 264.7 cells but it showed no effect on LPS-induced TNF-α in RAW 264.7 cells. The plant components in Benjakul, *Z. officinale, P. indica*, *P. interruptum*, *P. longum* and *P. sarmentosum* not only exhibited the strong inhibitory effect on β-hexosaminidase release but also revealed the potent inhibitory effect on NO production. Particularly, *P. interruptum* and *P. sarmentosum* displayed inhibitory effect on TNF-α release. Only 6-shogaol which is pure compound isolated from Benjakul showed the highest inhibitory effects on β-hexosaminidase release and TNF-α production, and also displayed the potent inhibitory effect on NO production. Moreover, plumbagin showed a significant effect on β-hexosaminidase release and also showed the greatest inhibitory effect on NO production but it had no inhibitory effect on TNF-α production.

In vivo anti-allergic activity, the allergic mice that were treated with the ethanolic extract of Benjakul at concentration of 37.5 mg/kg tended to relieve the OVA-induced allergic rhinitis in the modeled BALB/c mice. This extract had decreased the number of grades of inflammatory cells and reduced the amount of mast cells migration into nasal tissues. The ethanolic extract of Benjakul (37.5 mg/kg)-treated allergic mice had decrease Th2 cytokine expressions such as IL-5 and IL-13, and also slightly increase Treg cytokine such as IL-10. Nevertheless, this extract did not induce a Th1 response. For Prasaprohyai remedy, the ethanolic extract of Prasaprohyai at concentration of 100 mg/kg tended to lessen the OVA-induced allergic rhinitis in the modeled BALB/c mice. Treatment with the ethanolic extract of Prasaprohyai at concentration 100 mg/kg had reduced the number of grades of mucus glands in nasal cavity, reduction of Th2 cytokine expressions such as IL-4, IL-5 and IL-13, only

up-regulation of IL-12p35 which Th1 cytokine and also induction of immunosuppressive cytokine response, Treg cytokine, such as IL-10.

The ethanolic extract of *Atratylodes lancea* was separated by VLC and continuously separated by MPLC. One compound as taraxerol acetate was isolated from this plant. Taraxeral acetate and two authentic pure compounds from *A. lancea* namely atractylodin TS and β-eudesmol showed no effect on LPS-stimulated NO production in RAW 264.7 cells. Nevertheless, atractylodin TS presented the most effect on β-hexosaminidase release in RBL-2H3, followed by β-eudesmol but taraxerol acetate had no anti-allergic activity.

The stability study of the ethanolic extract of Prasaprohyai under accelerated conditions (40ºC, 75% RH for 6 months) showed no change on β-hexosaminidase release and NO production. Whereas, the ethanolic extract of Benjakul under accelerated conditions (40ºC, 75% RH for 6 months) displayed no change only on nitric oxide production but β-hexosaminidase release decreased on days 120, 150 and 180 with a quickly reduction of plumbagin in Benjakul ethanolic extract.

Three capsule formulations were obtained from the ethanol extracts of Prasaprohyai (P3-capsule formulation) and Benjakul (B1- and B4-capsule formulations). These capsules composed of 10% w/w of extract, Avecel® PH-101, Avecel® PH-102, lactose, Aerosil®, magnesium stearate and talcum by total weight of 500 mg per capsule. The weight variation and disintegration time of the capsules were performed in accordance with the BP 2013 requirements. After stability test under accelerated condition (40ºC, 75% RH for 6 months), the moisture content and NO production in RAW 264.7 cells of P3-, B1- and B4-capsule formulations had not changed. But the β-hexosaminidase release in RBL-2H3 of P3-capsule formulation had increased on days 150 and 180. Thus, it is concluded that P3-capsule formulation could be stored for two years at room temperature without loss of the anti-inflammatory activity, but it could be kept for 1.5 years without loss of the anti-allergic activity. Additionally, B1- and B4-capsule formulations could be stored for

two years at room temperature without loss of both the anti-allergic and anti-inflammatory activities.

From the present study, these results provided evidence that the ethanolic extract of Prasaprohyai showed *in vivo* anti-allergic effect and also showed to be stable for two years at room temperature without loss of *in vitro* anti-allergy and anti-inflammation. Moreover, the P3-capsule formulation from Prasaprohyai showed to be stable for two years at room temperature without loss of *in vitro* anti-inflammation. The ethanolic extract of Benjakul showed *in vitro* and *in vivo* anti-allergic activities and *in vitro* anti-inflammatory activity. Also the ethanolic extract of Benjakul showed to be stable for two years at room temperature without loss of *in vitro* anti-inflammation. In addition, the B1- and B4-capsule formulations from Benjkaul showed to be stable for two years at room temperature without loss of *in vitro* anti-allergy and anti-inflammation. Therefore, these results support the using of the ethanolic extract of Prasaprohyai for treatments of fever, cold and allergy. The ethanolic extract of Benjakul used in traditional medicine is proved to have adaptogenicity for the weather change because it has both anti-allergic and anti-inflammatory activities.

The further study should be to study of pure compounds which are the active makers of Prasaprohyai and Benjakul on allergic rhinitis in mouse model. The clinical efficacy of these remedies should be further evaluated to confirm this activity. Additionally, the mechanism of anti-inflammatory and anti-allergic activities of these remedies should be investigated. For allergic rhinitis mouse model, the duration of the experiment should be extended from 1 month to 2 months in order to obtain clear results. Thus, the extension of the allergic rhinitis mice experiment to 2 months should be further studied.

REFERENCES

Books and Book Articles

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2012). *Cellular and molecular immunology* (7th ed.). Philadelphia: Saunders.

Chandrasoma, P., & Taylor, C. R. (1998). *Concise pathology* (3rd ed.). Connecticut: Appleton & Lange.

Department of Medical Sciences, Ministry of Public Health. (2000). *Thai herbal Pharmacopoeia volume II*. Bangkok: Prachachon.

Department of Medical Sciences, Ministry of Public Health. (2011). *Supplement to Thai herbal Pharmacopoeia*. Bangkok: Prachachon.

Gentile, D. A., & Skoner, D. P. (2010). Pediatric allerfy principles and practice (2nd ed.). In D. Y. M. leung, H. A. Sampson, R. Geha, & S. J. Szefler (Eds.), *Chapter 28: Allergic rhinitis* (pp. 291-300). Edinburgh: Saunders.

Hou, W., Xu, G., & Wang, H. (2011). Treating autoimmune disease with Chinese medicine. In J. M. Gould (Ed.), *Chapter 1: Basic immunology and immune system disorders* (pp. 1-12). Edinburgh: Churchill Livingstone.

Kumar, V., Abbas, A. K., & Aster, J. C. (2013). Robbins basic pathology (9th ed.). *Chapter 2: Inflammation and repair* (pp.29-74). Philadelphia: Saunders.

Leung, A. Y., & Foster, S. (1996). *Encyclopedia of common natural ingredients used in food, drugs, and cosmetics* (2nd ed.). New York: John Wiley & Sons.

Makchuchit, S. (2010). *Anti-inflammatory and anti-allergic activities of Thai traditional medicine preparation called Prasaprohyai*. (Master's thesis). Thammasat University, Faculty of Medicine.

Malamed, S. F. (2015). Medical emergencies in the dental office (7th ed.). *Chapter 24: Allergy* (pp. 384-415). Missouri: Mosby.

Oettgen, H., & Broide, D. H. (2012). Allergy (4th ed.). In S. T. Holgate, M. K. Church, D. H. Broide, & F. D. Martinez (Eds.), *Chapter 1: Introduction to mechanisms of allergic disease* (pp. 1-32). Edinburgh: Saunders.

Pooma, R., & Suddee, S. (Eds.). (2014). *Thai plant names Tem Smitinand revised edition 2014*. Bangkok: Office of the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation.

Rattarom, R. (2013). *Regulation of apoptosis on lung cancer cells through caspase-dependent mechanism by Benjakul extract and its ingredients*. (Doctoral dissertation). Thammasat University, Faculty of Medicine.

Sakpakdeejaroen, I. (2009). *Study on cytotoxicity and chemical fingerprint of ethanolic extract of Benjakul preparation*. (Master's thesis). Thammasat University, Faculty of Medicine.

Saralamp, P., Chuakul, W., Temsiririrkkul, R., & Clayton, T. (1996). *Medicinal plants in Thailand volume I*. Bangkok: Amarin Printing and Publishing Public.

Scadding, G. K., Church, M. K., & Borish, L. (2012). Allergy (4th ed.). In S. T. Holgate, M. K. Church, D. H. Broide, & F. D. Martinez (Eds.), *Chapter 10: Allergic rhinitis and rhinosinusitis* (pp. 203-226). Edinburgh: Saunders.

Sireeratawong, S. (2008). *Anti-inflammatory, anti-pyretic, analgesic and anti-tumor effects of Benjakul extract in animal*. Bangkok: National Research Council of Thailand.

Ward, P. A. (2010). Fundamentals of inflammation. In C. N. Serhan, P. A. Ward, & D. W. Gilroy (Eds.) *Part I: The inflammatory response-an overview, Chapter 1: Acute and chronic inflammation* (pp. 1-16). Cambridge: Cambridge University Press.

World Health Organization. (1999). *WHO monographs on selected medicinal plants-Volume 1*. Geneva: Malta.

Zhengtao, W., & Peishan, X. (Eds.). (2015). *Monograaphs for quality evaluation of Chinese crude drugs*. New York: SCPG Publishing Corporation.

Articles

Abdel-Aziz, M., Abass, A., Zalata, K., Al-Galel, T. A., Allam, U., & Karrouf, G. (2014). Effect of dexamethasone and *Nigella sativa* on inducible nitric oxide synthase in the lungs of a murine model of allergic asthma. *Iranian Journal of Allergy, Asthma and Immunology, 13*(5), 324-334.

Abramson, J., Licht, A., & Pecht, I. (2006). Selective inhibition of the FcεRI-induced *de novo* synthesis of mediators by an inhibitory receptor. *The EMBO Journal, 25*(2), 323-334.

Ahmed, S., Zhan, C., Yang, Y., Wang, X., Yang, T., Zhao, Z., ... Hu, X. (2016). The transcript profile of a traditional Chinese medicine, *Atractylodes lancea*, revealing its sesquiterpenoid biosynthesis of the major active components. *PLoS ONE, 11*(3), e0151975. DOI: 10.1371/journal.pone.0151975

Ahui, M. L. B., Champy, P., Ramadan, A., Van, L. P., Araujo, L., André, K. B., ... Herbelin, A. (2008). Ginger prevents Th2-mediated immune responses in a mouse model of airway inflammation. *International Immunopharmacology, 8*(12), 1626-1632.

Ali, B. H., Blunden, G., Tanira, M. O., & Nemmar, A. (2008). Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale* Roscoe): a review of recent research. *Food and Chemical Toxicology, 46*, 409-420.

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.

Anuthakoengkun, A., & Itharat, A. (2014). Inhibitory effect on nitric oxide production and free radical scavenging activity of Thai medicinal plants in osteoarthritic knee treatment. *Journal of the Medical Association of Thailand, 97*(Suppl. 8), S116-S124.

Ashley, N. T., Weil, Z. M., & Nelson, R. J. (2012). Inflammation: mechanisms, costs, and natural variation. *Annual Review of Ecology, Evolution, and Systematics, 43*, 385-406.

Aswar, U., Shintre, S., Chepurwar, S., & Aswar, M. (2015). Antiallergic effect of piperine on ovalbumin-induced allergic rhinitis in mice. *Pharmaceutical Biology, 53*(9), 1358-1366.

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

Bajaj, S., Singla, D., & Sakhuja, N. (2012). Stability testing of pharmaceutical products. *Journal of Applied Pharmaceutical Science, 2*(3), 129-138.

Balaha, M. F., Tanaka, H., Yamashita, H., Rahman, M. N. A., & Inagaki, N. (2012). Oral *Nigella sativa* oil ameliorates ovalbumin-induced bronchial asthma in mice. *International Immunopharmacology, 14*, 224-231.

Barnes, P. J. (2001). Th2 cytokines and asthma: an introduction. *Respiratory Research, 2*, 64-65.

Barnes, P. J. (1999). Therapeutic strategies for allergic diseases. *Nature, 402*(Suppl. 6760), B31-B38.

Blantz, R. C., & Munger, K. (2002). Role of nitric oxide in inflammatory conditions. *Nephron, 90*, 373-378.

Blessy, M., Patel, R. D., Prajapati, P. N., Agrawal, Y. K. (2014). Development of forced degradation and stability indicating studies of drugs – a review. *Journal of Pharmaceutical Analysis, 4*(3), 159-165.

Bokesch, H. R., Gardella, R. S., Rabe, D. C., Bottaro, D. P., Linehan, W. M., McMahon, J. B., & McKee, T. C. (2011). A new hypoxia inducible factor-2 inhibitory pyrrolinone alkaloid from roots and stems of *Piper sarmentosum*. *Chemical and Pharmaceutical Bulletin (Tokyo), 59*(9), 1178-1179.

Boskabady, M.-H., Keyhanmanesh, R., Khameneh, S., Doostdar, Y., & Khakzad, M.-R. (2011). Potential immunomodulation effect of the extract of *Nigella sativa* on ovalbumin sensitized guinea pigs. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology), 12*(3), 201-209.

Bousquet,J., Van Cauwenberge, P., & Khaltaev, N. (2004). ARIA in the pharmacy: management of allergic rhinitis symptoms in the pharmacy. Allergic rhinitis and its impact on asthma. *Allergy, 59*, 373-387.

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *Journal of Pathology, 214*, 149-160.

Brozek, J. L., Bousquet, J., Agache, I., Agarwal, A., Bachert, C., Bosnic-Anticevich, S., ... Schünemann, H. J. (2017). Allergic rhinitis and its impact on asthma (ARIA) guidelines-2016 revision. *American Academy of Allergy, Asthma & Immunology, 140*(4), 950-958.

Bunjean, K., Sukkasem, K., Noppacroh, N., Yamkaew, N., Janthayanont, D., Theerapancharern, W., ... Moungthong, G. (2012). Prevalence of allergic rhinitis and types of sensitized allergen in adult at Wat Intaram community, Hua Raeu, Phra Nakhon Si Ayutthaya district, Phra Nakhon Si Ayutthaya province, Thailand. *Journal of the Medical Association of Thailand, 95*(Suppl. 5), S63-S68.

Bunnag, C., Jareoncharsri, P., Tantilipikorn, P., Vichyanond, P., & Pawankar, R. (2009). Epidemiology and current status of allergic rhinitis and asthma in Thailand – ARIA Asia-Pacific Workshop report. *Asian Pacific Journal of Allergy and Immunology, 27*(1), 79-86.

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.

Chae, H.-S., Kim, Y.-M., & Chin, Y.-W. (2016). Atractylodin inhibits interleukin-6 by blocking NPM-ALK activation and MAPKs in HMC-1. *Molecules, 21*(9), 1169. DOI: 10.3390/molecules21091169

Checker, R., Patwardhan, R. S., Sharma, D., Menon, J., Thoh, M., Sandur, S. K., ...Poduval, T. B. (2014). Plumbagin, a vitamin K3 analogue, abrogates lipopolysaccharide-induced oxidative stress, inflammation and endotoxic shock *via* NF-**κ**B suppression. *Inflammation, 37*(2), 542-554.

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*, 949-958.

Chen, B.-H., Wu, P.-Y., Chen, K.-M., Fu, T.-F., Wang, H.-M., & Chen, C.-Y. (2009). Antiallergic potential on RBL-2H3 cells of some phenolic constituents of *Zingiber officinale* (ginger). *Journal of Natural Products, 72*, 950-953.

Chen, Y., Wu, Y., Wang, H., & Gao, K. (2012). A new 9-nor-atractylodin from *Atractylodes lancea* and the antibacterial activity of the atractylodin derivatives. *Fitoterapia, 83*, 199-203.

Cheng, C., Ng, D. S. W., Chan, T. K., Guan, S. P., Ho, W. E., Koh, A. H. M., ... Wong, W. S. F. (2013). Anti-allergic action of anti-malarial drug artesunate in experimental mast cell-mediated anaphylactic models. *Allergy, 68*(2), 195-203.

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. DOI: 10.1155/2013/914563

Choudhary, N., & Singh, V. (2017). *Piper longum* Linn: a review of its phytochemicals and their network pharmacological evaluation. *bioRxiv 169763*. Advance online publication. DOI: https://doi.org/10.1101/169763

Chung, F. (2001). Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-γ. *Mediators of Inflammation, 10*, 51-59.

Clark, D. A., & Cokerb, R. (1998). Molecules in focus transforming growth factor-beta (TGF-β). *The International Journal of Biochemistry & Cell Biology, 30*(2), 293-298.

Commins, S. P., Borish, L., & Steinke, J. W. (2010). Immunologic messenger molecules: cytokines, interferons, and chemokines. *Journal of Allegy and Clinical Immunology, 125*(2 Suppl. 2), S53-S72.

Corren, J. (2012). Inhibition of interleukin-5 for the treatment of eosinophilic diseases. *Discovery Medicine, 13*(71), 305-312

Couper, K. N., Blount, D. G., & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *The Journal of Immunology, 180*(9), 5771-5777.

Deng, Y., Liu, Z., & Geng, Y. (2016). Anti-allergic effect of *Artemisia* extract in rats. *Experimental and Therapeutic Medicine, 12*, 1130-1134.

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.

Dhanalakshmi, D., Umamaheswari, S., Balaji, D., Santhanalakshmi, R., & Kavimani, S. (2016). Phytochemistry and pharmacology of *Piper longum* – a systematic review. *World Journal of Pharmacy and Pharmaceutical Sciences, 6*(1), 381-398.

Dhanik, J., Arya, N., & Nand, V. (2017). A review on *Zingiber officinale*. *Journal of Pharmacognosy and Phytochemistry, 6*(3), 174-184.

Dinda, B., Das, S. K., Hajra, A. K., Bhattacharya, A., De, K., Chel, G., & Achari, B. (1999). Chemical constituents of *Plumbago indica* roots and reactions of plumbagin: part II. *Indian Journal of Chemistry, 38B*, 577-582.

Duan, J.-a, Wang, L., Qian, S., Su, S., & Tang, Y. (2008). A new cytotoxic prenylated dihydrobenzofuran derivative and other chemical constituents from the rhizomes of *Atractylodes lancea* DC. *Archives of Pharmacal Research, 31*(8), 965-969.

Dugasani, S., Pichika, M. R., Nadarajah, V. D., Balijepalli, M. K., Tandra, S., & Korlakunta, J. N. (2010). Comparative antioxidant an anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol. *Journal of Ethnopharmacology, 127*(3), 515-520.

Feng, T., Su, J., Ding, Z.-H., Zheng, Y.-T., Li, Y., Leng, Y., & Liu, J.-K. (2011). Chemical constituents and their bioactivities of "Tongling white ginger" (*Zingiber officinale*). *Journal of Agricultural and Food Chemistry, 59*(21), 11690-11695.

Förstermann, U., & Sessa, W. C. (2012). Nitric oxide synthase: regulation and function. *European Heart Journal, 33*, 829-837.

Franceschi, C., & Campisi, J. (2014). Chronic inflammation (inflammaging) and its potential contribution to age-associated disease. *The Journals of Gerontology: Series A, 69*(Suppl. 1), S4-S9.

Franks, F. (1994). Accelerated stability testing of bioproducts: attractions and pitfall. *Trends in Biotechnology, 12*(4), 114-117.

Funk, J. L., Frye, J. B., Oyarzo, J. N., & Timmermann, B. N. (2009). Comparative effects of two gingerol-containing *Zingiber officinale* extracts on experimental rheumatoid arthritis. *Journal of Natural Products, 72*, 403-407.

Furie, M. B. (2017). An overview of inflammation. *Pathobiology of Human Disease, 2014*, 226-230.

Galli, S. J., Tsia, M., & Piliponsky, A. M. (2008). The development of allergic inflammation. *Nature, 454*(7203), 445-454.

Garbo, G., Tessema, B., & Brown, S. M. (2013). Complementary and integrative treatments allergy. *Otolaryngologic Clinics of North America, 46*, 295-307.

Gour, N., & Wills-Karp, M. (2015). IL-4 and IL-13 signaling in allergic airway disease. *Cytokines, 75*, 68-78.

Greenfeder, S., Umland, S. P., Cuss, F. M., Chapman, R. W., & Egan, R. W. (2001). Th2 cytokines and asthma: the role of interleukin-5 in allergic eosinophilic disease. *Respiratory Research, 2*(2), 71-79.

Gupta, R. A., Motiwala, M. N., Dumore, N. G., Danao, K. R., & Ganjare, A. B. (2015). Effect of piperine on inhibition of FFA induced TLR4 mediated inflammation and amelioration of acetic acid induced ulcerative colitis in mice. *Journal of Ethanopharmacology, 164*, 239-246.

Gupta, S. K., & Sharma, A. (2014). Medicinal properties of *Zingiber officinale* Roscoe – a review. *IOSR Journal of Pharmacy and Biological Sciences, 9*(5), 124-129.

Gutiérrez-Cardona, N., Sands, P., Roberts, G., Lucas, J. S., Walker, W., Salib, R., ... Ismail-Koch, H. (2017). The acceptability and tolerability of nasal douching in children with allergic rhinitis: a systematic review. *International Journal of Pediatric Otorhinolaryngology, 98*, 126-135.

Guzik, T. J., Korbut, R., & Adamek-Guzik, T. (2003). Nitric oxide and superoxide in inflammation and immune regulation. *Journal of Physiology and Pharmacology, 54*(4), 469-487.

Hamza, T., Barnett, J. B., & Li, B. (2010). Interleukin 12 a key immunoregulatory cytokine in infection applications. *International Journal of Molecular Sciences, 11*, 789-806.

Hawrylowicz, C. M., & O'Garra, A. (2005). Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nature Reviews Immunology, 5*, 271-283.

Hayat, K., Asim, MB. R., Nawaz, M., Li, M., Zhang, L., & Sun, N. (2011). Ameliorative effect of thymoquinone on ovalbumin-induced allergic conjunctivitis in Balb/c mice. *Current Eye Research, 36*(7), 591-598.

Hematpoor, A., Liew, S. Y., Chong, W. L., Azirun, M. S., Lee, V. S., & Awang, K. (2016). Inhibition and larvicidal activity of phenylpropanoids from *Piper sarmentosum* on acetylcholinesterase against mosquito vectors and their binding mode of interaction. *PLoS ONE, 11*(5), e0155265. DOI: 10.1371/journal.pone.0155265

Hong, J.-F., Song, Y.-F., Liu, Z., Zheng, Z.-C., Chen, H.-J., & Wang, S.-S. (2016). Anticancer activity of taraxerol acetate in human glioblastoma cells and a mouse xenograft model *via* induction of autophagy and apoptotic cell death, cell cycle arrest and inhibition of cell migration. *Molecular Medicine Reports, 13*(6), 4541-4548.

Hossen, M. J., Yang, W. S., Kim, D., Aravinthan, A., Kim, J.-H., & Cho, J. Y. (2017). Thymoquinone: An IRAK1 inhibitor with *in vivo* and *in vitro* anti-inflammatory activities. *Scientific Reports, 7*, 42995. DOI: 10.1038/srep42995

Hotha, K. K., Reddy, S. P. K., Raju, V. K., & Ravindranath, L. K. (2013). Forced degradation studies: practical approach – overview of regulatory guidance and literature for the drug products and drug substances. *International Research Journal of Pharmacy, 4*(5), 78-85.

Huang, J., Zhang, T., Han, S., Cao, J., Chen, Q., & Wang, S. (2014). The inhibitory effect of piperine from *Fructus piperis* extract on the degranulation of RBL-2H3 cells. *Fitoterapia, 99*, 218-226.

Huang, Q., Huang, X., Deng, J., Liu, H., Liu, Y., Yu, K., & Huang, B. (2016). Differential gene expression between leaf and rhizome in *Atractylodes lancea*: a comparative transcriptome analysis. *Frontiers in Plant Science, 7*, 348. DOI: 10.3389/fpls.2016.00348

Hussain, K., Hashmi., F. K., Latif, A., Ismail, Z., & Sadikun, A. (2012). A review of the literature and latest advances in research of *Piper sarmentosum*. *Pharmaceutical Biology, 50*(8), 1045-1052.

Hussain, K., Ismail, Z., Sadikun, A., & Ibrahim, P. (2009). Evaluation of extracts of *Piper sarmentosum* for accelerated stability by metabolomics fingerprint profiling. *Pharmacognosy Research, 1*(4), 185-191.

Imm, J., Zhang, G., Chan, L.-Y., Nitteranon, V., & Parkin, K. L. (2010). [6]-Dehydroshogaol, a minor component in ginger rhizome, exhibits quinone reductase inducing and anti-inflammatory activities that rival those of curcumin. *Food Research International, 43*(8), 2208-2213.

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-S203.

Iwalewa, E. O., McGaw, L. J., Naidoo, V., & Eloff., J. N. (2007). Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology, 6*(25), 2868-2885.

Iyer, S. S., & Cheng, G. (2012). Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Critical Reviews in Immunology, 32*(1), 23-63.

Jin, Y., Wi, H. J., Choi, M.-H., Hong, S.-T., & Bae, Y. M. (2014). Regulation of anti-inflammatory cytokines IL-10 and TGF-β in mouse dendritic cells through treatment with *Clonorchis sinensis* crude antigen. *Experimental & Molecular Medicine, 46*, e74. DOI: 10.1038/emm.2013.144

Kaewbumrung, S., & Panichayupakaranant, P. (2012). Isolation of three antibacterial naphthoquinones from *Plumbago indica* roots and development of a validated quantitative HPLC analytical method. *Natural Product Research, 26*(21), 2020-2023.

Kaushik, D., Rani, R., Kaushik, P., Sacher, D., & Yadav, J. (2012). *In vivo* and *in vitro* antiasthmatic studies of plant *Piper longum* Linn. *International Journal of Pharmacology, 8*(3), 192-197.

Kawamoto, Y., Ueno, Y., Nakahashi, E., Obayashi, M., Sugihara, K., Qiao, S., ... Takeda, K. (2016). Prevention of allergic rhinitis by ginger and the molecular basis of

immunosuppression by 6-gingerol through T cell inactivation. *The Journal of Nutritional Biochemistry, 27*, 112-122.

Kawase, M., He, F., Kubota, A., Hata, J.-y., Kobayakawa, S.-i., & Hiramatsu, M. (2006). Inhibitory effect of *Lactobacillus gasseri* TMC0356 and *Lactobacillus* GG on enhanced vascular permeability of nasal mucosa in experimental allergic rhinitis of rats. *Bioscience, Biotechnology, and Biochemistry, 70*(12), 3025-3030.

Khan, A. M., Shahzad, M., Asim, M. B. R., Imran, M., & Shabbir, A. (2015). *Zingiber officinale* ameliorates allergic asthma *via* suppression of Th2-mediated immune response. *Pharmaceutical Biology, 53*(3), 359-367.

Kim, H. G., Han, E. H., Jang, W.-S., Choi, J. H., Khanal, T., Park, B. H., ... Jeong, H. G. (2012). Piperine inhibits PMA-induced cyclooxygenase-2 expression through downregulating NF-κB, C/EBP and AP-1 signaling pathways in murine macrophages. *Food and Chemical Toxicology, 50*(7), 2342-2348.

Kim, K. Y. (2018). Anti-inflammatory and ECM gene expression modulations of β-eudesmol *via* NF-κB signaling pathway in normal human dermal fibroblasts. *Biomedical Dermatology, 2*, 3. DOI: 10.1186/s41702-017-0014-3

Kondelková, K., Vokurková, D., Krejsek, J., Borská, L., Fiala, Z., & Ctirad, A. (2010). Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disoeders. *Acta Medica (Hradec Králové), 53*(2), 73-77.

Kondo, S., Sattaponpan, C., Phongpaichit, S., Srijan, A., & Itharat, A. (2010). Antibacterial activity of Thai medicinal plants Pikutbenjakul. *Journal of the Medical Association of Thailand, 93*(Suppl. 7), S131-S135.

Koonrungsesomboon, N., Na-Bangchang, K., & Karbwang, J. (2014). Therapeutic potential and pharmacological activities of *Atractylodes lancea* (Thunb.) DC. *Asian Pacific Journal of Tropical Medicine*, 421-428.

Korhonen, R., Lahti, A., Kankaanranta, H., & Moilanen, E. (2005). Nitric oxide production and signaling in inflammation. *Current Drug Targets-Inflammation & Allergy, 4*, 471-479.

Kotawong, K., Chaijaroenkul, W., Muhamad, P., & Na-Bangchang, K. (2018). Cytotoxic avtivities and effects of atracylodin and β-eudesmol on the cell cycle arrest and apoptosis on cholangiocarcinoma cell line. *Journal of Pharmacological Sciences, 136*, 51-56.

Kumar, S., Singhal, V., Roshan, R., Sharma, A., Rembhotkar, G. W., & Ghosh, B. (2007). Piperine inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer through suppression of Nf-κB and IκB kinase activation. *European Journal of Pharmacology, 575*(1-3), 177-186.

Lantz, R. C., Chen, G. J., Sarihan, M., Sólyom, A. M., Jolad, S. D., & Timmermann, B. N. (2007). The effect of extracts from ginger rhizome on inflammatory mediator production. *Phytomedicine, 14*, 123-128.

Larché, M., Akdis, C. A., & Valenta, R. (2006). Immunological mechanisms of allergen-specific immunotherapy. *Nature Reviews Immunology, 6*(1), 761-771.

Laroux, F. S., Pavlick, K. P., Hines, I. N., Kawachi, S., Harada, H., Bharwani, S., ... Grisham, M. B. (2001). Role of nitric oxide in inflammation. *Acta Physiologica Scandinavica, 173*, 113-118.

Larsen, J. N., Broge, L., & Jacobi, H. (2016). Allergy immunotherapy: the future of allergy treatment. *Drug Discovery Today, 21*(1), 26-37.

Lauw, F. N., Florquin, S., Speelman, P., Van Deventer, S. J. H., & Van Der Poll, T. (2001). Role of endogenous interleukin-12 in immune response to staphylococcal enterotoxin B in mice. *Infection and Immunity, 69*(9), 5949-5952.

Lee, M.-Y., Seo, C.-S., Lee, J.-A., Lee, N.-H., Kim, J.-H., Ha, H., ... Shin, H.-K. (2011). Anti-asthmatic effects of *Angelica dahurica* against ovalbumin-induced airway inflammation *via* upregulation of heme oxygenase-1. *Food and Chemical Toxicology, 49*, 829-837.

Li, F., Nitternon, V., Tang, X., Liang, J., Zhang, G., Parkin, K. L., & Hu, Q. (2012). *In vitro* antioxidant and anti-inflammatory activities of 1-dehydro-[6]-gingerdione, 6-shogaol, 6-dehydroshogaol and hexahydrocurcumin. *Food Chemistry, 135*(2), 332-337.

Li, J., Hua, Y., Ji, P., Yao, W., Zhao, H., Zhong, L., & Wei, Y. (2010). Effects of volatile oils of *Angelica sinensis* on an acute inflammation rat model. *Pharmaceutical Biology, 54*(9), 1881-1890.

Lim, M. Y., & Leong, J. L. (2010). Allergic rhinitis: evidence-based practice. *Singapore Medical Journal, 51*(7), 542-550.

Liu, R., Tao, E., Yu, S., Liu, B., Dai, L., Yu, L., ... Lai, X. (2016). The suppressive effects of the petroleum ether fraction from *Atractylodes lancea* (Thunb.) DC. on a collagen-induced arthritis model. *Phytotherapy Research, 30*, 1672-1679.

Mahavorasirikul, W., Viyanant, V., Chaijaroenkul, W., Itharat, A., & Na-Bangchang, K. (2010). Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. *BMC Complementary and Alternative Medicine, 10*, 55. DOI: 10.1186/1472-6882-10-55

Majdalawieh, A., & Ro, H.-S. (2010). Regulation of IκBα function and NF-κB signaling: AEBP1 is a novel proinflammatory mediator in macrophages. *Mediators of Inflammation, 2010*, 823821. DOI: 10.1155/2010/823821

Makchuchit, S., Itharat, A., & Tewtrakul, S. (2010). Antioxidant and nitric oxide inhibition activities of Thai medicinal plants. *Journal of the Medical Association of Thailand, 93*(Suppl. 7), S227-S235.

May, J. R., & Dolen, W. K. (2017). Management of allergic rhinitis: a review for the community pharmacist. *Clinical Therapeutics, 39*(12), 2410-2419.

Medzhitov, R. (2010). Inflammation 2010: new adventures of an old flame. *Cell, 140*, 771-776.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*, 428-435.

Meechan, P., Tungtrongchitr, A., Chaisri, U., Maklon, K., Indrawattana, N., Chaicumpa, W., & Sookrung, N. (2013). Intranasal, liposome-adjuvanted cockroach allergy vaccines made of refined major allergen and whole-body extract of *Periplaneta americana*. *International Archives of Allergy and Immunology, 161*, 351-362.

Mo, J.-H., Lee, S.-E., Wee, J. H., Lee, J.-E., Rhee, C.-S., Lee, C. H., & Kim, D.-Y. (2013). Anti-allergic effects of So-Cheong-Ryong-Tang, a traditional Korean herbal medicine, in an allergic rhinitis mouse model. *European Archives of Oto-Rhino-Laryngology, 270*(3), 923-930.

Motwani, M. P., Newson, J., Kwong, S., Richard-Loendt, A., Calas, R., Dalli, J., & Gilroy, D. W. (2017). Prolonged immune alteration following resolution of acute inflammation in humans. *PLoS ONE, 12*(10), e0186964. DOI: 10.1371/journal.pone.0186964

Na, J.-Y., Song, K., Lee, J.-W., Kim, S., & Kwon, J. (2016). Pretreatment of 6-shogaol attenuates oxidative stress and inflammation in middle cerebral artery occlusion-induced mice. *European Journal of Pharmacology, 788*, 241-247.

Nakai, Y., Kido, T., Hashimoto, K., Kase, Y., Sakakibara, I., Higuchi, M., & Sasaki, H. (2003). Effect of the rhizomes of *Atractylodes lancea* and its constituents on the delay of gastric emptying. *Journal of Ethnopharmocology, 84*(1), 51-55.

Naseri, M., Mojab, F., Khodadoost, M., Kamalinejad, M., Davati, A., Choopani, R., ... Emtiazy, M. (2012). The study of anti-inflammatory activity of oil-based dill (*Anethum graveolens* L.) extract used topically in formalin-induced inflammation male rat paw. *Iranian Journal of Pharmaceutical Research, 11*(4), 1169-1174.

Nathan, C. (2002). Points of control in inflammation. *Nature, 420*, 846-852.

Ngo, S. T., & Li, M. S. (2013). Top-leads from natural products for treatment of Alzheimer's disease: docking and molecular dynamics study. *Molecular Simulation, 39*(4), 279-291.

Nurtjahja-Tjendraputra, E., Ammit, A. J., Roufogalis, B. D., Tran, V. H., & Duke, C. C. (2003). Effective anti-platelet and COX-1 enzyme inhibitors from pungent constituents of ginger. *Thrombosis Research, 111*, 259-265.

O'Conner, C., & Nichol, A. Inflammation, immunity and allergy. *Anaesthesia and Intensive Care Medicine, 16*(7), 328-333.

Ojewole, J. A. O. (2006). Analgesic, anti-inflammatory and hypoglycaemin effects of ethanol extract of *Zingiber officinale* (Roscoe) rhizomes (Zingiberaceae) in mice and rats. *Phytotherapy Research, 20*, 764-772.

Olmos, G., & Lladó, J. (2014). Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. *Mediators of Inflammation, 2014*, 861231. DOI: 10.1155/2014/861231

Ormstad, H., Groeng, E.-C., Duffort, O., & Løvik, M. (2003). The effect of endotoxin on the production of IgE, IgG1 and IgG2a antibodies against the cat allergen Fel d 1 in mice. *Toxicology, 188*, 309-318.

Ouyang, Z., Zhang, L., Zhao, M., Wang, P., Wei, Y., & Fang, J. (2012). Identification and quantification of sesquiterpenes and polyacetylenes in *Atractylodes lancea* from various geographical origins using GC-MS analysis. *Revista Brasileira de Farmacognosia Brazilian Journal of Pharmacognosy, 22*, 957-963.

Ozdoganoglu, T., & Songu, M. (2012). The burden of allergic rhinitis and asthma. *Therapeutic Advances in Respiratory Disease, 6*(1), 11-23.

Pan, M.-H., Chiou, Y.-S., Tsai, M.-L., & Ho, C.-T. (2011). Anti-inflammatory activity of traditional Chinese medicinal herbs. *Journal of Traditional and Complementary Medicine, 1*(1), 8-24.

Pan, M.-H., Hsieh, M-C., Hsu, P.-C., Ho, S.-Y., Lai, C.-S., Wu, H., ... Ho, C.-T. (2008). 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Molecular Nutrition & Food Research, 52*, 1467-1477.

Panthong, S., & Itharat, A. (2014). Effect of *Piper chaba* Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz. on natural killer cell activity and lymphocyte proliferation. *Journal of the Medical Association of Thailand, 97*(Suppl. 8), S133-S139.

Park, G., Oh, D.-S., Lee, M. G., Lee, C. E., & Kim, Y.-u. (2016). 6-Shogaol, an active compound of ginger, alleviates allergic dermatitis-like skin lesions *via* cytokine inhibition by activating the Nrf2 pathway. *Toxicology and Applied Pharmacology, 310*, 51-59.

Parmar, V. S., Jain, S. C., Bisht, K. S., Jain, R., Taneja, P., Jha, A., ... Boll, P. M. (1997). Phytochemistry of the genus *Piper*. *Phytochemistry, 46*(4), 597-673.

Passante, E., Ehrhardt, C., Sheridan, H., & Frankish. (2009). RBL-2H3 cells are imprecise model for mast cell mediator release. *Inflammation Research, 58*(9), 611-618.

Passante, E., & Frankish, N. (2009). The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflammation Research, 58*(11), 737-745.

Pawankar, R., Mori, S., Ozu, C., & Kimura, S. (2011). Overview on the pathomechanisms of allergic rhinitis. *Asia Pacific Allergy, 1*, 157-167.

Plengsuriyakarn, T., Karbwang, J., & Na-Bangchang, K. (2015). Anticancer activity using positron emission tomography-computed tomography and pharmacokinetics of β-eudesmol in human cholangiocarcinoma xenografted nude mouse model. *Clinical and Experimental Pharmacology and Physiology, 42*(3), 293-304.

Plengsuriyakarn, T., Viyanant, V., Eursitthichai, V., Picha, P., Kupradinun, P., Itharat, A., & Na-Bangchang, K. (2012). Anticancer activities against cholangiocarcinoma, toxicity and pharmacological activities of Thai medicinal plants in animal models. *BMC Complementary and Alternative Medicine, 12*, 23. DOI: 10.1186/1472-6882-12-23

Popa, C., Netea, M. G., Van Riel, P. L. C. M., Van Der Meer, J. W. M., & Stalenhoef, F. H. (2007). The role of TNF-α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *The Journal of Lipid Research, 48*, 751-762.

Rachawat, P., Pinsornsak, P., Kanokkangsadal, P., Itharat, A. (2017). Clinical efficacy and safety of Benjakul remedy extract for treating primary osteoarthritis of knee compared with diclofenac: double blind, randomized controlled trial. *Evidence-Based Complementary and Alternative Medicine, 2017*, 9593580. DOI: 10.1155/2017/9593580

Rahman, S. F. S. A., Sijam, K., & Omar, D. (2016). *Piper sarmentosum* Roxb. : a mini review of ethnobotany, phytochemistry and pharmacology. *Journal of Analytical & Pharmaceutical Research, 2*(5), 00031. DOI: 10.15406/japlr.2016.02.00031

Rahman, U. ur., Ali, S., Ubaidullah, Khan, I., Khan, M. A., Arif, S., & Rahman, S. U. (2016). Anti-inflammatory activity of taraxerol acetate. *Journal of Medical Sciences, 24*(4), 216-219.

Rahman, U. ur., Durrani, S., Ubaidullah, Ali, S., & Rahman, S. u. (2016). Anti-pyretic activity of taraxerol acetate. *Kaohsiung Journal of Medical Sciences, 9*(2), 165-167.

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

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*(Suppl. 8), S70-S75.

Rattarom, R., Sakpakdeejaroen, I., & Itharat, A. (2010). Cytotoxic effects of the ethanolic extract from Benjakul formula and its compounds on human lung cancer cells. *Thai Journal of Pharmacology, 32*(1), 99-101.

Rehman, U. U., Shah, J., Khan, M. A., Shah, M. R., Ishtiaq, & Khan, I. (2013). Molecular docking of taraxerol acetate as a new COX inhibitor, *A Journal of the Bangladesh Pharmacological Society, 8*, 194-197.

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-S134.

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.

Saporta, D. (2012). Efficacy of sublingual immunotherapy *versus* subcutaneous injection immunotherapy in allergic patients. *Journal of Environmental and Public Health, 2012*, 492405. DOI: 10.1155/2012/492405

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

Sattaponpan, C., & Kondo, S. (2011). Antibacterial activity of crude extracts of Prasaprohyai formula and its components against pathogenic bacteria. *Journal of the Medical Association of Thailand, 94*(Suppl. 7), S153-S161.

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology, 75*, 163-189.

Seo, M.-J., Kim, S.-J., Kang, T.-H., Rim, H.-K., Jeong, H.-J., Um, J.-Y., ... Kim, H.-M. (2011). The regulatory mechanism of β-eudesmol is through the suppression of caspase-1 activation in mast cell-mediated inflammatory response. *Immunopharmacology and Immunotoxicology, 33*(1), 178-185.

Sharma, J. N., Al-Omran, A., & Parvathy, S. S. (2007). Role of nitric oxide in inflammatory diseases. *Inflammopharmacology, 15*(6), 252-259.

Sharma, P. K., Singh, V., & Ali, M. (2016). Chemical composition and antimicrobial activity of fresh rhizome essential oil of *Zingiber officinale* Roscoe. *Pharmacognosy Journal, 8*(3), 185-190.

Shim, S., Kim, S., Choi, D.-S., Kwon, Y.-B., & Kwon, J. (2011). Anti-inflammatory effect of [6]-shogaol: potential roles of HDAC inhibition and HSP70 induction. *Food and Chemical Toxicology, 49*, 2734-2740.

Singh, N., Kumar, S., Singh, P., Raj, H. G., Prasad, A. K., Parmar, V. S., & Ghosh, B. (2008). *Piper longum* Linn. extract inhibits TNF-α-induced expression of cell adhesion molecules by inhibiting NF-κb 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. *International Scholarly Research Network Pharmacology, 2012*, 480265. DOI: 10.5402/2012/480265

Sireeratawong, S., Vannasiri, S., Sritiwong, S., Itharat., A., & Jaijoy, K. (2010). Anti-inflammatory, anti-nociceptive and antipyretic effects of the ethanol extract from root of *Piper sarmentosum* Roxb. *Journal of the Medical Association of Thailand, 93*(Suppl. 7), S1-S6.

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-κB and phosphorylation of JNK in HMC-1 cells. *Immunopharmacology and Immunotoxicology, 35*(4), 462-470.

Sookrung, N., Indrawattana, N., Tungtrongchitr, A., Karuhassuwan, C., Chaisri, U., & Chaicumpa, W. (2008). A murine model of allergy caused by American cockroach (CR), *Periplaneta americana*. *Asian Pacific Journal of Allergy and Immunology, 26*, 143-139.

Stankov, S. V. (2012). Definition of inflammation, causes of inflammation and possible anti-inflammatory strategies. *The Open Inflammation Journal, 5*, 1-9.

Stôhr, J. R., Xiao, P.-G., & Bauer, R. (2001). Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*. *Journal of Ethnopharmacology, 75*, 133-139.

Stuehr, D. J., Kwon, N. S., Nathan, C. F., & Griffith, O. W. (1991). N^{ω}-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *The Journal of Biological Chemistry, 266*(10), 6259-6263.

Sukkasem, N., Chatuphonprasert, W., Tatiya-aphiradee, N., & Jarukamjorn, K. (2016). Imbalance of the antioxidative system by plumbagin and *Plumbago indica* L. extract induces hepatotoxicity in mice. *Journal of Intercultural Ethanopharmacology, 5*(2), 137-145.

Sun, J., Zhang, X., Broderick, M., & Fein, H. (2003). Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors, 3*, 276-284.

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

Suthanurak, M., Sakpakdeejaroen, I., Rattarom, R., & Itharat, A. (2010). Formulation and stability test of Benjakul extract tablets: a preliminary study. *Thai Journal of Pharmacology, 32*(1), 160-163.

Tan, B., Shi, H. L., Ji, G., & Xie, J. Q. (2011). Effects of taraxerol and taraxerol acetate on cell cycle and apoptosis of human gastric epithelial cell line AGC. *Journal of Chinese Integrative Medicine, 9*(6), 638-642.

Tang, F., Fan, K., Wang, K., & Bian, C. (2018). Atractylodin attenuates lipopolysaccharide-induced acute lung injury by inhibiting NLPR3 inflammasome and TLR4 pathways. *Journal of Pharmacological Sciences, 2018*. DOI: 10.1016/j.jphs.2017.11.010

Tasaniyananda, N., Chaisri, U., Tungtrongchitr, A., Chaicumpa, W., & Sookrung, N. (2016). Mouse model of cat allergic rhinitis and intranasal liposome-adjuvanted refined Feld1 vaccine. *PLoS ONE, 11*(3), e0150463. DOI: 10.1371/journal.pone.0150463

Tewtrakul, S., & Itharat, A. (2007). Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. *Journal of Ethnopharmacology, 190*(3), 412-416.

Tewtrakul, S., & Subhadhirasakul, S. (2007). Anti-allergic activity of some selected plants in the Zingiberaceae family. *Journal of Ethnopharmacology, 109*(3), 535-538.

Tewtrakul, S., Subhadhirasakul, S., & Kummee, S. (2008). Anti-allergic activity of compounds from *Kaempferia parviflora*. *Journal of Ethnopharmacology, 116*(1), 191-193.

Thebpatiphat, S., Pengprecha, S., & Ternai, B. (1988). 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.

Thomson, M., Al-Qattan, K. K., Al-Sawan, S. M., Alnaqeeb, M. A., Khan, I., & Ali, M. (2002). The use of ginger (*Zingiber officinale* Rosc.) as a potential anti-inflammatory and antithrombotic agent. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 67*(6), 475-478.

Tjendraputra, E., Tran, V. H., Liu-Brennan, D., Roufogalis, B. D., & Duke, C. C. (2001). Effect of ginger constituents and synthetic analogues on cyclooxygenase-2 enzyme in intact cells. *Bioorganic Chemistry, 29*, 156-163.

Tripathi, S., Bruch, D., & Kittur, D. S. (2008). Ginger extract inhibits LPS induced macrophage activation and function. *BMC Complementary and Alternative Medicine, 8*, 1. DOI: 10.1186/1472-6882-8-1

Tungsukruthai, P., Nootim, P., Worakunphanich, W., & Tabtong, N. (2018). Efficacy and safety of herbal steam bath in allergic rhinitis: a randomized controlled trial. *Journal od Integrative Medicine, 16*, 39-44.

Tuntiwachwuttikul, P., Phansa, P., Pootaeng-on, Y., & Taylor, W. C. (2006). Chemical constituents of the roots of *Piper sarmentosum*. *Chemical and Pharmaceutical Bulletin, 54*(2), 149-151.

Umar, S., Sarwar, A. H. M. G., Umar, K., Ahmad, N., Sajad, M., Ahmad, S., ... Khan, H. A. (2013). Piperine ameliorates oxidative stress, inflammation and histological outcome in collagen induced arthritis. *Cellular Immunology, 284*, 51-59.

Van Breemen, R. B., Tao, Y., & Li, W. (2011). Cyclooxygenase-2 inhibitors in ginger (*Zingiber officinale*). *Fitoterapia, 82*(1), 38-43.

Veeresham, C. (2012). Natural products derived from plants as a source of drugs. *Journal of Advanced Pharmaceutical Technology & Research, 3*(4), 200-201.

Vieira, G. C., Gadelha, F. A. A. F., Pereira, R. F., Ferreira, L. K. D. P., Barbosa-Filho, J. M., Bozza, P. T., & Piuvezam, M. R. (2018). Warifteine, an alkaloid of *Cissampelos sympodialis*, modulates allergic profile in a chronic allergic rhinitis model. *Revista Brasileira de Farmacognosia, 28*(1), 50-56.

Vongthoung, K., Kamchansuppasin, A., Temrangsee, P., Munkong, N., & Kaendee, N. (2016). Effects of Benjakul water extract on pancreas in high-fat fed rats. *Thammasat Medical Journal, 16*(2), 161-175.

Wambre, E., James, E. A., & Kwok, W. W. (2012). Characterization of $CD4^+$ T cell subsets in allergy. *Current Opinion in Immunology, 24*(6), 700-706.

Wang, A., Xiao, Z., Zhou, L., Zhang, J., Li, X., & He, Q. (2016). The protective effect of atractylenolide I on systemic inflammation in the mouse model of sepsis created by cecal ligation and puncture. *Pharmaceutical Biology, 54*(1), 146-150.

Wang, H., Zhang, J., Gao, C., Zhu, Y., Wang, C., & Zheng, W. (2007). Topical levamisole hydrochloride therapy attenuates experimental murine allergic rhinitis. *European Journal of Pharmacology, 577*(1-3), 162-169.

Wang, T., Wu, F., Jin, Z., Zhai, Z., Wang, Y., Tu, B., ... Tang, T. (2014). Plumbagin inhibits LPS-induced inflammation through the inactivation of the nuclear factor-kappa B and mitogen activated protein kinase signaling pathways in RAW 264.7 cells. *Food and Chemical Toxicology, 64*, 177-183.

Wei, M., Xie, X., Chu, X., Yang, X., Guan, M., & Wang, D. (2013). Dihydroartemisinin suppresses ovalbumin-induced airway inflammation in mouse allergic asthma model. *Immunopharmacology and Immunotoxicology, 35*(3), 382-389.

Wheatley, L. M., & Togias, A. (2015). Allergic rhinitis. *The New England Journal of Medicine, 372*(5), 456-463.

Williams, C. M. M., Rahman, S., Hubeau, C., & Ma, H.-L. (2012). Cytokine pathways in allergic disease. *Toxicologic Pathology, 40*(2), 205-215.

Wu, T.-F., Chan, Y.-Y., Shi, W.-Y., & Jhong, M.-T. (2017). Uncovering the molecular mechanism of anti-allergic activity of silkworm pupa-grown *Cordyceps militaris* fruits body. *The American Journal of Chinese Medicine, 45*(3), 497-513.

Xu, K., Feng, Z.-M., Jiang, J.-S., Yang, Y.-N., & Zhang, P.-C. (2017). Sesquiterpenoid and C14-polyacetylene glycosides from the rhizomes of *Atractylodes lancea*. *Chinese Chemical Letters, 28*, 597-601.

Yamai, H., Sawada, N., Yoshida, T., Seike, J., Takizawa, H., Kenzaki, K., ... Tangohu, A. (2009). Triterpenes augment the inhibitory effects of anticancer drugs on growth of human esophageal carcinoma cells in vitro and suppress experimental metastasis in vivo. *International Journal of Cancer, 125*(4), 952-960.

Yin, M., Xiao, C.-C., Chen, Y., Wang, M., Guan, F.-q., Wang, Q.-z., ... Feng, X. (2015). A new sesquiterpenoid glycoside from rhizomes of *Atractylodes lancea*. *Chinese Herbal Medicines, 7*(4), 371-374.

Young, H.-Y., Luo, Y.-L., Cheng, H.-Y., Hsieh, W.-C., Liao, J.-C., & Peng, W.-H. (2005). Analgesic and anti-inflammatory activities of [6]-gingerol. *Journal of Ethnopharmacology, 96*(1-2), 207-210.

Yu, C., Xiong, Y., Chen, D., Li, Y., Xu, B., Lin, Y., ... Wang, L. (2017). Ameliorative effects of atractylodin on intestinal inflammation and co-occurring dysmotility in both constipation and diarrhea prominent rats. *The Korean Journal of Physiology & Pharmacology, 21*(1), 1-9.

Zhang, J.-l., Huang, W.-m., & Zeng, Q.-y. (2015). Atractylenolide I protects mice from lipopolysaccharide-induced acute lung injury. *European Journal of Pharmacology, 765*, 94-99.

Zhang, Y., Liu, C., Dong, B., Ma, X., Hou, L., Cao, X., & Wang, C. (2015). Anti-inflammatory activity and mechanism of surfactin in lipopolysaccharide-activated macrophages. *Inflammation, 38*(2), 756-764.

Other Materials

Bielory, L. (2014). *Complementary and alternative therapies for allergic rhinitis and conjunctivitis*. Retrieved from https://www.uptodate.com/contents/complementary-and-alternative-therapies-for-allergic-rhinitis-and-conjunctivitis

British Pharmacopoeia Commission. (2013). *British pharmacopoeia 2013 volume III*. Retrieved from https://youngpharmacists.com/2014/04/11/british-pharmacopoeia-2013-by-british-pharmacopoeia-commission/

Flora of China. (1999). *Piperaceae*. Retrieved from http://flora.huh.harvard.edu/china/mss/volume04/PIPERACEAE.published.pdf

International Conference on Harmonization. (2003). *ICH harmonized tripartite guideline: Stability testing of new drug substances and products Q1A (R2)*. Retrieved from

http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf

National Drug Committee. (2013). *National list of essential medicines (NLEM)*. Retrieved from http://drug.fda.moph.go.th:81/nlem.in.th/sites/default/files/attachments/herbal_book_56.pdf

Rhee, C.-S. (2013). *Allergic rhinitis model*. Retrieved from http://www.allergy.or.kr/journal/abst/2013s/19.pdf

APPENDICES

APPENDIX A

CHEMICAL REAGENTS FOR *IN VITRO* EXPERIMENTS

1. Reagents for cell culture

1.1 Fetal bovine serum (FBS, inactivated)

Slowly thaw the frozen FBS at 4ºC overnight, heat-inactivated at 56ºC for 30 min, mix gently, aliquot and store at -20ºC.

1.2 Penicillin Streptomycin (P/S)

Slowly thaw the frozen P/S at 37ºC until completely thaw, gently mix, aliquot and store at -20ºC.

1.3 0.5% Trypsin-EDTA

Slowly thaw the frozen 0.05% trypsin-EDTA at 37ºC until completely thaw, gently mix, aliquot and store at -20ºC.

1.4 Basic minimum essential medium (MEM)

Reagents

MEM medium powder	9.7	g
$NaHCO_3$	2.2	g
Sterile deionized water	1,000	ml

Directions

Dissolve MEM powder and $NaHCO_3$ with sterile deionized water. Adjust pH to 7.2-7.6 with 10% HCl or 10% NaOH, filter through 0.2 μm membrane filter and store at 4ºC.

1.5 Complete MEM medium for RBL-2H3 cells

Add 15% FBS (inactivated) and 1% P/S into basic MEM medium.

1.6 Basic RPMI medium 1640

Reagents

RPMI medium 1640 powder	10.4	g
$NaHCO_3$	2.0	g
Sterile deionized water	1,000	ml

Directions

Dissolve RPMI powder and $NaHCO_3$ with sterile deionized water. Adjust pH to 7.2-7.6 with 10% HCl or 10% NaOH, filter through 0.2 μm membrane filter and store at 4ºC.

1.7 Complete RPMI medium 1640 for RAW 264.7 cells

Add 10% FBS (inactivated) and 1% P/S into basic RPMI medium 1640.

1.8 Phosphate buffered saline (PBS)

Reagents

PBS	1	tablet
Deionized water (DI water)	100	ml

Directions

Dissolve 1 tablet of PBS in 100 ml of DI water, sterilize by autoclaving at 121ºC for 15 min and store at 4ºC.

2. Reagents for β-hexosaminidase assay

2.1 Stock anti-DNP IgE solution and working anti-DNP IgE solution

Stock anti-DNP IgE solution (50 μg/ml)

Reagents

Anti-DNP IgE	0.5	ml
Sterile PBS	9.5	ml

Directions

Dissolve 0.5 ml of anti-DNP IgE in 9.5 ml of sterile PBS, aliquot 100 μl per micro tube and store at -20ºC.

Working anti-DNP IgE solution (Freshly prepare)

Regents

Stock DNP-IgE solution	100	μl
Complete MEM medium	900	μl

Directions

Add 900 μl of complete MEM medium into 100 μl of stock DNP-IgE solution

2.2 DNP-BSA solution (0.1 mg/ml)

Reagents

DNP-BSA	10.0	mg
Buffer A	100	ml

Directions

Dissolve 10.0 mg of DNP-BSA in 100 ml of buffer A, aliquot 1 ml per micro tube and store at -20°C.

2.3 Citric/citrate buffer (0.1 M, pH 4.5)

Reagents

Citric acid monohydrate (0.1 M)	10.51	g/500 ml DI water
Trisodium citrate dihydrate (0.1 M)	14.71	g/500 ml DI water

Directions

Mix citric acid monohydrate solution and trisodium citrate dihydrate solution, adjust pH to 4.5 and store at room temperature.

2.4 Carbonate/bicarbonate buffer (0.1 M stop solution, pH 10.0)

Reagents

Na_2CO_3 (0.1 M)	5.3	g/500 ml DI water
$NaHCO_3$ (0.1 M)	4.2	g/500 ml DI water

Directions

Mix Na_2CO_3 solution and $NaHCO_3$ solution together, adjust pH to 10.0 and store at room temperature.

2.5 Buffer A (Siraganian buffer, pH 7.2)

Reagents

NaCl (119 mM)	6.954	g
KCl (5 mM)	0.373	g
D-(+)-glucose (5.6 mM)	1.009	g
$MgCl_2.6H_2O$ (0.4 mM)	0.081	g

$CaCl_2.2H_2O$ (1 mM)	0.147	g
PIPES (25 mM)	8.396	g
Bovine serum albumin (BSA) (0.1%)	1.000	g
DI water	1,000	ml

Directions

Dissolve and mix thoroughly. Adjust pH to 7.2 with 0.1 N NaOH and store at room temperature.

3. Reagents for nitric oxide assay

3.1 Griess reagent

Reagents

Sulfanilamide	1.0	g
N-(1-Naphthyl)ethylenediamine dihydrochloride	0.1	g
Phosphoric acid	2.5	g

Directions

Dissolve all reagents in 100 ml of DI water and store at 4°C.

3.2 MTT solution (5 mg/ml)

Reagents

Thiazolyl blue tetrazolium bromide	1.0	g
Sterile PBS	200	ml

Directions

Dissolve 1.0 g of MTT in 200 ml of sterile PBS and store at 4°C (protect from light).

3.3 0.04 M HCl in isopropanol

Reagents

Hydrochloric acid (37%)	0.83	ml
Isopropanol	249.17	ml

Directions

Adjust volume to 250 ml with isopropanol and store at room temperature.

4. Reagents for tumor necrosis factor-alpha ELISA kit

4.1 Washing buffer

Reagents

Wash buffer	20	ml
Sterile deionized water	475	ml

Directions

Dissolve and mix gently and store at 4°C for 30 days.

4.2 Mouse TNF-α control

Reagents

Mouse TNF-α control	1	vial
Sterile DI water	1.0	ml

Directions

Add 1.0 ml of sterile DI water into mouse TNF-α control vial, mix thoroughly (freshly prepared).

4.3 Mouse TNF-α standard

Reagents

Mouse TNF-α standard	1	vial
Sterile DI water	1.0	ml

Directions

Dissolve mouse TNF-α standard with sterile DI water to producing a stock solution of 7,000 pg/ml and mix gently prior to making dilutions (freshly prepared).

4.4 Substrate solution

Reagents

Color reagent A

Color reagent B

Directions

Mix color reagents A and B together in equal volumes within 15 min of use (protect from light).

5. Forced degradation study

5.1 3N Hydrochloric acid (HCl)

Reagents

Hydrochloric acid (37%)	24.88	ml
DI water	75.12	ml

Directions

Adjust volume to 100 ml with DI water and store at room temperature.

5.2 3N Sodium hydroxide (NaOH)

Reagents

Sodium hydroxide	12.0	g
DI water	100	ml

Directions

Dissolve 12.0 g of sodium hydroxide in 100 ml of DI water and store at room temperature.

5.3 30% Hydrogen peroxide (H_2O_2)

Reagents

Hydrogen peroxide (50%)	60	ml
DI water	40	ml

Directions

Adjust volume to 100 ml with DI water and store at room temperature.

APPENDIX B

CHEMICAL REAGENTS FOR *IN VIVO* EXPERIMENTS

1. Reagents for indirect ELISA

1.1 Phosphate buffered saline (PBS) (0.01 M, pH 7.4)

Reagents

NaCl (137 mM)	8.00	g
KCl (2.7 mM)	0.20	g
Na_2HPO_4 (10 mM)	1.44	g
KH_2PO_4 (2 mM)	0.24	g
Ultrapure deionized water (UDW)	1,000	ml

Directions

Dissolve and mix thoroughly. Adjust pH to 7.4 with HCl. Sterilize by autoclaving at 121°C for 15 min and store at room temperature.

1.2 Coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6)

Reagents

$NaHCO_3$	2.10	g
Na_2CO_3 (0.05 M)	0.53	g/100 ml UDW
Ultrapure deionized water	500	ml

Directions

Dissolve 2.1 g of $NaHCO_3$ in 400 ml of UDW, adjust pH to 9.6 with 0.05 M Na_2CO_3, adjust volume to 500 ml with UDW and store at 4°C.

1.3 PBST buffer (0.05% Tween-20)

Reagents

PBS (0.01 M)	1,000	ml
Tween-20	0.5	ml

Directions

Suspend PBS with 0.05% tween-20. Mix thoroughly and store at room temperature.

1.4 Diluent (0.2% gelatin and 0.2% BSA)

Reagents

PBS (0.01 M)	100	ml
Gelatin type B from bovine skin	0.2	g
BSA (code A8022)	0.2	g

Directions

Dissolve 0.2 g of gelatin in PBS, heat at 60ºC until completely dissolving. Keep at room temperature before add 0.2 g of BSA and store at 4ºC.

1.5 Blocking solution (1% BSA)

Regents

PBS (0.01 M)	100	ml
BSA (code A8022)	1.0	g

Directions

Dissolve 1.0 g of BSA in 100 ml of PBS, vortex and store at 4ºC.

1.6 Stop solution (1% SDS)

Reagents

Ultrapure deionized water	100	ml
Sodium dodecyl sulfate (SDS)	1.0	g

Directions

Dissolve 1.0 g of SDS in 100 ml of ultrapure deionized water, vortex and store at room temperature.

2. Reagents for histopathology of nasal cavity

2.1 Fixative (5% paraformaldehyde and 4% sucrose)

Reagents

Paraformaldehyde (PFA)	50.0	g
Sucrose	40.0	g
PBS (0.01 M)	1,000	ml
NaOH	one-by-one	

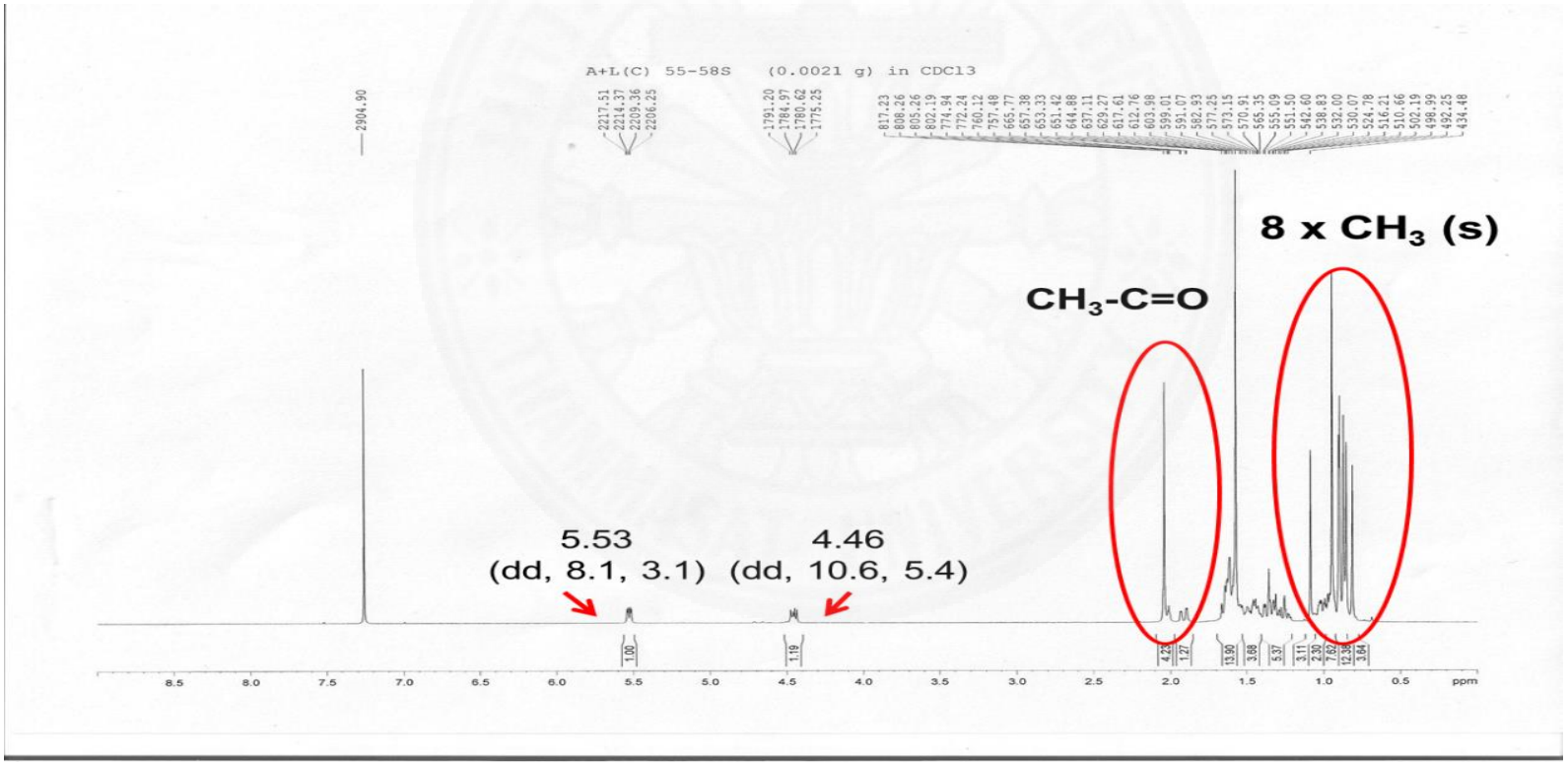
Directions

Dissolve 50.0 g of PFA in PBS. Stir and heat at 60ºC then add one-by-one of NaOH into the solution until completely dissolving. Add 40.0 g of sucrose into the solution then adjust pH to 7.4 with HCl (37%). Filter through 0.2 μm membrane filter and store at 4ºC (freshly prepared).

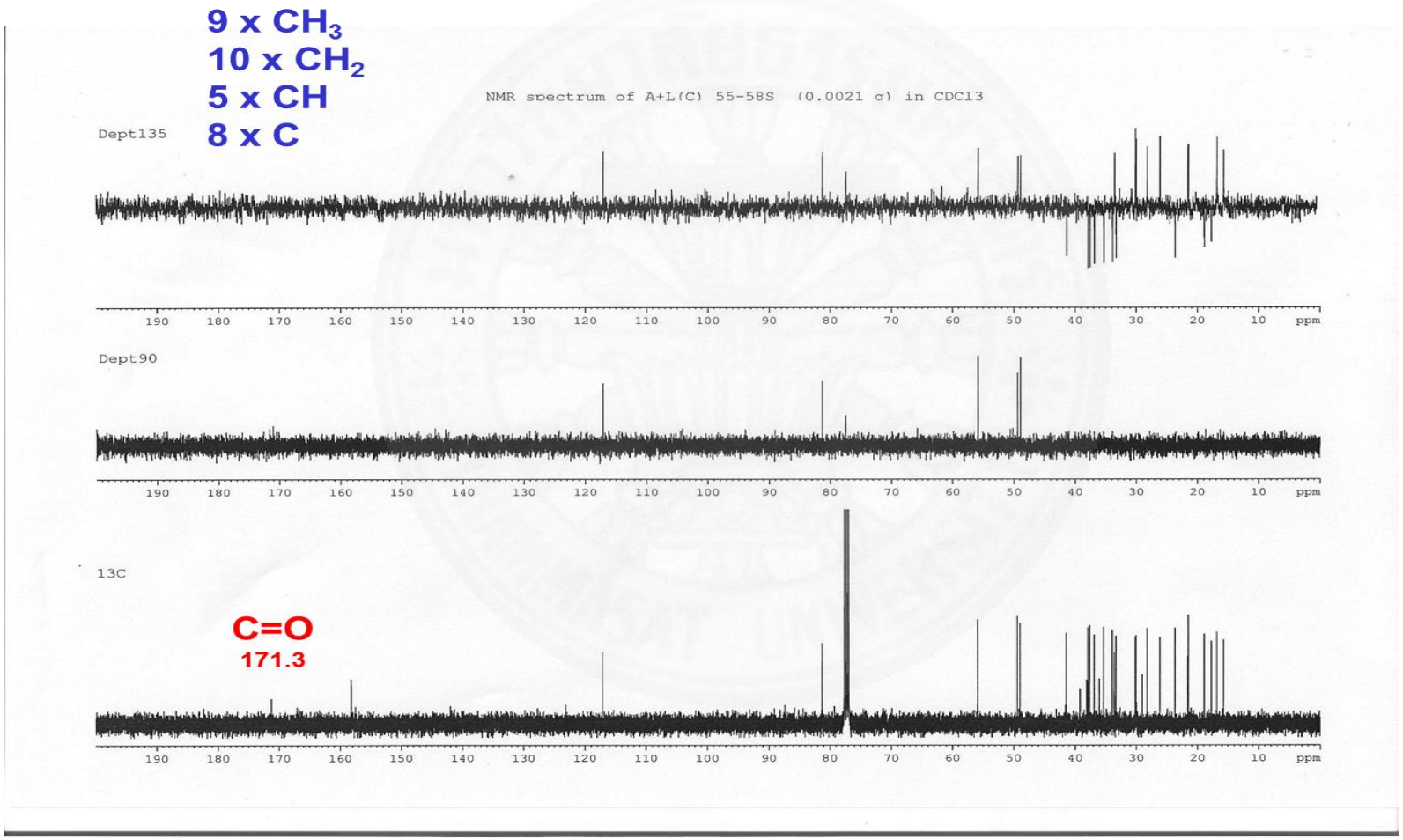
APPENDIX C

^{1}H NMR AND ^{13}C NMR OF TARAXEROL ACETATE

1. ^{1}H NMR (400 MHz, $CDCl_3$) of taraxerol acetate



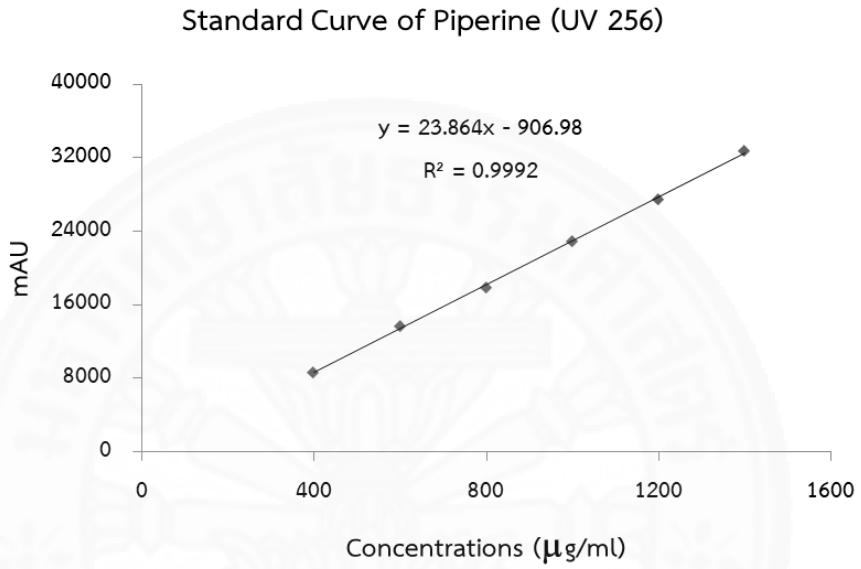
2. ^{13}C NMR (100 MHz, $CDCl_3$) of taraxerol acetate



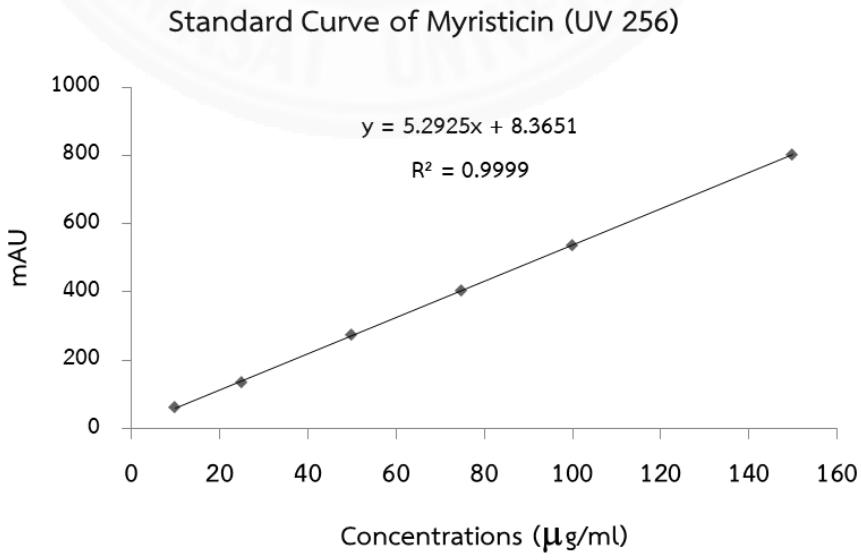
APPENDIX D

STANDARD CURVES OF PURE COMPOUNDS ANALYZE BY HPLC

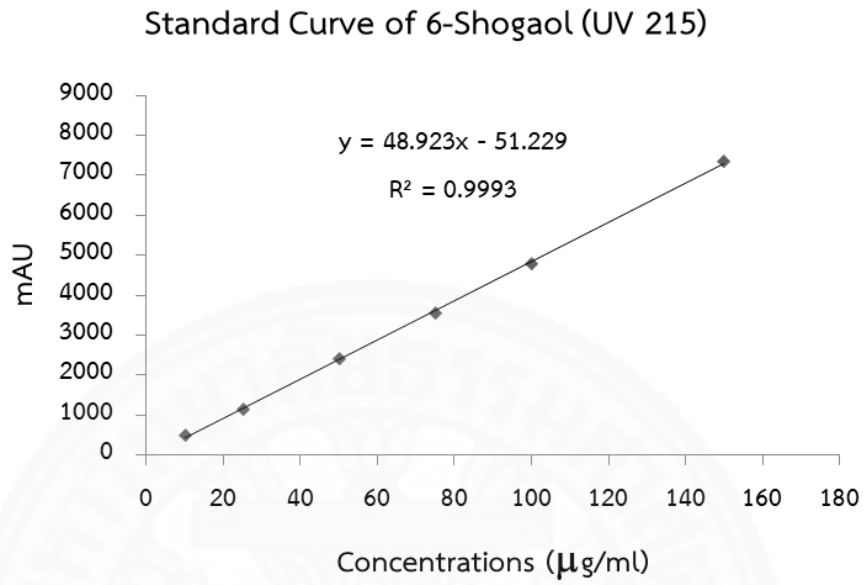
1. Standard curve of piperine



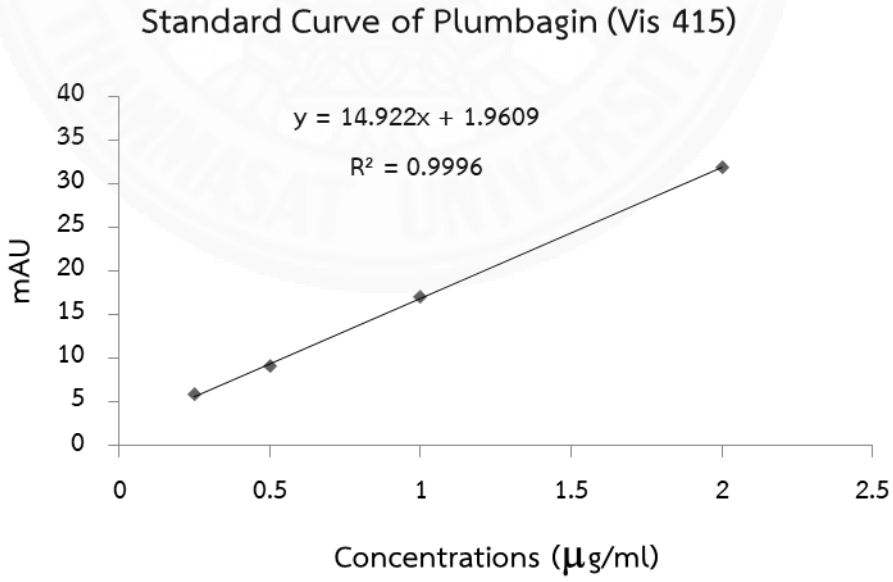
2. Standard curve of myristicin



3. Standard curve of 6-shogaol



4. Standard curve of plumbagin



BIOGRAPHY

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Publications

Makchuchit, S., Rattarom, R., & Itharat, A. (2017). The anti-allergic and anti-inflammatory effects of Benjakul extract (a Thai traditional medicine), its constituent plants and its some pure constituents using *in vitro* experiments. *Biomedicine & Pharmacotherapy, 89*, 1018-1026. (Ph.D. Publication)

Itharat, A., Uttama, S., & **Makchuchit, S.** (2016). Anti-inflammatory activities of constituents in Sang Yod rice extracts, γ-oryzanol, vitamins E, B1, B2 and B3, using inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS) activated RAW 264.7 murine macrophage cells. *Medicinal & Aromatic Plants, 5*(3), 1000244.

Jai-aue, A., **Makchuchit, S.,** Juckmeta, T., & Itharat, A. (2014). Anti-allergic, anti-inflammatory and antioxidant activities of the different extracts of Thai

traditional remedy called Prabchompoothaweep for allergic rhinitis treatment. *Journal of the Medical Association of Thailand, 97*(Suppl. 8), S140-S148.

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. *Journal of the Medical Association of Thailand, 97*(Suppl. 8), S125-S132.

Sakpakdeejaroen, I., **Makchuchit, S.,** & Itharat, A. (2014). Nitric oxide inhibitory activity of herbal extract formulae for anti-inflammation. *Thammasat Medical Journal, 14*(1), 7-12.

Mukkasombut, N., Chanvimalueng, W., **Makchuchit, S.,** & Itharat, A. (2013). Correlation of biological activities on different extraction period times and stability study of Pra-sa-prao-yai remedy. *Thammasat Medical journal, 13*(4), 504-512.

Kanokkangsadal, P., Pinsornsak, P., **Makchuchit, S.,** & Itharat, A. (2013). Correlation of extraction time with biological activities and stability test of crude Sahastara remedy. *Thammasat Medical journal, 13*(4), 496-503.

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.

Kakatum, N., Jaiarree, N., **Makchucit, S.,** & Itharat, A. (2012). Antioxidant and anti-inflammatory activities of Thai medicinal plants in Sahasthara remedy for muscle pain treatment. *Journal of the Medical Association of Thailand, 95*(Supply. 1), S120-S126.

Tappayuthpijarn, P., Itharat, A., & **Makchuchit, S.** (2011). Acetylcholinesterase inhibitory activity of Thai traditional nootropic remedy and its herbal ingredients. *Journal of the Medical Association of Thailand, 94*(Supply. 7), S183-S189.

Makchuchit, S., Itharat, A., & Tewtrakul, S. (2010). Antioxidant and nitric oxide inhibition activities of Thai medicinal plants. *Journal of the Medical Association of Thailand, 93*(Supply. 7), S227-S235.

Makchuchit, S., & Itharat, A. (2010). Nitric oxide inhibitory activity of Thai medicinal plants called Kod-Tung-Ha. *Thai Journal of Pharmacology, 32*(1), 188-191. (Proceeding)

Itharat, A., **Makchuchit, S.,** & Tewtrakul, S. (2009). Anti-inflammatory activity of Thai traditional medicine preparation called Prasaprohyai. *Journal of Medicinal Plant and Natural Product Research, 75*, 1043. (Abstract)

Makchuchit, S., Itharat, A., & Tewtrakul, S. (2009). Anti-allergic activity of Thai medicinal plants. *Journal of Medicinal Plant and Natural Product Research, 75*, 956. (Abstract)

Presentations

Makchuchit, S., & Itharat, A. (2017). Inhibitory effect of chemical constituents from Benjakul ethanolic extract on β-hexosaminidase release in RBL-2H3 cells and nitric oxide production in RAW 264.7 cells. Books of program and abstracts of the 2nd ONE HEALTH International Conference 2017 on One Health Concept: How can we collaborate?, 14-15 December 2017, Centara Grand at Central Plaza Ladprao, Bangkok, Thailand. (Ph.D. Oral Presentation)

Makchuchit, S., & Itharat, A. (2017). Determination of anti-allergic and anti-inflammatory effects of Benjakul extract after accelerated storage conditions. Abstracts of RGJ-Ph.D. Congress 18 on Global Sustainability: Lesson Learned from the Royal Projects, 8-10 June 2017, Richmond Stylish Convention Hotel, Nonthaburi, Thailand. (Ph.D. Poster Presentation)

Makchuchit, S., & Itharat, A. (2016). Effect of Benjakul extract on nitric oxide production in RAW264.7 cells after being subjected to accelerated storage conditions. (p.200). Book of abstracts of 20th World Congress on Clinical Nutrition (WCCN) “Traditional Medicine, Functional Food, Nutrition, Natural Health Product and Spiritual Healing: Additional Tools for Healthcare Delivery”, 14-16 December 2016, Bangkok, Thailand. (Ph.D. Poster Presentation)

Makchuchit, S., & Itharat, A. (2015). The anti-allergic activity of Prasaprohyai preparation extract after accelerated stability testing. (p.3084). Book of abstracts of World Academy of Science Engineering and Technology: International Scholarly and Scientific Research & Innovation, 28-29 May 2015, Tokyo, Japan. (Ph.D. e-Poster Presentation)

Lairungruang, K., **Makchuchit, S.,** & Itharat, A. (2014). Cytotoxic and antioxidant activities of *Achasma sphaerocephalum* Holtt. rhizomes extracts. (p.144). Book of program and abstracts of 18th World Congress on Clinical Nutrition (WCCN) "Agriculture, Food and Nutrition for Health and Wellness", 1-3 December 2014, Ubon Ratchathani, Thailand.

Makchuchit, S., & Itharat, A. (2014). The inhibitory effect of *Piper interruptum*, *Piper longum*, and *Piper sarmentosum* on nitric oxide and tumor necrosis factor-alpha release by lipopolysaccharide-activated RAW 264.7 cells. (p.78). Book of program and abstracts of 18th World Congress on Clinical Nutrition (WCCN) "Agriculture, Food and Nutrition for Health and Wellness", 1-3 December 2014, Ubon Ratchathani, Thailand. (Ph.D. Oral Presentation)

Lairungruang, K., Itharat, A., **Makchuchit, S.,** & Panthong, S. (2014). Biological activities of *Areca catechu* L. extracts for oral cancer and laryngeal cancer. (pp.389-390). Book of abstracts of MED TU Conference Knowledge on the run for Smart physicians, 16-18 July 2014, Faculty of Medicine, Thammasat University, Pathumthani, Thailand.

Juckmeta, T., Itharat, A., & **Makchuchit, S.** (2014). Cytotoxic, anti-inflammatory and antioxidant activities of *Tiliacora triandra* (Yanang). (p.77). Book of abstracts of 1st Joint ACS AGFD-ACS ICSCT Symposium on Agricultural and Food Chemistry, 4-5 March 2014, Montien Riverside Hotel Bangkok, Thailand.

Makchuchit, S., Sakpakdeejaroen, I., & Itharat, A. (2014). Anti-inflammatory activity of *Piper interruptum* extract by inhibition of nitric oxide and tumor necrosis factor-alpha production in lipopolysaccharide-activated RAW 264.7 murine macrophage cells. (p.74). Book of abstracts of 1st Joint ACS AGFD-ACS ICSCT Symposium on Agricultural and Food Chemistry, 4-5 March 2014, Montien Riverside Hotel Bangkok, Thailand. (Ph.D. Poster Presentation)

Makchuchit, S., & Itharat, A. (2012). Anti-allergic and anti-inflammatory activities of Prasaprohyai formula. (p.56). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012, Thammasat University, Pathumthani, Thailand. (Ph.D. Oral Presentation)

Kanokkangsadal, P., Pinsornsak, P., **Makchuchit, S.,** & Itharat, A. (2012). Study on extraction time of crude Sahastara remedy for standardization analysis. (p.55). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012, Thammasat University, Pathumthani, Thailand.

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, Pathumthani, Thailand.

Mukkasombut, N., Chanvimalueng, W., **Makchuchit, S.,** & Itharat, A. (2012). DPPH free radical scavenging and total phenolic content values of Pr-sa-prao-yai on different extraction time periods. (p.51). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012, Thammasat University, Pathumthani, Thailand.

Anuthakoengkun, A., **Makchuchit, S.,** & Itharat, A. (2012). Inhibitory effect on nitric oxide production and antioxidant activity of Thai medicinal plants in Zingiberaceae family for osteoarthritis treatment. (p.46). Book of abstracts of 1st Conference on Graduate Student Network of Thailand, 18 December 2012, Thammasat University, Pathumthani, Thailand.

Thongdeeying, P., **Makchuchit, S.,** Panthong, S., Ruangnoo, S., & Itharat, A. (2012). Inhibitory effect on LPS-induced PGE_2 release in RAW264.7 cells of *Dioscorea membranacea* extract and its ingredient. (p.440). Book of abstracts and proceedings NATPRO 4: the 4th International Conference on Natural Products for Health and Beauty, 28-30 November 2012, Chiang Mai, Thailand.

Makchuchit, S., Sakpakdeejaroen, I., & Itharat, A. (2012). Anti-allergic activity of Benjakul formula and its component (p.293). Book of abstracts and proceedings NATPRO 4: the 4th International Conference on Natural Products

for Health and Beauty, 28-30 November 2012, Chiang Mai, Thailand. (Ph.D. Poster Presentation)

Anuthakoengkun, A., Itharat, A., & **Makchuchit, S.** (2012). Nitric oxide inhibitory and antioxidant activities of Thai medicinal plants used for osteoarthritis patients. (p.288). Book of abstracts and proceedings NATPRO 4: the 4th International Conference on Natural Products for Health and Beauty, 28-30 November 2012, Chiang Mai, Thailand.

Itharat, A., Ruangnoo, S., **Makchuchit, S.,** Thongdeeying, P., & Panthong, S. (2012). Anti-inflammatory compounds of *Smilax corbularia* Kunth. (p.49). Book of abstracts of MED TU Conference from the Best Practice to Patient Education, 10-12 July 2012, Faculty of Medicine, Thammasat University, Pathumthani, Thailand.

Makchuchit, S., & Itharat, A. Nitric oxide inhibitory activity of Thai medicinal plants called Kod-Tung-Ha. (pp.188-191). Book of proceedings of 32th Pharmacological and Therapeutic Society of Thailand Meeting, 25-26 March 2010, Thammasat University, Pathumthani, Thailand. (Poster Presentation)

Makchuchit, S., & Itharat, A. (2009). Anti-allergic activity of Thai traditional medicine preparation called Prasaprohyai. (p.127). Book of program and abstracts of 6th National Herb Expo, 2-6 September 2009, Bangkok, Thailand. (Poster Presentation)

Makchuchit, S., Itharat, A., & Tewtrakul, S. (2009). Anti-allergic activity of Thai medicinal plants. (p.956). Book of abstracts of 57th International Congress and Annual Meeting of the GA, 16-20 August 2009, Geneva, Switzerland. (Poster Presentation)

Makchuchit, S., & Itharat, A. (2009). Anti-allergic activity of Thai traditional medicine preparation called Prasaprohyai. (p.188). Book of abstracts of MED TU FORUM 2009 Changes: New Trends in Medicine, 14-17 July 2009, Thammasat University, Pathumthani, Thailand. (Oral Presentation)

Awards

The outstanding award for poster presentation on “Effect of Benjakul extract on nitric oxide production in RAW264.7 cells after being subjected to accelerated storage conditions” in the 20th World Congress on Clinical Nutrition (WCCN), 14-16 December 2016, Bangkok, Thailand. (Ph.D. Award)

The 2nd award for oral presentation of graduate student on “Anti-allergic activity of Thai traditional medicine preparation called Prasaprohyai” in MED TU FORUM 2009, 17 July 2009, Thammasat University, Pathumthani, Thailand.