ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF THAI TRADITIONAL REMEDY FOR STROKE CALLED LOM-AM-MA-PRUEK AND ITS PLANT INGREDIENTS

BY

MR. PUN THONGMEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPILED THAI TRADITIONAL MEDICINE

FACULTY OF MEDICINE

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ENTITLED

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Thesis Title	ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF THAI TRADITIONAL REMEDY FOR STROKE CALLED LOM-AM-MA-PRUEK AND ITS PLANT INGREDIENTS
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ABSTRACT

The Thai national list of essential medicine specifiesLom-Am-Ma-Pruek (LAMP) is Thai traditional medicine for stroke and an analgesic in the musculoskeletal system. It is in Chawadarn scripture, it consists of this medicinal plants as follows: *Allium sativum* Linn, *Alpinia galanga* (L.) Willd, *Cinnamomum camphora* (L.) Presl, *Cleome viscosa* Linn, *Crateva adansonii* DC, *Crateva religiosa* G.Forst, *Curcuma zedoaria* (Berg) Roscoe, *Erythrina variegata* Linn, *Myristica fragrans* Houtt (seed and fruit), *Piper nigrum* Linn, *Plumbago indica* Linn, *Zingiber cassumunar* Roxb, Sodium Chloride (NaCl).When mixed together, the remedy has a spicy taste. Thai traditional medicine practitioners believed that spicy herbs can help balance the body. Therefore, LAMP should be investigated and confirmed as an anti-inflammatory preparation to support using it for treatment of stroke and muscle pain.Thus the, aims of this study were to investigate antioxidant activity by NBT assay and anti-inflammatory effects of LAMP and its plant ingredients. In this study, Griess's reagent was used to measure the anti-inflammatory activity by inhibition effects of all extracts on nitric oxide production activated by lipopolysaccharide in RAW 264.7 cell lines. TNF-αand COX-2 inhibitor activities which cause inflammation in many organs were also

determined foranti-inflammatory activity. The chemical fingerprint of LAMP was analyzed by high performance liquid chromatography.

LAMP remedy and its plant ingredients were extracted by two methods namely, maceration in 95% ethanol and decoction, to obtain ethanolic and aqueous extracts. The yield of aqueous extractof LAMP was higher than the ethanolic extract (11.37% and 7.37%, respectively). The ethanolic extract of *Myristica fragrans* Houtt (mace) showed highest percentage of yield (8.70%) andthe water extract of*Allium sativum* Linn (42%) showed highest percentage of yield

Raw material of plant ingredients were standardized by following Thai Herbal Pharmacopoeia standard (THP). All plant ingredients of LAMP remedy met standard values except *Cinnamomum camphora* and Sodium Chloride. However, LAMP remedy values all met THP criteria (the moisture content of LAMP remedy was 8.64±0.36%, total ash as 7.34±0.43% and acid insoluble ash as 1.76±0.05%).

In vitro anti-inflammatory activities were tested by inhibition to NO production, inhibition effect on LPS-induced TNF- α release and LPS-stimulated PGE_2 release on RAW 264.7 cell lines. Antioxidant activity was by scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction. For the LAMP remedy extracts, the 95% ethanolic extract (LAMPE) possessed potent inhibitory effect on NO production with IC_{50} value 24.90±0.86 μg/ml. However, LAMPE exhibited less anti-inflammatory activity than Prednisolone (IC_{50} value 1.31±0.05 μg/ml). The aqueous extract of Lom-Am-Ma-Preuk remedy (LAMPW) exhibited weak anti-inflammatory activity on this path way (IC_{50} valuemore than 100 μg/ml). The ethanolic extract of *Alpinia galanga* showed the most effect on NO inhibitory assay (IC_{50} value 6.99±0.4 μg/ml).The second was the ethanolic extract of*Curcuma zedoaria* (IC_{50} was value 14.38±1.4 μg/ml). For the LAMP remedy extracts, the 95% ethanolic extract (LAMPE) possessed potent activity against TNF-α release but was less effective than prednisolone, a positive control with IC_{50} values 35.01±2.61 and 0.95±0.19 μg/ml, respectively. On the other hand, aqueous extract of LAMP remedy exhibited weak inhibitory TNF-αrelease activity. The 95% ethanolic extract also showed the higher activity on PEG_2 release thanthe aqueous extract (IC_{50} value 4.77±0.03 and more than 100 μg/ml, respectively). For ethanolic extract of plant ingredients, it

was interesting that more than half of all extracts possessed potent inhibitory effect on LPS-stimulated PGE_2 release. The ethanolic extract of *A. galangal*, *M. fragrans* (Mace), *P. nigrum* and *Z. cassumunar* exhibited the strongest potent on anti-inflammatory activity (IC_{50} values 1.23±0.01, 1.57±0.37, 2.95±0.49 and 3.08±0.34 µg/ml, respectively). It was not significantly different from positive control prednisolone IC_{50} value 0.96±0.01µg/ml (*p-value*>0.05). The second were most potent significantly different *P. indica*, *M. fragrans* (Nutmeg), *E. variegate* (IC_{50} values 4.09±0.32, 4.6±0.19 and 9.27±0.72µg/ml, respectively). Theethanolic extract (LAMPE) had higher antioxidant activity by NBT assay than aqueous extract (LAMPW) with EC_{50} values 40.13±1.1 and more than 100 µg/ml, respectively.It was significantly different from positive control or propyl gallate EC_{50} value7.15±1.06µg/ml (*p-value*< 0.05). *A. galanga* ethanolic extract exhibited the strongest antioxidant activity with EC_{50} value 3.94±0.35µg/ml. It was not significantly different when compared with positive control or propyl gallate(*p-value*> 0.05).

Analysis by HPLC showed that myristicin as a compound in the ethanolic extract of LAMP showed the highest content (297.84±5.42 mg/g of extract). Next were piperine, eugenol and plumbagin (189.66±2.56, 58.75±0.13 and 45.01±2.35 mg/g of extract, respectively). From this result, new knowledge and scientific report of chemical fingerprint and quantification of LAMP remedy and RP-HPLC method could be considered for quality control of LAMP extract.

LAMP ethanolic extract was tested for stability under accelerated conditions (40±2^{o}C with 75±5% RH for 6 months) and evaluated for anti-inflammation effect by inhibition of Nitric oxide production release from RAW 264.7 cell lines. The result revealed that anti-inflammation effect of the ethanolic extract of LAMP by inhibition of nitric oxide production release from RAW 264.7 cell lines at Day15, 30, 60, 90, 120, 150 and 180 were not significantly different from day 0 (*p-value*> 0.05).

In conclusion, the 95% ethanolic extract of Lom-Am-Ma-Pruek remedy possessed potent anti-inflammatory activity on three pathways. All of these findings support the traditional use of LAMP remedy for the treatment of stroke and an analgesic in the musculoskeletal system. Thus, its ethanolic extract should be continuously studied in animal model for anti-inflammation, safety and product development using myristicinas an anti-inflamatory marker to analyse product.

Keywords: Lom-Am-Ma-Pruek remedy, anti-inflammatory, antioxidant

หัวข้อวิทยานิพนธ์	ฤทธิ์ต้านการอักเสบ และฤทธิ์ต้านอนุมูลอิสระ ของสมุนไพรที่ใช้ในการรักษาโรคอัมพฤกษ์อัมพาต ของตำรับยาแก้ลมอัมพฤกษ์ และสมุนไพรในตำรับ
ชื่อผู้เขียน	นายปัญญ์ ทองมี
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บทคัดย่อ

บัญชียาหลักแห่งชาติ ของการแพทย์แผนไทยนั้นระบุไว้ว่า ยาแก้ลมอัมพฤกษ์ มีสรรพคุณในการรักษาโรคอัมพฤกษ์อัมพาต ช่วยลดการปวดในระบบกล้ามเนื้อและกระดูก ซึ่งอยู่ในคัมภีร์ชวดาร ประกอบไปด้วยพืชสมุนไพรจำนวน 14 ชนิด คือ หัวกระเทียม (*Allium sativum* Linn.), เหง้าข่า (*Alpinia galanga* (L.) Willd.), การบูร (*Cinnamomum camphora* (L.) Presl.), ผักเสี้ยนผีทั้งต้น (*Cleome viscosa* Linn), เปลือกกุ่มบก (*Crateva adansonii* DC.), เปลือกกุ่มน้ำ (*Crateva religiosa* G.Forst), เหง้าขมิ้นอ้อย (*Curcuma zedoaria* (Berg) Roscoe.), เปลือกทองหลาง (*Erythrina variegata* Linn.), ดอกจันทน์ (mace of *Myristica fragrans* Houtt.), ลูกจันทน์ (nutmeg of *Myristica fragrans* Houtt.), ลูกพริกไทย (*Piper nigrum* Linn.), รากเจตมูลเพลิงแดง (*Plumbago indica* Linn.), เกลือ (Sodium Chloride), เหง้าไพล (*Zingiber cassumunar* Roxb.) สมุนไพรในตำรับส่วนใหญ่จะมีรสเผ็ดร้อน แพทย์แผนไทยเชื่อว่า สมุนไพรที่มีรสเผ็ดร้อนจะช่วยปรับสมดุลให้ร่างกาย ตำรับลมอัมพฤกษ์ยังไม่มีการศึกษาฤทธิ์ทางชีวภาพมาก่อน ดังนั้นจึงควรได้รับการศึกษาเพื่อยืนยันยาตำรับแก้ลมอัมพฤกษ์สามารถเป็นยาต้านการอักเสบ เพื่อสนับสนุนให้มีการใช้ยาสำหรับการรักษาโรคอัมพฤกษ์อัมพาต และโรคในระบบกล้ามเนื้อกระดูก วัตถุประสงค์ของการศึกษา คือการตรวจสอบฤทธิ์ต้านอนุมูลอิสระโดยวิธี NBT assay และการตรวจสอบฤทธิ์การอักเสบของสมุนไพรเดี่ยวและสมุนไพรทั้งตำรับ โดยดูฤทธิ์ยับยั้งการสร้าง nitric oxide การหลั่ง TNF-α และ COX-2 ที่ถูกกระตุ้นด้วย lipopolysaccharideใน

เซลล์ RAW 264.7 และนำสารสกัดตำรับมาตรวจหาพิมพ์ลายนิ้วมือ (fingerprint) โดยเทคนิคโครมาโตกราฟีของของเหลวสมรรถนะสูง (HPLC) และการศึกษาความคงตัวของสารสกัด ภายใต้สภาวะเร่งที่อุณหภูมิ 40±2^{o}C ความชื้นสัมพัทธ์ 75±5% RH เป็นเวลา 6 เดือน

ตำรับแก้ลมอัมพฤกษ์ถูกนำมาสกัดด้วยกัน 2 วิธี คือ การหมักด้วย 95% เอทานอล และการต้มด้วยน้ำ พบว่าการต้มด้วยน้ำมีปริมาณของ % yield มากกว่าวิธีหมักด้วย 95% เอทานอล (11.37% และ7.37% ตามลำดับ) สมุนไพรเดี่ยวที่นำมาหมักด้วย 95% เอทานอล ทั้งหมดพบว่าดอกจันทน์ (mace of *Myristica fragrans*) มีปริมาณ % yield สูงที่สุด เท่ากับ 8.70% และสมุนไพรเดี่ยวที่นำมาสกัดด้วยวิธีการต้มน้ำ พบว่า กระเทียม (*Allium sativum*) มีปริมาณ % yield สูงที่สุด เท่ากับ 42%

การควบคุมคุณภาพสมุนไพรตำรับ และส่วนประกอบของตำรับ โดยการทดสอบปริมาณความชื้นของสมุนไพร ปริมาณเถ้ารวม ปริมาณเถ้าที่ไม่ละลายในกรด ตามหลักของมาตราฐานสมุนไพรไทย ส่วนประกอบของสมุนไพรในตำรับผ่านเกณฑ์มาตราฐาน ยกเว้น การบูร (*Cinnamomum camphora* (L.) Presl) และเกลือ (Sodium Chloride) แต่อย่างไรก็ตาม เมื่อนำส่วนประกอบทุกอย่างมารวมเป็นตำรับแก้ลมอัมพฤกษ์พบว่าผ่านเกณฑ์ของมาตรฐานกำหนด โดยมีค่า (ค่าความชื้น เท่ากับ 8.64±0.36%, ปริมาณเถ้ารวม เท่ากับ 7.34±0.43% และ ปริมาณเถ้าที่ไม่ละลายในกรด 1.76±0.05%)

นำสารกสัดที่ได้มาทดสอบฤทธิ์ทางชีวภาพในหลอดทดลอง ด้วยวิธีการทดสอบฤทธิ์ต้านการอักเสบโดยยับยั้งการหลั่งไนตริกออกไซด์ (NO), TNF-α และ COX-2ที่ถูกกระตุ้นด้วย LPS ในเซลล์ RAW 264.7และทดสอบฤทธิ์ต้านอนุมูลอิสระโดยวิธี NBT assayจากการศึกษาพบว่าสารสกัดชั้น 95% เอทานอลของตำรับแก้ลมอัมพฤกษ์มีฤทธิ์ในการยับยั้งการหลั่งไนตริกออกไซด์ได้ดีโดยมีค่า IC_{50} เท่ากับ 24.90±0.86 μg/ml. แต่ก็มีค่าน้อยกว่าสารมาตราฐานคือ Prednisolone (IC_{50} เท่ากับ1.31±0.05 μg/ml) สารสกัดชั้นน้ำของตำรับไม่มีฤทธิ์ในการยับยั้งการหลั่งไนตริกออกไซด์ สารสกัดชั้นเอทานอลของสมุนไพรในตำรับที่มีฤทธิ์ในการยับยั้งการหลั่งไนตริกออกไซด์มากที่สุดคือ ข่า (*Alpinia galanga* (L.) Willd.) โดยมีค่า IC_{50} เท่ากับ 6.99±0.4μg/ml รองลงมาคือ ขมิ้นอ้อย (*Curcuma zedoaria* (Berg) Roscoe.) โดยมีค่า IC_{50} เท่ากับ14.38±1.4μg/ml ส่วนฤทธิ์ในการยับยั้งการหลั่งTNF- αพบว่า สารสกัดชั้นเอทานอลของตำรับมีฤทธิ์ในการยับยั้งการหลั่ง TNF- αแต่มีฤทธิ์น้อยกว่ายามาตรฐาน Prednisolone (IC_{50} เท่ากับ 35.01±2.61 และ 0.95±0.19 μg/ml ตามลำดับสารสกัดชั้นเอทานอลของตำรับมีฤทธิ์ในการยับยั้ง

เอนไซม์ COX-2 มีค่าIC_{50} เท่ากับ 4.77±0.03µg/ml สมุนไพรของตำรับชั้นเอทานอลมากกว่าครึ่งหนึ่งมีฤทธิ์ในการยับยั้งเอนไซม์ COX-2 เช่น ข่า (*Alpinia galanga* (L.) Willdใ), ดอกจันทน์ (mace of *Myristica fragrans* Houttใ), พริกไทย (*Piper nigrum* Linn.) และไพล (*Zingiber cassumunar* Roxb.) มีฤทธิ์ที่ดี (IC_{50} เท่ากับ 1.23±0.01, 1.57±0.37, 2.95±0.49 และ 3.08±0.34 µg/ml, ตามลำดับ) การศึกษาฤทธิ์ต้านอนุมูลอิสระพบว่าสารสกัดชั้นเอทานอลของตำรับ (EC_{50} เท่ากับ 40.13±1.1 µg/ml) มีฤทธิ์ต้านอนุมูลอิสระมากกว่าสารสกัดชั้นน้ำ (EC_{50}>100 µg/ml) ส่วนสมุนไพรเดี่ยวในตำรับที่มีฤทธิ์ต้านอนุมูลอิสระมากที่สุด คือ ข่า (*Alpinia galanga*) ซึ่งมีค่าไม่แตกต่างกันกับสารมาตราฐานคือ Propyl Gallate (IC_{50} เท่ากับ 3.94±0.35 และ 7.15±1.06 µg/mlตามลำดับ)

การตรวจหาปริมาณสารสำคัญในตำรับด้วยเทคนิคโครมาโตกราฟีของของเหลวสมรรถนะสูง (HPLC) พบว่า myristicin ที่พบในตำรับชั้นเอทานอล มีปริมาณสารมากที่สุด (297.84±5.42 mg/g ของสารสกัด) ตามด้วยสาร piperine, egenol และ plumbagin มีปริมาณสารสำคัญที่อยู่ในตำรับ เท่ากับ 189.66±2.56, 58.75±0.13และ45.01±2.35 mg/g ของสารสกัดตามลำดับ ผลการศึกษาที่ได้เป็นความรู้ใหม่ ซึ่งนำนำสารสกัดตำรับมาตรวจหาพิมพ์ลายนิ้วมือ (fingerprint) และหาปริมาณสารสำคัญในสารสกัดตำรับแก้ลมอัมพฤกษ์

สารสกัดชั้นเอทานอลของตำรับถูกนำมาทดสอบความคงตัวถายใต้สภาวะเร่งที่อุณหภูมิ 45±2^{o}C ความชื่นสัมพัทธ์ 75±5% RH เป็นเวลา 6 เดือนและเก็บตัวอย่างทุก (0, 15, 30, 60, 90, 120, 150 และ 180 วัน) แล้วนำมาทดสอบฤทธิ์ต้านการอักเสบ ฤทธิ์ในการยับยั้งการหลั่ง nitric oxide พบว่าสารสกัดชั้นเอทานอลของตำรับมีความคงตัวที่ดีเมื่อทดสอบฤทธิ์ต้านการอักเสบผลที่ได้ไม่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญ (*p-value*> 0.05) เมื่อเปรียบเทียบกับวันที่0

จากผลการทดลองในหลอดทดลองพบว่าสมุนไพรชั้น 95% เอทานอลของตำรับ มีฤทธิ์ต้านการอักเสบที่ดีทั้ง 3 วิธีการ และยังมีฤทธิ์ในการต้านอนุมูลอิสระ จากผลสรุปทั้งหมดสามารถสนับสนุนการใช้ยาตำรับแก้ลมอัมพฤกษ์ในการรักษาโรคอัมพฤกษ์อัมพาต และการปวดของกล้ามเนื้อกระดูก เพราะว่าการอักเสบและอนุมูลอิสระ ส่งผลโดยตรงที่ทำให้เกิดโรคอัมพฤกษ์อัมพาตและทำให้เกิดการปวดของกล้ามเนื้อได้ สุดท้ายนี้การศึกษาเป็นเพียงแค่หลอดทดลอง และอนาคอาจมีการศึกษาความปลอดภัยในสัตว์ทดลอง ความเป็นพิษต่อสัตว์ทดลอง และขั้นตอนในการพัฒนาเป็นผลิตภัณฑ์โดยมี myristicin เป็น markerในการวิเคราะห์ต่อไป

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MR.PunThongmee

2016

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ATCC	American type culture collection
$CHCl_3$	Chlorofrom
cm	Centimeter
Cm^3	Cubic centimeter
CO_2	Carbondioxide
conc.	Concentration
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMSO	Dimethy sulfide
e^-	Electron
EC_{50}	Concentration causing 50% effective activity
EDTA	Ethylendiamintetraacetic acid
e.g.	Example gratis, for example
ELISA	Enzyme-linked immunosorbent assay
eNOS	Epithelial nitric oxide synthase
et al	Etalii, and other
etc	Et cetera, and other things
EtOH	Ethanol
EtOAc	Ethylacetate
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GAE	Gallic acid equivalents
g/l	Gram per liter
g/ml	Gram per milliliter

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
GC-MS	Gas chromatography-mass spectrometry
hr	Hour
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
H_2O	Water
IC_{50}	Concentration causing 50% inhibition effect
i.e.	Id est, than is
IFN-γ	Interferon- γ
IL-1,6	Interlukin-1,6
iNOS	Inducible nitric oxide
KCl	Potassium chloride
LPS	Lipopolysaccharide
m	Meter
M	Molar (concentration)
MeOH	Methanol
mg/ml	Milligram per milliliter
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
MQ	Milli-Q
MTT	Thiazolyl blue tetrazolium bromide or 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide
N	Normality
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
NADPH	Nicotinamide adenine dinucleotide phosphase
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium
NED	*N*-(1-Naphthyl)ethylenediamine dihydrochloride
ng	Nanogram
nm	Nanometer
nNOS	Neuronal nitric oxide synthesis
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffer saline
PGE_2	Prostaglandin E_2
pg/ml	Picogram per milliliter
pH	Potential of hydrogen ion
PMA	Phorbol 12-myristate 13-acetate
P/S	Penicillin/Streptomycin solution
RAW 264.7	Murine macrophage leukemia
RH	Relative humidity
RNS	Reactive nitrogen species
rpm	Revolution per minute
RPMI 1640	Roswell Park Memorial Institute 1640
ROS	Reactive oxygen species
SA	Sulfanilamide
SEM	Standard error of mean
TNF- α	Tumor necrosis factor-alpha

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
U/ml	Unit per milliliter
UV	Ultraviolet
VCAM-1	Vascular call adhesion molecule-1
w/v	Weight by volume
w/w	Weight by weight
^{o}C	Degree Celsius
%	Percent
&	And
/	Per
<	Less than
=	Equal
>	More than
µg	Microgram
µl	Microliter
µg /ml	Microgram per milliliter
µM	Micromolar

CHAPTER 1
INTRODUCTION

1.1 General introduction

A stroke, sometimes referred to as a cerebrovascular accident (CVA), cerebrovascular insult (CVI), or colloquially called a brain attack, is the loss of brain function due to a disturbance in the blood supply to the brain. This disturbance is due to either ischemia (lack of blood flow) or hemorrhage (Sims and Muyderman, 2010). Stroke is the primary cause of adult disability in developed countries and ranks only behind cancer and cardiac disease as a major cause of death (Flynn *et al*., 2008). Data from the Health Information Unit, Bureau of Health Policy and Strategy in year 2006-2010 showed that the number and rate of death from hypertension and cerebrovascular disease increased every year. (Bureau of Policy and Strategy 2006-2010). Inflammation is characterized by the accumulation of inflammatory cells and mediators in the ischemic brain. After ischemia onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulate within the brain tissue subsequently leading to inflammatory injury. Increasing evidence shows that astrocytes may also act as inflammatory cells responding to ischemic stroke. Inflammation is increasingly recognized to be the key element in pathological progression of ischemic stroke. (Danton and Dietrich, 2003)

While haemorrhagic stroke triggers cerebral edema and inflammation, this short review focuses on the more common ischemic stroke. Importantly, while ischemia at first induces only a loss of function at its very early stages, structural damage appears rapidly thereafter and progresses as minutes and hours go by. Reduction of cerebral blood flow leads to a lack of oxygen and glucose supply to the brain parenchyma.This nutrient deficiency triggers multiple events including a dramatic depletion of ATP, perturbation of the cellularionic homeostasis, neurotransmitter release and activation of many cytotoxic enzymes. The release of the excitatory neurotransmitter glutamate leads to excessive excitotoxic stimulation of glutamate receptors in energy deprived neurons. Excitotoxicity is a major mechanism

in the early stages of the progression of ischemic brain injury. Other detrimental events include peri-infarct depolarisation, apoptosis and inflammation (Dirnagl *et al*., 1999).

Traditional medicine from the Thai National List of Essential medicine specifies Lom-Am-Ma-Pruek as a remedy for stroke and an analgesic in the musculoskeletal system. In the textbook of Chawadarn (Old medical texts of Thailand, 2007), it consists of fourteen medicinal plants as follows: *Allium sativum* Linn, *Alpinia galanga* (L.) Willd, *Cinnamomum camphora* (L.) Presl, *Cleome viscosa* Linn, *Crateva adansonii* DC, *Crateva religiosa* G.Forst, *Curcuma zedoaria* (Berg) Roscoe, *Erythrina variegata* Linn, *Myristica fragrans* Houtt (seed and fruit), *Piper nigrum* Linn, *Plumbago indica* Linn, *Zingiber cassumunar* Roxb, Sodium Chloride (NaCl).When mixed together, the remedy has a spicy taste. Thai traditional medicine practitioners believed that spicyherbscan help balance the body. Therefore, it should be investigated and confirmed that Lom-Am-Ma-Pruek preparation can be an anti-inflammatory medicine for treatment of stroke and muscle pain. Thus, the aim of this study was to investigate anti-inflammatory effects of Lom-Am-Ma-Pruek and extracts of each component plant which have been used to reduce inflammation. In this study Griess reagent was used to measure the anti-inflammatory activity by inhibition effects of all extracts on nitric oxide production activated by lipopolysaccharide in RAW 264.7 cell lines.Inhibitor activity of TNF-α and COX-2 which cause inflammation in many organs were also investigated to determine anti-inflammatory activity.The results from this research are expected to support the use of a Thai traditional medicine called Lom-Am-Ma-Pruek for treatment of stroke and as an analgesic in the musculoskeletal system.

1.2 Objectives

1.2.1 Overall aims

The overall aims of this research are to study the anti-inflammatory and antioxidant activites of ethanolic andaqueous extracts of Lom-Am-Ma-Pruek remedy and its plant ingredients.

1.2.2 Specific aims

1.2.2.1 To investigate anti-inflammatory activity by nitric oxide inhibition induced by lipopolysaccharide in Murine Macrophage RAW 264.7 cells of the extracts of Lom-Am-Ma-Pruek remedy and its plant ingredients.

1.2.2.2 To investigate anti-inflammatory activity by inhibition of the enzyme COX-2 by the extracts of Lom-Am-Ma-Pruek remedy and its plant ingredients.

1.2.2.3 To investigate anti-inflammatory activity by inhibition of the TNF-α by the extracts of Lom-Am-Ma-Pruek remedy and its plant ingredients.

1.2.2.4 To investigate antioxidant activity of the extract of Lom-Am-Ma-Pruek remedy and its plant ingredients.

1.2.2.5 To study the chemical fingerprints of ethanolic extract of Lom-Am-Ma-Pruek remedy using high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS).

1.2.2.6 To study the stability of Lom-Am-Ma-Pruek remedy extract under accelerated conditions.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Stroke (Cerebrovascular accident)

A stroke, or "brain attack," occurs when blood circulation to the brain fails. Blood flow decrease and oxygen lack can cause brain death. There are two broad categories of stroke: those caused by a blockage of blood flow and those caused by bleeding into the brain. An ischemic stroke, a blockage of a blood vessel in the brain or neckis the most frequent cause of stroke and is responsible for about 80 percent of strokes. These blockages stem from three conditions: thrombosis which forms a clot within blood vessel to the brain in the neck, embolism which moves a clot to another part such as from heart to brain and stenosiswhich is a narrowed artery leading to brain. If there is any bleeding in brain or brain space, it can cause hemorrhagic stroke, the second type of stroke. Although different mechanisms are involved in the pathogenesis of stroke, increasing evidence shows that ischemic injury and inflammation may account for its pathogenic progression (Muir*et al.,* 2007). Cerebral ischemia triggers the pathological pathways of the ischemic cascade and ultimately causes irreversible neuronal injury in the ischemic core within minutes of the onset (Dirnagl *et al.,* 1999). However, a much larger volume of brain tissuesurrounding this ischemic core, known as the penumbra, can be salvaged if cerebral blood flow is promptly restored. Thus, the original definition of the ischemic penumbra referred to areas of brain that were damaged but not yet dead, offering the promise that if proper therapies could be found, one could rescue brain tissue after stroke and reduce post-stroke disability (Lakhan*et al*., 2009).

In addition, current therapeutic, including anti-platelet and thrombolytic drugs, only partially ameliorate the clinical outcome of stroke patients because such drugs are aimed at preserving or restoring cerebral blood flow rather than at preventing the actual mechanisms associated with neuronal cell death (Gladstone*et al*., 2002; Lo*et al*., 2003).

2.1.1Mechanisms ofischemic inflammation

Cerebral blood flow reduces more than 80% in the core of region a few minutes, causes cell damage and death rapidly, which develop as a consequence of the acute energy failure and loss of ionic gradients associated with permanent and anoxic depolarization (Hossmann, 1994; Dirnagl, 1999). A few hours later, the infarct expands into the penumbra, an area of partially preserved energy metabolism, as a result of peri-infarct spreading depression and molecular injury pathways that are activated in the cellular and extracellular compartments. At this stage, cellular damage is mainly triggered by excitotoxicity, mitochondrial disturbances, reactive oxygen species production and programmed cell death (Lo*et al*., 2005). These phenomena, such as vasogenic edema and delayed inflammatory processes, are the result of the evolution of tissue damage prolonged for days or weeks (Hossmann, 2006). There is increasing evidence demonstrating that neuroinflammatory processes play an important role in the pathophysiology of brain ischemia. The inflammatory process initiated a few hours after stroke and may last for days and weeks as a delayed tissue reaction to injury and is characterized by an immediate phase (Dirnagl*et al*., 1999; Stoll *et al*., 1998). In addition to their deleterious contribution to ischemic tissue damage, inflammatory mediators may also exert beneficial effects on stroke recovery (Del*et al*., 2001; Kriz, 2006; Denes*et al*., 2007).

Inflammation is characterized by the accumulation of inflammatory cells and mediators in the ischemic brain. After ischemia onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulate within the brain tissue subsequently leading to inflammatory injury. Increasing evidence shows that astrocytes may also act as inflammatory cells responding to ischemic stroke (Qing*et al*., 2007).

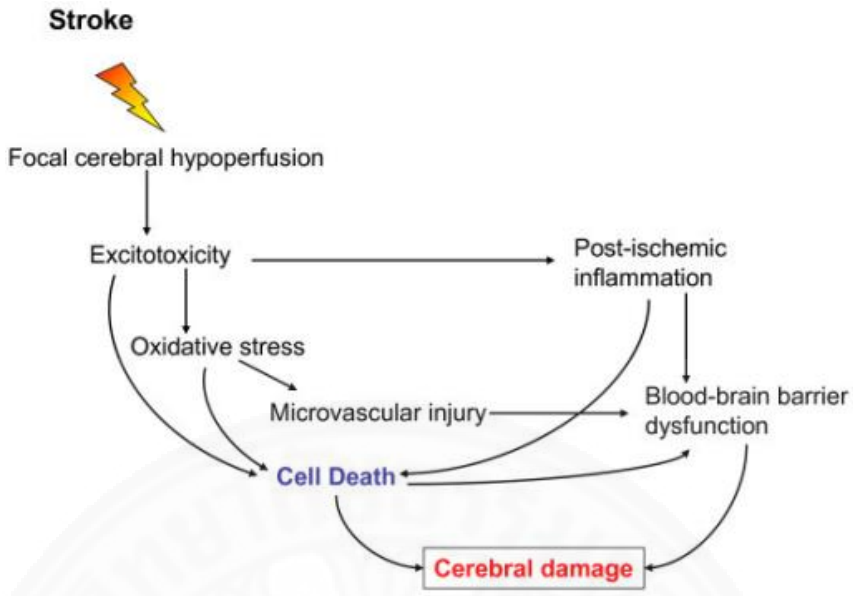


Figure 2.1 Ischemic stroke leads to hypoperfusion of a brain area that initiates a complex series of events. Excitotoxicity, oxidative stress, microvascular injury, blood-brain barrier dysfunction and postischemic inflammation lead ultimately to cell death of neurons, glia and endothelial cells. The degree and duration of ischemia determines the extent of cerebral damage (Lakhan *et al.*, 2009).

1.1.2.1 Leukocytes

4–6 h hours after ischemia onset, leukocytes which adhere vessel wall are leading to migration and accumulation into ischemic brain tissue with subsequent release of proinflammatory mediators. These mediators lead to secondary injury of potentially salvageable tissue within the penumbra surrounding the infarct core. The first leukocyte subtype recruited to the ischemic brainis the neutrophil and may potentiate injury by directly secreting deleterious substances or other inflammatory mediators (Hallenbeck, 1996).

2.1.1.2 Microglia/Macrophages

Microglia, the resident macrophages of the brain, play a critical role as resident immunocompetent and phagocytic cells in the CNS (Kreutzberg, 1996), and serve as scavenger cells in the event of infection, inflammation, trauma, ischemia, and neurodegeneration (ElKhoury *et al*., 1998; Thomas, 1992). Once activated, microglia can undergo morphologic transformation into phagocytes, making them virtually indistinguishable from circulating macrophages. Whether microglia/macrophages are necessarily damaging following brain ischemia is unclear, but a few lines of evidence suggest that activated microglia may contribute to injury. Edaravone, a novel free radical scavenger, significantly reduced the infarct volume and improved the neurological deficit scores for ischemic mice by reducing microglial activation (Zhang *et al*., 2005).

2.1.1.3 Astrocytes

Astrocytes are specialized glial cells that outnumber neurons by over fivefold. They contiguously tile the entire central nervous system (CNS) and exert many essential complex functions in the healthy CNS. Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions. Substantial progress has been made recently in determining functions and mechanisms of reactive astrogliosis and in identifying roles of astrocytes in CNS disorders and pathologies. Aside from traditional inflammatory cells, astrocytes are known to express different kinds of inflammatory mediators (Benveniste, 1998; Che *et al*., 2001). Following ischemia, Brain astrocyte, called “reactive gliosis,” was characterized by specific structural and functional changes and activated resulting in increased glial fibrillary acidic protein (GFAP) expression (Pekny & Nilsson, 2005). Astrocytes also participate in brain inflammation by expressing major histocompatibility complex (MHC) and costimulatory molecules, developing Th2 (anti-inflammatory) immune responses and suppressing interleukin-12 (IL-12) expression, though this has yet to be demonstrated in ischemia models. Inflammatory factors, cytokines, chemokines and inducible nitric oxide synthase (iNOS) are secreted by astrocytes. Following 10 minutes of transient

global ischemia, iNOS expression was found in reactive astrocytes of hippocampus but not in uninjured hippocampal astrocytes (Dong and Benveniste, 2001).

2.1.1.4 Adhesion molecules

Neuroinflammatory mediators induce the expression of receptors and adhesion molecules to promote the recruitment and infiltration of leukocytes into the brain which are rapidly released from injured tissue following ischemic insult. Indeed, focal ischemia is associated with significantly elevated levels of cyto- kines, such as tumor necrosis factor (TNF)-a, interleukin (IL)-1b and IL-6 (Liu*et al*., 1994; Wang *et al*., 1994), and chemokines, such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (Kim*et al*., 1995; Wang *et al*., 1995; Che X *et al* 2001). These mediators induce the expression of the adhesion molecules ICAM-1 [27–30], P-selectin and E-selectin and integrins (Zhang *et al*., 1998; Huang *et al*., 2000; Che X *et al* 2001; Kim*et al*., 1995) on endothelial cells and leukocytes, which promote the adhesion and transendothelial migration of leukocytes (Lindsberg *et al*., 1996). By this mechanism, activated neutrophils and platelets accumulate in cerebral capillaries and further impair blood perfusion of the injured tissue (Mori *et al*., 1992).

2.2.1Inflammatory mediators

2.2.1.1Interleukin-1 (IL-1)

One of several proteins is important for lymphocyte proliferation. Macrophages produce Interleukin-1 (IL-1) and IL-1 induces the production of interleukin-2 by T cells which are stimulated by antigen or mitogen. Interleukin-2 (IL-2), produced by T cells, stimulates the proliferation of T cells bearing specific receptors for IL-2; these receptors are expressed in response to antigenic stimulation. Furthermore, IL-2 also seems to induce the production of interferon and is used as an anticancer drug for treatment of a wide variety of solid malignant tumors. Another interleukin, interleukin-3 (IL-3) is necessary for the differentiation of suppressor T cells. IL-1's two isoforms, IL-1α and IL-1β and its endogenous inhibitor, IL-1 receptor antagonist (IL-1ra) have been the most studied in experimental stroke. IL-1beta mRNA elevations have been documented within 15–30 min after ischemia (Buttini *et al*., 1994) with increased protein a few hours later (Davies *et al*., 1999)

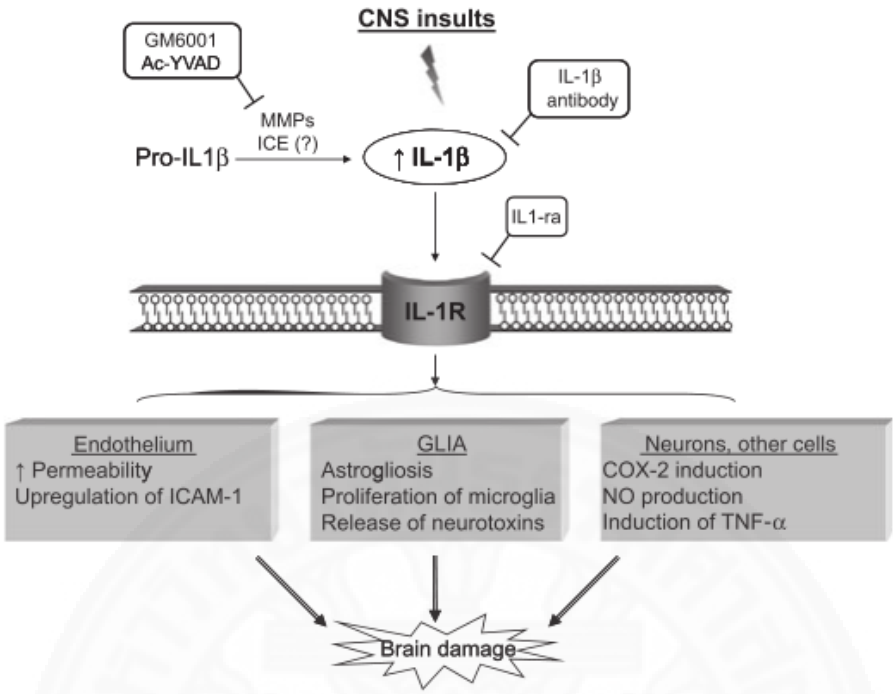


Figure 2.2 Putative mechanisms implicated in IL-1b-induced neuroinflammation after stroke injury. CNS, central nervous system (Amantea*et al*., 2008)

2.2.1.2Tumor necrosis factor-alpha (TNF-α)

TNF was identified as the key regulation of inflammatory response which can cause necrosis of tumors. This review describes the known signaling pathways and cell biological effects of TNF, and our understanding of the role of TNF in human disease. TNF-α is also upregulated in the brain after ischemia with similar expression patterns as IL-1beta. Initial increases are seen 1–3 h after ischemia onset (Liu *et al*., 1994), and, like IL-1beta, has a biphasic pattern of expression with a second peak at 24–36 h (Murakami *et al*., 2005; Offner *et al*., 2006). TNF-α expression was initially observed in neurons (Liu *et al*., 1994), then later in microglia and some astrocytes (Uno *et al*., 1997) as well as in the peripheral immune system (Offner *et al*., 2006).

2.2.1.3 Cyclooxygenase (COX)

Cyclooxygenase (COX) converted Arachidonic acid which released from brain, to prostaglandin H_2 (PGH_2) in Cyclooxygenase pathway during ischemia/reperfusion. COX has two isoforms. COX-1 is constitutively expressed in many cell types, including microglia and leukocytes during brain injury (Schwab *et al*., 2002).

The roles of various COX metabolites are protean, but accumulated data suggest that those downstream of COX-2 are likely deleterious. Recent work has shown that prostaglandin E (2) EP1 receptors may be the downstream effectors responsible for neurotoxicity in ischemic stroke (Kawano *et al*., 2006). The treatment with COX-2 inhibitors after stroke can improve neurological outcomes (Nogawa *et al*., 1997; Sugimoto and Iadecola, 2003). Furthermore, COX-2 deficient mice have reduced injury after N-methyl-D-aspartate (NMDA) exposure (Iadecola *et al*., 2001), whereas COX-2 overexpression exacerbates brain injury (Dore *et al*., 2003). Interestingly, COX-2 mediates its toxic effect through PGE 2 rather than ROS, even though COX-2 can generate both (Manabe *et al*., 2004).

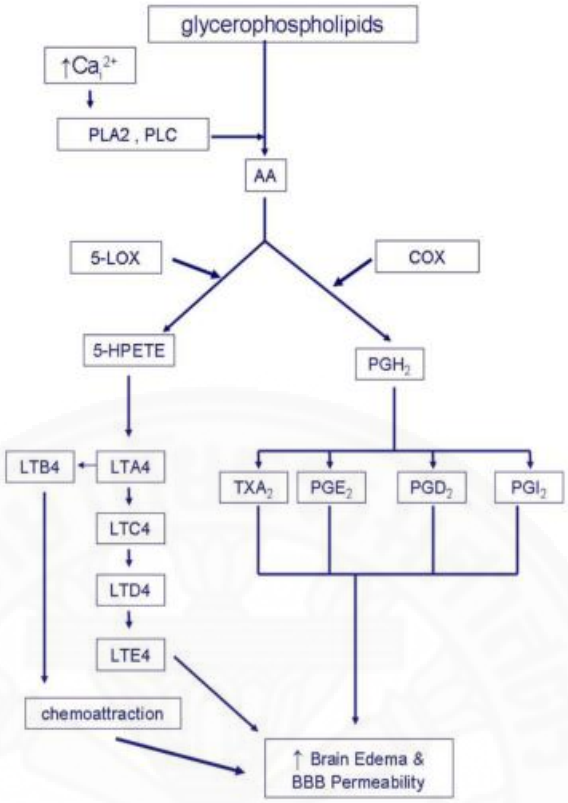


Figure 2.3 Ischemic activation of the arachidonic acid cascade (Qing*et al*., 2007).

2.2.1.4Nitric Oxide/Nitric Oxide Synthase

Nitric oxide (NO), a signaling molecule, plays an important role in physiological processes as neuronal communication, host defense, and regulation of vascular tone. This relatively stable gas readily diffuses into cells and cell membranes where it reacts with molecular targets. Three nitric oxide synthases (NOS) isoforms exist; endothelial NOS (also known as type III, NOS-III and NOS-3), neuronal NOS (also known as type I, NOS-I and NOS-1), and inducible NOS (also known as type II, NOS-II and NOS-2). Ischemic injury might be caused by iNOS that is relevant to inflammatory cells and its expression is thought to be restricted to cells involved in inflammatory responses such circulating leukocytes, microglia and astrocytes. In the brain, an increase of NO production and iNOS enzymatic activity were associated with ischemia-induced upregulation of iNOS mRNA and protein (Iadecola *et al*., 1995). DNA might be damaged by NO in cerebral ischemia through the formation of peroxynitrite (Cui *et al*., 2000; Huang *et al*., 2000).

2.2.1.5Reactive Oxygen Species

Generation of reactive oxygen species (ROS) by inflammatory cells occurs via several enzyme systems. Superoxide is generated via COX, xanthine dehydrogenase, xanthine oxidase and NADPH oxidase, whereas myeloperoxidase (MPO) and monoamine oxidase (MAO) generate hypochlorous acid and H_2O_2. Among all the oxidants in the brain parenchyma after MCAO, superoxide anion is a major one, causing direct injury to ischemic brain or by reacting with NO to generate peroxynitrite (Chan, 2001). The switch to glycolysis in the O_2-depleted cell results in lactic acid and H^+ build-up in the mitochondria and the subsequent reversal of the H^+ uniporter on the mitochondrial membrane which causes excess cytosolic H^+ accumulation and acidosis (Gerhard*et al*., 2000).

Acidosis contributes to oxidative stress by providing H^+ for the conversion of $\bullet O_2^-$ into H_2O_2 or the more reactive hydroxyl radical (•OH). In addition, in the O_2^- depleted cell the potent protein and lipid oxidant peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide (NO) and $\bullet O_2^-$, rapidly exhausting the NO bioavailability. Activation of NMDA receptors (NMDARs) by glutamate also increases intracellular NO and subsequent $ONOO^-$ production in the ATP depleted post-synaptic cell. Neuronal nitric oxide synthase (nNOS) is physically anchored to NMDARs and following activation and influx of Ca^{2+}, Ca^{2+} binds calmodulin and rapidly activates nNOS generating NO (Stanika*et al*., 2012).

2.2 Stroke in Thai traditional medicine

2.2.1 Pathogenesis of stroke in Thai traditional medicine.

The principle theory of Thai traditional medicine is the knowledge of four elements namely earth or Pathavi (consisting of 20 components), water or Apo (Semha)(consisting 12 of components), wind or Wayo (Wata) (consisting of 6 types) and fire or Techo (consisting of 4 components). Disease occurs when there is animbalance of elements in the body and involves many factors, for example season, environment and behavior all bring about the imbalance. The concept of stroke in Thai traditional medicine is similar to hypertension and hypercholesterolemia. Accordingly the etiology of diseases caused by wind imbalance (Wata disorder) in textbook of Chawadarn (Old medical texts of Thailand, 2007), ancient menuscript practice was concerned with increasing, decreasing and restoring proper interaction between the 4 elements.

There are many factors affecting stroke such as food, age, cigarette, alcohol, weather and stress. The beginning of a stroke is an increase of Pitha from various risk factors. These effects result in increase of Wata in the abdomen. (Athokamawataand Uthangkamawata), the Semha (disorder of blood and Wata, bubble is blood)resolting inincrease of Wata and Pittha. First, the effects result in increases of Wata moving upward producing high blood pressure. Second, the effects result in increases of Pitha drying of blood producing hyperlipidemia. Third, when Wata and Pittha increase there are results in the nervous system (Sumanar and Ammapruek Ammapatth line), caused abnormal brain function or the imbalance of body elements (result may be complete paralysis of one side of the body, problems with balance and co-ordination), and the Thai traditional remedy which treats these symptoms is hot and mild for decreasing Wata and Pittha. Lom-Am-Ma-Pruek remedy controls abnormalities of the wind and fire elements in the body. The mechanism is shown in **Figure 2.4.**

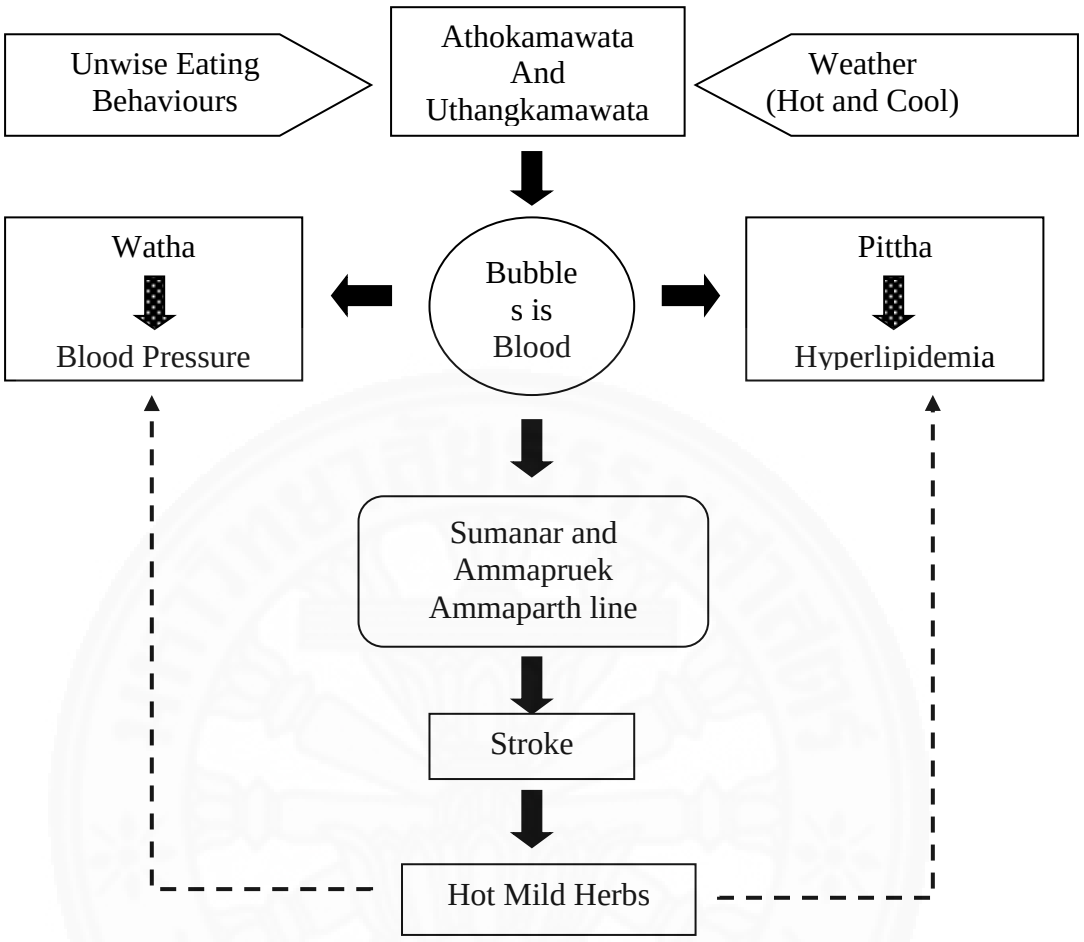


Figure 2.4 The concept of stroke as seen in Thai traditional medicine terms

2.3 Lom-Am-Ma-Pruek remedy and plant ingredients

2.3.1 Lom-Am-Ma-Pruek remedy in Thai traditional medicine

Thai traditional medicine from Thai National List of Essential Medicine specifies Lom-Am-Ma-Pruek as a remedy for stroke and an analgesic in the musculoskeletal system. In the textbook of Chawadarn (Old medical texts of Thailand, 2007), it consists of fourteen medicinal plants as follows: *Allium sativum* Linn,*Alpinia galanga* (L.) Willd, *Cinnamomum camphora* (L.) Presl, *Cleome viscosa* Linn, *Crateva adansonii* DC,*Crateva religiosa* G.Forst, *Curcuma zedoaria* (Berg) Roscoe, *Erythrina variegata* Linn, *Myristica fragrans* Houtt (seed and fruit), *Piper nigrum* Linn, *Plumbago indica* Linn, *Zingiber cassumunar* Roxb, Sodium Chloride (NaCl).When mixed together, the remedy has a spicy taste. Thai traditional medicine

practitioners believed that spicyherbscan help balance the body.The classification of ingredients in this remedy are shown in **Figure2.5** and the biological activities data shown in **Table 1.1**

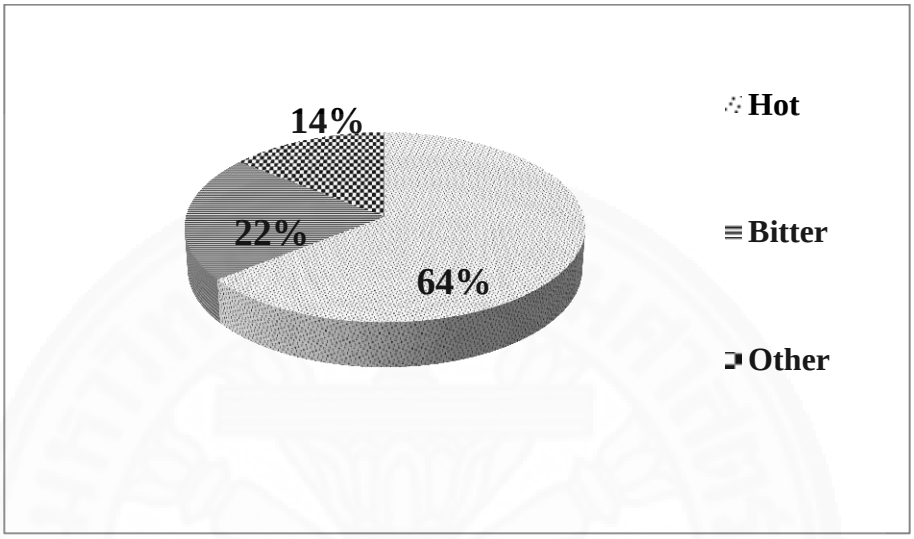


Figure 2.5 The classification of ingredients in this remedy in Thai traditional medicine

Table 2.1 Plants and parts of plants comprising Lom-Am-Ma-Pruek preparation

Species	Family	Thai name	Plant part	Flavor	Voucher specimen number	Percentage	Thai traditional
Allium sativum Linn.	ALLIACEAE	Kratiem	Bulbs	Hot and spicy	SKP 006 01 19 01	7.14	Antipyretic, Carminative, Antipruritic
Alpinia galanga (L.) Willd.	ZINGIBERACEAE	Kra	Rhizome	Hot and spicy	SKP 206 01 07 01	7.14	Aperient, Analeptic, Gynecology, Carminative
Cinnamomum camphora (L.) Presl.	LAURACEAE	Karaboon	Crystal	Hot	SKP 096 03 03 01	7.14	Cardio tonic, Diuretic agents
Cleome viscosa Linn.	CLEOMACEAE	Pukseanphee	All of trunk	Bitter	SKP 039-1 03 22 01	7.14	Vermicide, Cardio tonic, Promote blood circulation
Crateva adansonii DC.	CAPPARACEAE	Koombouk	Bark	Bitter and Fragrant	SKP 039-1 03 01 01	7.14	Analgesic, Analeptic, Neurotropic drugs
Crateva religiosa G.Forst.	CAPPARACEAE	Koomnum	Bark	Bitter and Fragrant	SKP 039-1 03 18 01	7.14	Perspire drugs, Analeptic, Promote blood circulation
Curcuma zedoaria (Berg) Roscoe.	ZINGIBERACEAE	Kaminaoi	Rhizome	Hot and Astringent	SKP 206 03 26 01	7.14	Liniment, Analgesic, Carminative
Erythrina variegata Linn.	LEGUMINOSAE	Thonglarng	Bark	Bitter	SKP 098 05 22 01	7.14	Antipyretic, Analgesic

* Modified from Thai traditional characteristics and reviewed from the explanatory text of Osot Pra Narai (Phichianshunthorn *et al.*, 2005)

Table 2.1 Table 8-5.Plants and parts of plants comprising Lom-Am-Ma-Pruek preparation (Continued)

Species	Family	Thai name	Plant part	Flavor	Voucher specimen number	Percentage	Thai traditional
Myristica fragrans Houtt.	MYRISTICACEAE	Lokchan	Seed	Hot and Fragrant	SKP 121 13 06 01	7.14	Tonic, Cardio tonic, Carminative
Myristica fragrans Houtt.	MYRISTICACEAE	Dokchan	Aril of Seed	Hot and Fragrant	SKP 121 13 06 01	7.14	Tonic, Cardio tonic, Carminative
Piper nigrum Linn.	PIPERACEAE	Pikthai	Seed	Hot and spicy	SKP 146 16 14 01	7.14	Tonic, Cardio tonic, Carminative, Tonic for longevity
Plumbago indica Linn.	PLUMBAGINACEAE	Jattamoonplengdang	Root	Hot and spicy	SKP 148 16 09 01	7.14	Promote blood circulation, Gynecology, Carminative
Sodium Chloride(NaCl)		Salt	Crystal	Salty		7.14	Skin Care
Zingiber cassumunar Roxb.	ZINGIBERACEAE	Phai	Rhizome	Hot and Astringent	SKP 206 26 03 01	7.14	Analgesic, Gynecology

* Modified from Thai traditional characteristics and reviewed from the explanatory text of Osot Pra Narai (Phichianshunthorn *et al.*, 2005)

2.3.2 General data of the ingredients in Lom-Am-Ma-Pruek remedy

Figure 2.6*Allium sativum* Linn. (ALLIACEAE)

2.3.2.1 *Allium sativum* Linn. (ALLIACEAE)

(1) Thai Common Name: kratiem

(2) Traditional and Ethnopharmacological uses of *Allium sativum* Linn.

Garlic has been widely used in Thailand for centuries both as food and as medicine. Other cultures have also made use of garlic, especially those in Asia as well as the Mediterranean region. Garlic in the form of supplementary pills is widely popular now due to its well-known health benefits.

In Thai traditional medicine garlic is used as a carminative, antipyretic, antipruritic and few treating wind colic, skin disease also as an ingredient Leung-Pid-Samud remedy (diarrhea), Prasapai remedy (postpartum care), That-Bun-Job remedy (antiflatulent, diarrhea).

(3) Description:

Stem underground bulb, in clusters. Bulb with 8-16 bulblets, which are white, oval in shape, and adventitious.Leaves simple, green, fleshy, hollow, cylindrical, and radical. Flower small, white umbellate. Fruit capsular (Abbasi *et al.*, 2012).An image of*Allium sativum* Linn is shown in **Figure 2.6**.

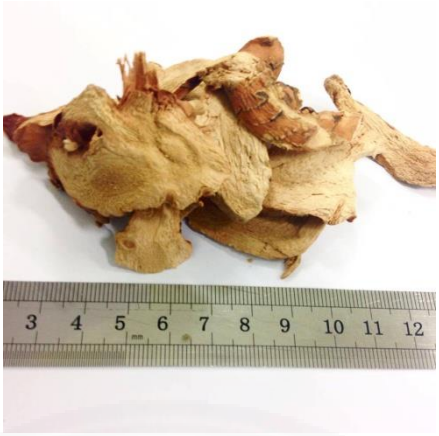


Figure 2.7*Alpinia galanga* (L.) Willd. (ZINGIBERACEAE)

2.3.2.2 *Alpinia galanga* (L.) Willd. (ZINGIBERACEAE)

(1) Thai Common Name: kra

(2) Traditional and Ethnopharmacological uses ofAlpinia galanga (L.) Willd.

Marketed plants are very important items of trade in many parts of the world. The community uses these for a variety of purposes such as food, cosmetics, flavors, spices, and medicines. It seems that plants used for medicinal purposes form the most common category (Juntachote *et al*., 2006). Galangal (also called galanga, galingale, or galangale) is a species of the ginger family (Zingiberaceae) which is composed of 47 genera and 1400 species distributed world wide (Pothisiri, 1996). Three species of galangal, *A. officinarum* Hance, *A. galanga Linnaeus* Willd. And *A. conchigera* Griff.are commonly known and found in Thailand.

The rhizomes of galangal and its derivatives have long been used for its aromatic stimulant, carminative, and condiment properties much like ginger (the dried rhizome of *Zingiber officinale*). Galangal oil is used to flavor French liqueurs and some tobaccos. Galanga (greater galangal), containing the volatileessential oil essence d'Amali, is used in China and northern India for various respiratory complaints in children, particularly bronchial (mucous membrane inflammation) (Lewis and Elvin 1977).

In Thai traditional medicine, rhizomes are used for healing contusion, stomach discomfort, abdominal discomfort, and squeamishness, expectorant, blood tonic while leaves and flowers are used for treatmentof ring worm (Athamaprasangsa *et al.,* 1994; Mallavarapu, 2002; Kanjanapothi *et al*., 2004).

(3) Description:

A perennial tuberous herb with elongate leafy stems and slightly aromatic rootstock; leaves cauline, 22.5-45 cm long, 3.7-11.2 cm broad, oblong-lanceolate, acute, glabrous, green above, paler beneath with slightly callous white margins, sheaths long, glabrous, ligule about I cm long and rounded; flowers irregular, bisexual, greenish white in dense-flowered panicles 15-30 cm long, branches short, rachis pubescent, pedicels 0.3-0.4 cm long, bracts 1 cm long, ovate-lanceolate; calyx 1 cm long, tubular, irregularly 3-toothed; corolla gamopetalous, 3cm long, tube 1.2 cm long, lobes oblong, obtuse,subequal, 0.6 cm broad, lip 2.1 cm long, claw green, 6 mm long, 2.5 mm broad, blade white striated with red, about 1.2 cm long, broadly elliptic, shortly 2-lobed at apex with a pair of subulate glands at the base of the claw; stamen 1, perfect, 1.8 cm long, filament flattened, anther cells diverging at the top occasionally with an orbicular crest, lateral staminodes minute or obsolete; ovary inferior, 3-locular, ovules few on an axile placenta, style filiform, stigma subglobose; fruit orange red, indehiscent (Arambewela and Wijesinghe, 2006). The image of *Alpinia galanga* is shown in **Figure 2.7**.

Figure 2.8*Cinnamomum camphora* (L.) Presl. (LAURACEAE)

2.3.2.3 *Cinnamomum camphora* (L.) Presl. (LAURACEAE)

(1) Thai Common Name: karaboon

(2) Traditional and Ethnopharmacological uses of *Cinnamomum camphora* (L.) Presl.

Camphor has a long-valued history for its extensive and diverse uses in the East. Traditionally used for the treatment of toxication caused by food, alcohol or drugs, as well as diarrhea, abdominal pain, hypertension, skin itching and cancer (Lee*et al.*, 2002)

(3) Description:

Tree or shrub (Cassytha a twining parasitic perennial herb with leaves reduced or absent), mostly evergreen (deciduous in temperate regions), sometimes dioecious.Bark and foliage usually aromatic.Leaves usually alternate, occasionally opposite or subopposite or verticillate, simple, usually entire, rarely lobed (Sassafras), mostly pinninerved and subpalmately veined, or often triplinerved, usually punctate and leathery, estipulate.Flowers in usually axillary, occasionally subterminal, panicles, spikes, racemes, or pseudoumbels, generally bisexual, sometimes unisexual, actinomorphic, mostly 3-merous, also 2-merous, small, greenish, yellowish, or white.Perianth biseriate, of usually 4 or 6 basally connate usually undifferentiated sepal-like segments, deciduous or persistent; perianth tube usually persisting as a cupule at base of fruit. Androecium typically of 4 whorls of 3 stamens each, adnate to perianth tube, innermost whorl rarely united, usually reduced to staminodes; filaments usually free, 3rd whorl usually bearing 2 usually sessile and distinct basal glandular protuberances; anthers basifixed, 2-celled or 4-celled at anthesis, those of 2 outer

whorls mostly introrse, inner 3rd whorl extrorse, dehiscing by flaplike valves opening upward. Pistil 1; ovary usually superior, 1-loculed; ovule solitary, anatropous, pendulous, placentation parietal; style 1; stigma 1, occasionally 2- or 3-lobed. Fruit a drupe or berry usually surrounded at base by enlarged and often persistent perianth tube seated on a large receptacle or pedicel. Seed with large straight embryo; endosperm absent (Starr and Loope 2003). The image of *Cinnamomum camphora* is shown in **Figure 2.8.**

Figure 2.9*Cleome viscosa* Linn. (CLEOMACEAE)

2.3.2.4 *Cleome viscosa* Linn. (CLEOMACEAE)

(1) Thai Common Name: pukseanphee

(2) Traditional and Ethnopharmacological used of *Cleome viscosa* Linn.

The natives and traditional healers of India use *C. viscosa* Linn. for various therapeutic purposes. In traditional system of medicine, this plant is used to treat various disorders such as diarrhea, fever, inflammation, liver diseases, bronchitis, skin diseases and malarial fever. The juice is useful in piles, lumbago, and earache (Kirtikar and Basu**,** 1975).The plant is good for malarial fevers and useful in blood diseases, uterine complaints. The leaves are also used in for wounds and ulcers.*C. viscosa* leaves and young shoots used to cook like a vegetable, which is having sharp mustard like flavour. The pungent seeds and seed pods can be used as a mustard substitute in curries (Williams*et al.*, 2003).

(3) Description:

C. viscosa plant is erect, grooved, aromatic glandular and contains sticky shoots. Stem is rounded, solid, (glandular) hairy while root is taproot branched white or brown. Plant bears, elliptic-oblong, obovate leaflets of variable in size (1.5–2.5 cm) broad and petiole up to 5 cm long. Leaves are sessile, compound, trifoliolate, alternate, spiral,stalked, foliate in shape and contain glandular hairs onboth sides, leaflets are elliptic, (glandular) hairy on bothside, margin entire, apex acute, base acute, pinnatelyveined. Flowers are pedicelate, bisexual, single, axillary,stalked, yellow, petals 4 and free. These are white oryellow in color. Flowering occurs from May to Septemberand fruiting in August to November.Inflorescence is racemose or corymbose. Stipules are absent. Pedicels are 6–20 mm long; bracts foliaceous, petals 8–15 mm long, 2–4 mm broad, oblong‐obovate. Stamens are 10–12 and rarely occur up to 20 and not exceeding the petals; gynophores absent. Sepals are oblong‐lanceolate, 3–4 mm. long, 1–2 mm wide, glandular pubescent. Fruits aredark brown 30–75 mm long and 3–5 mm broad in size,a capsule, either a slender, linear‐oblong capsule, erect,obliquely striated and tapering at both ends. Its twovalves contain hundreds of seeds that are oil producing (Upadhyay, 2015). The image of *Cleome viscosa* is shown in **Figure 2.9**.

Figure 2.10*Crateva adansonii* DC. (CAPPARACEAE)

2.3.2.5 *Crateva adansonii* DC. (CAPPARACEAE)

(1) Thai Common Name: koombouk

(2) Traditional and Ethnopharmacological uses of *Crateva adansonii* DC.

In India, different parts of the plant are extensively used in folklore medicine for the cure of many disease conditions. The powdered bark is used in the treatment of urinary, renal tubules, gastro-intestinal, and uterine affection (Gitte *et al.*, 2012). In South Africa, the plant is used in inflammatory conditions, asthma, snakebites, and as astringent (Akanji *et al.*, 2013).

(3) Description: Deciduous shrub or small tree up to 10(–15) m tall; bole usually irregular and short, up to 50 cm in diameter; bark surface smooth and grey to brown, inner bark thin, yellow-brown with brown streaks; crown rounded, more or less open; twigs glabrous, brown with grey lenticels. Leaves alternate but clustered near end of twigs, compound with 3 leaflets; stipules minute, soon falling; petiole 2.5–8.5 cm long; petiolules up to 8 mm long; leaflets elliptical to ovate or lanceolate, the lateral ones asymmetrical, 3–12.5 cm × 1–5 cm, cuneate at base, acuminate at apex, papery, glabrous, pinnately veined with up to 15 pairs of indistinct lateral veins. Inflorescence a short terminal raceme up to 2.5(–7.5) cm long, glabrous, up to 15 flowered. Flowers bisexual, nearly regular, 4-merous; pedicel 1.5–4 cm long; sepals free, deltoid to lanceolate, 3–9 mm long, equal; petals free, ovate, slightly unequal, 1.5–3 cm long, yellowish white, sometimes red-purple tipped; stamens 15–20, free, 2–3.5(–5) cm long, with purplish anthers; ovary superior, long-stalked, ellipsoid, glabrous, 1-celled, stigma sessile, knob-shaped. Fruit a distinctly stalked,

globose berry 4–5(–8) cm, smooth, yellow to brown, with mealy whitish pulp, up to 15 (–20) seeded. Seeds are kidney-shaped, 0.5–1 cm long, brown to black. The image of *C. adansonii* is shown in **Figure 2.10**.

Figure 2.11*Crateva religiosa* G.Forst. (CAPPARACEAE)

2.3.2.6 *Crateva religiosa* G.Forst. (CAPPARACEAE)

(1) Thai Common Name: koomnum

(2) Traditional and Ethnopharmacological uses of *Crateva religiosa* G.Forst:

C. religiosa bark contains saponins and sugars. The plant parts used for medicinal purposes include stem barks, leaves and root barks. The plant is used ethnopharmacologically as diuretic, laxative, lithonotriptic, antirheumatic, antiperiodic, bitter tonic, rubifacient and counterirritant. In folklore the bark is specifically used in urinary disorders including kidney and bladder stones, antiemetic and as an antidote in snake bite. The ethnopharmacological information regarding its use against urinary disorders and pathological skin conditions prompted us to select some of the fungal pathogens including *Candida albicans, Candida tropicalis, Candida krusei, Cryptococcus marinu* and *Aspergillus niger* (Saho *et al.*, 2008).

(3) Description:

C. religiosa grows as a deciduous tree, height of 3 to 15 feet and trunk diameter of 40 cm. The plant parts have no hairs. The bark of the branches is light green to yellowish-green when dry with gray elongated lenticels. The alternate arranged leaves are divided into petiole and leaf blade. Most 6 to 7 (5 to 10) cm long

petioles have near the rachis tiny triangular glands. The imparipinnate leaf blades are composed of three leaflets. The leaflets have a first thin, later become thicker, usually 3 to 5 (rarely to 7) mm long stem. The thin and leathery leaflets are with a length of usually 5.5 to 7 (4 to 10) cm long and (often 2 to) 3 to 4 cm wide. On both sides of reddish central nerve are five to ten page nerve, the nerve network are clearly visible. The Endfiederblatt has an asymmetrical base. The stipules are small and triangular. The image of *C. religiosa* is shown in **Figure 2.11**.

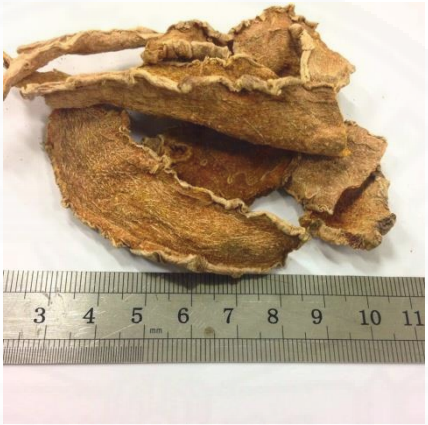


Figure 2.12*Curcuma zedoaria* (Berg) Roscoe. (ZINGIBERACEAE)

2.3.2.7 *Curcuma zedoaria* (Berg) Roscoe. (ZINGIBERACEAE)

(1) Thai Common Name: kaminaoi

(2) Traditional and Ethnopharmacological uses of *Curcuma zedoaria* (Berg) Roscoe:

C.zedoaria has been used traditionally in many countries especially in South-East Asia as a folk medicine for many centuries and is a valuable medicinal plant. In the traditional way, the dried rhizomes of *C. zedoaria* were selected to make drinks or to be extracted as medicine. *C. zedoaria* rhizome extracts which contains Curcumin have been used to treat stomach diseases, blood stagnation, hepato protection, diarrhea, coryza, dermatosis disorders and rheumatism and promoting menstruation as a traditional medicine. Antimicrobial activity, anti-inflammatory anti-hepatotoxic, neuroprotective activity and cytotoxic effects against human ovarian cancer cells are all regarded as abilities of curcumin productions from *C.zedoaria* and furthermore, zedoaria natural products are used as spices, tonics and also in perfumery

with great luxurious foliage that has high commercial value in floriculture. The rhizomes are also used in food industry as condiment and dye. There have been many reports on the anti-allergic effects of some plants in the Zingiberaceae family, such as *C. longa* and *C.zedoaria* for treatment of itching and other skin diseases, whereas *C. zedoaria* has been used as a substitute for *C.longa* and has recently been reported to show anti-allergic activity as well

In Thai traditional medicine, rhizomes are spicy and the hot flavor is used for treatment as liniment, analgesic, carminative. Flowers are used for treatment of ring worm. Fruit are used for treatment of nausea, vomiting.

(3) Description:

C. zedoaria is a rhizome that grows in tropical and subtropical wet forest regions. The fragrant plant bears yellow flowers with red and green bracts and the underground stem section is large and tuberous with numerous branches. The leaf shoots of zedoaria are long and can reach 1 meter (3 feet) in height.The image of *C. zedoary*is shown in **Figure 2.12**.

Figure 2.13*Erythrina variegata* Linn. (LEGUMINOSAE)

2.3.2.8 *Erythrina variegata* Linn. (LEGUMINOSAE)

(1) Thai Common Name: thonglarng

(2) Traditional and Ethnopharmacological uses of *Erythrina variegata* Linn:

Leaves of *E. variegata* (Indian coral tree) and eaten as a pot herb, and used as an antiobesity drug in Siddha medicine. It has folklore reputation as anti-inflammatory in India, China and South East Asia, and different parts of the plant are reported with insecticidal, hemaglutinating, curaric, skeletal muscle relaxant, feeding deterrent, antispasmodic, antimycobacterial and antiosteoporotic activities (Mangathayaru*et al.,* 2010).

In Pohnpei the leaves are reportedly used to make a drink to nullify curses, and the smoke from smoldering leaves, bark, or roots is inhaled for the same purpose. In Yap the leaves and bark are reportedly used as a potion to treat stomachache. In Tonga the bark is mixed with others and used to treat stomachache. In Samoa the leaves are occasionally used to treat eye ailments, and the bark is applied to swellings. In India, China, and Southeast Asia, the bark and leaves are used in many traditional medicines, including one said to destroy pathogenic parasites and relieve joint pain; the juice from the leaves is mixed with honey and ingested to treat tapeworm, roundworm, and threadworm in India; women take this juice to stimulate lactation and menstruation; it is commonly mixed with castor oil to treat dysentery; a warm poultice of the leaves is applied externally to relieve rheumatic joints; and the bark is used as a laxative, diuretic, and expectorant **(**Whistler *et al.,* 2006**).**

(3) Description:

It is typically found on sandy soil in littoral forest, and sometimes in coastal forest up to 250 m (800ft) in elevation. The coral tree is cultivated particularly as an ornamental tree and as a shade and soil improvement tree (it fixes nitrogen) for other tree crops. The most attractive type, var. variegata, is grown for its variegated leaves, as well as its seasonal showy red flowers.This fast-growing, 50-60 feet tall and wide deciduous tree with green and yellow-variegated, 6-inch-long leaves creates a broad canopy but has spiny branches. In spring, before the leaves appear, coral tree is decorated with showy red blossoms, each flower 2.5 inches long and arranged in dense, six-inch-long racemes. These blooms are followed by 12-inch-long, red/brown seedpods which contain poisonous seeds. The image of *E. variegata* is shown in **Figure 2.13**.

Figure 2.14 *Myristica fragrans* Houtt. (MYRISTICACEAE)

2.3.2.9 *Myristica fragrans* Houtt. (MYRISTICACEAE)

(1) Thai Common Name: lokchan and dokchan

(2)Traditional and Ethnopharmacological uses of *Myristica fragrans* Houtt:

M. fragrans (Houtt.), commonly called nutmeg, is an aromatic evergreen tree that grows 30–39 ft high with spreading branches and yellow fleshy fruits, having an appearance like apricot or peach. Many other species of the plant exist, but the most common one is *M. fragrans*. Both the nutmeg and mace have been used as general condiments and to flavour many foods such as soups, gravies, milk products, fruits juices, sweet sauces, gelatins, snacks foods, and breakfast cereals (Nagano, 2008). *M. fragrans* has been used to treat rheumatism and stomach complaints in Indonesia, Malaysia, England, and China (Chirathaworn *et al*., 2007).The image of *M. fragrans* is shown in **Figure 2.14**.

Figure 2.15*Piper nigrum* Linn. (PIPERACEAE)

2.3.2.10 *Piper nigrum* Linn. (PIPERACEAE)

(1) Thai Common Name: prikthai

(2) Traditional and Ethnopharmacological uses of *Piper nigrum* Linn.:

Pepper (*Piper nigrum* Linn.), the king of spice, is one of the oldest and most popular spices in the world. It belongs to the family Piperaceae and is used in many Asian countries as a stimulant in the treatment of colic, rheumatism, headache, diarrhoea, dysentery, menstrual pain, removing excessive gas from gastrointestinal tract and increasing flow of urine. It is used in folk medicine for stomach disorders, digestive problems, neuralgia and scabies **(**Koul and Kapil, 1993**)**.

(3) Description: is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. When dried, the fruit is known as a peppercorn. When fresh and fully mature, it is approximately 5 millimetres (0.20 in) in diameter, dark red, and, like all drupes, contains a single seed. Peppercorns, and the ground pepper derived from them, may be described simply as pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit) and white pepper (ripe fruit seeds).The image of *Piper nigrum* is shown in **Figure 2.15**.

Figure 2.16 *Plumbago indica* Linn (PLUMBAGINACCEAE)

2.3.2.11 *Plumbago indica* Linn (PLUMBAGINACCEAE)

(1) Thai Common Name: jattamoonplengdang

(2) Traditional and Ethnopharmacological uses of *Plumbago indica* Linn:

Plumbago (family plumbaginaceae) is an evergreen shrub with colored flowers widely grown in a tropical climate, especially in Southeast Asia, South Asia and South African. There are many species of Plumbago e.g. *P. indica* L., syn. *P. rosea* with red flowers, *P. zeylanica* with white flowers and *P. auriculata* with blue flowers. Their major ingredients are napthoquinones such as plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), 3, 3'-biplumbagin and elliptinone, 4-hydroxybenzal-dehyde (V), trans-cinnamic acid (VI), vanillic acid (VII), lupenone and trilinolein (Nguyen, 2004; Zhang *et al.*, 2007).

In Thai traditional medicine, ethanolic extracts of *P. indica* roots are commonly used to treat hemorrhoids and also used as carminative to stimulate appetite by increasing digestive enzyme secretion and intestinal absorption. It also commonly used to combine with other types of herbs to make new recipes for various traditional medical treatments. For example, the pikutbenjakul recipe which contains a *P. indica* root extract as one-fifth of its ingredients. Pikutbenjakul has been used as an adaptogenic drug for cancer patients especially breast cancer (Durga *et al.*, 1992).

(3) Description: *P. indica* is an erect or spreading, more or less branched, hearbaceous or half-woody plant, 1.5 meters or less in height. Leaves are ovate to oblong-ovate, 8 to 13 centimeters long, smooth, slightly drooping, with entire, undulate or wavy margins, with a pointed or blunt tip and a pointed base. Spikes are 15 to 30 centimeters long. Calyx is tubular, 8 to 10 millimeters long, covered with stalked, sticky glands. Corolla is bright red, tube is slender, about 2.5 centimeters long, and the spreading limb, about 3 centimeters in diameter (Nisha*et al.*, 2002). The image of *P. indica*is shown in **Figure 2.16**.

Figure 2.17 Sodium Chloride (NaCl)

2.3.2.12 Sodium Chloride (NaCl)

Sodium chloride is an ionic compound found in various foods and medical treatments. More commonly referred to as “salt” or “table salt,” sodium chloride is used as a seasoning in many foods.

Medically, sodium chloride solutions are used in catheter flush injections or intravenous infusions, and for cleaning objects such as contact lenses in the form of saline. Sodium chloride inhalation can remove certain bacteria in body secretions.The image of Sodium chloride is shown in **Figure 2.17**.

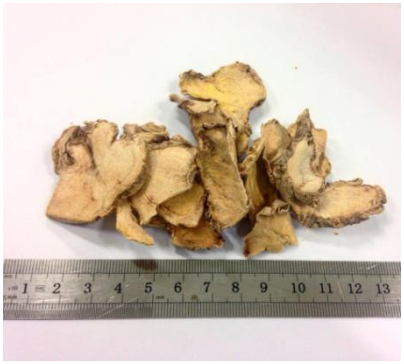


Figure 2.18 *Zingiber cassumunar* Roxb. (ZINGIBERACEAE)

2.3.1.13 *Zingiber cassumunar* Roxb. (ZINGIBERACEAE)

(1) Thai Common Name: phai

(2) Traditional and Ethnopharmacological uses of *Zingiber cassumunar* Roxb:

Ethnobotany is concerned with local people's interaction with plants and medicinal uses in treating human health problems, social and economic support systems and benefits in our day today life. From the variety of many medicinal plants, *Z. cassumnar* has traditionally been widely used to beautify the skin, to ward off asthma, chronic, colds, nausea poultice, decoction, and medicinal massage treatment. *Z. cassumnar* is used in relieving abdominal pain, headache, stomachache, anodyne, constipation, colic, cramps, constipation, fever, flatulence, gonorrhoea, jaundice, malaria, numbness, parturition, vermifuge. It has also been used in joint and muscle inflammation and helps to reduce fever generation. It has antiviral, antiseptic, analgesic and antibacterial properties as well. It also contains cassumunarin that has special antioxidant properties. It has been reported that *Z. cassumnar* oil relieves muscle pain, used as a rubbing or poultice, a decoction, analcoholic tincture, a massage or eaten fresh and is a treatment for asthma in traditional Thaimedicine **(**Piromrat *et al.*, 1980).

(3) Description:

Grows in tropical Asia, from India to Indonesia.Commonly planted in the yard in places that get enough sunlight.In flooded or muddy soil, its growth will be disturbed and rhizome rots quickly. It has a creeping rhizome and fleshy, the shape is almost round to elliptic or irregularly, 2-5 mm thick. Outer surface is not flat, lined, sometimes with shredded leaves, yellowish brown color, when young yellow halved until golden brown. The image of *Z. cassumnar* is shown in **Figure 2.18**.

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Allium sativum Linn. (ALLIACEAE)	Hypolipidae-mic agents	Bulbs	- A 2013 meta-analysis concluded that garlic preparations may effectively lower total cholesterol by 11–23 mg/dL and LDL cholesterol by 3–15 mg/dL in adults with high cholesterol if taken for longer than two months. The same analysis found that garlic had a marginally positive effect on HDL cholesterol, no significant effect on blood triglyceride levels, and that garlic preparations were generally well tolerated with very few side effects.Garlic may reduce platelet aggregation	Ried, 2013; Rahman, 2007
Alpinia galanga (L.) Willd. (ZINGIBERACEAE)	Anti-inflammatory	Rhizome	- 95% ethanolic extract from rhizomes of *A. galanga* extract possessed moderate anti-inflammatory activity on LPS-induce NO production from RAW264.7 cell line (IC_{50} value 21.50±0.09 μg/ml)	Anuthakoengkun *et al*., 2012
			- *A. galangal*, isolated compounds such as galanganal, galanganols B, galanganols C and trans-p-coumaryl diacetate have shown potent inhibitory effects on NO production induced by lipopolysaccharide in mouse peritoneal macrophages (IC_{50} value 68mM, 88mM, 33mM and 19mM, respectively).	Morikawa *et al*., 2005

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Alpinia galanga (L.) Willd. (ZINGIBERACEAE)	Anti-inflammatory	Rhizome	- In addition, the ethanolic extract of *M.fragrans* is an ingredient in Yapogdoodpis remedy. Anti-inflammatory effect on nitric oxide inhibition induced by lipopo-lysaccharide in murine macrophage RAW 264.7 cells, anti-inflammatory effect by inhibition of the enzyme COX-2 (IC_{50} values of21.50±0.09 and 14.95±0.13µg/ml, respectively)	Jaiarree, 2011
Cinnamomum camphora (L.) Presl. (LAURACEAE)	Anti-inflammatory	Crystal	- 80%MeOH leaf extract of *C. camphora* (100µg/ml) and its subfractions such as hexane and EtoAc extracts (100µg/ml) significantly blocked the production of IL-1, IL-6 and TNF-α of stimulated by LPSfrom RAW264.7 cell by 65%	Lee *et al.,* 2006
Cleome viscosa Linn. (CLEOMACEAE)	Anti-inflammatory	All of trunk	-95% ethanolic extract from leaf of *C. viscosa* extract possessed moderate anti-inflammatory activity on LPS-induce NO production and inhibition of the enzyme COX-2 and TNF-αfrom RAW264.7 cell line (IC_{50} value26.42±2.17, 78.07±0.18and 78.07 ± 0.18 µg/ml, respectively)	Jaiarree, 2011

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Cleome viscosa Linn. (CLEOMACEAE) (Continued)	Analgesic	*Seeds*	-Fixed oil from the seeds of *C. viscosa* showed analgesic and antiemetic activity in acetic acid induced writhing mice (intraperitoneally) and chick emetic model (oral treatment) respectively.	Ahmad*et al.,* 2011
	Antioxidant	leaves	-The total phenolic, flavonoid content of *C. viscosa* leaf extract was66.38±0.82mg/g and 0.54±0.04mg/g respectively DPPH assay showed EC_{50} value 77.30 µg/ml	Gupta*et al.,* 2011
Crateva adansonii DC. (CAPPARACEAE**)**	Antioxidant	Bark	- The extract produced a minute concentration-dependent increase in free radical scavenging activities. Effects of the extract were significantly ($P < 0.05$) lower when compared to the effects of the ascorbic acids. The IC_{50} of the extract is >400 µg/ml.	Udeh and Onoja, 2015
Crateva religiosa G. Forst. (CAPPARACEAE)	Antifungal	Bark	-The petroleum ether, chloroform, ethanolic and aqueous extracts of *C. religiosa* were active against *C. albicans, C. tropicalis, C. krusei* by disc diffusion method and the ethanolic extract showed good activity of minimum inhibitory concentrationsin the range of 0.062 n 0.5mg/disc.	Saho *et al.,* 2008

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Curcuma zedoaria (Berg) Roscoe. (ZINGIBERACEAE)	Anti-inflammatory	Rhizomes	- *C. zedoaria*, the two compounds, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one and procurcumenol inhibited the production of TNF-α by lipopolysaccharide (LPS)-activated macrophages (IC_{50} values of 12.3 and 310.5 μM, respectively).	Jang, 2001
		Rhizomes	-Inhibitory activity of prostaglandin E_2 production using lipopolysaccharide (LPS)-induced of mouse macrophage cell RAW 264.7 cell lines of two compounds from rhizome of *C.zedoaria*, Beta-turmerone and ar-turmerone and sesquiterpenoids exhibited considerable activity (IC_{50} values 7.3, 24.0 and μM, respectively).	Hong, 2002
	Antioxidant	Rhizomes	-The ethanolic extract and ethyl acetate and water extracts from rhizome of *C.zedoaria*at concentration of 100 μg/ml showed antioxidant efficacy of 85.41, 97.9 and 98.95 % inhibition of DPPH free radicals respectively.	Himaja *et al.*, 2010

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
*Erythrina variegata*Linn. (LEGUMINOSAE) (Continued)	Antioxidant	*Seeds*	-The levels of *E. variegata*showed antioxidant enzymes such as SOD and CAT were reduced significantly (P<0.001) in animals treated with high-fat diet (HFD). The administration of *E.variegata* seeds at doses 200 and 400 mg/kg significantly restored the reduced enzymic activity (P<0.001).	Balamurugan and Shantha, 2010
	Hypolipidae-mic agents	*Seeds*	-Treatment with *E.variegata* seeds at 200 and 400 mg/kg produced a reduction (24.15% and 32.32%) in total cholesterol; (14.43% and 19.24%) in triglycerides; (28.7% and 38.9%) in LDL in the respective doses with statistical significance (P<0.001).	
	Anti-inflammatory	Bark	-Ethanolic extract of *E. variegata* showed potential analgesic activity in tail immersion and hot plate test which is also comparable to the standard drug morphine (5 mg/kg b.w.) and studies show that phaseollin of *E.variegata* has the best fitness score against the COX-1 which is 56.64 and 59.63 for COX- 2 enzyme.	Mir *et al.*, 2010

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Myristica fragrans Houtt. (MYRISTICACEAE)	Anti-inflammatory	Aril of seed and seed	- In addition, the ethanolic extract of *M.fragrans* is an ingredient in Sahasthara. Anti-inflammatory effect on nitric oxide inhibition induced by lipopolysaccharide in murine macrophage RAW 264.7 cells, anti-inflammatory effect by inhibition of the enzyme COX-2 and anti-inflammatory effect by inhibition of the TNF-α (IC_{50} values of46.36±1.53μg/ml, 41.46 ± 1.06 μg/ml and IC_{50} > 50μg/ml, respectively).	Kakatum, 2011
	Antioxidant	Fresh leaves	-The scavenging activity of freeze-dried water extract of *M. fragrans* was observed to be better than that of BHA, as indicated by 0.20 mg/ml against 0.34 mg/ml of half maximal inhibitory concentration (IC_{50}) values, respectively. However, BHT produced the highest DPPH free radical scavenging activity, as it caused 50% inhibition of free radicals at 0.04 mg/ml.	Akinboro *et al.*, 2011

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive compounds	Biological activities	References
Myristica fragrans Houtt. (MYRISTICACEAE) (Continued)	Antioxidant	Seeds	-Isolated compound of *M. fragrans*, 1(dilignan, argen-teane), 2 (meso-dihydroguaiaretic acid) 3 and (erythroaustro-bailignan-6) for lipid peroxidation inhibition, compound 2 is twice as active as 3 (IC_{50} = 0.41 and 0.82 lM, respectively), compound 1 is an intermediate inhibitor (IC_{50} = 0.68 lM).	Calliste *et al.*, 2010
Piper nigrum Linn. (PIPERACEAE)	Anti-inflammatory	*Seeds*	-The 95% ethanolic extract from seed of *P. nigrum* extract inhibited LPS-activated NO production in RAW264.7 cells (IC_{50} = 32.0 μg/ml).	Kakatum, 2011
	Antioxidant	*Seeds*	-The 95% ethanolic extract from seed of *P. nigrum* extract showed an antioxidation effect on DPPH radical (EC_{50} = 91.16 ±1.15μg/ml).	Anuthakoengkun *et al*., 20012
			-The 95% ethanolic extract from seed of *P. nigrum* extract showed an antioxidation effect on NBT assay (EC_{50} = 51.24 ±2.12μg/ml).	Inprasit, 2014

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/Bioactive Compounds	Biological activities	References
Piper nigrum Linn. (PIPERACEAE) (Continued)	Analgesic	Piperine	- Piperine at a dose of 5 mg/kg and ethanol extract at a dose of 15 mg/kg after 120 min and hexane extract at a dose of 10 mg/kg after 60 min exhibited significant ($P<0.05$) analgesic activity by tail immersion method, in comparison to ethanol extract at a dose of 10 mg/kg using analgesy-meter in rats. However, with hotplate method, piperine produced significant ($P<0.05$) analgesic activity at lower doses (5 and 10 mg/kg) after 120 min.	Koul and Kapil, 1993
Plumbago indica Linn (PLUMBAGINACCEAE)	Anti-inflammatory	Root	-In addition, the ethanolic extract of *Plumbago indica* is an ingredient in Sahasthara. Anti-inflammatory effect on nitric oxide inhibition induced by lipopolysaccharide in murine macrophage RAW 264.7 cells and anti-inflammatory effect by inhibition of the enzyme COX-2 (IC_{50} values of36.22 and 31.20 μg/ml, respectively).	Kakatum, 2011

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/ Bioactive compounds	Biological activities	References
Plumbago indica Linn (PLUMBAGINACCEAE) (Continued)	Antioxidant	*Root*	- Methanolic extract from root of *P. indica* extract showed total phenolic content 116.6±32.08 mg/g and total flavonoid content is 39.2±640 mg/g and the total antioxidant is 683±885 mg/g. - The *P. indica* root extracts had also detectable hydroxyl radical scavenging activity compared to quercetin (IC_{50} values 78.2 µg/ml and 20.9µg/ml, respectively).	Eldhose *et al.*, 2013
			-The 95% ethanolic extract from seed of *P. indica* extract showed an antioxidation effect on NBT assay (EC_{50} = 41.91±2.12µg/ml).	Inprasit, 2014

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/ Bioactive compounds	Biological activities	References
Zingiber cassumunar Roxb. (ZINGIBERACEAE) (Continued)	Anti-inflammatory	Rhizome	-The 95% ethanolic extract from rhizome of *Z. cassumunar* inhibited LPS-activated NO production in RAW264.7 cells (IC_{50} = 4.35±0.00 μg/ml).	Anuthakoengkun *et al*., 2012
			-95% ethanolic extract from leaves of *C. viscosa* extract possessed stronger anti-inflammatory activity on LPS-induce the enzyme COX-2 from RAW264.7 cell line (IC_{50} value 2.4 ± 0.14 μg/ml).	Jaiarree, 2011
	Antioxidant	Rhizome	-The 95% ethanolic extract from rhizome of *Z. cassumunar* showed an antioxidation effect on DPPH radical (EC_{50} = 27.39±1.35μg/ml).	Anuthakoengkun *et al*., 2012

Table 2.2 Biological activities of ingredients of Lom-Am-Ma-Pruek remedy (Continued)

Scientific name	Activities	Part used/ Bioactive compounds	Biological activities	References
Zingiber cassumunar Roxb. (ZINGIBERACEAE) (Continued)	Anti-inflammatory	Rhizome	- The COX-2 inhibitory assay was performed by measuring prostaglandin E2production in lipopolysaccharide-stimulated mouse macrophage RAW 264.7 cells of four isolated compound from Chloroform extracts of *Z. cassumunar*, (±) *trans*-3-(3,4dimethoxyphynel) -4-[(E)-3-4dimetthoxystyryl] cyclohex-1-ene (1) and (±) *trans*-3-(4-hydeoxy-3-met-hoxyphenyl)-4-[(E)-3-4-dimetthoxystyryl] cyclohex-1-ene (2), 4-(2,4,5-trimethoxyphynyl) -but-1,3-diene (3), 4-(3,4-di-metthoxystyryl)-but-1,3diene (4). Two phenylbutenoid dimers, 1 and 2 exhibited considerable activity with IC_{50}values of 2.71 and 3.64mM while Two phenylbutenoid monomers, 3 and 4,showed moderate activity (IC_{50}14.97, 20.68mM, respectively). Celecoxib, a positive control showed IC_{50}values 0.52 nM.	Han *et al.*, 2005

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials and Methodology

3.1.1 Plant materials

The parts of plants used to treat stroke and an analgesic in the musculoskeletal system, the place of collection of and voucher specimens of Lom-Am-Ma-Pruek remedy are shown in **Table 3.1**. The voucher specimens were obtained from the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkla, Thailand.

Table 3.1 Plants and parts of plants comprising Lom-Am-Ma-Pruek preparation

Species	Family	Thai name	Plant part	Flavor	Voucher specimen number	Percentage
Allium sativum Linn.	ALLIACEAE	Kratiem	Bulbs	Hot and spicy	SKP 006 01 19 01	7.14
Alpinia galanga (L.) Willd.	ZINGIBERACEAE	Kra	Rhizome	Hot and spicy	SKP 206 01 07 01	7.14
Cinnamomum camphora (L.) Presl.	LAURACEAE	Karaboon	Crystal	Hot	SKP 096 03 03 01	7.14
Cleome viscosa Linn.	CLEOMACEAE	Pukseanphee	All of trunk	Bitter	SKP 039-1 03 22 01	7.14
Crateva adansonii DC.	CAPPARACEAE	Koombouk	Bark	Bitter and Fragrant	SKP 039-1 03 01 01	7.14

Table 3.1 Plants and parts of plants comprising Lom-Am-Ma-Pruek preparation (continued)

Species	Family	Thai name	Plant part	Flavor	Voucher specimen number	Percentage
Crateva religiosa G.Forst.	CAPPARACEAE	Koomnum	Bark	Bitter	SKP 039-1 03 18 01	7.14
Curcuma zedoaria (Berg) Roscoe.	ZINGIBERACEAE	Kaminaoi	Rhizome	Hot and spicy	SKP 206 03 26 01	7.14
Erythrina variegata Linn.	LEGUMINOSAE	Thonglarng	Bark	Bitter	SKP 098 05 22 01	7.14
Myristica fragrans Houtt.	MYRISTICACEAE	Lokchan	Nutmeg	Hot and Fragrant	SKP 121 13 06 01	7.14
Myristica fragrans Houtt.	MYRISTICACEAE	Dokchan	Mace	Hot and Fragrant	SKP 121 13 06 01	7.14
Piper nigrum Linn.	PIPERACEAE	Pikthai	Seed	Hot and spicy	SKP 146 16 14 01	7.14
Plumbago indica Linn.	PLUMBAGINACEAE	Jattamoonplengdang	Root	Hot and spicy	SKP 148 16 09 01	7.14
Sodium Chloride(NaCl)	-	Salt	Crystal	Salty	-	7.14
Zingiber cassumunar Roxb.	ZINGIBERACEAE	Phai	Rhizome	Hot and Astringent	SKP 206 26 03 01	7.14

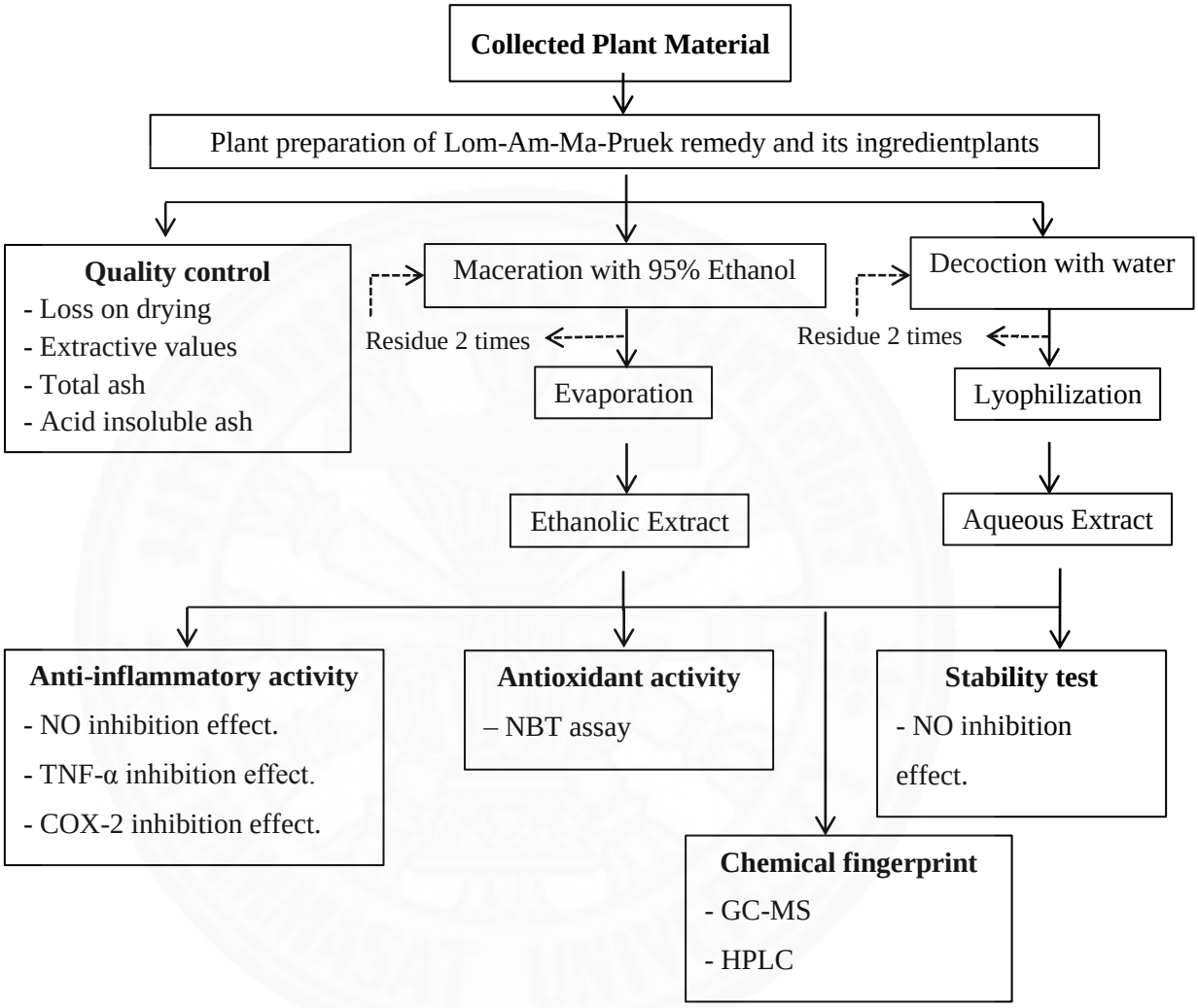


Figure 3.1 Conceptual framework of this research

3.2 Chemicals, reagents, instruments, plastics and glasswares

3.2.1 Extraction

Ethanol 95%, commercial grade	C.M.J Anchor company, Thailand
Distilled water (Milli-Q≥18 Mega Ohm)	Milford, USA

3.2.2 Quality controls

3.2.2.1 Acid insoluble ash

10% Hydrochloric acid (HCl)	Merck, Germany
Distilled water (Milli-Q≥18 Mega Ohm)	Milford, USA

3.2.2.2 Extractive value

Ethanol 95%, commercial grade	C.M.J Anchor company, Thailand
Chloroform ($CHCl_3$), analytical grade	RCI labscan, Thailand
Distilled water (Milli-Q≥18 Mega Ohm)	Milford, USA

3.2.3 *In vitro* assay for anti-inflammatory assay

3.2.3.1 The chemicals and reagents of assay for NO inhibitory effect

Dimethyl sulfoxide [$(CH_3)_2SO$](DMSO)	RCL Labscan, Thailand
Fetalbovineserum (FBS)	Biochem, Germany
Hydrochloric acid	Univar, Australia
N-(1-Naphthy)ethylenediamine dihydrochloride	Sigma, USA
Penicillin-Streptomycin (P/S)	Gibco, USA
Phosphate buffered saline (PBS)	Amresco, USA
Phosphoric acid solution	Sigma, USA
RPMI medium 1640	Gibco, USA
Sodium bicarbonate (NaHCO3)	BHD, England
Sodium hydroxide (NaOH)	Univar, Australia
Sulfanylamide	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trypan blue stain 0.4%	Gibco, USA
Trypsin - EDTA	Gibco, USA

3.2.3.2 The chemicals and reagents of assay for inhibitory effect on LPS-induced TNF-α release from RAW 264.7 cell lines

Calibrator diluents RD5K	R&D systems, USA
Dimethyl sulfoxide [$(CH_3)_2SO$](DMSO)	RCL Labscan, Thailand
Fetalbovineserum (FBS)	Biochem, Germany
Hydrochloric acid	Univar, Australia
Lipopolysaccharide from E.coli 055:B5 (LPS)	Sigma-Aldrich, USA
N-(1-Naphthy)ethylenediamine dihydrochloride	Sigma, USA
Penicillin-Streptomycin (P/S)	Gibco, USA
Phosphate buffered saline (PBS)	Amresco, USA
Phosphoric acid solution	Sigma, USA
RPMI medium 1640	Gibco, USA
Sodium bicarbonate (NaHCO3)	BHD, England
Sodium hydroxide (NaOH)	Univar, Australia
Stop solution for TNF-α kit	R&D systems, USA
Sulfanylamide	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
TNF-α conjugate	R&D systems, USA
TNF-α kit control	R&D systems, USA
TNF-α standard	R&D systems, USA
Trypan blue stain 0.4%	Gibco, USA
Trypsin - EDTA	Gibco, USA
Wash buffer	R&D systems, USA

3.2.3.3 The chemicals and reagents of assay for inhibitory effect on LPS-induced Prostaglandins (PEG_2) from RAW 264.7 cells line.

Dimethyl sulfoxide [$(CH_3)_2SO$](DMSO)	RCL Labscan, Thailand
EIA Buffer	Cayman, USA
Ellman'man Reagent	Cayman, USA
Fetalbovineserum (FBS)	Biochem, Germany
Hydrochloric acid	Univar, Australia
Lipopolysaccharide from E.coli 055:B5 (LPS)	Sigma-Aldrich, USA
N-(1-Naphthy) ethylenediamine dihydrochloride	Sigma, USA
Penicillin-Streptomycin (P/S)	Gibco, USA
Phosphate buffered saline (PBS)	Amresco, USA
Phosphoric acid solution	Sigma, USA
Prostaglandin E_2 AChE Tracer	Cayman, USA
Prostaglandin E_2 Monoclonal Antibody	Cayman, USA
RPMI medium 1640	Gibco, USA
Sodium bicarbonate ($NaHCO_3$)	BHD, England
Sodium hydroxide (NaOH)	Univar, Australia
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trypan blue stain 0.4%	Gibco, USA
Trypsin - EDTA	Gibco, USA
Wash buffer	Cayman, USA

3.2.4 *In vitro* assay for antioxidant activity

3.2.4.1 The chemicals and reagents of Nitroblue tetrazolium (NBT) dye reduction assay

Dimethyl sulfoxide [$(CH_3)_2SO$](DMSO)	RCL Labscan, Thailand
Fetalbovineserum (FBS)	Biochem, Germany
Hanks'balanced salt solution (HBSS)	Sigma, USA
Hydrochloric acid	Univar, Australia
N-(1-Naphthy)ethylenediamine dihydrochloride	Sigma, USA
Nitrotetrazolium blue chloride (NBT)	Sigma, USA
Penicillin-Streptomycin (P/S)	Gibco, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma, USA
Phosphate buffered saline (PBS)	Amresco, USA
RPMI medium 1640	Gibco, USA
Trypan blue stain 0.4%	Gibco, USA

Table 3.2 List of equipments, plastics and glasswares used in the studies

Name	Source
96-well plate flat, bottom with lid	Costar Corning, USA
96-well plate flat, bottom without lid	Costar Corning, USA
Autoclave	Hirayama, Japan
Balance 0.01 mg - 41 g	Mettler-Toledo, Swizerland
Balance 0.01 g - 220 g	Precica, Swizerland
Balance 0.5 mg - 3100 g	Mettler-Toledo, Swizerland
Cell culture flask, canted neck 25, 75 cm^3	Corning, USA
Centrifuge machine	Boeco, Germany
CO_2 humidified incubator	Forma, USA
Crucibles	Coorstex, USA
Cryogenic tube 2 ml	Corning, USA
Disposable pipette 2,5,10 and 25 ml	Corning, USA
Eppendorf	Costar Corning, USA
Filter paper no.1 (125 mmØ)	Whatman, USA
Filter paper no.40 (125 mmØ)	Whatman, USA
Freezer	Sanyo, Japan
Glass bottles	Schott Duran, Germany
Glasswares	Schott Duran, Germany
Hematocytometer	Boeco, Germany
High Performance Liquid Chromatography (HPLC)	Algelent technologies, USA
Hot air oven	Memmert, Germany
Hot plate	Thermolyne, USA
HPLC colunm	Phenomenex, USA
Inverted microscope	Nikon, Japan
Laminar air flow	Faster, Italy
Lyophilizer	Telster, Spain
Micropipettes	Eppendrof, Germany
Microplate reader	Bio Tek, USA

Table 3.2 List of equipments, plastics and glasswares used in the studies (continued)

Name	Source
Microplate washer	Bio Tek, USA
Moisture analyzer	Scaltec instrument, Germany
Muffle furnace	Nabertherm, Germany
Multi-channels pipette	Costar Corning, USA
PGE_2 Enzyme Immuno-Assay Kit	Cayman, USA
pH meter	WTW inolab, Germany
Pipette tips	Costar Corning, USA
Pipette boy	Brand, USA
Quantikine mouse TNF- α ELISA test kit	R&D systems, USA
Reagent reservoir (Sterile)	Costar Corning, USA
Refrigerrator (-20 ^{o}C)	Sanyo, Japan
Rotary evaporator	Buchi, Swizerland
Shaking incubator	Vision Scientific, Korea
Sonicator	Elma, Germany
Syringes	Nipro, Thailand
Vacuum Desiccator	Simax, USA
Vacuum pump	Rocker, Taiwan
Vortex	Scientific industries, USA
Water bath	Memmert, Germany
Water purification machine	Elga, UK

3.3 Preparation of crude extracts

Plant parts, of Lom-Am-Ma-Pruek remedy were collected and purchased from several regions of Thailand. These were washed, sliced thinly, dried in an oven at 50^{o}C and powdered. After that, these plants were weighed and mixed to be Lom-Am-Ma-Pruek remedy. The extracts would be obtained by two methods, namely maceration and decoction.

3.3.1 Maceration Plants of Lom-Am-Ma-Pruek remedy were macerated with 95% ethanol for 3 days. Next, filtered and concentrated to dryness under pressure. The maceration was repeated. 2 times (total 3 times) and dried by using an evaporator. Percentages of yield were calculated.

3.3.2Decoction Plants of Lom-Am-Ma-Pruek remedy were boiled in distilled water for 15 minutes. Then filtered using Whatman NO.1 filter paper and concentrated to dryness by lyophilizer. This was repeated 2 times (total 3). Percentages of yields from all extracts were calculated.

$$\%\text{Yield} = \frac{\text{Weight of the extract (g) x100}}{\text{Weight of dried powder (g)}}$$

The crude extracts were tested for anti-inflammatory activities by assay for NO inhibitory effect, COX-2 and TNF-α. The stability of Lom-Am-Ma-Pruek remedy was determined by storageunder accelerated conditions (40±2^{o}C with 75±5% RH) for 6 months.

3.4 Assay for quality control of Lom-Am-Ma-Pruek remedy and its plant ingredients.(Thai Herbal Pharmacopeia, 2000)

The quality control methods used for the plant ingredients followed the protocols set by the Thai Herbal Pharmacopoeia. The parameters used in this study were loss on drying, extractive value, total ash and acid insoluble ash.

3.4.1 Loss on drying

Moisture analyzers provide a rapid and accurate method for moisture content and dry weight analysis of ingredients. In this study, the electronic moisture analyzer was used for analysis of loss on drying. 2 grams of ingredient were put into the moisture analyzer, at 120°C. The weight of dried sample was taken and analyzed by using the following equation.

$$\% \text{ Moisture content} = \frac{\text{weight of start sample} - \text{weight dried sample (g)} \times 100}{\text{weight of start sample (g)}}$$

3.4.2 Extractive value

3.4.2.1 Ethanol soluble extractive value: 5 grams of drug powder was macerated with 100ml of ethanol in a flask closed with foil for 24 hours and was occasionally shaken over the first 6hours, then allowed to stand for 18hours. After filtration 25ml of the filtrate was evaporated to dryness at 105°C in a tared flat-bottomed shallow dish, and weighed. Percentage of ethanol-soluble extractive value was calculated with reference to the air dried drug.

3.4.2.2 Aqueous soluble extractive value : 5 grams of plant powder was macerated with 100 ml of 0.25% chloroform in water (instead of ethanol) in a flask closed with foil for 24 hours and occasionally shaken oven 6hours period and then allowed to stand for 18 hours. After filtration 25 ml of the filtrate was evaporated to dryness at 105°C in a tared flat-bottomed shallow dish, and weighed. The water soluble extractive value was calculated with reference to the air dried drug.

$$\% \text{ Extractive value} = \frac{\text{weight of extract (g.)} \times 100}{\text{weight of dried powder of plant (g.)}}$$

3.4.3 Total ash

Total ash was determined by weighing 2 grams powder into pre-weighed a crucible and burning in a muffle furnace at 450°C for 5 hours. The crucible was placed into a desiccator to cool down and weighed again. This was repeated until the cruciblеweight was stable, and the total ash calculated using the following equation.

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

3.4.4 Acid insoluble ash

The ash obtained from the previous process was boiled with 25ml of 10% HCl for 5 min. The insoluble matter was collected on ash-less filter paper and was washed with hot water until pH=7, dried, ignited, cooled in a desiccator and weighed. Percentage of acid insoluble ash was calculated with reference to the air dried drug.

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

3.5 Assay for anti-inflammatory activity

3.5.1 Assay for NO inhibitory effects from RAW 264.7 cell lines.

(Tewtrakul and Itharat, 2007)

3.5.1.1 Human cell lines.

Murine macrophage leukemia (RAW 264.7) cell line,which was obtained from American Type Culture Collecyion (ATTC TIB-71), is the most commonly used mouse macrophage cell line in medical research. Cell culture of RAW 264.7 were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50μg/ml streptomycin. The cells were maintained at 37 ^{o}C in a 5% CO_2 atmosphere with 95% a humidity.

3.5.1.2 Preparation of sample solution

Each sample was initially dissolved in a quantity of sterile dimethylsulfoxide (DMSO) for ethanolic extracts and the aqueous extracts was dissolved in sterile water and then filtered by 0.22 μl to concentration 50 mg/ml. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentrationwere added to each well of plates to obtain final concentrations of 0.01-100 μg/ml.

3.5.1.3 Testing procedure

The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with $1x10^6$cells/well and allowed to adhere for 24 hours at 37 ^oC in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 100μg/ml of LPS together with test sample at various concentrations and then incubated for 24 hours. NO production was determined by measuring the accumulation of nitrite in the supernatant using the Griess reagent (100 μl). Griess reagent was added to 96-well plates and the opacity determined with a microplate reader at 570 nm. The inhibition (%) was calculated by using the following equation. IC_{50} values were determined by Prism program.

Inhibition (%) = (C-S)/(C-B) x100

Control (C) : 0.2%DMSO + LPS

Sample (S) : Sample + LPS

Blank (B) : medium (RPMI)

3.5.2 MTT assay

Cytotoxicity was determined by MTT assay of cell viability by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. Briefly, after 24 hours incubation with test samples, MTT solution (10 μl, 5 mg/ml in PBS) was added to the wells and then incubated at 37 ^oC at 5% CO_2 atmosphere with 95% humidity for 2 hours. After that, the medium was removed, and isopropanol added containing 0.04 M HCl to dissolve the formazan solution. The optical density was then read with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample treated group was less than 70%. The inhibition (%) was calculated by using the following equation. IC_{50} values were determined by Prism program (n=3)

% Toxicity = [(Control O.D. – Sample O.D.)/Control O.D.] x 100

3.5.3 Assay for inhibitory effect on LPS-induced TNF- α release from RAW 264.7 cell line. (R&D Systems, 2012; Tewtrakul and Itharat, 2007)

3.5.3.1 Principle of determination TNF- α

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-**α** was pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-**α** present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-**α** was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of TNF-**α** bound in the initial step. The color development is stopped and the intensity of the color measured.

3.5.3.2 Human cell lines.

Murine macrophage leukemia (RAW 264.7) cell line is the most commonly used mouse macrophage cell line in medical research. Culture of RAW 264.7 was in RPMI 1640 medium (BIOCHROM^{AG}) supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50μg/ml streptomycin. The cells were maintained at 37 ^{o}C in a 5% CO_2 atmosphere with 95% a humidity.

3.5.3.3 Preparation of sample solution

Each sample was initially dissolved in a quantity of sterile dimethylsulfoxide (DMSO) for ethanolic extracts and the aqueous extracts were dissolved in sterile water and then filtered by 0.22 μl to concentration 50 mg/ml. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration was added to each well of plates to obtain final concentrations of 0.01-100 μg/ml.

3.5.3.4 Procedure of inhibitory effects on LPS-induce TNF- α release from RAW 264.7 cells line assay.

Theinhibitory effects on the release of TNF-α fromRAW 264.7 cells were evaluated using Quantikine mouse TNF-α ELISA test kit. Briefly, the cells were seeded in 96-well plates with $1x10^5$ cells/well and allowed to adhere for 24 hours at 37^oC in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 100μg/ml of LPS together with test sample at various concentrations and then incubated for 24 hours.

The supernatant was diluted in calibrator diluents (RD5K), stock of Mouse TNF- α standard (7000 pg/ml) and Mouse TNF- α control diluted in 1.0 ml of distilled water. Then, assay diluents (RD1-63) were added to each well of ELISA plate. The calibrated supernatant (50 μl) and standard (50 μl) were added to 96-wells ELISA plate, and then the plate was covered and incubated 2 hours at room temperature. After that, each well was washed 5 times with wash buffer (400 μl) (25 ml wash buffer 600 ml diluted in distilled water) and Mouse TNF- α conjugate (100 μl)added in each well, covered and incubated 2 hours at room temperature. Each well was washed 5 times and 100 μl substrate solution(color reagent A and color reagent B) added, and incubated 30 minutes at room temperature and protected from light. In the last step, stop solution (diluted hydrochloric acid) was added to each well and optical density read at 450 nm by spectrophotometer. The inhibition of TNF-α production was calculated using the following equation and IC_{50} values calculated by using Prism Program.

$$\text{Inhibition (\%)} = \frac{(A-B)}{(A-C)} \text{x} 100$$

A-C: TNF-α concentration (μg/ml) [A: LPS (+), Sample (-); B: LPS (+), Sample (+); C: LPS (-), Sample (-)].

3.5.4Assay inhibitory effect on LPS-induced Prostaglandins (PEG_2) from RAW 264.7 cells line. (Cayman, 2013; Tewtrakul and Itharat, 2007)

3.5.4.1 Principle of determination PEG_2

This assay is based on the competition between PGE_2 and a PGE_2-actylcholinesterase (AChE) conjugate (PGE_2 Tracer) for a limited amount of PGE_2 Monoclonal Antibody. Because the concentration of the PGE_2 Tracer is held constant while the concentration of PGE_2 varies, the amount of PGE_2 Tracer that is able to bind to the PGE_2 Monoclonal Antibody will be inversely proportional to the concentration of the PGE_2 in the well. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent is added to the well. The product of this enzymatic reaction is a distinct yellow color and absorbs light strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PGE_2 Tracer bound to the well, which is inversely proportional to the amount of free PGE_2 present in the well during the incubation.

3.5.4.2 Human cell lines.

Murine macrophage leukemia (RAW 264.7) cell line is the most commonly used mouse macrophage cell line in medical research. Cells culture of RAW 264.7 were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were maintained at 37 ^{o}C in a 5% CO_2 atmosphere with 95% a humidity.

3.5.4.3 Preparation of sample solution

Each sample was initially dissolved in a quantity of sterile dimethylsulfoxide (DMSO) for ethanolic extracts and the aqueous extracts was dissolved in sterile water and then filtered by 0.22 μl to concentration 50 mg/ml. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration was added to each well of plates to obtain final concentrations of 0.01-100 μg/ml.

3.5.4.4 Procedure ofinhibitory effect on LPS-induced Prostaglandins (PEG_2) from RAW 264.7 cells line.

RAW264.7 macrophage cells were maintained in RPMT supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50µg/ml streptomycin. The cells were harvested with trypsin-EDTA and diluted to a suspension in fresh medium. The cells were seeded in 96-well plates with $1x10^6$cells/well and allowed to adhere for 18-20 hours at 37 ^oC in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 5µg/ml of LPS together with test sample at various concentrations and then incubated for 24 hours. The supernatant (50 µl) was then transferred into 96-well PGE_2 Enzyme Immuno-Assay (Cayman Chemical Company). Then PGE_2 Tracer (50 µl) was added and antibody PGE_2(50 µl) to each well of ELISA plate,and incubated 24 hours in dark condition at 4 ^oC. Then each waswashed 5 times with wash buffer (400 µl) (wash buffer 2.5 ml and Polysorbate 0.5 ml diluted in 1000 ml distilled water) and Ellman's Reagent (200 µl) added to each well, covered and incubated 1.3 hours at at 37 ^oC. Ater that, the absorbance was measured by spectrophotometer at 412nm.The inhibition of LPS-induced Prostaglandins (PEG_2**)** was determained using the following equation and IC_{50} values calculated by using Prism Program.

$$\text{Inhibition (\%)} = \frac{Y}{X}x100$$

X: OD (Inactive) – OD (Active)

Y: OD (Samples) – OD (Active)

3.6 Assay for antioxidant activity

3.6.1 Nitroblue tetrazolium (NBT) dye reduction assay (Makishima *et.,al* 1996; Srisawat *et.,al* 2010)

3.6.1.1 Human cell lines

Human promyelocytic leukemia cell line (HL-60) was cultured in RPMI 1640 supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50μg/ml streptomycin. The cells were maintained at 37 ^{o}C in an incubator with 5% CO_2 and 95% humidity. To induce myeloid differentiation, HL-60 cells were cultivated for 6 days in RPMI1640 containing 1.3% DMSO.

3.6.1.2 Preparation of sample solution

Each sample was initially dissolved in a quantity of sterile dimethylsulfoxide (DMSO) for ethanolic extracts and the aqueous extracts were dissolved in sterile water and then filtered by 0.22 μl to concentration 10 mg/ml. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration was added to each well of plates to obtain final concentrations of 0.01-100 μg/ml.

3.6.1.3 Procedure ofinhibitory effect by Nitroblue tetrazolium (NBT) dye reduction assay

Intracellular superoxide formation was quantified by nitroblue tetrazolium reduction assay (NBT) according to the method of Makishima *et.,al* 1996. In short, HL-60 $1x10^6$ cells were incubated with various dilutions of the extract dissolved in Hanks'balanced salt solution (HBSS) 500 μl at 37 ^{o}C in an incubator with 5% CO_2 and 95% humidity for 15 minutes. After that, the plateswere incubated with 500 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 1.25 mg/ml nitrotetrazolium blue chloride (NBT) solution for another 1 hour. At the end of the incubation time, 2 ml of 1N HCl were added. After vortexing and centrifugation at 4,000 rpm for 10 minutes, supernatant was removed and dissolved in 300 ml DMSO. Then, 100 μl of sample solution was added into 96 well plates. The absorbance was measured at 572 nm using a microplate reader. The inhibition of each concentration of the extract against superoxide formation measured by NBT reduction was calculated by the following equation.

NBT reduction (%) = [(ODcontrol-ODsample)/ODcontrol) x100]

3.7Study on chemical fingerprint of Lom-Am-Ma-Pruek extract

3.7.1Chemicals and Reagents

Authentic compounds: plumbagin purchased from Sigma, piperine purchased from Meack, eugenolpurchased from Fluka, myristicin purchased from Fluka, acetonitrile, methanol and purified water (HPLC grade) from Labscan (Bangkok, Thailand).

3.7.2 Apparatus and chromatographic conditions

3.7.2.1 RP-HPLC analysis

The chemical fingerprint study was carried out using high performance liquid chromatography (HPLC) system, with ultraviolet visible (UV-vis) detector (spectromonitor® 4100) and automatic injector (Spectra System AS3500). Data were analyzed with TSP PC1000 software. A reversed-phase column, Phenomenex Luna 5 µ C18(2) 100A analytical column (150 x 4.60 mm. 5 micron), with guard column of the same material was used.The mobile phase was composed with gradient elution followed method described by Rattarom (2013, pp. 89-90) of water-acetonitrile: 0 min, 90:10; 30 min, 50:50; 40 min, 5:95; 45 min,5:95; 45.1 min, 90:10; 50 min 90:10. 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 210 and 256 nm. The operating temperature was maintained at room temperature.

3.7.2.2 Preparation of Lom-Am-Ma-Pruek for HPLC analysis

Ten milligrams of Lom-Am-Ma-Pruek ethanolic extract was dissolved in 1 ml methanol, and then sonicated for 15 minutes. This solution was filtered through a membrane filter (pore size 0.45 µm) prior to analysis.

3.7.2.3 Preparation of standard solutions

A stock solution of eugenol, plumbagin, piperine and myristicinwas prepared at a concentration of 1.0 mg/ml with DMSO and stored at -20°C until use

3.8Study on gas chromatography-mass spectrometry (GC-MS) of Lom-Am-Ma-Pruek extract

3.8.1 Apparatus and chromatographic conditions

3.8.1.1 GC-MS analysis

The 95% ethanolic extract of Lom-Am-Ma-Pruek was analyzed by using a Thermo focus GC, Polaris Q and auto injector gas chromatography-mass spectrometry with capillary column TG-5 slims (Thermo Fisher Scientific). The ionization energy was achieved by electron impact at 70 eV. Helium (He) gas was carrier with flow rate 1.0 ml/min. The initial temperature of column oven was programmed 60^{o}C, and then heated to 300 ^{o}C with a rate of 5^{o}C/min and kept constant at 300^{o}C for 10 min. The mass spectrometrum of each peak was recorded in the positive ion current mode of mass spectrometer within a mass range of 35-400. Identification of oil constituent was achieved using the National Institute of Standards and Technology (NIST).

3.8.1.2 Preparation of Lom-Am-Ma-Pruek for GC-MS analysis

Fiftymilligrams of Lom-Am-Ma-Pruekwere transferred into 10 ml volumetric flask, anddiluted to volume with methanol.

3.9 The stability test on Lom-Am-Ma-Preuk (LAP) extract

Stability testing was done using transparent vials. LAP extract was put in these vials and exposed under 40±2^{o}C with 75±5% RH as accelerated testing for 6 a periods. All samples were tested for nitric oxide inhibition effect and cytotoxicity activities on days 0, 15, 30, 60, 90, 120, 150, 180.

3.10 Statistical analysis

All data are the mean of three replications. Values of different parameters are expressed as the mean ± standard error of mean. Statistical analysis was performed using SPSS (SPSS 13 for windows) statistical software.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Extraction of Lom-Am-Ma-Pruek (LAMP) remedy and its ingredients

4.1.1 Percentage of yield

4.1.1.1 The percentage of yield of Lom-Am-Ma-Pruek remedy

LAMP remedy was extracted by maceration with 95% ethanol and decoction. The yield of ethanolic extract of LAMP was 7.37%. The yield of aqueous extract of LAMP was 11.37%. The results showed that the yield of the aqueous extract of LAMP was higher than the ethanolic extract.

4.1.1.2 The percentage of yield from the ethanolic extract of ingredients plants

Each ingredient plant was extracted by 95% ethanol. *Myristica fragrans* (mace) showed high percentage of yield (8.70%) followed by *Alpinia galanga*, *Curcuma zedoaria*, *Myristica fragrans* (nutmeg), *Plumbago indica*, *Piper nigrum*, *Zingiber cassumunar*, *Cleome viscosa*, *Allium sativum*, *Erythrina variegata*, *Crateva adansonii* and *Crateva religiosa* (8.70, 8.53, 7.39, 7.16. 6.75, 4.47, 3.64, 3.20, 3.16, 1.95, 1.60 and 1.49%, respectively).

4.1.1.3 The percentage of yield from the aqueous extract of ingredients plants

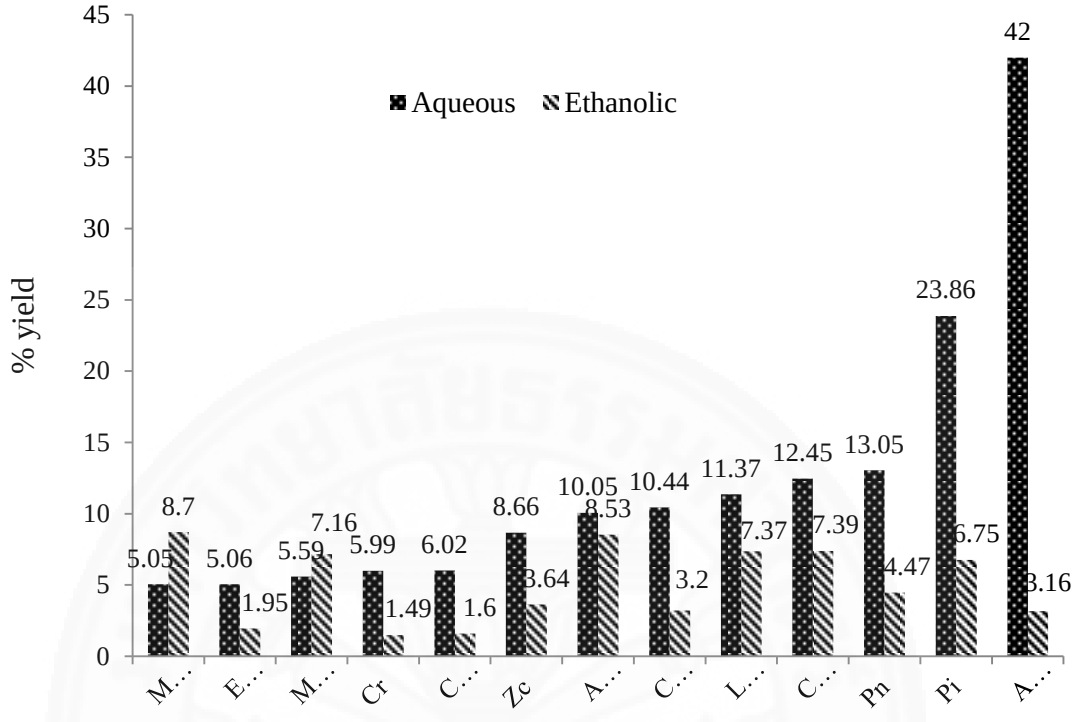
Each ingredient plant was extracted by decoction. *Allium sativum*showed high percentage of yield (42%) followed by *Plumbago indica*, *Piper nigrum*,*Curcuma zedoaria*, *Cleome viscosa*, *Alpinia galanga*,*Zingiber cassumunar*, *Crateva adansonii*, *Crateva religiosa*, *Myristica fragrans* (nutmeg), *Erythrina variegata* and *Myristica fragrans* (mace) (42, 23.86, 13.05, 12.45, 11.37, 10.44, 10.05, 8.66, 6.02, 5.99, 5.59, 5.06 and 5.05%, respectively).

Table 4.1 The yield (%) of Lom-Am-Ma-Pruek remedy extracts and its ingredients

Sample	Extraction	Code	% Yield
Allium sativum Linn.	Aqueous	AsH	42
	Ethanolic	AsE	3.16
Alpinia galanga (L.) Willd.	Aqueous	AgH	10.05
	Ethanolic	AgE	8.53
Cinnamomum camphora (L.) Presl.	Aqueous	CcH	-
	Ethanolic	CcE	-
Cleome viscosa Linn.	Aqueous	CvH	10.44
	Ethanolic	CvE	3.2
Crateva adansonii DC.	Aqueous	CaH	6.02
	Ethanolic	CaE	1.6
Crateva religiosa G.Forst.	Aqueous	CrH	5.99
	Ethanolic	CrE	1.49
Curcuma zedoaria (Berg) Roscoe.	Aqueous	CzH	12.45
	Ethanolic	CzE	7.39
Erythrina variegata Linn.	Aqueous	EvH	5.06
	Ethanolic	EvE	1.95
Myristica fragrans Houtt. (Mace)	Aqueous	MfmH	5.05
	Ethanolic	MfmE	8.7
Myristica fragrans Houtt. (Nutmeg)	Aqueous	MfnH	5.59
	Ethanolic	MfnE	7.16

Table 4.1 The yield (%) of Lom-Am-Ma-Pruek remedy extracts and its ingredients (continued)

Sample	Extraction	Code	% Yield
Piper nigrum Linn.	Aqueous	PnH	13.05
	Ethanolic	PnE	4.47
Plumbago indica Linn.	Aqueous	PiH	23.86
	Ethanolic	PiE	6.75
Sodium Chloride (NaCl).	Aqueous	ScH	-
	Ethanolic	ScE	-
Zingiber cassumunar Roxb.	Aqueous	ZcH	8.66
	Ethanolic	ZcE	3.64
Lom-Am-Ma-Pruek	Aqueous	LAMPH	11.37
	Ethanolic	LAMPE	7.37



The yield (%) of Lom-Am-Ma-Pruek remedy and its ingredient plants

Figure 4.1 The yield (%) of Lom-Am-Ma-Pruek remedy and its ingredientplants

4.2 Quality controls of raw material of Lom-Am-Ma-Pruek remedy and its ingredient plants

4.2.1 Result of quality standardization; moisture content, total ash, acid insoluble ash and extractive values

LAMP remedy was tested for quality standard, namely moisture content, total ash, acid insoluble ash and extractive values according to the standard values set by the Thai Herbal Pharmacopoeia (THP). The standard value of THP indicated that moisture content is not more than 10%, total ash is not more than 10% and acid insoluble ash is not more than 2%. The results of quality parameters of LAMP and its ingredients are show in **Table 4.2**. LAMP remedy met all quality standards. It registered within standard values for moisture content, total ash and acid insoluble ash (8.64±0.36, 7.34±0.43, 1.76±0.05%, respectively). The standard values of all of its ingredientplants were within those set by THP except *Cinnamomum camphora* which has moisture content more than standard criterion (34.76±0.12%). The total ash values for all ingredients were within standard criteria except Sodium Chloride (NaCl) (28.19±0.31). The acid insoluble ash values for all ingredients were within standard. Although two from fourteen plant ingredients were outside standard criteria, all standardized values of LAMP remedy were accepted as complying with following by THP standard criteria.

Extractive value of ethanolic extract of LAMP showed a lower value than aqueous extract (5.3±0.15 and 19.2±1.19%, respectively). Ethanolic extract of *Myristica fragrans*(Nutmeg) showed the highest yield (18.8±0.12%) and the ethanolic extract of *Cinnamomum camphora* showed the lowest yield(0.04±0.01%). The extractive value of aqueous extract of Allium sativum and Sodium Chloride (NaCl) showed the highest yield (81.46±1.32 and 80.03±1.01%, respectively). Cinnamomum camphora showed the lowest yield (0.03±0.01%). The result revealed that the standard values of LAMP remedy and its ingredient plants for this experiment within THP standards.

Table 4.2 Results of quality controls of Lom-Am-Ma-Pruek remedy and its ingredients; moisture content, total ash, acid insoluble ash and extractive values (mean ± SD), (n=3)

Species	%Moisture Content	% Ash Content		% Extractive Values	
		Total Ash	Acid Insoluble	Ethanolic Soluble	Aqueous Soluble
Allium sativum Linn.	3.96±0.66	3.49±0.05	1.82±0.07	0.9±0.02	81.46±1.32
Alpinia galanga (L.) Willd.	9.70±0.09	3.74±0	1.87±0.01	6±0.04	15.4±0.72
Cinnamomum camphora (L.) Presl.	34.76±0.12	1.31±0.13	0.41±0.03	0.04±0.01	0.03±0.01
Cleome viscosa Linn.	8.55±0.31	9.66±0.12	0.24±0.03	3.7±0.06	14.45±0.06
Crateva adansonii DC.	7.43±0.37	7.77±0.13	0.78±0.01	16.8±0.17	11.4±0.03
Crateva religiosa G.Forst.	8.06±0.30	9.74±0.06	0.21±0.01	1.1±0.01	17.2±0.06
Curcuma zedoaria (Berg) Roscoe.	8.88±0.10	8.14±0.01	1.13±0.13	7.15±0.04	24.3±0.12
Erythrina variegata Linn.	8.47±0.33	9.69±0.25	1.81±0.11	3.3±0.03	4.55±0.04
Myristica fragrans Houtt. (Mace)	6.59±0.29	6.27±0.12	0.71±0.06	7.65±0.06	6.04±0.04
Myristica fragrans Houtt. (Nutmeg)	6.81±0.27	2.48±0.04	1.17±0.02	18.8±0.12	4.51±0.02
Piper nigrum Linn.	7.13±0.37	2.43±0.01	0.51±0.01	4.55±0.04	1.55±0.01
Plumbago indica Linn.	6.69±0.34	9.02±0.43	1.34±0.02	8.75±0.01	39±0.04
Sodium Chloride(NaCl)	7.68±0.11	28.19±0.31	1.93±0.04	0.98±0.04	80.03±1.01
Zingiber cassumunar Roxb.	8.56±0.06	5.47±0.13	1.78±0.01	5.35±0.08	15.85±0.02
Lom-Am-Ma-Pruek	8.64±0.36	7.34±0.43	1.76±0.05	5.3±0.15	19.2±1.19

Criteria of Thai Herbal Pharmacopoeia (THP): Acceptable standardization values of quality of crude drug not more than 10% for moisture content, 10% for total ash and 2% for acid insoluble ash.

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4.3 Assay for anti-inflammatory activity

4.3.1 Assay for NO inhibitory effects from RAW 264.7 cell lines.

Effects of Lom-Am-Ma-Pruek remedy (LAMP) and its ingredients on the pro-inflammatory mediator, nitric oxide (NO) in activated murine macrophage cell lines (RAW 264.7) were measured as anti-inflammatory properties compared with positive control (Prednisolone). It was found that lipopolysaccaride (LPS) stimulated the highest NO production in RAW 264.7 cell lines at concentration of 10 ng/ml. Thus, 10 ng/ml of LPS was used to induce NO production in RAW cells in this study. The measurement of nitrite accumulation was by Griess's reagent. The method for analyzing the inhibition effect on NO production is described in section 3.5.1. The results of the inhibition activity against LPS induced NO production of Lom-Am-Ma-Pruek remedy and its ingredients are shown in **Table 4.3**.

For the LAMP remedy extracts, the 95% ethanolic extract (LAMPE) possessed potent inhibitory effect on NO production with IC_{50} value 24.90±0.86 µg/ml. However, LAMPE exhibited less anti-inflammatory activity than Prednisolone (IC_{50} value 1.31±0.05 µg/ml). The aqueous extract of Lom-Am-Ma-Preuk remedy (LAMPW) exhibited weak anti-inflammatory activity on this pathway (IC_{50}> 100µg/ml).

The investigation of inhibitory effects on NO production among ingredient plants showed that most extracts possessed anti-inflammatory activity. The ethanolic extract which was the most effective was *Alpinia galanga* (L.) Willd.(IC_{50} value6.99±0.4µg/ml). It was significantly different from positive control or prednisolone withIC_{50} value1.31±0.05µg/ml (*p-value*< 0.05). Second was *Curcuma zedoaria* (Berg) Roscoe which exhibited strong anti-inflammatory activity (IC_{50} value14.38±1.4µg/ml) followed by *Piper nigrum* Linn.and*Plumbago indica* Linn. **(**IC_{50} value16.42±1.19 and 24.54±1.97µg/ml, respectively) but *Plumbago indica* Linn.has toxicity at concentration 30 µg/ml.

Moderate anti-inflammatory activity by inhibition of NO production in RAW 264.7 cell lines were *Zingiber cassumunar* Roxb. (IC_{50} value 45.34±1.33μg/ml), *Erythrina variegata* Linn. (IC_{50} value 47.1±0.21μg/ml), *Myristica fragrans* Houtt.(Nutmeg) (IC_{50} value 68.84±1.18μg/ml), *Myristica fragrans* Houtt. (Mace) (IC_{50} value 82.46±0.59μg/ml)

Weak anti-inflammatory activity were *Allium sativum* Linn., *Crateva adansonii* DC., *Crateva religiosa* G.Forst., *Cinnamomum camphora* (L.) Presl., *Cleome viscosa* Linn. and Sodium Chloride .(IC_{50} value more than 100 μg/ml).

Among aqueous extracts of LAMP ingredients, there were five for which IC_{50} value can be calculated. The aqueous extract which was the most effective was *E. variegate* (IC_{50} value 37.22±0.33 μg/ml) followed by *Z. cassumunar* (IC_{50} value 46.2±2.4μg/ml), *M. fragrans* (Mace) (IC_{50} value 44.86±1.51μg/ml), *C. zedoaria* (IC_{50} value 51.29±2.36μg/ml) and *M. fragrans* (Nutmeg) (IC_{50} value 67.63±4.14μg/ml).

Previous studies of 8 species including *A. galanga, C.camphora, C. viscosa, C. zedoaria, M. fragrans, P. nigrum, P. indica, Z. cassumunar* and Sodium Chloride, showed the ethanolic extract from rhizome of *A. galanga* had potent anti-inflammatory effect on RAW264.7 murine macrophage leukemia cells (Anuthakoengkun *et al*., 2012) and the aqueous acetone extract of *Alpinia galanga* rhizomes shows inhibitory effects on NO production induced by lipopolysaccharide in mouse peritoneal macrophages. Among them, galanganal (IC_{50}=68mM), galanganols B (88mM) and C (33mM),) and trans-p-coumaryl diacetate (19mM) were found to show inhibitory activity (Morikawa *et al*., 2005). For *C.camphora*, 80% MeOH leaf extract of *C. camphora* and its subfractions including EtoAc and hexane extracts (100μg/ml) significantly blocked the production of NO in LPS/IFN from RAW264.7 cell by 65% (Lee et al., 2006). The ethanolic extract of *C. viscosa,Z. cassumunar* and Sodium Chloridewhich are ingredients in Ya-Pok-Dud-Pit remedy possess potent anti-inflammatory activities by nitric oxide inhibition induced by lipopolysaccharide in murine macrophage RAW 264.7 cell lines(Anuthakoengkun *et al*., 20012). For *C. zedoaria*, isolated compoundsfrom the rhizome such as Beta-turmerone, ar-turmerone and sesquiterpenoids haveinhibited lipopolysaccharide (LPS)-induced prostaglandin E_2 production in cultured mouse macrophage cell RAW 264.7 in a dose-dependent manner. In addition, these

compounds exhibited inhibitory effects on LPS-induced nitric oxide production in the cell system (Hong *et al.,* 2002). The ethanolic extract of *M. fragrans, P. nigrum, P. indica*which are ingredients in Sahasthara possess potent anti-inflammatory activities by nitric oxide inhibition induced by lipopolysaccharide in murine macrophage RAW 264.7 cell lines with IC_{50} values of46.36±1.53, 32.0 and 36.22µg/ml, respectively (Kakatum, 2011).

The results are consistent with many previous studies showing that *A. galangal, C. zedoaria, M. fragrans, P. nigrum, P. indica* and *Z. cassumunar* possess anti-inflammatory activity by inhibition of NO production. From this result, new knowledge and scientific data of Lom-Am-Ma-Pruek remedy and its four ingredients *A. sativum, C. adansonii, C. religiosa* and *E.variegata*are a positive outcome and support continued use of this remedy according to Thai traditional medicine.

Table 4.3 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced NO production from RAW264.7 cells and cytotoxicity of plant extracts (mean±SEM), (n=3)

Plant name	Code	%Inhibition of Nitric Oxide Production and (%cytotoxicity)							IC_{50}
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
A. sativum	AsE	-	-	-	-	-	4.78±2.16 (15.82±6.57)	7.30±4.63 (-7.24±11.79)	>100
	AsH	-	-	-	-	-	14.47±10.93 (-16.13±3.43)	14.39±8.75 (-9.18±0.92)	>100
A. galanga	AgE	(-0.10±0.30) (-7.50±6.17)	1.17±2.27 (-0.55±5.73)	7.13±2.83 (6.42±4.20)	80.76±4.31 (20.34±3.57)	-	-	-	6.99±0.4*
	AgH	-	-	-	-	-	10.80±0.58 (7.69±3.62)	22.07±0.63 (8.98±0.57)	>100
C. camphora	CaE	-	-	-	-	-	-0.44±5.71 (-1.6±6.57)	3.13±4.63 10.82±11.79)	>100
C. viscosa	CvE	-	-	-	-	-	25.22±1.93 (7.56±0.90)	44.17±4.68 (11.84±2.08)	>100

	CvH	-	-	3.86±4.91 (-21.52±4.62)	8.48±7.01 (-13.25±4.8)	16.06±0.57 (12.97±3.54)	23.09±3.49 (14.93±3.7)	51.12±0.31 (13.29±1.95)	97.93±0.92*

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table 4.3 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced NO production from RAW264.7 cells and cytotoxicity of plant extracts (mean±SEM), (n=3) (continued)

Plant name	**Code**	**%Inhibition of Nitric Oxide Production and (%cytotoxicity)**							**IC_{50} (µg/ml)**
		0.01 µg/ml	**0.1 µg/ml**	**1 µg/ml**	**10 µg/ml**	**30 µg/ml**	**50 µg/ml**	**100 µg/ml**	
C. adansonii	CaE	-	-	-	-	-	4.78±2.16 (15.82±6.57)	7.30±4.63 (-7.24±11.79)	>100
	CaH	-	-	-	-	-	7.23±1.05 (-6.02±0.13)	9.39±0.57 (8.66±0.51)	>100
C. religiosa	CrE	-	-	-	-	-	3.849±1.180 (-1.71±8.22)	11.433±1.321 (-18.57±5.86)	>100
	CrH	-	-	-	-	-	(-0.81±1.62)	9.47±0.81	>100

							(-15.53±1.04)	(-1.86±1.34)	
C. zedoaria	CzE	-1.74±2.24 (-16.14±2.46)	-1.29±3.04 (-11.16±3.79)	-1.82±3.3 (-16.28±4.23)	29.66±4.07 (-2.38±4.34)	97.8±2.28 (4.33±0.98)	-	-	14.38±1.4*
	CzH	-	-	4.5±3.52 (-13.2±1.55)	4.73±3.63 (-10.89±1.93)	28.19±6.22 (10.44±1.68)	48.61±1.17 (11.65±6.07)	54.16±0.53 (15.95±3.42)	51.29±2.36*
E. variegata	EvE	-	-	2.87±0.72 (-29.34±2.42)	10.75±1.59 (-17.56±3.29)	30.65±0.44 (-15.54±1.76)	53.02±0.26 (7.33±4.43)	85.24±2.27 (16.19±1.2)	47.1±0.21*
	EvH	-	-	4.99±4.74 (12.57±1.01)	-3.82±3.1) (-6.18±1.58)	39.09±0.45 (-1.39±5.71)	58.52±2.83 (13.58±1.83)	72.04±4.35 (17.83±1.14)	37.22±0.33*

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table 4.3 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced NO production from RAW264.7 cells and cytotoxicity of plant extracts (mean±SEM), (n=3) (continued)

Plant name	**Code**	**%Inhibition of Nitric Oxide Production and (%cytotoxicity)**							**IC_{50} (µg/ml)**
		0.01 µg/ml	**0.1 µg/ml**	**1 µg/ml**	**10 µg/ml**	**30 µg/ml**	**50 µg/ml**	**100 µg/ml**	
M. fragrans	Mfm E	-	-	6.21± 3.57 (-	-1.2±2.71 (-	5.37±0.1 (-8.26±5.82)	20.87±2.03 (-	67.36±3.32 (-34.58±6.93)	82.46±0.59*

(Mace)				12.95±3.19)	11.51±4.48)		31.92±7.78)		
	Mfm H	-	-	-1.87±7.87 (-23.81±3.28)	6.59±1.55 (-9.18±0.99)	22.29±6.92 (0.1±0.72)	55.6±6.69 (13.29±0.41)	73.71±3.23 (19.62±1.59)	44.86±1.51*
M. fragrans (Nutmeg)	MfnE	-	-	-8.44±7.49 (-9.6±3.31)	0.65±5.19 (-7.52±2.04)	12.56±2.79 (-10.49±7.58)	30.90±2.11 (-12.09±4.88)	80.54±4.03 (-23.37±7.21)	68.84±1.18*
	MfnH	-	-	-4.15±5.66 (1.08±2.43)	3.44±3.94 (-9.89±3.84)	7.42±1.16 (1.9±2.47)	31.66±2.44 (7.11±3.05)	67.95±4.55 (15.32±3.75)	67.63±4.14*
P. nigrum	PnE	0.67±3.92 (-19.24±2.07)	3.26±3.31 (-8.70±1.06)	11.76±2.30 (-4.76±1.53)	32.92±4.50 (0.20±0.59)	81.69±3.37 (21.80±2.98)	-	-	16.42±1.19*
	PnH	-	-	-	-	-	2.68±12.67 (-10.37±0.20)	-2.24±3.97 (-8.29±0.32)	>100
P. indica	PiE	3.92±2.54 (-11.33±3.73)	2.12±2.66 (-18.07±4.5)	-0.12±2.09 (-0.13±3.38)	13.07±3.12 (-10.14±0.36)	67.49±3.61 (-46.65±3.81			24.54±1.97*
	PiH	-	-	-	-	-	2.30±0.54	3.10±0.78	>100

(2.86±0.79) (-21.86±0.66)

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table 4.3 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced NO production from RAW264.7 cells and cytotoxicity of plant extracts (mean±SEM), (n=3) (continued)

Plant name	Code	%Inhibition of Nitric Oxide Production and (%cytotoxicity)							IC_{50} (µg/ml)
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
Sodium chloride	NaCl	-	-	-	-	-			>100
Z. cassumunar	ZcE	-	-	1.22±3.58 (-9.78±4.50)	6.09±4.06 (-10.38±5.90)	27.99±3.08 (-17.33±2.86)	55.95±1.51 (-18.53±4.80)	91.29±1.23 (-12.08±5.21)	45.34±1.33*
	ZcH	-	-	2.69±3.24 (-7.33±5.36)	9.27±3.46 (-2.49±6.16)	38.27±2.76 (12.25±0.68)	51.2±0.94 (21.19±1.47)	57.91±1.43 (21.16±2.52)	46.2±2.4*
Lom-Am-Ma-Pruek	LAPME	-	-16.36±4.86 (-26.70±2.00)	-7.42±2.48 (-22.53±1.67)	16.54±1.65 (-28.22±5.20)	66.70±4.76 (-10.71±0.31)	94.04±0.77 (-11.17±6.50)	-	24.90±0.86*
	LAPMH	-	-	-	-	-	1.84±1.56 (-15.02±3.98)	3.80±0.64 (-7.29±2.11)	>100
Prednisolone	Pred		32.35±1.87 (3.97±1.29)	46.11±0.48 (7.44±3.18)	58.45±1.17 (8.71±1.8)	64.86±2.03 (11.79±1.07)	74.03±1.17 (6.57±6.65)		1.31±0.05

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

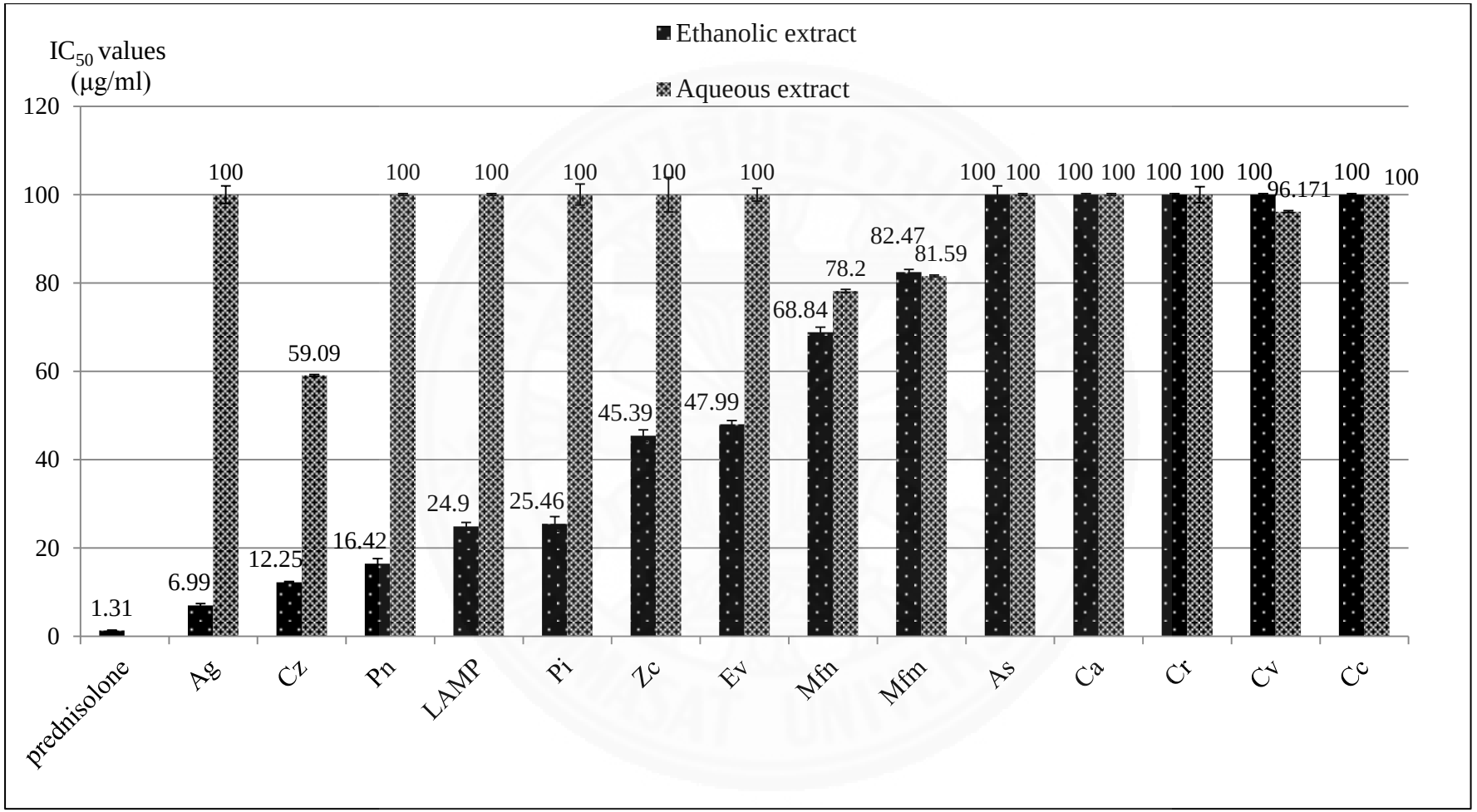


Figure 4.2 The IC_{50} value of 95% ethanolic and aqueous extracts of Lom-Am-Ma-Pruek and its plant ingredients

4.3.2 Assay for Inhibitory effects on LPS-induced TNF- α release from RAW 264.7 cells line.

The result of inhibition effects on LPS-induced TNF- α release from RAW 264.7 cells line using Quantikine mouse TNF- α ELISA test kit of Lom-Am-Ma-Pruek remedy and its ingredients are shown in **Table 4.4**. For the LAMP remedy extracts, the 95% ethanolic extract (LAMPE) possessed potent activity against TNF-α release but was less effective than prednisolone, a positive control with IC_{50} value 35.01±2.61 and 0.95±0.19 μg/ml, respectively. On the other hand, the aqueous extract of LAMP remedy exhibited weak inhibitory activity on this pathway. In addition, *Z. cassumunar* exhibitedmoderate activity with IC_{50} value 16.90±3.54 μg/ml. *M. fragrans* (nutmeg) has weak activity with IC_{50} value 49.36±0.42 μg/ml.All of these had no toxicity with RAW 264.7 cells at low concentration.

Although the extract of *C. viscosa*and *C. camphora* had no anti-inflammatory activity by LPS-induced TNF- α release, there have been previous reportsrecording anti-inflammatory activity.This may be of because differences in time of collection and stage of growth of the herbs (Anuthakoengkun *et al*., 20012; Lee *et al.,* 2006).

Table 4.4 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced TNF-α from RAW264.7 cells and IC_{50} values (mean±SEM), (n=2)

Plants name	Code	%Inhibition of TNF-α from RAW264.7 cells							IC_{50}
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
A. sativum	AsE	-	-	-	-	-	-	-42.07±2.32	>100
	AsH	-	-	-	-	-	-	20.54±3	>100
A. galanga	AgE	-	-	-	-33.69±0.7	-	-	-	>10
	AgH	-	-	-	-	-	-	20.07±3.69	>100
C. camphora	CaE	-	-	-	-	-	-	32.4±1.85	>100
C. viscosa	CvE	-	-	-	-	-	-	25.89±7.45	>100
	CvH	-	-	-	-	-	-	12.06±1.14	>100
C. adansonii	CaE	-	-	-	-	-	-	-35.34±5.16	>100
	CaH	-	-	-	-	-	-	19.92±3.42	>100
C. religiosa	CrE	-	-	-	-	-	-	-38.31±7.31	>100
	CrH	-	-	-	-	-	-	16.33±2.04	>100
C. zedoaria	CzE	-	-	-	-	-38.66±6.46	-	-	>30
	CzH	-	-	-	-	-	-	13.12±3.83	>100

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table 4.4 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced TNF-α from RAW264.7 cells and IC_{50} values (mean±SEM), (n=2) (continued)

Plants name	Code	%Inhibition of TNF-α from RAW264.7 cells							IC_{50}
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
E. variegata	EvE	-	-	-	-	-	-	19.82±7.81	>100
	EvH	-	-	-	-	-	-	-25.74±7.59	>100
M. fragrans	MfnE	-	-	-	18.73±6.02	13.41±3.65	51.17±0.71	64.08±1.46	49.36±0.42
(nutmeg)	MfnH	-	-	-	-	-	-	15.58±1.5	>100
M. fragrans	MfmE	-	-	-	-	-	-	30.42±1.07	>100
(mace)	MfmH	-	-	-	-	-	-	18.98±0.27	>100
P. nigrum	PnE	-	-	-	-	31.49±7.83	-	-	>30
	PnH	-	-	-	-	-	-	23.02±0.91	>100
P. indica	PiE	-	-	-	-	-58.38±10.89	-	-	>30
	PiH	-	-	-	-	-	-	33.31±3	>100
Z. cassumunar	ZcE	-	-	-	42.01±4.07	63.1±3.8	72.86±3.25	65.07±0.8	16.90±3.54
	ZcH	-	-	-	-	-	-	31.61±7.44	>100
Sodium chroline	NaClH	-	-	-	-	-	-	13.42±1.5	>100

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table 4.4 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced TNF-α from RAW264.7 cells and IC_{50} values (mean±SEM), (n=2) (continued)

Plants name	Code	%Inhibition of TNF-α from RAW264.7 cells							IC_{50}
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
Lom-Am-	LAMPE	-	-	8.55±5.79	6.20±2.10	40.58±4.48	71.06±2.79	-	35.01±2.61
Ma-Pruek	LAPMH	-	-	-	-	-	-	1.15±8.84	>100
Prednisolone	Pred	-	34.16±4.09	50.93±3.14	70.5±4.07	-	86.83±2.21	-	0.95±0.19

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

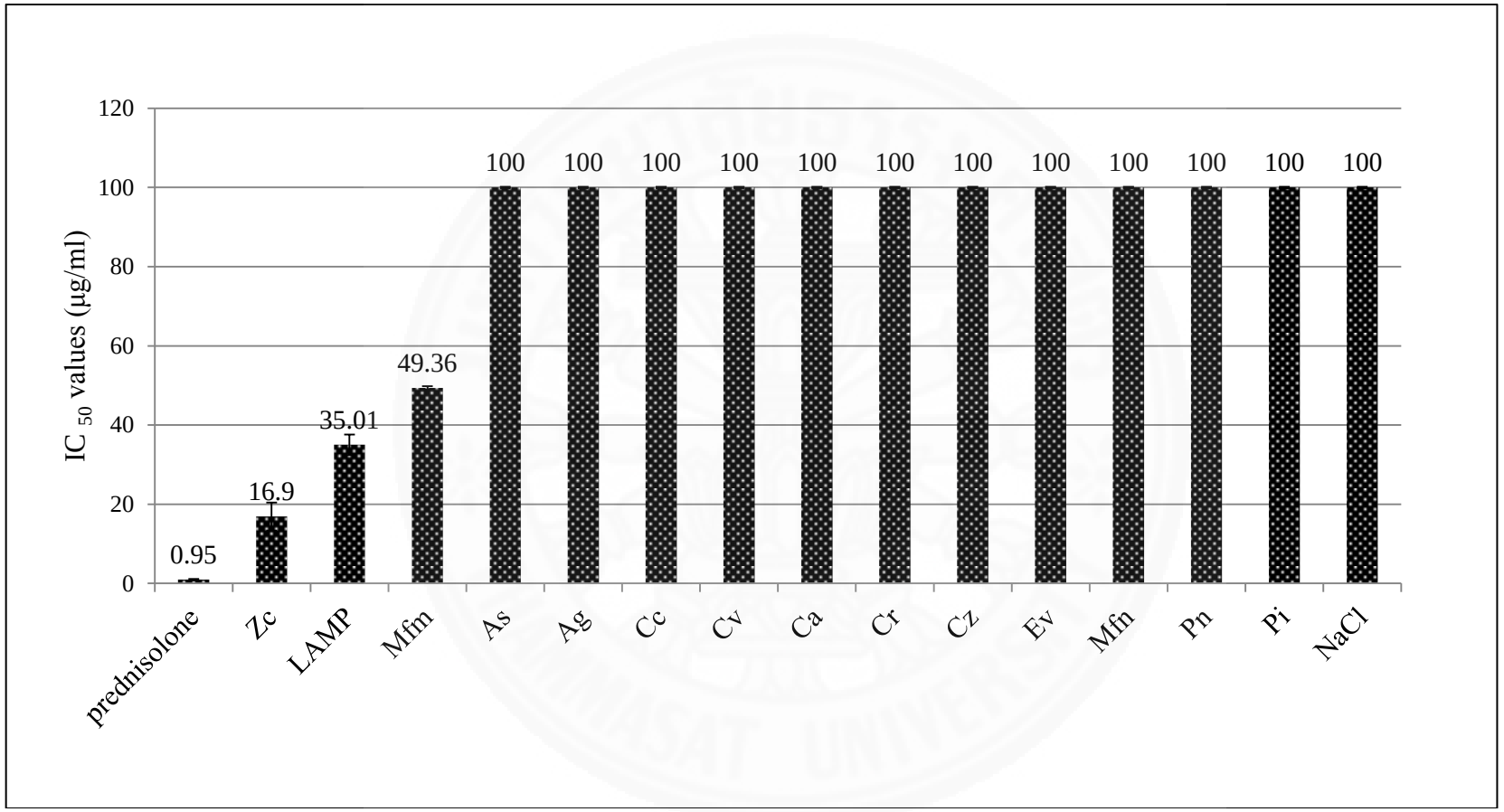


Figure 4.3 IC_{50} values of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced TNF-α from RAW264.7 cells

4.3.3Assay for inhibitory effect on LPS-induced Prostaglandins (PEG_2) from RAW 264.7 cells line.

The assay which determined inhibition effects of Lom-Am-Ma-Pruek remedy and its plant ingredients on LPS-stimulated PGE_2 release is described in section 3.5.4. The results are shown in **Table 4.5**. The result of Lom-Am-Ma-Pruek remedy revealed that among the extracts, 95% ethanolic extract was the best crude extract which gave the lowest IC_{50} value compared with the aqueous extract which possessed a higher IC_{50} value (IC_{50} value 4.77±0.03 and more than 100 μg/ml, respectively).

For ethanolic extracts of ingredient plants, it was interesting because more than half of all extracts possessed potent inhibitory effects on LPS-stimulated PGE_2 release. The ethanolic extracts of *A. galangal*, *M. fragrans* (Mace), *P. nigrum* and *Z. cassumunar* exhibited the strongest anti-inflammatory activity (IC_{50} value 1.23±0.01, 1.57±0.37, 2.95±0.49 and 3.08±0.34μg/ml, respectively). They were not significantly different from positive control or prednisolone with IC_{50} value0.96±0.01μg/ml (*p-value*>0.05).*P. indica*, *M. fragrans* (Nutmeg), *E. variegata* also exhibited potent anti-inflammatory activity (IC_{50} values 4.09±0.32, 4.6±0.19 and 9.27±0.72μg/ml, respectively).

Moderate anti-inflammatory activity by inhibition effect on LPS-stimulated PGE_2 release were *C. adansonii* (IC_{50} value 16.78±1.13μg/ml), *C. zedoaria* (IC_{50} value 8.36±1.05μg/ml) and *C. viscosa* (IC_{50} value 39.75±1.46μg/ml).

Weak anti-inflammatory activity was *A. sativum*, *C. religiosa*, *C.camphora* and Sodium Chloride (IC_{50} values more than 100 μg/ml).

These results agree with the past investigations which found that the exthanol extract of *M. fragrans* (Mace), *P. nigrum*, *P. indica* and *M. fragrans* (Nutmeg) has an inhibitory effect on LPS-stimulated PGE_2 release (Han *et al.*,2005; Kakatum, 2011; Anuthakoengkun *et al*., 20012). In addition, there have been reports on anti-inflammatory activity of the extracts. For example, rhizome extract of *C.zedoaria* showed inhibitory activity of prostaglandin E_2 production using lipopolysaccharide (LPS)-induced of mouse macrophage cell RAW 264.7 cell lines of three compounds from rhizome of *C.zedoaria*, Beta-turmerone and ar-turmerone and sesquiterpenoids exhibited considerable activity (Hong, 2002). Ethanolic extract of

E. variegata showed potential analgesic activity in tail immersion and hot plate test which is also comparable to the standard drug morphine (5 mg/kg b.w.) and studies shows that phaseollin of *E. variegata* had the best fitness score against the COX-1 which is 56.64 µg/ml and 59.63 µg/ml for COX- 2 enzyme (Mir *et al.*, 2010).

Table4.5 Percentage of inhibition of Prostaglandins (PEG_2) release from RAW 264.7 cells (IC_{50} µg/ml±SEM), (n=2)

Plants name	Code	%Inhibition of Prostaglandins (PEG_2) from RAW264.7 cells							IC_{50} (µg/ml)
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
A. sativum	AsE	-	-	-	-	-	-	36.09±2.9	>100
	AsH	-	-	-	-	-	-	24.46±4.33	>100
A. galanga	AgE	-	4.75±1.9	41.66±0.09	84.73±3.51	81.12±3.13	-	-	1.23±0.01
	AgH	-	-	-	-	-	-	41.22±2.08	>100
C. adansonii	CaE	-	-	-	28.51±0.48	82.13±6.76	86.96±2.26	107.09±1.45	16.78±1.13
	CaH	-	-	-	-	-	-	-11.07±2.59	>100
C. camphora	CcE	-	-	-	-	-	-	33.5±1.29	>100
C. religiosa	CrE	-	-	-	-	-	-	22.74±2.18	>100
	CrH	-	-	-	-	-	-	-0.91±2.3	>100
C. viscosa	CvE	-	-	-	25.96±0.34	43.29±1.11	55.93±0.52	81.06±0.52	39.75±1.46
	CvH	-	-	-	-	-	-	7.29±0.09	>100
C. zedoaria	CzE	-	17.55±3.38	25.77±0.97	44.93±1.77	74.72±2.26			18.36±1.05
	CzH	-	-	-	-	-	-	34.45±3.06	>100

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table4.5 Percentage of inhibition of Prostaglandins (PEG_2) release from RAW 264.7 cells (IC_{50} µg/ml±SEM), (n=2)

Plants name	Code	%Inhibition of Prostaglandins (PEG_2) release from RAW264.7 cells							IC_{50} (µg/ml)
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
E. variegata	EvE	-	-	-7.55±0.19	54.62±4.57	-	75.02±2.05	81.55±0.19	9.27±0.72
	EvH	-	-	-	-	-	-	-5.49±4.11	>100
M. fragrans (Mace)	MfnE	-	-	47.94±1.41	77.24±0.3	-	103.43±0.91	99.7±1.21	1.57±0.37
	MfnH	-	-	-	-	-	-	47.82±1.29	>100
M. fragrans (Nutmeg)	MfmE	-	-	37.97±4.33	65.66±4.23	-	85.4±3.83	91.74±1.11	4.6±0.19
	MfmH	-	-	-	-	-	-	32.25±2.24	>100
P. nigrum	PnE	-	-4.99±5.96	16.11±0.33	94.53±0.49	96.62±1.61	-	-	2.95±0.49
	PnH	-	-	-	-	-	-	-11.04±1.45	>100
P. indica	PiE	3.7±2.18	7.12±1.05	23.44±2.38	96.49±5.79	-	-	-	4.09±0.32
	PiH	-	-	-	-	-	-	31.5±2.14	>100
Z. cassumunar	ZcE	-	-	40.38±1.71	78.55±1.01	-	90.03±2.42	102.62±2.92	3.08±0.34
	ZcH	-	-	-	-	-	-	28.65±2.2	>100
Sodium chloride	NaClH	-	-	-	-	-	-	-32.38±1.1	>100

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

Table4.5 Percentage of inhibition of Prostaglandins (PEG_2) release from RAW 264.7 cells (IC_{50} µg/ml±SEM), (n=2)

Plants name	Code	%Inhibition of Prostaglandins (PEG_2) release from RAW264.7 cells							IC_{50}
		0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
Lom-Am-Ma-Pruek	LAMPE	-	-	15.33±0.08	88.44±0.33	97.74±0.42	96.9±1.76	-	4.77±0.03
	LAPMH	-	-	-	-	-	-	10.14±1.43	>100
Prednisolone	Pred	-3.45±0.19	-3.73±0.28	53.31±0.09	81.83±0.84	-	-	-	0.96±0.01

Asterisk (*) indicates a significant difference at p value < 0.05, compared with prednisolone, a positive control

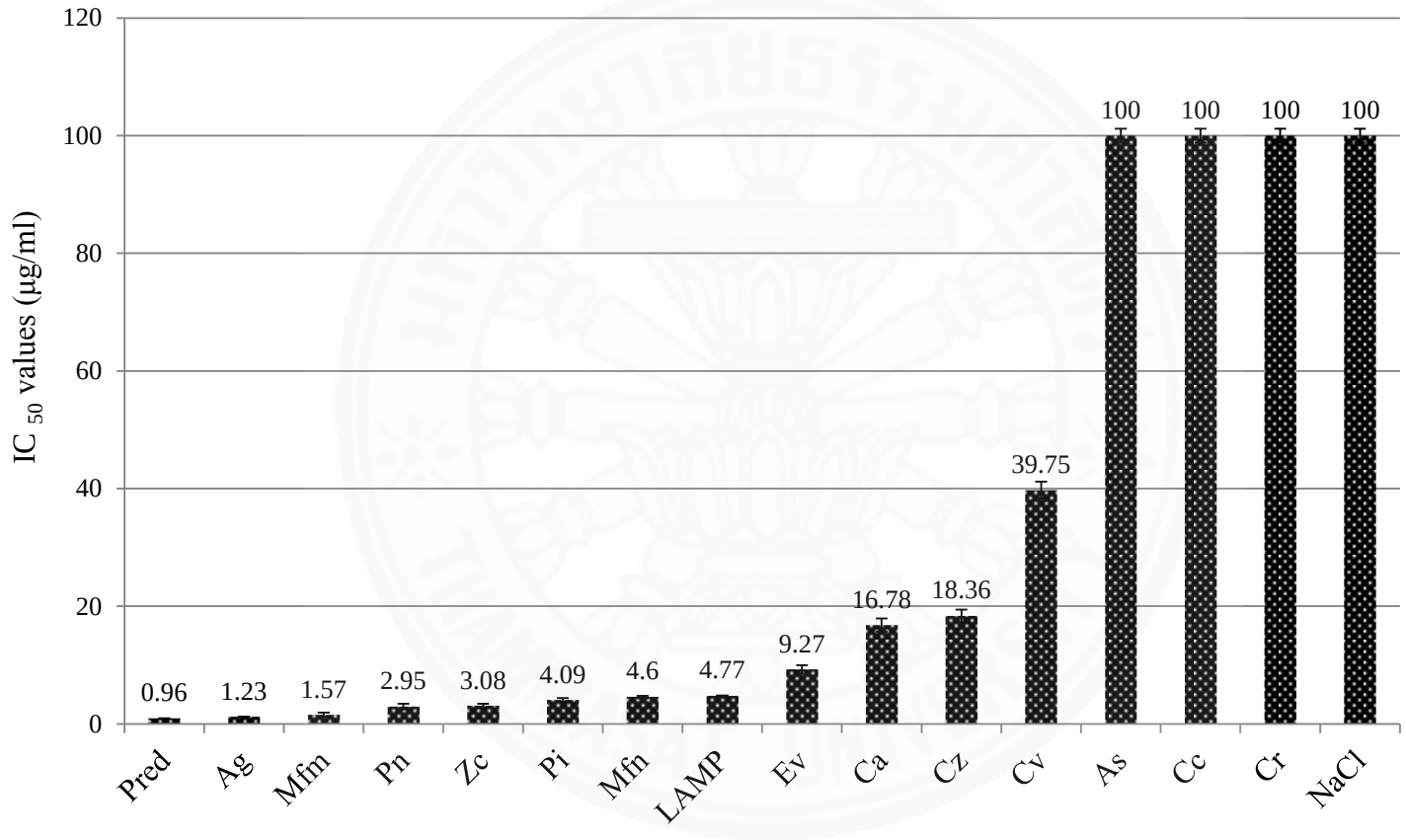


Figure 4.4IC_{50} values of inhibition of ethanolic extracts Lom-Am-Ma-Pruek remedy and its ingredients of Prostaglandins (PEG_2) release from RAW 264.7 cells

Table4.6 The comparison of anti-inflammatory activities of ethanolic extracts Lom-Am-Ma-Pruek remedy and its ingredients by three path ways (inhibition effect on NO production, PEG_2 release and TNF-α release)

Plant extract	Code	IC_{50} of inhibitory effects stimulated by LPS (µg/ml)		
		NO production	TNF-α release	PEG_2 release
A. sativum	As	>100	>100	>100
A. galanga	Ag	6.99±0.4	>100	1.23±0.01*
C. adansonii	Ca	>100	>100	16.78±1.13
C. camphora	Cc	>100	>100	>100
C. religiosa	Cr	>100	>100	>100
C. viscosa	*Cv*	97.93±0.92	>100	39.75±1.46
C. zedoaria	Cz	14.38±1.4	>100	18.36±1.05
E. variegata	Ev	47.1±0.21	>100	9.27±0.72
M. fragrans (Mace)	Mfm	82.46±0.59	>100	1.57±0.37*
M. fragrans (Nutmeg)	Mfn	68.84±1.18	49.36±0.42	4.6±0.19
P. nigrum	Pn	16.42±1.19	>100	2.95±0.49*
P. indica	Pi	24.54±1.97	>100	4.09±0.32
Z. cassumunar	Zc	45.34±1.33	16.90±3.54	3.08±0.34*
Sodium chloride	NaCl	>100	>100	>100
Lom-Am-Ma-Pruek	LAMP	24.90±0.86	35.01±2.61	4.77±0.03
Prednisolone	Pred	1.31±0.05	0.95±0.19	0.96±0.01

Asterisk (*) indicates a not significant difference at p value > 0.05, compared with prednisolone, a positive control

The comparison of anti-inflammatory activities on three path ways (as meansured by 3 assay) revealed that 95% ethanolic extract of Lom-Am-Ma-Pruek remedy (LAMP) possessed potent anti-inflammatory activity on three path ways. It can inhibit nitric oxide production, releasing of TNF-α and releasing of PEG_2 very well with low IC_{50}values. 95% ethanolic extract of the remedy could inhibit NO production and releasing of TNF-α with high potency but less was effective than prednisolone, a positive control. Interestingly, potency of 95% ethanolic extract of remedy on inhibition of prostaglandins (PEG_2) release approximate to prednisolone but, not significantly different from prednisolone. Inhibitory effect on PEG_2 release was more effective than a fivefold dose of prednisolone. However, prednisolone is one of the steroidal anti-inflammatory drugs.

It may be summarized that 95% ethanolic extract of LAMP exhibited potent anti-inflammatory activity on the pro-inflammatory mediator, nitric oxide which acts as an important signaling molecule involved in physiological processes such as neuronal communication, host defense, and regulation of vascular tone. Furthermore, TNF-α which represents chronic inflammation and appears to exacerbate cerebral injury can be inhibited by 95% ethanolic extract of LAMP remedy. In addition, prostaglandins which respond in acute phase of inflammation by producing fever and COX-2-derived prostaglandin E_2 may contribute to ischemic cell damage by disrupting Ca^{2+} homeostasis in neurons via activation of prostaglandin E_2 receptors can also be inhibited by 95% ethanolic extract of LAMP remedy. These results form a basis of scientific knowledge which supports the potency of Lom-Am-Ma-Pruek remedy for treatment of stroke and an analgesic in the musculoskeletal system according to Thai traditional medicine.

Plant ingredients which can inhibit three path ways of inflammatory mediators were *M. fragrans* (Nutmeg) and *Z. cassumunar*. *M. fragrans* (Nutmeg) possessed moderate potency on NO production and releasing of TNF-α, but also higher potency than a fourfold dosage of prednisolone on releasing of PEG_2. *Z. cassumunar* possessed the strongest potency on all three pathways. The pure compounds myristicin, curcumin and (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD), all have anti-inflammatory properties.

4.4 Assay for antioxidant activity

4.4.1 Nitroblue tetrazolium (NBT) dye reduction assay

The assay which determined inhibition effects of Lom-Am-MaPruek remedy and its plant ingredients in scavenging PMA-stimulatedsuperoxide production in HL-60 cells measured by the NBT reduction is described in section 3.6.1. The results are shown in **Table 4.7**. The results shown Lom-Am-Ma-Pruek remedy ethanolic extract (LAMPE) had stronger antioxidant activity than the aqueous extract (LAMPW) with EC_{50} values 40.13±1.1 and more than 100 μg/ml, respectively.It is significantly different from positive control or propyl gallate of EC_{50} value 7.15±1.06 μg/ml (*p-value*< 0.05).

Nine ethanolic extracts showed antioxidant activity. *A. galanga* exhibited the strongest antioxidant activity with EC_{50} value 3.94±0.35μg/ml. It was not significantly different from positive control or propyl gallate (*p-value*>0.05). Second, were mace of *M. fragrans, C. zedoaria* andnutmeg of *M. fragrans* which showed high activity with EC_{50} values18.75±2.47, 19.16±1.72 and 19.5±2.51μg/ml, respectively. *P. indica, Z. cassumunar* and *P. nigrum* exhibited moderate antioxidant activity with EC_{50} values 23.9±1.1, 27.43±3.8 and 35.81±0.72μg/ml, respectively. *E. variegate* and *C. viscosa* exhibited low antioxidant activity with EC_{50} values 85.47±3.3 and 82.47±1.07μg/ml, respectively.

Two aqueous extracts showed antioxidant activity. *C. viscosa* and *A. galangal* exhibited low antioxidant activity with EC_{50} values 83.38±1.76 and 83.51±3.14 μg/ml, respectively.

These results relate with previous investigations which found that the exthanol extract of *P. nigrum* and *P. indica* inhibited $O_2^{\cdot-}$ productionin HL-60 cells (Inprasit, 2014). Past phytochemical studies on these plants, except for *C. viscosa, E. variegata,* Mace of *M. fragrans,* Nutmeg of *M. fragrans*, *P. nigrum* and *P. indica* have indicated the presence of flavonoid and total phenolic (Balamurugan and Shantha, 2010; Calliste *et al.,* 2010; Gupta*et al.,* 2011, Akinboro *et al.,* 2011;Jaiarree, 2011; Anuthakoengkun *et al*., 2012; Eldhose *et al.,* 2013).

Table 4.7 The inhibition (%) of Lom-Am-Ma-Pruek remedy extracts and its ingredients in scavenging PMA-stimulated superoxide production in HL-60 cells measured by NBT reduction (mean±SEM), (n=3)

Plant name	Code	%inhibition of $O_2^{\cdot-}$ production						EC_{50}
		0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
A. sativum	AsE	-	-	-	-	-	33.41±4.25	>100
	AsH	-	-	-	-	-	32.61±4.61	>100
A. galanga	AgE	7.32±9.62	17.85±6.6	102.05±0.49	101.29±1.95	-	-	3.94±0.35*
	AgH	-	-1.86±2.04	10.19±1.49	-	33.43±2.98	59.50±1.32	83.51±3.14
C. camphora	CcE	-	-	-	-	-	27.46±4.44	>100
C. viscosa	CvE	-	14.17±1.94	22.53±1.86	-	38.38±1.51	57.61±1.27	82.47±1.07
	CvH	-	6.33±2.64	16.6±3.08	-	37.52±2.31	56.94±1.81	83.38±1.76
C. adansonii	CaE	-	-	-	-	-	18.48±3.17	>100
	CaH	-	-	-	-	-	-0.33±2	>100
C. religiosa	CrE	-	-	-	-	-	19.64±1.43	>100
	CrH	-	-	-	-	-	15.1±0.33	>100

Asterisk (*) indicates a not significant difference at p value > 0.05, compared with propyl gallate, a positive control

Table4.7 The inhibition (%) of Lom-Am-Ma-Pruek remedy extracts and its ingredients in scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction (mean±SEM), (n=3)

Plant name	Code	%inhibition of $O_2^{\cdot-}$ production						EC_{50} (µg/ml)
		0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
C. zedoaria	CzE	12±0.75	16.96±2.11	38.93±0.72	61.19±5.67	-	-	19.16±1.72
	CzH	-	-	-	-	-	19.98±4.93	>100
E. variegata	EvE	-	17.57±2.74	13.74±4.61	-	20.25±2.14	68.42±7.88	85.47±3.3
	EvH	-	-	-	-	-	44.14±3.19	>100
M. fragrans (Mace)	MfmE		13.6±3.71	32.4±3.24		99.34±1.43	99.94±2.68	18.75±2.47
	MfmH	-	-	-	-	-	8.42±0.71	>100
M. fragrans (Nutmeg)	MfnE	-	15.27±1.49	32.42±3.57	-	95.42±2.93	100.45±0.82	19.5±2.51
	MfnH	-	-	-	-	-	11.93±1.02	>100
P. nigrum	PnE	-	-6.33±0.56	28.21±3.58	45.89±0.56	68.04±3.21	-	35.81±0.72
	PnH	-	-	-	-	-	13.64±0.95	>100
P. indica	PiE	-3.65±2.97	11.64±1.17	23.55±3.04	86.026±2.03	-	-	23.9±1.1
	PiH	-	-	-	-	-	20.29±0.98	>100
Sodium Chloride	NaCl	-	-	-	-	-	11.41±1.4	>100

Asterisk (*) indicates a not significant difference at p value > 0.05, compared with propyl gallate, a positive control

Table4.7 The inhibition (%) of Lom-Am-Ma-Pruek remedy extracts and its ingredients in scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction (mean±SEM), (n=3)

Plants name	Code	%inhibition of $O_2^{\cdot-}$ production						EC_{50} (µg/ml)
		0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
Z. cassumunar	ZcE	-	-0.34±4.69	24.22±1.86	-	58.57±2.58	70.32±1.83	27.43±3.8
	ZcH	-	-	-	-	-	29.2±1.34	>100
Lom-Am-Ma-Pruek	LAMPE	-	-4.8±1.82	3.03±6.31	30.8±2.72	67.73±2.87	-	40.13±1.1
	LAMPH	-	-	-	-	-	26.46±1.53	>100
Propyl Gallate	PG	-	22.79±5.27	60.97±4.33	-	90.24±1.95	94.58±0.4	7.15±1.06

Asterisk (*) indicates a not significant difference at p value > 0.05, compared with propyl gallate, a positive control

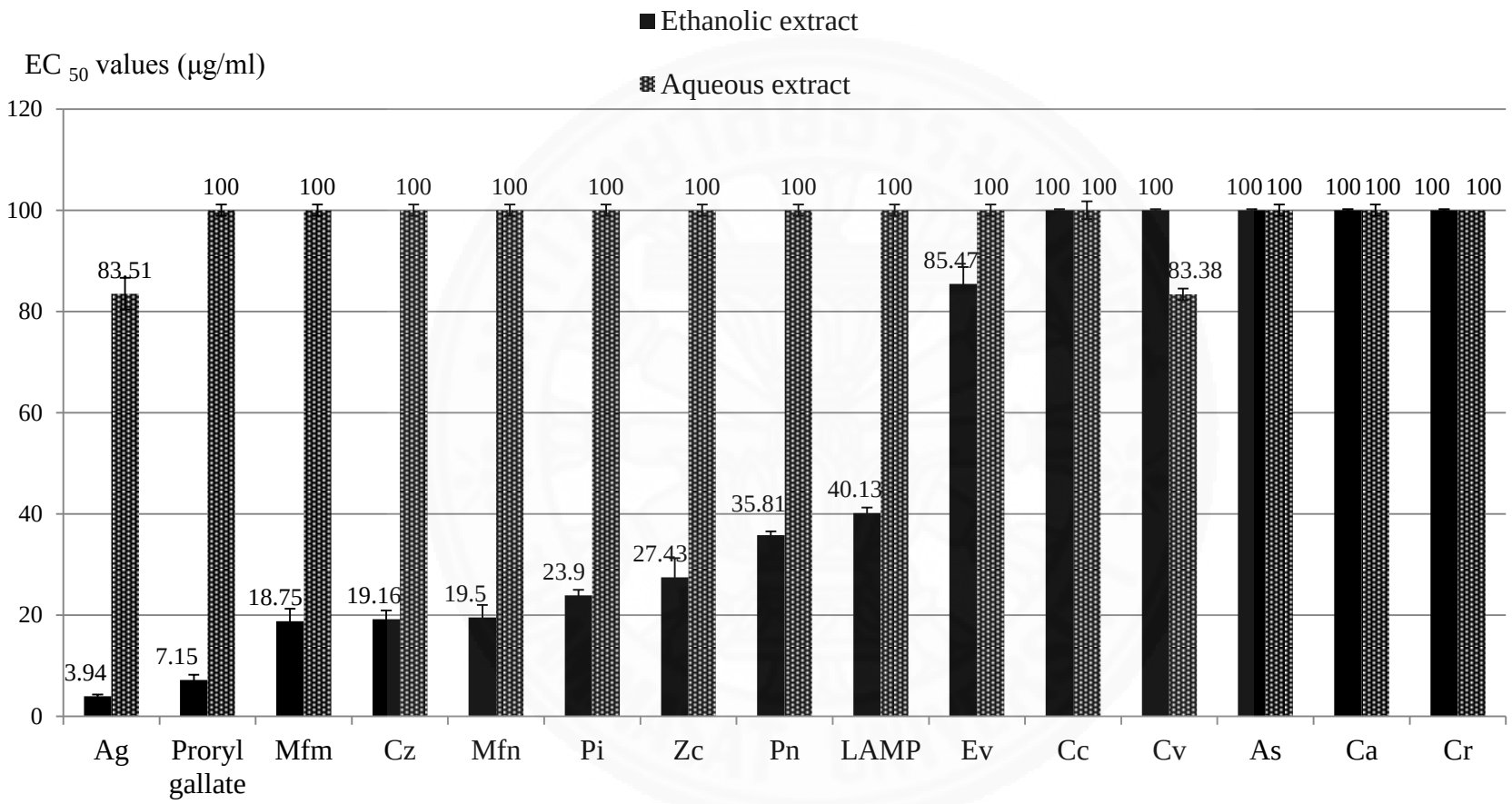


Figure 4.5 EC_{50} of Lom-Am-Ma-Pruek remedy extracts and its ingredients in scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction (mean±SEM), (n=3)

Table 4.8 Summary of the biological activities of Lom-Am-Ma-Pruek remedy ethanolic extracts, and its ingredients. (Inhibition effect on NO production, PEG_2 release, TNF-α release and inhibition of $O_2^{\cdot-}$ production)

Plants name	Code	IC_{50} of inhibitory effects stimulated by LPS (μg/ml)			Antioxidant EC_{50} (μg/ml)
		NO production	TNF-α release	PEG_2 release	
A. sativum	AsE	>100	>100	>100	>100
	AsH	>100	>100	>100	>100
A. galanga	AgE	6.99±0.4	>10	1.23±0.01	3.94±0.35
	AgH	>100	>100	>100	83.51±3.14
C. adansonii	CaE	>100	>100	16.78±1.13	>100
	CaH	>100	>100	>100	>100
C. camphora	CcE	>100	>100	>100	>100
C. religiosa	CrE	>100	>100	>100	>100
	CrH	>100	>100	>100	>100
C. viscosa	CvE	>100	>100	39.75±1.46	82.47±1.07
	CvH	97.93±0.92	>100	>100	83.38±1.76
C. zedoaria	CzE	14.38±1.4	>100	18.36±1.05	19.16±1.72
	CzH	51.29±2.36	>100	>100	>100

Table 4.8 Summary of the biological activities of Lom-Am-Ma-Pruek remedy ethanolic extracts, and its ingredients. (Inhibition effect on NO production, PEG_2 release, TNF-α release and inhibition of $O_2^{\cdot-}$ production), (continued)

Plants name	Code	IC_{50} of inhibitory effects stimulated by LPS (µg/ml)			Antioxidant IC_{50} (µg/ml)
		NO production	TNF-α release	PEG_2 release	
E. variegata	EvE	47.1±0.21	>100	9.27±0.72	85.47±3.3
	EvH	37.22±0.33	>100	>100	>100
M. fragrans	MfnE	82.46±0.59	>100	1.57±0.37	18.75±2.47
(Nutmeg)	MfnH	44.86±1.51	>100	>100	>100
M. fragrans	MfmE	68.84±1.18	49.36±0.42	4.6±0.19	19.5±2.51
(Mace)	MfmH	67.63±4.14	>100	>100	>100
P. nigrum	PnE	16.42±1.19	>100	2.95±0.49	35.81±0.72
	PnH	>100	>100	>100	>100
P. indica	PiE	24.54±1.97	>100	4.09±0.32	23.9±1.1
	PiH	>100	>100	>100	>100
Z. cassumunar	ZcE	45.34±1.33	16.90±3.54	3.08±0.34	27.43±3.8
	ZcH	46.2±2.4	>100	>100	>100
Sodium chloride	NaClH	>100	>100	>100	>100

Table4.8 Summary of the biological activities of Lom-Am-Ma-Pruek remedy ethanolic extracts, its and ingredients. (Inhibition effect on NO production, PEG_2 release, TNF-α release and inhibition of $O_2^{\cdot-}$ production), (continued)

Plants name	Code	IC_{50} of inhibitory effects stimulated by LPS (µg/ml)			Antioxidant IC_{50} (µg/ml)
		NO production	TNF-α release	PEG_2 release	
Lom-Am-Ma-	LAMPE	24.90±0.86	35.01±2.61	4.77±0.03	40.13±1.1
Pruek	LAPMH	>100	>100	>100	>100
Prednisolone	Pred	1.31±0.05	0.95±0.19	0.96±0.01	-
Propyl gallate	PG	-	-	-	7.15±1.06

4.5 Study on chemical fingerprint of Lom-Am-Ma-Pruek remedy preparation using high performance liquid chromatography

The aim was to develop a reversed phase high performance liquid chromatography (RPHPLC) method to control quality of Lom-Am-Ma-Pruek remedy in two aspects namely chemical fingerprint and quantification. In this method, piperine, plumbagin, myristicin and eugenol as the compoundshaving the most potent inhibition of anti-inflammatory (**Table4.10**) were used to be markers because this method has good sensitivity, precision, and accuracy. Preparation of Lom-Am-Ma-Pruek sample and pure compounds is shown in section3.7.2.1. The liquid chromatographic conditions are summarized in **Table 4.9**. Representative chromatograms of the ethanolic extract of Lom-Am-Ma-Pruek and contents of pure compounds are shown in **Figure 4.4-4.6** and **Table4.11**. Standard curves and chromatograms of pure compounds are shown in **Figure 4.7** and **4.8.**

The result showed that myristicin in the ethanolic extract of Lom-Am-Ma-Pruek was present in the highest content (297.84±5.42 mg/g of extract), followed by piperine, eugenol and plumbagin, with content of 189.66±2.56, 58.75±0.13 and 45.01±2.35mg/g of extract, respectively. From this result, new knowledge and scientific dataof the chemical fingerprint and quantification of Lom-Am-Ma-Pruek remedy and RP-HPLC method may be considered for quality control of Lom-Am-Ma-Pruek extract.

Table 4.9 HPLC condition for analysis of ethanolic extract of Lom-Am-Ma-Pruek remedy

Operating parameters	**Conditions**
Stationary Phase	Phenomenex Luna 5 µ C18(2) 100A analytical column (150 x 4.60 mm. 5 micron)
Mobile Phase	Water-acetronitrile with gradient elution as follows: 0 min, 90:10; 30 min, 50:50; 40 min, 5:95; 45 min, 5:95; 45.1 min, 90:10; 50 min 90:10.
Flow Rate	1.0 ml/min
Wavelength	210 and 256 nm.
Injection Volume	10 µl

Table 4.10 The comparison of anti-inflammatory activities of pure compounds on two path ways

Sample	**IC_{50} of inhibitory effects stimulated by LPS (µg/ml)**	
	NO production	**PGE_2 relese**
Piperine	70.25 µg/ml	>100 µg/ml
Plumbagin	0.002 µg/ml	0.08 µg/ml
Myristicin	>100 µg/ml	1.80 µg/ml
Eugenol	>100 µg/ml	0.37 µM

Table 4.11 The concentration range, linearity (r^2), retention time (RT), and content (mg/g of extract) of bioactive markers from Lom-Am-Ma-Pruek extract analyzed by using HPLC.

Compounds	Wavelength (nm)	Concentration range (µg/ml)	Linearity (r^2)	RT (min)		Content (mg/g of extract)
				Standard	Lom-Am-Ma-Pruek extract	HPLC
Eugenol	210	5-25	0.9985	26.722	26.481	58.75±0.13
Plumbagin	256	5-25	0.9968	29.458	29.172	45.01±2.35
Piperine	256	100-500	0.9921	32.449	32.285	189.66±2.56
Myristicin	210	20-100	0.9982	34.978	33.818	297.84±5.42

(A)

(B)

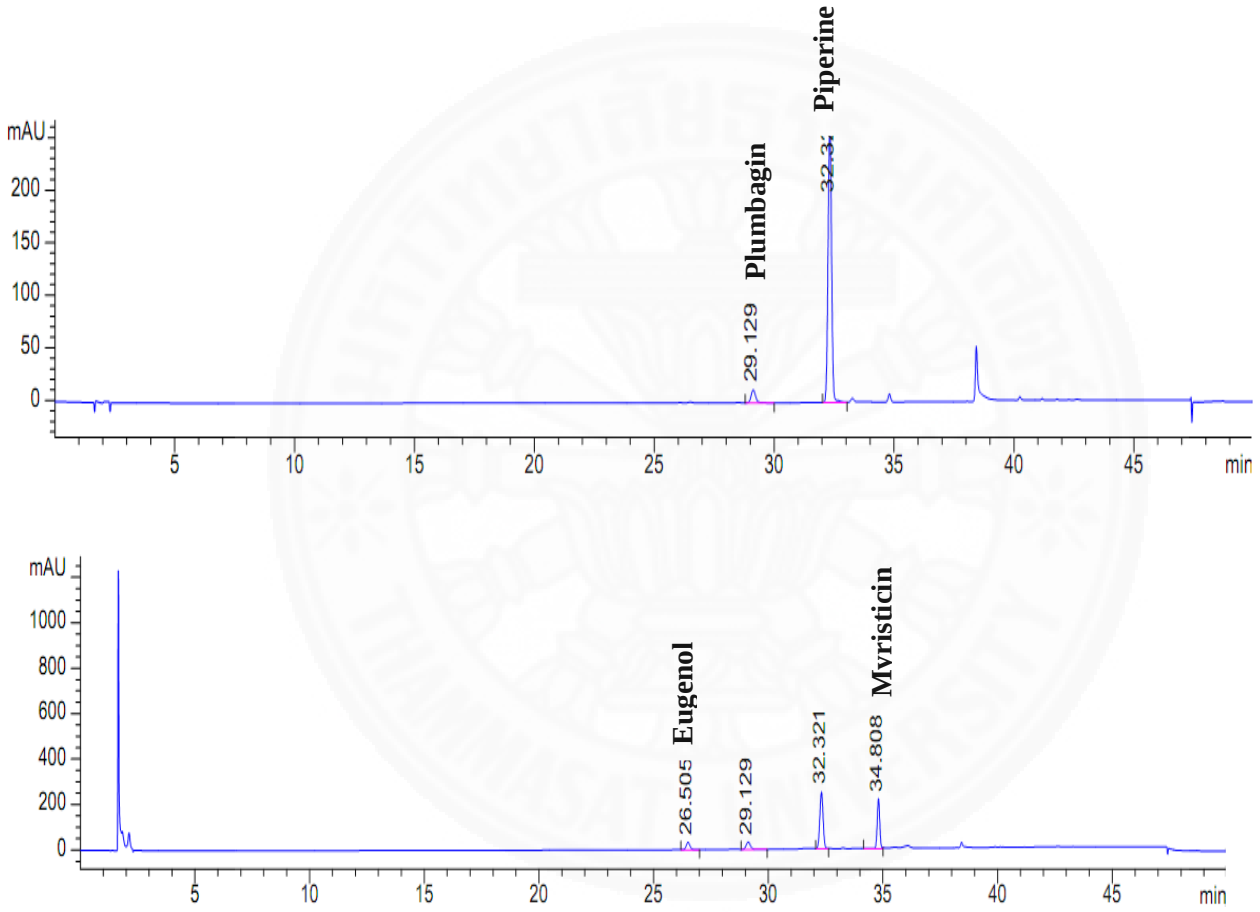
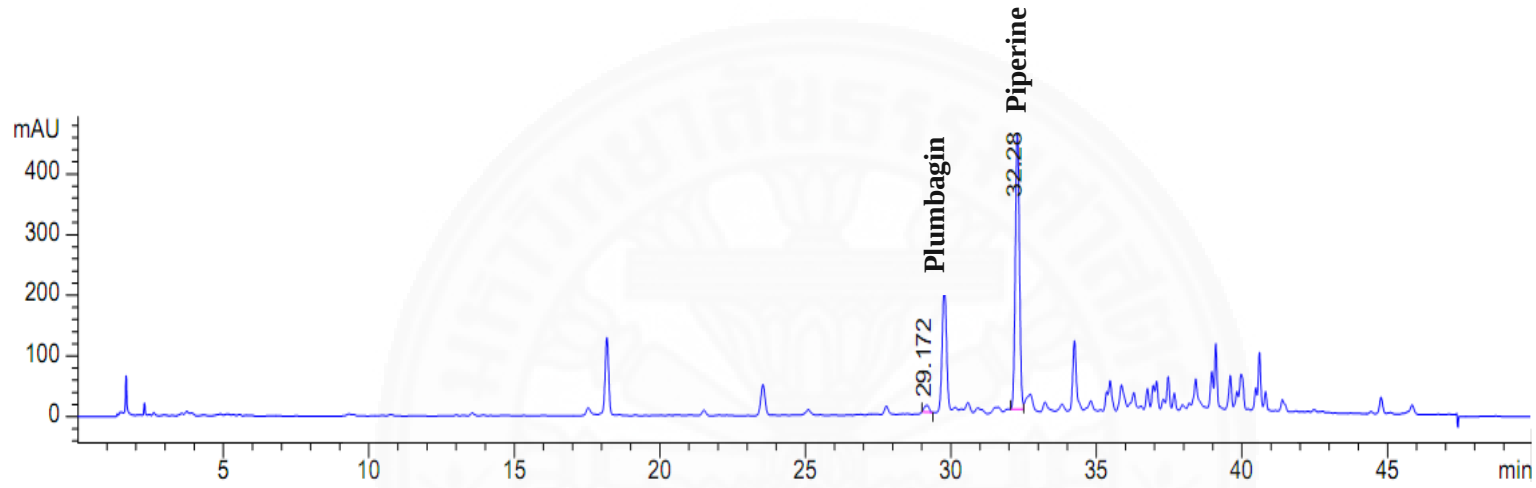


Figure 4.6HPLC chromatogram of standard (A) plumbagin and piperine in wavelength at 256 nm and (B) eugenol and myristicin in absorption at 210 nm

(A)



(B)

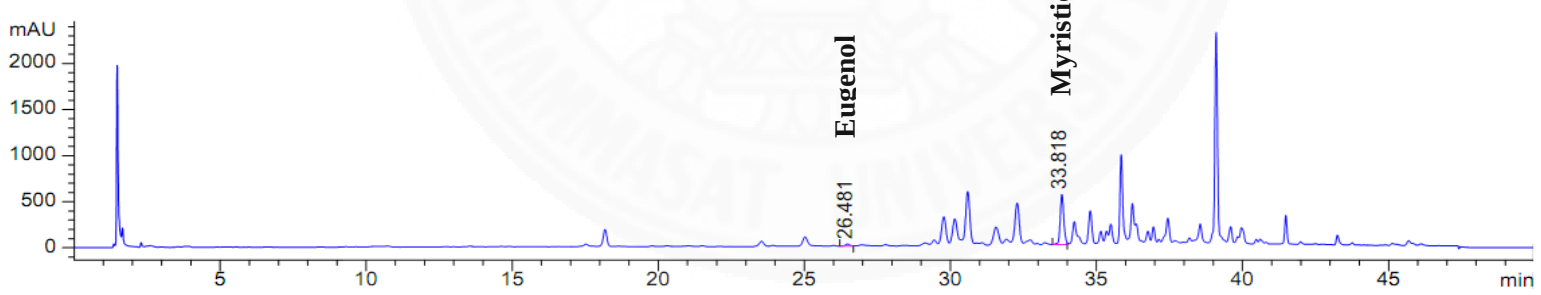
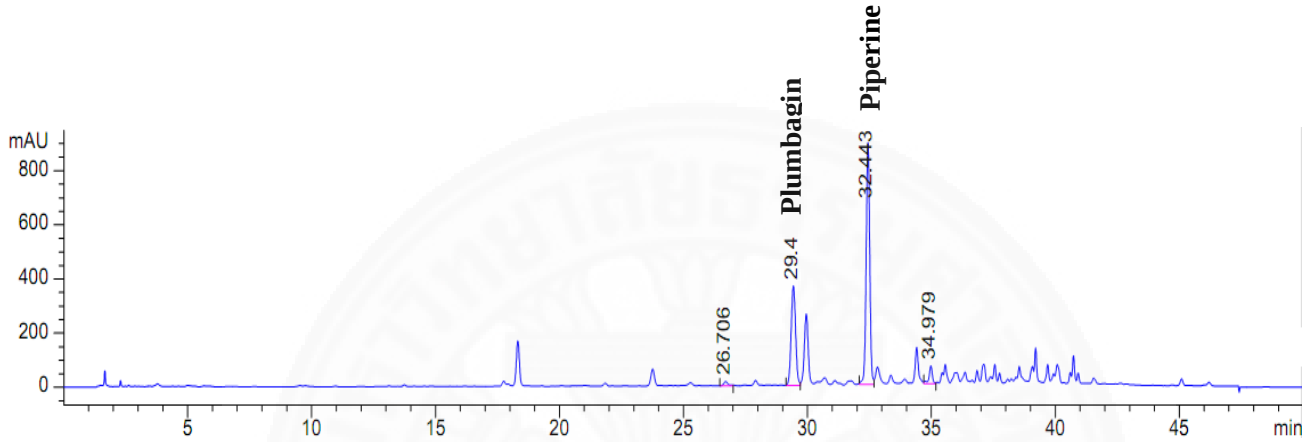


Figure 4.7 Chromatogram of ethanolic extract of Lom-Am-Ma-Pruek (A) wavelength at 256 nm. (B) wavelength at 210 nm.

(A)



(B)

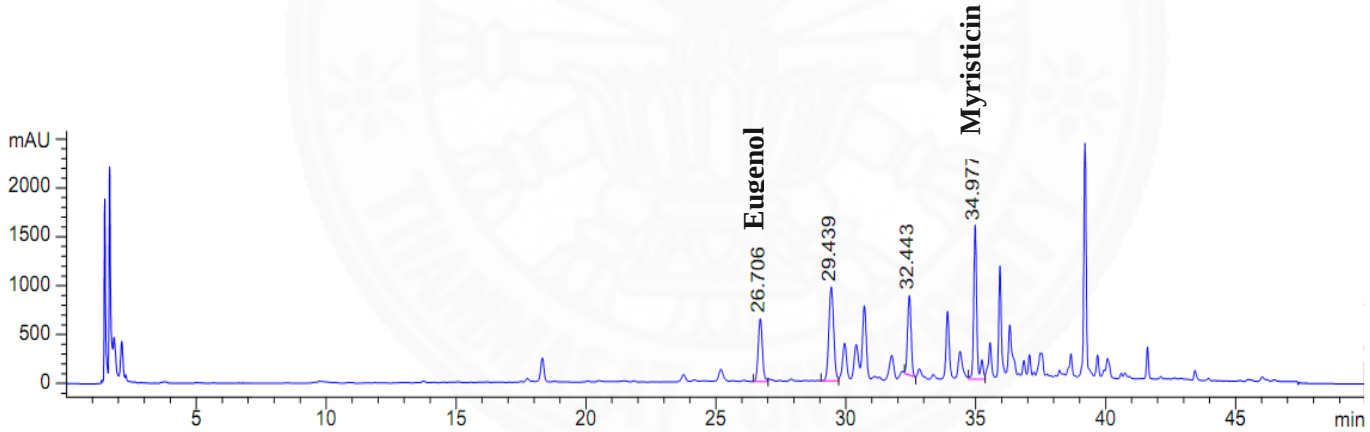


Figure 4.8HPLC chromatogram the standard of (A) plumbagin and piperine in wavelength at 256 nm and (B) eugenol and myristicin in absorption at 210 nm. known amount was spiked to ethanolic extract of Lom-Am-Ma-Pruek sample solution.

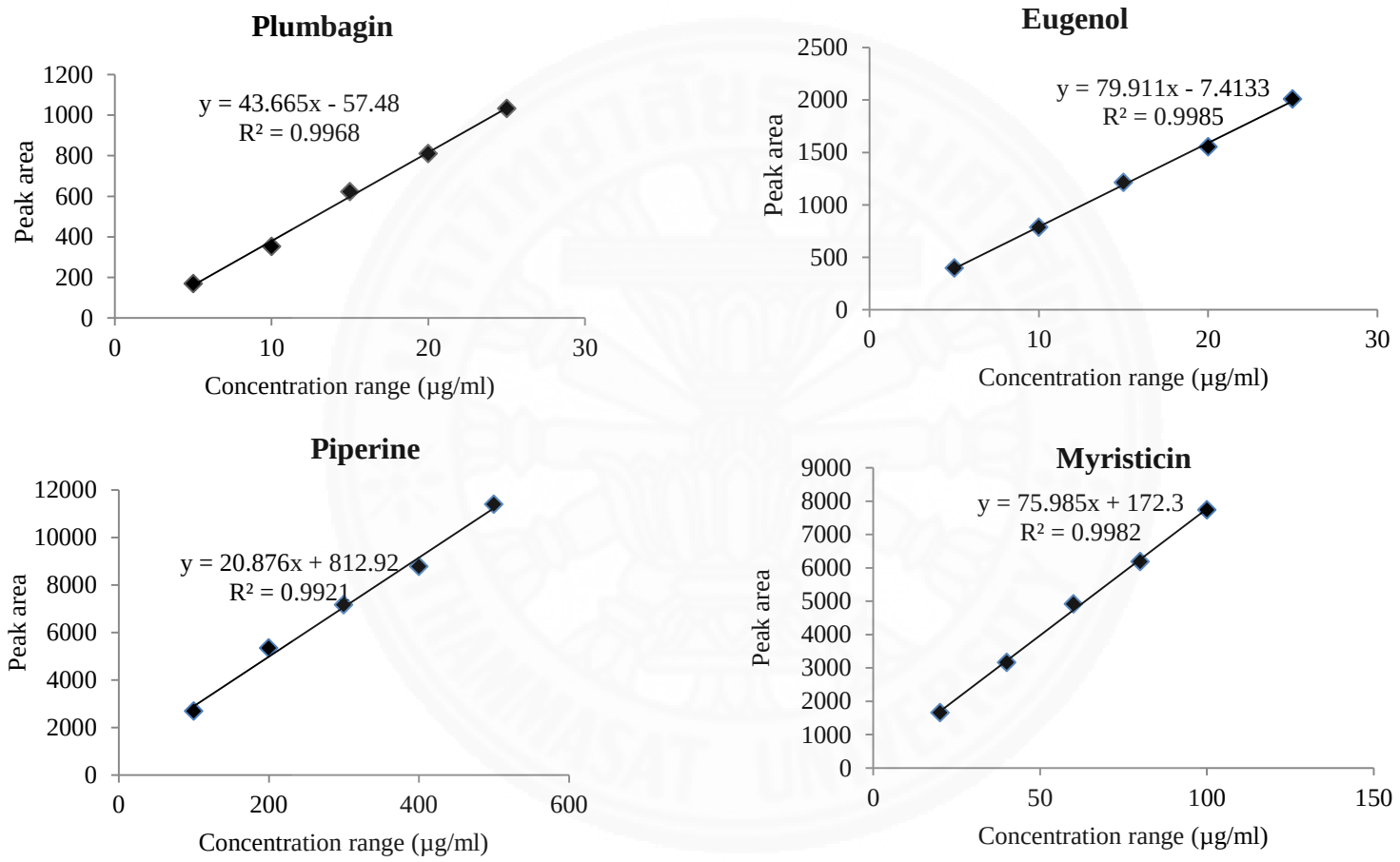


Figure 4.9 Calibration curve (HPLC pattern of standard)

4.6 Phytochemical test of Lom-Am-Ma-Pruek ethanolic extract by using gas chromatography-mass spectrometry (GC-MS)

The 95% ethanolic extract of LAMP was analyzed by using a Thermo focus GC Gas Chromatography-Mass Spectrometry with capillary column TG-5 slims (30 m x 0.25 mm x 0.25 µm), (Thermo Fisher Scientific). The ionization energy was achieved by electron impact at 70 eV. Helium (He) gas was the carrier with flow rate 1.0 ml/min. The initial temperature of column oven was programed 60^{o}C, and then heated to 300 ^{o}C with a rate of 5^{o}C/min and kept constant at 300^{o}C for 10 min. The mass spectrometrum of each peak was recorded in the positive ion current mode of mass spectrometer within a mass range of 35 to 400. Identification of oil constituents was achieved using the National Institute of Standards and Technology (NIST).

Table 4.12 Chemical composition of Lom-Am-Ma-Pruek remedy by using GC-MS

No.	Compounds	RI	Area (%)
1	Camphor	14.19	5.36
2	Terpinene-4-ol	15.24	0.16
3	Iso bornyl acetate	15.68	0.21
4	1,3-Benzodioxole, 5-(1-propenyl)- (CAS)	18.39	1.55
5	Benzene	18.81	0.07
6	Pulegone	19.71	0.41
7	Eugenol	20.08	0.43
8	Methyleugenol	21.34	1.59
9	beta-caryophyllene	21.93	1.03
10	Phenol	22.56	0.17
11	Humulene	22.84	0.76
12	(+)-Cuparene	23.43	1.54
13	BETA-SELINENE	23.68	0.32
14	Zingiberene	23.78	2.39
15	Alloaromadendrene	24.11	1.01
16	1-ALLYL-3,4-METHYLEN-DIOXY-5-METHOXY-BENZENE	24.37	4.35

Table 4.12 Chemical composition of Lom-Am-Ma-Pruek remedy by usingGC-MS (Continued)

No.	Compounds	RI	Area (%)
17	GAMMA-MUUROLEN	24.49	3.36
18	cis-Asarone	24.99	4.89
19	DMPBD	25.43	0.36
20	Isoaromadendrene epoxide	25.92	2.58
21	2-ALLYL-1,4-DIMETHOXY-3-METHYL-BENZENE	26.03	0.53
22	Methoxyeugenol	26.13	1.71
23	Triquinacene	26.90	5.83
24	Chroman-4-ol acetate	27.07	15.57
25	1-Butyn-3-one, 1-(6,6-dimethyl-1,2-epoxycyclohexyl)-	27.61	0.31
26	Ar-tumerone	27.72	3.19
27	Tumerone	27.84	3.43
28	(3E)-5-Isopropyliden-6-methyl-3,6,9-decatrien-2-one	28.26	0.19
29	Curlone	28.57	1.78
30	Triquinacene	29.08	0.24
31	Naphthalene	29.48	0.37
32	Tetradecanoic acid	29.93	4.58
33	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)	30.14	0.43
34	Palmitic acid	30.54	1.54
35	Vetiverylacetate3	30.86	0.70
36	3,5,7-Nonatrien-2-one, 8-methyl-7-(1-methylethyl)-, (E,E)- (CAS	31.48	0.42
37	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	32.04	1.93
38	Cyclopentanecarboxylic acid	32.04	1.93

Table 4.12 Chemical composition of Lom-Am-Ma-Pruek remedy by using GC-MS (Continued)

No.	Compounds	RI	Area (%)
39	Hexadecanoic acid	33.22	0.16
40	l-(+)-Ascorbic acid 2,6-dihexadecanoate	33.98	2.54
41	1,4-Dimethoxytriquinacene	34.40	6.23
42	Nonanedioic acid	34.55	1.97
43	2,3-Dihydroxypropyl elaidate	36.52	0.25
44	9-Octadecenoic acid	37.25	0.79
45	Linolein,	37.61	0.55
46	Oleic acid	37.74	1.92
47	Adamantane-1-carboxylic acid	44.47	0.94
48	Austrobailignan-6	45.70	6.26
49	3-(3-Hydroxy-4-methoxyphenyl)-l-alanine	46.00	1.16

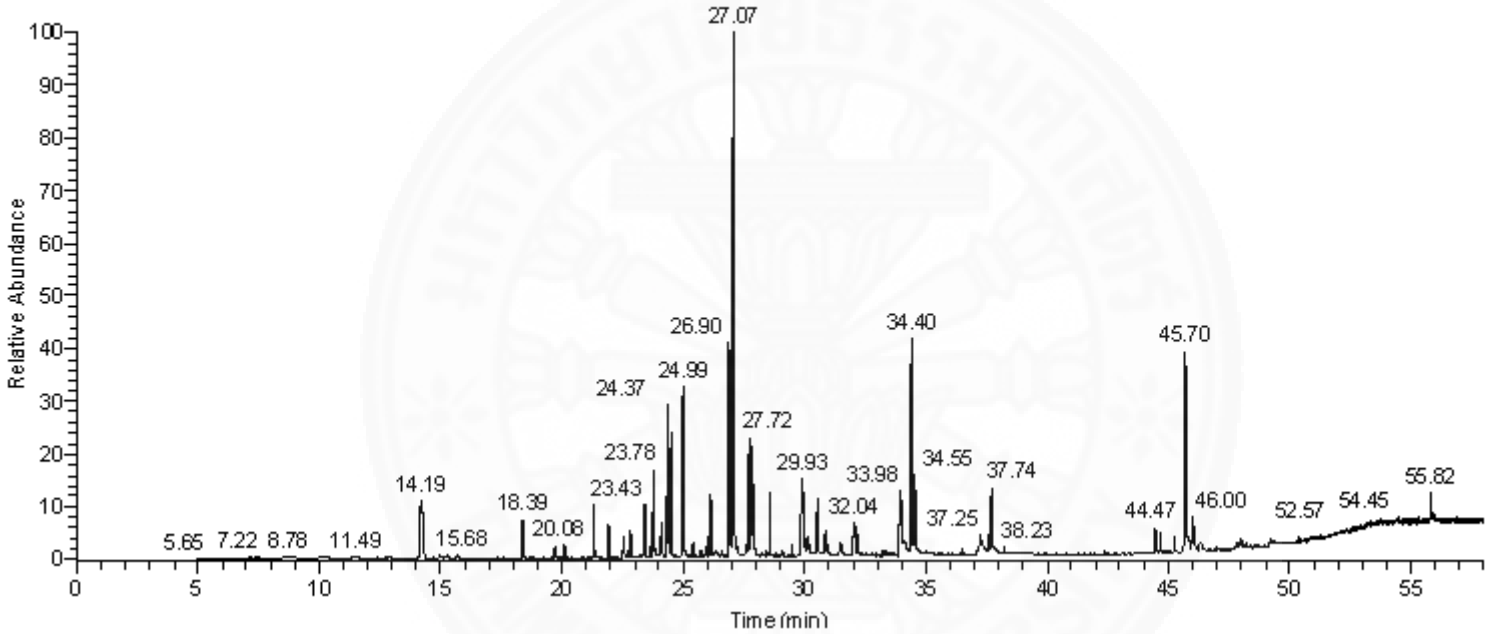


Figure 4.10 Chromatogram of Lom-Am-Ma-Pruek remedy by using GC-MS

Camphor ($C_{10}H_{16}O$) is a natural product derived from the wood of the camphor laurel (*Cinnamomum camphora* L.) trees through steam distillation and purification by sublimation. Camphor has a counter-irritant, mild analgesic action, and is a major component of liniments for relief of fibrosis, neuralgia and similar conditions. It can be used as a mild expectorant; when ingested camphor has irritant and carminative properties. Camphorated-oil, a solution in oil given through intramuscular or subcutaneous way, can be used as a circulatory and respiratory stimulant, but this use is considered hazardous. When in combination with menthol and chenodeoxycholic acid it has been used to aid dispersal of bile duct stones, although this is no longer recommended (Zuccarini, 2009).

Eugenol ($C_{10}H_{12}O_2$) a phenylpropanoid, is an allyl chain-substituted guaiaco. Eugenol is a component of *Alpinia galanga* (L.) Willd. and other essential oils. At lower concentrations, eugenol possesses anti-inflammatory, (Wargovich *et al*., 2001), antibacterial activity (Singh and Maurya, 2007; Laekeman *et al*., 1990), antifungal activity (Ahmad *et al*., 2010; Khan *et al*., 2012) and anti-oxidant activity (Ito *et al*., 2005).

β-asarone or cis-asarone (cis-isomer of 2, 4, 5-trimethoxy-l-propenyl-benzene) may be a potential candidate for development as a therapeutic agent to manage cognitive impairment associated with conditions such as Alzheimer's disease (Geng *et al*., 2010).

Phenol (hydroxybenzene) is a colourless, crystalline substance of characteristic odour, soluble in water and organic solvents. Phenol is 2-[(2’E)-3’,7’-dimethy-2’,6’-octadieny]-4-methoxy-6-methylphenol which has strong anti-inflammatory activity(Jie *et al*., 2004).

DMPBD stands for (E)-1-(3', 4'-dimethoxyphenyl) butadiene found in essential oil and shows strong anti-inflammatory effect. DMPBD dose-dependently inhibited the rat ear edema induced by ethyl phenylpropiolate (EPP), arachidonic acid (AA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) and it was more potent than any other standard drugs being used. In EPP-induced edema IC (50) of DMPBD and oxyphenbutazone were 21 and 136 nM per ear, respectively (Jeenapongsa *et al*., 2003).

Piperine is an alkaloid found naturally in plants belonging to the Piperaceae family, such as *Piper nigrum* L, commonly known as black pepper, and *Piper longum* L, commonly known as long pepper. Piperine at a dose of 5 mg/kg and ethanol extract at a dose of 15 mg/kg after 120 min and hexane extract at a dose of 10 mg/kg after 60 min exhibited significant ($P<0.05$) analgesic activity by tail immersion method, in comparison to ethanol extract at a dose of 10 mg/kg using analgesy-meter in rats. However, with hotplate method, piperine produced significant ($P<0.05$) analgesic activity at lower doses (5 and 10 mg/kg) after 120 min (Koul and Kapil, 1993).

Turmerone is the principle flavouring compound of turmeric (*Curcuma zedoaria* (Berg) Roscoe.). Turmerone and ar-turmerone are known to be the character impact compounds of turmeric contributing to the dry turmeric aroma. The anti-inflammatory activity of prostaglandin E_2 production using lipopolysaccharide (LPS)-induced of mouse macrophage cell RAW 264.7 cell lines of two compounds from rhizome of *C.zedoaria*, Beta-turmerone and ar-turmerone and sesquiterpenoids exhibited considerable activity (IC_{50} values 7.3, 24.0 and μM, respectively) (Hong, 2002).

4.7 Stability test

4.7.1 *In vitro,* anti-inflammatory activity by inhibition of nitric oxide production from RAW 264.7 cell lines of Lom-Am-Ma-Pruek remedy from stability test

The result revealed that all Lom-Am-Ma-Pruek ethanolic extracts from the stability test (Days 15, 30, 60, 90, 120, 150 and 180) were not significantly different in anti-inflammation effect by inhibition of nitric oxide production release from RAW 264.7 cell lines compared with day 0 (*p-value*> 0.05). The data is shown in **Table 4.13**.

Table 4.13 The percentage of inhibition of Lom-Am-Ma-Pruek remedy extracts and its ingredients on LPS induced NO production from RAW264.7 cells and cytotoxicity from stability test (mean±SEM), (n=3) (continued)

Sample	%inhibition of nitric oxide Production and (%cytotoxicity)				IC_{50}
	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	(µg/ml)
Day 0	-7.42±3.01 (-12.62±4.48)	28.54±4.73 (-3.18±1.11)	51.7±3.09 (12.02±4.88)	94.04±2.45 (19.42±2.21)	28.16±1.57
Day 15	-5.91±0.99 (-1.9±4.47)	30.47±2.39 (3.77 ±1.07)	50.11±0.65 (10.65±3.03)	87.36±1.36 (26.58±1.59)	29.92±1.46
Day 30	-3.17±5.27 (-8.01±1.95)	55.1±0.81 (-0.23±8.31)	49.01±0.98 (1.32±4.72)	77.57±1.84 (17.49±2.26)	23.07±2.44
Day 60	1.04±3.12 (-21.22±3.38)	35.02±2.64 (-4.61±2.4)	48.47±1.77 (-4.01±3.88)	79.63±3.03 (9.56±2.58)	31.73±1.45
Day 90	-2.82±1.51 (-6.62±6.12)	30.79±0.89 (-1.39±5.73)	49.91±2.04 (10.25±1.41)	73.82±1.69 (18.52±4.95)	31.96±1.78
Day 120	-3.82±4.66 (-10±0.1)	28.63±2.41 (-13.56±3.92)	56.66±2.22 (10.82±2.51)	82.52±2.38 (20.66±3.35)	30.14±2.35
Day 150	-2.83±3.84 (3.16±5.1)	29.22±2.93 (11.18±3.75)	49.01±1.74 (14.35±1.9)	87.36±0.95 (19.2±3.16)	22.25±1.88
Day 180	-3.71±1.72 (4.05±6.27)	27.79±1.82 (-1.99±2.11)	53.42±4.55 (21.51±2.78)	77.57±0.11 (26.16±1.93)	26.12±0.3

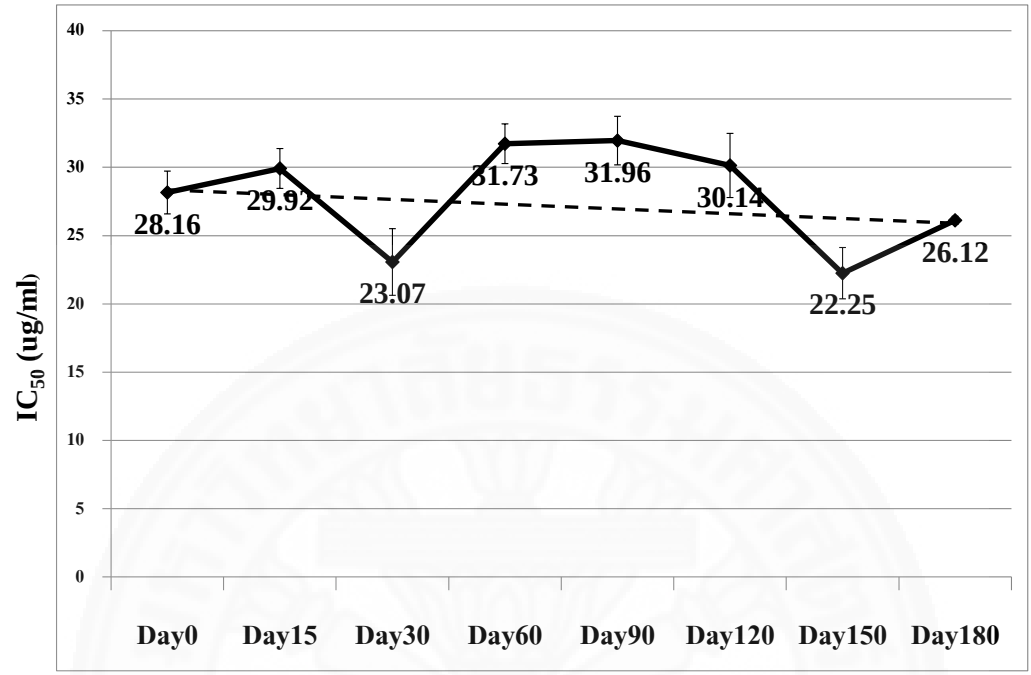


Figure 4.11 Anti-inflammatory activity by inhibition of nitric oxide production from RAW 264.7 cell lines of Lom-Am-Ma-Preuk (LAMP)remedy from stability test (mean ± SEM), (n=3)

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Lom-Am-Ma-Pruek remedy (LAMP) has been used for stroke and an analgesic in the musculoskeletal system in Thai traditional medicine for a long time.Thai national list of essential medicine also specified Lom-Am-Ma-Pruek (LAMP) as a remedy for stroke and an analgesic in the musculoskeletal system. It is in Chawadarn scripture, it consists of fourteen medicinal plants as follows: *Allium sativum* Linn, *Alpinia galanga* (L.) Willd, *Cinnamomum camphora* (L.) Presl, *Cleome viscosa* Linn, *Crateva adansonii* DC, *Crateva religiosa* G.Forst, *Curcuma zedoaria* (Berg) Roscoe, *Erythrina variegata* Linn, *Myristica fragrans* Houtt (seed and fruit), *Piper nigrum* Linn, *Plumbago indica* Linn, *Zingiber cassumunar* Roxb, Sodium Chloride (NaCl).When most ofthe plants are mixed, they produce a spicy taste. Thai traditional medicine practitioners believe that spicy herbal flavorscan help balance elements of the body. There is no previous report on the biological activity of LAMP extract. Its effectiveness has never been determined by scientific method which is the most important for authorized safety and efficacy in clinical use according toThai traditional medicine. Therefore, this study aimed to investigate necessary procedures for quality control of plant materials, *in vitro* anti-inflammatory assay by inhibition effects on NO production, PEG_2 release and TNF-α release and antioxidant assay by inhibition of $O_2^{\cdot -}$ productionactivities (NBT assay) and stability testing of LAMP extract.Development of chemical fingerprint was also carried out using high performance liquid chromatography (HPLC) of LAMP extract.

LAMP and its ingredient plants extracts were prepared by maceration and decoction methods. The percentage of yields are shown as percentage weight for weight. For the LAMP, the aqueous extract has the highest percentage of yield (11.37%) followed by 95% ethanolic extract (7.37%). Each of the ingredient plants was extracted by 95% ethanol. *M. fragrans* (mace) showed highest percentage of yield (8.70%) followed by the ethanolic extract of *A. galangal* (8.70%) and *C. zedoaria* (8.53%). The three least percentage of yields are *E. variegata* (1.95%), *C. adansonii* (1.60%) and *C. religiosa* (1.49).

Each of the ingredients was extracted by decoction. *A. sativum* showed highest percentage of yield (42%) followed by the aqueous extract of*P. indica* (42%) and *P. nigrum* (23.86%).The three least percentage of yields were nutmeg of *M. fragrans* (5.59), *E. variegata* (5.06%) and mace of *M.fragrans* (5.05).

Quality controls of the crude drug were determined to standardize the plant materials following the Thai Herbal Pharmacopoeia (THP) protocols. Moisture content of the crude drug of LAMP and its ingredient plants had percentage values less than 10% which were accepted by THP, except *C. camphora* which had a high moisture content because it is hygroscopic in air. However, if all ingredients were combined as LAMP, its values were accepted by THP (8.64±0.36%). Ash content analysis which is used for determination of inorganic contamination consists of total ash and acid insoluble ash. Both ash contents of LAMP and its ingredient plants were accepted by THP criteria. Percentage of total ash was 7.34±0.43 and percentage of acid insoluble ash was1.76±0.05 although sodium chloride showed total ash was 28.19±0.31 which was higher than criteria of THP. Extractive values which indicated quality of crude drug were performed using two solvent extracts. First, the ethanol extract of LAMP gave extractive value percentage of 5.3±0.15%. The alcoholic extracts which showed the highest percentage of yield were *M. fragrans* (Nutmeg), *C. adansonii* and *P. indica* (18.8±0.12, 16.8±0.17 and 8.75±0.01, respectively), and the extracts which showed the lowest percentage of extractive value were Sodium Chloride (NaCl), *A. sativum*, *C. camphora* (0.98±0.04, 0.9±0.02 and 0.04±0.01, respectively). The aqueous extractive value of LAMP was 19.2±1.19%. The highest percentages of component aqueous extracts were *A. sativum*, Sodium Chloride (NaCl) and *P. indica* (81.46±1.32, 80.03±1.01 and 39±0.04, respectively). The aqueous extracts which exhibited lowest percentages extractive value were *P. nigrum*, *C. camphora* and *M. fragrans* (Nutmeg) (1.55±0.01, 0.03±0.01and 4.51±0.02, respectively).

Anti-inflammatory activity was assessed by three related path ways including inhibitory effect on nitric oxide (NO) production, tumor necrosis factor-alpha (TNF-α) and prostaglandin E_2 (PGE_2) release against lipopolysaccharide (LPS) induced mouse leukemia monocyte/macrophage cell lines (RAW 264.7). The 95% ethanolic extract (LAMPE) possessed a potent inhibitory effect on NO production

with IC_{50} value 24.90±0.86 μg/ml. However, LAMPE exhibited less anti-inflammatory activity than Prednisolone (IC_{50} value 1.31±0.05 μg/ml). The aqueous extract of Lom-Am-Ma-Preuk remedy (LAMPW) exhibited weak anti-inflammatory activity on this path way (IC_{50}> 100μg/ml). Investigation of the inhibitory effect on NO production among plant ingredients showed that the most extracts possessed anti-inflammatory activity. The ethanolic extract which was the most effective on NO inhibitory effect was *A. galanga* (IC_{50} value6.99±0.4 μg/ml). It was significantly different from positive control or prednisolone which has IC_{50} value1.31±0.05μg/ml (*p-value*< 0.05). Second was *C. zedoaria* which exhibited strong anti-inflammatory properties (IC_{50} value 14.38±1.4 μg/ml) followed by *P.nigrum* and *P. indica* **(**IC_{50} values 16.42±1.19 and 24.54±1.97μg/ml, respectively). *P. indica* had toxicity at concentration 30 μg/ml.

In addition, anti-inflammatory activity by the inhibitory effect on TNF-α release of LAMP and its plant ingredients was also determined.For the LAMP remedy extracts, the 95% ethanolic extract (LAMPE) possessed potent activity against TNF- α release but was less effective than prednisolone, a positive control with IC_{50} value as 35.01±2.61 and 0.95±0.19 μg/ml, respectively. On the other hand, aqueous extract of LAMP remedy exhibited weak inhibitory activity on this path way. In addition, *Z. cassumunar* exhibitedmoderate activity with IC_{50} value 16.90±3.54 μg/ml and *M. Fragrans* (nutmeg) had weak activity with IC_{50} value 49.36±0.42 μg/ml.

Investigation of the inhibitory effect of PEG_2 release, of LAMP revealed that, 95% ethanolic extract was the best crude extract which gave the lowest IC_{50} value compared with aqueous extract which possessed higher IC_{50} value (IC_{50} value 4.77±0.03 and more than 100 μg/ml, respectively). For ethanolic extract of ingredient plants, it was interesting that more than half of all extracts possessed potent inhibitory effect on LPS-stimulated PGE_2 release. The ethanolic extract of *A. galangal*, *M. fragrans* (Mace), *P. nigrum* and *Z. cassumunar* exhibited the strongest anti-inflammatory activity (IC_{50} value 1.23±0.01, 1.57±0.37, 2.95±0.49 and 3.08±0.34μg/ml, respectively). They were not significantly different from positive control or prednisolone which has IC_{50} value 0.96±0.01 μg/ml (*p-value*>0.05). Second were *P. indica*, *M. fragrans* (Nutmeg), *E. variegata* which exhibited strong

potency in anti-inflammatory activity (IC_{50} value 4.09±0.32, 4.6±0.19 and 9.27±0.72 μg/ml, respectively).

It can be summarized that 95% ethanolic extract of LAMP exhibited potent anti-inflammatory activity on the pro-inflammatory mediator, nitric oxide which acts as an important signaling molecule involved in physiological processes such as neuronal communication, host defense, and regulation of vascular tone (Wang *et al.* 2007). Furthermore, TNF-α which represents chronic inflammation and which appears to exacerbate cerebral injury (Wang *et al.* 2007) is also inhibited by 95% ethanolic extract of LAMP remedy. In addition, prostaglandins which respond in acute phase of inflammation by producing fever and COX-2-derived prostaglandin E2 may contribute to ischemic cell damage by disrupting Ca 2^{+} homeostasis in neurons via activation of prostaglandin E_2 receptors (Kawano *et al.*, 2006) may also be inhibited by 95% ethanolic extract of LAMP remedy. These results are basic scientific knowledge which supports the potency of Lom-Am-Ma-Pruek remedy for treatment of stroke and as an analgesic in the musculoskeletal system according to Thai traditional medicine.

Antioxidant activity of LAMP and its plant ingredients in scavenging PMA-stimulatedsuperoxide production in HL-60 cells was measured by the NBT reduction assay. The result showed Lom-Am-Ma-Pruek remedy ethanolic extract (LAMPE) had higher antioxidant activity than the aqueous extract (LAMPW) with EC_{50} value 40.13±1.1 and more than100 μg/ml, respectively.It was significantly different from positive control or propyl gallate which has EC_{50} value 7.15±1.06 μg/ml (*p-value*< 0.05). Nine ethanolic extracts also showed antioxidant activity. *A. galanga* exhibited the strongest antioxidant activity with EC_{50} value 3.94±0.35μg/ml. It was not significantly different when compared with positive control propyl gallate (*p-value*> 0.05). Second were Mace of *M. fragrans*, *C. zedoaria* and Nutmeg of *M. fragrans* which showed high activity with EC_{50} values 18.75±2.47, 19.16±1.72 and 19.5±2.51 μg/ml, respectively. *P. indica*, *Z. cassumunar* and *P. nigrum* exhibited moderate antioxidant activity with EC_{50} values 23.9±1.1, 27.43±3.8 and 35.81±0.72 μg/ml, respectively. *E. variegata*and *C. viscosa* exhibited low antioxidant activity with EC_{50} values 85.47±3.3 and 82.47±1.07μg/ml, respectively. Two aqueous extracts

showed antioxidant activity. *C. viscosa* and *A. galangal* exhibited low antioxidant activity with EC_{50} values 83.38±1.76 and 83.51±3.14 μg/ml, respectively.

The result was to develop a reversed phase high performance liquid chromatography (RPHPLC) method to control quality of LAMP remedy in two aspects namely chemical fingerprint and quantification. In this method, piperine, plumbagin, myristicin and eugenol as the compounds having the most potent inhibition of anti-inflammatoryeffects were used to be markers because this method has good sensitivity, precision, and accuracy.The result showed that myristicin in the ethanolic extract of LAMP was present in the highest content (297.84±5.42 mg/g of extract). Piperine, eugenol and plumbagin contents followed with 189.66±2.56, 58.75±0.13 and 45.01±2.35 mg/g of extract, respectively. A RP-HPLC method for studying chemical fingerprints of ethanolic extract of LAMP and quantifying piperine, plumbagin and myristicin has been presented by Rattarom (2013). From this result, new knowledge and scientific dataof chemical fingerprint and quantification of LAMP remedy and RP-HPLC method may be considered for quality control of LAMP extract. Thus, a method of HPLC should be validated in future studies.

From these results, the detection of chemical fingerprints of LAM using GC-MS is not recommended in chemical analysis of this extract, because this method cannot detect the peaks of compounds which can not be vaporized. Analysis of chemical fingerprint with another chromatography method or one with changed condition of the same system is recommended.

The stability of 95% ethanolic extract of LAMP was investigated by storing the extract under accelerated conditions at 40±2^{o}C with 75±5% RH for 6 months period and evaluated for inhibition of NO production from RAW264.7 cell lines. The result revealed that LAMP ethanolic extract was highly stable because the antiinflammation activities of all sample days were not significantly different from Day 0. It showed that the extract can be stored for two years at room temperature without loss of antiinflammation activity.

In conclusion, the 95% ethanolic extract of Lom-Am-Ma-Pruek remedy possessed potent anti-inflammatory activity on three path ways. All of these findings can support the traditional use of LAMP remedy for the treatment of stroke and an analgesic in the musculoskeletal system. Thus, its ethanolic extract should be

furtherstudied in the animal model for anti-inflammation and safety.Product development which uses myristicine as an anti-inflammatory marker is recommended.

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APPENDICES

APPENDIX A
CHEMICAL REAGENTS

1. Reagents for determination Nitric Oxide

1.1 Griess reagent

Sulfanilamide	1.0 g
N-(1-Naphthyl)ethylenediamine dihydrochloride	0.1 g
Phosphoric acid	2.5 g
Adjust volume with MQ water to	100 ml

(Stored at 4 ^{o}C)

1.2 MTT solution (5 mg/ml)

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide or Thiazolyl blue tetrazolium bromide	200 mg
PBS	40 ml

(Wrapped in foil and stored at 4 ^{o}C)

1.3 0.04 M HCl in Isopropanol

HCl	0.83 ml
Adjust volume with Isopropanol to	250 ml

2. Reagent for determination TNF- α

2.1 Wash buffer solution

Wash buffer	25 ml
Distilled water	600 ml

(stored at 4 ^{o}C)

2.2 Substrate solution

Color reagent A and B should be mixed together in equal volumes

(Freshly prepared, Wrapped in foil)

3. Reagent for determination COX-2

3.1 EIA buffer solution

Diluted the content of EIA buffer concentrate (10x) with 90 ml of ultra pure water

(stored at 4 ^{o}C)

3.2 Wash buffer solution

Wash buffer concentrate (400x)	5 ml
Ultra pure water	2000 ml
Tween 20	1 ml

(stored at 4 ^{o}C)

3.3 Prostaglandin E_2 AChE Tracer solution

Reconstituted PGE_2 AChE tracer 100 dtn with 6 ml of EIA buffer

(stored at 4 ^{o}C)

3.4 Prostaglandin E_2 monoclonal antibody solution

Reconstituted PGE_2 monoclonal antibody 100 dtn with 6 ml of EIA buffer (stored at 4 ^oC)

3.5 Ellman's Reagent

Reconstituted PGE_2Ellman's Reagent100 dtn with 20 ml of ultra pure water (stored at 4 ^oC)

4. Reagent for cell cuture

4.1 RPMI 1640 (incomplete media)

RPMI 1640 with L-glutamine	10.43 g
$NaHCO_3$	2 g
Ultra pure water	1000 ml

Adjust pH to 7.00-7.20 with 10% NaOH or 1% HCl

Filter through sterile membrane at a pore size of 0.2 micron

(Stored at 4 ^oC)

4.2 RPMI 1640 (complete media)

RPMI 1640 (incomplete media)	1000 ml
FBS	100 ml
Penicilin-Sreptomycin	10 ml

(stored at 4 ^oC)

4.3 PBS (Phosphate buffer saline)

PBS	1 Tablet
Distilled water	100 ml

(stored at 4 ^oC)

4.4 FBS (Fetal bovine serum)

Slowly thaw the FBS (inactivate), heat 56 ^{o}C, 60 mins

(Aliquot, Stored at -20 ^{o}C)

4.5 P/S (Penicilin-Sreptomycin)

Slowly thaw the frozen P/S in water bath at 37 ^{o}C till completely thawed

(Aliquot, Stored at -20 ^{o}C)

4.6 Trysin-EDTA

Slowly thaw the frozen 0.5% trypsin-EDTA, in water bath at 37 ^{o}C till completely thawed

(Aliquot, Stored at -20 ^{o}C)

5. Reagent for Nitroblue tetrazolium (NBT) dye reduction assay

5.1 Nitroblue tetrazolium (NBT) solution

NBT	1.25 mg
Hank's buffer to	1 ml

(Freshly prepared, Wrapped foil)

5.2 Phorbol 12 myristate 13-actate (PMA)

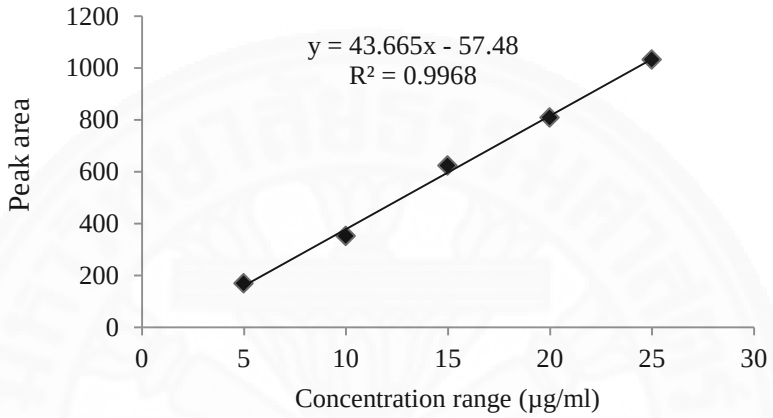
Dilution stock PMA conc. 2 mg/ml to 250 ng/ml with Hank'sBalanced Salt solution

(Freshly prepared, Wrapped foil)

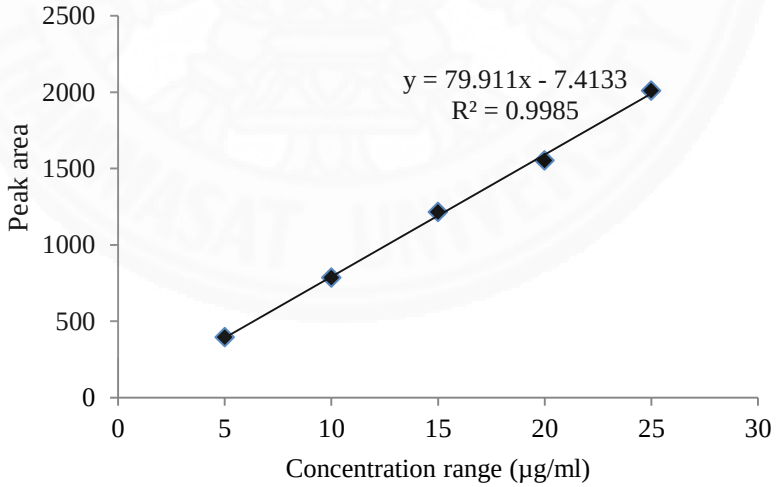
APPENDIX B

STANDARD CURVES OF PURE COMPOUNDS ANALYZED BY HPLC

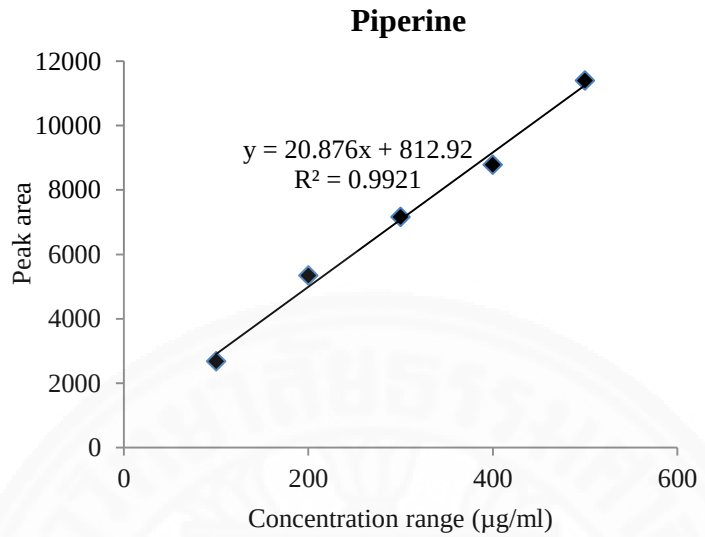
Plumbagin

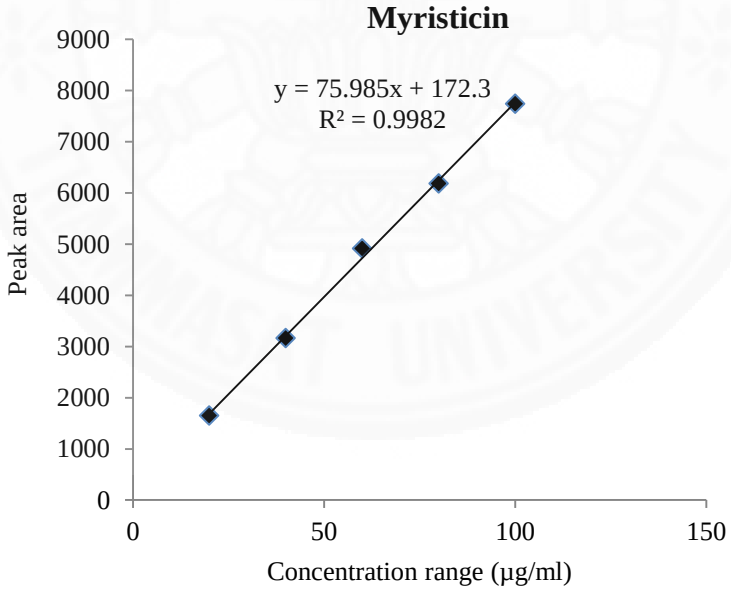


Eugenol



APPENDIX B (CONTINUE)





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	High School Certificate in Science-Mathematics
2011	Rajabhat Suan Sunanta University
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	Faculty of Science

List of Publication and Proceeding

Thongmee, P., Itharat, A. (2016). Anti-inflammatory activities of *Erythrina variegata* bark ethanolic extract. *Journal of the Medicinal Association of Thailand*, 99, (Suppl.).

Conference and Presentation

Thongmee, P., Itharat, A. (2014). Anti-inflammatory Activity of a Thai Traditional Medicine Called Lom-Am-Ma-Pruek. *18th World Congress on Clinical Nutrition (WCCN)*. Ubon Ratchathani, Thailand. (Poster presentation)

Thongmee, P., Itharat, A. (2015). Anti-inflammatory and Antioxidant Activities of Thai Traditional Medicine Called Lom-Am-Ma-Pruek. *25th Misconception in everyday practice*. Thammasat University, Thailand. (Oral presentation)