ANTI-ALLERGIC ACTIVITY OF TRIKATUK, TRIPHALA AND TRISARN REMEDIES

BY

MISS NAPAPORN PATTANACHAROENCHAI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN MEDICAL SCIENCES

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was approved as partial fulfillment of the requirements for
the degree of Master of Science Program in Medical sciences

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ABSTRACT

Trikatuk remedy (TK) has long been used in Thai traditional medicine for adaptogen and for treating disease in the rainy season. It comprises of *Piper nigrum* seed, *Piper retrofractum* flower and *Zingiber officinale* rhizome. Triphala remedy (TP) is used to adjust patients' elements in summer, is composed of dried fruit of three medicinal plants, namely *Phyllanthus emblica*, *Terminalia bellirica* and *Terminalia chebula*. Trisarn remedy (TS) is a Thai traditional medicine used in winter, consist of three plants namely *Piper interruptum* vine, *Piper sarmentosum* root and *Plumbago indica* root. There is no report to comparative the anti-allergic activity of three remedies and the activity related to allergy such as anti-inflammatory. Therefore, the objectives of this research were to study the anti-allergic activity of TK, TP, TS and its ingredients. Then, the remedy which show the strongest anti-allergic properties was choosed to study anti-inflammatory effects. Three remedies were extracts boiling in water and macerated in 95% ethanol, plant ingredients were extracts with 95% ethanol to obtain 15 extracts. The extract which showed the highest anti-allergic and anti-inflammatory activities was selected to study for stability. The results of screening on antiallergy indicated that the ethanolic extract of Trikatuk remedy exhibit the highest anti-allergic

activity against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells, with an IC_{50} value of 38.02±1.34 μg/ml, followed by the ethanolic extract of *Piper nigrum*, *Piper retrofractum*, *Plumbago indica*, *Piper interruptum* and *Zingiber officinale* (IC_{50} value of 44.97±6.16, 50.91±6.44, 63.55±3.77, 78.30±3.09 and 81.85±12.00 μg/ml respectively). The water extract of Trikatuk Triphala and Trisarn remedies and other plants were apparently inactive (IC_{50} > 100 μg/ml). Thus, this research selected the ethanolic extract of Trikatuk which showed the best anti-allergic activity to continuously studied for anti-inflammatory and stability test. The results of new extract of Trikatuk remedy and its ingredients show that the ethanolic extract of *Piper nigrum* exhibited the highest anti-allergic activity with an IC_{50} value of 22.4±2.35 μg/ml. It also showed higher anti-allergic activity than Chlorpheniramine (CPM) (IC_{50} value 26.13±1.89 μg/ml) but not significantly different (p-value < 0.05), followed by the ethanolic extract of Trikatuk with an IC_{50} value 28.87±1.13 μg/ml, the ethanolic extract of *Piper retrofractum* (IC_{50} value 47.49±1.03 μg/ml) and the ethanolic extract of *Zingiber officinale* (IC_{50} value 50.07±4.33 μg/ml). The ethanolic extract of *Zingiber officinale* and the ethanolic extract of Trikatuk exhibited the most potent anti-inflammation by inhibitory effect against nitric oxide (NO) production in RAW 264.7 cells, with an IC_{50} value 19.41±1.19 and 24.35±0.81 μg/ml, which was not significantly different prednisolone (IC_{50} value 21.93±0.37 μg/ml) (p-value < 0.05), followed by the ethanolic extract of *Piper nigrum* and the ethanolic extract of *Piper retrofractum* (IC_{50} value 33.23±2.33 and 35.89±2.51 μg/ml, respectively). The study on chemical fingerprint was carried out using Reverse Phase High Performance Liquid Chromatography (HPLC) and including the study on specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy for validate the HPLC method. The results exhibited that HPLC method showed good specificity, linearity, lower LOD and LOQ, precision and accuracy. The ethanolic extract of Trikatuk remedy was evaluated for stability under accelerated conditions (40ºC, 75±5%RH for 6 months) and evaluated for inhibitory effect of β-hexosaminidase from RBL-2H3 cells showed that Trikatuk was

significantly since day 120 from day 0, and inhibition of NO production from RAW 264.7 cells showed the highly stable as the activities was not significantly different from day 0 (p-value < 0.05). Piperine, 6-gingerol and 6-shogaol determined contents as marker compounds by using HPLC method. Furthermore, the amount of piperine at day 90 and day 180 were increase than day 0 (p-value < 0.05) but 6-gingerol was more quickly reduced (34.57%) after day 180 and 6-shogaol content on day 15, day 30, day 60, day 90 and day 180 were not significantly different from day 0, while the amount of 6-shogaol at day 120 and day 150 were increase than day 0 and also showed significant difference (p-value < 0.05).

In conclusion, these findings indicated that the ethanolic extract of Trikatuk remedy showed the highest *in vitro* anti-allergic and anti-inflammatory activities, and active compounds of Trikatuk remedy are stable except 6-gingerol. These results can support the use of Trikatuk as a adaptogenic drug for treatment of allergic and inflammatory related diseases Trikatuk can be used instead of allergic steroid drug. The ethanolic of Trikatuk should be developed into modern medicine for anti-allergic treatment in the future. However, 6-gingerol was unstable. Thus, Trikatuk preparation should be kept in freezer for use.

Keywords: Anti-allergic, Anti-inflammatory, Adaptogenic drug, Thai traditional medicine, HPLC validation method

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บทคัดย่อ

พิกัดยาตรีกฏุกทางการแพทย์แผนไทยใช้เป็นยาปรับธาตุในฤดูฝน ประกอบด้วยพืช 3 ชนิดคือ ผลพริกไทย ดอกดีปลี และเหง้าขิง พิกัดยาตรีผลาใช้ปรับธาตุในฤดูร้อน ประกอบด้วยผลของมะขามป้อม สมอไทย และสมอพิเภก พิกัดยาตรีสารใช้ปรับธาตุในฤดูหนาว ประกอบด้วยเถาสะค้าน รากช้าพลู และรากเจตมูลเพลิงแดง ปัจจุบันยังไม่มีรายงานการศึกษาเพื่อเปรียบเทียบฤทธิ์ต้านการแพ้ของพิกัดยาทั้ง 3 การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านการแพ้ของพิกัดตรีกฏุก ตรีผลา ตรีสาร และเลือกพิกัดยาที่มีฤทธิ์ต้านการแพ้เพื่อมาศึกษาฤทธิ์ต้านการอักเสบ สารสกัดที่มีฤทธิ์ต้านการแพ้และต้านการอักเสบได้ดีจะถูกนำมาศึกษาองค์ประกอบทางเคมีและความคงตัวของสารสกัดภายใต้สภาวะเร่งเพื่อใช้ในการควบคุมคุณภาพของยาสมุนไพร พิกัดยาทั้ง 3 นำมาสกัดโดยการต้มน้ำและหมักด้วยเอทานอล 95% สมุนไพรเดี่ยวแต่ละตัวในตำรับถูกนำมาสกัดด้วยเอทานอล 95% ได้สารสกัดทั้งหมด 15 ตัวอย่าง สารสกัดทั้งหมดที่ได้ถูกนำมาทดสอบฤทธิ์ทางชีวภาพโดยศึกษาฤทธิ์ต้านการแพ้โดยการยับยั้งการหลั่งเอนไซม์ β-hexosaminidase พบว่า สารสกัดด้วยเอทานอล 95% ของพิกัดตรีกฏุก เมล็ดพริกไทยดำ ผลดีปลี รากเจตมูลเพลิงแดง เถาสะค้านและเหง้าขิงมีฤทธิ์ต้านการแพ้ได้ดี โดยมีค่าความเข้มข้นของสารสกัดที่ยับยั้งการหลั่งเอนไซม์ β-hexosaminidase ได้ 50% (IC_{50}เท่ากับ 38.02±1.04, 44.97±6.16, 50.91±6.44, 63.55±3.77, 78.30±3.09 และ 81.85±12.00 ไมโครกรัม/มิลลิลิตร ตามลำดับ) ส่วนสารสกัดโดยการต้มน้ำ และสารสกัดด้วยเอทานอลตัวอื่นไม่มีฤทธิ์ยับยั้งเอนไซม์ดังกล่าว ดังนั้นจึงเลือกพิกัดตรีกฏุก และสมุนไพรเดี่ยวในตำรับ มาสกัดใหม่ด้วยเอทานอล 95% เพื่อศึกษาฤทธิ์ต้านการแพ้ ฤทธิ์ต้านการอักเสบ ศึกษาองค์ประกอบทางเคมีและความคงตัวของสารสกัดภายใต้สภาวะเร่ง พบว่าเมล็ดพริกไทยดำ มีฤทธิ์ต้านการแพ้ได้ดีที่สุดโดยมีค่าความเข้มข้นของสารสกัดที่ยับยั้งการหลั่งเอนไซม์ β-hexosaminidase ได้ 50% (IC_{50} เท่ากับ 22.4±2.35 ไมโครกรัม/มิลลิลิตร) โดยไม่มีความแตกต่างกับ Chlorpheniramine ซึ่งมีค่า IC_{50} เท่ากับ 26.13±1.89 ไมโครกรัม/มิลลิลิตร (p-value<0.05) รองลงมาคือสารสกัดพิกัดตรีกฏุก ผลดีปลี และเหง้าขิง (IC_{50}

เท่ากับ 28.87±1.13, 47.49±1.03 และ 50.07±4.33 ไมโครกรัม/มิลลิลิตร ตามลำดับ) การศึกษาฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่งไนตริกออกไซด์พบว่าสารสกัดของเหง้าขิงและพิกัดตรีกฏุกมีฤทธิ์ในการยับยั้งการหลั่งไนตริกออกไซด์ที่ดีที่สุดโดยมีค่า IC_{50} เท่ากับ 19.41±1.19 และ 24.35±0.81 ไมโครกรัม/มิลลิลิตร โดยไม่มีความแตกต่างกับสารมาตรฐาน Prednisolone ซึ่งมีค่า IC_{50} เท่ากับ 21.93±0.37 ไมโครกรัม/มิลลิลิตร (p-value<0.05) รองลงมาคือพริกไทยดำและดีปลี โดยมีค่า IC_{50} เท่ากับ 33.23±2.33 และ 35.89±2.51 ไมโครกรัม/มิลลิลิตร ตามลำดับ การศึกษาองค์ประกอบทางเคมีของสารสกัดตรีกฏุก ด้วยเทคนิคโครมาโตรกราฟีของเหลวสมรรถนะสูง (HPLC) โดยใช้สารสำคัญที่เป็นตัวเทียบ (marker) คือ piperine, 6-gingerol และ 6-shogaol นอกจากนั้นยังต้องมีการตรวจสอบความใช้ได้ของวิธีวิเคราะห์ (validate method) โดยพิจารณาจากความจำเพาะ (specificity) ความตรง (linearity and range) ปริมาณต่ำสุดของสารที่สามารถตรวจวัดได้ (LOD) ปริมาณต่ำสุดของสารที่สามารถวิเคราะห์ได้ (LOQ) ความเที่ยง (precision) และความถูกต้อง (accuracy) พบว่าวิธีวิเคราะห์ที่นำมาใช้นั้นมีความจำเพาะ ความตรง ปริมาณสารต่ำสุดของสารที่สามารถตรวจวัดและปริมาณต่ำสุดของสารที่สามารถวิเคราะห์ได้ก็มีค่าต่ำ มีความเที่ยงและมีความถูกต้อง การศึกษาความคงตัวของสารสกัดตรีกฏุกภายใต้สภาวะเร่งที่อุณหภูมิ 40°C ความชื้นสัมพัทธ์ 75±5% เป็นเวลา 6 เดือน แล้วนำมาทดสอบฤทธิ์ต้านการแพ้และฤทธิ์ต้านการอักเสบ พบว่าสารสกัดตรีกฏุกมีฤทธิ์ในการยับยั้งยับยั้งการหลั่งเอนไซม์ β-hexosaminidase ลดลงที่ 120 วัน และยังคงมีฤทธิ์ต้านการอักเสบอยู่ไม่เปลี่ยนแปลงจนถึง 180 วัน จากนั้นนำมาหาปริมาณสารสำคัญในพิกัดตรีกฏุกพบว่า piperine มีความคงตัวที่ดี และมีปริมาณเพิ่มขึ้นที่ 90 และ 180 วัน 6-gingerol ลดลงจนเหลือปริมาณ 34.57% เมื่อสิ้นสุดการทดลอง (180) วัน ส่วน 6-shogaol มีปริมาณเพิ่มขึ้น ที่ 120 และ 150 วัน

จากผลการทดลองทั้งหมดสรุปได้ว่าสารสกัดด้วยเอทานอล 95% ของพิกัดตรีกฏุกมีฤทธิ์ต้านการแพ้และมีฤทธิ์ต้านการอักเสบได้ดี สารสำคัญในตำรับมีความคงตัวสูงยกเว้น 6-gingerol ซึ่งข้อมูลเหล่านี้สนับสนุนการใช้พิกัดยาตรีกฏุกในการปรับสมดุลของร่างกายที่เกี่ยวข้องกับโรคภูมิแพ้ พิกัดยาตรีกฏุกมีความคงตัวสูงสามารถนำใช้แทนยาสเตียรอยด์ และพัฒนารูปแบบยาให้ใช้ง่ายขึ้น อย่างไรก็ตามในส่วนของการควบคุมคุณภาพและทดสอบความคงตัวแสดงให้เห็นว่า สาร 6-gingerol ไม่คงตัว ดังนั้นจึงควรเก็บสารสกัดตรีกฏุกไว้ในที่อุณหภูมิต่ำ หรืออุณหภูมิ -20°C เพื่อป้องกันการสูญเสียของสารสำคัญในตำรับ

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Miss Napaporn Pattanacharoenchai

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ACN	Acetonitrile
ANOVA	Analysis of variance
ATCC	American type culture collection
Anti DNP IgE	Anti-dinitrophenyl-Immunoglobulin E
BSA	Bovine serum albumin
ºC	Degree Celsius
C18	Covalently-bonded octadecylsilane
$CaCl_2$	Calcium chloride
CC	Column chromatography
$CHCl_3$	Chloroform
cm	Centimeter
cm^3	Cubic centimeter
CO_2	Carbon dioxide
Conc.	Concentration
DI	Deionized water
DMSO	Dimethyl sulfoxide
DNP-BSA	Albumin dinitrophenyl
e.g.	Example gratia, for example
et al	Et alii, and other
etc.	Et cetera, and other things
EtOH	Ethanol
EtOAc	Ethyl acetate
FBS	Fetal bovine serum
FcεRI	High-affinity IgE receptor
g	Gram
g/l	Gram per liter

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
hr	Hour
H_2O	Water
IC_{50}	Concentration causing 50% inhibitory effect
IgE	Immunoglobulin E
IL	Interlukin
KCl	Potassium chloride
Kg	Kilogram
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
m	Meter
M	Molar (concentration)
MEM	Minimum essential medium eagle
MeOH	Methanol
mg	Milligram
$MgCl_2$	Magnesium chloride
mg/ml	Milligram per milliliter
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mol	Mole

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
N	Normality
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NF-kB	Nuclear factor-kappa B
ng	Nanogram
NK	Natural killer cell
nm	Nanometer
NO	Nitric oxide
NO_2	Nitrite
OD	Optical density
PBS	Phosphate buffer saline
PGE2	Prostaglandin E_2
pH	Potential of hydrogen ion
PIPES	Piperazine-*N*, *N*'- bis (2-ethanesulfonic acid)
PNAG	p-nitrophenyl *N*-acetyl-β-D-glucosaminide
P/S	Penicillin/streptomycin
RAW264.7	Murine macrophage leukemia
RBL-2H3	Rat basophilic leukemia
RP	Reverse phase

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
rpm	Revolution per minute
RPMI1640	Roswell Park Memorial Institute 1640
RT	Retention time (for HPLC)
SA	Sulfanilamide
SEM	Standard error of the mean
Th1	T helper 1 cell
Th2	T helper 2 cell
TNF-α	Tumor necrosis factor-alpha
UV	Ultraviolet
WHO	World health organization
w/v	Weight by volume
w/w	Weight by weight
%	Percent
>	More than
<	Less than
=	Equal
/	Per
&	And
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microliter
µg/ml	Microgram per milliliter
µM	Micromolar

CHAPTER 1
INTRODUCTION

1.1 Introduction

Allergy is an immune dysfunction, which is a serious health problem worldwide. Substances that cause of allergic reaction are called allergens including food, pollen, dust mites, cosmetics, mold spores and animal hairs (Tewtrakul *et al.*, 2009). The incidence of asthma and allergy, defined as immunologically mediated hypersensitivity, is increasing. It is estimated that over 20% of the world population suffers from IgE-mediated allergic diseases, such as asthma, rhinitis, conjunctivitis, atopic eczema/atopic dermatitis, and anaphylaxis. Asthma is estimated by the World Health Organization (WHO) to affect about 150 million people worldwide (WHO, 2002), hundreds of millions of people in the world have rhinitis and it is estimated that 235 million people have asthma (Larsen *et al.*, 2015). Asthma is a chronic inflammatory disease with high incidence, about 300 million people worldwide. Its prevalence is expected to increase particularly in the pediatric population. In Europe asthma affects around 30 million people and the total cost of this disease is estimated to be 17.7 billion euro/year with a productivity loss of 9.8 billion euro/year. In particular, the European Lung Foundation reports that in UK 3.4 million people (1:7 in the 2-15 years old group and 1:25 in adults) need asthma therapy. Different asthma phenotypes have been identified on to basic of various types of inflammatory cells with a potential critical role in the pathogenesis of this disease by secreting cytokines and pro-inflammatory molecules (Chini *et al.*, 2014). Asthma is characterized by airway inflammation mediated through infiltration of eosinophils, neutrophils and mast cells in the airway wall and related airway smooth muscle constriction. Chronic and/or recurrent airway inflammation, mucous hyper secretion, and airway smooth muscle mediated bronchoconstriction conspire to make the airflow limitation, symptoms and signs of

asthma (Gaffin *et al.*, 2014). In Thailand the prevalence of allergic disorders in children was estimated to be as high as 38% (7 million people), and 20% in adults. About 15% and 7% of children and adults, respectively suffer from allergic asthma. About 80% of allergic rhinitis patients could be related to allergic asthma, whereas about 40% of allergic asthma patients could be related to allergic rhinitis (Bunnak, 2007).

Hypersensitivity type I, an allergic reaction, is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. The histamine causes smooth muscle contraction, increases vascular permeability and vasodilation. The early phase reaction of allergy occurs within minutes after allergen exposure, whereas the late phase reaction occurs hours later and involves cytokines secretion such as TNF-α and IL-4 (Goldsby & Kuby, 2002).

Mast cells have long been regarded as central to the initiation and mediation of the early phase of allergic inflammation and may also be responsible for the initiation of chronic allergic inflammation. Mast cell-derived histamine, PGD_2 and LTC_4 together produce the symptoms of the early response to allergen challenge. The role of the mast cell in the initiation of chronic allergic inflammation is less well established. Mast cells contain IL-3, IL-4, IL-5, IL-6 and TNF_α (Church & Holgate, 1993) and that they may release during both early and late phase of hypersensitivity and are required for both production of Th2 cytokine and the migration of Th2 cells to the sites of allergic inflammation are released by mast cells and macrophages. This release is dependent on the antigen-IgE complex (Mo *et al.*, 2011). These mediators induce the enzyme inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO) in nasal mucosa (Hanazawa *et al.*, 2000) nitric oxide is present in high concentrations in patients with rhinitis and asthma (Cobos Barroso *et al.*, 2008). The inhibition of the release of histamine, interleukins, mediators, TNF-α and nitric oxide are the role of treatments for allergic inflammation. Therefore, this study is to investigate the anti-allergic and anti-inflammatory effects of Trikatuk, Triphala and Trisarn which are used as adaptogens. In this study, anti-allergic activity was determined by inhibitory activity of the extracts

on antigen-induced β-hexosaminidase release as a marker of degranulation in rat basophilic leukemia (RBL-2H3) cells (Tewtrakul *et al.*, 2009). Griess reagent was used to measure the anti-inflammatory activity by inhibitory effects of all extracts on nitric oxide (NO) production activated by lipopolysaccharide (LPS) in RAW264.7 cell lines (Tewtrakul & Itharat, 2007).

Trikatuk is a Thai traditional medicine, used as an adaptogenic drug for treating diseases of the rainy season such as flatulence, sweating and anorexia. It comprises *Piper nigrum* (black pepper), *Piper retrofractum* (long pepper) and *Zingiber officinale* (ginger).

Triphala, used to adjust the four basic elements (i.e. earth, water, wind and fire which make up the fundamental principle in TTM) in summer, is composed of dried fruits of three medicinal plants, namely *Phyllanthus emblica* (Malacca tree, Ma-kham-pom), *Terminalia bellirica* (Beleric Myrobalan, Sa-mor-pi-pek) and *Terminalia chebula* (Myrobalan Wood, Sa-mor-thai).

Trisarn, a Thai traditional medicine used in winter, consists of three plants, namely *Piper interruptum* (Sa-kan), *Piper sarmentosum* (Cha-phlu) and *Plumbago indica* (Chettamun-phloeng-daeng).

There are many reports on the anti-allergic activity of black pepper (Kraithep *et al.*, 2008); piperine, a major pungent substance in the fruit of the black pepper (Kraithep *et al.*, 2008; Huang *et al.*, 2014); ginger (Tewtrakul & Subhadhirasakul, 2007; Kawamoto *et al.*, 2016); and there are many reports on the anti-inflammatory properties of Trikatuk (Murunikkara & Rasool, 2014); piperine (Bae *et al.*, 2011; Kim *et al.*, 2012; Shrivastava *et al.*, 2013; Ying, X. *et al.*, 2013; Ying, Xiaozhou *et al.*, 2013) and (Kumar *et al.*, 2007) ; ginger (Dugasani *et al.*, 2010; Hsiang *et al.*, 2013; Justo *et al.*, 2015; Li *et al.*, 2013; Pan *et al.*, 2008; Ramadan & El-Menshawy, 2013; Tripathi *et al.*, 2007; van Breemen *et al.*, 2011) and (Young *et al.*, 2005) ; *Terminalia chebula* (Gautam *et al.*, 2013; Reddy *et al.*, 2009); *Phyllanthus emblica* (Dang *et al.*, 2011; Sripanidkulchai

& Junlatat, 2014); Triphala remedy (Sireeratawong *et al.*, 2013); *Piper interruptum* (Sireeratawong *et al.*, 2012) and *Piper sarmentosum* (Zakaria *et al.*, 2010). However, there is no report on comparison study of antiallergy of Trikatuk, Triphala and Trisarn. Therefore, the objectives of this study are to investigate the anti-allergic activity of Trikatuk, Triphala, Trisarn and choose the remedy which showed the strongest anti-allergic properties study on anti-inflammatory effects, identify its biomarker and investigate for stability.

1.2 Aims of this study

1.2.1 Overall aims

Overall aims of this research are to study on the anti-allergic activity of Trikatuk, Triphala, Trisarn and select the best anti-allergic formulations which is also continuously studied for anti-inflammatory activity and stability test.

1.2.2 Specific aims

1.2.2.1 To compare the anti-allergic activity of Trikatuk, Triphala and Trisarn remedies against rat basophilic leukemia (RBL-2H3) mast cells.

1.2.2.2 To study the anti-inflammatory activity of the strongest anti-allergic remedy against murine macrophage leukemia (RAW264.7) cell lines.

1.2.2.3 To study the strongest anti-allergic compounds as biomarker for analysis and for stability test.

1.2.2.4 To study the chemical fingerprints of the strongest anti-allergic compounds by high performance liquid chromatography method.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Overview of allergy

Allergy or type I hypersensitivity is inflammatory reaction mediated largely by immunoglobulin E (IgE), reactions are occurring within minutes after exposure to an antigen, and always involve IgE mediated degranulation of basophils or mast cells. Allergy, like several other diseases, is the result of an interplay between genetic and environmental factors. When atopic individuals are exposed to allergens, sensitization occurs in a T-helper type-2 (Th2) dependent pathway that is characterized by the production of several cytokines, principally interleukin IL-4 and IL-13. This, in turn, causes the generation of allergen-specific IgE antibodies by plasma cells (Gangwar *et al.*, 2015).

Allergic reactions are classified into four types: Type I reactions are occurring within minutes of exposure to antigen, and IgE-mediated degranulation of basophils or mast cells; Type II, antibody binding to a cell membrane (cytotoxic); Type III, interaction of antigen complement; and Type IV, T cell and cytokines mediated hypersensitivity reactions (Table 2.1) (Lydyard *et al.*, 2011).

Classification of hypersensitivities reactions consist immediate, intermediate and delayed. Hypersensitivities reactions occur at different times after coming into contact with allergens can cause mast cell and basophil degranulation release of chemical and immune mediators resulting in tissue damage and pathology. Factors to the development of hypersensitivity include genetics, environment and age (Lydyard *et al.*, 2011).

Table 2.1 Classification of Hypersensitivities

Type	Time of appearance	Disorders	Mechanism
I	2-30 min (immediate)	Allergic reaction, asthma, anaphylaxis	Cross-linking of FcRε-bound IgE antibodies on mast cell cause degranulation and release of vasoactive amines (histamine)
II	5-8 h (cytotoxic)	*Erythroblastosis fetails*, Goodpasture' syndrome, autoimmune hemolytic anemia	IgM or IgG antibody complement
III	2-8 h (immune complex)	Immune complex disease (e.g., systemic lupus erythematosus, SLE	Antibody-antigen complexes
IV	24-72 h (delayed)	Contact dermatitis, tuberculosis, chronic graft rejection	Antibody mediated stimulation

(Lydyard *et al*., 2011 and Doan *et al*., 2008)

The inflammatory response due to an allergic reaction (allergic inflammation) usually occurs in two phases. The early-phase reaction involves mast cell degranulation and release of histamine, prostaglandins, tryptases and leukotrienes after IgE bound to the mast cell surface is exposed to the sensitized antigen within seconds to minutes. The late-phase reaction involves eosinophils, basophils, T cells, macrophages and neutrophils infiltrating into the conjunctiva 6 - 72 h after exposure to the allergen (Abelson *et al.,* 2015), and is due to cross-linking of allergen specific IgE molecules bound to their high affinity receptors FcεRI expressed on mast cells.

Mast cells activation results in the onset of a late phase reaction in which various inflammatory cells and mainly the eosinophils are recruited from the blood circulation to the inflammation of tissue and assume an activated phenotype. The classic pathway of the early phase is characterized by IgE bound FcεRI activated mast cells, Other non-IgE dependent mechanisms of stimulation are also known to take place especially in the late phase and chronic outcome of allergic inflammation (Gangwar *et al.*, 2015). Since, β-hexosaminidase is normally released along with histamine from mast cells or basophils, this enzyme is therefore used as the marker for mast cell degranulation in RBL-2H3 cell line (Tewtrakul *et al.*, 2009).

2.2 Mast cell and Basophils

Mast cells are found throughout in connective tissues close to blood vessels and particularly in the subepithelial areas of the respiratory, gastrointestinal and genitourinary tracts. Basophils are granulocytes which stain with basic dyes and are present in very low numbers in the circulation (<0.2%). (Lydyard *et al.*, 2011). Mast cells store in granules with chemical mediators of inflammation and originate from the bone marrow (Merluzzi *et al.*, 2015). These mediators are released when high affinity FcεRI specific for immunoglobulin E (IgE) are brought into close proximity (Balseiro-Gomez *et al.*, 2016; Spendier, 2016). FcεRI are expressed on mast cells, that reside in most vascularized tissues in mammals and other vertebrates, and on basophilic granulocytes (basophils), that ordinarily circulate in very low numbers in the blood but which can be recruited to sites of inflammation. When mast cell- or basophil-bound IgE antibodies remember antigens that are at least bivalent, rapid aggregation of the FcεRI initiates complex intra-cellular signaling pathways (Galli *et al.*, 2016). Mast cell can be activated both in a receptor-dependent or -independent manner. Upon their activated they release a wide spectrum of mediators which can be classify into three groups according to the time kinetic of their release. The first group contains preformed mediators like histamine, proteases as well as some cytokines, e.g. tumor necrosis factor α (TNF-α), that

are stored in the numerous mast cell granules and can be released immediately after cell activation. The products of the second group are also released relatively fast and comprise rapidly synthesized bioactive metabolites of arachidonic acid such as prostaglandins and leukotrienes. The final group contains products which are newly synthesized via unregulated gene expression in response to stimulation, including most cytokines and chemokines. The different biological functions of these products characterize mast cell not only as simple effector immune cells but enables them to regulate both innate and adaptive immunity(Yu *et al.*, 2015).

2.3 Inflammation

Nitric oxide (NO) is produced in mammalian cells from L-arginine and oxygen by a family of enzymes known as NO synthases (NOS). The three NOS isoforms are the neuronal (nNOS or type I), endothelial (eNOS or type III) and inducible (iNOS or type II) types. The nNOS and eNOS are constitutively expressed, whereas the iNOS can be induced by bacterial lipopolysaccharide or certain cytokines such as tumor necrosis factor-α, interleukin-1 and interferon γ. These three NOS isoforms have been observed in rat peritoneal eosinophils, human peripheral blood eosinophils and in dermal eosinophilic pustular folliculitis (Ferreira *et al.*, 2002). Nitric oxide is thought to be an important inflammatory mediator in several atopic diseases. NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities. However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cells (Tewtrakul & Itharat, 2007).

2.4 Macrophages

Macrophages play a dual role in allergic responses and inflammation in the airways. They may be present at various stages of activation and, therefore, will express

different functional properties. On the one hand, macrophages are recruited to the airways of allergic subjects following allergen challenge. As effector cells, they have proinflammatory functions, having the ability to migrate to sites of inflammation and being involved in the elimination of foreign materials and cellular debris. On the other hand, alveolar macrophages are among the first cells to encounter inhaled compounds, and can produce many different mediators that can have a putative role in asthma. An excessive inflammatory response caused by macrophages might disturb gas exchange. This means that alveolar macrophages must be capable of both enhancing and suppressing inflammatory responses, and must be programmed to implement the effector responses appropriate to the needs of the moment. Overall, the experimental evidence indicates that alveolar macrophages have the potential to inhibit the immune activation and inflammatory cell influx into the lungs caused by the inhalation of respiratory allergens. Among possible anti-asthmatic substances elaborated by alveolar macrophages, there are factors promoting Th1 polarization, such as interferon (IFN)-α, IL-12, IL-18, and nitric oxide (NO), or those with generalized anti-inflammatory activity, such as transforming growth factor (TGF)-β, IL-10, PGE2, and 15hydroxyeicosatetraenoic acid (15-HETE) (Verstraelen *et al.*, 2008).

2.5 General data of plant in Trikatuk remedy

2.5.1 *Piper nigrum* Linn. (PIPERACEAE)

Piper nigrum Linn. Is a flowering vine in PIPERACEAE family, its common names in various countries are Prik Thai Dam (Thailand), peppercorn and black pepper (English). It is a native plant of Southeast Asia. Black pepper is grown at South Thailand and Malaysia. It is the source of hot and pungent peppercorns, one of the most popular spices in the world. *P. nigrum* has several uses such as helping in pain relief, rheumatism, chills, flu, colds, muscular aches and fever. Recent reports show the anti-oxidant properties of *P. Nigrum* (Saha & Verma, 2015), and piperine, a major

pungent substance in the fruit of the black pepper inhibits platelet aggregation of COX-1 and inhibits lipopolysaccharide-induced generation of prostaglandin PGE_2 and PGD_2 in RAW264.7 cells by suppressing the activity of COX-2 and anti-oxidant (Bagheri *et al.*, 2014; Son *et al.*, 2014) and anti-fungal (Chithra *et al.*, 2014). *P. nigrum* exhibited anxiolytic and anti-depressant activity (Hritcu *et al.*, 2015) and also showed anti-bacterial (Venkat Reddy *et al.*, 2004).

Piper nigrum Linn, shown in figure 2.1 is an aromatic woody, climber, branches stout, trailing and rooting at the nodes. Leaves, simple, very variable in length from 12.5-17.5 cm and width from 5.0-12.5 cm, sometimes glaucous beneath; base acute rounded or cordate, equal or unequal having 5-9 basal nerves with another pair higher up which run to the tip; apex acuminate; petiole 1.2-3.7 cm, stout. Flowers: usually dioecious, but often the female bears 2 anthers, and the male a pistillade, anther 2-celled. Fruiting spikes 10-12 cm or more long. Fruits: drupaceous, globose or ovoid, sessile, 1-seeded, 4-6 mm in diameter, orange red to reddish when ripe, seeds usually globose, testa thin (THP 1, 1998) description shown in figure 2.2.

(http://www.webindia123.com/garden/herb_spi/pepper.htm)

Figure 2.1 *Piper nigrum* Linn. (PIPERACEAE)

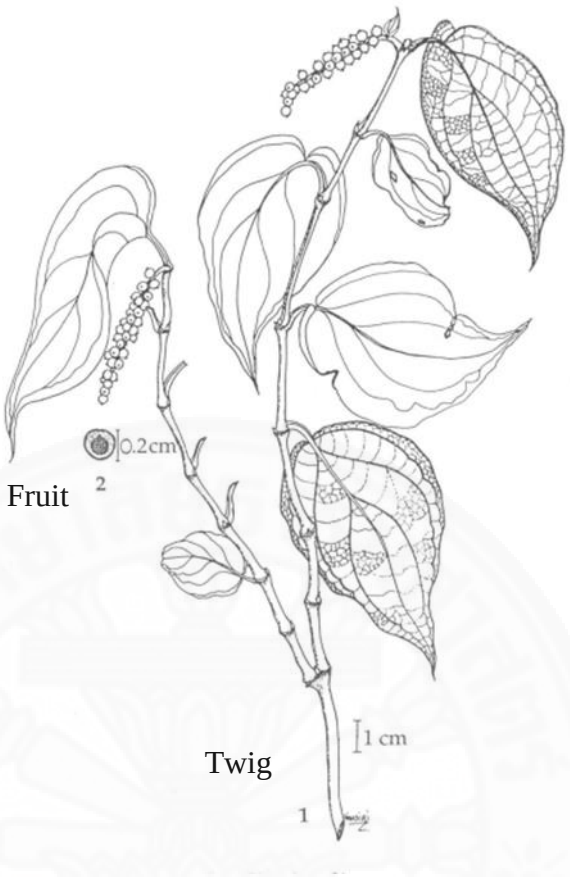


Figure 2.2 Description of *Piper nigrum* Linn. (PIPERACEAE)

2.5.2 *Piper retrofractum* Vahl. (PIPERACEAE)

Piper retrofractum or *Piper chaba* Hunter (PIPERACEAE) is widely distributed in Southeast Asia. Its common names are various countries are Dee-plee, Deeplee-chueag, Prik-hang (Thailand) and Long pepper (English). In Thailand it is used as an anti-flatulent, expectorant, antitussive, antifungal, uterus-contracting agent, sedative-hypnotic, appetizer, and counter-irritant in traditional medicine (Matsuda *et al.*, 2009). The anti-obesity effects of the plant have been reported (Kim *et al.*, 2011).

Piper retrofractum is cultivated for its fruit, which is usually dried and used as a spice and seasoning. The fruit of *Piper retrofractum* is similar in appearance and taste to that of the *Piper longum*, shown in figure 2.3.

http://puechkaset.com

Figure 2.3 *Piper retrofractum* Vahl. (PIPERACEAE)

2.5.3 *Zingiber officinale* Roscoe. (ZINGIBERACEAE)

Zingiber officinale (ZINGIBERACEAE), its common names in various country are Khing (Thailand) and Ginger (English). It is a medicinal plant that has been widely used all over the world and is indigenous to tropical Asia, probably to southern China or India. The rhizomes of the plant have a powerful aroma and are extensively used as a spice and as medicine. Ginger is well known for its nutraceutical value, which can be ascribed to a variety of bioactive compounds, including the gingerols, zingiberene and the shogaols. Biological activities reported include prevention of allergic rhinitis in mouse model (Kawamoto *et al.*, 2016), anti-diabetes in rats (Kazeem *et al.*, 2015), anthelmintic (Lin *et al.*, 2014), antibacterial (Mesomo *et al.*, 2013), antioxidant (Mukherjee *et al.*, 2014), anti-gastrointestinal cancer (Prasad & Tyagi, 2015) and 6-shogaol inhibits breast cancer cells (Ray *et al.*, 2015).

A specimen of *Zingiber officinale* is shown in figure 2.3 It is a perennial herb, up to 1.5 meter in height, with asymmetric flowers. Due to the long period of breeding in different continents, different types of the species have developed. *Zingiber officinale* is not known to occur in the wild state. It is assumed that it originated in south-east Asia.

http://www.medicinalplantsindia.com/ginger.html

Figure 2.4 *Zingiber officinale* Roscoe. (ZINGIBERACEAE)

2.6 General data of plant in Triphala remedy

2.6.1 *Phyllanthus emblica* Linn. (EUPHORBIACEAE)

Phyllanthus emblica (EUPHORBIACEAE) is called Ma-Kham-Pom (Thailand) and Embric Myrobalan (English). It can be used as a gastroprotective agent in nonsteroidal anti-inflammatory drug (NSAID)-induced gastropathy. This plant has been reported to exhibit antioxidant (Chatterjee *et al.*, 2011; Liu *et al.*, 2008); anti-inflammatory (Dang *et al.*, 2011), anticancer activity (Liu *et al.*, 2012) and antidiarrheal in vivo properties (Mehmood *et al.*, 2011) fruits shown in figure 2.5 and fruiting twig are shown in figure 2.6.

http://herbnaturals.blogspot.com/2015/12/nelli-fruit-phyllanthus-emblica.html

Figure 2.5 *Phyllanthus emblica* Linn. (EUPHORBIACEAE)

Figure 2.6 Fruiting twig of *Phyllanthus emblica* Linn.

2.6.2 *Terminalia bellirica* Roxb. (COMBRETACEAE)

Terminalia bellirica (COMBRETACEAE) is called Sa-Mow-Phi-Phek (Thailand) and Beleric myrobalan (English). It is mainly used to treat heat rash, diarrhea, and liver and gall diseases. This plant has been reported to exhibit anti-fibrotic activity (Chen *et al.*, 2015) and angiogenesis activity in vivo (Prabhu *et al.*, 2012). It contains tannins which are chebulagic acid, ellagic acid, gallic acid, etc. It also contains *β*-sitosterol and a green fixed oil fruits shown in figure 2.7 (THP II, 2000).

Figure 2.7 *Terminalia bellirica* Roxb. (COMBRETACEAE)

2.6.3 *Terminalia chebula* Retz. (COMBRETACEAE)

Terminalia chebula (COMBRETACEAE) is called Sa-Mow-Thai (Thailand) and Chebulic Myrobalan (English). It is a native plant in India and Southeast Asia and is extensively cultivated in Taiwan. Its dried ripe fruit, also called medicinal terminalia fruit, has traditionally been used to treat various ailments in Asia. This plant has been reported to exhibit a variety of biological activity, including anti-cancer on colon adenocarcinoma HT-29 cancer cell lines (Vangalapati *et al.*, 2013); antioxidant (Ali *et al.*, 2013), neuroprotective effect (Chang & Lin, 2012), anti-fertility (Ghosh *et al.*, 2015), anti-caries (Jagtap & Karkera, 1999) and anti-mutagenicity (Kaur *et al.*, 1998). It contains tannins which are chebulinic acid, chebulic acid, tannic acid, gallic acid, etc. It also contains β-sitosterol, saponin and a fixed oil containing principally esters of palmitic, oleic and linoleic acids fruits shown in figure 2.8.

http://www.homeremediess.com/terminalia-chebula-benefits-and-pictures/

Figure 2.8 *Terminalia chebula* Retz. (COMBRETACEAE)

2.7 General data of plant in Trisarn remedy

2.7.1 *Piper interruptum* Opiz. (PIPERACEAE)

Piper interruptum, it's common names is Sa-kan (Thailand). This plant has been reported as exhibiting anti-Inflammatory, analgesic, and antipyretic activities as the ethanol extract (Sireeratawong *et al.*, 2012).

http://www.bankaset-foodfarm.com/product/387/

Figure 2.9 *Piper interruptum* Opiz. (PIPERACEAE)

2.7.2 *Piper sarmentosum* Roxb. (PIPERACEAE)

Piper sarmentosum is called Cha-phlu (Thailand). This plant has been reported as showing antihypertensive, antioxidant (Mohd Zainudin *et al.*, 2015) human gingival fibroblast proliferation activities (Ab Rahman *et al.*, 2014). It exhibits antidiabetic properties in rat model (Thent *et al.*, 2012), antinoniceptive and antiinflammatory in vivo (Zakaria *et al.*, 2010), anticarcinogenic effects (Zainal Ariffin *et al.*, 2009), antituberculosis and antiplasmodial (Rukachaisirikul *et al.*, 2004) and *P. Sarmentosum* Roxb. has a hypoglycemic effect in rats (Peungvicha *et al.*, 1998).

http://picssr.com/tags/kudak

Figure 2.10 *Piper sarmentosum* Roxb. (PIPERACEAE)

2.7.3 *Plumbago indica* Linn. (PLUMBAGINACEAE)

Plumbaga indica is called Chettamoon-phloeng-daeng (Thailand). Thai traditional medicine uses the root a carminative and digestive tonic and for treatment of eczema, chloasma, stomachache, diarrheal and hemorrhoids. Plumbagin, a pure compound from *Plumbago indica* has shown anthelminthic (Atjanasuppat *et al.*, 2009) and antimalarial effects (Sumsakul *et al.*, 2014).

http://navigate.botanicgardens.org/weboi/oecgi2.exe

Figure 2.11 *Plumbago indica* Linn. (PLUMBAGINACEAE)

http://tropical.theferns.info/image.php?id=Plumbago+indica

Figure 2.12 Flower of *Plumbago indica* Linn. (PLUMBAGINACEAE)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-allergic activity	Ethanol extract showed inhibition of the release of β-hexosaminidase by IC_{50}= 14.0 µg/ml.	(Kraithep *et al.*, 2008)
			Piperine, the bioactive compound in black pepper possessed anti-allergic activity IC_{50} = 16.0 µg/ml.	(Kraithep *et al.*, 2008)
			Piperine inhibited expression levels of IL-4, IL-13, and TNF-α. Piperine inhibited the expression of cytokines, and the release of both β-hexosaminidase and histamine, which could be stimulated by antigen in RBL-2H3 mast cells and found that the levels of intracellular $Ca2_+$ also decreased, and piperine inhibited IgE-mediated signaling pathways, including the phosphorylation of Lyn, p38, Erk, and Ras.	(Huang *et al.*, 2014)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-inflammatory activity	In vivo, Piperine reduces the severity of cerulein-induced acute pancreatitis and reduces the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in mast cells and inhibited the activation of mitogen-activated protein kinases (MAPKs).	(Bae *et al.*, 2011)
			Piperine (10, 50 and 100 μg/ml) significantly inhibited the production of NO and prostaglandin E2 (PGE2) induced by LPS, decreased gene expression and production of tumor necrosis factor-alpha (TNFα), inducible NO synthase (iNOS) and COX-2 in RAW264.7 cell, and inhibited nuclear factor-kappa B (NF-kB).	(Ying, Xiaozhou *et al.*, 2013)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-inflammatory activity (continued)	Piperine inhibited the production of PGE2 and NO. Piperine significantly decreased the IL-1β-stimulated gene expression and production of MMP-3, MMP-13, iNOS and COX-2 in human osteoarthritis chondrocyte, and inhibited NF-kB by suppressing the degradation of its inhibitory protein $I_\kappa B_\alpha$ in the cytoplasm.	(Ying, X. *et al.*, 2013)
			Piperine depletes inflammatory markers, TNF-α and IL-1β in 6-OHDA-induced Parkinson's rats.	(Shrivastava *et al.*, 2013)
			In vivo, Piperine (10, 50, or 100 mg/kg) decreased interstitial edema and reduced inflammatory cell infiltration. Piperine also reduced cerulein-induced activity of myeloperoxidase, a marker of neutrophil	(Bae *et al.*, 2011)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-inflammatory activity (continued)	infiltration and piperine inhibited the activation of mitogen activated protein kinases (MAPKs).	
			Piperine inhibited collagen- and AA-induced platelet aggregation in a concentration-dependent manner, with IC_{50} values of 158.0 and 134.2 μM, respectively. It also significantly inhibited the activity of TXA2 synthase, but not of COX-1, in platelets. Piperine significantly inhibited lipopolysaccharide-induced generation of both PGE2 and PGD2 in RAW264.7 cells by suppressing the activity of COX-2, without effect on cPLA2, in a concentration-dependent manner, with IC_{50} values of 7.7 and 10.1 μM, respectively.	(Son *et al.*, 2014)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-inflammatory activity (continued)	Piperine suppressed PMA-induced COX 2 mRNA expression and protein production in a dose-dependent manner at concentrations of 10–100 μM in RAW264.7 cells.	(Kim *et al.*, 2012)
			Piperine inhibited TNF-α induced expression of cell adhesion molecules i.e. intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1). Piperine blocks the phosphorylation and degradation of $I_{\kappa}B_{\alpha}$ by attenuating TNF-α induced IkB kinase activity.	(Kumar *et al.*, 2007)
		Antibacterial	The petroleum ether extract of *P. nigrum* afforded 2E, 4E, 8Z-*N*- isobutyleicosatrienamide, pellitorine, trachyone, pergumidiene and isopiperolein B. All the isolated compounds were active	(Venkat Reddy *et al.*, 2004)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Antibacterial (continued)	against *Bacillus subtilis*, *Bacillus sphaericus*, and *Staphylococcus aureus* amongst Gram + ve bacteria, and *Klebsiella aerogenes* and *Chromobacterium violaceum* among Gram – ve bacterial strains.	
		Antioxidant	*P. nigrum* extract showed inhibition of anti-oxidant activity by DPPH with IC_{50} value 24 ± 0.02 µg/mL	(Saha & Verma, 2015)
		Antixiolytic and antidepressant	In vivo, the methanolic extract of *P. nigrum* fruits has anxiolytic and antidepressant effects, (50 and 100 mg/kg) increased the percentage of elevated plus-maze and forced swimming tests.	(Hritcu *et al.*, 2015)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper nigrum Linn. (PIPERACEAE)	Prik-Thai-Dam	Anti-inflammatory activity	Trikatuk, significant anti-inflammatory effects were observed in trikatuk treated adjuvant induced arthritic rats (1000 mg/kg/b.wt.) by a reduction in the levels of circulating immune complexes and inflammatory mediators (TNFα and IL1β).	(Murunikkara & Rasool, 2014)
Piper retrofractum Vahl. (PIPERACEAE)	Dee-plee	Anti-obesity	In the animal model, oral piperidine alkaloids administration (50, 100, or 300 mg/kg/day for 8 weeks) significantly reduced high-fat diet -induced body weight gain.	(Kim *et al.*, 2011)
		Antioxidant and α-glucosidase inhibitory activity	The compounds isolated from the leaves of *P. retrofractum* exhibited moderate α-glucosidase inhibitory and antioxidant activities (4.60 ± 1.74% to 11.97 ± 3.30%).	(Luyen *et al.*, 2014)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper retrofractum Vahl. (PIPERACEAE)	Dee-plee	Anti-hepatitis	A new amide constituent named piperchabamide E from the fruit of *P. chaba* inhibited of the D-galactosamine (D-GalN) /TNFα-induced death of hepatocytes Furthermore, a principal amide constituent, piperine, dose-dependently inhibited the increase in serum GPT and GOT at doses of 2.5–10 mg/kg in D-GalN/LPS-treated mice, and this inhibitory effect was suggested to depend on the reduced sensitivity of hepatocytes to TNFα.	(Matsuda *et al.*, 2008)
		Anti-hepatoprotective activity	In the 80% aqueous acetone extract from the fruit of *Piper chaba*, three new amides, piperchabamides E, G, and H were found to show hepatoprotective activities, showed inhibitory effects on the increase in serum aspartate	(Matsuda *et al.*, 2009)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper retrofractum Vahl. (PIPERACEAE)	Dee-plee	Anti-hepatoprotective activity (continued)	aminotransaminase (sAST) and alanine aminotransaminase (sALT), as markers of liver injury, induced by D-galac-tosamine (D-GalN)/LPS in mice at doses of 25–50 mg/kg, and inhibited the cytotoxicity induced by both D-GalN and D-GalN/TNFα in hepatocytes (IC_{50} value = 18 and 11 µg/mL, respectively).	
Zingiber officinale Roscoe. (ZINGIBERACEAE)	Khing	Anti-allergic activity	The ethanolic (EtOH) extract of *Zingiber officinale* exhibited anti-allergic effect against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells, with an IC_{50} value of 40.3 µg/ml.	(Tewtrakul & Subhadhirasakul, 2007)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Zingiber officinale Roscoe. (ZINGIBERACEAE)	Khing	Anti-allergic activity (continued)	Ginger and 6-gingerol, using a mouse allergy model and primary cell line culture system shows anti-allergic rhinitis.	(Kawamoto *et al.*, 2016)
		Anti-inflammatory activity	Ginger inhibited LPS-induced NO production by peritoneal macrophages and J774 cells.	(Justo *et al.*, 2015)
			Purified 10-gingerol, 8-shogaol and 10- shogaol inhibited COX-2 with IC_{50} values of 32 µM, 17.5 µM and 7.5 µM, respectively but not COX-1.	(van Breemen *et al.*, 2011)
			Ginger-turmeric rhizomes mixture showed efficacy against rheumatoid arthritis severity and complications as shown in rat adjuvant-induced arthritis model.	(Ramadan & El-Menshawy, 2013)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Zingiber officinale Roscoe. (ZINGIBERACEAE)	Khing	Anti-inflammatory activity (continued)	S-[6]-gingerol reduced IL1β-induced COX2 upregulation and oxidative stress in HuH7 cells as well as NFkB activity.	(Li *et al.*, 2013)
			In vivo ginger and zingerone ameliorated 2,4,6-trinitrobenzene sulphonic acid (TNBS) induced colonic injury in mice and significantly regulated cytokine-related pathways and suppressed TNBS-induced NF-kB activation and IL-1β protein level in the colon.	(Hsiang *et al.*, 2013)
			Zingiber officinale (100 µg/ml) decreased cytokine gene TNFα and IL-6 expression in high-fat diet (HFD)-fed rats on human hepatocyte (HuH-7) cells, reduced NF-kB activity to 28.9 ± 6.6%, and reduced IKK activity to 70.9 ± 9.7%.	(Li *et al.*, 2012)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Zingiber officinale Roscoe. (ZINGIBERACEAE)	Khing	Anti-inflammatory activity (continued)	Ginger (200 mg/kg body weight) significantly suppressed the incidence and severity of arthritis by increasing anti-inflammatory and decreasing the production of pro-inflammatory cytokines.	(Ramadan *et al.*, 2011)
			[6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol exhibited with IC_{50} values of 4.05, 2.5, 1.68 and 0.85 µM against superoxide radical and IC_{50} values of 4.62, 1.97, 1.35 and 0.72µM against hydroxyl radical. On the other hand, all the compounds at a concentration of 6 µM have significantly inhibited N-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulated oxidative burst in human polymorphonuclear neutrophils (PMN), and	(Dugasani *et al.*, 2010)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Zingiber officinale Roscoe. (ZINGIBERACEAE)		Anti-inflammatory activity (continued)	significantly inhibited production of NO and PGE2.	
			6-shogaol significantly blocked protein and mRNA expression of inducible NOS (iNOS) and COX-2 in LPS-induced macrophages by inhibiting the activation of NFkB by interfering with the activation PI3K/Akt/IkB kinases IKK and MAPK.	(Pan *et al.*, 2008)
			6-gingerol inhibited the production of pro-inflammatory cytokines, TNF-α, IL-12, and IL- 1 from LPS stimulated macrophages.	(Tripathi *et al.*, 2007)

Table 2.2 Biological activity of Trikatuk remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Zingiber officinale Roscoe. (ZINGIBERACEAE)	Khing	Analgesic and anti-inflammatory activities (continued)	[6]-gingerol (25 mg/kg–50 mg/kg) produced an inhibition of acetic acid-induced writhing response and formalin-induced licking time in the late phase. [6]-Gingerol (50-100 mg/kg) also produced an inhibition of paw edema induced by carrageenin.	(Young *et al.*, 2005)
		Antioxidant	[6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol exhibited substantial scavenging activities with IC_{50} values of 26.3, 19.47, 10.47 and 8.05 µM against DPPH radical.	(Dugasani *et al.*, 2010)

Table 2.3 Biological activity of Triphala remedy and its ingredients

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Phyllanthus emblica Linn. (EUPHORBIACEAE)	Ma-Kham-Pom	Antioxidant	Maceration of 50 % ethanolic extract (EPE) and methanolic extract (MPE) of *P. emblica* branches showed high total phenolic content (608.80 ± 5.75 and 626.95 ± 10.58 TAE mg/g) respectively and strong antioxidative activity (EC_{50} of DPPH at 9.48 and 7.23 µg/ml respectively and FRAP values at 7.63 ± 0.10 and 9.95 ± 0.19 mmol/g) respectively.	(Sripanidkulchai & Junlatat, 2014)
		Anti-inflammatory activity	EPE suppressed the expression of LPS-induced pro-inflammatory genes (COX-2, iNOS, TNF-α, IL-16 and IL-6) in RAW 264.7 murine macrophage cells and significantly suppressed the carrageenan-induced paw edema in rats in a dose-dependent manner	(Sripanidkulchai & Junlatat, 2014)

Table 2.3 Biological activity of Triphala remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Phyllanthus emblica Linn. (EUPHORBIACEAE)	Ma-Kham-Pom	Anti-inflammatory activity (continued)	Triphala recipe (4 mg/ear) significantly expressed on the ear edema formation induced by ethyl phenylpropiolate-induced. Triphala recipe at the doses of 300, 600 and 1,200 mg/kg significantly reduced carrageenan-induced hind paw edema and Triphala recipe (300, 600, 1,200 mg/kg), had a significant inhibitory effect on both phases, especially in late phase.	(Sireeratawong *et al.*, 2013)
Terminalia bellirica Roxb. (COMBRETACEAE)	Sa-Mow-Phi-Phek	Anti-proliferative and apoptotic activities	Ethyl acetate fraction (EF) of *Terminalia bellirica* showed that 31.25–250 µg/mL exhibited cytotoxic and antiproliferative effects on HSC-T6 cells. EF at 50	(Chen *et al.*, 2015)

Table 2.3 Biological activity of Triphala remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Terminalia bellirica Roxb. (COMBRETACEAE)	Sa-Mow-Phi-Phek	Anti-proliferative and apoptotic activities (continued)	μg/mL significantly decreased the levels of collagen I, collagen III, TGF-β1, and hydroxyproline.EF suppressed the gene expression of Smad2, PDGFR, α-SMA, TIMP-1, and TIMP-2 but elevated that of MMP-2.	
Terminalia chebula Retz. (COMBRETACEAE)	Sa-Mow-Thai	Antioxidant	*Terminalia chebula* extract decreased free radicals and myeloperoxidase activities affected in acetic acid-induced colitis.	(Gautam *et al.*, 2013)
		Anti-inflammatory activity	*Terminalia chebula* extract (300, 600 and 1,200 mg/kg), showed decrease in colonic damage score and weight and adhesions from 43.4 to 68.3 %, 25.4 to 39.1 and 50.0 to 75.0 %, respectively.	(Gautam *et al.*, 2013)
			Chebulagic acid, a compounds of *Terminalia chebula* showed potent COX–LOX dual inhibition activity with	(Reddy *et al.*, 2009)

Table 2.3 Biological activity of Triphala remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Terminalia chebula Retz. (COMBRETACEAE)	Sa-Mow-Thai	Anti-inflammatory activity (continued)	IC_{50} values of 15±0.288, 0.92±0.011 and 2.1±0.057 µM for COX-1, COX-2 and 5-LOX respectively.	
		Anti-proliferative activity	Chebulagic acid showed anti-proliferative activity against HCT-15, COLO-205, MDA-MB-231, DU-145 and K562 cell lines and induced apoptosis in COLO-205 cells.	(Reddy *et al.*, 2009)

Table 2.4 Biological activity of Trisarn remedy and its ingredients

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper interruptum Opiz. (PIPERACEAE)	Sa-Kan	Analgesic activity	The ethanol extract of *P. interruptum* inhibited ethyl phenylpropiolate-induced ear edema and carrageenan-induced hind paw edema in rats and reduced transudative and granuloma weights as well as body weight gain and thymus weight of the chronic inflammatory model using cotton pellet-induced granuloma formation in rats.	(Sireeratawong *et al.*, 2012)
		Antipyretic Activity	*Piper interruptum* extract at doses of 300, 600, 1,200 mg/kg significantly decreased the rectal temperature of hyperthermia rats.	(Sireeratawong *et al.*, 2012)
Piper sarmentosum Roxb. (PIPERACEAE)	Cha-Phlu	Anti-nociceptive activity	The aqueous extract of the leaves of *Piper sarmentosum* in doses of 30, 100 and 300 mg/kg exerted analgesia of 18.1, 45.2 and 61.6%, respectively.	(Zakaria *et al.*, 2010)

Table 2.4 Biological activity of Trisarn remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper sarmentosum Roxb. (PIPERACEAE)	Cha-Phlu	Anti-inflammatory activity	The aqueous extract of the leaves of *Piper sarmentosum* in doses of 30, 100 and 300mg/kg assessed using the carrageenan-induced paw edema test in rats had significant anti-inflammatory effect in a dose-dependent manner.	(Zakaria *et al.*, 2010)
		Antihypertensive activity	In vivo *Piper sarmentosum* leaf aqueous extract showed a significant reduction in systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP), increase NO production and was able to reduce blood pressure and cholesterol level.	(Mohd Zainudin *et al.*, 2015)
		Proliferative activity	The leaf extracts (100 µg/mL) of *P. sarmentosum* exhibited human gingival fibroblast (HGF) proliferative activity at 28.6%	(Ab Rahman *et al.*, 2014)

Table 2.4 Biological activity of Trisarn remedy and its ingredients (Continued)

Scientific name (Family)	Thai name	Activities	Detail on Biological activities	References
Piper sarmentosum Roxb. (PIPERACEAE)	Cha-Phlu	Anti-diabetic	*Piper sarmentosum* treated diabetic group showed increase in body weight and decrease in fasting blood glucose and urine glucose level compared to the diabetic group.	(Thent *et al.*, 2012)
Plumbago indica Linn. (PLUMBAGINACEAE)	Chettamoon-Phloeng-Daeng	Anthelmintic activity	Plumbagin, a pure compound from *Plumbago indica*, had the strongest activity against *Caenorhabditis elegans*.	(Atjanasuppat *et al.*, 2009)
		Antimalarial activity	Plumbagin exhibited promising antimalarial activity with in vitro IC_{50} against 3D7 chloroquine-sensitive *Plasmodium falciparum* clones of 580 and 370 nM, respectively.	(Sumsakul *et al.*, 2014)

CHAPTER 3
METHODOLOGY

3.1 Materials

3.1.1 Plant Materials

The required parts of three remedies were purchased from a traditional herbal drug store in Bangkok. Classifications of the plant materials were quotable from the herbarium of The Royal Forest Department, Bangkok, Thailand the plants of three remedies are exhibited in Table 3.1 to 3.3.

Table 3.1 Plant materials of Trikatuk remedy

Species	Family	Part used	Voucher specimen number
Piper nigrum Linn.	PIPERACEAE	Seed	SKP 146161401
Piper retrofractum Vahl.	PIPERACEAE	Flower	SKP 146161801
Zingiber officinale Roscoe.	ZINGIBERACEAE	Rhizome	SKP 206261501

Figure 3.1 Dried seed of *Piper nigrum* Linn

Figure 3.2 Dried flower of *Piper retrofractum* Vahl.

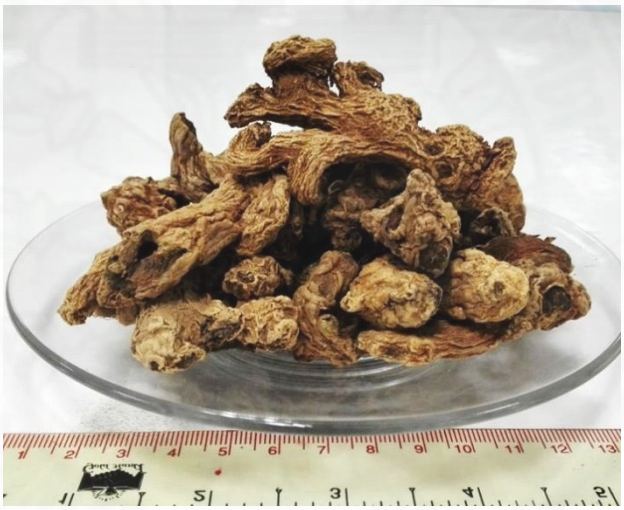


Figure 3.3 Dried rhizome of *Zingiber officinale* Roscoe.

Table 3.2 Plant materials of Triphala remedy

Species	Family	Pare used	Voucher specimen number
Phyllanthus emblica Linn.	EUPHORBIACEAE	Fruit	SKP 071160501
Terminalia bellirica Roxb.	COMBRETACEAE	Fruit	SKP 049200201
Terminalia chebula Retz.	COMBRETACEAE	Fruit	SKP 049200301

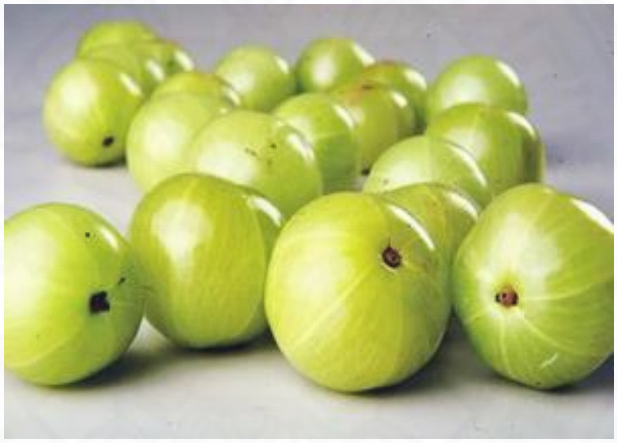


Figure 3.4 Dried fruit of *Phyllanthus emblica Linn.*

https://medthai.com

Figure 3.5 Dried fruit of *Terminalia bellirica* Roxb.

Figure 3.6 Dried fruit of *Terminalia chebula* Retz.

Table 3.3 Plant materials of Trisarn remedy

Species	Family	Part used	Voucher specimen number
Piper interruptum Opiz.	PIPERACEAE	Vine	SKP 146160901
Piper sarmentosum Roxb.	PIPERACEAE	Root	SKP 146161901
Plumbago indica Linn.	PLUMBAGINACEAE	Root	SKP 148161901

Figure 3.7 Dried vine of *Piper interruptum* Opiz.

Figure 3.8 Dried root of *Piper sarmentosum* Roxb.

http://www.biogang.net/knowledge_detail.php?id=184

Figure 3.9 Dried root of *Plumbago indica* Linn.

3.1.2 Animal cell lines

Anti-allergic activity: RBL-2H3 Rat basophilic leukemia cell line [cell no. CRL-2256 was obtained from American Type Culture Collection (ATCC CRL-2256)] and cultured in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of penicillin (100 units/ml) and streptomycin (100 units/ml). Cultured were incubated in 5% CO_2 at 37º C with 95%% humidity in tissue

culture flasks. Cell were detached with 0.25% Trypsin-EDTA solution. After the cells were washed with PBS, they were resuspended in medium and used for subsequent experiments.

Anti-inflammatory activity: RAW 264.7 Murine leukemia macrophage cell line [cell no.TIB-71 was obtained from American Type Culture Collection (ATCC TIB-71)] and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin (100 units/ml), streptomycin (100 units/ml) and incubated in 5% CO_2 at 37° C with 95% humidity and changed cultured medium three times a week.

3.2 Chemical and reagents

3.2.1 Extraction

95 % Ethanol, commercial grade	(C.M.J. Anchor company, Thailand)
Distilled water	(Milford, USA)

3.2.2 Cell culture

Fetal bovine serum	(Biochrom, Germany)
Minimum Essential Medium (MEM)	(Biochrom, Germany)
Penicillin-Streptomycin (P/S)	(Biochrom, Germany)
Phosphate-buffer saline (PBS)	(Biochrom, Germany)
RPMI medium 1640	(Biochrom, Germany)
Sodium bicarbonate ($NaHCO_3$)	(BDH, England)
Trypan blue stain 0.4%	(Gibco, USA)
Trypsin EDTA	(Gibco, USA)

3.2.3 *In vitro* anti-allergic assay

Anti-dinitrophenylated bovine albumin (DNP-BSA)	(Sigma, USA)
Anti-DNP IgE (Monoclonal Anti-IgE)	(Sigma, USA)
Calcium chloride ($CaCl_2.2H_2O$)	(Merck, Germany)
Chlorpheniramine maleate salt	(Sigma-Aldrich, USA)
Citric acid monohydrate	(Merck, Germany)
D-(+)-glucose	(Fluka, Germany)
Dimethyl sulfoxide (DMSO)	(Sigma, USA)
Distilled water	(Milford, USA)
Magnesium chloride ($MgCl_2.6H_2O$)	(Merck, Germany)
PIPES	(Amresco, USA)
PNAG (4-Nitrophenyl *N*-acetyl-β-D glucosaminide)	(Sigma, USA)
Potassium chloride (KCl)	(Merck, Germany)
Sodium bicarbonate ($NaHCO_3$)	(Merck, Germany)
Sodium carbonate (Na_2CO_3)	(Merck, Germany)
Sodium chloride (NaCl)	(Univar, Australia)
Trisodium citrate dehydrate	(Merck, Germany)

3.2.4 *In vitro* anti-inflammatory assay

3.2.4.1 NO inhibitory effect using the Griess reagent

Dimethyl sulfoxide (DMSO)	(Fluka, Germany)
Hydrochloric acid (HCl)	(Merck, Germany)
Isopropanol	(RCI Labscan, Thailand)
Lipopolysaccharide from *E.coli* (LPS)	(Sigma-Aldrich, USA)
N-(1-Naphthyl) ethylenediamine	(Sigma-Aldrich, USA)

Dihydrochloride	
Phosphate-buffered saline (PBS)	(Biochrom, Germany)
Phosphoric acid (H_3PO_4)	(Sigma-Aldrich, USA)
Prednisolone	(Sigma-Aldrich, USA)

3.2.4.2 MTT assay

3-(4, 5-Dimethyl-2-thiazolyl)	(Sigma-Aldrich, USA)
-2, 5-diphenyl-2H-tetrazolium	
bromide (MTT)	
Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)	(Sigma-Aldrich, USA)

3.2.5 High Performance Liquid Chromatography (HPLC)

Acetonitrile (ACN)	(RCI labscan, Thailand)
Methanol (CH_3OH)	(RCI labscan, Thailand)
Water analytical grade	(RCI labscan, Thailand)
Piperine	(Merck, Germany)
6-Gingerol	(ChromaDex, USA)
6-Shogaol	(ChromaDex, USA)
Dimethyl sulfoxide (DMSO)	(Fluka, Germany)

3.2.6 Instruments

3.2.6.1 Instruments and plastic wares

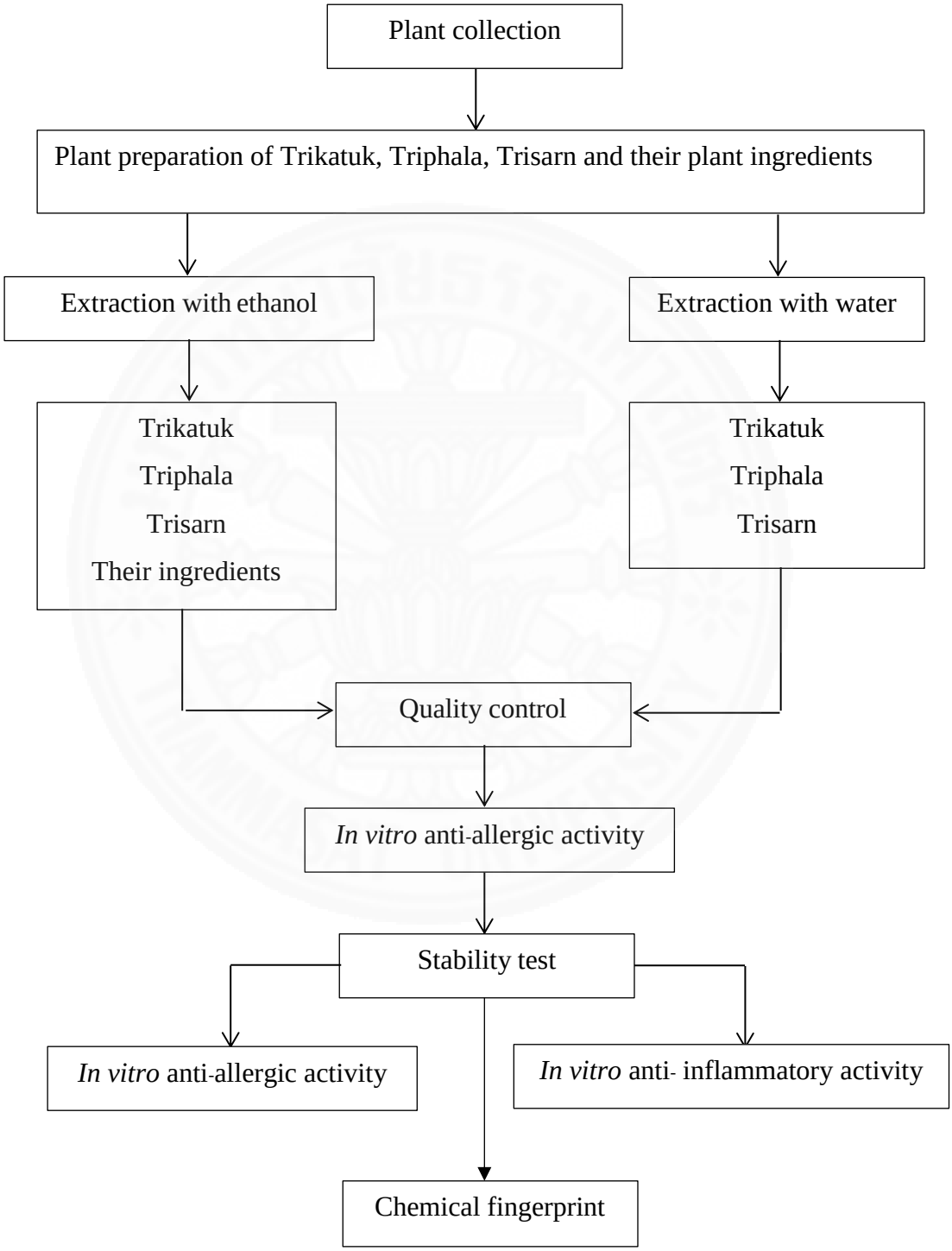
24-well plates flat, bottom	Costar Corning, USA
75 cm^2 plastic tissue culture flasks	Costar Corning, USA
96-well microplates flat, bottom	Costar Corning, USA
with lid	
96-well microplates flat, bottom	Costar Corning, USA
Without lid	
Autoclave	Hirayama, Japan
Beaker 10, 50, 100, 600, 1000 ml	Schott Duran, Germany
Buchner Funnel	Schott Duran, Germany

Centrifugation	Beckman Coulter, USA
Centrifuge tube 15, 50 ml	Costar Corning, USA
CO_2 humidified incubator	Shel lab, USA
Crucibles	Coorstex, USA
Disposable pipette	Costar Corning, USA
Erlenmeyer flasks	Schott Duran, Germany
Eppendorf	Costar Corning, USA
Filter paper no.1 (125 mm)	Whatman, USA
Filter paper no.40 (125 mm)	Whatman, USA
Freezer	Sanyo, Japan
Glass bottle 50, 250, 500, 1000 ml	Schott Duran, Germany
Hematocytometer	Boeco, Germany
High Performance Liquid Chromatography (HPLC)	Agilant technology, USA
Hot air oven	Memmert, Germany
Hot plate	Thermolyne, USA
HPLC Analytical column C18, 5U, 250 mm × 4.6 mm (Luna)	Phenomenex, USA
Inverted microscope	Nikon, Japan
Laminar air flow	Boss tech, Thailand
Liquid nitrogen tank	Taylor-Wharton, USA
Lyophilizer	Telster, Spain
Membrane filter 0.22 micron	Millipore, Germany
Micropipettes 2, 20, 200, 1000 µl	Gilson, USA
Microplate reader	Bio Tek, USA
Multi-channels pipette	Costar Corning, USA
pH buffer	Thermo Scientific, USA
pH meter	WTW inolab, Germany
Pipette tips	Costar Corning, USA
Pipette boy	Integra biosciences, Switzerland

Refrigerator (-20°C)	Sanyo, Japan
Rotary evaporator	Buchi, Switzerland
Shaking incubator	Vision Scientific, Korea
Sonicator	Elma, Germany
Stability incubator	Termarks, Norway
Vacuum Desiccator	Simax, USA
Vacuum pump	Rocker, Taiwan
Water bath	Memmert, Germany
Water purification machine	Elga, UK

3.3 Methods

3.3.1 Conceptual frameworks



3.3.2 Quality control of plant materials

The quality control methods were performed following Thai Herbal Pharmacopeia (THP 1, 1998). Loss on drying, total ash, acid insoluble ash and extractive value were determined.

3.3.2.1 Loss on drying

Moisture content is one of the most important factors for material quality and storability. It can be determined either by air oven or moisture meter. In this process, an electronic moisture analyzer, and 2 g sample were used. After the automatic process, the sample was reweighed and the percentage of loss on drying calculated using the equation.

$$\text{(\%) Moisture content} = \frac{\text{(Start weight-finish weight)} \times 100}{\text{Start weight}}$$

3.3.2.2 Total ash

This method examined the physiological ash and non-physiological ash or inorganic compound that contaminated with raw material. First, cleaned and dried the crucible until the weight of crucible was stable. Then, 2 grams of sample weighed in crucible and burned at 450 °C until the ash chance to gray or white and put in desiccators until cool down. Next, weighed and burned the crucible until stable and calculated total ash using the equation.

$$\text{(\%) Total ash} = \frac{\text{Stable weight after burning (g)} \times 100}{\text{Weight before burning (g)}}$$

3.3.2.3 Acid insoluble ash

This method was continued from total ash method. Boiled the total ash with 25 ml of 10% HCl for 5 minutes, collect the insoluble matter on an ashless filter

paper (Whatman no. 40), diluted to pH 7 by distilled water, dried the ashless filter paper and burned at 450 °C for 9 hours and calculated acid insoluble ash using the equation.

$$\% \text{ Acid insoluble ash} = \frac{\text{Stable weight after burning (g)} \times 100}{\text{Weight before burning (g)}}$$

3.3.2.4 Extractive values

Extractive values were performed in 95% Ethanol, and water to determine the quantity of active constituents.

Ethanol-soluble extractive value

Dry powder 5 g was macerated in 100 ml of 95% ethanol, shacked during the first 6 hours and standing at room temperature for 18 hours. Then, 20 ml of the extract were filtered and evaporated. After that, dry at 105°C to constant weight. calculate the percentage of ethanol extract using the equation.

Water-soluble extractive value

Process as same as ethanol-soluble extractive but using chloroform water for aqueous extract.

$$\text{Extractive value} = \frac{\text{Weight of the extract (g)} \times 100}{\text{Weight of dry powder (g)}}$$

3.3.3 Preparation of plant extracts

All plants were cleaned thoroughly in water and dried by hot air oven at 50 °C for 24h. The plants of three remedies formulated to a coarse powder. The preparation of each remedy and plant ingredients were macerated with 95% ethanol and formulated of each remedy were decocted in water. All extracts stored at -20 ºC until used.

Maceration: For the ethanolic extract, one hundred grams of dried powder of each ingredient were produced. Each remedy was made using one hundred grams of

dried powder of each of its ingredients. Three hundred grams of dried mixed powder of each remedy were macerated with 95% ethanol for 3 days, and filtered through a Whatman No.1 paper. The solvent was removed under reduced pressure by rotary evaporator and repeated 2 more times, using the previous residue each time. Percentage yield of all extracts of each part were calculated and extracts stored in a freezer at -20 °C until use.

Decoction: The dried powder was boiled in distilled water for 30 minutes, filtered and dried by freeze-drying with a lyophilizer. The percentage of yield was calculated using the following equation:

$$\% \text{ Yield} = \frac{\text{Weight of the extract (g)} \times 100}{\text{Weight of dried powder (g)}}$$

3.3.4 Anti-allergic activity

3.3.4.1 *In vitro* assay of inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cell lines

The release of β-hexosaminidase from stimulated RBL-2H3 cells was measured as previously reported (Tewtrakul & Subhadhirasakul, 2007). Briefly, RBL-2H3 cells were collected in 24-well plates (2×10^5 cells/well) using Minimum Essential Medium Eagle (MEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 units/ml) and anti-dinitrophenyl immunoglobulin E (anti- DNP IgE) (0.45 µg/ml), then incubated overnight at 37°C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 µl of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*, *N*''-bis (2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2] and then incubated in 160 µl of Siraganian buffer for an additional 10 min at 37°C in 5% CO_2. Subsequently, 20 µl of test sample solution were added to each well and incubated for 10 min, followed by the addition of 20 µl of

antigen (DNP-BSA, fc. 10 µg/ml) and incubated at 37°C in 5% CO_2 for 20 min to stimulate the cells to degranulate, equal volumes (50 µl each) of supernatant and p-NAG (1mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) were transferred into 96-well microtiter plates and incubated at 37°C for 1 h. The reaction was stopped by adding 200 µl of 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 ethanolic extract dissolved in dimethyl sulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1%), aqueous extract dissolved in sterile distilled water. Chlorpheniramine, a clinically used drug, were used as a positive control. The inhibition (%) of the release of β-hexosaminidase by the test samples were calculated by the equation shown below, and the samples whose activity at 100 µg/ml was more than 80% inhibition, were further evaluated for IC_{50} values. The IC_{50} values were determined graphically:

Inhibition (%) = [1-(T-B-N)/(C-N)] ×100

Control (C): DNP-BSA (+), Test sample (-)

Test (T): DNP-BSA (+), Test sample (+)

Blank (B): DNP-BSA (-), Test sample (+)

Normal (N): DNP-BSA (-), Test sample (-)

3.3.5 Anti-inflammatory activity

3.3.5.1 Assay of NO production and viability of LPS-stimulated RAW 264.7 cells

The inhibition of NO production from RAW264.7 cells were evaluated using the following modified method (Tewtrakul & Itharat, 2007) Briefly, subcultured of the cells with 0.25% trypsin-EDTA and diluted to a suspension in fresh medium. The cells were cultured in 96-well microtiter plates with 100 µl complete RPMI (1×10^5 cells/well) and incubated at 37°C in 5% CO_2 for 24 hours. Complete RPMI (100 µl/well) contained with 10 ng/ml of lipopolysaccharide (LPS) were add to the

complete RPMI wells to total volume 100 µl/well. Control was complete RPMI without LPS added. After that, the cells were tested with samples (100 µl/well) and incubated for 24 hours. Next, equal volumes (100 µl each) of supernatant and Griess reagent (0.1% *N*-(1-Naphthyl) ethylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4) were transferred into 96-well microtiter plates. The NO production was determined by measuring the accumulation of nitric oxide which interacted with Griess reagent. Cytotoxicity was also determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) colorimetric assay. Briefly, after 24 hours of incubation with test sample, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells. After 2 hours of incubation, the medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan production in the cells. The absorbance was measured at 570 nm using a microplate reader. The test sample was considered cytotoxic when the optical density of the sample-treated group is less than 70% of that in the control group. Indomethacin was used as reference standard. The inhibition (%) of the release of NO by the sample was calculated by the following equation, and IC_{50} value graphically determined.

% inhibition = [A-B/A-C] ×100

A-C: NO_2 concentration (µM)

A: LPS (+), test sample (-)

B: LPS (+), test sample (+)

C: LPS (-), test sample (-)

3.3.6 Stability study

3.3.6.1 Stability testing under accelerated condition

The crude extracts were carried out in triplicate using transparent vials with screw cap, then kept at 40ºC with 75±5 % RH (ICH,2004) as accelerated conditions for 6 months of period and samples withdrawn on the following schedule: 15, 30, 60, 90, 120, 150 and 180 days. The method of testing for stability were to

determine anti-allergic activity by inhibitory effects on the release of β-hexosaminidase in RBL-2H3 cells, and determine anti-inflammatory activity by inhibitory effects on the release of NO from RAW264.7 cell lines. The content of markers compound (piperine, 6-gingerol and 6-shogaol) was evaluated by using HPLC method. The sample stability values were calculated and compared with control samples (0 day: fresh ethanolic extracts that kept in a freezer at -20 ºC). No significant difference indicate that the extract was stable for at least two years when kept in a closed container protected from light and stored at room temperature.

3.4 Chemical fingerprint of Trikatuk preparation stability test by using High Performance Liquid Chromatography (HPLC)

3.4.1 Chemicals and reagents

Standard piperine was purchased from Merck (Darmstadt, Germany), with 6-gingerol and 6-shogaol purchased from Sigma-Aldrich (USA), acetronitrile and purified water (HPLC grade) from Labscan (Bangkok, Thailand).

3.4.2 Apparatus and chromatographic conditions

The study on chemical fingerprint was carried out following modified method (Sakpakdeejaroen., 2009) using High performance liquid chromatography (HPLC) system (Agilent 1100 series, USA). A reversed-phase column was Phenomenex Luna 5µ C18 (2) 100A analytical column (250 x 4.60 mm 5 micron; Phenomenex, Inc., USA), protected by a Security Guard Cartridge (C18, 4 x 3.0 mm; Phenomenex, Inc., USA). The mobile phase was composed of water: acetonitrile at the following gradient: 0-25 min, 60:40; 25-40 min, 50:50; 40-45 min, 5:95; 45-45.10 min, 0:100; 45.10-50 min, 60:40. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate was 1 ml/min with UV absorbance detection at 227 nm. The operating temperature was maintained at room temperature.

3.4.3 Trikatuk remedy preparation

The sample solutions were prepared by accurately weighing; 10 mg of crude extract was dissolved with methanol. The solutions were sonicated for 10 min and filtered through a 0.45 µm membrane filter before analysis. The sample solutions 10 µl were directly injected into the HPLC column and separated under above chromatographic condition. The analysis was performed in triplicated.

3.4.4 Standard preparation

A stock solution of the standard piperine10 mg/ml, was weighed accurately and dissolved in 1 ml of methanol and diluting to be serial concentration. The stock solution was serially diluted to the concentrations of 40-2,000 µg/ml.

A stock solution of the standard 6-gingerol; 10 mg dissolved in 1 ml of DMSO and diluting to be serial concentration. The stock solution was serially diluted to the concentrations of 1-100 µg/ml.

A stock solution of the standard 6-shogaol; 10 mg dissolved in 1 ml of DMSO and diluting to be serial concentration. The stock solution was serially diluted to the concentration of 1-100 µg/ml. All solutions were stored under refrigeration. The sample solutions, 10 µl, were directly injected into the HPLC column and separated under above chromatographic condition. The mean peak areas for each concentration were calculated and standard calibration curves were constructed by plotting concentrations against peak areas.

3.5 Validation of HPLC method

The study on chemical fingerprint of ethanolic extract of Trikatuk preparation and the validation of the analytical method for piperine, 6-gingerol and 6-shogaol of the active compound in crude extract were including the study on specificity, linearity and range, limit of detection (LOD) and limit of quantitation (LOQ), precision

and accuracy for validate the HPLC method that describes below (Itharat & Sakpakdeejaroen, 2010).

3.5.1 Specificity

For specificity validation, standard piperine solution (200µg/ml), 6-gingerol solution (5µg/ml), 6-shogaol solution (5 µg/ml) and the sample solution of the ethanolic extract of Trikatuk preparation (1 mg/ml) were prepared with methanol. The methanol was as used a control. A volume of 10 µl was injected into the HPLC column.

3.5.2 Linearity and range

For linearity validation, preparing the standard solutions (piperine, 6-gingerol and 6-shogaol) at least 5 concentrations were prepared in methanol in the range of piperine of 40-2000 µg/ml (40, 200, 400, 800, 1200, 1600 and 2000 µg/ml), 6-gingerol of 1-100 µg/ml (1, 5, 10, 25, 50, 80 and 100 µg/ml) and 6-shogaol of 1-100 µg/ml (1, 5, 10, 25, 50, 80 and 100 µg/ml). A volume of 10 µl of each concentration was injected into the HPLC column. Triplicated analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

3.5.3 Limit of detection and limit of quantitation

For limit of detection (LOD) and limit of quantitation (LOQ), serial dilutions of piperine, 6-gingerol and 6-shogaol were prepared in methanol and then analyzed using the HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

3.5.4 Precision

For precision validation, standard compound of piperine, 6-gingerol and 6-shogaol solutions at least 3 concentrations were prepared and 10 µl of each concentration was injected into the HPLC column. Concentrations of standard compound from the experiments were calculated with a linear equation of the standard curve. Triplicate analyses were conducted. The intra- and inter-day precisions were

obtained by triplicate analyses in a day and per day over 3 days, respectively. Coefficient of variation (CV) was calculated as standard deviation (SD) to the mean value from the results of triplicate testing.

3.5.5 Accuracy

The standard of piperine, 6-gingerol and 6-shogaol with known concentration were spiked to the ethanolic extract of Trikatuk sample solution, where the contents of piperine, 6-gingerol and 6-shogaol had been previously determined before adding the standard compounds. The three injections for each concentration were performed per day over three different days (3 injections × 3 concentrations × 3 days) and calculated % recovery. The percentage recovery was calculated using the following equation:

$$\%\ \text{Recovery} = [(\text{Spike}) - (\text{Sample}) / (\text{Standard}) \times 100]$$

3.5.6 Statistical analysis

All experiments carried out in triplicate. Values of different parameters are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using Graphpad Prism 5 statistical software and significance (p-value < 0.05) was determined by one-way analysis of variance (ANOVA), following Dunnett's test for each activity.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening of anti-allergic activity

4.1.1 Anti-allergic activity of Trikatuk, Triphala and Trisarn remedies

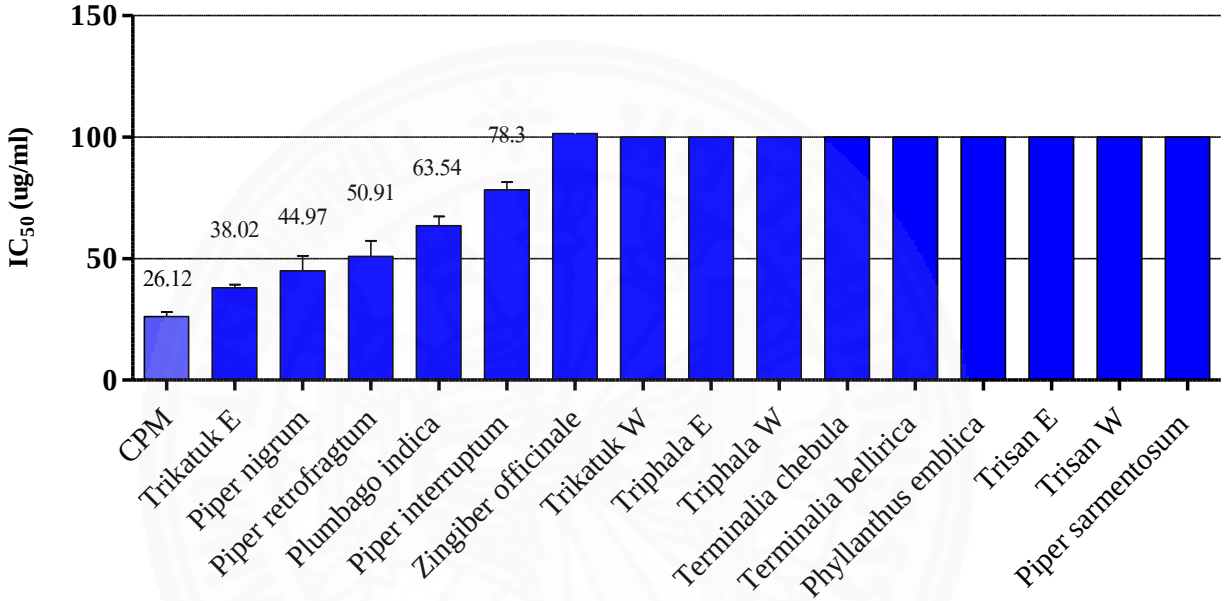
Trikatuk, Triphala and Trisarn remedies were extracted by decoction with water and maceration with 95% ethanol. Both extracts of all remedies extract and the ethanolic extract of all ingredients of three remedy were evaluated antiallergic activity by determination of inhibitory effect on the release of β-hexosaminidase from RBL-2H3 cell lines (Tewtrakul & Subhadhirasakul, 2007). The results of anti-allergic activity of the extracts (screening) are shown in Tables 4.1.

The results of anti-allergic evaluation of all plant extracts showed that the ethanolic extract of Trikatuk remedy possessed the highest anti-allergic activity against antigen-induced β-hexosaminidase with IC_{50} value 38.02±1.34 µg/ml and was not significantly different from standard chlorpheniramine (CPM) with IC_{50} value 26.12±1.89 µg/ml (p-value<0.05) calculated by one-way analysis of variance from Prism program. The second was the ethanolic extract of *Piper nigrum* (IC_{50} value 44.97±6.16) following by the ethanolic extract of *Piper retrofractum*, *Plumbago indica*, *Piper interruptum* and *Zingiber officinale* (IC_{50} value 50.91±6.44, 63.54±3.77, 78.30±3.09 and 81.85±12.00 µg/ml respectively). The water extract of Trikatuk, Triphala, Trisarn remedies, the ethanolic extract of Triphala and Trisarn remedy, the ethanolic extract of *Terminalia bellirica*, the ethanolic extract of *Terminalia chebula*, the ethanolic extract of *Phyllanthas emblica* and the ethanolic extract of *Piper sarmentosum* were apparently inactive (IC_{50} >100 µg/ml).

Furthermore, this finding of results, they are concluded that the ethanolic extract of Trikatuk remedy and its ingredients were selected to study the anti-allergic and anti-inflammation activities and quality control of the extract.

Table 4.1 The IC_{50} and inhibition (%) of Trikatuk Triphala Trisarn remedies and its ingredients on antigen-induced degranulation from RBL-2H3 cells at various concentrations (mean ± SEM), (n=3)

Plant species	Extracts	% inhibition at 100 µg/ml	IC_{50}±SEM (µg/ml)
Trikatuk remedy	95% ethanol	93.91 ± 1.47	38.02±1.34
	water	0	>100
Piper nigrum Linn.	95% ethanol	91.70 ± 1.91	44.97±6.16
Piper retrofractum Vahl.	95% ethanol	81.68 ± 5.6	50.91±6.44
Zingiber officinale Roscoe.	95% ethanol	56.15 ± 1.92	81.85±12.00
Triphala remedy	95% ethanol	35.05 ± 0.84	>100
	water	6.43 ± 1.38	>100
Phyllanthas emblica Linn.	95% ethanol	36.48 ± 19.51	>100
Terminalia bellirica Roxb.	95% ethanol	44.64 ± 13.13	>100
Terminalia chebula Retz.	95% ethanol	18.20 ± 4.35	>100
Trisarn remedy	95% ethanol	27.75 ± 0.54	>100
	water	0.95 ± 0.43	>100
Piper interruptum Opiz.	95% ethanol	71.39 ± 3.79	78.30±3.09
Piper sarmentosum Roxb.	95% ethanol	0.81 ± 6.44	>100
Plumbago indica Linn.	95% ethanol	72.59 ± 6.53	63.55±3.77
Chlorpheniramine		97.81 ± 6.48	26.13±1.89



*p-value < 0.05 compared with standard chlorpheniramine (CPM)

Figure 4.1 The IC_{50} value (µg/ml) of Trikatuk, Triphala, Trisarn remedies extracts and all plants on the release of β-hexosaminidase from RBL-2H3 cells (mean ± SEM), (n=3)

4.2 Extraction of Trikatuk remedy and its ingredients

4.2.1 Percentage of yield

Trikatuk remedy, *Piper nigrum*, *Piper retrofractum* and *Zingiber officinale* were macerated with 95% ethanol prepare as explain in section 3.3.3. The percentage of yields of the extracts are shown in Table 4.2.

Table 4.2 The yield (%) of Trikatuk remedy extract and its ingredients

Plant species	Extracts	Code	%Yield
Piper nigrum Linn	EtOH	PN	6.31
Piper retrofractum Vahl.	EtOH	PR	8.88
Zingiber officinale Roscoe.	EtOH	ZO	4.73
Trikatuk remedy	EtOH	TK	9.99

4.3 Quality controls of raw material of Trikatuk remedy and its ingredients

4.3.1 Results of quality standardization; loss on drying, total ash, acid insoluble ash and extractive values

Trikatuk remedy and its ingredients were tested for quality standard including loss on drying (moisture content), total ash, acid insoluble ash and extractive values according to standard value set of the Thai Herbal Pharmacopoeia (THP 1, 1998; THP 2, 2000; THP 3, 2009). The standard value of THP indicated that moisture content is < 10 %, total ash < 10 % and acid insoluble ash < 2 %. The results were shown in table 4.3.

Table 4.3 Results of quality controls of Trikatuk remedy and its ingredients; moisture content, total ash, acid insoluble ash and extractive values (mean ± SEM), (n=3)

Sample	% Moisture content	% Ash		Extractive values	
		Total ash	Acid insoluble ash	Ethanol soluble	Water soluble
Piper nigrum Linn.	6.78	4.24±0.10	0.41±0.06	5.65	7.01
Piper retrofractum Vahl.	5.97	7.00±0.20	0.28±0.05	8.63	8.21
Zingiber officinale Roscoe.	5.92	7.18±0.09	0.76±0.04	3.38	10.08

4.4 *In vitro* assay for anti-allergic activity

4.4.1 Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells

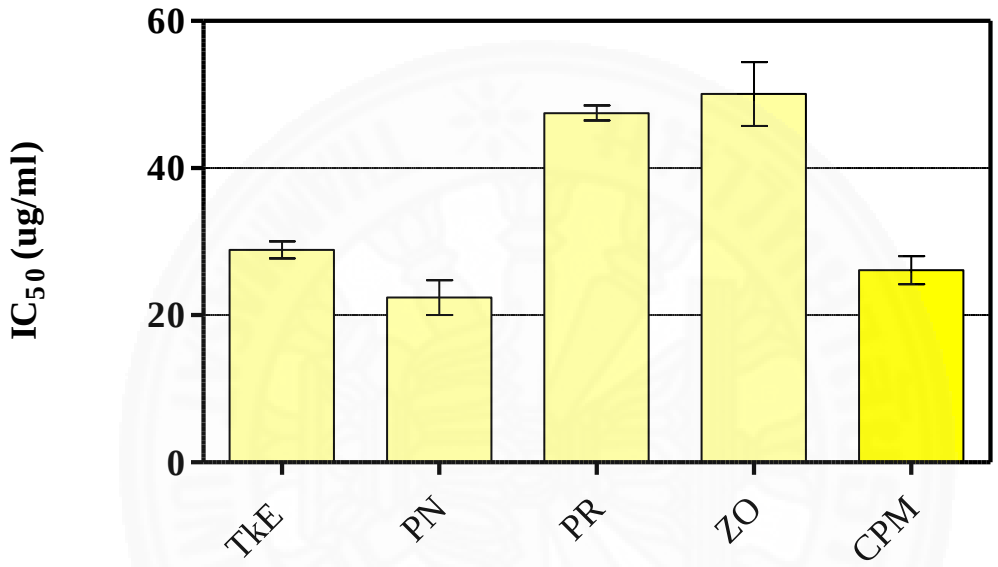
Results from the preliminary assays for anti-allergic evaluation of all plant indicated that the ethanolic extract of trikatuk remedy and its ingredients possessed the highest anti-allergic activity against antigen-induced β-hexosaminidase. Therefore, Trikatuk and its ingredients were selected to extract to be the ethanolic extract for studying on its anti-allergic, anti-inflammation, quality control and stability.

The results of anti-allergic evolution of Trikatuk and its ingredients extract are shown in Table 4.4.and figure 4.2. This data showed that the ethanolic extract of *Piper nigrum* exhibited the highest anti-allergic activity against antigen-induced β-hexosaminidase with IC_{50} value 22.40±2.36 µg/ml. The second by Trikatuk remedy with IC_{50} value 28.87±1.13 µg/ml and were not significantly different from standard chlorpheniramine (CPM) with 26.12±1.89 µg/ml (p-value<0.05) calculated by one-way ANOVA analysis of variance from Prism program. Chlorpheniramine was used as a positive control in this study. Following by *Piper retrofractum* and *Zingiber officinale* showed with IC_{50} value 47.49±1.03 and 50.07±4.33 µg/ml respectively.

These finding of anti-allergic activity *in vitro* were related many previously reports (Kraithep *et al.*, 2008; Huang *et al.*, 2014; Tewtrakul & Subhadhirasakul, 2007; Kawamoto *et al.*, 2016), the main chemical constituents of Trikatuk remedy are alkaloids and phenolic compounds.

Table 4.4 The inhibition (%) at various concentrations and IC_{50} values of ethanolic extract of Trikatuk remedy and its ingredients on the release of β-hexosaminidase from RBL-2H3 cells (mean ± SEM), (n=3)

Plant species	**Inhibition (%) at various concentrations (µg/ml)**					**IC_{50}±SEM (µg/ml)**
	1	**10**	**20**	**50**	**100**	
Piper nigrum	8.87±1.43	16.15±1.73	22.90±0.1	90.51±0.11	98.73±0.61	22.40±2.35
Piper retrofractum	1.10±3.33	5.68±4.11	11.24±0.1	53.31±1.34	95.00±1.61	47.49±1.03
Zingiber officinale	16.75±0.1	18.62±2.67	29.57±2.07	42.29±3.42	71.63±1.55	50.07±4.33
Trikatuk remedy	5.80±0.35	14.13±3.60	27.45±0.1	75.32±3.11	97.91±0.54	28.87±1.13
Chlorpheniramine	-	17.94±0.21	38.63±1.17	72.04±3.71	97.81±6.49	26.13±1.89



*p-value < 0.05 compared with standard chlorpheniramine (CPM)

Figure 4.2 The IC_{50} value (µg/ml) of the ethanolic extract of Trikatuk and ingredients on the release of β-hexosaminidase from RBL-2H3 cells (mean ± SEM), (n=3)

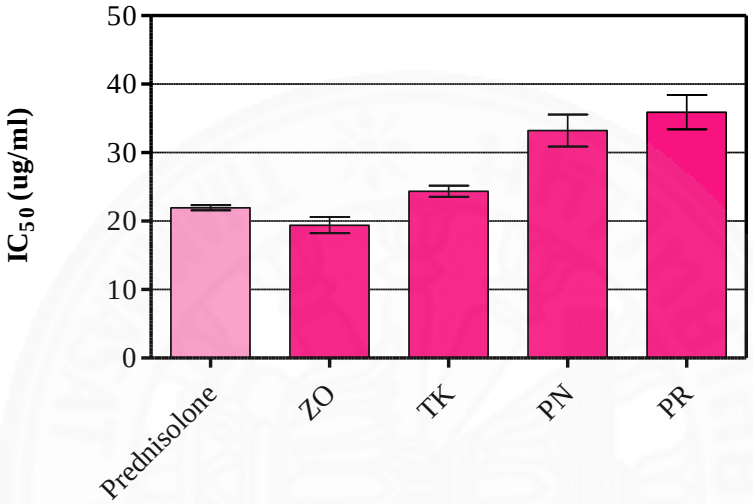
4.5 *In vitro* assay for anti-inflammatory activity

4.5.1 Inhibitory effects on LPS-induced Nitric oxide release from RAW 264.7 cells

Nitric oxide (NO), an inflammatory mediator, they play a critical causative role in the pathogenesis of inflammation. Under physiological conditions, NO is synthesized from L-arginine by nitric oxide synthase (NOS). In the family of NOS, inducible NOS (iNOS) is involved in pathological aspects, and can be expressed in response cytokines or mediators to pro-inflammatory such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) as well as nitric oxide synthase are induced immune cells by lipopolysaccharide (LPS). NO plays an important physiological role as a defense molecule in the immune system, while the excess production of NO by macrophages contributes to numerous pathological processes (Jiang *et al.*, 2015). The ethanolic extract of Trikatuk remedy and its ingredients are the best anti-allergic formulation which is also continuously studied for anti-inflammatory. Anti-inflammatory properties of Trikatuk remedy and its ingredients were evaluated by measuring their inhibitory activity against LPS induced NO production in RAW264.7 cell lines. NO was determined by the griess reaction assay. All extracts at concentration 1, 10, 20, 50 and 100µg/ml inhibited NO production in dose dependent manner.

Table 4.5 The inhibition (%) at various concentrations and IC_{50} values of ethanolic extract of Trikatuk remedy and its ingredients on the LPS-induced of NO production from RAW264.7 cells and percentage of cell viability at several concentrations (mean ± SEM), (n=3)

Plant species	**Inhibition (%) at various concentrations and Cytotoxicity (%) at various concentrations (µg/ml)**					**IC_{50} ± SEM (µg/ml)**
	1	**10**	**20**	**50**	**100**	
Trikatuk remedy	6.25±4.64	20.34±2.68	39.18±1.22	76.49±1.58	96.82±0.26	24.35±0.81
	-4.75±1.46	-4.46±1.5	3.06±1.30	5.89±061	32.92±9.16	
Piper nigrum	6.68±0.45	17.87±0.56	26.78±2.35	61.98±4.62	97.36±0.85	33.23±2.33
	-3.11±1.85	-3.49±2.29	-4.24±4.71	8.23±3.71	92.60±0.07	
Piper retrofractum	2.77±4.72	16.71±2.77	25.05±3.08	59.11±1.06	91.56±1.45	35.89±2.51
	0.88±2.39	1.46±2.78	3.65±3.40	16.06±5.81	23.19±6.22	
Zingiber officinale	1.94±2.65	21.10±3.71	52.44±3.28	84.80±1.58	96.53±0.30	19.41±1.19
	-8.90±3.03	4.30±1.12	19.86±2.21	34.59±3.30	74.13±2.35	
Prednisolone	20.19±0.44	31.69±0.50	40.22±0.36	67.59±0.33	94.11±0.65	21.93±0.37
	15.55±11.27	20.16±10.23	21.51±8.61	34.93±8.17	41.53±6.13	



*p-value < 0.05 compared with standard Prednisolone

Figure 4.3 The IC_{50} value (µg/ml) of the ethanolic extract of Trikatuk and ingredients on the release of NO from RAW264.7 cells (mean ± SEM), (n=3)

The results of inhibitory activity against LPS induced NO production using Griess reagent of this data showed in Table 4.5 and Figure 4.3. The ethanolic extract of *Zingiber officinale* exhibited high anti-inflammatory activity on inhibitory effect on LPS stimulated NO production with IC_{50} value 19.41±1.19 µg/ml and the ethanolic extract of Trikatuk with IC_{50} value 24.35±0.81 µg/ml were not significantly different from standard Prednisolone with IC_{50} value 21.91±0.37 µg/ml (p-value<0.05) calculated by one-way analysis of variance from Prism program. Following by the ethanolic extract of *Piper nigrum* and *Piper retrofractum* with IC_{50} value 33.23±2.33 and 35.89±2.51 µg/ml respectively.

The cell viability of all ethanolic extracts of Trikatuk and its ingredients by using MTT assay showed no cytotoxicity at low concentration.

4.6 Study on chemical fingerprint of Trikatuk preparation using High Performance Liquid Chromatography technique

Results from the anti-allergic and anti-inflammatory activities of the ethanolic extract of Trikatuk remedy give evidence of the potential for production this preparation in manufacturing level for using in adaptogenic drug for anti-allergic. Piperine, 6-gingerol and 6-shogaol has been identified as main compound in Trikatuk remedy, which can promote as a marker and these results were used for standardization of ethanolic extract of Trikatuk preparation. High performance liquid chromatography (HPLC) method has been choosen for investigation chemical fingerprint and quality control is good sensitivity, precision and accuracy. The chemical characteristics of the ethanolic extract of Trikatuk remedy were Piperine, 6-gingerol and 6-shogaol, in Trikatuk which isolated by HPLC technique exhibited as anti-allergic and anti-inflammatory activities (Makchuchit and Itharat, 2017).

4.6.1 Development of chromatographic method

The ethanolic extract of Trikatuk remedy was studied on chemical fingerprint by high performance liquid chromatography which is shown in section 3.4. The system of chromatographic conditions was summarized in Table 4.6. A representative chromatogram is shown in Figure 4.4.

Table 4.6 HPLC conditions for analysis of ethanolic extract of Trjkatuk remedy.

Operating parameters	Conditions
Stationary Phase	Phenomenex Luna 5µ C18 column
Mobile Phase	Water: Acetonitrile with gradient elution as follow: 0 min, 60:40; 25 min, 50:50, 30 min, 5:95, 35 min, 0:100, 35.10 min, 60:40
Flow rate	1.0 ml/min
Wavelength	227 nm
Injection Volume	10 µl

The chromatographic conditions described above and was used to analyzed the chemical characteristics of the ethanolic extract of Trikatuk remedy as the HPLC fingerprints. Retention time of the 6-gingerol (11.43 min), piperine (14.64 min) and 6-shogaol (28.30 min) (Figure 4.4)

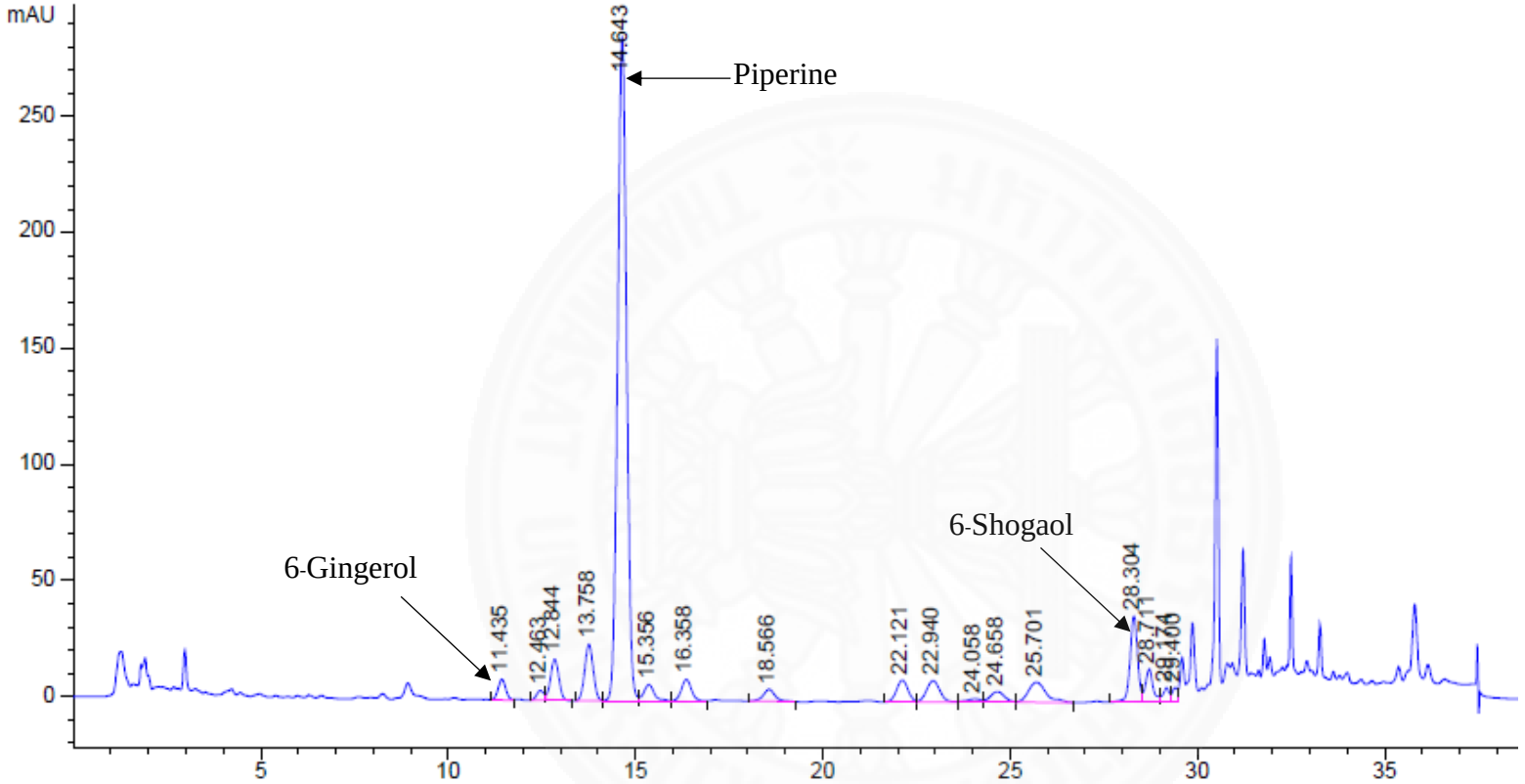


Figure 4.4 HPLC chromatogram of ethanolic extract of Trikatuk remedy (1 mg/ml). Mobile phase; water: acetonitrile with gradient elution as follow 0 min, 60:40; 25 min, 50:50; 30 min, 5:95; 35 min, 0:100; 35.10 min, 60:40; Flow rate 1.0 min/ml; UV detector at 227 nm.

4.7 HPLC method validation

Chromatographic method that was developed and described in section 4.6 and was validated following in section 3.5.

4.7.1 Specificity validation

The results of the developed HPLC method, chromatograms for specificity validation are shown in Figure 4.5. In Figure 4.5, it is apparent that piperine is a major compound of ethanolic extract of Trikatuk remedy with retention time of 14.64 min. 6-shogaol is a minor compound of ethanolic extract of Trikatuk remedy with retention time of 28.30 min, followed by 6-gingerol found the smallest amount in ethanolic extract of Trikatuk remedy with retention time of 11.43 min.

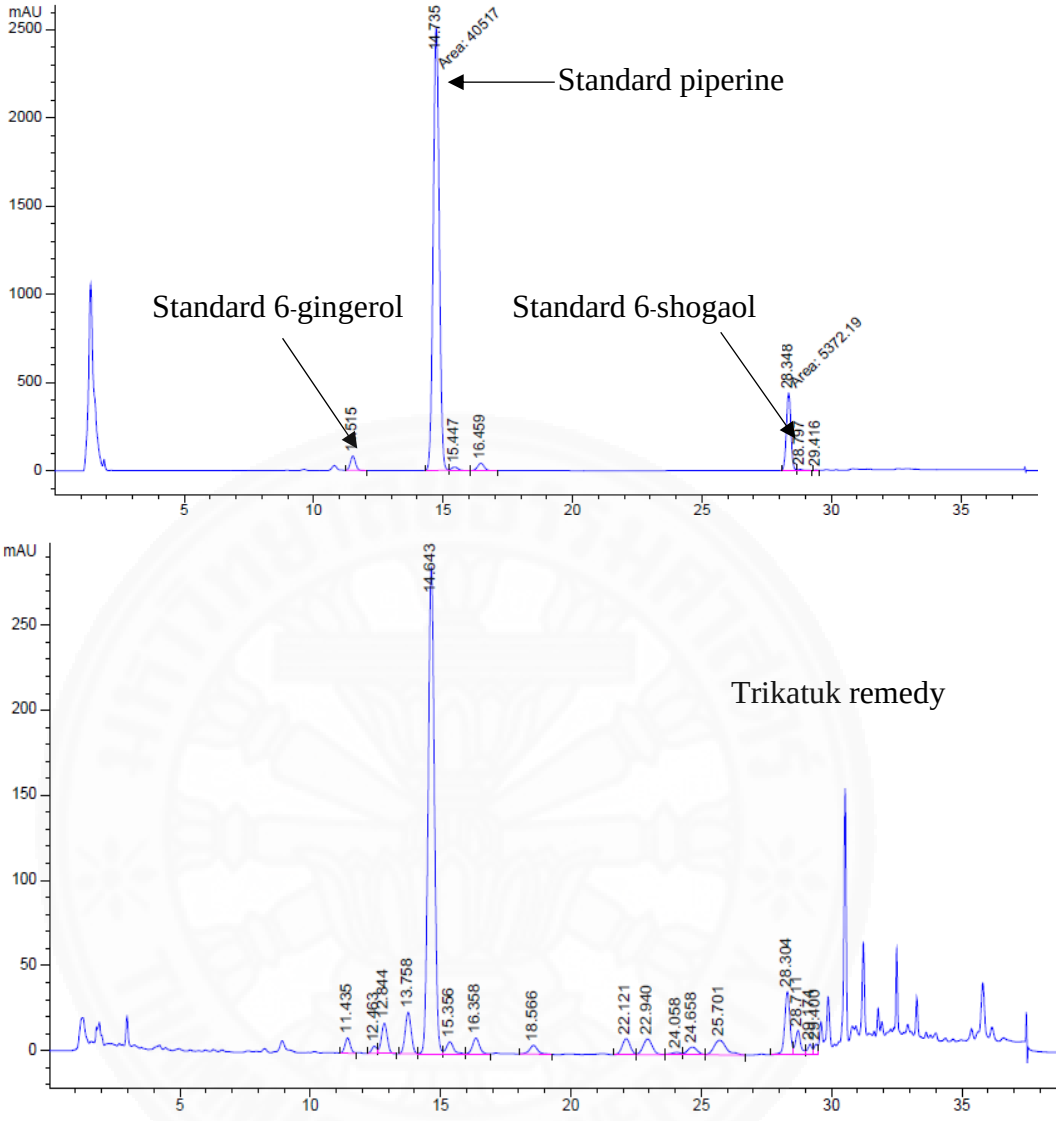


Figure 4.5 The specificity validation for the HPLC analytical method for piperine, 6-gingerol and 6-shogaol: (A) standard mixed of piperine, 6-gingerol and 6-shogaol (B) ethanolic extract of Trikatuk remedy sample solution.

4.7.2 Quantitation parameters

The calibration curve of piperine, 6-gingerol and 6-shogaol were created by plotting the peak area versus concentrations (Figure 4.6, 4.7 and 4.8). The chromatographic signals indicated a linear dependence with the concentration of piperine, 6-gingerol and 6-shogaol. Thus, piperine, 6-gingerol and 6-shogaol concentration was able to calculated from regression equation: Serial dilutions of

standard piperine (40-2000 µg/ml), 6-gingerol (1-100 µg/ml) and 6-shogaol (1-100 µg/ml) were analyzed as describe in section 3.4.4 for studying the linearity. Three separate calibration curves of each standard obtained on different days by plotting the peak area versus concentration. The results are shown in Table 4.7.

Table 4.7 Parameter of quantitative evaluation for piperine, 6-gingerol and 6-shogaol

Parameter	Piperine	6-gingerol	6-shogaol
Linear range (µg/ml)	40-2000	1-100	1-100
Equation	Y=19.649X+290.81[a]	Y=10.511X+7.2385[a]	Y=57.464X+27.167[a]
Linearity (r^2)	0.9999	0.9999	0.9999
LOD (µg/ml) [b]	0.5	0.5	0.1
LOQ (µg/ml) [c]	1	1	0.2

[a] Y=AX+B, where Y is peak area, X is the concentration of analyzed sample

[b] Limit of detection (LOD): signal to noise ratio=3

[c] Limit of quantitation (LOQ): signal to noise ratio=10

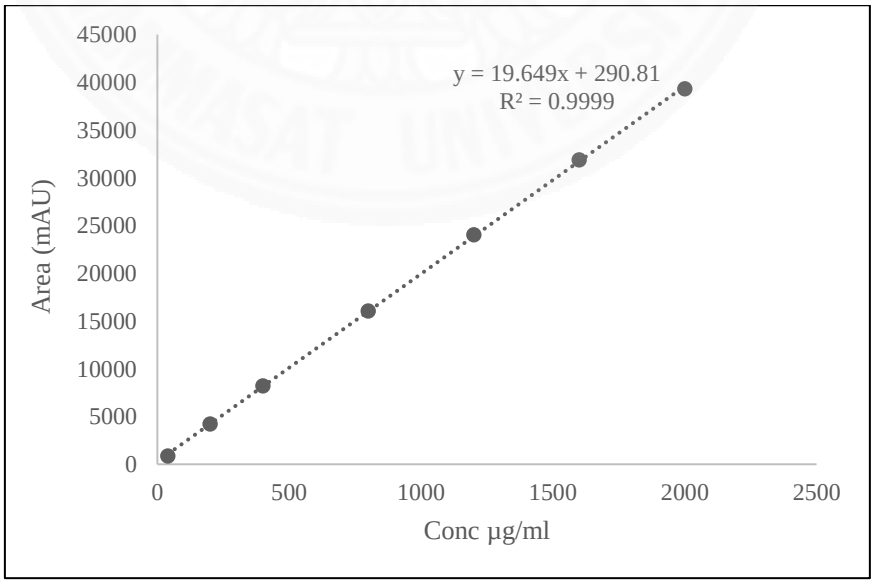


Figure 4.6 Calibration curve of standard piperine

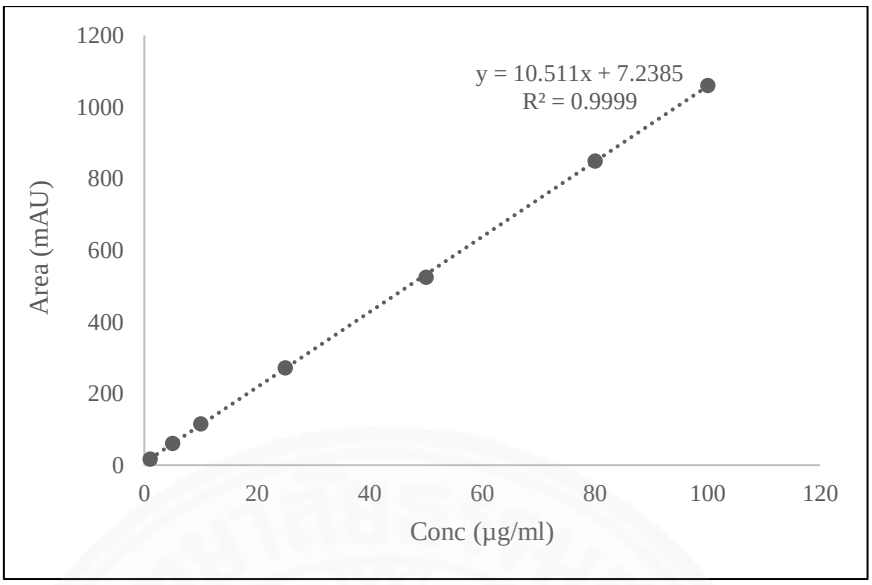


Figure 4.7 Calibration curve of standard 6-gingerol

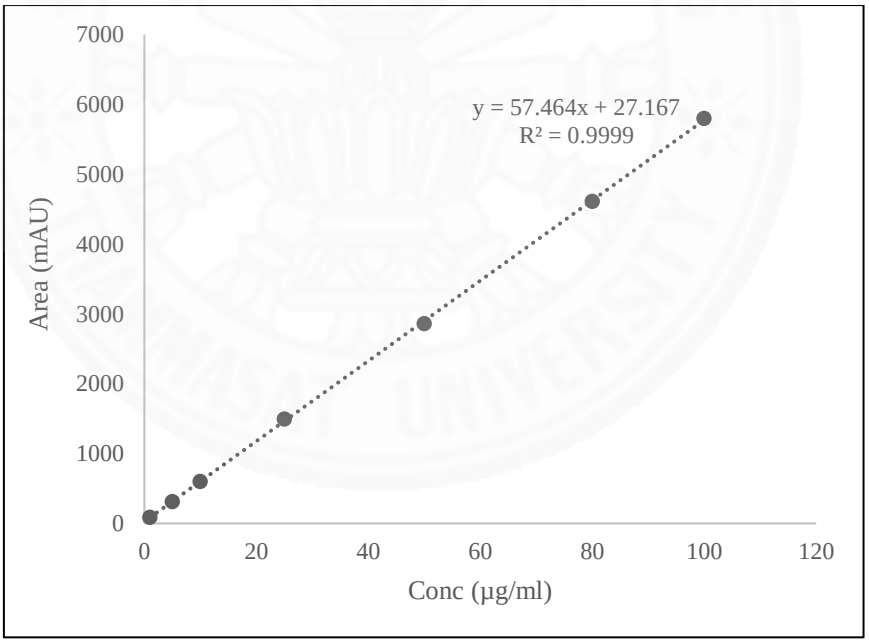


Figure 4.8 Calibration curve of standard 6-shogaol

The linearity of piperine was observed in the range of 40-2000 µg/ml, 6-gingerol and 6-shogaol were obtained in the range of 1-100 µg/ml. The results exhibited that the correlation between the peak area and the concentration of piperine,

6-gingerol and 6-shogaol were found to be linear when evaluated by linear regression analysis. The linear equation and correlation coefficient (r^2) of piperine (Y=19.649X+290.81, r^2 =0.9999), 6-gingerol (Y=10.511X+7.2385, r^2 =0.9999 and 6-shogaol (Y=57.464X+27.167, r^2 =0.9999) were obtained.

The limit of detection (LOD) represents the lowest concentration of piperine, 6-gingerol and 6-shogaol that can be detected by the instrument and the analytical method, whereas the limit of quantitation (LOQ) represents the lowest concentration of piperine, 6-gingerol and 6-shogaol that can be determined with acceptable precision and accuracy by the instrument and validated method. The result of LOD and LOQ analysis of piperine (LOD= 0.5 and LOQ= 1.0 µg/ml) 6-gingerol (LOD= 0.5 and LOQ= 1.0 µg/ml) and 6-shogaol (LOD= 0.1 and LOQ= 0.2 µg/ml) indicated the analytical method for the quantitation of piperine 6-gingerol and 6-shogaol of ethanolic extract of Trikatuk preparation exhibited good sensitivity.

4.7.3 Precision validation

The precision of the method was expressed as relative standard deviation (RSD) of a series of measurements. The experimental values obtained in the determination of piperine 6-gingerol and 6-shogaol in the samples solution are presented in Table 4-8 4.9 and Table 4.10. Both of the intra- and inter-day precisions of the analytical method were studied, which obtained by triplicated analyze in a day and per day over three days, respectively. The results showed in Table 4.8, 4.9 and Table 4.10.

Table 4.8 Validation of precision of the analytical method for piperine

Concentration (µg/ml)	Intra-day[a] (n=3)		Inter-day[b] (n=9)	
	Measured concentration (µg/ml)	RSD[c] (%)	Measured concentration (µg/ml)	RSD[c] (%)
200	209.72±0.51	0.2	200.50±3.83	1.91
800	802.62±8.88	1.1	811.13±14.58	1.80
1600	1596.26±15.02	0.9	1614.19±22.60	1.40

[a] All values are mean ± SD as obtained by triplicate analyses in a day. [b] All values are mean ± SD as obtained by triplicate analyses per day over three days. [c] Relative standard deviation (RSD) = SD/mean ×100%.

Table 4.9 Validation of precision of the analytical method for 6-gingerol

Concentration (µg/ml)	Intra-day[a] (n=3)		Inter-day[b] (n=9)	
	Measured concentration (µg/ml)	RSD[c] (%)	Measured concentration (µg/ml)	RSD[c] (%)
5	4.99±0.08	1.7	4.94±0.09	1.88
25	24.68±0.19	0.8	24.98±0.35	1.42
80	80.72±1.17	1.4	80.80±1.56	1.93

[a] All values are mean ± SD as obtained by triplicate analyses in a day. [b] All values are mean ± SD as obtained by triplicate analyses per day over three days. [c] Relative standard deviation (RSD) = SD/mean ×100%.

Table 4.10 Validation of precision of the analytical method for 6-shogaol

Concentration (µg/ml)	**Intra-day[a] (n=3)**		**Inter-day[b] (n=9)**	
	Measured concentration (µg/ml)	**RSD[c] (%)**	**Measured concentration (µg/ml)**	**RSD[c] (%)**
5	5.06±0.08	1.5	4.98±0.09	1.75
25	25.33±0.39	1.5	25.16±0.45	1.80
80	78.73±0.96	1.2	79.76±1.27	1.60

[a] All values are mean ± SD as obtained by triplicate analyses in a day. [b] All values are mean ± SD as obtained by triplicate analyses per day over three days. [c] Relative standard deviation (RSD) = SD/mean ×100%.

4.7.4 Accuracy validation

The accuracy of the method was determined by investigating the recovery of samples of spiking known of standard piperine 6-gingerol and 6-shogaol of the ethanolic extract of Trikatuk remedy and comparing the found value to the true value, which recoveries between 90- 110% indicating a good accuracy of this method obtained. The results showed in Table 4.11, 4.12 and Table 4.13.

Table 4.11 Validation of the accuracy of the analytical method for piperine

Spike level (µg/ml)	Recovery (%)			Mean (%)[a]	RSD (%)[b]
	1	2	3		
200	97.32	95.89	95.37	96.19±1.01	1.05
800	96.52	97.32	95.33	96.39±1.00	1.04
1600	105.34	104.18	107.72	105.88±1.77	1.68

[a] All values are mean ± SD

[b] Relative standard deviation (RSD) = SD/mean ×100%.

Table 4.12 Validation of the accuracy of the analytical method for 6-gingerol

Spike level (µg/ml)	Recovery (%)			Mean (%)[a]	RSD (%)[b]
	1	2	3		
5	98.19	97.78	100.4	98.79±1.41	1.43
25	95.5	92.35	94.89	94.25±1.67	1.77
80	103.92	100.89	103.19	102.67±1.58	1.54

[a] All values are mean ± SD

[b] Relative standard deviation (RSD) = SD/mean ×100%.

Table 4.13 Validation of the accuracy of the analytical method for 6-shogaol

Spike level (µg/ml)	Recovery (%)			Mean (%)[a]	RSD (%)[b]
	1	2	3		
5	98.87	96.63	98.17	97.89±1.15	1.17
25	101.46	102.93	100.42	101.60±1.26	1.24
80	102.55	99.61	102.99	101.72±1.84	1.81

[a] All values are mean ± SD as obtained by triplicate analyses.

[b] Relative standard deviation (RSD) = SD/mean ×100%.

The results from Table 4.11, 4.12 and Table 4.13 found that piperine, 6-gingerol and 6-shogaol have good recoveries, for which ranging from 94.25 to 105.88 %, with 1.04 to 1.81 % of Relative standard deviation (RSD). It demonstrates that the analytical method has good accuracy.

4.8 Stability of ethanolic extract of Trikatuk remedy

4.8.1 Stability study under accelerated condition

The crude extract of Trikatuk remedy was investigated under accelerated condition. The crude extract was store in transparent vials with screw cap, then kept at 40ºC with 75±5 % RH for 6 months. The changes of amount of piperine, 6-gingerol and 6-shogaol, anti-allergic and anti-inflammatory activities characteristics of the extract in various storage times (0, 15, 30, 60, 90, 120, 150, 180 days) were determined.

4.8.2 Stability test of *in vitro* assay for anti-allergic activity

The ethanolic extract of Trikatuk remedy exhibited the most potent anti-allergic effect against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cell, with an IC_{50} value of 28.87±1.13 µg/ml. Thus, the ethanolic extract of Trikatuk remedy was determined anti-allergic activity under

acceleration condition. The results showed in Table 4.14. These findings indicate that the anti-allergic activity of TK decreased under the accelerated condition. The IC_{50} value of TK showed that the anti-allergic activity was significant decrease since day 120 (p<0.05). This result was described that TK extract is stable on antiallergic activity for one years and 4 months.

Table 4.14 The inhibition (%) at various concentrations and IC_{50} values of ethanolic extract of Trikatuk remedy from stability test on the release of β-hexosaminidase from RBL-2H3 cells (mean ± SEM), (n=3).

Sample	**Inhibition (%) at several concentrations (μg/ml)**				**IC_{50} (μg/ml)**
	1	**10**	**50**	**100**	
Day 0	5.80±0.35	14.13±3.60	75.32±3.11	97.91±0.54	28.87±1.13
Day 15	12.22±0.49	18.10±0.65	62.64±1.45	93.11±0.82	29.92±1.07
Day 30	12.01±0.36	18.18±0.69	65.49±0.41	93.63±0.83	28.40±0.30
Day 60	9.91±0.28	14.27±0.21	68.73±1.91	96.07±0.36	29.29±0.93
Day 90	9.89±0.55	10.49±0.33	61.32±2.61	93.75±0.44	37.30±1.99
Day 120	6.43±0.23	8.99±0.79	63.37±3.75	95.02±0.58	38.43±4.05*
Day 150	3.65±0.19	9.55±0.45	57.00±0.97	93.31±0.18	42.95±1.03***
Day 180	4.80±0.47	10.15±0.51	38.30±1.36	84.76±1.03	58.00±1.07***

*Significant difference from Day 0 (p-value<0.05)

4.8.3 Stability test of *in vitro* assay for anti-inflammatory activity

The ethanolic extract of Trikatuk remedy exhibited the most potent anti-inflammatory effect against LPS induced NO production in RAW264.7 cells, with an IC_{50} value of 24.35±0.81 μg/ml. Thus, the ethanolic extract of Trikatuk remedy was determined anti-inflammatory activity under acceleration condition. The results showed in Table 4.15. The results of that ethanolic extract of Trikatuk under the accelerated

condition is stable on anti- inflammatory activity within 2 years because antiinflammatory of TK extract at day 180 is not changed when compared with day 0 (*p*-value < 0.05).

Table 4.15 The inhibition (%) at various concentrations and IC_{50} values of ethanolic extract of Trikatuk remedy from stability test on the LPS-induced of NO production from RAW 264.7 cells (mean ± SEM), (n=3)

Sample	Inhibition (%) at several concentrations (µg/ml)					IC_{50} (µg/ml)
	1	10	20	50	100	
Day 0	6.25±4.64	20.34±2.68	39.18±1.22	76.49±1.58	96.82±0.26	24.35±0.81
Day 15	1.28±1.83	25.25±0.19	55.45±0.36	92.06±0.89	98.59±0.39	17.39±0.12
Day 30	-1.75±2.28	23.93±0.36	54.97±1.61	88.51±2.69	94.92±1.63	18.16±0.71
Day 60	-4.73±7.85	17.19±14.19	49.27±8.52	88.99±0.53	97.83±0.20	19.04±3.13
Day 90	-25.41±1.98	-0.38±0.97	33.53±1.84	82.75±0.83	92.65±1.29	27.23±0.66
Day 120	7.23±2.77	17.31±0.75	34.86±1.61	75.80±1.82	98.63±0.45	26.31±0.93
Day 150	6.91±0.49	21.90±1.93	38.11±2.47	74.71±3.06	97.83±0.43	24.78±1.81
Day 180	8.95±0.87	17.95±1.97	29.05±1.39	66.71±0.81	97.12±0.21	30.61±1.19

4.8.4 Stability of piperine, 6-gingerol and 6-shogaol in ethanolic extract of Trikatuk remedy

The stability of amount of piperine, 6-gingerol and 6-shogaol in ethanolic extract of Trikatuk remedy were evaluated after keeping under the accelerated condition as describe in section 3.3.6 and determined content with method in section 3.4. The results of stability testing exhibited that the piperine content on day 0, day 15, day 30, day 60, day 120 and day 150 were not significantly different with day 0, while the amount of piperine at day 90 and day 180 were increase when was compared with day 0 (p-value<0.05) simultaneously 6-gingerol was slightly reduced (34.57% after day 180) and 6-shogaol content on day 15, day 30, day 60, day 90 and day 180 were not significantly different from day 0 (p-value<0.05), while the amount of 6-shogaol at day 120 and day 150 were higher than day 0 also showed significant difference (p-value<0.05) data shown in Table 4.16 and Figure 4.9, 4.10 and Figure 4.11.

Table 4.16 Amount of piperine, 6-gingerol and 6-shogaol of the ethanolic extract after stored under accelerated condition (40ºC, 75% RH)

Day	Piperine content		6-Gingerol content		6-shogaol content	
	mg/g	%remaining	mg/g	%remaining	mg/g	%remaining
0	213.55±6.55	100%	10.99±0.09	100%	10.53±0.15	100%
15	249.43±17.87	116.80%	9.03±0.52	82.16%	10.12±0.51	96.11%
30	250.96±23.29	117.51%	8.86±0.48	80.61%	11.17±0.27	106.07%
60	289.45±7.65	135.54%	6.42±0.07	58.41%	11.21±0.15	106.45%
90	320.65±12.49*	150.15%	5.94±0.30	54.04%	11.74±0.49	111.49%
120	222.79±7.41	104.32%	5.71±0.51	51.95%	13.25±0.31*	125.83%
150	232.53±30.04	108.89%	5.89±0.61	53.59%	13.56±0.55*	128.77%
180	305.85±25.84*	143.22%	3.80±0.27	34.57%	10.80±0.79	102.56%

*Significant difference from Day 0 (p-value<0.05)

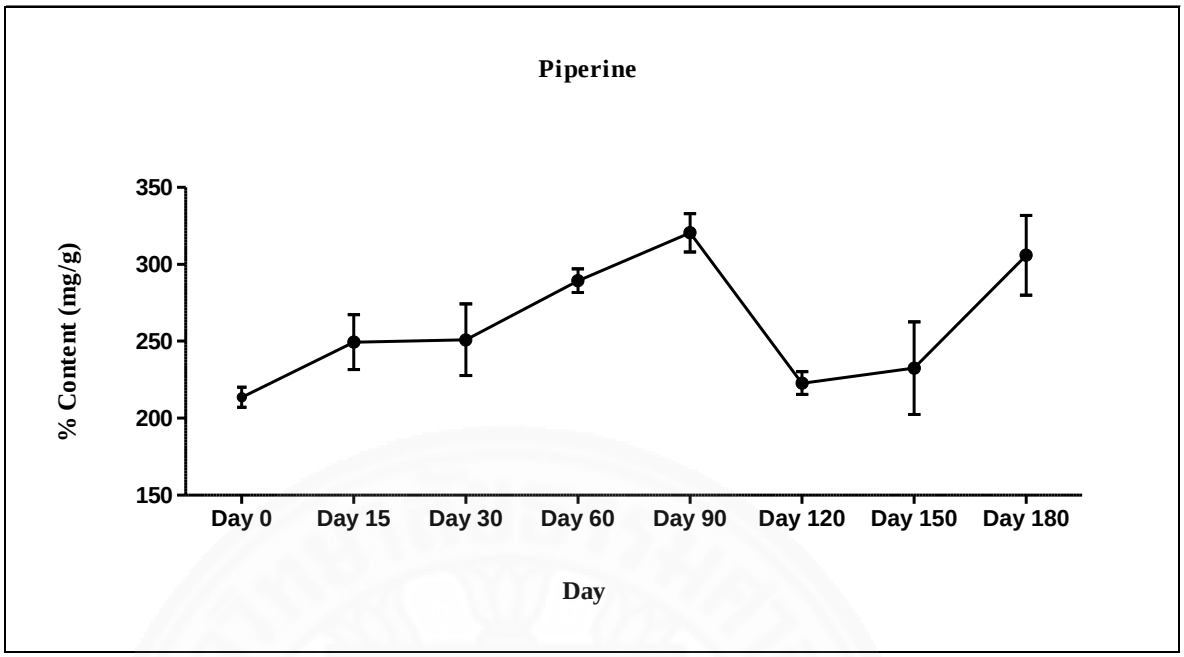


Figure 4.9 The stability of piperine (% content) in the ethanolic extract of Trikatuk remedy under accelerated condition (40ºC, 75% RH)

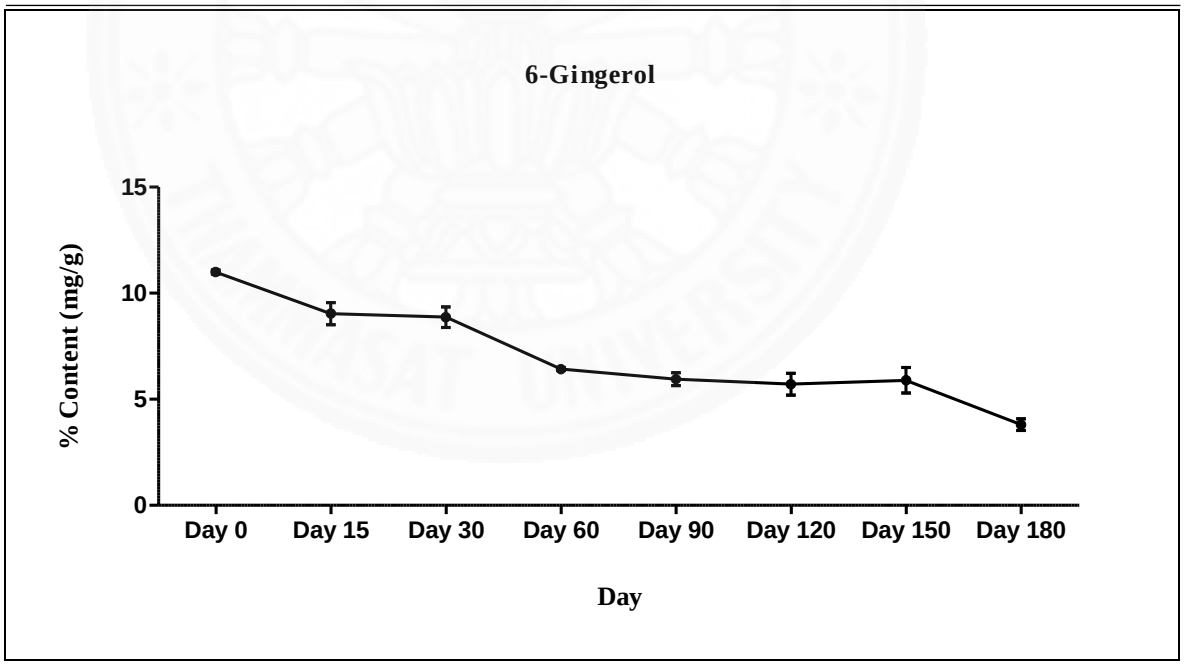


Figure 4.10 The stability of 6-gingerol (% content) in the ethanolic extract of Trikatuk remedy under accelerated condition (40ºC, 75% RH)

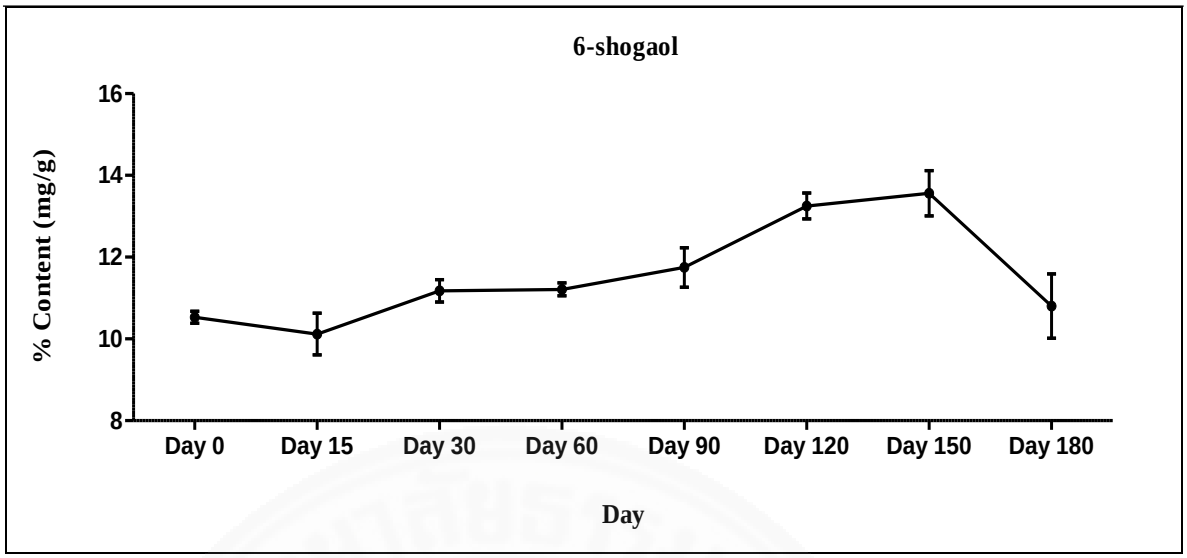


Figure 4.11 The stability of 6-shogaol (% content) in the ethanolic extract of Trikatuk remedy under accelerated condition (40ºC, 75% RH)

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

The investigation of Trikatuk remedy (TK) was based on their use by Thai traditional medicine for adaptogen and for treating disease in the raining season. Triphala remedy (TP) was based on their use to adjust patients' elements in summer. Trisarn remedy (TS) was based on their use by Thai traditional medicine used in winter. There is no previous report to comparative the anti-allergic activity of three remedies and the activity related to allergy such as anti-inflammatory. Thus, the objectives of this research were to study the biological activity based on their folk medicinal use include which anti-allergic activity of TK, TP, TS and its ingredients extracts by different methods. Then, the formulations which exhibit the strongest anti-allergic properties was selected to be a preliminary study on anti-inflammatory effects. In addition, the chemical fingerprints of the formulations having the strongest anti-allergic and anti-inflammatory properties was selected for using high performance liquid chromatography for quality control and stability testing.

Three remedies were extracts by different method: as followed decoction with water and maceration in 95% ethanol, three ingredients were maceration in 95% ethanol to obtain 15 extracts. Anti-allergic activities of all extracts were tested by inhibitory effects on the release of β-hexosaminidase in stimulated rat basophilic leukemia RBL-2H3 cells. This results on this test revealed that the water extract of TK, TP, TS remedies and the ethanolic extract of TP, TS, *Terminalia bellirica*, *Terminalia chebula*, *Phyllanthus emblica* and *Piper sarmentosum* exhibited no antiallergic activity. The ethanolic extract of Trikatuk remedy showed the highest anti-allergic activity (IC_{50} = 38.02 µg/ml) and was not significantly different from standard chlorpheniramine (CPM) (IC_{50} = 26.12 µg/ml (p-value<0.05) followed by *Piper nigrum*, *Piper retrofractum*, *Plumbago indica*, *Piper interruptum* and *Zingiber officinale* with IC_{50} = 44.97, 50.91, 63.54, 78.30 and 81.85 µg/ml respectively. Thus, the ethanolic extract of Trikatuk

remedy and its ingredients support TK formulation having high anti-allergic potency by inhibition on the release of β-hexosaminidase.

Trikatuk remedy and its ingredients were extracted by maceration in 95% ethanol. TK remedy showed the highest yield of 9.99%, followed by *Piper retrofractum*, *Piper nigrum* and *Zingiber officinale* with the yield of 8.88%, 6.31% and 4.73%, respectively.

The raw materials of ingredients from TK were tested for standardization following the Thai Herbal Pharmacopoeia (THP) protocols. All plant ingredients of TK which showed moisture content not more than standard index (<10%), total ash not more than standard index (<10%), acid insoluble ash not more than standard index (<2%). Their raw materials were accepted by standard criteria of Thai Herbal Pharmacopoeia.

Anti-allergic activity of the ethanolic extract of TK and its plant ingredients were tested by measuring their effects on the release of β-hexosaminidase in stimulated rat basophilic leukemia RBL-2H3 cells. The results showed the ethanolic extract of *Piper nigrum* exhibited the highest anti-allergic activity (IC_{50} = 22.40 µg/ml), followed by TK (IC_{50} = 28.87 µg/ml), *Piper retrofractum* (IC_{50} = 47.49µg/ml) and *Zingiber officinale* (IC_{50} = 50.07 µg/ml). The results also indicated that the anti-allergic effects of the ethanolic extract of *Piper nigrum* and TK were not significantly different from standard chlorpheniramine (IC_{50} = 26.12 µg/ml) (p-value<0.05).

Anti-inflammatory activity of the ethanolic extract of TK and its ingredients were evaluated by inhibition of nitric oxide (NO) in stimulated macrophages RAW 264.7 cells. The determination of nitric oxide by colorimetric Griess reagent revealed that the ethanolic extract of *Zingiber officinale* exhibited potent activity against LPS stimulated NO production (IC_{50} = 19.41µg/ml) and TK (IC_{50} = 24.35 µg/ml) was not significantly different from standard Prednisolone (IC_{50} = 21.91 µg/ml (p-value<0.05), followed by *Piper nigrum* (IC_{50} = 33.23 µg/ml) and *Piper retrofractum* (IC_{50} = 35.89 µg/ml).

A reverse phase high performance liquid chromatography (RP-HPLC) procedure was used for studying chemical fingerprint of the ethanolic extract of

Trikatuk remedy. The method was validated and showed good linearity, precision, accuracy and recovery. The calibration curves were linear over the ranges of 40-2000 µg/ml for piperine, 1-100 µg/ml for 6-gingerol and 6-shogaol, respectively with $r^2 = 0.999$. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.5 and 1.0 µg/ml for piperine, 0.5 and 1.0 µg/ml for 6-gingerol and 0.1 and 0.2 µg/ml for 6-shogaol, respectively. The precision of the HPLC method for determining piperine, 6-gingerol and 6-shogaol, confirmed by both of intra- and inter-day analysis, All the relative standard deviation (RSD) for piperine, 6-gingerol and 6-shogaol were less than 1.93%. The accuracy of the method for piperine, 6-gingerol and 6-shogaol were studied by spiking standard piperine, 6-gingerol and 6-shogaol into the ethanolic extract of Trikatuk remedy. The percentage recoveries for piperine, 6-gingerol and 6-shogaol were found to be ranging from 94.25 to 105.88 %, with 1.04 to 1.81 % of relative standard deviations. These results demonstrated that the proposed method has good precision and accuracy.

The stability of the ethanolic extract of Trikatuk remedy was investigated by monitoring the anti-allergic effect on antigen induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells and anti-inflammatory effect were evaluated by inhibition of nitric oxide (NO) in stimulated macrophages RAW 264.7 cells. TK remedy were demonstrated to decrease in anti-allergic activity under the accelerated condition (40ºC, 75% RH for 6 months) and were demonstrated to be highly stable with anti-inflammatory activity under the accelerated condition (40ºC, 75% RH for 6 months) was not significantly different from day 0 (p-value < 0.05).

The stability of the ethanolic extract of TK remedy was evaluated under 40±2 ºC with 75±5% RH as an accelerated condition by determining content of piperine, 6-gingerol and 6-shogaol using HPLC methods. The result of stability testing showed that the amount of piperine was increase from 213.55 mg/g (100%) at day 0 to 320.65 mg/g (150.15%) at day 90 and 305.85 mg/g (143.22%) at day 180. Because, piperine is the

main compound in *Piper nigrum* Linn and *Piper retrofractum* Vahl (Rattarom, R., 2013). Moreover, methyl piperate, which as derivative of piperine (Olsen, R. A. & Spessard, G. O, 1981) can also be found in both plants. Thus, the extracts of TK are kept long and high temperatures, methyl piperate may be converted to piperine but the exact mechanism is unknown. The chemical structures of piperine and methyl piperate showed in Figure 5.1.

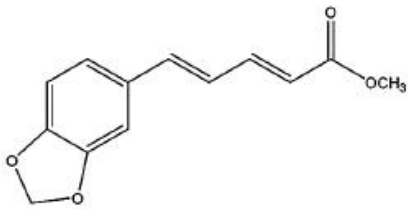
In contrast 6-gingerol was more quickly reduced. At day 0, the amount of 6-gingerol was 10.99 mg/g (100%) and reduced to 3.80 mg/g (34.57%) after day 180, indicating that 6-gingerol was unstable and amount of 6-shogaol was increase from 10.53 mg/g (100%) at day 0 to 13.25 mg/g (125.83%) at day 120 and 13.56 mg/g (128.77%) at day 150. These results illustrated that the amount of 6-gingerol was significantly reduced under high temperature because of 6-gingerols, is thermally labile due to the presence of a β-hydroxy keto group in the structure, and undergo dehydration readily to form the corresponding 6-shogaols (Bhattarai S,2001). The reaction showed in Figure 5.2.

Therefore 6-gingerol can be evaporated more easily than piperine and 6-shogaol. The stability results indicated that the extract could be stored for at least two years without loss activity. Moreover, it was concluded that piperine as a bioactive marker for anti-allergic and anti-inflammatory activities of the ethanolic extract of TK remedy.

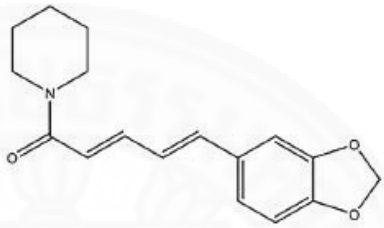
In contrast, the anti-allergic activity of the ethanolic extract of TK preparation was decrease significantly at day 120 (p-value<0.05). It may be due to 6-gingerol an active ingredient of TK changed to another form or evaporated in the sample. Therefore, it was concluded that the anti-allergic activity of TK was low stability under the accelerated condition (40 ºC, 75% RH for 6 months), whereas the ethanolic extract of TK strong activity for anti-inflammatory because it showed the highest NO production.

In conclusion, Trikatuk as a Thai traditional medicine which was normally used to adaptogen for treating diseases in rainy season such as flatulence, sweating, anorexia, cold and allergy. All of these findings indicated that Trikatuk remedy can treat allergic-related diseases and inflammatory-related diseases because TK extract and its ingredients possessed strong anti- allergic activity against antigen induced β-hexosaminidase in RBL-2H3 cell lines, and also possessed strong anti- inflammatory activity against LPS stimulated NO production in RAW 264.7 cell lines. TK is herbal medicine which can be used instead of steroid drug using in allergic treatment because steroid drug has harmful side effects in the long term. The information from this study may be useful for further studies and the development of this traditional medicine to be modern products for treatment of cold, fever, allergic- related diseases and inflammatory-related diseases in the future. However, the ethanolic extract of TK remedy should be studied extensively in animal model for immunomodulatory.

Thus, in the future study should be continued to isolate active compounds from the ethanolic extract of TK remedy by bioassay guided isolation method and developed the health product for oral use such as tablets and capsules. Finally, development of TK product should be investigated safety, efficacy and clinical trial should be undertaken in the future.



Methyl piperate



Piperine

Figure 5.1 Chemical structures of piperine and methyl piperate

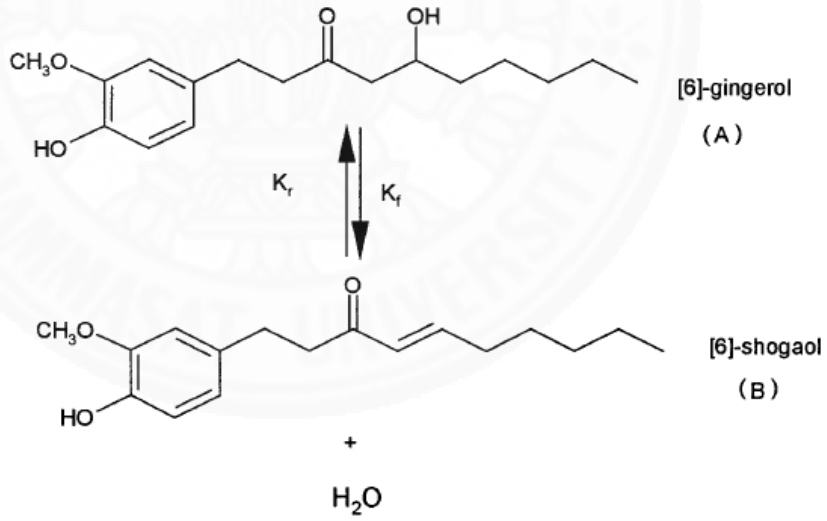


Figure 5.2 Degradation process of gingerol to shogaol

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APPENDICES

APPENDIX A
CELL CULTURE, SUPPLEMENT AND REAGENT

1.Reagent for cell culture

- FBS (inactivated)

  Prepared by heating at 56°C for 30 minutes Aliquot and stored at -20°C.

- Stock Minimum essential medium (MEM)

MEM powder with Earle's salts, L-glutamine	1	Pack
$NaHCO_3$	2.2	g

  Adjust pH to 7.4 by 1 N NaOH or 1 M HCl then adjust volume to 1,000 ml and filtered stored at 4°C.

- MEM (complete media)

Stock MEM	400	ml
10% FBS	40	ml
1% Penicillin-Streptomycin	4	ml

  Stored at 4°C

- Stock RPMI 1640 medium

RPMI 1640 with L-glutamine	1	Pack
$NaHCO_3$	2.0	g

  Adjust pH to 7.4 by 1 N NaOH or 1 M HCl then adjust volume to 1,000 ml and filter stored at 4°C.

- RPMI 1640 (complete media)

Stock RPMI 1640	400	ml

10% FBS	40	ml
1% Penicillin-Streptomycin	4	ml

Stored at 4°C

- Phosphate buffer saline (PBS)

Na_2HPO_4	1.42	g
$NaH_2PO_4.H_2O$	1.76	g
NaCl	8.76	g
Distilled water	900	ml

Adjust pH to 7.4 by 1 N NaOH or 1 M HCl then adjust volume to 1,000 ml sterile by autoclave and stored at 4°C.

APPENDIX B
CHEMICAL REAGENT PREPARATION

1.Reagent for inhibitory effect of β-hexosaminidase assay

- Siraganian buffer (BufferA) pH 7.2

NaCl	119 mM	6.954 g/l
KCl	5 mM	0.373 g/l
Glucose	5.6 mM	1.009 g/l
$MgCl_2.6H_2O$	0.4 mM	0.081 g/l
$CaCl\ 2H_2O$	1 mM	0.147 g/l
NaOH	40 mM	1.6 g/l
PIPES	25 mM	8.396 g/l
BSA	0.1%	1.0 g/l
Distilled water	900	ml

Adjust pH to 7.2 by 0.1 N NaOH, then adjust volume to 1,000 ml and stored at 4°C.

- 0.1 M Citric buffer pH 4.5

Citric acid monohydrate ($C_6H_8O_7.H_2O$)	0.1 M	10.51 g/500 ml
Trisodium citrate dihydrate ($C_6H_5O_7Na_3.2H_2O$)	0.1 M	14.71 g/500 ml

Adjust pH 4.5 by $C_6H_8O_7.H_2O$ / $C_6H_5O_7Na_3.2H_2O$ and stored at room temperature.

- M Na_2CO_3 buffer pH 10.0

Na_2CO_3	0.1 M	5.3 g/500 ml
$NaHCO_3$	0.1 M	4.2 g/500 ml

Adjust pH 10.0 by Na_2CO3/ $NaHCO_3$ and stored at room temperature.

- Stock Anti-DNP-IgE solution (50 µg/ml)

Anti-DNP-IgE solution	0.5	ml
PBS	9.5	ml

Aliquot 100 µl/tube and stored at -20°C.

- Working Anti-DNP-IgE solution

Stock of Anti-DNP-IgE solution	100	µl
Working MEM	900	µl

Working solution were prepared fresh time.

- DNP-BSA solution (0.1 mg/ml)

DNP-BSA	1.0	mg
Distilled water	10	ml

Aliquot and stored at -20°C

2. Reagent for inhibitory effect of Nitric oxide (NO) assay

- Griess reagent

Sulfanilamide	1.0	g
N-(1-Napthyl) ethylenediamine dihydrochloride	0.1	g
Phosphoric acid	2.5	g

Adjust volume to 100 ml and stored at 4 °C.

- MTT solution (5 mg/ml)

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	5	mg

PBS 1 ml

Avoided from light and stored at 4 °C.

- 0.04 M HCl in Isopropanol

HCl (37%) 0.83 ml

Adjust volume with isopropanol to 250 ml and stored at room temperature.

BIOGRAPHY

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