ฤทธิ์ระงับปวดและฤทธิ์ต้านการอักเสบของกรดโรสมารินิกและสารสกัดใบรางจืด

นางสาววรรณวิษา บุญญะริกพันธุ์ชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF ROSMARINIC ACID AND *THUNBERGIA LAURIFOLIA* LEAF EXTRACT

Miss Wanvisa Boonyarikpunchai

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacology

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2013

Thesis Title	ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF ROSMARINIC ACID AND *THUNBERGIA LAURIFOLIA* LEAF EXTRACT
By	Miss Wanvisa Boonyarikpunchai
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Flying Officer Pasarapa Towiwat, Ph.D.
Thesis Co-advisor	Associate Professor Police Captain Suchada Sukrong, Ph.D.

Accepted by the Faculty of Pharmaceutical Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Pharmaceutical Science
(Assistant Professor Rungpetch Sakulbumrungsil, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Police Lieutenant Colonel Somsong Lawanprasert, Ph.D.)

.....Thesis Advisor
(Assistant Professor Flying Officer Pasarapa Towiwat, Ph.D.)

.....Thesis Co-advisor
(Associate Professor Police Captain Suchada Sukrong, Ph.D.)

.....Examiner
(Assistant Professor Rataya Luechapudiporn, Ph.D.)

.....External Examiner
(Associate Professor Siriporn Fungwithaya, M.S.)

วรรณวิษา บุญญะริกพันธุ์ชัย: ฤทธิ์ระงับปวดและฤทธิ์ต้านการอักเสบของกรดโรสมารินิกและสารสกัดใบรางจืด. (ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF ROSMARINIC ACID AND *THUNBERGIA LAURIFOLIA* LEAF EXTRACT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ร.ท.หญิง ดร.ภัสราภา โตวิวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, 154 หน้า.

รางจืด (*Thunbergia laurifolia* Lindl.) จัดอยู่ในวงศ์ Acanthaceae มีสรรพคุณใช้เป็นยาถอนพิษ และใช้รักษาอาการปวด บวม อักเสบ และปวดศีรษะ กรดโรสมารินิกซึ่งเป็นสารสำคัญของรางจืดสกัดได้จากสารสกัดเอทานอลของใบรางจืด การทดสอบนี้ทำการประเมินฤทธิ์ระงับปวดของกรดโรสมารินิกและสารสกัดใบรางจืดในหนูเมาส์ด้วยวิธี hot-plate, acetic acid-induced writhing และ formalin และทำการทดสอบฤทธิ์ต้านการอักเสบของกรดโรสมารินิกในหนูเมาส์ด้วยวิธี carrageenan-induced paw edema และcotton pellet-induced granuloma formation และทำการทดสอบฤทธิ์ต้านการอักเสบของสารสกัดใบรางจืดด้วยวิธี carrageenan-induced paw edema พบว่า กรดโรสมารินิกขนาด 50, 100 และ 150 มก./กก. และสารสกัดใบรางจืดทุกขนาด (500-2500 มก./กก.) ที่ให้โดยการป้อนมีฤทธิ์ระงับปวดอย่างมีนัยสำคัญทางสถิติเมื่อทดสอบด้วยวิธี hot-plate และฤทธิ์ระงับปวดนั้นถูกยับยั้งได้ด้วยนาลอกโซน แสดงว่ากลไกการออกฤทธิ์ระงับปวดเกี่ยวข้องกับตัวรับโอปิออยด์ กรดโรสมารินิกขนาด 50 และ 100 มก./กก.สามารถลดการบิดงอลำตัวของหนูที่เกิดจากการเหนี่ยวนำด้วยกรดอะซิติกได้ 52.05% และ 84.53% ตามลำดับอย่างมีนัยสำคัญทางสถิติ สารสกัดใบรางจืดขนาด 1000, 1500, 2000 และ 2500 มก./กก. สามารถลดจำนวนครั้งของการบิดงอลำตัวได้ 32.11%, 45.04%, 47.81%, และ 67.89% ตามลำดับอย่างมีนัยสำคัญทางสถิติ แสดงว่าทั้งกรดโรสมารินิกและสารสกัดใบรางจืดมีฤทธิ์ระงับปวดต่อความปวดที่เกิดจากการอักเสบ กรดโรสมารินิกขนาด 100 มก./กก.และสารสกัดใบรางจืดขนาด 1500 และ 2000 มก./กก. ยังสามารถยับยั้งความปวดที่เกิดจากการเหนี่ยวนำด้วยฟอร์มาลินในระยะแรกและระยะหลังได้อย่างมีนัยสำคัญทางสถิติ ผลการทดสอบด้วยวิธี formalin สอดคล้องกับผลที่ได้จากการทดสอบด้วยวิธี hot-plate และ acetic acid-induced writhing แสดงให้เห็นว่ากรดโรสมารินิกและสารสกัดใบรางจืดออกฤทธิ์ระงับปวดได้ทั้งในระบบประสาทส่วนกลางและระบบประสาทส่วนปลาย กรดโรสมารินิกและสารสกัดใบรางจืดในขนาดที่มีประสิทธิภาพสูงสุดไม่มีผลคลายกล้ามเนื้อหรือสงบระงับในการทดสอบด้วย rota-rod กรดโรสมารินิกขนาด 100 มก./กก. สามารถยับยั้งการบวมของอุ้งเท้าที่ถูกเหนี่ยวนำด้วยคาราจีแนนที่เวลา 3, 4, 5 และ 6 ชั่วโมง ได้อย่างมีนัยสำคัญทางสถิติ ในขณะที่สารสกัดใบรางจืดทุกขนาดสามารถลดการบวมของอุ้งเท้าที่เวลา 4, 5 และ 6 ชั่วโมงหลังจากฉีดคาราจีแนน ได้อย่างมีนัยสำคัญทางสถิติ แสดงให้เห็นว่ากรดโรสมารินิกและสารสกัดใบรางจืดออกฤทธิ์ต้านการอักเสบในช่วงระยะที่ 2 ของการอักเสบ ทั้งกรดโรสมารินิกและสารสกัดใบรางจืดมีฤทธิ์ต้านการบวมของอุ้งเท้าที่ถูกเหนี่ยวนำด้วยพรอสตาแกลนดินอี 2 ได้อย่างมีนัยสำคัญทางสถิติ ซึ่งการบวมของอุ้งเท้าที่ถูกเหนี่ยวนำด้วยพรอสตาแกลนดินอี 2 ที่ลดลงสามารถอธิบายผลการยับยั้งของกรดโรสมารินิกและสารสกัดใบรางจืดในการทดสอบด้วยวิธี acetic acid-induced writhing และ formalin ได้บางส่วน กรดโรสมารินิกขนาด 100 มก./กก. สามารถยับยั้งการเกิด granuloma จากการเหนี่ยวนำด้วยก้อนสำลีได้อย่างมีประสิทธิภาพแสดงให้เห็นว่า กรดโรสมารินิกมีฤทธิ์ต้านการอักเสบในระยะเรื้อรังของการอักเสบ ผลการประเมินความเป็นพิษเฉียบพลันยืนยันถึง ความปลอดภัยของกรดโรสมารินิกและสารสกัดใบรางจืดทุกขนาดที่ใช้ในการทดสอบ จากผลการทดสอบทั้งหมดแสดงให้เห็นว่า กรดโรสมารินิกและสารสกัดใบรางจืด มีฤทธิ์ระงับปวดทั้งในระบบประสาทส่วนกลางและระบบประสาทส่วนปลาย กลไกการออกฤทธิ์ระงับปวดน่าจะมีความเกี่ยวข้องกับวิถีของโอปิออยด์ กรดโรสมารินิกและสารสกัดใบรางจืดมีฤทธิ์ต้านการอักเสบแบบเฉียบพลันและแบบเรื้อรัง กลไกการออกฤทธิ์ต้านการอักเสบของกรดโรสมารินิกและสารสกัดใบรางจืด บางส่วนเกิดจากการยับยั้งโดยตรงที่ตัวรับของพีจีอี 2 ซึ่งฤทธิ์ระงับปวดและฤทธิ์ต้านการอักเสบของสารสกัดใบรางจืดบางส่วนเกิดจากกรดโรสมารินิกซึ่งเป็นสารสำคัญในสารสกัดใบรางจืด ข้อมูลที่ได้จากการศึกษานี้อาจนำไปสู่การใช้กรดโรสมารินิกและรางจืดในการรักษาอาการปวดและความผิดปกติของการอักเสบอย่างสมเหตุผล

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5476218933 : MAJOR PHARMACOLOGY

KEYWORDS : ROSMARINIC ACID / *THUNBERGIA LAURIFOLIA* / ANTINOCICEPTION / ANTI-INFLAMMATION / HOT-PLATE TEST / ACETIC ACID-INDUCED WRITHING TEST / FORMALIN TEST / CARRAGEENAN-INDUCED PAW EDEMA / PROSTAGLANDIN E_2-INDUCED PAW EDEMA / COTTON PELLET-INDUCED GRANULOMA

WANVISA BOONYARIKPUNCHAI: ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF ROSMARINIC ACID AND *THUNBERGIA LAURIFOLIA* LEAF EXTRACT. ADVISOR: ASST. PROF. FLG. OFF. PASARAPA TOWIWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. POL. CAPT. SUCHADA SUKRONG, Ph.D., 154 pp.

Rang Chuet (*Thunbergia laurifolia* Lindl., family Acanthaceae) is traditionally used as an antidote for poisons and for treatment of pain, edema, inflammation, and headache. Rosmarinic acid (RA), a main constituent of Rang Chuet, was isolated from an ethanolic extract of *T. laurifolia* leaves. The antinociceptive activities of RA and *T. laurifolia* leaf extract (TLE) were assessed in mice using hot-plate, acetic acid-induced writhing, and formalin tests. The anti-inflammatory effect of RA was determined in mouse models of carrageenan-induced paw edema and cotton pellet-induced granuloma formation. The anti-inflammatory effect of TLE was determined in carrageenan-induced paw edema. Orally administered RA (50, 100, and 150 mg/kg) and all doses of TLE (500-2500 mg/kg) showed significant antinociceptive activity in the hot-plate test and these effects were reversed by naloxone suggesting opioid-mediated mechanism. RA at doses of 50 and 100 mg/kg significantly reduced acetic acid-induced writhing by 52.05% and 84.53%, respectively. TLE at doses of 1000, 1500, 2000, and 2500 mg/kg significantly decreased the number of writhes by 32.11%, 45.04%, 47.81%, and 67.89%, respectively. These data indicate that both RA and TLE have antinociceptive effects on inflammatory pain. RA at 100 mg/kg and TLE at doses of 1500 and 2000 mg/kg also caused significant inhibition of formalin-induced pain in the early and late phases. The results from the formalin test were in agreement with those obtained from the hot-plate and writhing tests, thereby indicating that RA and TLE act both centrally and peripherally to achieve pain relief. Most effective doses of RA and TLE showed no detectable relaxant or sedative effects in the rota-rod test. RA at 100 mg/kg significantly suppressed carrageenan-induced paw edema at 3, 4, 5, and 6 h, while all doses of TLE significantly decreased paw edema at 4, 5, and 6 h after carrageenan injection. These results indicate that RA and TLE exerted anti-inflammatory action during the second phase of inflammation. Both RA and TLE showed significant activity against PGE_2-induced paw edema. The reduction of PGE_2- induced paw edema could explain, at least in part, the inhibitory effects of RA and TLE in the acetic acid-induced writhing and formalin tests. RA at 100 mg/kg effectively inhibited cotton pellet-induced granuloma formation which indicated that RA has anti-inflammatory activity in the chronic phase of inflammation. An acute toxicity assessment confirmed the safety of all doses of RA and TLE used in the study. Taken together, these results show that RA and TLE possess both central and peripheral antinociceptive activity. The analgesic mechanisms of action of RA and TLE are most likely involved with the opioid pathway. RA and TLE have anti-inflammatory effects against acute and chronic inflammation. The anti-inflammatory mechanisms of action of RA and TLE are partly due to direct blockage of prostaglandin receptors. The antinociceptive and anti-inflammatory effects of TLE are partly resulted from its main constituent, rosmarinic acid. These data provide a basis for the rational use of RA and *T. laurifolia* for treatment of pain and inflammatory disorders.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

Academic Year : 2013 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

The author wish to express her sincere gratitude and appreciation to her advisor, Assistant Professor Flying Officer Dr. Pasarapa Towiwat, for her intensive advice, guidance, suggestions and encouragement throughout her study. The author wish to thank Associate Professor Police Captain Dr. Suchada Sukrong, her thesis co-advisor for her valuable advice and providing rosmarinic acid and *T. laurifolia* leaves extract for this study. Thanks are also extended to the committee members.

The author wish to thank Mr. Pipob Suwanchaikasem for his help with the extractions, isolation and identification of rosmarinic acid and herbal extracts for this study.

The author would like to thank all staff members in the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their help and support.

The author also would like to thank her lab partners especially Ms. Anusara Jongchanapong and Ms. Chayanin Kiratipaiboon, and her friends for their help, advice and encouragement throughout her thesis study.

Finally, the author would like to thank her parents and her family for everything in her life.

CONTENTS

Page

LIST OF TABLES

Table Page

Table Page

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
κ	=	kappa
δ	=	delta
μL	=	microliter
μM	=	micro molar
/	=	per
%	=	percent
% MPE	=	percentage of the maximum possible effect
°C	=	celsius degree
5-HT	=	5- hydroxytryptamine
AA	=	ascorbic acid
AUC	=	area under the curves (area of analgesia)
CAA	=	caffeic acid
cAMP	=	cyclic adenosine monophosphate
cm	=	centimeter
CNCP	=	chronic noncancer pain
CNS	=	central nervous system
Co.	=	company
COA	=	coumaric acid
COMT	=	catechol-*o*-methyltransferase
COX	=	cyclooxygenase
CR	=	corticosteroid receptor
CVF	=	cobra venom factor
DEP	=	diesel exhaust particle
DH	=	dorsal horn
DPPH	=	diphenyl-picryl-hydrazyl

DRG	=	dorsal root ganglia
DS	=	dorsal columns
ED_{50}	=	median effective dose
e.g.	=	example gratia
et al.	=	et alii (and other)
FA	=	ferulic acid
g	=	gram
g/L	=	gram per liter
GABA	=	gamma-aminobutyric acid
GI	=	gastrointestinal
GlcA	=	glucuronide moiety
GRE	=	glucocorticoid response element
h	=	hour
HO-1	=	heme oxygenase-1
HUVEC	=	human umbilical vein endothelial cells
IASP	=	International Association for the Study of Pain
IFN	=	interferons
IL	=	interleukins
IND	=	Indomethacin
iNOS	=	inducible nitric oxide
i.p.	=	intraperitoneal
JEV	=	Japanese encephalitis virus
LD_{50}	=	median lethal dose
LOX	=	lipoxygenase
LPS	=	lipopolysaccharide
LT_s	=	leukotrienes
m	=	meter
Meth	=	methyl moiety
mg	=	milligram

mg/kg	=	milligram per kilogram
mg/ml	=	milligram per milliliter
MI	=	myocardial infraction
min	=	minute
ml	=	milliliter
ml/kg	=	milliliter per kilogram
MO	=	morphine sulphate
mm	=	millimeter
m/sec	=	meter per second
N	=	sample size
NAL	=	naloxone
NO	=	nitric oxide
NSAIDs	=	non-steroidal anti-inflammatory drugs
NSS	=	normal saline solution
PAF	=	Platelet-activating factor
PAG	=	periaqueductal gray
PE	=	perilla extract
PGE_2	=	prostaglandin E_2
PGI_2	=	Prostacyclin
PI3K	=	phosphatidylinositol-3-kinase
PKA	=	protein kinase A
p.o.	=	per os
PST	=	phenolsulfotransferase
RA	=	rosmarinic acid
ROS	=	reactive oxygen species
sec	=	second
SRS-A	=	slow reacting substance of anaphylaxis
STT	=	spinothalamic tract
SW	=	sterile water

Sulf	=	sulfate moiety
$T_{1/2}$	=	half-life
TL	=	*Thunbergia laurifolia*
TLE	=	*Thunbergia laurifolia* leaf extract
TNF	=	tumor necrosis factor
TPA	=	12-*O*-tetradecanoylphorbol 13-acetate
TXA_2	=	thromboxane A_2
UGT	=	UDP-glucuronosyltransferase
VEGF	=	vascular endothelial growth factor
vs	=	versus
W_r	=	mean number of writhing response
w/w	=	weight by weight

CHAPTER I
INTRODUCTION

Background and Rational

Pain is a subjective experience, hard to define exactly. Pain can affect all areas of a person's life including sleep, thought, emotion and activities of daily living. Additionally, pain is the most common symptom prompting patients to seek medical attention and is reported by more than 80% of individuals who visit their primary care provider. Despite the frequency of pain symptoms, individuals often do not obtain satisfactory pain relief. This has led to recent initiatives in health care to make pain the fifth vital sign (O'Neil, 2010).

Inflammation is an important protective response, designed to get rid of the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the consequences of such injury (e.g. necrotic cells and tissues). Without inflammation infection would go unchecked, and injured tissues might remain permanent festering sores. Sometimes mechanisms designed to destroy foreign invaders and necrotic tissues have an intrinsic ability to injure normal tissues. When it is inappropriately directed against self-tissues or is not adequately controlled, it becomes the cause of injury and disease such as rheumatoid arthritis, atherosclerosis and fibrosis, as well as life-threatening hypersensitivity reactions to insect bites, drugs and toxins. Therefore, great attention is given to the damaging consequences of inflammation (Kumar, Abbas and Fausto, 2005).

Pain and inflammation are major problems in the general population that affect lifestyle and health. Many analgesic and anti-inflammatory drugs are available for treating these symptoms, but these drugs can cause adverse effects when used for long term treatment. Therefore, there is considerable interest in discovery and development of new analgesic and anti-inflammatory drugs from natural sources with high efficacy and low side effects.

Nonopioid analgesics, including acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), are generally used for mild to moderate pain. Nonopioid

analgesics relieve various types of acute and chronic pain (e.g., trauma, postoperative, cancer and arthritis pain) and are especially effective for certain types of somatic pain (e.g., muscle and joint pain, inflammatory pain, and postoperative pain; Berry et al., 2006). Adverse effects of NSAIDs include gastrointestineal problem, bleeding, kidney dysfunction and hypersensitivity reactions.

Opioid analgesics, such as morphine, fentanyl and meperidine, are recommended for moderate to severe pain that does not respond to nonopioid analgesics alone. Opioid analgesics play a major role in the treatment of acute pain (e.g., trauma, postperativepain), breakthrough pain, cancer pain, and some types of noncancer pain (Berry et al., 2006). These drugs are associated with several adverse effects including sedation, confusion, respiratory depression, nausea, vomiting, constipation, pruritus, and urinary retention.

Thunbergia laurifolia Lindl. belongs to the Acanthaceae family and is known in Thai as “Rang Chuet” (Chan and Lim, 2006). This plant is commonly used for relief of symptoms including pain, inflammation, edema, headache, and excessive thirst, and as an antidote for poisons in Thai traditional medicine (นิจศิริ และธวัชชัย, 2547). An aqueous extract preparation of *T. laurifolia* leaves has anticholinergic effects and decreases mortality in rats treated with folidol, an organophosphate insecticide (Tejasen and Thongthapp, 1980). This extract also has hepatoprotective activity against ethanol-induced liver injury *in vitro* and *in vivo* (Pramyothin et al., 2005). Topical application of alcohol and hexane extracts of *T. laurifolia* leaves also produces anti-inflammatory activity, with significant inhibition of carrageenin-induced paw edema in mice (Chanrumanee et al., 1998). Subcutaneous administration of an ethanol extract of *T. laurifolia* leaves has also been shown to have antinociceptive and anti-inflammatory effects in several animal models (Phosri et al., 2008). However, the active compounds responsible for these effects of *T. laurifolia* leaf extracts have not been determined.

An ethanolic extract of *T. laurifolia* leaves was shown to have antioxidant activity in a DPPH radical scavenging assay (Suwanchaikasem et al., 2011). TLC bioautography used for separation of the bioactive constituents indicated that rosmarinic acid (RA) was

responsible for the antioxidant activity (Suwanchaikasem et al., 2011). The antioxidative effect of RA is also apparent in its reduction of liver injury induced by D-galactosamine (Won et al., 2003). Rosmarinic acid also has an additive effect in treating inflammatory diseases such as rheumatoid arthritis due to its free radical-scavenging capacity (Youn et al., 2003). The anti-inflammatory activity of RA is mainly attributable to inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) activities and complement activation (Sahu et al., 1999). In animal models, RA significantly inhibited paw edema induced by *Bothrops jararacussu* snake venom (Ticli et al., 2005) and RA given intraperitoneally reduced the number of total exudate cells in a carrageenan-induced pleurisy model, but was ineffective on tail-flick latencies in a tail-flick assay in rats (Gamaro et al., 2001).

Therefore, the aim of the present study was to investigate the effects of orally administered RA, a major compound isolated from *T. laurifolia* leaves, and *T. laurifolia* leaf extract on nociception and inflammation in mice. We also examined the mechanisms of actions of RA and *T. laurifolia* leaf extract underlying these effects. The findings may lead to the development of treatment options from natural compounds for pain and inflammation.

Purpose of study

To investigate the antinociceptive and anti-inflammatory effects of RA and *T. laurifolia* leaf extract. In addition, the possible mechanisms of actions involved were also examined.

Hypothesis

RA and *T. laurifolia* leaf extract have antinociceptive and anti-inflammatory effects in various animal models.

Research design

Experimental Research

Expected benefit and application

The findings obtained from the present study may eventually lead to the development of the novel therapy with minor adverse effects from natural compounds in treating pain and inflammation. Furthermore, this study may provide scientific evidence to support the use of RA and *T. laurifolia* leaf extract as the analgesic and anti-inflammatory agents.

Key words

Rosmarinic acid

Thunbergia laurifolia

Antinociception

Anti-inflammation

Hot-plate test

Acetic acid-induced writhing test

Formalin test

Carrageenan-induced paw edema

Prostaglandin E_2-induced paw edema

Cotton pellet-induced granuloma

CHAPTER II
LITERATURE REVIEWS

Pain

The current definition of pain as proposed by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bugduk, 1994).

Pain can be classified base on pain duration (e.g., acute vs. chronic pain) and underlying pathophysiology (e.g., nociceptive vs. neuropathic pain).

Duration of Pain

Acute pain

Acute pain is usually elicited by injury to body tissues and activation of nociceptive stimuli at the site of local tissue damage. It is generally of short duration and remits when the underlying pathologic process has resolved (Turk and Okifuji, 2001). Its biological function is protective, acting as a warning that an external threat is noxious or signaling organ malfunction. The pain’s location, radiation, intensity, and duration, as well as those factors that aggravate or relieve it, provide essential diagnostic clues. Common causes of acute pain include surgery, acute illness, trauma, labor and medical procedures. The best way of managing acute pain is to diagnose and treat the cause (Greene and Harris, 2008).

Chronic pain

Chronic pain is usually elicited by an injury but may be perpetuated by factors that are both pathologically and physically remote from the originating cause. Chronic pain extends for more than 6 months and generally represents low levels of an underlying pathologic process that does not explain the presence or extent of the pain (Turk and Okifuji, 2001). Chronic pain can be quite variable. Chronic pain is a leading cause of disability in the United States. Unlike acute pain, persistent chronic pain usually

serves no useful function. The biologic factors that contribute to chronic pain include peripheral mechanisms, peripheral-central mechanisms, and central mechanisms (Jacobson and Mariano, 2001). Persons with chronic pain may not exhibit the somatic, autonomic, or affective behaviors often associated with acute pain. As painful conditions become prolonged and continuous, autonomic nervous system responses tend to decrease. However, chronic pain often is associated with loss of appetite, sleep disturbances, and depression (Grichnick and Ferrante, 1991).

Pathophysiology of Pain

Nociceptive pain

Nociceptive pain typically is classified as either somatic pain and visceral pain. Somatic pain arises from superficial structures, such as skin, bone, joint, muscle, or connective tissue and well localize, constant, aching, or gnawing in character. Whereas visceral pain arises from visceral organs such as the large intestine or pancreas and poorly localize but is constant and aching in character and is referred to cutaneous sites (Foley, 1985).

Neuropathic pain

The current definition of neuropathic pain (NP) according to the International Association for the Study of Pain (IASP) is “pain initiated or caused by a primary lesion or dysfunction of the nervous System (Merskey and Bogduk, 1994). Neuropathic pain represents unusual and sometimes intractable sensory disturbances associated with disease or injury of the peripheral or central nervous system (Jensen et al., 2001). These include numbness, paresthesias, and pain. Depending on the cause, few or many axons could be damaged, and the condition could be unilateral or bilateral. Causes of neuropathic pain can be classified according to the extent of peripheral nerve involvement. Conditions that can lead to pain by causing damage to peripheral nerve in a single area include nerve entrapment, nerve compression from a tumor mass, and various neuralgias such as trigeminal, postherpetic, and post-traumatic (Vaillancourt and Langevin, 1999). Neuropathic pain can vary with the extent and location of disease

or injury. There may be allodynia or pain that is stabbing, jabbing, burning, or shooting. The pain may be persistent or intermittent.

Nociceptors

Nociception is defined as "the neural processes of encoding and processing noxious stimuli including intense thermal, mechanical, and chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors. Nociceptors, (also called pain receptors), are sensory receptors that are activated by noxious insults to peripheral tissues. Structurally, the receptive endings of peripheral pain fibers are free nerve endings. These receptive endings, which are widely distributed in the skin, dental pulp, periosteum, meninges, and some internal organs, translate the noxious stimuli into action potentials that are transmitted by a dorsal root ganglion to the dorsal horn of spinal cord. The cell bodies of nociceptors are located in dorsal root ganglia (DRG) for the body and the trigeminal ganglia for the face and have both a peripheral and central axonal branch that innervates their target tissue and the spinal cord, respectively.

Nociceptive action potentials are transmitted through two types of afferent nerve fibers: myelinated Aδ fibers and unmyelinated C fibers (Kandel et al., 2000; Bear et al., 2001; Berne and levy, 2000; Guyton and Hall, 2005). The larger Aδ fibers have considerably greater conduction velocities, transmitting impulses at rate of 6 to 30 m/sec. The C fibers are the smallest of all peripheral nerve fibers; they transmit impulses at the rate of 0.5 to 2.5 m/sec (Guyton and Hall, 2005). Pain conducted by Aδ fibers traditionally is called *fast pain* or first pain (sharp or bright pain) and typically is elicited by mechanical or thermal stimuli. C fiber pain often is described as *slow-wave pain* or second pain because it is slower in onset and longer in duration. It typically is incited by chemical stimuli or by persistent mechanical or thermal stimuli (Figure 1).

Unlike other sensory receptors, nociceptors respond to several forms of stimulation, including mechanical, thermal, and chemical. Some receptors respond to a single type of stimuli (mechanical or thermal) and others, called *polymodal receptors*, respond to all three types of stimuli (mechanical, thermal, and chemical; Kandel et al.,

2000). Therefore, the membranes of nociceptors contain ion channels that are activated by these types of stimuli. Mechanical stimuli can arise from intense pressure applied to skin or from the violent contraction or extreme stretch of muscle. Both extremes of heat and cold can stimulate nociceptors. Chemical stimuli arise from a number of sources, including tissue trauma, ischemia, and inflammation.

A wide range of chemical mediators are released from injured and inflamed tissues, including hydrogen and potassium ions, prostaglandins, leukotrienes, histamine, bradykinin, acetylcholine, and serotonin (Figure 2; Guyton and Hall, 2005). These chemical mediators produce their effects by directly stimulating nociceptors or sensitizing them to the effects of nociceptive stimuli: perpetuating the inflammatory responses that lead to the release of chemical agent that act as nociceptive stimuli or increase the response to nociceptive stimuli. For example, bradykinin, histamine, serotonin, and potassium activate and also sensitize nociceptors (Cross, 1994; Julius and Basbaum; 2001; McHugh and McHugh, 2000).

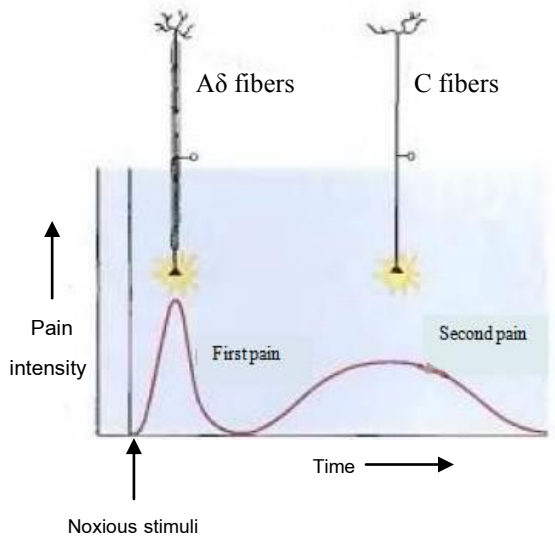


Figure 1 The first pain sensation registered by noxious stimulation is mediated by myelinated Aδ fibers. The second, longer-lasting pain sensation is mediated by unmyelinated C fibers (Bear et al., 2001)

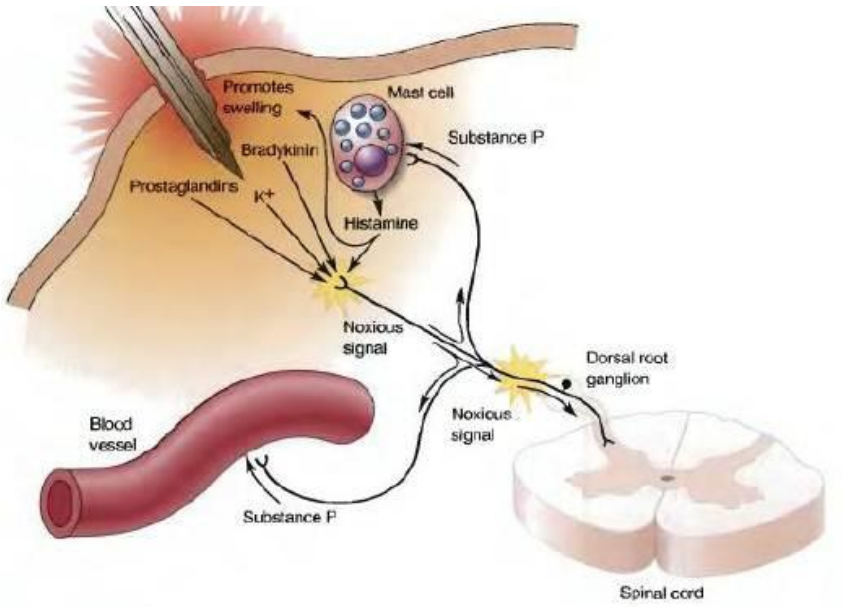


Figure 2 Chemical mediators of injured and inflamed tissues (Bear et al., 2001).

Mechanisms of pain

Nociception refers to the process by which information about tissue damage is conveyed to the central nervous system (CNS). As with other forms of somatosensation, the pathways are composed of first-, second- and third-order neurons. The first order neurons and their receptive endings detect stimuli that threaten the integrity of innervated tissues. Second-order neurons are located in the spinal cord and process nociceptive information. Third-order neurons project pain information from the thalamus to the somatosensory cortex where the perception and subjective meaning of pain take place. The thalamus and somatosensory cortex integrate and modulate pain as well as the person's subjective reaction to the pain experience (Nestler et al., 2001; Porth and Sommer, 2009). The process of pain pathway consists of 4 steps, including transduction, transmission, Modulation and perception (Figure 3).

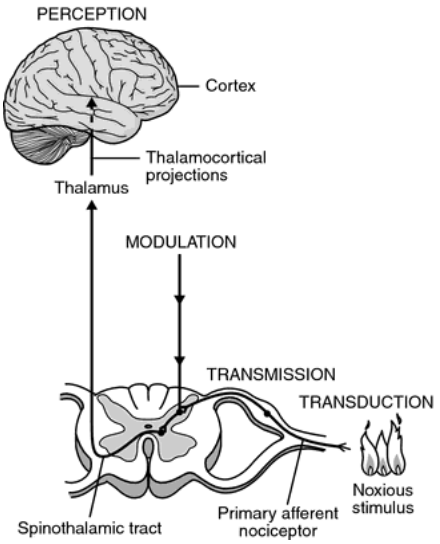


Figure 3 Mechanisms of the nociceptive circuit (Ferrante and VadeBoncouer, 1993).

Transduction

Transduction is the process by which noxious stimuli are translated into a nociceptive impulse at peripheral receptor sites called nociceptors. Nociceptors are sensory receptors that are preferentially sensitive to tissue trauma. (Merskey and Bugduk,1994). These receptors are the free endings of (primary afferent) nerve fibers distributed throughout the periphery (Figure 5). Signals from these nociceptors travel primarily along two fiber types: slowly conducting unmyelinated C-fibers and small, myelinated, and more rapidly conducting Aδ-fibers (Figure 4).

Injury to tissue causes cells to break down and release various tissue byproducts and mediators of inflammation (e.g., prostaglandins, substance P, bradykinin, histamine, serotonin, and cytokines; Byers and Bonica, 2001; Meyer et al., 1994). Some of these substances activate nociceptors (e.g., cause them to generate nerve impulses) and most sensitize nociceptors (e.g., increase their excitability and discharge frequency) (Woolf, 1989; Costigan and Woolf, 2000). Ongoing activation of nociceptors may cause nociceptive pain. Peripheral (nociceptor) sensitization amplifies signal transmission and thereby contributes to central sensitization and clinical pain states (Woolf, 1993).

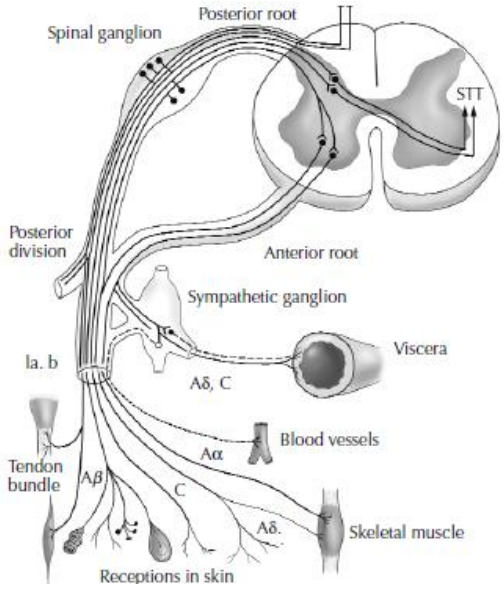


Figure 4 A simplified schema of a spinal nerve and the different types of fibers contained therein. (DC; dorsal columns, STT; spinothalamic tract; Terman and Bonica, 2001)

Transmission

Transmission is the process by which nociceptive impulses are sent to the dorsal horn of spinal cord via nerve pathways (primary sensory afferents, the faster Aδ-fibers and the slower C-afferent nerve fibers) toward the brain. The primary afferent neurons, nociceptive pain fibers synapse in various layers of the dorsal horn in spinal cord, releasing a variety of neurotransmitters, including glutamate, substance P and aspartate. Their axons terminate in the dorsal horn of spinal cord, where they have connections with many spinal neurons. In turn, spinal neurons have input from many primary afferents. These spinal neurons project axons to the contralateral thalamus, which in turn projects to the somatosensory pathway, frontal cortex, and other areas. The somatosensory cortex is thought to be involved in the sensory aspects of pain, such as the intensity and quality of pain, whereas the frontal cortex and limbic system are thought to be involved with the emotional responses to it (Berry et al. 2006; Baumann and Strickland, 2008).

Modulation

Modulation is the process of dampening or amplifying these pain-related neural signals. Modulation of nociceptive transmission occurs at multiple (peripheral, spinal, supraspinal) levels. Yet, historically, modulation has been viewed as the attenuation of dorsal horn transmission by descending inhibitory input from the brain. Melzack and Wall's Gate Control Theory brought this notion to the forefront in 1965 (Melzack and Wall, 1965). Models of descending pain systems now include both inhibitory and facilitory descending pathways.

Multiple brain regions contribute to descending inhibitory pathways (Terman and Bonica, 2001). Nerve fibers from these pathways release inhibitory substances (e.g., endogenous opioids, serotonin, norepinephrine, GABA) at synapses with other neurons in the dorsal horn (DH). These substances bind to receptors on primary afferent and/or DH neurons and inhibit nociceptive transmission. Such endogenous modulation may contribute to the wide variations in pain perception observed among patients with similar injuries (Fields, 1987; Basbaum and Fields, 1984; Hammond, 1986).

Pain perception

The perception of pain is an uncomfortable awareness of some part of the body, characterized by a distinctly unpleasant sensation and negative emotion best described as threat. Both cortical and limbic system structures are involved (Chapman, 2001). Nociceptive information from some dorsal horn projection neurons travels via the thalamus to the contralateral somatosensory cortex (Figure 5; Terman and Bonica, 2001), where input is somatotopically mapped to preserve information about the location, intensity, and quality of the pain (Guilbaud et al., 1994; Covington, 2000). The thalamus relays other nociceptive input to the limbic system. This input joins input from the spinoreticular and spinomesencephalic tracts to mediate affective aspects of pain (Fields, 1987). Immediate social and environmental context influences the perception of pain, as do past experience and culture. Consequently, a standard cause of pain (e.g., surgery) can generate enormous individual differences in pain perception.

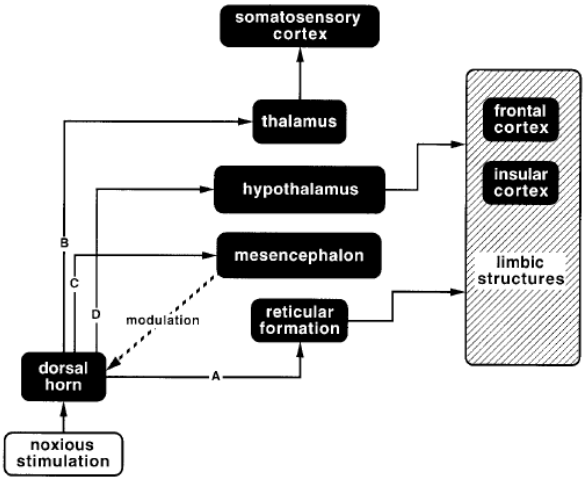


Figure 5 Multiple pathways of nociceptive transmission for the spinal cord to central structures. There are four major pathways the A; spinoreticular, B; spinothalamic, C; spinomesencephalic, and D; spinohypothalamic tracts (Chapman and Nakamura, 1999).

The gate theory of pain

Various theories have tried to integrate the anatomical pain pathway and the psychological and neurological components that contribute to the perception of pain. The generally accepted model is the “gate control theory” in Figure 6. This was first described by Ronald Melzack and Patrick Wall in 1965. They proposed that neuronal impulses generated by noxious stimuli are modified in the dorsal horn of the spinal cord by a specialized mechanism (“gate”), which can tend to either inhibit or facilitate transmission of the pain impulse from peripheral organs to the brain. The gate is not an “all-or-none” mechanism, and a balance between opposing factors determines how much of the initial impulse is transmitted through it (Green and Harris, 2008).

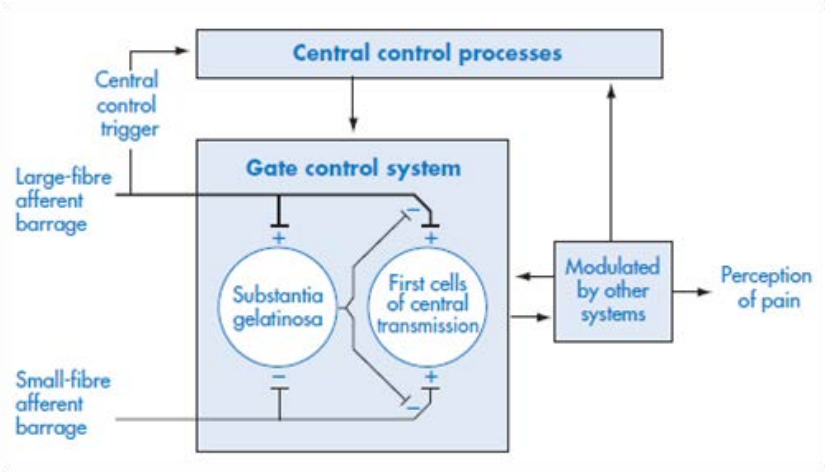


Figure 6 Gate control theory of the transmission of pain impulses (+ excitation; - inhibition; Greene and Harris, 2008).

Neurotransmitters involved in pain

Opioid receptors and endogenous opioids

The important discoveries of stereospecific opioid receptors and endogenous opioids further increased our understanding of the biochemical mechanisms involved in pain transmission and perception.

Several families of endogenous opioids have been classified such as the endorphins, enkephalins, and dynorphins. Each family is derived from a distinct precursor polypeptide and has a characteristic anatomical distribution.

Other transmitters and mediators

Physical or chemical insult can stimulate nociceptors. Inflammation, ischemia or other pain-inducing stimuli cause the release of noxious chemicals (e.g. bradykinin, histamine, and 5-HT) in the site of injury.

Prostaglandins (PGs), although not directly inducing pain, appear to sensitize nociceptors to various chemical and pressure stimuli. This explains why NSAIDs, which block PG synthesis, are effective analgesics in some situations.

Substance P (neurokinin-1), a polypeptide probably released by the small-diameter c-fibers, is believed to be involved in pain transmission in the dorsal horns of

the spinal cord. It is probably not the actual transmitter, but initiates a series of events leading to the recruitment of proinflammatory agent. The latter release mediators, e.g. PGs, LTs, 5-HT, and histamine, which stimulate the nerve endings and cause sensitization. Sensitization involves a lowering of the trigger threshold, producing hyperalgesia. Excitatory amino acid transmitters, e.g. glutamate and aspartate, may also be involved.

Pain transmission may be blocked if opioid receptors have already been occupied by endorphins at the spinal level. If successful in passing through the gating mechanisms, and several are probably involved in the total pathway, the pain impulse is transmitted via the reticular activating system of the pons and midbrain to the thalamus. They are directed to the appropriate part of the cerebral cortex where the impulses are perceived as pain. The limbic system, which is anatomically close to these areas, is thought to be responsible for the emotional component of pain. Transmission of the pain impulse may be modified in the CNS by the presence of 5-HT and other chemical mediators (Greene and Harris, 2008).

Pharmacologic Therapy

Non-narcotic analgesics (acetaminophen, aspirin, and other Nonsteroidal anti-inflammatory drugs (NSAIDs))

Acetaminophen is often selected as initial therapy for mild-to-moderate pain and is considered first-line in several pain situations such as low back pain and osteoarthritis.

NSAIDs have analgesic, antipyretic and anti-inflammatory properties. They appear to act peripherally at pain receptor level. They are particularly useful in treating patients with chronic disease accompanied by both pain and inflammation and for short-term treatment of mild to moderate acute pain, including musculoskeletal injuries and bone pain. Particular indications include the relief of pain accompanying dysmenorrhea, and that associated with neoplastic bone metastases, but they are of minimal use in neuropathic pain (Griffin and Woolf, 2005; O'Neil, 2010; Greene and Harris, 2008).

Mode of action

Acetaminophen (paracetamol), preferentially reduces central prostaglandin synthesis by an uncertain mechanism and blocks pain impulses in the periphery, as a result produces analgesia and antipyresis but has relatively little anti-inflammatory efficacy.

NSAIDs inhibit the activity of cyclooxygenase enzymes (COX-1 and COX-2) that are necessary for the production of prostaglandins (PGs) which results in reduced nociceptors sensitization and the recruitment of leukocytes, which produce other inflammatory mediators, and increased pain threshold (Griffin and Woolf, 2005; O'Neil, 2010). The relatively recent discovery that COX has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues (O'Neill and Ford-Hutchinson, 1993), but plays an especially important role in the gastrointestinal (GI) tract, kidneys, and platelets; COX-1 primarily produces prostaglandins with beneficial effects (e.g., regulation of blood flow to the gastric mucosa and kidneys; Miyoshi, 2001; Griswold and Adams, 1996). Activation of COX-1 leads to the formation of autacoids, e.g. protective prostacyclins, in the gastric mucosa and vascular endothelium, prostaglandin E_2 (PGE_2) in the kidney and thromboxane A_2 (TXA_2) in the platelets. In contrast, COX-2 is normally not present but may be induced in response to inflammatory stimuli; COX-2 is involved in fever, the central modulation of pain and the initiation of uterine contractions and fetal expulsion in childbirth, but its physiological roles are not fully defined. COX-2 is mostly inducible by cytokines and other pro-inflammatory stimuli that cause an inflammatory response (Greene and Harris, 2008).

Side effects

Acetaminophen is well tolerated at usual dose and has few clinically significant drug interactions except causing increased hypoprothrombinemic response to warfarin in patients receiving acetaminophen dose of more than 2,000 mg per day. Hepatotoxicity has been reported with excessive use and overdose, and risk of this adverse effect increases in those with hepatitis or chronic alcohol use, as well as those who binge drink or are in fasting state. Regular chronic use of acetaminophen has been

associated with chronic renal failure, but reports are conflicting. For these reasons, the maximum dose should be reduced by 50% to 75% in patients with renal dysfunction or hepatic disease and in those who engage in excessive alcohol use (O'Neil, 2010).

NSAIDs demonstrated a flat-dose response curve, with higher doses producing no greater efficacy than moderate dose but resulting in increased incidence adverse effects (e.g., gastrointestinal (GI) irritation, hepatic dysfunction, renal insufficiency, platelet inhibition, sodium retention, and CNS dysfunction). NSAIDs are classified as nonselective (inhibit COX-1 and COX-2) or selective (inhibit only COX-2) based on degree of COX inhibition. COX-2 inhibition is responsible for the anti-inflammatory effects, while COX-1 inhibition contributes increased GI and renal toxicity.

Adverse effects of nonselective NSAIDs as a class include GI problems (such as dyspepsia, ulcers, perforation, bleeding, and liver dysfunction), bleeding, kidney dysfunction, hypersensitivity reactions, and CNS effects (Max et al., 1999). The cardiovascular safety of the COX-2 inhibitor (coxibs) has been questioned due to the increased risk of myocardial infarction (MI) and stroke noted in several trials. This increased cardiovascular risk has been confirmed by case-control studies. Consequently, rofecoxib, valdecoxib and parecoxib have been withdrawn by manufacturer. Other coxibs have also produced an increased cardiovascular risk. It has been suggested that the basis for these observations is that selective COX-2 inhibitors significantly reduce levels of prostacyclin (PGI_2), an inhibitor of platelet aggregation, and do not affect thromboxane (TXA_2), a potent vasoconstrictor) formation by COX-1. There is therefore an increased tendency for vascular obstruction (Greene and Harris, 2008; O'Neil, 2010).

Opioid analgesics

Opioids are used to treat moderate to severe pain that does not respond to non-opioids alone (Max et al., 1999). They are often combined with nonopioids because this permits use of lower doses of the opioid (i.e., dose-sparing effect). Opioids play a major role in the treatment of acute pain (e.g., trauma, postoperative pain), breakthrough pain, cancer pain, and some types of chronic noncancer pain (CNCP; Max et al., 1999). For

chronic pain, their use was once highly controversial, however, use of opioids in chronic pain is now gaining acceptance. Opioids are classified by their activity at site, usual pain intensity treated and duration of action (O'Neil, 2010).

Opioid receptors

Direct evidence that opioids are recognized by specific receptors came from binding studies by Snyder and his colleagues in 1973, though the existence of specific antagonists had earlier suggested that such receptors must exist. Various pharmacological observations implied that more than one type of receptor was involved, the origin suggestion of multiple receptor types arising from *in vivo* studies of the spectrum of actions (e.g., analgesia, sedation, pupillary constriction and bradycardia) produced by different drugs. It was also found that some opioids, but not all, were able to relieve withdrawal symptoms in morphine dependent animals, and this was interpreted in terms of distinct receptor subtypes. The conclusion from these and many subsequent pharmacological studies, now confirmed by receptor cloning, is that three types of opioid receptor, termed mu, delta and kappa (all of them are typical G-protein-coupled receptors; Hunter, 2003).

Opioid agonists and antagonists

Pure agonists (e.g., morphine, pethidine, methadone and fentanyl) include most of the typical morphine-like drugs. They all have affinity for mu receptors and generally lower affinity for delta and kappa sites.

Partial agonist (e.g., buprenorphine) can produce a partial response irrespective of the concentration, and there may even be a decreased response if the optimum concentration is exceeded. They bind with the mu receptor and compete with the agonists, both naturally occurring and exogenous. If they are used in combination with a complete agonist, they may act as competitive antagonists and level of analgesia may be reduced so that they show only limited activity.

Mixed agonists-antagonists (e.g., pentazocine, nalbuphine and butorphanol) are antagonists at the mu receptor but are still effective as analgesics through agonist effects at the kappa receptor, the agonist effect being either complete or partial.

Pure antagonists are used to reverse respiratory depression post-operatively, to treat opioid poisoning (e.g., naloxone) and to prevent relapse in detoxified opioid addicts (e.g. naltrexone; Hunter, 2003; Greene and Harris, 2008).

Mode of action

Opioids bind to opioid receptors in the central nervous system, inhibit the transmission of nociceptive input from the periphery to the spinal cord, activate descending inhibitory pathways that modulate transmission in the spinal cord, and alter limbic system activity (Duggan and North, 1983; Benedetti, 1979; Stein, 1995; Reisine and Pasternak, 1996). Thus, opioids modify sensory and affective aspects of pain. The different actions of opioids (i.e., agonist and antagonist) at various opioid receptors (e.g., mu, kappa, and delta) provide one means of classification. In this system, opioids are broadly classified as mu agonists or agonist-antagonists. Because experts do not recommend use of agonist-antagonists as first-line analgesics (Max et al., 1999).

Opioid receptors belong to the family of G-protein-coupled receptors and inhibit adenylate cyclase, so reducing the intracellular cAMP. All three receptor subtypes exert this effect and they also exert effects on ion channels through a direct G-protein coupling to the channel. By these means, opioids promote the opening of potassium channels and inhibit the opening of voltage-gated calcium channels, which are the main effects seen at the membrane level. These membrane effects reduce both neuronal excitability (since the increased K^+ conductance causes hyperpolarization of the membrane) and transmitter release (owing to inhibition of Ca^{2+} entry; Hunter, 2003).

Side effects

Binding of mu agonist opioids to receptors in various body regions (e.g., CNS, GI tract) results in therapeutic effects and side effects. Side effects of mu agonist opioids as a class include sedation, mental clouding or confusion, respiratory depression, nausea, vomiting, constipation, pruritus (itching), and urinary retention. With the exception of constipation, these side effects tend to subside with time. Sedation and nausea are common when initiating therapy and when increasing doses. Nausea can be prevented with centrally acting antiemetic. Sedation usually improves with continued therapy but might become intractable at high doses, and stimulants such as methylphenidate might be needed. Respiratory depression is a serious adverse effect, and usually occurs after acute administration in opioid-naïve patients. Tolerance to respiratory depression develops rapidly with repeated doses, and respiratory depression is rarely a clinically significant problem in pain patients even those with respiratory impairment. Constipation is a significant adverse effect which tolerance to develop and prophylaxis with stimulant laxatives and stool softeners is recommended (O'Neil, 2010).

Most opioids should be used with caution in patients with impaired ventilation, bronchial asthma, liver failure, or increased intracranial pressure (Max et al., 1999). Opioid-induced respiratory depression is usually short-lived, antagonized by pain, and most common in the opioid-naive patient.

Inflammation

Definition

The function of an inflammatory reaction is a protective response intended to eliminate the initial course of cell injury as well as the necrotic cells and tissues resulting from that injury. Excessive inflammation may lead to tissue injury and cause physiologic decompensation, organ dysfunction and death. Inflammation is divided into two basic patterns, including acute and chronic inflammation (Murphy and Ward, 2005; Kumar, Abbas and Fausto, 2005)

Acute inflammation

Acute inflammation is of relatively short duration, lasting from a few minutes up to a few days, and is characterized by the exudation of fluid and plasma components and emigration of leukocytes (predominantly neutrophils) into the extravascular tissues. (Sherwood and Toliver-Kinsky, 2004; Horton-Szar, 2007).

Cardinal signs of acute inflammation

The cardinal signs of acute inflammation are redness, heat, swelling and pain, described by Celsus in the first century AD. If the injury has been excessive a fifth sign, loss of function, may occur, described by Galen in the second century AD (Green and Harris, 2008). Acute inflammation involves two major components: vascular and cellular response (Murphy and Ward, 2005; Kumar, Abbas and Fausto, 2005; Chandrasoma and Taylor, 1998). Both the vascular and cellular reactions of inflammatory response are mediated by chemical factors that are derived from plasma proteins or cell and are produced in response by inflammatory stimulus.

Vascular stage

The vascular response of acute inflammation, which leads to an increase in blood flow and changes in the small blood vessels of the microcirculation.

Hyperaemia

An essential function of acute inflammation is to provide an increased blood flow to the damaged area, facilitating the transport of agents involved in defence or repair. After a brief reflex constriction, and possibly also clotting to minimize local bleeding, the local arterioles dilate, flushing the capillary network with blood. (Figure 7; Green and Harris, 2008).

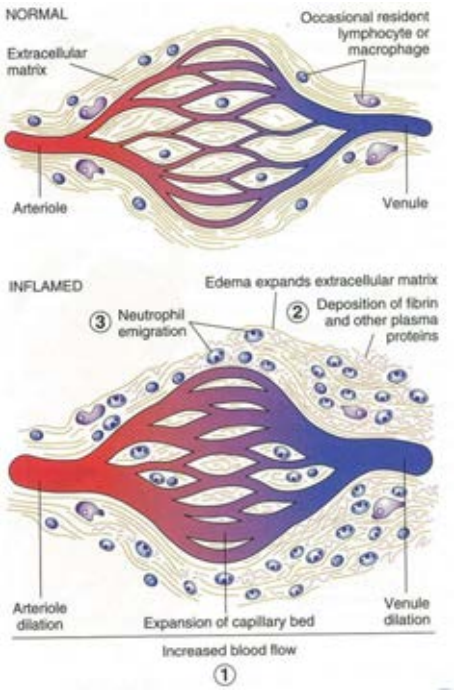


Figure 7 The major local manifestations of acute inflammation: (1) vascular dilation (causing erythema and warmth), (2) extravasation of plasma fluid and proteins (edema), and (3) leukocyte emigration and accumulation in the site of injury (Kumar et al., 2002).

The redness and heat associated with inflammation: the rise in local temperature is partly the result of an increase in local blood flow and partly of a higher metabolic rate in the inflamed area involved some mediators (Table 1).

Table 1 Some mediators involved in acute inflammation

Mediator	Source	Effect
Vasoactive amines		
• Histamine	Mast cells	Vascular dilation
• Serotonin	Platelets	Vascular leakage
Platelet activating factor	Various WBCs	Chemotaxis
IL-8	Monocytes	Chemotaxis
	Lymphocytes	
CRP (an 'acute phase protein')	Liver	Opsonization
Prostaglandins and leukotrienes	Phospholipid membrane of	Vascular leakage
	tissue cells	Chemotaxis
		Pain
		Anti-inflammatory
Vasoactive polypeptides	Plasma	Vascular dilation
		Vascular leakage
		Pain
Complement	Plasma	Chemotaxis
		Vascular leakage
		Cell lysis
		Opsonization

IL; interleukin, CRP; C-reactive protein, WBC; white blood cell.

Exudation

The swelling observed is caused by leakage of blood plasma through the vessel wall into the tissue interstitial space, producing swelling, pain, and impaired function (Figure 8). In normal capillaries the hydrostatic pressure of the blood forces fluid into the interstitial space. This pressure is partly offset by the oncotic pressure exerted by plasma proteins, which are too large to pass through normal capillary walls

and so are retained in the blood stream. In inflammation this balance is upset. Arterial vasodilatation results in an increased capillary hydrostatic pressure and hence an increased volume of interstitial fluid into the tissue space, thus increasing tissue oncotic pressure and further facilitating the movement of fluid from the blood to the interstitial space. A more diffuse vascular leakage from venules distant from the site of injury, adding to the exudate volume, is caused by chemical mediators such as histamine, bradykinin, nitric oxide and leukotriene B_4 (Kumar, Abbas and Fausto, 2005; Horton-Szar, 2007; Greene and Harris, 2008).

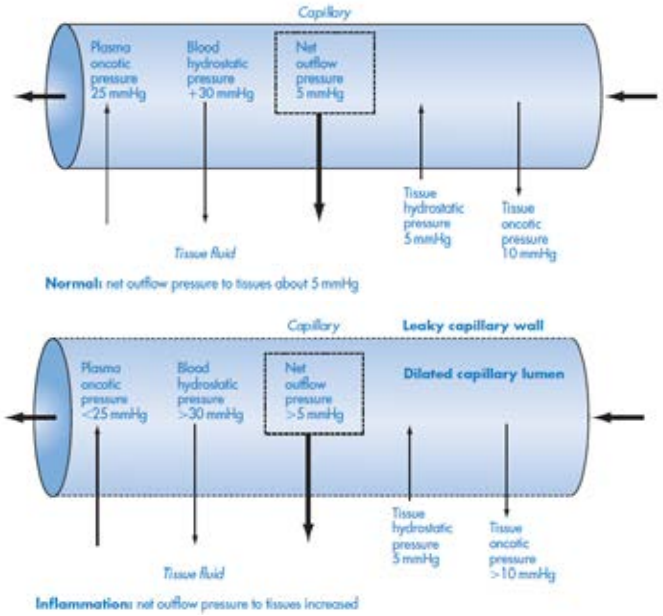


Figure 8 Exudation from capillaries (Greene and Harris, 2008).

Cellular stages

The cellular response of acute inflammation, which leads to emigration of leukocytes from the microcirculation and their activation to eliminate the injurious agent. The most important leukocyte in acute inflammation is neutrophils. The cellular response to inflammation focused on the recruitment of leukocytes from the blood to site of infection or injury, and a rapid response also requires the release of chemical mediators from tissue cells (mast cells and macrophages) that are prepositioned in the tissues (Nathan, 2000).

Leukocyte response

The sequence of stages in the leukocyte responses to inflammation includes leukocyte (1) margination and adhesion, (2) emigration, (3) chemotaxis, and (4) activation and phagocytosis (Figures 9). During the early stages of the inflammatory response, fluid leaves the capillaries, causing an increase in blood viscosity. The release of chemical mediators and cytokines affects the capillaries and causes the leukocytes to increase their expression of adhesion molecules. As this occurs, the leukocytes slow their movement and begin to accumulate along the endothelial surface. This process of leukocytes accumulation is called *margination.* As the leukocytes accumulate, they also begin to adhere to the vessel endothelium (Greene and Harris, 2008).

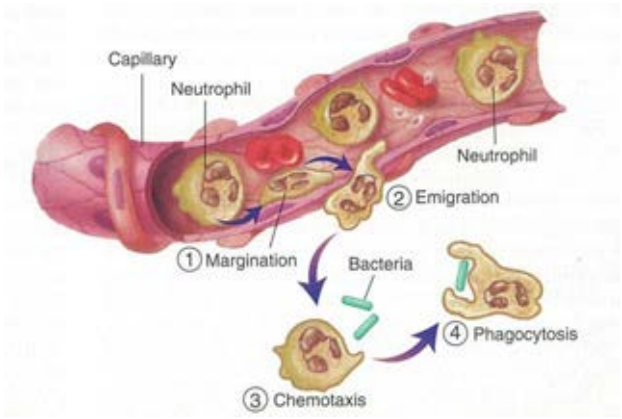


Figure 9 The cellular phase of acute inflammation (Porth and Sommer, 2009).

Emigration is a mechanism by which the leukocytes change shape, insert pseudopods into the junctions between the endothelial cells, and squeeze through the interendothelial junctions by amoeboid movement into the tissue spaces. The emigration of leukocytes also may be accompanied by an escape of red blood cells. Once they have exited the capillary, the leukocytes wander through the tissue guided by cytokines, bacterial and cellular debris, and complement fragments (e.g., C3a, C5a). The process by which leukocytes migrate in response to a chemical signal is called c*hemotaxis* (Porth and Sommer, 2009).

The next and final stage of the cellular response consists of leukocyte activation and elimination of the injurious agent. This is accomplished largely by *phagocytosis*, a process in which activated leukocytes engulf and degrade the bacteria and cellular debris. Phagocytosis involves three distinct steps: (1) adherence plus opsonization, (2) engulfment, and (3) intracellular killing (Figures 12). Contact of the bacteria or antigen with the phagocyte cell membrane is essential for trapping the agent and triggering the final steps of phagocytosis. If the antigen is coated with antibody or complement, its adherence is increased because of binding to complement. The enhanced binding of an antigen due to antibody or complement is called *opsonization*. Engulfment follows the recognition of the agent as foreign. During the process of engulfment, the cytoplasm move around and eventually enclose the particle in a membrane-surrounded phagosome. The phagosome then fuses with membrane of a lysosome, resulting in the formation of a phagolysosome (Porth and Sommer, 2009).

Intracellular killing of pathogens by phagocytotic cells is accomplished through several mechanisms, including lysosomal enzymes and oxygen-dependent mechanisms. The oxygen-dependent mechanisms, which involve the generation of reactive oxygen intermediates (free radicals), require a number of metabolic enzymes. Individuals born with genetic defects affecting the enzymes needed for generation of the reactive oxygen intermediates have increased susceptibility to recurrent bacterial infection (Porth and Sommer, 2009).

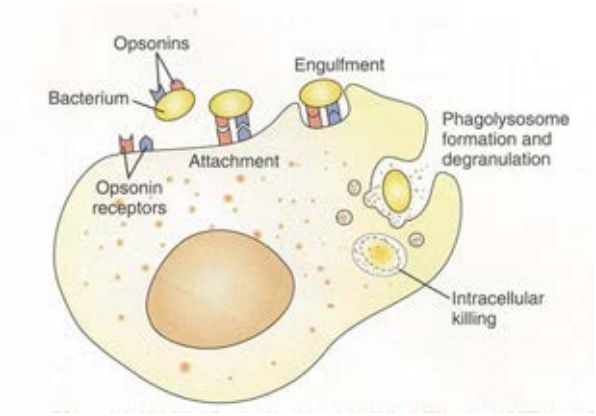


Figure 10 Phargocytosis of a particle (e.g., bacterium): opsonization, attachment, engulfment, and intracellular killing (Porth and Sommer, 2009).

Inflammatory mediators

Inflammation is precipitated by injury, its signs and symptoms produced by the release of chemicals mediators that stimulate a vascular response that force fluid and leukocytes to flow to the site of the injury. Stimulated nerve endings signal the brain that there is an injury. Plasma-derived mediators are present in the plasma in precursor forms that must be activated, usually by a series of proteolytic enzymes. The cell-derived mediators are normally sequestered in intracellular granules that need to be secreted, or they are newly synthesized in response to a stimulus. The production of active mediators is triggered by microbes or host proteins, such as the complement, kinins, or coagulation systems; those are themselves activated by microbes or damaged tissues (Figure 11). Mediators can act on one target cell or have diverse targets. Once activated and released from the cell, most mediators are short-lived. They may be transformed into inactive metabolites, inactive by enzymes or otherwise scavenged or degraded (Sherwood and Toliver-Kinsky, 2004; Kumar, Abbas and Fausto, 2005; Horton-Szar, 2007; Porth and Sommer, 2009; Greene and Harris, 2008).

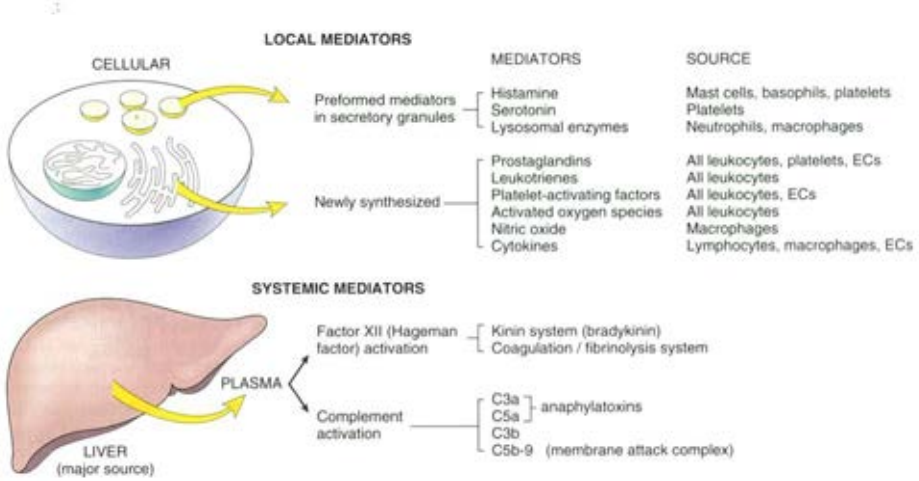


Figure 11 Chemical mediators of inflammation (ECs; endothelial cells; Kumar et al., 2002).

Plasma-derived mediators of inflammation

The plasma is the source of three major mediators of inflammation, including the kinins, the products of the coagulation/fibrinolysis system, and the proteins of the

complement system (Porth and Sommer, 2009; Greene and Harris, 2008; Kumar, Cotran and Robbins, 2003).

Kinin system

The kinin system generates vasoactive peptide from plasma proteins called kininogens, by the action of proteases called kallikreins. The kinin system is stimulated by activated coagulation factor XII (The Hageman factor; Figure 12). Activation of kinin system results in release of bradykinin. The kinin system, cause increased capillary permeability and pain. Both effects are cardinal features of acute inflammation. Activation of bradykinin is short lived because it is quickly inactivated by an enzyme called kininase.

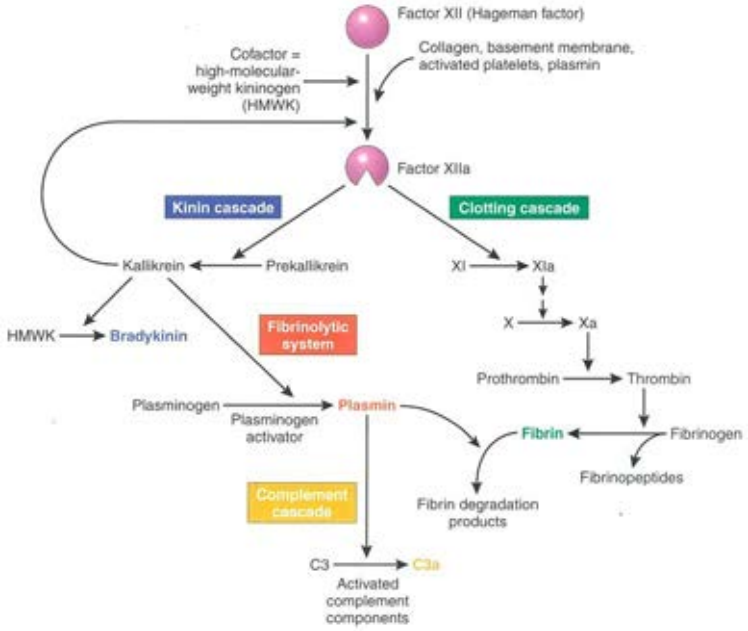


Figure 12 Interrelationships between the four plasma mediator systems triggered by activation of factor XII (Hageman factor; Kumar et al., 2002).

The clotting system

There are two potential inflammatory mediators derived from the clotting system: 1) Fibrinopeptides (release from fibrinogen molecules by the action of thrombin during clotting). These may induce vascular leakage and be chemotactic for neutrophils.

2) Fibrin degradation products (release during the proteolysis of fibrin by plasmin) may well be chemotactic for neutrophils.

The complement system

The complement system consists of a cascade sequence of plasma proteins is made up of more than 20 components that plays an important role in both immunity and inflammatory response by (1) causing vasodilation and increasing vascular permeability, (2) promoting leukocyte activation, adhesion, and chemotaxis, and (3) augmenting phagocytosis.

Cell-derived mediators of inflammation

The cell-derived mediators include histamine and serotonin, which are preformed and stored in intracellular granules, and arachidonic acid metabolites, platelet-activating factors, cytokines, and nitric oxide, all of which are rapidly synthesized in response to an appropriate stimulus.

Vasoactive amines (Histamine, 5-Hydroxytryptamine)

These are preformed inflammatory mediators and so can be rapidly released by inflammatory cells. Histamine is widely distributed throughout the body. Preformed histamine is found in high concentrations in the mast cell of connective tissues adjacent to blood vessels. It is also found in circulating blood platelets and basophils. Preformed histamine is present in the granules of mast cells and is released in response to a variety of stimuli. Histamine produces dilation of arterioles and increased vascular permeability of venules.

5-Hydroxytryptamine (5-HT or serotonin) is also a preformed vasoactive mediator with effects similar to those of histamine. It is present in platelets. Release of serotonin (and histamine) from platelets is stimulated when platelets aggregate after contact with collagen, thrombin, adenosine diphosphate, and antigen-antibody complexes.

Arachidonic acid metabolites; Prostaglandins, Leukotrienes, and Lipoxins.

Arachidonic acid is a 20-carbon unsaturated fatty acid found in phospholipids of cell membranes. The release of arachidonic acid from membrane phospholipids by the enzymatic action of phospholipases. The synthesis of arachidonic acid mediators follows one of two pathways: the cyclooxygenase pathway, which culminates in the synthesis of the prostaglandins, and the lipooxygenase pathway, which culminates in the synthesis of the leukotrienes (Figures 13).

Several prostaglandins are synthesized from arachidonic acid through the cyclooxygenase pathway. The stable prostaglandins (PGE_1 and PGE_2) induce inflammation and potential the effects of histamine and other mediators. The prostaglandins, thromboxane A_2 promotes platelet aggregation and vasoconstriction.

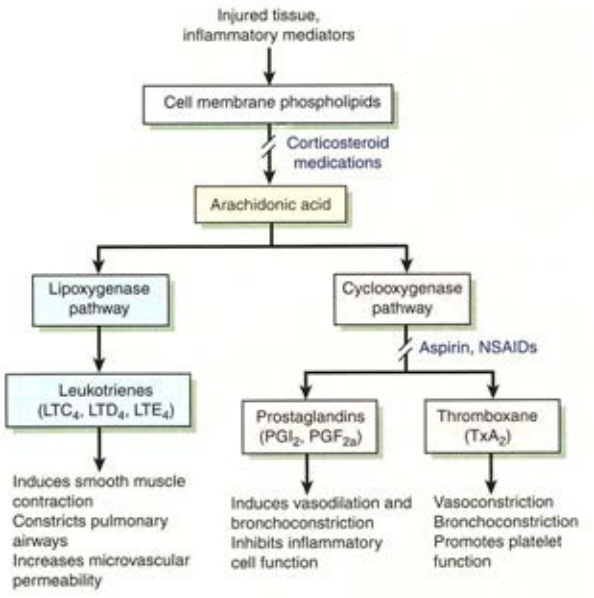


Figure 13 Biosynthesis of the prostaglandins, thromboxanes and leukotrienes by the cyclooxygenase and lipoxygenase pathway (Lüllmann et al., 2000)

Like the prostaglandins, the leukotrienes are formed from arachidonic acid, but through the lipoxygenase pathway. The leukotrienes also have been reported to affect the permeability of the postcapillary venules, the adhesion properties of endothelial

cells, and the chemotaxis and the extravasation of neutrophil, eosinophils and monocytes. The leukotrienes LTC_4, LTD_4, and LTE_4, collectively known as the *slow reacting substance of anaphylaxis* (SRS-A), cause slow and prolonged contraction of the bronchioles and are important inflammatory mediators in bronchial asthma and anaphylaxis.

Platelet-activating factor

Platelet-activating factor (PAF), a potent inflammatory mediator, is generated from a complex lipid stored in cell membranes. It is synthesized by virtually all inflammatory cells, endothelial cells, and injured tissue cells. It cause platelet aggregation, primes and enhances the functions of neutrophils and monocytes, and is a potent eosinophil chemoattractant. It is also an extremely potent vasodilator; increase the permeability of small blood vessels at the site of injury.

Cytokines

Cytokines are a family of chemical messengers that act over short distances (autocrine and/or paracrine) by binding specific receptors on target cell surfaces. Cytokines are proteins produced by many cell types, principally activated macrophages and lymphocytes that modulate function of other cells. They include the colony-stimulating factors and the interleukins (IL), interferons (IFN), and Tumor necrosis factor (TNF) that are important in the inflammatory response.

Interleukin 1 and Tumor Necrosis Factor

Although historically associated with cellular immune responses, various cytokines, in particular TL-1 and TNF, have additional effects that are important in inflammatory responses. Both IL-1 and TNF are produced by activated macrophages (IL-1 can also be synthesized by other cell types), and secretion is stimulated by endotoxin, immune complexes, toxins, physical injury, or a variety of inflammatory mediators. Both IL-1 and TNF induce endothelial activation with increased expression of adhesion molecules, secretion of additional cytokines and growth factors, production of

eicosanoids and nitric oxide (NO) and increased endothelial thrombogenicity. TNF also causes aggregation and activation of neutrophils and the release of proteolytic enzymes from mesenchymal cells, thus contributing to tissue damage. Both cytokine activate tissue fibroblasts, resulting in increased proliferation and production of extracellular matrix.

Nitric oxide

Nitric oxide (NO) is a short-lived, soluble, free radical gas which is produced by a variety of cells and capable of mediating a bewildering number of effector functions, plays multiple roles in inflammation. NO is a potent vasodilator that is released from endothelial cells and macrophages. NO causes vascular smooth muscle relaxation via cyclic GMP-mediated mechanism resulting in vasodilation at the site of inflammation. It is produced by the action of nitric oxide synthase on L-arginine. NO plays an important role in the inflammatory response, including smooth muscle relaxation and reduces platelet aggregation and adhesion and, serves a regulator of leukocyte recruitment, and aids in the killing of microbial agents by phagocytic cells.

Chronic inflammation

Chronic inflammation can be considered to be inflammation of longer duration (weeks to months to years) in which active inflammation, tissue injury, and healing proceed simultaneously. Chronic inflammation is characterized by the following (Kumar, Cotran and Robbins, 2003; Porth and Sommer, 2009).

(1) Infiltration with mononuclear (chronic inflammatory) cells, including macrophages, lymphocytes, and plasma cells.
(2) Tissue destruction, largely directed by the inflammatory cells.
(3) Repair, involving new vessel proliferation (angiogenesis) and fibrosis.

Characteristic of chronic inflammationis an infiltration by mononuclear cells (macrophages) and lymphocytes instead of the neutrophils commonly seen in acute inflammation. Chronic inflammation also involves the proliferation of fibroblasts instead of exudates. As a result, the risk of scarring and deformity usually is considered greater

than in acute inflammation. Agents that evoke chronic inflammation typically are low-grade, persistent irritants that are unable to penetrate deeply or spread rapidly. Immunologic mechanisms are throught to play an important role in chronic inflammation. The two patterns of chronic inflammation are a nonspecific chronic inflammation and granulomatous inflammation.

Nonspecific chronic inflammation

Nonspecific chronic inflammation involves a diffuse accumulation of macrophages and lymphocytes at the site of injury. Ongoing chemotaxis causes macrophages to infiltrate the inflamed sited, where they accumulate owing to prolonged survival and immobilization. These mechanisms lead to fibroblast proliferation, with subsequent scar formation that in many cases replaces the normal connective tissue or the functional parenchymal tissues of the involved structures.

Granulomatous inflammation

A granulomatous lesion is a distinctive form of chronic inflammation. A granuloma typically is a small, 1-2 mm. lesion in which there is a massing of macrophages surrounded by lymphocytes. These modified macrophages resemble epithelial cells and sometimes are called epithelioid cells. Like other macrophages, these epithelioid cells are derived originally from blood monocytes. These types of agents have one thing in common: they are poorly digested and usually are not easily controlled by other inflammatory mechanisms. The epithelioid cells in granulomatous inflammation may clump in a mass or coalesce, forming the multinucleated giant cell that attempts to surround the foreign agent. A dense membrane of connective tissue eventually encapsulates the lesion and isolates it. These cells are often referred to as foreign body giant cells.

If the reaction is prolonged, healing and repair will often accompany the inflammation, rather than follow it. Thus fibroblasts have an important role in chronic inflammation, and fibrosis is the main cause of residual damage, as in organization and repair.

Pharmacological treatment of inflammation

Although inflammation is a natural response to injury, this process can be uncomfortable for patient, especially when there is fever, pain, and swelling. Anti-inflammatory medication can be given to reduce the inflammatory process and bring comfort to the patient. Anti-inflammatory medication stops the production of prostaglandins resulting in a decrease in the inflammatory process (Kamienski and Keogh, 2006).

Glucocorticoids are frequently used to suppress inflammation, allergy and immune response. Anti-inflammatory therapy is used in many diseases (e.g., rheumatoid arthritis, ulcerative colitis, bronchial asthma, severe inflammatory conditions of the eye and skin). The powerful anti-inflammatory effects of glucocorticoids rest on their capacity to suppress the action of many proinflammatory mediators. Glucocorticoids bind to a corticosteroid receptor (CR) and the complex translocates to the nucleus where it bind to a glucocorticoid response element (GRE). This complex increases the transcription of a number of anti-inflammatory genes and interferes with the binding of transcription factors activating protein and NF-κB to their response element, this action inhibits the production of a range of proinflammatory mediators, including IL-1β and TNF-α. Moreover, glucocorticoids also increase synthesis of the polypeptide lipocortin-1, which inhibits phospholipase A2 and the synthesis of eicosanoids and PAF (Berry et al., 2006; Kamienski and Keogh, 2006; Bennett and Brown, 2008). Serious unwanted effects of glucocorticoids are unlikely if the daily dose is below the equivalent of hydrocortisone 50 mg or prednisolone 10 mg. Unwanted effects generally follow prolonged administration of glucocorticoids. The principle adverse effects of chronic glucocorticoids administration are Cushing's syndrome, tendon rupture, immunosuppression, peptic ulcer and glaucoma (Bennett and Brown, 2008).

There are other anti-inflammatory medications that are not chemically the same as corticosteroids medication. These are referred to as nonsteroidal anti-inflammatory drugs (NSAIDs). However, inhibition of prostaglandin synthesis by NSAIDs attenuates rather than abolishes inflammation. Nevertheless, the relatively modest anti-inflammatory

actions of the NSAIDs give, to most patients with rheumatoid arthritis, some relief from pain, stiffness, but they do not alter the course of the disease (Kamienski and Keogh, 2006; Neal, 2002).

ANIMAL MODELS

Antinociceptive activity testing

There are limitations in the use of human subjects for experimentation on pain mechanisms and pathways. Analgesic agents developed for humans have been routinely used in a broad range of animal species. Therefore, the basic science of pain and the preclinical research on pain treatments essentially depend on nociceptive tests that were done on naive animals. A few of the most commonly used methods will be described, which may illustrate important aspect of investigations of pain in animals.

Rodent models of pain have historically played a dominant role in the study of pain mechanisms (Negus et al. 2006; Walker et al. 1999). There are many good reasons for this, including the practicalities of low cost, simplified ethical concerns, and the scientific value of having a large database of prior research to provide context for new findings. In rodents, acute nociception is usually assessed using either a noxious heat stimulus (e.g., hot plate, tail withdrawal, Hargreaves test) or mechanical stimulus (Randall-Selitto test) applied to either a hindpaw or tail. While there are no reported quantitative assays to assess acute mechanical nociception in large animals

Hot-plate test

The hot-plate test was described by Woolfe and Mac Donald in 1944, and it is one of the most commonly used models of nociception and analgesia in rodents. The hot plate test has been used by many investigators and has been found to be suitable for evaluation of centrally acting analgesics.

This test consists of introducing a rat or mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid. A plate heated to a constant temperature produces two behavioral components

that can be measured in terms of their reaction times including paw licking and jumping. Both are considered to be supraspinally integrated responses.

As far as analgesic substances are concerned, the paw licking behavior is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50°C or less. The specificity and sensitivity of the test can be increased by measuring the reaction time of the first evoked behavior regardless of whether it is paw-licking or jumping.

The behavior is relatively stereotyped in the mouse but is more complex in the rat, which sniffs, licks its forepaws, licks its hind paws, straightens up, stamps its feet, starts and stops washing itself, among other things. These behaviors have been labeled “chaotic defensive movements”. Furthermore, this test is very susceptible to learning phenomena, which result in a progressive shortening of the jumping reaction time accompanied by the disappearance of the licking behavior. Thus, the animal may lick the paws and then jump during the first test but will jump almost immediately during subsequent tests. Similarly, even putting the animals on an unheated plate just once to watch the test leads in subsequent tests to a diminution in the reaction time under standard conditions with a constant noxious temperature. Finally, reiteration of the test once a day or once a week inevitably leads to a progressive decrease in the reaction time. In general, these measures are very variable, even within a single laboratory. All these factors make this test a very delicate one to use (Le bars et al., 2001).

Writhing test

In this test is widely used to evaluate both central and peripheral analgesic activity. Pain is induced by injection of irritants such phenylbenzoquinone, acetylcholine, dilute hydrochloric, acetic acid, bradykinin, adrenaline, adenosine triphosphate, potassium chloride, tryptamine, and oxytocin into the peritoneal cavity of mice.

The intraperitoneal administration of irritating agents that irritate serous membrane provokes a very stereotyped of stretching behavior in the mouse and rat which is characterized by abdominal contractions, movement of whole body, a wave of

constriction and elongation passing caudally along the abdominal wall, sometimes accompanied by twisting of the trunk and followed by extension of the hind limbs, and a reduction in motor activity and motor coordination. In this test, commonly it is known as the “writhing test” (Le bars et al., 2001). These behaviors are considered to be reflexes and involved with visceral pain. This test base on acetic acid stimulation of nociceptive neurons by liberation of several mediators such as histamine, serotonin, cytokines, and eicosanoids especially PGE_2 in the peritoneal fluid (Deraedt et al., 1980). In the peritoneal fluid of rats which had been injected with acetic acid that appeared the considerable increase of PGE and PGF which had been injected with acetic acid and disappeared in 90 min. After 90 min the PGE_2 level was less than $PGF_{2\alpha}$. This balance between PGEs, which are hyperalgesic, and $PGF_{2\alpha}$, which has often been shown to be a PGE antagonist, could regulate defense mechanisms. Moreover an examination of cells collected by washing the peritoneum revealed a large decrease between 15 and 30 min after injection of the acetic acid suggested that the prostaglandins could be produced by neutrophil polynuclear cells but also by destruction of macrophages. Various types of prostaglandin biosynthesis inhibitors (non-steroid anti-inflammatory agents, non-narcotic analgesics and some monoamine-oxidase inhibitors and antioxidants) prevented prostaglandin release (Deraedt et al., 1980).

Analgesic activity of the test compound is determined utilizing the number of writhes which are counted over an interval of time. Unfortunately, the number of writhes decrease spontaneously with time therefore it is impossible to evaluate the duration of action of an analgesic on a single animal. Furthermore, the number of cramps is subject to a great deal of variability (Le bars et al., 2001).

The advantage of this method is that it allows for the evidence to be obtained for effects produced by weak analgesics. On the other hand, it lacks of specificity because positive results are also produced by numerous other substances, including some that have no analgesic action, e.g., adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics (Pearl et al., 1969). Thus, a positive result with this test does not necessarily mean there is analgesic activity.

The specificity can be improved by undertaking a preliminary rota-rod test to detect and eliminate molecules that alter the motor performance of the animal (Pearl et al., 1969). Although the writhing test has a poor specificity, it is sensitive and predictive, as shown by the correlation between ED_{50} values obtained in rats using this test and analgesic doses in humans (Le bars et al., 2001).

Formalin test

The formalin test has been proposed as a model for chronic pain (Dubuisson and Dennis, 1977). The rodent was injected subcutaneously of dilute formalin into the plantar surface of the hind paw, resulting in the animals presented behavioral responses such as elevation, licking, biting, or shaking of the injected paw. In the rat and mouse, the formalin injections produce a biphasic behavioral response. Two distinct periods of high licking activity can be discriminated, an early phase lasting from 0-5 min and a late phase lasting from 20 to 30 min after the injection of formalin (Hunskaar and Hole, 1987; Rosland et al, 1990). The early phase is related to the direct stimulation of nociceptors and is sensitive to local anesthetics, whereas the late phase involves both inflammatory mechanisms and central sensitization within the dorsal horn (Tjølsen et al., 1992). Hunskaar and Hole (1987) demonstrated that centrally acting analgesics such as opioids inhibited the responses in both phases. On the other hand, the nonsteroidal anti-inflammatory drugs and the steroids inhibited the responses only the late phase. The results suggested that the early phase is due to a direct effect on nociceptors and that prostaglandins do not play an important role during this phase in contrast to late phase that seems to be an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs (Hunskaar and Hole, 1987). This test may allow the researchers to dissociate between inflammatory and non-inflammatory pain which is a rough classification of analgesics as well as to identify the possible mechanism of analgesics between central and peripheral component (Hunskaar and Hole, 1987; Shibata et al, 1989; Tjølsen et al., 1992).

Anti-inflammatory activity testing

Inflammatory process involves a series of events that can be elicited by numerous stimuli, e.g. infectious agent, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes.

Pharmacological methods for testing acute and subacute inflammation include UV-erythema in guinea pigs, vascular permeability, oxazolone-induced ear edema in rats and mice, croton-oil edema in rats and mice, paw edema in rats, pleurisy tests and granuloma pouch technique. The proliferative phase is measured by methods for testing granuloma formation such as cotton wool granuloma, Glass rod granuloma, and PVC sponge granuloma (Vogel, 2008).

Paw edema

Among many methods used for screening the anti-inflammatory drugs, one of the most commonly employed techniques is based on the ability of agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin or carrageenan.

The carrageenan-induced edema formation has been accepted as a useful phlogistic tool for investigating systemic anti-inflammatory agent and first presented by Winter et al., in 1962. The rat paw was elicited by subplantar injection of 0.05 ml of 1% carrageenan suspension in saline. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighted. Usually, the volume of the injected paw was measured before and 3 or 6 h after application of the irritant. The difference was recorded as volume of edema. The difference of average values between treated animals and control groups is calculated for each time interval and

statistically evaluated. The difference at the various time intervals gives some hints for the duration of the anti-inflammatory effect (Vogel, 2008).

Carrageenan-induced inflammatory reaction has a biphasic response (Vinegar et al., 1969). The early phase (1–2 h) is associated with release of histamine, serotonin and kinins, while prostaglandins appear to be the most important mediators in the late phase (3–5 h) after intraplantar injection of carrageenan (Di Rosa and Sorrentino, 1968; Di Rosa, 1972). The edema at 3 h after the application of carrageenan was considered to reach the highest response (Andrade et al., 2007; Kale et al., 2007). The rats have been the animal of choice for the study of carrageenan-induced paw edema response for many years because it has been fully established. Previous study demonstrated the time course pattern in carrageenan-induced mouse paw edema resembles that observed in the rat (Levy, 1969; Posadas et al., 2004). The mouse paw edema responds to anti-inflammatory drugs that causes a reduction in inflammatory responses. Therefore an advantage to using mice rather than rats is the amount of compound needed because the difference between the usual rat and mouse weights is between five and ten fold (Levy, 1969). Thus, mouse paw oedema has been widely used to test new anti-inflammatory drug candidates and to study the mechanisms involved in inflammation (Posadas et al., 2004).

Cotton pellet-induced granuloma

The cotton pellet-induced granuloma used for investigated the effect of drugs on a chronic inflammation characterized by cellular proliferation and granuloma formation. The repair phase of the inflammatory process begins with proliferation of fibroblasts as well as multiplication of small blood vessels. Such proliferating cells penetrate the exudate producing a highly vascularized reddened mass known as granulation tissue (Swingle, 1974).

The cotton pellet-induced granuloma model has been introduced first by Meier et al. in 1950. The rats were implanted subcutaneously of cotton pellets induced granuloma formation. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly

formed connective tissue can be measured by weighing the dried pellets after removal (Vogel, 2008).

Rosmarinic acid

Rosmarinic acid (RA) is a water-soluble polyphenolic compound which commonly found in substantial sources in the family of Lamiaceae, which has many important species such as oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosmary (*Rosmarinus officinalis*), holy basil (*Ocimum sanctum*), sweet basil (*Ocimum basilicum*), perilla or shiso (*Perilla frutescens*), spearmint (*Mentha spicata*). Scarpati and Oriente in 1958, identified rosmarinic acid for the first time, as a pure compound from rosmary (*Rosmarinus officinalis*).

Phytochemicals

The structure of rosmarinic acid was elucidated as an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid in figure 14 (Scarpati and Oriente, 1958).

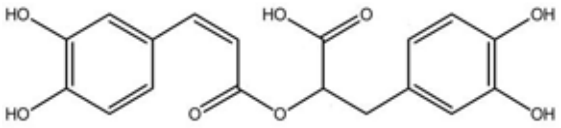


Figure 14 Chemical structure of rosmarinic acid (RA; Scarpati and Oriente, 1958).

Pharmacological activities

Analgesic, anti-inflammatory and antipyretic activity

. Rosmarinic acid (RA) suppressed synovitis in murine collagen induced arthritis model, this effect may be beneficial for treatment of rheumatoid arthritis. RA was also reported to have an additive effect in treating inflammatory diseases due to free radical-scavenging capacity (Youn et al., 2003).

RA isolated from the methanolic extract of *Cordia verbenacea* significantly inhibited paw edema induced by *Bothrops jararacussu* snake venom and its main phospholipase A_2 homologs (Ticli et al., 2005)

Oral administration of RA inhibits lung injury induced by diesel exhaust particle (DEP). DEP enhanced expression of iNOS mRNA and formation of nitrotyrosine and 8-OHdG in the lung, which was also inhibited by RA. RA inhibits DEP-induced lung injury by the inhibition of expression of inflammatory cytokines. Antioxidative activities of RA may also contribute to its protective effects (Sanbongi, 2003).

Topical application of RA on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema showed that RA reduced TPA-induced neutrophil infiltration (Osakabe et al., 2004)

RA inhibited several complement-dependent inflammatory processes including paw oedema induced by cobra venom factor (CVF) and ovalbumin/anti-ovalbumin-mediated passive cutaneous anaphylaxis (Englberger et al., 1988)

The effect of RA on anti-inflammatory and antinociceptive activities using carrageenan-induced pleurisy model and tail-flick assay in rats was determined. RA reduced the number of total exudate cells but ineffective on tail-flick latencies (Gamaro et al., 2011).

Antiviral activity

RA displayed a strong antiviral and anti-inflammatory activity against Japanese encephalitis. RA reduced the mortality of mice infected with Japanese encephalitis virus (JEV). JEV-infected animals treated with RA caused a significantly decrease ($p< 0.001$) in viral loads and proinflammatory cytokine levels (Swarup et al., 2007).

Hepatoprotective activity

Oral treatment with RA in perilla extract (PE) reduced liver injury induced by D-galactosamine and LPS in mouse, through the scavenging of superoxide molecules produced by Kupffer cells and inhibition of peroxynitrite formation induced by inducible nitric oxide synthase (iNOS) (Osakabe et al., 2002).

Neuroprotective activity

RA significantly attenuated H_2O_2-induced reactive oxygen species (ROS) generation and apoptotic cell death in human dopaminergic cell line, SH-SY5Y. RA effectively suppressed the up-regulation of Bax and down-regulation of Bcl-s and stimulated the antioxidant enzyme heme oxygenase-1 (HO-1) associated with the protein kinase A (PKA) and phosphatidylinositiol-3-kinase (PI3K) signaling pathway. RA should be clinically evaluated for the prevention of neurodegenerative diseases (Lee et al., 2008).

RA significantly protected neurons against oxidative stress, excitotoxicity and ischemia-reperfusion injury. These effects are mediated by the prevention of oxidative stress, intracellular Ca^{2+} overload, *c-fos* expression, inhibited nuclear factor-κB translocation and increased peroxisome-activated receptor-γ expression in SH-SY5Y cells not exposed to harmful stimuli (Fallarini et al., 2009).

Anti-angiogenic activity

RA inhibited angiogenesis processes including endothelial cell proliferation, migration, adhesion and tube formation of human umbilical vein endothelial cells (HUVEC) and reduced intracellular reactive oxygen species (ROS)-associated vascular endothelial growth factor (VEGF) expression and IL-8 release of endothelial cells (Huang and Zheng, 2006).

RA has an anti-angiogenic activity to retinal neovascularization in oxygen-induced retinopathy, 3a mouse model of retinopathy of prematurity, which related to cell cycle arrest with increase of $p21^{WAF1}$ expression. RA significantly inhibited the proliferation of retinal endothelial cells in a dose-dependent manner, and inhibited *in vitro* angiogenesis of tube formation; anti-proliferative activity of RA on retinal endothelial cells was related to G_2/M phase cell cycle arrest in dose-dependent manner (Kim et al., 2009).

Anti-proliferative activity

RA has been reported to possess anti-proliferative effect suggests that proliferative vascular diseases, molecular mechanisms responsible of RA could involve in cell cycle arrest in the G_0/G_1 and G_1/S phases (Makino et al., 2000).

Antioxidative activity

RA has a strong anti-apoptotic effect against ADR-induced cell death in H9C2 cardiac muscle cells by inhibiting ROS generation and JNK and ERK activation. RA should be viewed as a potential chemotherapeutic that inhibits cardiotoxicity in ADR-exposed patients (Kim et al., 2005).

RA has been isolated from *Perilla frutescens* Britton var. *acuta* f. *viridis* leaves as a scavenger in a xanthine/xanthine oxidase system. The scavenging activity of RA was significantly higher than that of ascorbic acid (AA) or other phenoliccarboxylic acids. RA effectively exhibited antioxidative activity in the biological systems through the scavenging of superoxide (Nakamura et al., 1998).

Tyrosinase inhibitor

Rosmarinic acid and its methyl ester, isolated from the ethyl acetate soluble fraction of the methanolic extract of *Salvia mittiorrhiza* were found to inhibit the oxidation of L-tyrosine catalyzed by mushroom tyrosine (Kang et al., 2004).

Anti-allergic activity

Orally administered RA from perilla extract (PE) in mice significantly prevented the increases in the numbers of eosinophils in bronchoalveolar lavage fluids and around murine airways. RA also inhibited the enhanced protein expression of IL-4 and IL-5, and eotaxin in the lungs of sensitized mice. RA is an effective intervention for allergic asthma, possibly through the amelioration of increases in cytokines, chemokines, and allergen-specific antibody (Sanbongi et al., 2004).

Metabolism of rosmarinic acid (RA)

RA is rapidly eliminated from the blood circulation after intravenous administration ($t_{1/2}$=9 min) and shows a very low toxicity with a LD_{50} in mice of 561 $mgkg^{-1}$ after intravenous administered (Parnham and Kesselring, 1985). Ritschel et al.,1989 showed that intravenously administered RA in mice was distributed in various tissues including lung, spleen, heart and liver and topical application of RA to skin of mice was absorbed percutaneously and became distributed in skin, blood, bone and muscle. These results show that in rats, RA becomes distributed in variety of tissues after topical and intravenous administration.

Orally administration of RA was excreted in urine as degraded forms contained seven metabolites such as *trans*-caffeic acid 4-*O*-sulfate, *trans*-*m*-coumaric acid 3-*O*-sulfate, *trans*-ferulic acid 4-*O*-sulfate, *trans*-caffeic acid, *m*-hydroxyphenylpropionic acid, *trans*-*m*-coumaric acid, and unchanged rosmarinic acid. The metabolites attributed to RA could not be found in the bile. Orally administered RA may thus be concluded to be excreted in the urine rather than in the bile, with cleavage of ester bonds, selective *para*-dehydroxylation, methylation, and sulfate-conjugation. The metabolites, *trans*-*m*-coumaric acid 3-*O*-sulfate, *trans*-ferulic acid 4-*O*-sulfate *m*-hydroxyphenylpropionic acid, and *trans*-*m*-coumaric acid were also found in the plasma. (Nakazawa and Ohsawa,1998). Moreover, Baba et al., 2004 reported that the main metabolites in urine following orally administered of RA in rats were RA, methyl-rosmarinic acid, caffeic acid, ferulic acid and *m*-coumaric acid within 8 h of RA administration. RA, methyl-rosmarinic acid and *m*-coumaric acid were detected in plasma, with peak concentrations being reached at 0.5, 1 and 8 h after RA administration, respectively. Approximately 83% of the total amount of these metabolites was excreted in the period 8-18 h after RA administration (Figure 15).

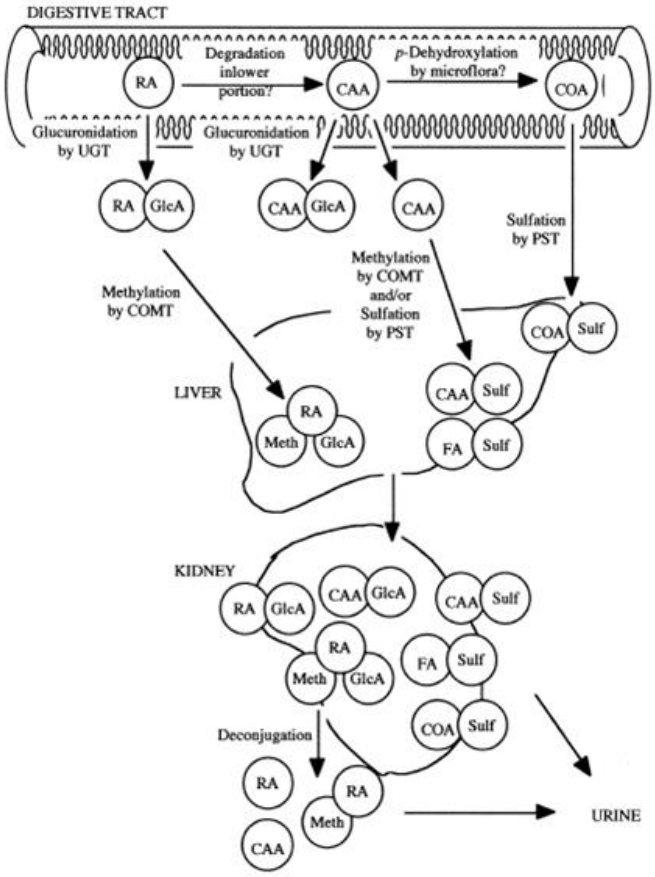


Figure 15 Proposed scheme of possible metabolic fate of rosmarinic acid administered orally in rats. (RA; rosmarinic acid, GlcA; glucuronide moiety, Sulf; sulfate moiety, Meth; methyl moiety, CAA; caffeic acid, FA; ferulic acid, COA; coumaric acid, UGT; UDP-glucuronosyltransferase, COMT; catechol-*o*-methyltransferase, PST; phenolsulfotransferase (Baba et al., 2004).

Thunbergia laurifolia **Lindl.**

Thunbergia laurifolia Lindl. is a large woody climber belong to the Acanthaceae family, and is known in Thai as “Rang Chuet” or “รางจืด” (Chan and Lim, 2006).

Local name: rang chuet, rang yen or nam nong.

Common names: Blue trumpet vine, purple allamanda, laurel-leaved thunbergia, laurel clock vine (Wagner et al., 1999)

Distribution

T. laurifolia is native to India (Wagner et al. 1999). Whistler in 2000 reported the native range of *T. laurifolia* in Myanmar (Burma) and Malaysia.

Uses

Thunbergia laurifolia Lindl. or rang chuet has been traditionally used in Southeast Asia for centuries. In Malaysia, the juice of the leaves is said to be efficacious in case of menorrhagia. It is also applied to the ears for deafness (Bor and Raizada, 1982). In Thailand, the leaves and roots of *T. laurifolia* are medicinal plant that is used for detoxification and as the first-aid treatment for several poisoning agents such as insecticides, ethyl alcohol, arsenic, and strychnine (นันทวันและอรนุช, 2543). It has also been applied for anti-inflammatory and antipyretic effects (วุฒิ, 2540).

Description

It is a climbing plant with smooth opposed leaves along the stem. The leaves are 8-10 cm long and 4-5 cm broad, broad-based, narrowing to a pointed tip, usually with scalloped lobes towards the base. The flowers are trumpet-shaped, about 5-8 cm in diameter, pale blue in color and produced during November-January. The seed pod is cone-shaped; Capsules are 1.5 cm wide, with beaks up to 3 cm long in Figure 16 A-C (Backer and Bakhuuzen, 1965).

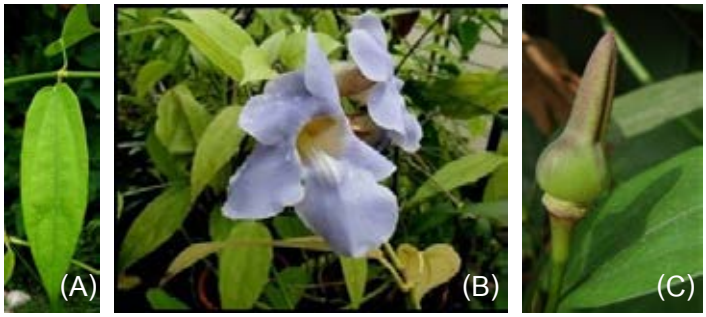
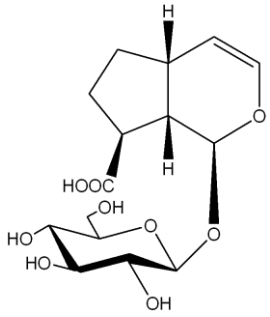


Figure 16 *Thunbergia laurifolia* Lindl. (A); leaves, (B); flower, and (C);fruit

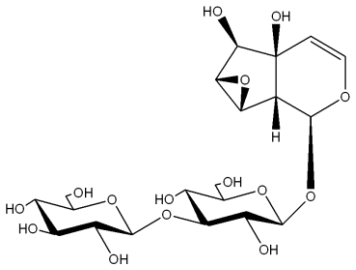
Phytochemical

The phytochemistry of *T. laurifolia* leaves has been studied. Iridoid glycosides, flavonoids, and phenolic acids are main constituents reported from this plant. Kanchanapoom et al. (2002) reported two iridoid glucosides of 8-*epi*-grandifloric acid and 3′-*O*-β-glucopyranosyl-stilbericoside isolated from the methanolic extract of the aerial part of *T. laurifolia* with seven glycosides of grandifloric acid, benzyl-β-glucopyranoside, (*E*)-2-hexenyl-β-glucopyranoside, hexanol-β-glucopyranoside, benzyl-β-(2′-*O*-β-glucopyranosyl)-glucopyranoside, 6,8-di-*C*-glucopyranosyl apigenin, apigenin-7-*O*-β-D-glucopyranoside.

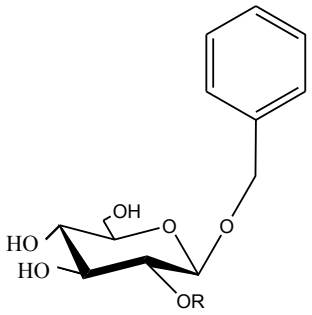
Both flowers and leaf materials of *T. laurifolia* have also been found to contain other bioactive phenolic constituents including delphinidin 3:5-di-*O*-β-D-glucopyranoside, apigenin, apigenin-7-*O*-β-D-glucopyranoside and chlorogenic acid (Purnima and Gupta, 1978; Thongsaard and Marsden, 2002). The structures of chemical components reported for *T. laurifolia* are shown in Figure 17.



grandifloric acid

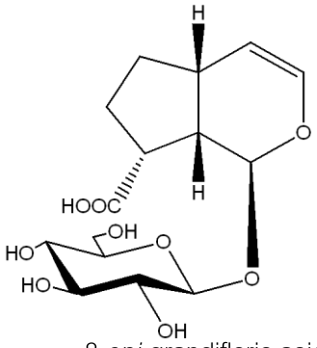


3′-*O*-β-glucopyranosyl-stilbericoside

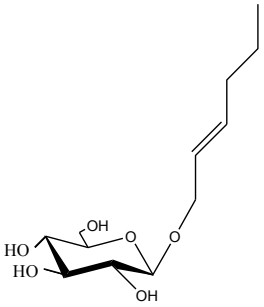


R = H ; benzyl-β-glucopyranoside

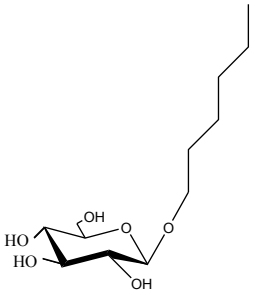
R = β-D-glucose ; benzyl-β-(2′-*O*-β-glucopyranosyl)-glucopyranoside



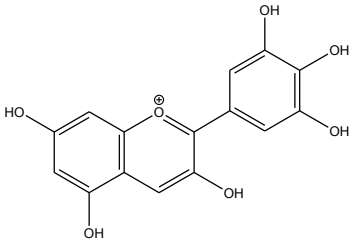
8-*epi*-grandifloric acid



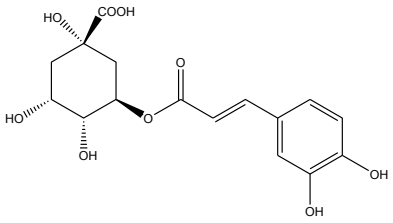
(*E*)-2-hexenyl-β-glucopyranoside



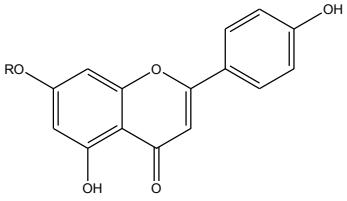
hexanol-β-glucopyranoside



delphinidin



chlorogenic acid



R = H ; apigenin

R = β-D-glucose ; apigenin-7-*O*-β-D-glucopyranoside

Figure 17 Structure of chemical constituents reported for *Thunbergia laurifolia* Lindl.

Pharmacological activities

Analgesic and anti-inflammatory activity

The ethanol extract of *T. laurifolia* were determined using acetic acid-induced writhing, formalin-induced hind paw and cotton pellet-induced granuloma in male mice. The mechanism of anti-inflammatory activity of extract was investigated using the inhibitory effect of extract on the production of proinflammatory mediators in lipopolysaccharide (LPS) activate murine macrophage (RAW 264.7). The anti-inflammatory activity of *T. laurifolia* leaf extract may result from the inhibition of NO and PGE_2 production probably by suppression of the iNOS and Cox-2 mRNA expression (Phosri et al., 2008).

Anti-inflammatory activity of *T. laurifolia* extract was investigated in mice using carrageenan-induced paw edema. The *T. laurifolia* extract showed the highest anti-inflammatory activity with 70% inhibition. *T. laurifolia* gel was measured by croton oil-induced ear edema in rats. *T. laurifolia* gel (40%) exhibited relatively the same efficacy as diclofenac gel. Chronic inflammation was investigated by formaldehyde-induced arthritis. Orally *T. laurifolia* extract significantly reduced arthritis within 10 days (Pongphasuk et al., 2003).

The ethanol extract of *T. laurifolia* leaves had anti-inflammatory activity. *T. laurifolia* extract significantly reduced paw edema and 30% *T. laurifolia* gel (30%) inhibited ear edema effectively as 1% diclofenac sodium (Charumanee et al., 2001). Moreover, anti-inflammatory activity of the methanol extract of *T. laurifolia* was investigated in ear edema in rat. *T. laurifolia* extract (30%) significantly reduced ear edema. *T. laurifolia* extract at 1 g/kg significantly inhibited paw edema in carrageenan-induced paw edema (มณเฑียร เปสี และคณะ, 2544).

Antidote activity

Rats were orally administered *T. laurifolia* (TL) leaf extract at 125 mg/kg before and after administration of 1.2 mg/kg of cadmium chloride solution ($CdCl_2$) subcutaneously for 5 days/week for 4 weeks. The leaf extract given to rats orally did not reduce the levels of Cd in blood and urine of the rats exposed to cadmium, but

prevented histological changes in the kidney. TL leaf extract can protect against Cd induced structural damage in rat kidney and also reduce other systemic toxicity (Morkmek et al., 2010).

Orally administration of aqueous *T. laurifolia* (TL) leaf extract did not affect levels of lead in blood and brain of mice given lead (1 g/L) in drinking water at for 8 weeks. However, co-treatment with aqueous TL leaf extract at 100 mg/kg or 200 mg/kg body weight was found to alleviate adverse effects of lead on learning deficit and memory loss, evaluated with water maze swimming test. Further, increased activity of the cell death marker enzyme caspase-3 was observed in the brain of mice treated with lead, Co-treatment with aqueous TL leaf extract at 100 mg/kg or 200 mg/kg body weight was found to restore the levels of caspase-3 activity and maintain total anti-oxidant capacity and anti-oxidant enzymes in the brain. TL leaf extract reduced neuronal cell death and memory loss caused by lead uptake in mice (Tangpong and Satarug, 2010).

T. laurifolia was investigated for detoxifying effects on paraquat-induced toxicity in rats. It was reported that the rats treated with the plant extract had higher survival rates and lower levels of plasma malonaldehyde (Ussanawarong et al., 2000).

Antidiabetic activity

Hypoglycemic properties of the aqueous leaves extract were evaluated in normoglycemic and alloxan-induced diabetic rats. The results showed that a 15-day-treatment with *T. laurifolia* extract at 60 mg/ml/day decreased levels of blood glucose in diabetic rats. The recovery of some β-cells was found in diabetic rats treated with the extract, although not completely normal. Alteration of the reproductive system was also observed in diabetic rats, but it was not improved by treatment with *T. laurifolia* extract (Aritajat et al., 2004).

Antiproliferative activity

Antiproliferative activity of *T. laurifolia* leaves on SKBR3 human breast cancer cell line using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay. The

extract did not show positive antiproliferative activity (Moongkarndi et al., 2004). Moreover, a dried leaf extract of *T. laurifolia* showed very weak cytotoxic activity against BHK and L929 normal cells, and HepG2 and Caco2 cancer cells using MTT assays (Oonsivilai et al., 2008).

Antioxidant activity

The aqueous extract of *T. laurifolia* yielded the highest total phenolic contents, free radical scavenging and ferric reducing effects as compared with the ethanol and acetone extracts (Oonsivilai et al., 2008). Total phenolic contents and antioxidant capacities of the commercial tea of *T. laurifolia* were significantly lower than those of crude material. There is significant difference in antioxidant values between different batches of the commercial *T. laurifolia* tea, suggesting that manufacturing procedures have not been standardized (Chan and Lim, 2006; Chan et al., 2011).

Hepatoprotective activity

Aqueous extract from leaves of *Thunbergia laurifolia* (TLE) were evaluated for hepatoprotective activity in *in vitro* and *in vivo* models. TLE increase viability of primary cultures of rat hepatocyte treated with ethanol up to 2-3 folds and decreased release of alanine transaminase (ALT) and aspartate transaminase (AST). TLE promote rat liver recovery after 14 days of ethanol treatment demonstrated by the decrease in severity of rat liver injury and the normalization in the levels of HTg, ALT and AST. These results suggest that TLE possess hepatoprotective activity against ethanol-induced liver injury both in primary cultures of rat hepatocyte and rats (Pramyothin et al., 2005).

Antimutagenicity activity

The aqueous, ethanol, and acetone extracts of *T. laurifolia* Lindl. or Rang Chuet (RC) were assayed for mutagenic and antimutagenic activity by bacterial reverse mutagenesis assay. The result indicated that all of three RC extracts exhibited strong dose-dependent antimutagenic activity inhibiting 2-aminoanthracene induced mutagenesis up to 87% in *Salmonella typhimurium* TA 98 (Oonsivilai et al., 2007).

Antimutagenic effect was also observed in the aqueous extract of *T. laurifolia* which significantly inhibited the induction of micronuclei in polychromatic erythrocytes induced by *Pueraria mirifica* (Saenphet et al., 2005).

Toxicity studies

In an acute toxicity study, animals were orally administered a single dose of three different Rang Chuet extracts such as water, ethanol, and acetone at doses of 2,000 and 15,000 mg/kg body weight,. An acute toxicity study was performed in both sexes of Wistar rats. All three Rang Chuet extracts did not produce mortality or general behavior changes and changes in gross morphology of rats' internal organs, as well as histological changes of visceral organs during 14 days of the tested period. The oral LD_{50} of three Rang chuet extracts by using different solvents including water, ethanol, and acetone in both sexes of rat were more than 15,000 mg/kg body weight (Posridee et al., 2011).

The chronic toxicity study of *T.laurifolia* aqueous extract (TLE) was studied in Wistar rats. The extract at doses ranging from 20 to 2,000 mg/kg/day for six months, did not affect the body weight, food consumption, behavior and general health. TLE did not cause any significant gross and histological lesions in the visceral organs of Wistar rats. The extract at doses of 1,000 and 2,000 mg/kg/day produced slight alterations of some hematological and clinical chemistry parameters of the rats (with in the normal range). However, the results suggest that prolonged use of high doses of *T. laurifolia* aqueous extract may affect the hematopoietic system and should be monitored (Chivapat et al., 2009). Moreover, Visitpongpant et al., 2003 reported that a 28-day repeated dose toxicity study of the *T. laurifolia* aqueous extract at an oral dose of 500 mg/kg revealed that the extract did not cause any histological changes of the organs; however, it may affect some hematological values and kidney weights.

CHAPTER III
MATERIALS AND MATHODS

EXPERIMENTAL ANIMALS

Male ICR mice weighing 18-25 g and 25-35 g were used in the experiments. The mice were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhonpathom, Thailand. The animals were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Chulalongkorn University at 25±2°C, 50-60% humidity, and under a 12/12 h light/dark cycle, with food and water provided *ad libitum.* The mice were kept for one week under laboratory conditions before use in experiments. At the end of each experiment, mice were sacrificed by carbon dioxide asphyxiation. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Appendix A).

DRUGS AND CHEMICALS

The following drugs and chemicals were used: normal saline solution (NSS; General Hospital Products Public Co., Thailand), 2% (w/v) Tween 80 solution (Srichansahaosoth Co., Thailand), sterile water (SW; General Hospital Products Public Co., Thailand), morphine sulfate (MO; Thai FDA), indomethacin (IND; Sigma Chemical Co., USA), acetic acid (Merck, Germany), formaldehyde (Merck, Germany), λ-carrageenan (Sigma Chemical Co., USA), prostaglandin E_2 (PGE_2; Sigma Chemical Co., USA), naloxone (NAL, Sigma Chemical Co., USA), pentobarbitone sodium (Ceva Sante Animale, France), rosmarinic acid (RA) isolated from *Thunbergia laurifolia* (RA; 12.50-150 mg/kg), and *T. laurifolia* leaf extract (TLE; 500-2500 mg/kg).

MO, acetic acid, formaldehyde, λ-carrageenan, naloxone, pentobarbitone sodium, and PGE_2 were dissolved in 0.9% NSS. IND and TLE were suspended in 2% (w/v) Tween 80 solution. RA was suspended in SW.

MO and IND were used as the standard analgesic drugs. IND was also used as a standard anti-inflammatory drug. The control animals were given with an equivalent volume of vehicle via the same route.

PLANT MATERIAL

T. laurifolia leaves were collected from Nakhonpathom Province, Thailand, and identified by Associate Professor Thatree Phadungcharoen, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The voucher specimen (SS-0510105) was deposited at the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

EXPERIMENTAL METHODS

Antinociceptive activity testing

Hot-plate test

Hot-plate test was conducted according to the method as previously described by Woolfe and Macdonald in 1944. Male ICR mice weighing 18-25 g were used and divided into groups of 10 animals. Mice were placed on the hot-plate (Harvard apparatus, USA; Figure 18) maintained the temperature at 55±0.5°C and were confined by a Plexiglas wall cylinder with 20 cm diameter and 30 cm height in order to keep the animal on the heated surface during testing. On the day of testing, animals were randomly assigned to one of seven or eight treatment groups and tested on the hot-plate for 3 pre-drug baseline trials conducted at 5-10 min intervals. The average of the last two pre-drug baseline trials was used as the baseline pre-drug latency measure for each animal. Only those animals which had a pretreatment hot-plate latency time less than 45 sec were used. Immediately, after the third baseline trial on the hot-plate, the animals were treated with NSS (10 ml/kg) or MO (10 mg/kg) intraperitoneally or 2% Tween 80 (10 ml/kg), SW (10 ml/kg), various doses of RA (12.5, 25, 50 100 and 150 mg/kg) or various doses of TLE (500, 1000, 1500, 2000, 2500 mg/kg) orally. The latency to the licking of a hind paw or jumping up from the surface of the hot-plate was

determined as the end point and recorded with a stopwatch. If this behavior was not observed within 45 sec, the animal was removed from the hot-plate to avoid tissue damage. The post drug latency was measured for 7 subsequent trials at 15, 30, 45, 60, 90, 120 and 240 min after drug administration.

The time-course of hot-plate latency was expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

$$\%\ \text{MPE} = \frac{(\text{post drug latency}) - (\text{pre-drug latency})}{(\text{cut-off time}) - (\text{pre-drug latency})} \times 100$$

where the cut-off time was set at 45 sec.

The area of analgesia for the hot-plate assays were obtained from computering the area under the corresponding 0-240 min time-course-%MPE curves; area were calculated using the trapezoidal rule (Tallarida and Murray, 1987).

Figure 18 Hot-plate analgesiometer (Harvard apparatus, USA)

Analysis of the mechanism of antinociceptive action of RA and TLE

The possible participation of the opioid system in the antinociceptive effect of RA and TLE were investigated using the model of mouse hot-plate test. Mice were pretreated with intraperitoneal injection of NAL (5 mg/kg), a short-acting opioid receptor

antagonist, 10 min before oral administration of RA (100 mg/kg) or TLE (2000 mg/kg) and the hot-plate latencies were measured.

Acetic acid-induced writhing test

Acetic acid-induced writhing in mice was conducted according to the method as previously described by Koster et al. in 1959. Male ICR mice weighing 18-25 g were used and divided into groups of 8 animals. On the day of testing, animals were randomly assigned to one of seven or eight treatment groups. Mice were pretreated with oral administration of 2% Tween 80 (10 ml/kg), IND (10 mg/kg), SW (10 ml/kg), various doses of RA (12.5, 25, 50 100 and 150 mg/kg) or various doses of TLE (500, 1000, 1500, 2000, 2500 mg/kg) 1 h before the intraperitoneal injection of 0.6% acetic acid solution at the volume of 10 ml/kg. The animals were then placed in an observation glass cylinder. The number of writhes (contraction of the abdominal muscles together with hind limb extension; Figure 19) were observed and counted at 5 min intervals for a period of 30 min after acetic acid administration. Antinociceptive activity was expressed as the percentage of inhibition of the writhing response compared with vehicle control group. The percentage of inhibition of the writhing response was calculated using the following formula:

$$\% \text{ Inhibition of writhing response} = \frac{W_r \text{ (control)} - W_r \text{ (test)}}{W_r \text{ (control)}} \times 100$$

where W_r = the mean number of writhing responses.

Figure 19 Writhing response.

Formalin test

Formalin test was conducted according to the method as previously described by Hunskaar and Hole in 1987. Male ICR mice weighing 18-25 g were used and divided into groups of 8 animals. Analgesic activity was determined using formalin-induced paw licking method. On the day of testing, animals were randomly assigned to one of seven or eight treatment groups. A 2.5% formalin solution (20 µL) was injected subcutaneously into the plantar surface of the left hind paw of each mouse 1 h after oral administration of 2% Tween 80 (10 ml/kg), IND (10 mg/kg), SW (10 ml/kg), various doses of RA (12.5, 25, 50 100 and 150 mg/kg) or various doses of TLE (500, 1000, 1500, 2000, 2500 mg/kg). Following the formalin injection, the animals were placed in an observation glass cylinder. Time of licking or biting the injected paw were recorded at 0-5 min and 15-30 min and expressed as the total number of paw licks in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection (Figure 20). The percentage of inhibition of early and late phases was analyzed using the following formula:

$$\% \text{ Inhibition of paw licking} = \frac{\text{Time (control)} - \text{Time (test)}}{\text{Time (control)}} \times 100$$

where time = mean time of paw licking (sec).

Figure 20 Formalin-induced paw licking.

Rota-rod test

To rule out the possibility of motor impairment or sedation effect of RA and TLE, a rota-rod test was performed as previously described by Dunham and Miya in 1957. Male ICR mice weighing 18-25 g were used and divided into groups of 8 animals. Mice were placed on a horizontal rod (35 mm.) rotating at a speed of 16 rpm (Ugo Basile, Italy; Figure 21). Mice capable of remaining on the rotating rod for 60 sec or more in three successive trials were selected for the study (Chattopadhyay et al., 2003). Each mouse was treated with oral administration of 2% Tween 80 (10 ml/kg), SW (10 ml/kg), RA (100 mg/kg) or TLE (2000 mg/kg) and placed on the rotating rod at 30, 60, 90, 120 and 240 min after drug administration. The results are expressed as the time in second for which the animal remained on the rota-rod (Pearl et al., 1969).

Figure 21 Rota-rod test.

Anti-inflammatory activity testing

Carrageenan-induced paw edema

The anti-inflammatory activity of RA and TLE were determined in a mouse model of carrageenan-induced paw edema as previously described by Levy in 1969. Male ICR mice weighing 18-25 g were used and divided into groups of 8 animals. On the day of testing, animals were randomly assigned to one of seven or eight treatment groups. Mice were pretreated with oral administration of 2% Tween 80 (10 ml/kg), IND (10 mg/kg), SW (10 ml/kg), various doses of RA (12.5, 25, 50, 100 and 150 mg/kg) or

various doses of TLE (500, 1000, 1500, 2000, 2500 mg/kg). One hour later, 1% carrageenan solution at the volume of 50 µL was injected subcutaneously into the plantar surface of the left hind paw of each mouse. Paw volume was measured using a plethysmometer (Ugo Basile, Italy; Figure 22) before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection. Edema was expressed as the mean increase in paw volume relative to the control. The percentage inhibition of paw edema was calculated using the following formula:

$$\% \text{ Inhibition of edema} = \frac{(V_c - V_t)}{V_c} \times 100$$

where V_c = edema volume in the control group; and V_t = edema volume in the test group.

Figure 22 Plethysmometer (Ugo Basile, Italy).

Analysis of the anti-inflammatory mechanism of action

The mechanism of anti-inflammatory activity of RA and TLE were examined using a mouse model of PGE_2-induced acute inflammation. Mice were pretreated with oral administration of 2% Tween 80 (10 ml/kg), IND (10 mg/kg), SW (10 ml/kg), RA (100 mg/kg) or TLE (2000 mg/kg). One hour later, animals were challenged by subcutaneous injection of 0.01% PGE_2 solution at the volume of 50 µL into the plantar surface of the left hind paw (Akkol et al., 2008; Castardo et al., 2008). Paw volume was

measured before and 0.5, 1, 1.5, 2, 3 and 4 h after PGE_2 administration. Edema was expressed as the mean increase in paw volume relative to the control. The percentage of inhibition of paw edema was analyzed using the formula as described above.

Cotton pellet-induced granuloma formation

The method was first described by Meier et al., in 1950. Male ICR mice weighing 25-30 g were used and divided into groups of 8 animals. On the day of testing, animals were randomly assigned to one of six treatment groups. Mice were anesthetized with pentobarbitone sodium (40 mg/kg). The back skin was shaved and sterile cotton pellets (weight = 10±0.5 mg) were implanted into subcutaneous tissue on the back. Animals were treated orally with 2% Tween 80 (10 ml/kg), IND (10 mg/kg), SW (10 ml/kg) or various doses of RA (50, 100 and 150 mg/kg) once daily for 7 consecutive days. On day 8, the mice were sacrificed and pellets surrounded with granulomatous tissue were removed and dried to constant weight at 60°C (Figure 23). The weight of granuloma was determined and the percentage of inhibition of granuloma was analyzed using the following formula:

$$\% \text{ Inhibition of weight of granuloma} = \frac{W_g \text{ (control)} - W_g \text{ (test)}}{W_g \text{ (control)}} \times 100$$

where W_g = mean weight of granuloma (mg).

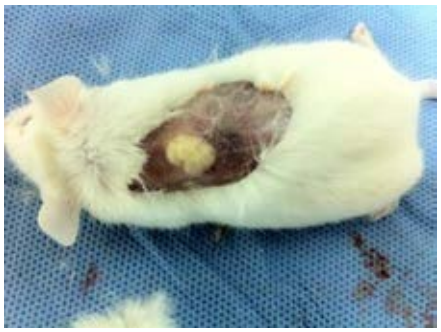


Figure 23 Cotton pellet induced granuloma in mice

ACUTE TOXICITY

Animals employed in the study were observed for 72 hs and morbidity or mortality was recorded, if happens, for each group at the end of observation period.

DATA TREATMENT AND STATISTICAL ANALYSIS

Results are expressed as the mean±S.E.M. Data were analyzed by Student's *t-test* and one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey test for multiple comparisons. The minimum level for significance was set at $p<0.05$.

CHAPTER IV
RESULTS

HOT-PLATE TEST

To demonstrate the validity of the hot-plate analgesic testing following drug administration, mice received MO (10 mg/kg; i.p.) and were tested during the subsequent 240 min period. As expected MO 10 mg/kg significantly (p<0.001) increased the hot-plate latency producing an area of analgesia at 15,292±734.84 %MPE-min compared with that of normal saline solution (NSS) (70.32±230.29 %MPE-min; Figure 24).

Initial studies utilizing the hot-plate test in mice to examine the efficacy of rosmarinic acid (RA) and *T. laurifolia* leaf extract (TLE) in producing analgesia. Mice were administered orally 2% Tween 80 (10 ml/kg), SW (10 ml/kg), various doses of RA (12.5-150 mg/kg), or various doses of TLE (500-2500 mg/kg). RA at doses of 50, 100 and 150 mg/kg significantly (p<0.001) increased the hot-plate latencies when compared to the vehicle group. RA 50 mg/kg significantly (p<0.001 and p<0.01, respectively) increased the hot-plate latencies when compared to RA 12.5 and 25 mg/kg. RA 100 mg/kg significantly (p<0.001, p<0.001, p<0.01, and p<0.01, respectively) increased the hot-plate latencies when compared to RA 12.5, 25, 50, and 150 mg/kg. RA 150 mg/kg significantly (p<0.001 and p<0.001, respectively) increased the hot-plate latencies when compared to RA 12.5 and 25 mg/kg. RA 100 mg/kg appears to be the most effective dose as shown in Figure 25. The analgesic peak effects of RA at doses of 50, 100 and 150 mg/kg were reached within 240, 240 and 30 min after oral administration, respectively. Individual time courses of the responses are shown in Figure 27.

All doses of TLE (500, 1000, 1500, 2000, and 2500 mg/kg) significantly (p<0.05, p<0.001, p<0.001, p<0.001, and p<0.001, respectively) increased the hot-plate latencies when compared to the vehicle group (Figure 26). TLE 2000 mg/kg significantly (p<0.05) increased the hot-plate latencies when compared to TLE 500 mg/kg. The analgesic peak effects of TLE at doses of 500, 1000, 1500, 2000, and 2500 mg/kg were

reached within 240, 240, 90, 240 and 240 min after oral administration, respectively. Individual time courses of the responses are shown in Figure 28.

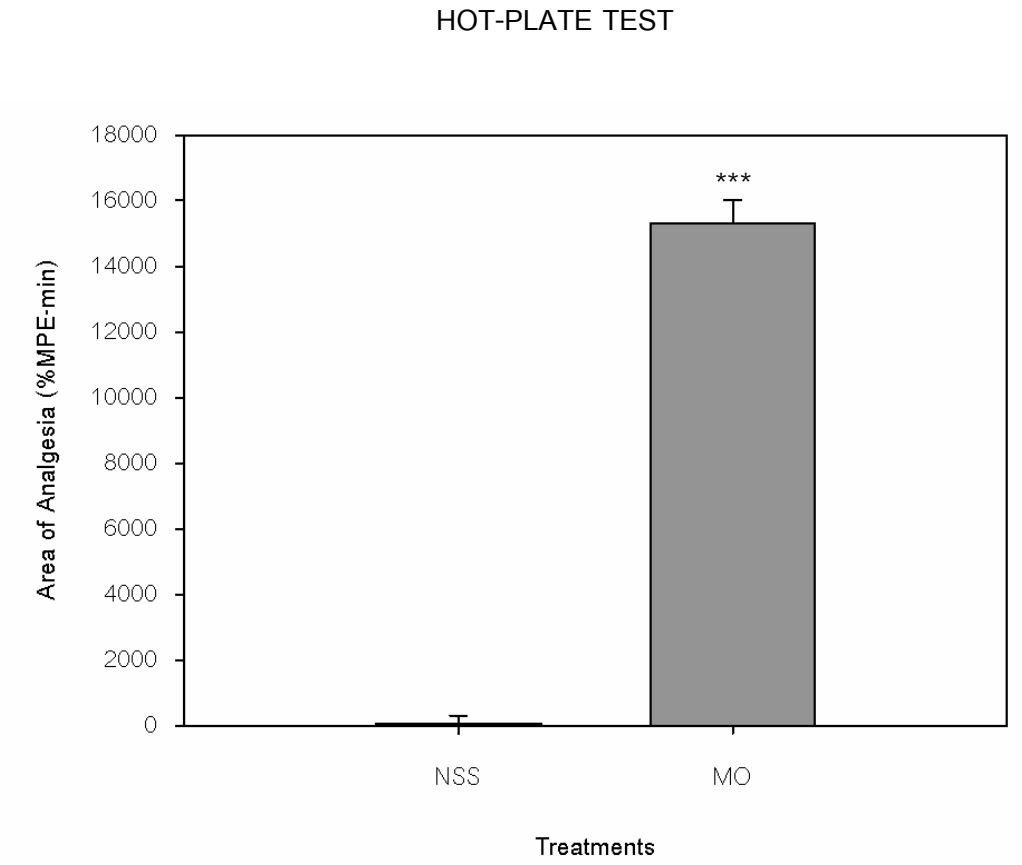


Figure 24 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of normal saline solution (NSS; 10 ml/kg) and morphine sulfate (MO; 10 mg/kg). N=10 for all groups.

***p<0.001 significantly different compared to NSS.

.

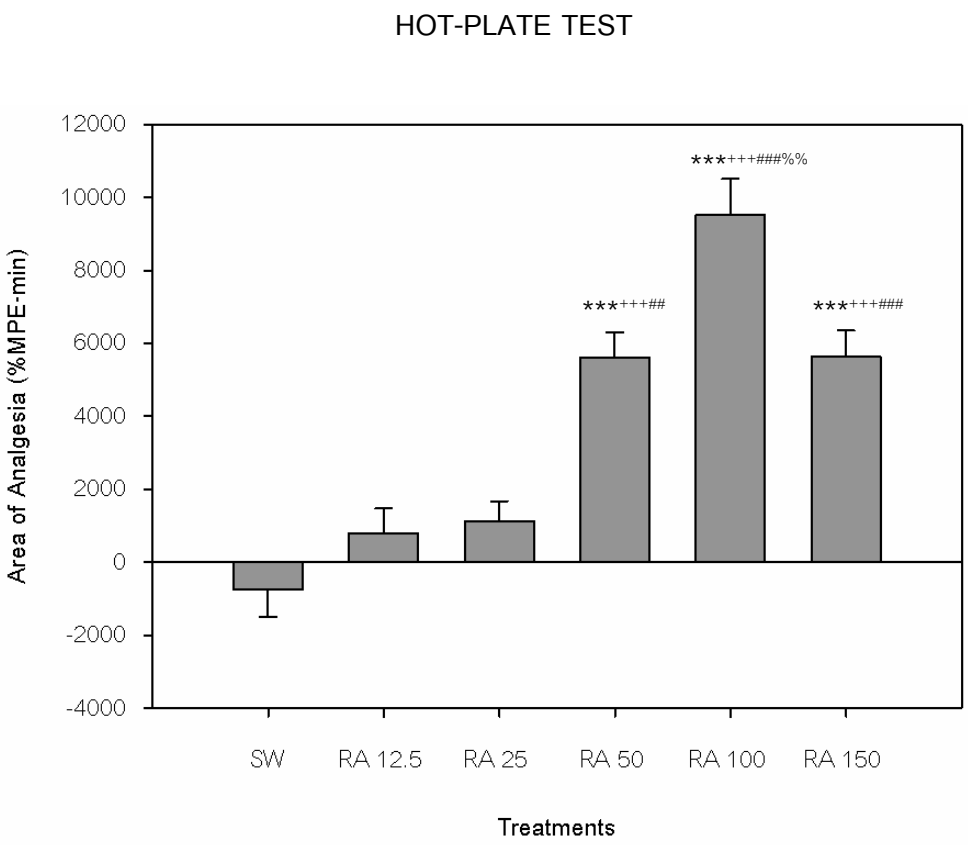


Figure 25 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=10 for all groups.

***$p<0.001$ significantly different compared to SW.

$^{+++}p<0.001$ significantly different compared to RA 12.5 mg/kg.

$^{##}p<0.01$, $^{###}p<0.001$ significantly different compared to RA 25 mg/kg.

$^{\%\%}p<0.01$ significantly different compared to RA 50 mg/kg.

HOT-PLATE TEST

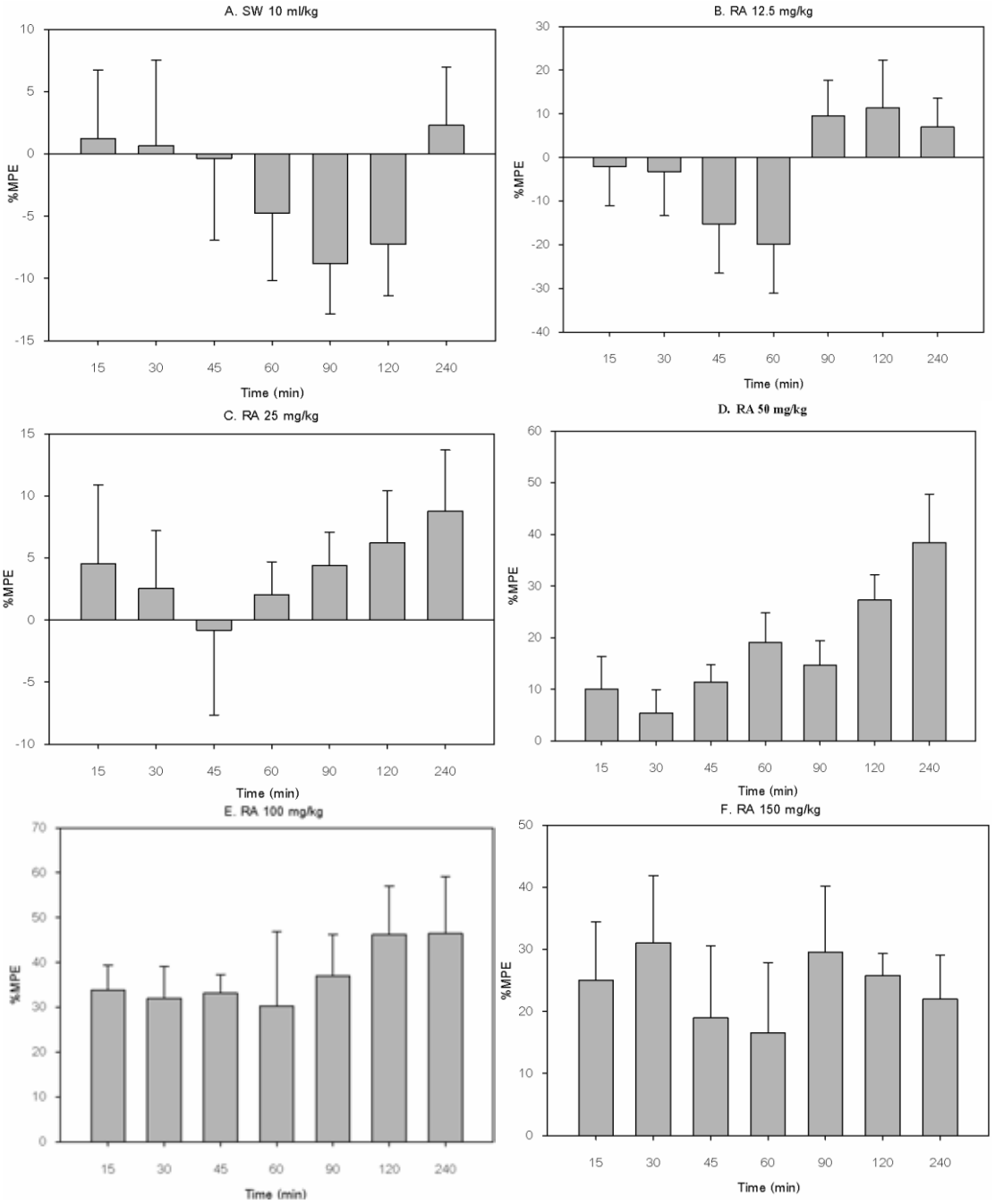


Figure 26 Individual time courses of the response (% MPE versus time (min)) after oral administration of sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5 -150 mg/kg). N=10 for all groups.

A. SW 10 ml/kg, B. RA 12.5 mg/kg, C. RA 25 mg/kg, D. RA 50 mg/kg, E. RA 100 mg/kg

F. RA 150 mg/kg

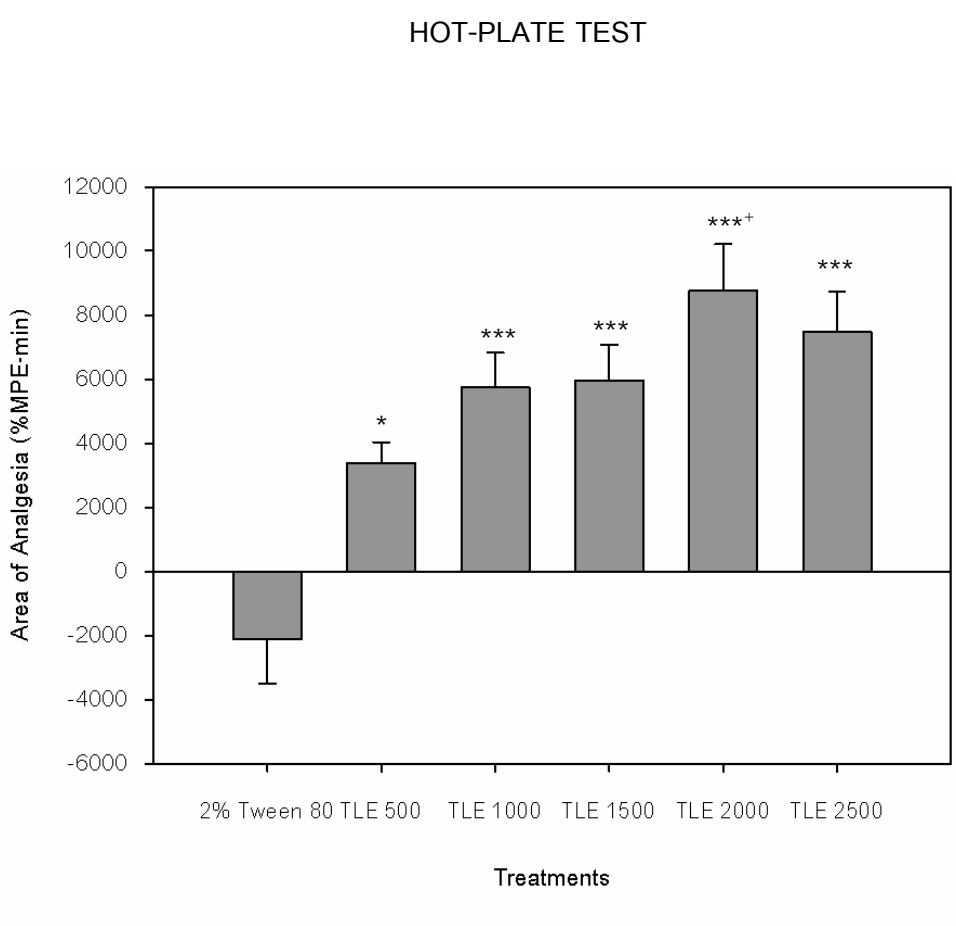


Figure 27 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=10 for all groups.

*$p<0.05$, ***$p<0.001$ significantly different compared to 2% Tween 80.

$^{+}p<0.05$ significantly different compared to TLE 500 mg/kg.

HOT-PLATE TEST

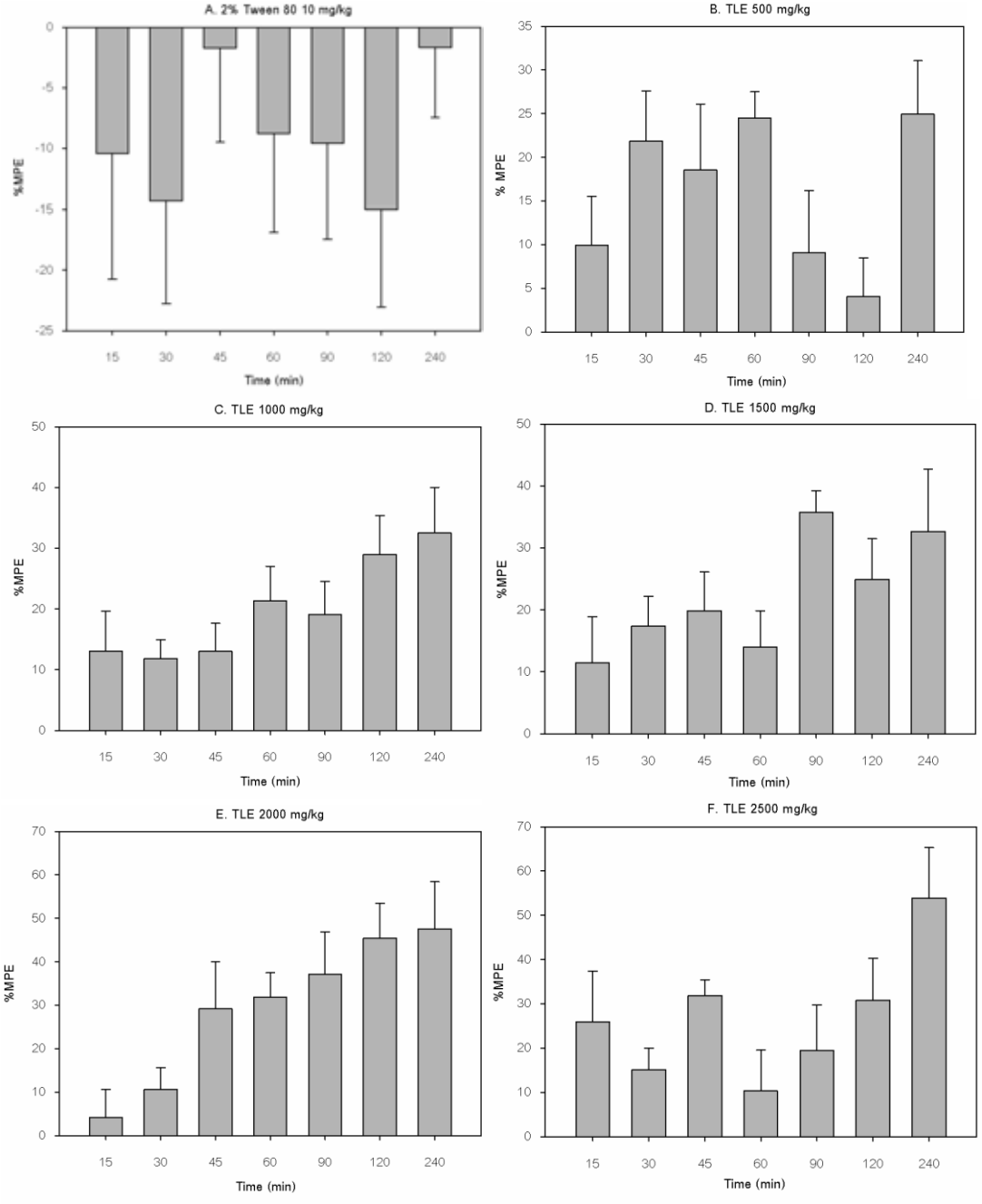


Figure 28 Individual time courses of the response (%MPE versus time (min)) after oral administration of 2% Tween 80 and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=10 for all groups.

A. 2% Tween 80 10 mg/kg, B. TLE 500 mg/kg, C. TLE 1000 mg/kg, D. TLE 1500 mg/kg,

E. TLE 2000 mg/kg, F. TLE 2500 mg/kg

Analysis of the mechanism of antinociceptive action of RA and TLE

The possible participation of the opioid system in the antinociceptive effects of RA and TLE actions were investigated using the model of mouse hot-plate test. Mice were then administered NSS (10 ml/kg; i.p.), NAL (5 mg/kg; i.p.), 2% Tween 80 (10 mg/kg; p.o.), SW (10 ml/kg; p.o.), RA (100 mg/kg; p.o.), TLE (2000 mg/kg; p.o.) or the combination of NAL and RA (5/100 mg/kg) and the combination of NAL and TLE (5/2000 mg/kg). NAL alone failed to produce significant response when compared to vehicle group. RA and TLE at the dose tested produced significant ($p<0.001$) response when compared to the vehicle group. The inclusion of naloxone with RA and TLE significantly ($p<0.01$, $p<0.001$, respectively) attenuated the analgesic response due to RA and TLE indicating the involvement of opioid receptors in the analgesic response produced by RA and TLE (Figure 29 and 31). Individual time courses of the responses are shown in Figure 30 and 32.

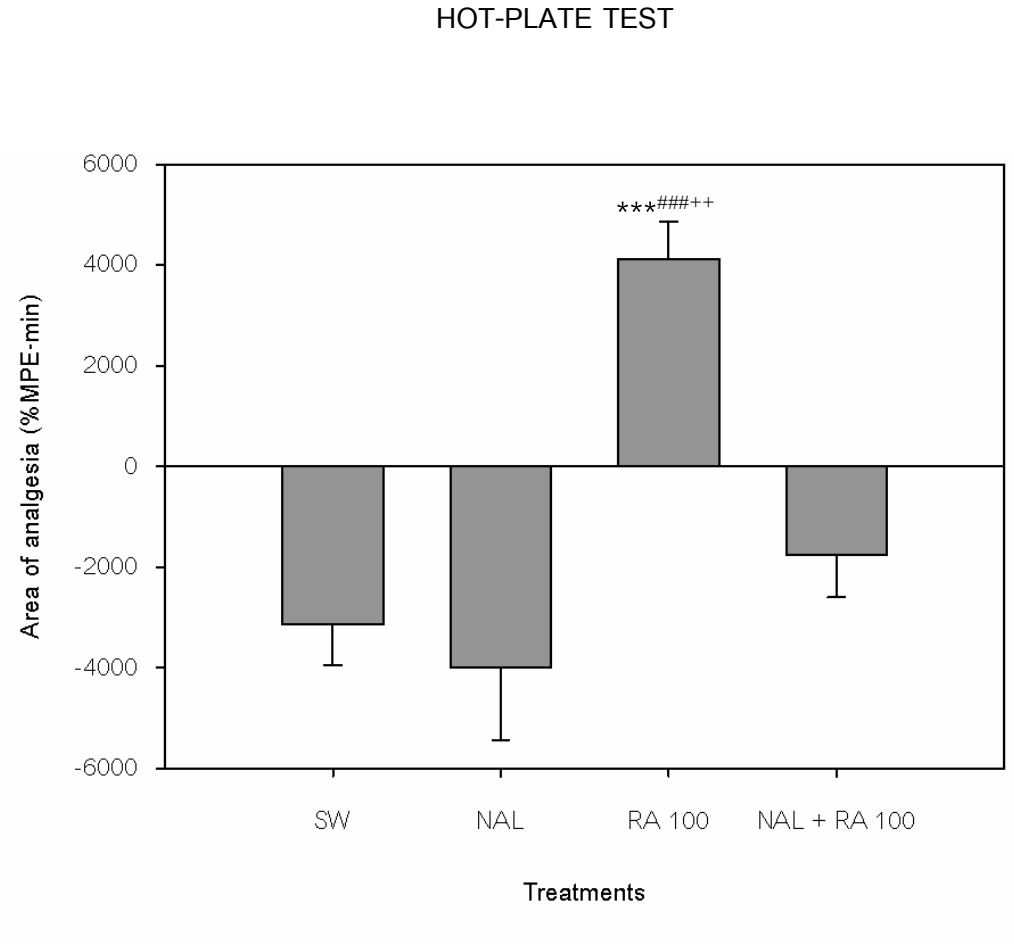


Figure 29 Area of analgesia (%MPE-min) from 0-240 minutes after administration of sterile water (SW; 10 ml/kg, p.o.), naloxone (NAL; 5 mg/kg, i.p.), rosmarinic acid (RA; 100 mg/kg, p.o.) and the combination of NAL and RA (5/100 mg/kg).

N=10 for all groups.

***p<0.001 significantly different compare to SW.

###p<0.001 significantly different compare to NAL.

++p<0.01 significantly different compare to NAL+RA 100.

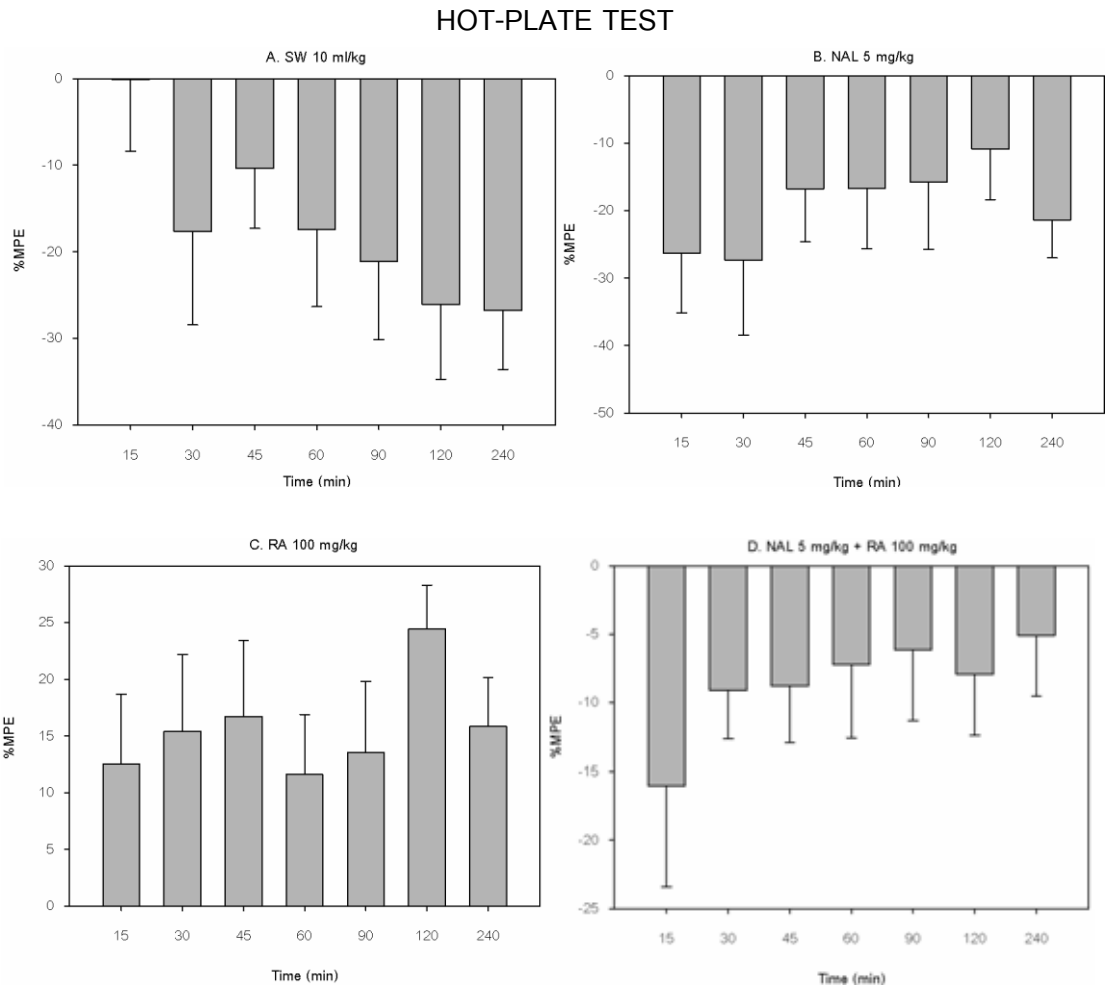


Figure 30 Individual time course of the response (%MPE versus time (min)) after administration of sterile water (SW; 10 ml/kg, p.o.), naloxone (NAL; 5 mg/kg, i.p.), rosmarinic acid (RA; 100 mg/kg, p.o.) and the combination of NAL and RA (5/100 mg/kg). N=10 for all groups.

A. SW 10 ml/kg, B. NAL 5 mg/kg, C. RA 100 mg/kg, D. NAL and RA (5/100 mg/kg).

HOT-PLATE TEST

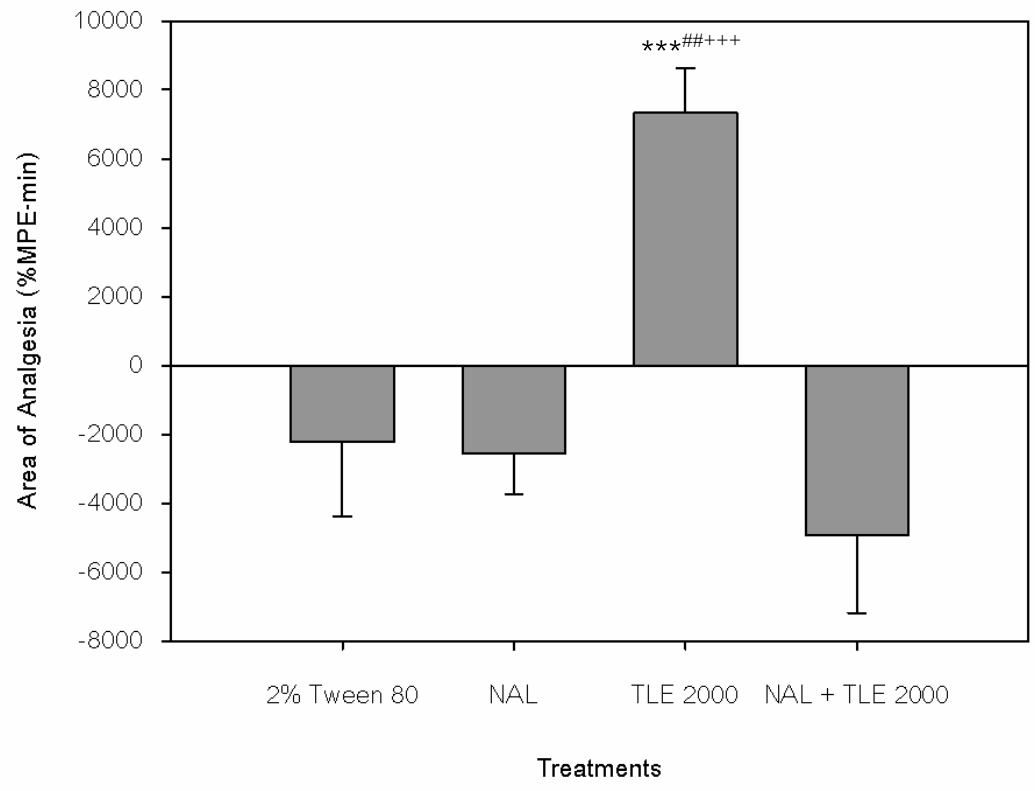


Figure 31 Area of analgesia (%MPE-min) from 0-240 minutes after administration of 2% Tween 80 (10 mg/kg; p.o.), naloxone (NAL; 5 mg/kg, i.p), *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg, p.o.) and the combination of NAL and TLE (5/2000 mg/kg). N=10 for all groups.

***$p<0.001$ significantly different compare to 2% Tween 80.

##$p<0.01$ significantly different compare to NAL.

+++$p<0.001$ significantly different compare to NAL+TLE 2000.

HOT-PLATE TEST

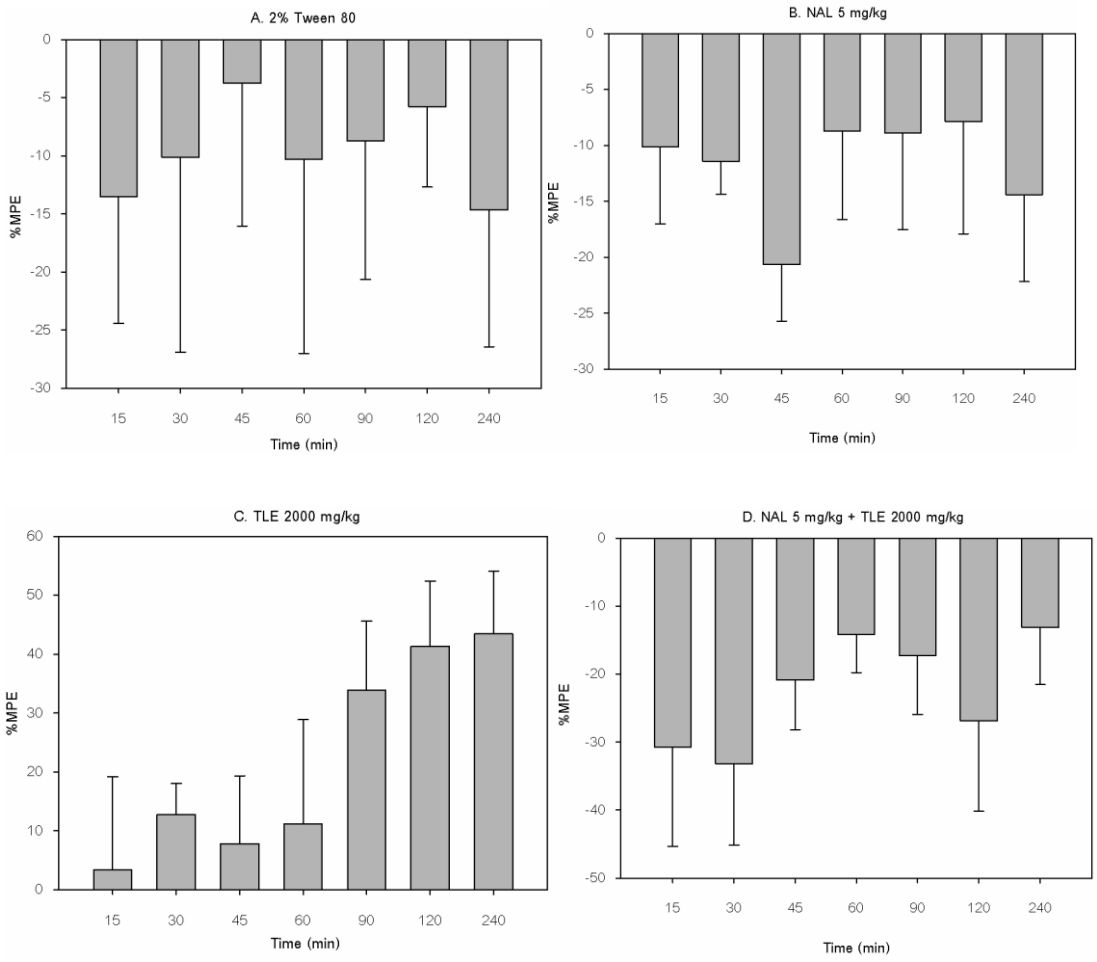


Figure 32 Individual time course of the response (%MPE versus time (min)) after administration of 2% Tween 80 (10 ml/kg; p.o.), naloxone (NAL; 5 mg/kg, i.p.), *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg, p.o.) and the combination of NAL and TLE (5/2000 mg/kg). N=10 for all groups.

A. 2% Tween 80 10 mg/kg, B. NAL 5 mg/kg, C. TLE 2000 mg/kg, D. NAL and TLE (5/2000 mg/kg).

ACETIC ACID-INDUCED WRITHING TEST

Studies then utilized the acetic acid-induced writhing method to examine the analgesic efficacy of RA and TLE. Each mouse was administered orally 2% Tween 80 (10 mg/kg), SW (10 ml/kg), IND (10 mg/kg), various doses of RA (12.5-150 mg/kg) or various doses of TLE (500-2500 mg/kg).

To demonstrate the validity of acetic acid-induced writhing method, IND 10 mg/kg was used as a positive control. As expected IND significantly ($p<0.01$) decreased writhing response by 60.81% compared with 2%Tween 80. RA at doses of 50 and 100 mg/kg significantly ($p<0.01$, $p<0.001$, respectively) decreased the number of writhes induced by acetic acid by 52.05% and 84.53% when compared to the vehicle control. RA at 50 mg/kg significantly ($p<0.05$) decreased the number of writhes when compared to RA 12.5 mg/kg. RA at 100 mg/kg significantly ($p<0.001$) decreased the number of writhes when compared to RA 12.5, 25 and 150 mg/kg. RA at doses of 12.5 and 25 mg/kg significantly ($p<0.05$) decreased the number of writhes when compared to IND. RA at 50 mg/kg showed an antinociceptive activity comparable to that of IND (Figure 33).

TLE at doses of 1000, 1500, 2000 and 2500 mg/kg significantly ($p<0.01$, $p<0.001$, $p<0.001$ and $p<0.001$, respectively) decreased the number of writhes by 32.11%, 45.04%, 47.81% and 67.89%, respectively when compared to the vehicle control. TLE 2500 mg/kg significantly decreased the number of writhes ($p<0.001$, $p<0.05$, respectively) when compared to TLE 500 and 1000 mg/kg. TLE 1500 and 2000 mg/kg significantly ($p<0.05$) decreased the number of writhes when compared to TLE 500 mg/kg. TLE 500, 1000, and 1500 mg/kg significantly ($p<0.001$, $p<0.001$, and $p<0.05$, respectively) decreased the number of writhes when compared to IND. IND significantly ($p<0.001$) decreased the number of writhes by 71.59% when compared to the vehicle control. TLE at 2500 mg/kg showed an antinociceptive activity comparable to that of IND (Figure 34).

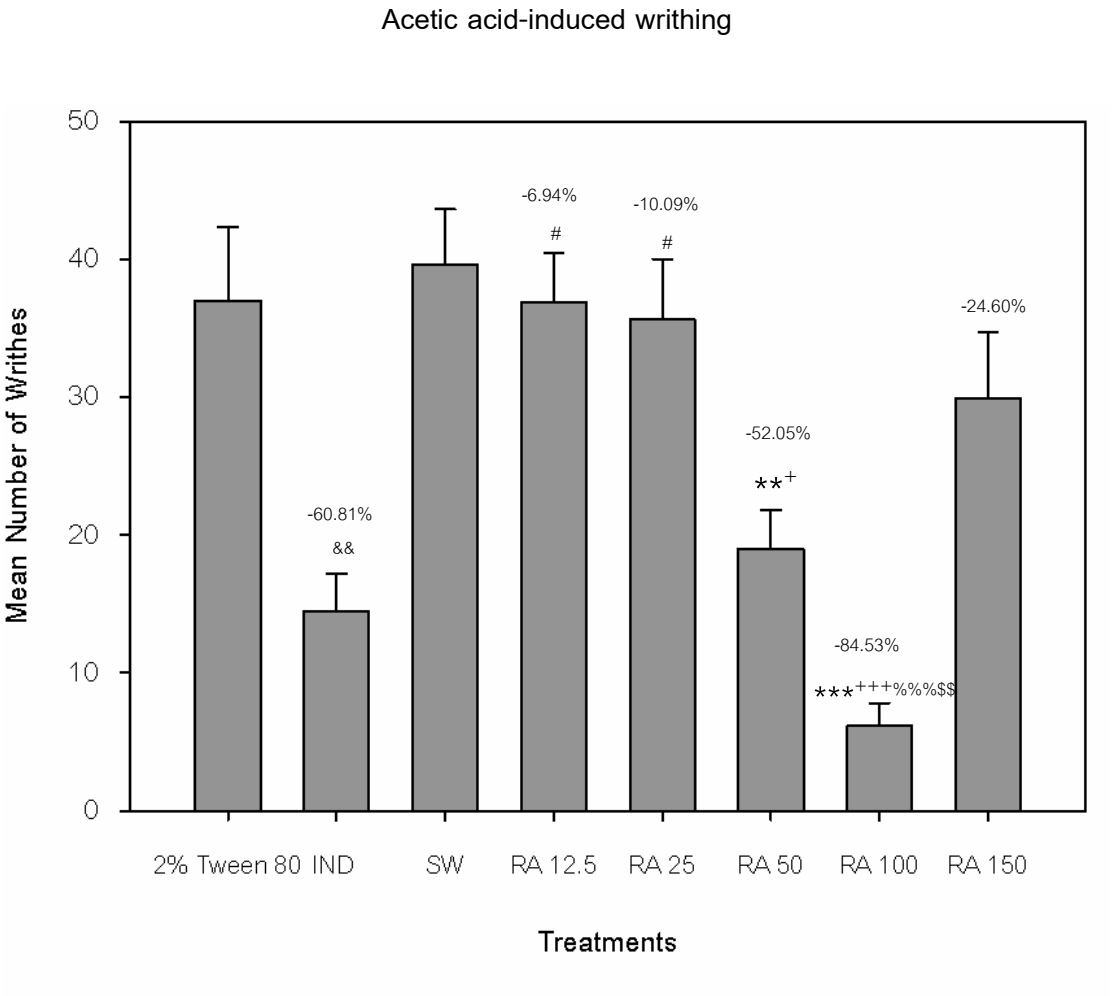


Figure 33 Mean number of writhes after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=8 for all groups.

&&$p<0.01$ significantly different compared to 2% Tween 80.

**$p<0.01$, ***$p<0.001$ significantly different compared to SW.

#$p<0.01$ significantly different compared to IND 10 mg/kg.

+$p<0.05$, +++$p<0.001$ significantly different compared to RA 12.5 mg/kg.

%%%$p<0.001$ significantly different compared to RA 25 mg/kg.

$$$p<0.001$ significantly different compared to RA 150 mg/kg.

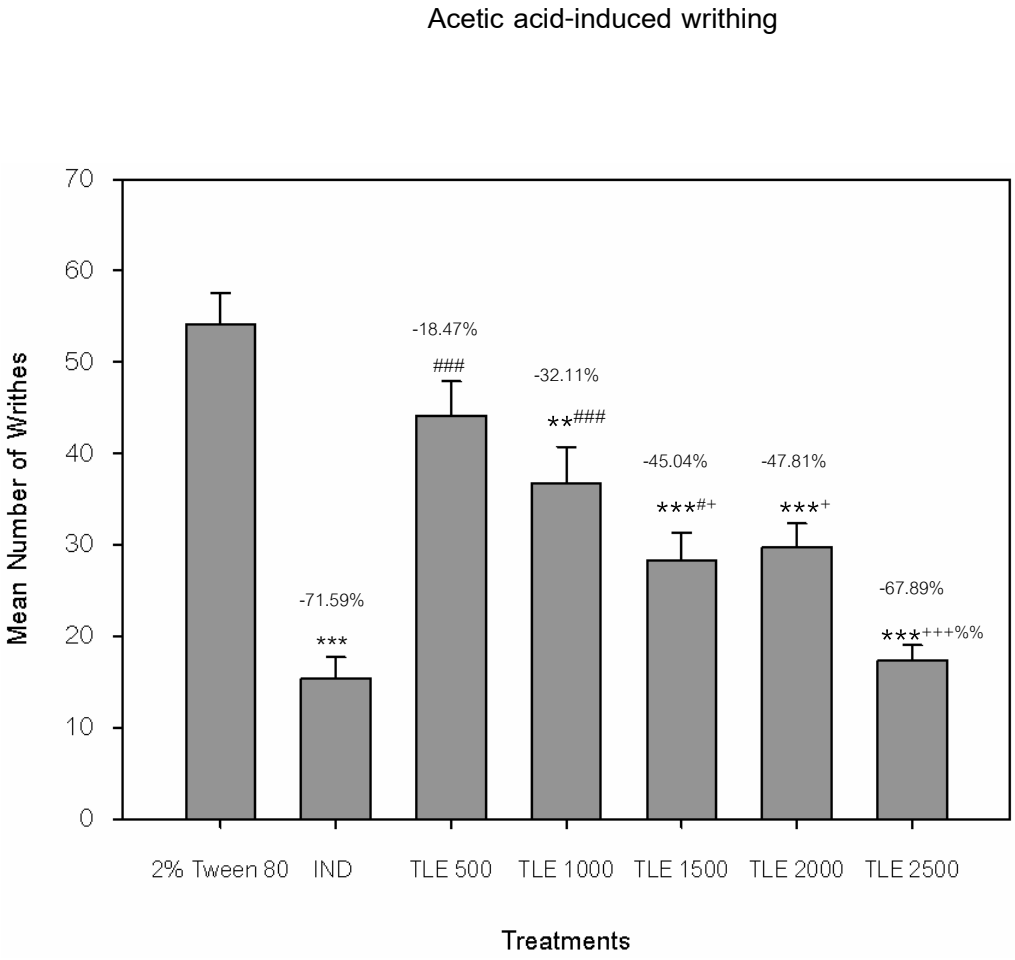


Figure 34 Mean number of writhes after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500 -2500 mg/kg). N=8 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\#}p<0.05$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

$^{+}p<0.05$, $^{+++}p<0.001$ significantly different compared to TLE 500 mg/kg

$^{\%\%}p<0.01$ significantly different compared to TLE 1000 mg/kg

FORMALIN TEST

To demonstrate the validity of formalin test following drug administration, mice were received MO (10 mg/kg) intraperitoneally or IND (10 mg/kg) orally and observed for paw licking in the early phase (0-5 min) and late phase (15-30 min). As expected MO significantly (p<0.001) decreased the licking time in both the early and late phases of the response by 89.24% and 95.81%, respectively producing mean time spent on paw licking of 9.45±1.60 and 5.51±1.18 sec compared with that of NSS (87.80±4.09 and 131.60±6.59 sec, respectively; Figure 35). IND significantly (p<0.001) reduced the time of licking and biting of injected paws during the late phase compared with that of the vehicle group (Figure 36-37). However, nociception during the early phase appeared to be unaffected by indomethacin.

Study then utilized the formalin test in mice to examine the efficacy of RA and TLE in producing analgesia. Mice were administered orally 2% Tween 80 (10 mg/kg), SW (10 ml/kg), IND (10 mg/kg), various doses of RA (12.5-150 mg/kg) or various doses of TLE (500-2500 mg/kg).

RA at 100 mg/kg caused significant inhibition of both phases of formalin-induced nociception by 55.36% and 58.76%, respectively compared to the vehicle group (p<0.01, p<0.001, respectively). Additionally, RA at 100 mg/kg significantly (p<0.001, p<0.001 and p<0.05, respectively) decreased the licking time compared with that of RA at doses of 12.5, 25, and 50 mg/kg during the early phase and significantly decreased the licking time compared with that of RA at doses of 12.5, 25, 50, and 150 mg/kg during the late phase. RA at 150 mg/kg significantly (p<0.05) reduced time spent on paw licking and biting during the early phase compared with that of RA at doses of 12.5 and 25 mg/kg. The reference drug, IND caused significant (p<0.001) inhibition by 68.42% during the late phase of formalin-induced nociception when compared to the vehicle group. The inhibitory effect of RA at 100 mg/kg on formalin-induced nociception during the late phase was comparable to that of IND. (Figure 36).

TLE at 1500 mg/kg significantly (p<0.001) decreased the licking time of both phase by 22.29% and 24.97%, respectively compared to the vehicle group. TLE at 2000

mg/kg significantly (p<0.001) decreased the licking time of both phases by 28.88% and 45.84%, respectively compared to the vehicle group. TLE at 2000 mg/kg also significantly (p<0.001, p<0.05 and p<0.01, respectively) reduced the licking time during the early phase when compared to TLE at doses of 500, 1000 and 2500 mg/kg and significantly (p<0.001, p<0.001 and p<0.05, respectively) reduced the licking time during the late phase when compared to TLE at doses of 500, 1000 and 1500 mg/kg. TLE at 2500 mg/kg significantly (p<0.001) decreased the licking time by 27.65% compared to the vehicle group and also significantly (p<0.001 and p<0.01, respectively) decreased the licking time compared to TLE at doses of 500 and 1000 mg/kg during the late phase. IND caused significant (p<0.001) inhibition of the late phase of formalin-induced nociception by 44.11% when compared to the vehicle group. The antinociceptive efficacy of TLE 2000 mg/kg on formalin-induced nociception during the late phase was comparable to that of IND (Figure 37).

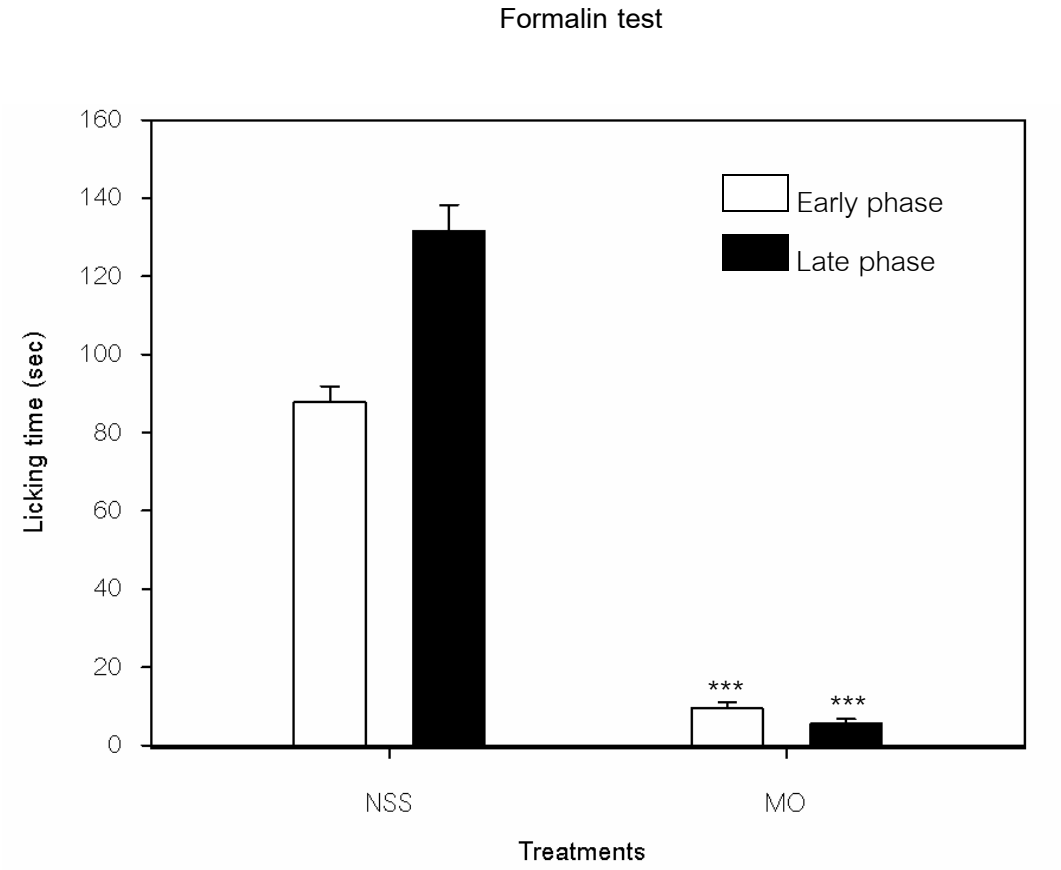


Figure 35 Time spent on paw licking after intraperitoneal administration of 0.9% normal saline solution (NSS; 10 ml/kg) and morphine sulfate (MO; 10 mg/kg). N=8 for all groups.

***p<0.001 significantly different compared to normal saline solution.

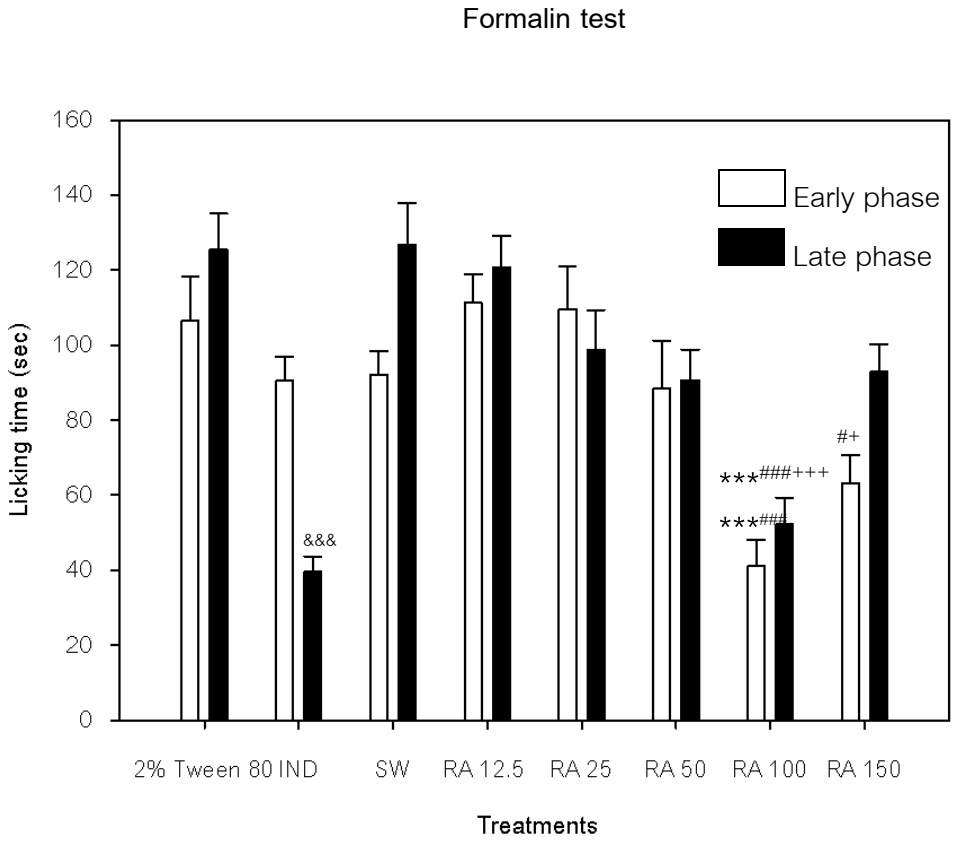


Figure 36 Time spent on paw licking after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=8 for all groups.

$^{\&\&\&}p<0.001$ significantly different compared to 2% Tween 80.

$^{***}p<0.001$ significantly different compared to sterile water.

$^{\#}p<0.05$, $^{\#\#\#}p<0.001$significantly different compared to RA 12.5 mg/kg.

$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$significantly different compared to RA 25 mg/kg.

$^{\%}p<0.05$ significantly different compared to RA 50 mg/kg.

$^{\$}p<0.05$ significantly different compared to RA 150 mg/kg.

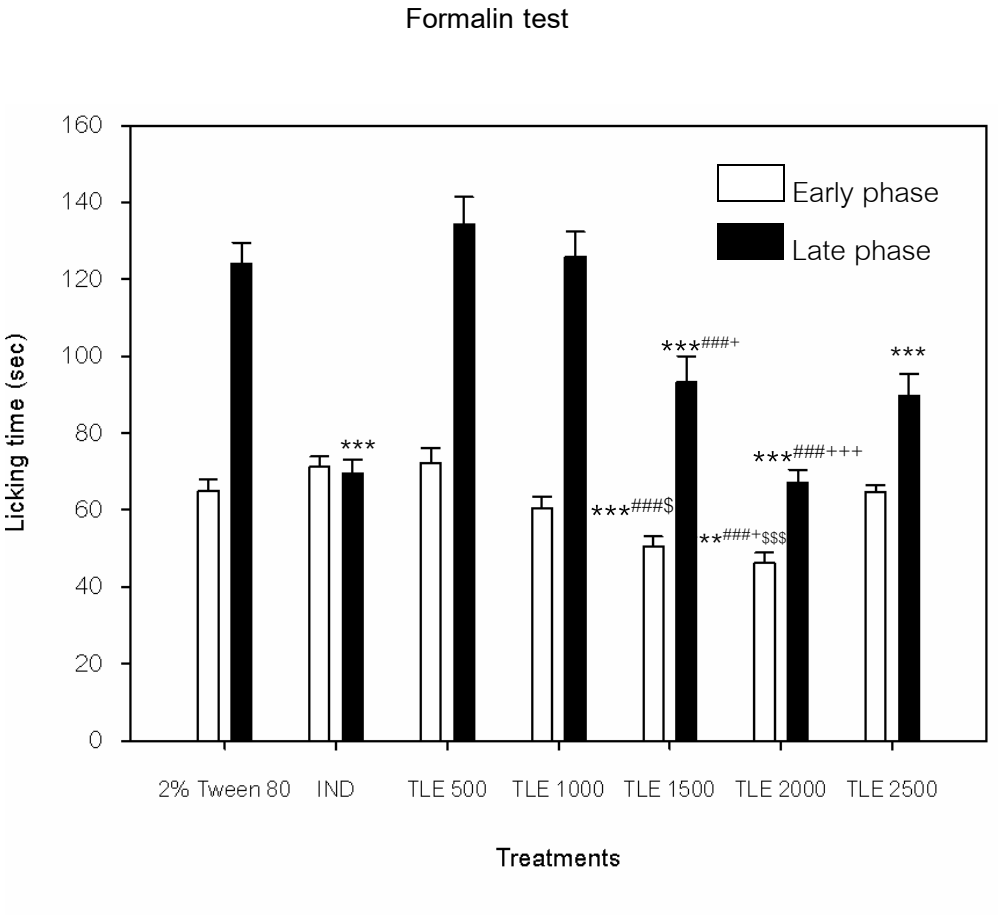


Figure 37 Time spent on paw licking after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg) and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=8 for all groups.

***$p<0.001$ significantly different compared to 2% Tween 80.

###$p<0.001$ significantly different compared to TLE 500 mg/kg.

+$p<0.05$, ++$p<0.01$, +++$p<0.001$ significantly different compared to TLE 1000 mg/kg.

$$p<0.05$, $$$ $p<0.01$significantly different compared to TLE 2500 mg/kg.

ROTA-ROD TEST

In order to determine the effect of RA and TLE on motor response, mice were orally administered SW (10 ml/kg), RA (100 mg/kg), 2% Tween 80, or TLE (2000 mg/kg) and tested on the rota-rod apparatus for 5 subsequent trials at 30, 60, 90, 120 and 240 min after drug administration. The results showed that oral administration of RA at 100 mg/kg and TLE at 2000 mg/kg did not affect the motor responses of the animals on the rota-rod test at all time tested (Figure 38-39).

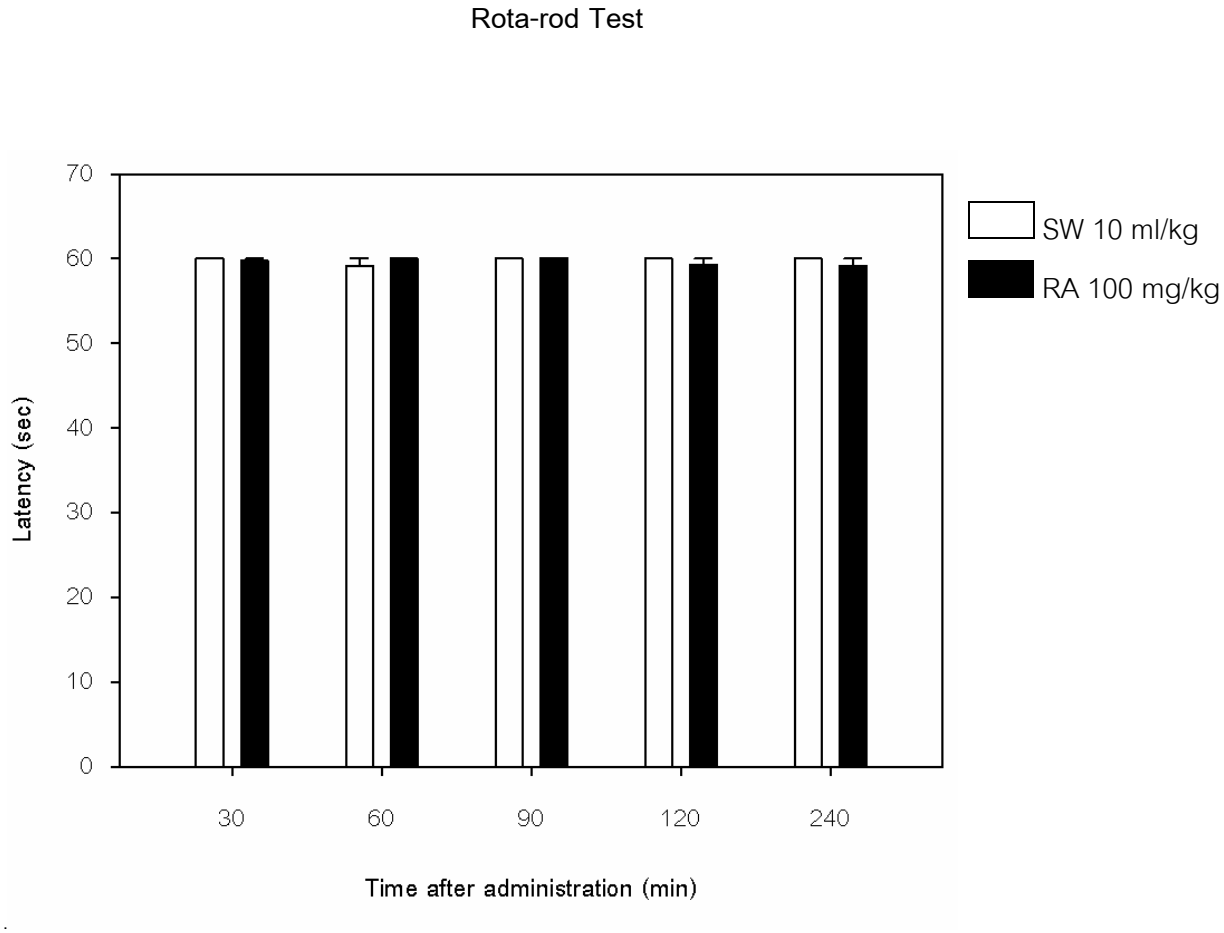


Figure 38 Rota-rod latency of rosmarinic acid (RA; 100 mg/kg) after oral administration compared to sterile water (SW; 10 ml/kg). N=8 for all groups.

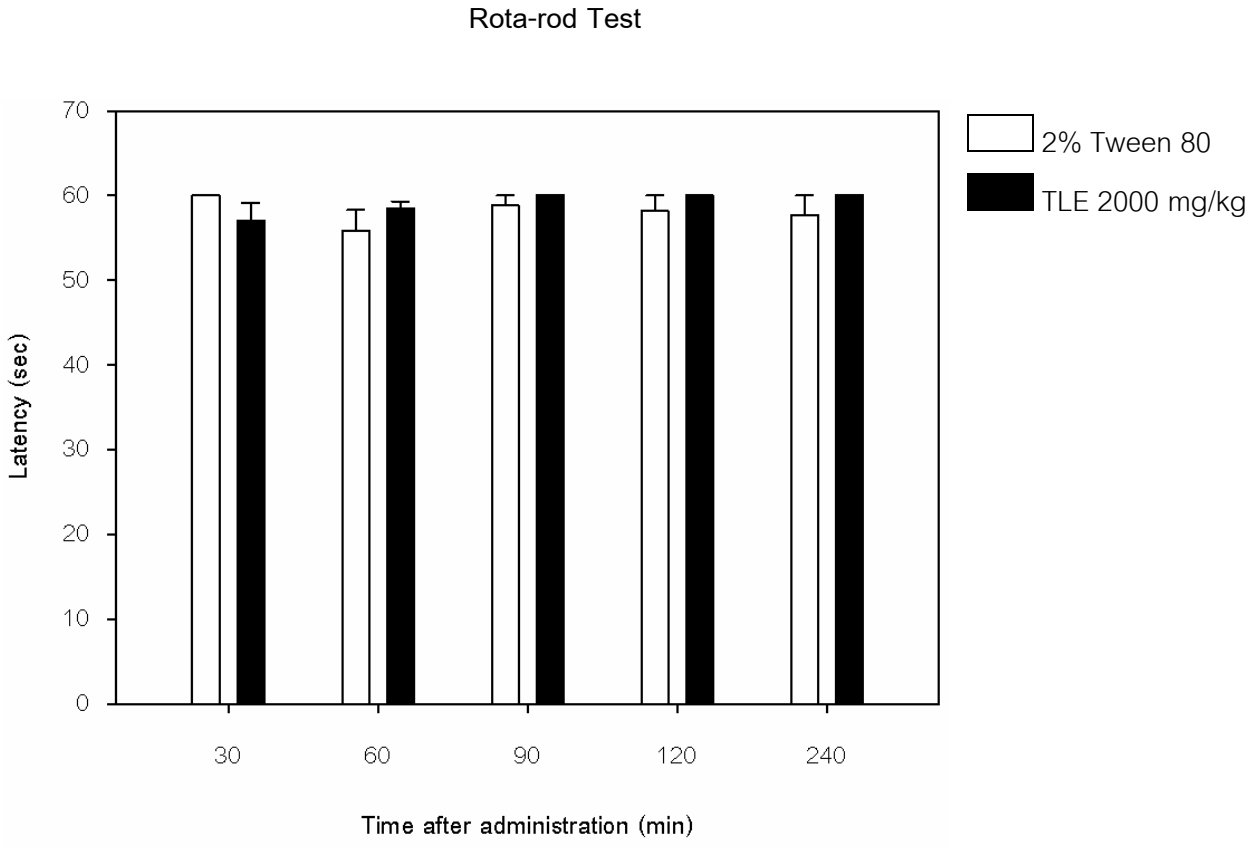


Figure 39 Rota-rod latency of *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg) after oral administration compared to 2% Tween 80 (10 mg/kg). N=8 for all groups.

CARRAGEENAN-INDUCED PAW EDEMA

Studies then utilized the carrageenan-induced mouse paw edema test to examine the anti-inflammatory efficacy of RA and TLE. Each mouse was administered orally 2% Tween 80 (10 mg/kg), SW (10 ml/kg), various doses of RA (12.5-150 mg/kg) or various doses of TLE (500-2500 mg/kg).

To demonstrate the validity of the carrageenan-induced mouse paw edema test, IND 10 mg/kg was used as a positive control. As expected IND caused a significant reduction of hind paw edema at 2, 3, 4, 5 and 6 h after carrageenan administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 68.29% at 4 h. RA at 50 mg/kg produced a significant ($p<0.05$) decreased in paw edema induced by carrageenan and produced an inhibition of paw edema of 51.32% only at 3 h after carrageenan administration compared with that of sterile water. However, RA 100 mg/kg significantly ($p<0.01$, $p<0.05$, $p<0.01$ and $p<0.05$, respectively) decreased paw edema at 3, 4, 5 and 6 h compared with that of the vehicle control. The percentage of inhibition of RA at 100 mg/kg appears to be sustainable during 3-6 hs after carrageenan injection and produced a maximum inhibition of paw edema of 57.04% at 3 h. In general, IND appeared to have a superior inhibitory activity on carrageenan-induced paw edema compared to RA at all doses used (Table 2).

All doses of TLE (500, 1000, 1500, 2000, and 2500 mg/kg) significantly decreased paw edema during 4-6 hs after carrageenan injection compared to 2% Tween 80. TLE at 500 mg/kg produced a significant ($p<0.05$, $p<0.01$, and $p<0.001$, respectively) inhibition of paw edema of 35.63%, 32.40% and 38.47%, respectively at 4, 5, and 6 h compared to 2% Tween 80. TLE at 1000 mg/kg produced a significant ($p<0.01$, $p<0.01$, and $p<0.001$, respectively) decrease in paw edema of 36.18%, 36.79%, and 37.45% , respectively at 4, 5, and 6 h compared to 2% Tween 80. TLE at 1500 mg/kg produced a significant ($p<0.05$, $p<0.05$, and $p<0.01$, respectively) decrease in paw edema of 32.74%, 28.57%, and 35.40% , respectively at 4, 5, and 6 h compared to 2% Tween 80. TLE at 2000 mg/kg produced a significant ($p<0.001$, $p<0.001$, and $p<0.001$, respectively) decrease in paw edema of 49.43%, 48.88%, and

47.70%, respectively at 4, 5, and 6 h compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 49.43% at 4 h. TLE at 2500 mg/kg produced a significant ($p<0.01$, $p<0.01$, and $p<0.001$, respectively) decrease in paw edema of 41.93%, 39.03%, and 40.53%, respectively at 4, 5, and 6 h compared to 2% Tween 80. Compared to the vehicle group, IND at 10 mg/kg also caused significant ($p<0.001$) reduction of hind paw edema at 2, 3, 4, 5 and 6 h after carrageenan administration and showed a maximum inhibition of paw edema of 74.71% at 4 h. IND appeared to have a superior inhibitory activity on carrageenan-induced paw edema compared to TLE at all doses used (Table 3).

Table 2 Change of edema volume (ml) of oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), SW (10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg) during 1-6 h after carrageenan administration.

N=8 for all groups. Inhibition is reported as percentage compared to the vehicle control.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different compared to the vehicle control.

Treatments (mg/kg)	Paw edema (ml)±S.E.M. (% inhibition)					
	1 h	2 h	3 h	4 h	5 h	6 h
2% Tween 80	0.1587±0.0159	0.2025±0.0180	0.2075±0.0226	0.2012±0.0165	0.2338±0.0122	0.2500±0.0226
IND 10 mg/kg	0.1050±0.0154 (-33.84%)	0.0800±0.0046*** (-60.49%)	0.0750±0.0093*** (-63.86%)	0.0638±0.0116*** (-68.29%)	0.0938±0.0122*** (-59.88%)	0.0925±0.0113*** (-63.00%)
SW	0.1438±0.0155	0.1650±0.0177	0.1513±0.0186	0.1425±0.0202	0.1813±0.0200	0.1775±0.0194
RA 12.5 mg/kg	0.0950±0.0176 (-33.93%)	0.1363±0.0206 (-17.39%)	0.1113±0.0182 (-26.43%)	0.1413±0.0226 (-0.84%)	0.1350±0.0192 (-25.54%)	0.1450±0.0246 (-18.31%)
RA 25 mg/kg	0.0888±0.0085 (-38.25%)	0.1175±0.0129 (-28.79%)	0.1275±0.0092 (-15.73%)	0.1325±0.0139 (-7.02%)	0.1400±0.0177 (-22.78%)	0.1550±0.0185 (-12.68%)
RA 50 mg/kg	0.1012±0.0093 (-29.62%)	0.1087±0.0085 (-34.12%)	0.0738±0.0118* (-51.32%)	0.0738±0.0139 (-48.21%)	0.1375±0.0181 (-24.16%)	0.1500±0.0239 (-15.49%)
RA 100 mg/kg	0.0938±0.0127 (-34.77%)	0.1063±0.0100 (-35.58%)	0.0650±0.0113** (-57.04%)	0.0650±0.0100* (-54.39%)	0.0875±0.0056** (-51.74%)	0.0925±0.0131* (-47.89%)
RA 150 mg/kg	0.1288±0.0095 (-10.43%)	0.1125±0.0096 (-31.82%)	0.1025±0.0128 (-32.25%)	0.0763±0.0138 (-46.46%)	0.1150±0.0089 (-36.57%)	0.1050±0.0113 (-40.85%)

Table 3 Change of edema volume (ml) of oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg) and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg) during 1-6 h after carrageenan administration. N=8 for all groups. Inhibition is reported as percentage compared to 2 % Tween 80.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	Paw edema (ml)±S.E.M. (%inhibition)					
	1 h	2 h	3 h	4 h	5 h	6 h
2% Tween 80	0.1538±0.0096	0.1950±0.01070	0.2063±0.0078	0.2175±0.0122	0.2275±0.0096	0.2438±0.0138
IND10 mg/kg	0.1125±0.0098 (-26.85%)	0.0925±0.0068** (-52.56%)	0.0763±0.0071*** (-63.02%)	0.0550±0.0078*** (-74.71%)	0.0788±0.0079*** (-65.36%)	0.0838±0.0075*** (-65.63%)
TLE 500 mg/kg	0.1138±0.0060 (-26.01%)	0.1850±0.0165 (-5.13%)	0.1912±0.0190 (-7.32%)	0.1400±0.0151* (-35.63%)	0.1538±0.0121** (-32.40%)	0.1500 ±0.0125*** (-38.47%)
TLE 1000 mg/kg	0.1188±0.0100 (-22.76%)	0.1800±0.0287 (7.69%)	0.1850±0.0212 (10.32%)	0.1388±0.0189** (36.18%)	0.1438±0.0235** (36.79%)	0.1525±0.0158*** (37.45%)
TLE 1500 mg/kg	0.1188±0.0055 (-22.76%)	0.1850±0.0080 (-5.13%)	0.1950±0.0200 (-5.48%)	0.1463±0.0135* (-32.74%)	0.1625±0.0131* (-28.57%)	0.1575±0.0114** (-35.40%)
TLE 2000 mg/kg	0.1175±0.0103 (-23.60%)	0.1475±0.0185 (-24.36%)	0.1363±0.0199 (-33.93%)	0.1100±0.0160*** (-49.43%)	0.1163±0.0112*** (-48.88%)	0.1275±0.0139*** (-47.70%)
TLE 2500 mg/kg	0.1200±0.0160 (-21.98%)	0.1613±0.0206 (-17.28%)	0.1637±0.0238 (-20.65%)	0.1263±0.0181** (-41.93%)	0.1387±0.0153** (-39.03%)	0.1450±0.0172*** (-40.53%)

PROSTAGLANDIN E_2-INDUCED MOUSE PAW EDEMA

The mechanisms of RA and TLE in producing anti-inflammation were examined utilizing the PGE_2-induced paw edema test. Mice were administered orally 2% Tween 80 (10 mg/kg), SW (10 ml/kg), IND (10 mg/kg), various doses of RA (12.5-150 mg/kg) or various doses of TLE (500-2500 mg/kg) 1 h before injection of PGE_2 into the plantar surface of the left hind paw.

To demonstrate the validity of the PGE_2-induced paw edema test, IND (a cyclooxygenase inhibitor) was used as a positive control. As expected IND at 10 mg/kg significantly ($p<0.001$) decreased paw edema at all time tested (0.5, 1, 1.5, 2, 3, and 4 h) after PGE_2 administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 61.08% at 2 h. RA at 100 mg/kg significantly decreased paw edema at all time tested (0.5, 1, 1.5, 2, 3, and 4 h; $p<0.01$, $p<0.001$, $p<0.001$, $p<0.01$, $p<0.01$, and $p<0.001$, respectively) after PGE_2 administration compared to the control group and showed a maximum inhibition of paw edema of 56.98% at 1 h. The anti-inflammatory effect of RA was comparable to IND at 1 and 4 h after PGE_2 administration (Table 4).

TLE at 2000 mg/kg significantly decreased paw edema at all time tested (0.5, 1, 1.5, 2, 3, and 4 h; ($p<0.01$, $p<0.05$, $p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively) after PGE_2 administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 76.64% at 2 hr. Compared to the vehicle group, IND at 10 mg/kg also caused significant reduction of hind paw edema at all time tested (0.5, 1, 1.5, 2, 3, and 4 h; $p<0.01$, $p<0.05$, $p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively) and showed a maximum inhibition of paw edema of 74.04% at 2 h. The anti-inflammatory effect of TLE was comparable to IND at 0.5 and 2 h (Table 5).

Table 4 Change of edema volume (ml) of oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and rosmarinic acid (RA; 100 mg/kg) during 0.5-4 h after prostaglandin E_2 administration.

N=8 for all groups. Inhibition is reported as percentage compared to the vehicle control.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to the vehicle control.

Treatments (mg/kg)	Paw edema (ml)±S.E.M. (% inhibition)					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
2% Tween 80	0.1675±0.0077	0.1563±0.0084	0.1588±0.0092	0.1575±0.0094	0.1538±0.0126	0.1338±0.0107
IND 10 mg/kg	0.0810±0.0164*** (-51.64%)	0.0700±0.0060*** (-55.21%)	0.0650±0.0068*** (-59.07%)	0.0613±0.0067*** (-61.08%)	0.0613±0. 0055*** (-60.14%)	0.0575±.0059*** (-57.03%)
SW	0.1375±0.0084	0.1425±0.0103	0.1200±0.0112	0.0913±0.0101	0.0987±0.0097	0.1175±0.0053
RA 100 mg/kg	0.0788±0.0058** (-42.69%)	0.0613±0.0081*** (-56.98%)	0.0550±0.0063*** (-54.17%)	0.0513±0.0055** (-43.81%)	0.0425±0.0068** (-56.94%)	0.0525±0.0041*** (-55.32%)

Table 5 Change of edema volume (ml) of oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg) and *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg) during 0.5-4 h after prostaglandin E_2 administration.

N=8 for all groups. Inhibition is reported as percentage compared to 2% Tween 80.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	Paw edema (ml)±S.E.M. (% inhibition)					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
2% Tween 80 10 mg/kg	0.1063±0.0091	0.0750±0.0144	0.1025±0.0100	0.0963±0.0091	0.1125±0.0062	0.1175±0.0041
IND 10 mg/kg	0.0700±0.0078** (-34.15%)	0.0375±0.0070* (-50.00%)	0.0288±0.0048*** (-71.90%)	0.0250±0.0054*** (-74.04%)	0.0438±0.0080*** (-61.07%)	0.0400±0.0054*** (-65.46%)
TLE 2000 mg/kg	0.0675±0.0056** (-36.50%)	0.0350±0.0042* (-53.33%)	0.0338±0.0063*** (-62.15%)	0.0225±0.00453*** (-76.64%)	0.0325±0.0045*** (-71.11%)	0.0350±0.0038*** (-70.21%)

COTTON PELLET-INDUCED GRANULOMA FORMATION

Studies then utilized the cotton pellet-induced granuloma formation test in mice to examine the chronic anti-inflammatory efficacy of RA. Each mouse was administered orally 2% Tween 80 (10 mg/kg), SW (10 ml/kg), IND (10 mg/kg) or various doses of RA (50-150 mg/kg).

To demonstrate the validity of the cotton pellet induced-granuloma formation test, IND was used as a positive control. As expected IND at 10 mg/kg caused significant ($p<0.05$) inhibitory effect on cotton pellet-induced granuloma formation by 47.13% compared to 2% Tween 80. Only RA at 100 mg/kg exerted a significant ($p<0.05$) inhibitory effect on cotton pellet-induced granuloma formation by 38.93%, compared to the control group. RA at 100 mg/kg also displayed an inhibitory action similar to that of the standard anti-inflammatory drug, IND (Table 6).

ACUTE TOXICITY

An acute toxicity study was carried out to evaluate the safety of RA and TLE. No acute toxicity or mortality was observed with oral administration of RA and TLE at all doses tested over an observation period of 72 h.

Table 6 Effect of oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 50-150 mg/kg) (for 7 consecutive days) on the weight of granuloma in mice. N= 8 for all groups. *$p<0.05$ significantly different compared to the vehicle control.

Treatments (mg/kg)	Cotton pellet (mg) ± S.E.M.	% inhibition
2% Tween 80	86.9000±7.1073	-
IND 10 mg/kg	45.9429±7.3702*	47.13
SW	95.4750±10.1653	-
RA 50 mg/kg	71.4000±9.8947	25.22
RA 100 mg/kg	58.3125±5.2141*	38.93
RA 150 mg/kg	69.9888±7.5498	26.70

Table 72 Summary of the effective doses of rosmarinic acid (RA) and *Thunbergia laurifolia* leaf extract (TLE) in various antinociceptive and anti-inflammatory activity testing models.

Treatments (mg/kg)	Antinociceptive Activity Testing Models				Anti-inflammatory Activity Testing Models		
	Hot-plate test	Writhing test	Formalin test		Carrageenan-induced paw edema test	PGE_2-induced paw edema test	Cotton Pellet-induced granuloma formation in mice
			Early phase	Late phase			
RA	50, 100, 150	50, 100	100	100	50, 100	100	100
TLE	500, 1000, 1500, 2000, 2500	1000, 1500, 2000, 2500	1500, 2000	1500, 2000, 2500	500, 1000, 1500, 2000, 2500	2000	-

CHAPTER V
DISCUSSION AND CONCLUSION

The antinociceptive and anti-inflammatory effects of rosmarinic acid (RA) isolated from an ethanolic extract of *Thunbergia laurifolia* leaves and *Thunbergia laurifolia* leaf extract (TLE) were evaluated in various animal models.

The antinociceptive properties of various doses of RA and TLE were studied using three laboratory models for assessment of behavioral responses to two different types of noxious stimuli including thermal (hot-plate) and chemically (writhing and formalin tests)-induced nociception stimuli (Le bars et al., 2001). The involvement of opioid receptors in the analgesic effects of RA and TLE were also investigated.

Firstly, the analgesic effects of RA and TLE were evaluated utilizing the standard mouse hot-plate test. The hot-plate test is a well-validated model for detection of centrally-acting analgesics, while peripherally-acting analgesics show little to no activity in this test. The hot-plate test measures the time taken to show behavioral responses including jumping with all four feet or hind paw licking. Both of these behaviors are considered to be supraspinal sensory integration responses and are thought to involve opioids (Woolfe and MacDonald, 1944; Le Bar et al., 2001). Morphine (MO) is commonly used as a reference drug. MO demonstrated a potent analgesic effect, indicating the sensitivity of the hot-plate test. RA and TLE were administered orally to the animals by suspending in sterile water and 2% Tween 80, respectively. The oral administration was chosen in order to imitate the normal consumption of *T. laurifolia* leaves and roots for detoxification of several poisoning agents in Thai traditional medicine.

Results from the hot-plate test indicated that RA at doses of 50, 100, and 150 mg/kg had significant effects on hot-plate latencies compared to controls, with the highest antinociceptive effect at 100 mg/kg. All doses of TLE also had significant effects on pain latency compared to controls, with the highest antinociceptive effect at 2000 mg/kg. These results suggested that both RA and TLE have central analgesic effects.The antinociceptive peak response of various doses of RA and TLE were

observed at different time points starting from 30-240 min after oral administration. A delay effect may partly due to variable absorption of RA and TLE from the gastrointestinal tract of rodents.

The mechanism of antinociceptive action of RA and TLE were further investigated using a specific opioid receptor antagonist, naloxone. The antinociceptive actions of the most effective doses of RA (100 mg/kg) and TLE (2000 mg/kg) were inhibited by naloxone. These results suggest that both RA and TLE have morphine-like antinociceptive activity.

The acetic acid-induced writhing test is used for screening peripheral analgesic effect of test compounds and considered to be a visceral pain model (Nakamura et al., 1983). This test is based on acetic acid stimulation of nociceptive neurons by liberation of several mediators such as histamine, serotonin, cytokines, and eicosanoids especially PGE_2 in the peritoneal fluid (Deraedt et al., 1980). Activation and sensitization of peripheral chemosensitive nociceptive receptors by these mediators lead to responses of constriction of the abdominal wall and twisting of the trunk followed by extension of hind limbs (Vander Wende and Margolin, 1956). These writhing responses are associated with development of peripheral inflammation (Kawabata, 2011). The nociceptive property of acetic acid may be due to the release of cytokines, including TNF-α, interleukin-1β, and interleukin-8 by resident peritoneal macrophages and mast cells (Ribeiro et al., 2000). This response can be prevented by various inhibitors of prostaglandin biosynthesis including nonsteroidal anti-inflammatory agents, non-narcotic analgesics, some monoamine oxidase inhibitors and antioxidants prevented prostaglandin release (Deraedt et al., 1980).

Indomethacin (IND), a nonsteroidal anti-inflammatory drug, produced significant analgesic response in the acetic acid-induced writhing test. RA at doses of 50 and 100 mg/kg and TLE at doses of 1000, 1500, 2000, and 2500 mg/kg showed significant activity against chemical pain induced by acetic acid, compared to the control group. Thus, these data indicated that RA and TLE have antinociceptive effects on inflammatory pain. This activity of RA and TLE may be related to reduced liberation of

inflammatory mediators or direct blockage of receptors. Several classes of drug including adrenergic receptor agonists and muscle relaxants, also effectively inhibit writhing (Le bars et al., 2001). However, due to the lack of specificity of this method, positive results in the writhing test should be confirmed in other experimental models. For this reason, the formalin test was further employed for this purpose.

The formalin test has been considered as a model of chronic pain (Dubuisson and Dennis, 1977). After intraplantar formalin injection, animals display a biphasic response consisting of flinching (rapid paw shaking), licking and biting of the injected paw (Franklin and Abbott, 1989). The early phase (neurogenic pain) initiates immediately after formalin injection and last for 5 minutes. This phase is caused by a direct effect of formalin on sensory C-fibers, and is thought to involve substance P, glutamate and bradykinin. The late phase (inflammatory pain) initiates 15 min after formalin injection and is associated with development of an inflammatory response and release of nociceptive mediators including histamine, serotonin, prostaglandins and bradykinin (Hunskaar and Hole, 1987). Centrally acting drugs inhibit both phases equally, while peripherally acting drugs inhibit only the late phase. Therefore, this test can be used to determine the possible mechanism of analgesia (Hunskaar and Hole, 1987; Shibata et al., 1989; Rosland et al., 1990).

In the current study, MO and IND were used as reference drugs. MO, a central analgesic drug, demonstrated potent analgesic effect in both early and late phases, but IND, a peripheral acting drug, demonstrated analgesic effect only in the late phase. RA at 100 mg/kg and TLE at doses of 1500 and 2000 mg/kg significantly reduced the time spent for paw licking in both phases of formalin-induced nociception. These results from the formalin test were in agreement with those obtained from the hot-plate and writhing tests, thereby indicating that both RA and TLE act both centrally and peripherally to achieve pain relief.

To exclude possible non-specific disturbances of motor coordination by RA and TLE, a rota-rod test was performed. This test is used to evaluate skeletal muscle relaxation, convulsions and depression of the central nervous system produced by a

test compound. The results of RA and TLE indicated no detectable relaxant or sedative effects at the most effective doses of RA (100 mg/kg) and TLE (2000 mg/kg). Therefore, the behavioral responses observed in the hot-plate, writhing and formalin tests were not due to motor dysfunction, but rather reflected a true antinociceptive effect.

To assess potential anti-inflammatory action, the effects of RA on the acute and chronic phases of inflammation were evaluated in mouse models of carrageenan-induced paw edema and cotton pellet-induced granuloma formation. The anti-inflammatory property of TLE was assessed in a mouse model of carrageenan-induced paw edema.

The carrageenan-induced paw edema test is a standard model for assessment of anti-inflammatory drugs and is widely used for evaluating the acute anti-inflammatory activities of natural compounds (Di Rosa and Willoughby, 1971). Carrageenan-induced rat paw edema was first described by Winter et al. in 1962 and carrageenan-induced mouse paw edema test was established by Levy in 1969. Since then, the mouse paw edema test has been increasingly used to evaluate the anti-inflammatory drug candidates (Posadas et al., 2004). Cardinal signs of inflammation (edema, hyperalgesia and erythema) develop immediately following subcutaneous carrageenan injection, resulting from actions of proinflammatory agents. The oedema at 3 h after the application of carrageenan was considered to reach the highest response (Andrade et al., 2007; Kale et al., 2007). The inflammatory response resulted from carrageenan can be modulated by inhibitors of specific molecules within the inflammatory cascade (Morris, 2003). Development of paw edema in mice after carrageenan injection is characterized by a biphasic response with involvement of multiple inflammatory mediators. The first phase (0-2 h after carrageenan injection) is mainly due to the release of pro-inflammatory agents, including histamine, serotonin and bradykinin, from damaged tissues; while the second phase of swelling (3-6 h after carrageenan injection) may correlate with release of prostaglandins (Di Rosa, 1972). More recently, the second phase has also been attributed to involvement of free radicals, nitric oxide and cyclooxygenases in the hind paw exudate (Iwata et al., 2010).

IND significantly reduced paw edema from 2-6 h (second phase) after carrageenan injection. The results are consistent with the previous study which showed that IND caused strong inhibition of the second phase without affecting the development of the first phase (Vinegar et al., 1969). The effect of IND during the second phase could be explained that IND is a cyclooxygenase inhibitor and contributes to the reduction of prostaglandins synthesis.

RA at 100 mg/kg significantly decreased mouse paw edema at 3, 4, 5, and 6 h after carrageenan injection. RA at 50 mg/kg had this effect only at 3 h and both RA doses ineffective at 1 and 2 h. These results indicated that RA exerted anti-inflammatory action during the second phase of inflammation. All doses of TLE showed significant reduction of paw edema at 4, 5, and 6 h after carrageenan injection, indicating that TLE produced anti-inflammatory effect during the second phase of inflammation. The mechanism underlying these anti-inflammatory activities of RA and TLE may therefore involve inhibition of the release of inflammatory mediators, such as prostaglandins, in local tissue or blockage of prostaglandin receptors.

Similar results were observed when paw edema was induced by prostaglandin E_2 (PGE_2) according to the method of Akkol et al. (2008) and Castardo et al. (2008). PGE_2 is generally considered as a key proinflammatory mediator and its role has been extensively studied in several inflammatory events. High levels of PGE_2 have been found in inflammatory exudates, and injection of PGE_2 directly into tissue has been shown to induce a number of classical signs of inflammation (Cluadino et al., 2006). IND, a positive control, significantly inhibited the edematogenic effect of PGE_2 from 0.5-4 h after PGE_2 injection. Both RA and TLE significantly reduced edema induced by PGE_2 at the same time period after PGE_2 injection indicating that these effects were due to direct blockage of prostaglandin receptors. These results complement those obtained from other models involving inflammatory mediators, including acetic acid-induced writhing and formalin licking responses. The reduction of PGE_2-induced paw edema could explain, at least in part, the inhibitory effect of RA and TLE in acetic acid-induced writhing and formalin tests.

The cotton pellet-induced granuloma formation test is a common method for evaluation of transudative, exudative and proliferative components of chronic inflammation because the dried pellet weights correlate well with the amount of granulomatous tissue (Swingle and Shideman, 1972). RA at 100 mg/kg effectively inhibited cotton pellet granuloma formation, which indicates that RA has anti-inflammatory activity in the chronic phase of inflammation. An acute toxicity assessment confirmed the safety of all doses of RA and TLE used in this study.

In conclusion, this study shows that RA isolated from an ethanolic extract of *T. laurifolia* leaves and TLE have antinociceptive properties in both central and peripheral models of nociception in mice. The analgesic mechanisms of action of RA and TLE are most likely involved with the opioid pathway. Moreover, RA showed anti-inflammatory properties in acute and chronic inflammation models. The anti-inflammatory mechanisms of action of RA and TLE were partly due to direct blockage of prostaglandin receptors. RA and TLE demonstrated similar results in all animal models, indicating that the antinociceptive and anti-inflammatory effects of TLE were partly resulted from its main constituent, rosmarinic acid. Further studies are required to understand the mechanisms of action of these effects. However, the results clarify the pharmacological actions of RA and TLE and highlight the potential use of these natural compounds for alleviating pain and treating inflammatory disorders.

FUTURE RESEARCH

The future research could comprise of several objectives as listed below

(1) To better understand the mechanisms of rosmarinic acid (RA) and *T. laurifolia* leaf extract (TLE) that involved in producing analgesic and anti-inflammatory effects.

(2) To better characterize the mechanisms of analgesic effects of RA and TLE using other opioid antagonists.

(3) To investigate the anti-inflammatory effect of RA and TLE in other standard experimental animal models of chronic inflammation.

(4) To investigate the potential use of RA and TLE in combination with other analgesics or anti-inflammatory drugs.

(5) To investigate other routes of administration that might be more appropriate and enhance the analgesic or anti-inflammatory effects of RA and TLE.

(6) To elucidate side effects and toxic effects of RA and TLE at high dosage or after chronic use.

These studies may provide important clues to help understand the mechanisms underlying the analgesic and anti-inflammatory effects of RA and TLE and further support the use of such compounds in clinical setting.

REFERENCES

นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร (บรรณาธิการ). 2543. สมุนไพร ไม้พื้นบ้าน. เล่มที่ 4. กรุงเทพมหานคร: ประชาชน.

นิจศิริ เรืองรังษี และธวัชชัย มังคละคุปต์. 2547. สมุนไพรไทย เล่ม 1. กรุงเทพมหานคร: สำนักพิมพ์ บี เฮลตี้.

มณเฑียร เปสี, วัชรี คุณกิตติ, ฉันทนา อารมย์ดี, มุกดา จิตต์เจริญธรรม (2544). การพัฒนาสารสกัดรางจืดเพื่อใช้รักษาแผลอักเสบในช่องปากในผู้ป่วยมะเร็ง. รายงานการวิจัย, คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น.

วุฒิ วุฒิธรรมเวช. 2540. สารานุกรมสมุนไพร รวมหลักเภสัชกรรมไทย. กรุงเทพมหานคร: โอเดียนสโตร์.

Akkol, E.K., Arif, R., Ergun, F., and Yesilada, E. Sesquiterpene lactones with antinociceptive and antipyretic activity from two *Centaurea* species. Journal of Ethnopharmacology 122 (2009): 210-215.

Andrade, S. F., Cardoso, L. G. V., Carvalho, J.C.T., and Bastos, J. K. Antiinflammatory and antinociceptive activities of extract, fractions and populnoic acid from bark wood of *Austroplenckia populnea*. Journal of Ethnopharmacology 109 (2007): 464–471.

Aritajat, S., Wutteerapol, S., and Saenphet, K. Anti-diabetic effect of *Thunbergia laurifolia* Linn. aqueous extract. Southeast Asian Journal of Tropical Medicine and Public Health 35 (2004): 53-58.

Baba, S. Osakabe, N. Natsume, M. and Terao, J. Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. Life Sciences 75 (2004): 165–178.

Backer, C.A., and Bakhuizen van den Brink, R.C. Acanthaceae. Flora of Java 2 (1965): 544-553.

Baumann, T.J. and Strickland, J. Pain Management. In Dipiro, J.T., Talbert, R.L., Yee, G.C., Matzke, G.R., Wells, B.G., and Posey, L.M. (eds.). Pharmacotherapy: A Pathophysiologic Approach, pp. 1089-1104. United States of America: MCGRAW-HILL Medical Publishing Division, 2008.

Bear, M.F., and Connors, B.W., and Paradiso, M.A. Neuroscience: Exploring the brain, pp. 397-435. Philadelphia: Lippincott Williams&Wilkins, 2001.

Benedetti, C. Neuroanatomy and biochemistry of antinociception. In Bonica, J.J., and Ventafridda, V., (eds.). Advances in Pain Research and Therapy pp. 31-44. New York: Raven Press, 1979.

Bennett, P.N., and Brown, M.J. Drug for inflammation and rheumatological disease. Clinical pharmacology, pp. 255-260. Spain: Churchill Livingstone Elseveir, 2008.

Berry, P.H., Covington, E.C., Dahl, J.L., Katz, J.A., and Miaskowski, C. Pain: Current Understanding of Assessment, Management, and Treatments, pp. 1-39. National Pharmaceutical Council: American Pain Society, 2006.

Basbaum, A.I., and Fields, H.L. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annual Review of Neuroscience 7 (1984): 309-338.

Bor, N.L., and Raizada, M.B. Some beautiful Indian climbers and shrubs. Oxford: Oxford University Press. 1982.

Byers, M., and Bonica, J.J. Peripheral pain mechanisms and nociceptor plasticity. In Loeser, J.D., Butler, S.H., Chapman, C.R., and Turk, D.C. (eds.). Bonica's Management of Pain, pp. 26-72. Baltimore, MD: Lippincott Williams & Wilkins, 2001.

Castardo, J.C., Prudente, A.S., Ferreira, J., Guimaraes, C.L., Monache, F.D., Filho, V.C., Otuki, M.F., and Cabrini., D.A. Antiinflammatory effects of hydroalcoholic extract and two biflavonoids from *Garcinia gardneriana* leaves in mouse paw Oedema. Journal of Ethnopharmacology 118 (2008): 405-411.

Chan, E.W.C., and Lim, Y.Y. Antioxidant activity of *Thunbergai laurifolia* tea. Journal of Tropical Forest Science 18 (2006): 130-136.

Chan, E.W.C., Eng, S.Y., Tan, Y.P., and Wong, Z.C. Phytochemistry and pharmacological properties of *Thunbergia laurifolia*: a review. Pharmacognosy Journal 3 (2011): 1-6.

Chanawirat, A., Toskulkao, C., Temcharoen, P., and Glinsukon, T. Protective effect of *Thunbergai laurifolia* extract on ethanol-induced hepatotoxicity in mice. Thesis, Faculty of Graduate Studies, Mahidol University, Bangkok, Thailand. 2000.

Chandrasoma, P., and Taylor, C.R. Concise pathology, pp. 31-92. Stamford, CT: Appleton & Lange, 1998.

Chanrumanee, S., Vejabhikul, S., Taesotikul, T., Netisingha, W., Sirisaard, P., and Leelapornpisit, P. Topical anti-inflammatory preparation from *Thunbergia lauriforia* Linn. In Pharma Indochina II (2001): 20-23.

Chapman, C.R., Nakamura, Y. A. passion of the Soul: an introduction topain for consciousness researchers, pp. 391-422. Conscious Cogn, 1999.

Chapman, C.R. The psychophysiology of pain. In: Loeser, J.D., Butler, S.H., Chapman, C.R., and Turk, D.C. (eds.). Bonica's Management of Pain. Bonica's Management of Pain, pp. 461-477. Baltimore, MD: Lippincott Williams & Wilkins, 2001.

Chattopadhyay, D., Arunachalam, G., and Mandal, S.C. CNS activity of the methanol extract of *Mallotus peltatus* (Geist) Muell Arg. Leaf: an ethnomedicine of Onge. Journal of Ethnopharmacology 85 (2003): 99-105.

Chivapat, S., Chavalittumrong, P., Attawish, A., Bansiddhi, J., and Pudungpat, S. Cronic toxicity of *Thunbergia laurifolia* Lindl. Extract. Journal of Thai Traditional & Alternative Medicine 7 (2009): 17-25.

Costigan, M., Woolf, C.J. Pain: molecular mechanisms. Journal of Pain 1 (2000): 35-44.

Covington, E.C. The biological basis of pain. International Review of Psychiatry 12 (2000):128-147.

Cross, S.A. Pathophysiology of pain. Mayo Clinic Proceeding 69 (1994): 375-383.

Deraedt, R., Jouquey, S., Delevallee, F., and Flahaut, M. Release of prostaglandins E and F in an algogenic readtion and its inhibition. European Journal of Pharmacology 61(1980): 17-24.

Di Rosa, M., Biological properties of carrageenan. Journal of Pharmacy and Pharmacology 24 (1972): 89-102.

Di Rosa, M., Willoughby, D., A screens for anti-inflammatory drugs. Journal of Pharmacy and Pharmacology 23 (1971): 297-300.

Di Rosa, M., and Sorrentino, L. The Mechanism of the Inflammatory Effect of Carrageenan. European Journal of Pharmacology 4 (1968): 340–342.

Dubuisson, D., and Dennis, S.G. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine and brain stimulation in rats and cats. Pain 4 (1977): 161-174.

Duggan, A.W., and North, R.A. Electrophysiology of opioids. Pharmacological Reviews 35 (1983): 219-281.

Dunham, N.W., and Miya, T.S. A note on a simple apparatus for detecting neurological deficit in rats and mice. Journal of the American Pharmacists Association 46 (1957): 208-210.

Englberger, W., Hadding, U., Etschenberg, E., Graf, E., Leyck, S., Winkelmann, J., and Parnham, M.J., Rosmarinic acid: a new inhibitor of complement C3-convertase with anti-inflammatory activity. International Journal of Immunopharmacology 10 (1988): 729-737.

Fallarini, S., Miglio, G., Paoletti, T., Minassi, A., Amoruso, A., Bardelli, C., Brunelleschi, S., and Lombardi, G. Clovamide and rosmarinic acid induce neuroprotective effects in *in vitro* models of neuronal death. British Journal of Pharmacology 157 (2009): 1072-1084.

França, S.C., Soares, A.M., Sampaio, S.V. Rosmarinic acid, a new snake venom phospholipase A2 inhibitor from Cordia verbenacea (Boraginaceae): antiserum action potentiation and molecular interaction. Toxicon. 46 (2005):318-327.

Franklin, K.B.J., and Abbott, F.V. Techniques for assessing the effects of drugs on nociceptive responses. Neuromethods Psychopharmacology 13 (1989):145-216.

Ferrante, F.M., and VadeBoncouer, T.R. Postoperative pain management. Postoperative pain; Analgesia; Pain, Postoperative; Treatment; Chemotherapy; therapy Churchill Livingstone (New York), 1993.

Foley, K.M. The treatment of cancer pain. The New England Journal of Medicine 313 (1985): 84–95.

Gamaro, G.D., Suyenaga, E., Borsoi, M., Lermen, J., Pereira, P., and Ardenghi, P. Effect of rosmarinic and caffeic acids on inflammatory and nociception process in rats. International Scholarly Research Network Pharmacol (2011): 1-6.

Greene, R.J., and Harris, N.D. Pathology and Therapeutics for Pharmacists, pp. 455-467. Great Britain: Pharmaceutical Press, 2008.

Grichnick, K., and Ferrante, F.M. The difference between acute and chronic pain. Mount Sinai Journal of Medicine 58 (1991): 217-220.

Griffin, R.S. and Woolf,C. Pharmacology of Analgesia. In Golan, D. E., Tashjian, A.H., Armstrong, E.J., Galanter, J.M., Armstrong, A.W., Arnaout, R.A. and Rose, H.S. (eds.). Principles of Pharmacology: The Patholophysiologic Basis of Drug Therapy, pp. 229-242. United States of America: Lippincott Williams & Wilkins, 2005.

Griswold, D.E., and Adams, J.L. Constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2): rationale for selective inhibition and progress to date. Medical Care Research and Review 12 (1996): 181-206.

Guilbaud, G., Bernard, J.F., Besson, J.M. Brain areas involved in nociception and pain. In: Wall, P.D., Melzack, R., (eds.). Textbook of Pain. Edinburgh: Churchill Livingstone, 1994.

Guyton, A., and Hall, J.E. Textbook of medical physiology pp. 598-604. Philadelphia: Elsevier Saunders, 2005.

Hammond, D.L. Control systems for nociceptive afferent processing: the descending inhibitory pathways. In Yasksh, T.L., (ed.). Spinal Afferent Processing, pp. 363-390. New York: Plenum Press, 1986.

Horton-Szar, D. Inflammation, Repair and cell death. Pathology. pp. 5-11. Chaina. Mosby Elsevier. 2007.

Huang, S.S., and Zheng, R.L. Rosmarinic acid inhibits angiogenesis and its mechanism of action in vitro. Cancer Letter 239 (2006): 271-280.

Hunskaar, S., and Hole, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30 (1987): 103-114.

Hunter, L. Analgesic drugs. Pharmacology, pp. 562-583. Scotland: Churchill Livingstone, 2003.

Iwata, M., Suzuki, S., Asai, Y., Inoue, T., and Takagi, K. Involvement of nitric oxide in a rat model of carrageenan-induced pleurisy. Mediators of Inflammation 682879 (2010).

Jacobson, L., and Mariano, A.J. General considerations in chronic pain. In Loser, J.D. (Ed), Bonica's management of pain, pp. 241-254. Philadephia: Lippincott Williams & wilkins, (2001).

Jensen, T.S., Gottup, H., Sindrup S.H., and **Bach, F.W.** The clinical picture of neuropathic pain. European Journal of pharmacology 429 (2001): 1-11.

Julius, D., and Basbuam, A.I. Molecular mechanisms of nociception. Nature 413 (2001): 203-210.

Kale, M., Misar, A. V., Dave, V., Joshi, M., and Mujumdar, A. M. Antiinflammatory Activity of *Dalbergia lanceolaria* Bark Ethanol Extract in Mice and Rats. Journal of Ethnopharmacology 112 (2007): 300–304.

Kamienski, M., and Keogh, J. Pharmacology Demystified. United State of America: McGraw-Hill, 2006.

Kanchanapoom, T., Kasai, R., and Yamasaki, K. Iridoid glucosides from *Thunbergia laurifolia*. Phytochemistry 60 (2002): 769-771.

Kang, H.S., Ki, H.R., Byun, D.S., Park, H.J., and Choi, J.S. Rosmarinic acid as a Tyrosinase Inhibitors from *Salvia miltiorrhiza.* Natural Product Sciences 10 (2004) : 80-84.

Kandel, E.R., Schwartz, J.H., and Jessell, T.M. Principles of neural science pp. 430-450. New York: McGraw-Hill, 2000.

Kim, D.S., Kim, H.R., Woo, E.R., Hong, S.T., Vhae, H.J., and Chae, S.W. Inhibitory effects of rosmarinic acid on Adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting reactive oxygen species and the activations of c-Jun N-terminal kinase and extracellular signal-regulated kinase. Biochemical Pharmacology 70 (2005): 1066-1078.

Kim, J.H., Lee, B.J., Kim, J.H., Yu, Y.S., Kim, M.Y., and Kim, K.W. Rosmarinic acid suppresses retinal neovascularization via cell cycle arrest with increase of $p21^{WAF1}$ expression. European Journal of Pharmacology 615 (2009): 150-154.

Kumar, V., Abbas, A.K. and Fausto, N. Pathologic Basis of Disease, pp. 48-80. Chaina: Elsevier Saunders, 2005.

Kumar, V., Cotran, R.S., Robbins, S.L. Acute and Chronic Inflammation. Basic Pathology, pp. 33-59. Chaina: Elsevier Saunders, 2002

Koster, R., Anderson, M., and de Beer, E.J. Acetic acid for analgesic screening. Federation proceedings 18 (1959): 412-418.

Le Bars, D., Gozariu, M., Cadden, S.W. Animal models of antinociception. Pharmacological Reviews. 53 (2001): 597-652.

Lee, H.J., Cho, H.S., Park, E., Kim, S., Lee, S.Y., Kim, C.S., Kim, D.K., Kim, S.J., and Chun, H.S. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology 250 (2008): 109-115.

Levy, L. Carrageenan Induced Paw Edema in the Mouse. Life Sciences 8 (1969): 801-808.

Loeser, J.D., and Treede, R.D. The Kyoto protocol of IASP Basic Pain Terminology. Pain 137 (2008): 473–7.

Makino, T., Ono, T., Muso, E., Yoshida, H., Honda, G., and Sasayama, S.T. Inhibitory effects of rosmarinic acid on the proliferation of cultured murine mesangial cells. Nephrology Dialysis Transplantation. 15 (2000): 1140-1145.

Max, M.B., Payne, R., and Edwards, W.T. Principles of Analgesic Use in the Treatment of Acute Pain and Cancer Pain,Glenview, IL: American Pain Society, 1999.

McHugh, J.M., and McHugh, W.B. Pain: Neuroanatomy, chemical mediators, and clinical implications. American Association of Critical-Care Nurses Clinical Issues 11 (2000): 168-179.

Meier, R., Schuler, W., and Desaulles, P.,. Zur Frage des Mechanismus der Hemmung des Bindergewebswachstums durch cortisone. Experientia 6 (1950): 469-471.

Melzack, R., and Wall, P.D. Pain mechanisms: a new theory. Science. 150 (1965): 971-979.

Merskey, H., and Bugduk, N. Classification of Chronic Pain. Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms. (eds.). Seattle, WA: IASP Press, 1994.

Meyer, R.A., Campbell, J.N., and Raja, S.N. Peripheral neural mechanisms of nociception. In: Wall, P.D., Melzack, R. (eds.). Textbook of Pain, Edinburgh: Churchill Livingstone, 1994.

Miyoshi, H.R. Systemic nonopioid analgesics. In Loeser, J.D., Butler, S.H., Chapman, C.R., et al, (eds.). Bonica's Management of Pain, pp. 1667-1681. Baltimore, M.D: Lippincott Williams & Wilkins, 2001.

Moongkarndi, P., Kosem, N., Luanratana, O., Jongsomboonkusol, S., and Pongpan, N. Antiproliferative activity of Thai medicinal plant extracts on human breast adenocarcinoma cell line. Fitoterapia 75 (2004): 375-377.

Morkmek, N., Chattaviriya, P., Lertprasertsuke ,N., Chuncharunee, S., and Ruangyuttikarn, W. Detoxification of Cadmium Induced Renal and Hepatic Injuries in Rats by *Thunbergia laurifolia* Lindl. Leaf Extract. Thai Journal of Toxicology 25 (2010): 115-123.

Morris, C.J. Carrageenan-Induced Paw Edema in Rat and Mouse. In Winyard, P.G. and Wiloughby, D.A. (eds.). Inflammation Protocols, pp. 115-121. United States of America: Human Press Inc., 2003.

Murphy, H.S., and Ward, P.A. Inflammtion. In Rubin, E., Gorstein, F., Rubin, R., et al. (Eds.). Rubin's pathology: clinicopathologic foundations of medicine, pp. 41-83. Philadelphia: Lippincott Williams & Wilkins, 2005.

Nakamura, H., Imazu, C., Ishii, K., Yokoyama, Y., Kadokawa, T., and Shimizu, M. Site of analgesic action of zomepirac sodium, a potent non-narcotic analgesic in experimental animals. The Japanese Journal of Pharmacology 33 (1983): 875-883.

Nakamura,Y., Ohto,Y., Murakami, A., and Ohigashi, H. Superoxide scavenging activity of rosmarinic acid from Perilla frutescens britton var. acuta f. viridis. The Journal of Agricultural and Food Chemistry 46 (1998): 4545-4550.

Nakazawa, T., and Ohsawa, K. Metabolism of rosmarinic acid in rats. Journal of Natural Products 61 (1998): 993–996.

Nathan, C. Points of control in inflammation. Nature 420 (2002): 848-852.

Neal, M.J. Medical Pharmacology at Glance. Great Britain: MPG Books Ltd, Bodmin Cornwall, 2002.

Negus, S., Vanderah, T.W., Brandt, M.R., Bilsky, E.J., Becerra, L., and Borsook, D. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. Journal of Pharmacology and Experimental Therapeutics 319 (2006): 507–514.

Nestler, E.J., Hyman, S.E. and Malenka, R.C. Pain. Molecular Neuropharmacology A Foundation for Clinical Neuroscience, pp. 434-451. United States of America: McGraw-Hill Companies, 2001.

O'Neil, C. Pain Management. In Chisholm-Burns, M.A., Schwinghammer, T.L., Wells, B.G., Malone, P.M., Kolesar, J.M. and Dipiro, J.T. (eds.). Pharmacotherapy

Principle & Practice, pp. 567-579. United States of America: McGraw-Hill Medical, 2010.

O'Neill, G.P., and Ford-Hutchinson, A.W. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Letters 330 (1993): 156-160.

Oonsivilai, R., Cheng, C., Bomser, J.A., Ferruzzi, M.G., and Ningsanond, S. Phytochemical profiling and phase II enzyme-inducing properties of *Thunbergia laurifolia* Lindl. (RC) extracts. Journal of Ethnopharmacology 114 (2007): 300-306.

Oonsivilai, R., Ferruzzi, M.G., and Ningsanond, S. Antioxidant activity and cytotoxicity of Rang chuet (*Thunbergia laurifolia* Lindl.) extracts. Asian Journal of Food and Agro-Industry 1 (2008): 116-128.

Osakabe, N., Yasuda, A., Natsume, M., Kato, Y., Osawa, T., and Yosikawa, T. Rosmarinic acid, a major polyphenolic component of *Perilla frutescens*, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. Free Radical Biology & Medicine 33 (2002): 798-806.

Osakabe, N., Yasuda, A., Natsume, M., and Yoshikawa, T. Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model. Carcinogenesis 25 (2004): 549-557.

Parnham, M.J., and Kesselring, K. Rosmarinic acid. Drug of the Future 10 (1985): 756-757.

Pearl, J., Stander, H., and McKean, D.B. Effects of Analgesics and Other Drugs on Mice in Phenylquinone and Rota-rod Test. Journal of Pharmacology and Experimental Therapeutics 167 (1969): 9-13.

Phosri, P., Mahakunakorn, P., and Srisodsai, A. Anti-inflammatory activity of *Thunbergia Laurifolia* leaf extract. Master of Sciences, Khon Kaen University, Khon K Thailand. 2008.

Pongphasuk, N., Khunkitti, W., Chitcharoenthum, M., and Aromdee, C. Development of Topical Anti-inflammatory Preparations from Medicinal Pants. Master of Pharmacy Thesis in Pharmaceuticals, Khon Kaen, Thailand. 2003.

Porth, C.M., and Sommer, C. Disorders of somatoswnsory function and pain. In Porth, C.M., and Matfin, G. (eds.). Pathophysiology concepts of altered health states, pp. 759-785. Chaina: Lippincott Williams & Wilkins, 2009.

Porth, C.M., and Sommer, C. Inflammation, tissue repair, and fever. In Porth, C.M., and Matfin, G. (eds.). Pathophysiology concepts of altered health states, pp. 759-785. Chaina: Lippincott Williams & Wilkins, 2009.

Posadas, I., et al. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. British Journal of Pharmacology 142 (2004): 331–338.

Posridee, K Sripa, B., Jitsomboon, B., and Oonsivilai, R. Acute Oral Toxicity Study of *Thunbergia Laurifolia* Linn. Extracts. The 12th Asean Food Conference, 2011.

Pramyothin, P., Chirdchupunsare, H., Runsipipat, A., and Chaichantipyuyh, C. Hepatoprotective activity of *Thunbergia laurifolia* Linn. extracts in rats treated

with ethanol: *In vitro* and *in vivo* studies. Journal of Ethnopharmacology 102 (2005): 408-411.

Purima, M., and Gupta, P.C. Colouring from the flowers of *Thunbergia laurifolia* Journal of Indian Chemistry Society 55 (1978): 622-623.

Reisine, T., and Pasternak, G. Opioid analgesics and antagonists. In Hardman, J.G., and Limbard, L.E. (eds.). Goodman & Gillman's the Pharmacologic Basis of Therapeutics, pp. 531-555. New York: McGraw-Hill, 1996.

Ritschel, W.A., Starzacher, A., Sabouni, A., Hussain, A.S., and Koch, H.P. Percutaneous absorption of rosmarinic acid in the rat. Methods and Findings in Experimental and Clinical Pharmacology 11 (1989): 345–352.

Rosland, J.H., Tjølsen, A., Maehle, B., and Hole, K., The formalin test in mice: effect of formalin concentration. Pain 42 (1990): 235-242.

Saenphet, K., Kantaoop, P., Saenphet, S., and Aritajat, S. Mutagenicity of *Pueraria mirifica* Airy Shaw & Suvatabandhu and antimutagenicity of *Thunbergia laurifolia* Linn. Southeast Asian Journal of Tropical Medicinal Public Health 36 (2005): 238-241.

Sahu, A., Rawal, N., and Pangburn, M.K. Inhibition of complement by covalent attachment of rosmarinic acid to activated C3b. Biochemical Pharmacology 57 (1999): 1439-1446.

Sanbongi, C., Takano, H., Osakabe, N., Sasa, N., Natsume, M., Yanagisawa, R., Inoue, K.I., Kato, Y., Osawa, T., and Yoshikawa, T. Rosmarinic acid inhibits lung injury

induced by diesel exhaust particles. Free Radical Biology & Medicine 34 (2003): 1060-1069.

Sanbongi, C., Takano, H., Osakabe, N., Sasa, N., Natsume, M., Yanagisawa, R., Inoue‡, K. –i., Sadakane§, K., Ichinose§, T., and Yoshikawa‡, T. Rosmarinic acid in perilla extract inhibits allergic inflammation induced by mite allergen in a mouse model. Clinical & Experimental Allergy 34 (2004): 971-977.

Scarpati, M., and Oriente, G., Isolation and constitution of rosmarinic acid from *Rosmarinus officinalis.* Ricerca in Science 28 (1958): 2329-2333.

Sherwood, E.R., and Toliver-Kinsky, T. Mechanisms of the inflammatory response. Best Practice & Research Clinical Anaesthesiology 18 (2004): 385-405.

Shibata, M., Ohkubo, T., Takahashi, H., and Inoki, R. Modified formalin test: characteristic biphasic pain response. Pain 38 (1989): 347-352.

Stein, C. The control of pain in peripheral tissues by opioids. The New England Journal of Medicine 332 (1995): 1685-1690.

Suwanchaikasem, P., Chaichantipyuth, C., and Sukrong, S.: Antioxidant-guided isolation of rosmarinic acid, a major constituent, from *Thunbergia laurifolia* and its use as a bioactive principle for standardization. Thesis, Faculty of Pharmaceutical Sciences, Chulalongkorn University,Bangkok, Thailand. 2012.

Swarup, V., Ghosh, J., Ghosh, S., Saxena, A., and Basu, A. Antiviral and anti-inflammatory effects of rosmarinic acid in an experimental murine model of Japanese encephalitis. Antimicrobial agent and Chemotherapy 51 (2007): 3367-3370.

Swingle, R.W. Evaluation for antiinflammatory activity. In Scherer, R.A., and Whitehouse, M.W. (eds.). Antiinflammatory Agents: Chemistry and Pharmacology, pp. 34–122. Academic Press: New York, 1974.

Swingle, K.F., and Shideman, F.E. Phases of the inflammatory response subcutaneous implantation of a cotton pellet and their modification by certain anti-inflammatory agents. The Journal of Pharmacology and Experimental Therapeutics 13 (1972): 226-234.

Tallarida, R.J., and Murray, R.B. Manual of pharmacologic calculation with computer programs. 2nd ed. New York: Springer-Verlag, 1987.

Tangpong, J., and Satarug, S. Alleviation of lead poisoning in the brain with aqueous leaf extract of the *Thunbergia laurifolia* (Linn.). Toxicology Letters 198 (2010): 83-88.

Tejasen, P., and Thongtharb, C. Experimental using *Thunbergia laurifolia* as antidote for insecticide poisoning. Chiang Mai Medical Bulletin19 (1980): 105-114.

Terman, G.W., and Bonica, J.J. Spinal mechanisms and their modulation. In: Loeser, J.D., Butler, S.H., Chapman, C.R., and Turk, D.C., (eds.). Bonica's Management of Pain 3rd ed., pp. 73-152. Baltimore, MD: Lippincott Williams & Wilkins, 2001.

Thongsaard, W., and Marsden, C.A. A herbal medicine used in the treatment of addiction mimics the action of amphetamine on in vitro rat striatal dopamine release. Neuroscience Letters 329 (2002): 129-132.

Ticli, F.K., Hage, L.I., Cambraia, R.S., Pereira, P.S., Magro, A.J., Fontes, M.R., Stábeli, R.G., Giglio, J.R., França, S.C., Soares, A.M., and Sampaio, S.V. Rosmarinic acid, a new snake venom phospholipase A2 inhibitor from *Cordia verbenacea* (Boraginaceae): antiserum action potentiation and molecular interaction. J 46(2005): 318-27.

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., and Hole, K. The formalin test: an evaluation of the method. Pain 38 (1992): 347-352.

Turk, D.C., and Okifuji, A. Pain terms and taxonomies of pain. In Loser, J.D. (Ed.). Bonica's management of pain, pp. 17-25. Philadelphia: Lippincott Williams&Wilkins, 2001.

Ussanawarong, S., Thesiri, T., Mahakunakorm, T., and Parasupattana, S. Effect of *Thunbergia laurifolia* Linn. on detoxication of paraquat. Khon Kaen University Research Journal 5 (2000) 11-17.

Vaillancourt, P.D., and Langevin, H.M. Painful peripheral neuropathies. Medical Clinics of North America 83 (1999): 627-643.

Vander Wende, C., and Margolin, S.. Analgesic tests based upon experimentally induced acute abdominal pain in rats. Federation proceedings 15 (1956): 494.

Vinegar, R., Scheriber, W., Hugo, R.: Biphasic development of carrageenan edema in rats. Journal of Experimental Pharmacology 166 (1969): 96-103.

Visitpongpant, W., Ruamgyuthikarn, V., Rujjanavej, C., Pantong, A., and Lertprasertsuk, N. Testing of toxicity of *Thunbergia laurifolia* Linn. abstract in mice. Journal of Herbal Medicine 10 (2003): 23-36.

Vogel, H.G. Drug Discovery and Evaluation: Pharmacological Assay, pp. 983-115. 3rd ed. Germany: Springer-Verlag, 2008.

Wagner, W.L., Herbst, D.R., and Sohmer, S.H. Manual of the Flowering Plants of Bishop Museum Special Publication 83, University of Hawaii and Museum Press, Honolulu, HI, 1999.

Walker, K., Fox, A.J., and Urban, L.A. Animal models for pain research. Journal of Molecular Medicine 7 (1999): 319-321.

Whistler, W.A. *Tropical Ornamenatals*. Timber Press, Portland, OR. 2000.

Winter, C.A., Risley, E.A., and Nuss, G.W. Carrageenan-induced oedema in hind paw of rat as an assay for anti-inflammatory drugs. Proceedings of the Society for Experimental Biology and Medicine 111 (1962): 544-547.

Won, J., Hur, Y.G., Hur, E.M., Park, S.H., Kang, M.A., Choi, Y., Park, C., Lee, K.H., and Yun, Y. Rosmarinic acid inhibits TCR-induced T-cell activation and proliferation in an Lck-dependent manner. European Journal of Immunology 33 (2003): 870-879.

Woolfe, G., and MacDonald A.D. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). Journal of Pharmacology and Experimental Therapeutics 80 (1944): 300-307.

Woolf, C.J. Recent advances in the pathophysiology of acute pain. British Journal of Anaesthesia 63 (1989):139-146.

Woolf, C.J. The pathophysiology of peripheral neuropathic pain-abnormal peripheral input and abnormal central processing. Acta Neurochirurgica Supplement 58 (1993): 125-130.

Youn, J., Lee, K.H., Won, J., Huh, S.J., Yun, H.S., Cho, W.G., and Paik, D.J. Beneficial effects of rosmarinic acid on suppression of collagen induced arthritis. The Journal of rheumatology 30 (2003): 1203-1207.

APPENDICES

Appendix A

Certificate of Project Approvel by the Institutional Animal Care and Use Committee,
Faculty of Pharmaceutical Sciences, Chulalongkhon University,
Bangkok, Thailand

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 13-33-014	**Approval No. 13-33-014**

Protocol Title
Antinociceptive and anti-inflammatory effects of rosmarinic acid and *Thunbergia laurifolia* leaf extract

Principal Investigator
PASARAPA TOWIWAT, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)
This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
March 4, 2013	March 4, 2015

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan
BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **NARUEPORN SUTANTHAVIBUL, Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

Appendix B

Antioxidant-guided isolation of rosmarinic acid (RA)

Antioxidant-guided isolation of rosmarinic acid (RA)

The extraction and isolation procedures have already been described by Suwanchaikasem et al., 2011. In brief, the dried *T. laurifolia* leaves (900 g) were macerated with 95% ethanol (8 L) at room temperature for 72 h. The extract was filtered and evaporated to dryness at 50 °C in a rotary evaporator. The extraction of the residue was repeated using the same conditions, and the two filtrates were combined. The 95% ethanolic extract (64.7 5 g) was chromatographied using ion-exchange resin eluted with water-acetone, silica gel column eluted with chloroform-methanol-formic acid (8.5:1.5:0.5) mixture, sephadex LH-20 with methanol, silica gel column with dichloromethane-methanol (7:3) to 100% methanol, respectively. Its structure was successfully determined using spectral analysis and NMR techniques. The chemical structure of rosmarinic acid. In addition, the structure was confirmed by mass spectroscopy using an AB-SCIEX QTRAP 5500 spectrometer.

Table 8 Raw data of DPPH-radical scavenging activities of the three “Rang Chuet” species.

Extract	**Concentration (µg/ml)**	**DPPH-scavenging activity (% ± SD)**
		T. laurifolia
Ethanol	150.00	57.96 ± 5.76
	100.00	43.29 ± 0.96
	67.00	40.25 ± 0.98
	44.00	33.19 ± 3.62
	30.00	22.30 ± 0.93
	20.00	28.80 ± 1.79
Aqueous	150.00	75.30 ± 3.33
	112.50	60.76 ± 1.45
	75.00	45.94 ± 2.79
	50.00	37.74 ± 0.21
	33.33	29.54 ± 1.81
	22.22	23.28 ± 3.04

Appendix C

Data of Hot-plate test

Table 9 Latency (sec) in the hot-plate test from 0-240 min after oral administration of sterile water (SW; 10 ml/kg) or various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=10 for all groups.

Treatments (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
Sterile water	15.82±1.30	16.94±1.94	17.06±1.58	17.10±0.88	15.69±0.87	14.23±1.30	14.74±1.12	17.07±1.9
RA 12.5	18.29±1.54	18.46±1.86	18.33±1.86	15.80±0.96	14.35±1.62	21.62±1.37	20.47±3.31	20.48±1.84
RA 25	15.46±0.74	17.12±1.39	16.52±0.93	15.57±1.50	16.10±1.07	16.75±1.16	17.29±1.47	18.33±1.09
RA 50	13.80±1.38	16.53±2.65	15.57±1.73	17.28±1.73	19.49±2.30	18.26±1.96	22.14±2.05	25.96±2.79
RA 100	12.39±1.28	23.67±2.21	23.62±2.28	23.16±2.25	24.39±4.12	25.14±2.87	27.17±3.46	28.74±3.72
RA 150	16.43±1.26	24.00±2.16	24.49±3.17	21.78±3.24	20.58±3.02	24.26±3.14	23.68±1.02	22.68±1.92

Table 10 Latency (sec) in the hot-plate test from 0-240 min after oral administration of 2% Tween 80 (10 mg/kg) or various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=10 for all groups.

Treatments (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
2% Tween 80	18.27±1.40	16.16±2.24	15.15±1.61	18.62±1.20	16.80±1.30	16.55±1.33	15.23±1.28	18.32±1.22
TLE 500	14.50±1.46	17.82±1.61	21.08±2.07	19.86±2.40	21.91±1.41	17.73±1.77	15.97±1.37	22.11±2.01
TLE 1000	13.50±1.15	18.16±1.62	17.39±1.27	17.85±1.49	20.58±1.66	19.99±1.26	22.75±2.08	24.25±2.23
TLE 1500	14.29±1.45	17.92±2.41	19.88±1.49	20.72±1.81	19.05±1.40	25.33±1.31	22.46±1.56	24.47±3.11
TLE 2000	16.06±1.38	17.07±2.23	19.21±1.56	24.09±3.20	25.14±1.97	26.40±2.89	29.10±2.56	29.26±3.31
TLE 2500	16.87±1.43	23.73±2.96	21.17±1.88	25.67±1.82	20.60±2.10	21.38±3.19	26.21±2.31	31.66±3.28

Table 11 %MPE-Time in hot-plate test from 0-240 min after oral administration of sterile water (SW; 10 ml/kg) or various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=10 for all groups.

Treatments (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
Sterile water	1.24±5.50	0.65±6.86	-0.36±6.59	-4.78±5.40	-8.80±4.06	-7.27±4.12	2.28±4.69	-756.65±757.58
RA 12.5	-2.07±9.02	3.29±10.06	-15.24±11.28	-19.89±11.25	9.57±8.12	11.37±10.96	6.95±6.59	800.34±673.19
RA 25	4.54±6.33	2.55±4.64	-0.82±6.82	2.02±2.66	4.37±2.71	6.22±4.20	8.78±4.91	1263±538.25
RA 50	10.02±6.31	5.32±4.62	11.37±3.39	11.02±5.78	14.61±4.77	27.33±4.87	38.41±9.37	5621.67±673.32
RA 100	33.79±5.58	31.99±7.04	33.12±4.15	30.27±16.59	37.03±9.19	46.18±10.81	46.42±12.70	9523.95±987.18
RA 150	25.03±9.35	31.00±10.83	18.94±11.63	16.55±11.31	29.51±10.69	25.76±3.52	21.97±7.07	5632.59±724.70

Table 12 %MPE-Time in hot-plate test from 0-240 min after oral administration of 2% Tween 80 (10 mg/kg) or various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=10 for all groups.

Treatments (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
2% Tween 80	-10.42±10.32	-14.36±8.40	-1.72±7.78	-8.80±8.09	-9.58±7.85	-14.99±8.04	-1.67±5.79	-2107.38±1792.84
TLE 500	9.96±5.56	21.85±5.73	18.54±7.54	24.49±3.03	9.05±7.15	4.05±4.44	24.93±6.11	3377.71±834.25
TLE 1000	13.04±6.59	11.84±3.07	13.01±4.64	21.36±5.63	19.09±5.41	28.92±6.41	32.52±7.51	5742.16±1396.29
TLE 1500	11.47±7.44	17.40±4.76	19.83±6.27	13.96±5.89	35.76±3.48	24.87±6.61	32.67±10.06	5942.68±1435.90
TLE 2000	4.15±6.53	10.59±5.09	29.17±10.85	31.86±5.60	37.17±9.73	45.38±8.08	47.58±10.83	8749.14±1879.47
TLE 2500	25.94±11.43	14.70±4.82	31.84±3.59	10.31±9.25	19.51±10.22	30.85±9.42	53.90±11.38	7458.60±1628.08

Table 13 Latency (sec) in hot-plate test from 0-240 min after administration of naloxone (NAL; 5 mg/kg, i.p.), sterile water (SW; 10 ml/kg, p.o.), rosmarinic acid (RA; 100 mg/kg, p.o.) and the combination of NAL and RA (5/100 mg/kg). N=10 for all groups.

Treatments (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
NAL 5 mg/kg	21.60±1.59	16.6520±1.08	16.68±1.35	18.12±2.08	18.88±1.21	19.18±1.26	19.68±1.67	17.13±1.65
SW 10 ml/kg	23.57±1.34	24.04±1.98	20.86±1.42	21.79±1.97	20.87±1.23	20.04±1.29	19.10±1.00	18.58±1.48
RA 100 mg/kg	16.28±0.80	19.93±1.78	20.83±1.83	21.14±1.93	19.76±1.39	20.14±1.87	23.32±1.20	20.92±1.26
NAL + RA 100 mg/kg	17.22±1.11	13.37±0.98	14.85±1.15	15.01±1.10	15.59±0.99	15.93±0.75	15.25±1.11	16.14±0.82

Table 14 Latency (sec) in mouse hot-plate test from 0-240 min after administration of naloxone (NAL; 5 mg/kg, i.p.), 2%Tween 80 (10 mg/kg; p.o.), *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg, p.o.) or the combination of NAL and TLE (5/2000 mg/kg).

N=10 for all groups.

Treatments (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
NAL 5 mg/kg	21.04±1.86	17.82±1.96	17.27±1.74	15.26±1.55	18.17±2.01	18.55±1.64	18.87±1.82	16.87±1.88
2% Tween 80 10 mg/kg	20.83±1.53	19.31±1.56	20.83±1.86	21.70±1.63	20.44±2.49	20.69±1.26	20.57±1.60	19.21±1.54
TLE 2000 mg/kg	19.83±2.19	24.10±1.06	23.94±1.70	22.75±2.66	25.06±2.18	27.20±3.65	28.82±3.80	30.67±2.95
NAL + TLE 2000 mg/kg	16.65±1.58	10.23±1.21	9.01±1.49	11.68±1.79	13.36±1.66	13.08±1.14	11.08±1.14	14.21±1.17

Table 15 % MPE-Time in hot-plate test from 0-240 min after administration of naloxone (NAL; 5 mg/kg, i.p.), sterile water (SW; 10 ml/kg, p.o.), rosmarinic acid (RA; 100 mg/kg, p.o.) or the combination of NAL and RA (5/100 mg/kg). N=10 for all groups.

Treatments (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
NAL 5 mg/kg	-26.30±8.88	-27.33±11.13	-16.79±7.84	-16.72±8.92	-15.80±9.97	-10.85±7.53	-21.40±5.61	-4004.28±1438.78
SW 10 ml/kg	-0.15±8.22	-17.65±10.81	-10.36±6.95	-17.41±8.95	-21.14±9.05	-26.11±8.61	-26.81±6.78	-3141.12±810.27
RA 100 mg/kg	12.51±6.16	15.39±6.79	16.70±6.71	11.59±5.30	13.56±6.22	24.44±3.86	15.84±4.30	4119.94±740.52
NAL + RA 100 mg/kg	-16.01±7.41	-9.13±3.51	-8.78±4.13	-7.24±5.33	-6.13±5.21	-7.94±4.47	-5.10±4.44	-1757.12±846.32

Table 13 %MPE-Time in hot-plate test from 0-240 min after administration of naloxone (NAL; 5 mg/kg, i.p.), 2%Tween 80 (10 mg/kg; p.o.), *Thunbergia laurifolia* leaf extract (TLE; 2000 mg/kg, p.o.) and the combination of NAL and TLE (5/2000 mg/kg).

N=10 for all groups.

Treatments (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
NAL 5 mg/kg	-10.11±6.94	-11.42±9.30	-20.68±5.03	-8.75±7.85	-8.87±8.65	-7.91±10.01	-14.41±7.78	-2553.68±1186.09
2% Tween 80 10 mg/kg	-13.52±10.93	-10.12±16.80	-3.76±12.30	-10.30±16.72	-8.71±11.95	-5.77±6.93	-14.68±11.76	-2217.47±2179.96
TLE 2000 mg/kg	3.36±15.85	12.74±5.31	7.81±11.49	11.15±17.72	33.87±11.70	41.32±11.06	43.44±10.68	7330.86±1307.08
NAL + TLE 2000 mg/kg	-30.74±14.55	-33.82±11.96	-20.89±7.28	-14.15±5.65	-17.27±8.66	-26.92±13.25	-13.14±8.42	-4915.99±2269.43

Appendix D

Data of Acetic acid-induced writhing test

Table 14 Dose-response and time-course effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg) on acetic acid-induced writhing in mice. N=8 for all groups.

Treatment(mg/kg)	Number of writhes (mean±S.E.M.)						Total
	0-5	5-10	10-15	15-20	20-25	25-30	
2% Tween 80	2.75±0.84	8.88±1.64	8.00±1.63	5.25±0.59	6.00±0.87	6.13±1.54	37.00±5.34
IND 10	0.38±0.14	4.13±1.23	3.75±0.88	3.00±0.63	2.63±0.87	0.63±0.38	14.50±2.67
SW 10	0.13±0.13	5.63±0.71	9.50±0.96	12.38±1.72	7.50±1.52	4.63±0.78	39.63±3.98
RA 12.5	1.13±0.35	9.50±0.89	8.75±1.42	7.50±0.87	5.50±0.82	4.50±0.76	36.88±3.56
RA 25	1.50±0.57	8.63±1.05	9.88±1.13	7.88±1.74	5.25±0.49	2.50±0.54	35.63±4.38
RA 50	0.50±0.19	5.50±1.32	6.25±1.44	2.50±0.73	2.00±0.50	2.25±0.70	19.00±2.77
RA 100	0.25±0.25	1.63±1.00	1.75±0.45	1.63±0.26	0.25±0.16	0.63±0.26	6.13±1.68
RA 150	1.75±0.49	5.75±0.84	7.75±1.22	7.00±1.20	4.75±0.73	4.13±1.16	29.88±4.80

Table 15 Dose-response and time-course effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg) or various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg) on acetic acid-induced writhing in mice. N=8 for all groups.

Treatment(mg/kg)	Number of writhes (mean±S.E.M.)						Total
	0-5	5-10	10-15	15-20	20-25	25-30	
2% Tween 80	2.88±0.40	13.13±1.14	13.13±1.68	11.13±0.70	9.13±0.67	4.00±0.70	54.13±3.41
IND 10	1.00±0.38	3.88±0.48	3.63±0.65	1.88±0.48	2.75±1.08	2.25±0.45	15.38±2.32
TLE 500	2.63±0.73	11.88±2.22	13.00±2.11	7.25±1.08	5.25±0.49	3.88±0.79	44.13±3.75
TLE1000	1.38±4.98	9.63±1.79	10.00±1.38	7.13±1.03	4.88±0.64	3.75±0.90	36.75±3.98
TLE 1500	1.50±0.63	6.25±1.13	6.88±0.95	4.50±0.98	5.25±0.70	4.00±0.71	28.25±3.06
TLE 2000	2.13±0.35	7.63±0.84	6.38±0.71	6.13±0.77	4.88±0.99	2.63±0.53	29.75±2.57
TLE 2500	1.25±0.31	4.75±0.68	4.13±0.52	2.88±0.69	3.25±0.59	1.13±0.35	17.38±4.63

Appendix E

Data of Formalin test

Table 19 Time spent on paw licking after intraperitoneal administration of 0.9% normal saline solution (NSS; 10 ml/kg) and morphine sulfate (MO; 10 mg/kg). N=8 for all groups.

Treatments	Licking time (sec)	
	Early phase	Late phase
NSS	87.80±4.09	131.60±6.59
MO	9.45±1.60	5.51±1.18

Table 20 Time spent on paw licking after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg). N=8 for all groups.

Treatments	Licking time (sec)	
	Early phase	Late phase
2% Tween 80	106.35±11.88	125.38±9.65
IND	90.40±6.43	39.60±4.07
Sterile water	92.06±6.39	126.67±11.22
RA	111.20±7.73	120.73±8.25
	109.65±11.38	98.79±10.52
	88.37±12.86	90.69±8.16
	41.09±7.03	52.24±7.12
	63.31±7.45	92.84±7.36

Table 21 Time spent on paw licking after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg) and various doses of *Thunbergia laurifolia* leaf extract (TLE; 500-2500 mg/kg). N=8 for all groups.

Treatments	Licking time (sec)	
	Early phase	Late phase
2% Tween 80	65.06±2.98	124.05±5.43
IND	71.16±2.81	69.33±3.67
TLE	72.22±3.78	134.17±7.15
	60.56±2.92	125±6.43
	50.56±2.50	93.18±6.75
	46.27±2.55	67.18±3.33
	64.70±1.85	89.75±5.74

Appendix F

Data of Rota-rod test

Table 22 Effect of rosmarinic acid (RA; 100 mg/kg) and *T. laurifolia* leaf extract (TLE; 2000 mg/kg) on motor impairment in Rota-rod test. N=8 per group.

Treatments (mg/kg)	Time (mean±S.E.M.)				
	30 min	60 min	90 min	120 min	240 min
SW	60±0	59.16±0.85	60±0	60±0	60±0
RA 100	57.50±0.25	60±0	60±0	59.30±0.70	59.08±0.92

Treatments (mg/kg)	Time (mean±S.E.M.)				
	30 min	60 min	90 min	120 min	240 min
2%Tween 80	60±0	60±0	59.5±0.5	59.62±0.38	59.62±0.38
TLE 2000	60±0	60±0	60±0	60±0	60±0

Appendix G

Data of Carrageenan-induced-paw edema test

Table 23 Effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and various doses of rosmarinic acid (RA; 12.5-150 mg/kg) on paw volume in the carrageenan-induced paw edema in mice. N=8 for all groups.

Treatment(mg/kg)	Paw edema (ml)±S.E.M.					
	1 h	2 h	3 h	4 h	5 h	6 h
2% Tween 80	0.1587	0.2025	0.2075	0.2012	0.2338	0.2500
IND 10	0.1050	0.0800	0.0750	0.0638	0.0938	0.0925
SW	0.1438	0.1650	0.1513	0.1425	0.1813	0.1775
RA 12.5	0.0950	0.1363	0.1113	0.1413	0.1350	0.1450
RA 25	0.0888	0.1175	0.1275	0.1325	0.1400	0.1550
RA 50	0.1012	0.1087	0.0738	0.0738	0.1375	0.1500
RA 100	0.0938	0.1063	0.0650	0.0650	0.0875	0.0925
RA 150	0.1288	0.1125	0.1025	0.0763	0.1150	0.1050

Table 24 Effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), and various doses of *T. laurifolia* leaf extract (TLE; 500-2500 mg/kg) on paw volume in the carrageenan-induced paw edema in mice. N=8 for all groups.

Treatment(mg/kg)	Paw edema (ml)±S.E.M.					
	1 h	2 h	3 h	4 h	5 h	6 h
2% Tween 80	0.1538	0.1950	0.2063	0.2175	0.2275	0.2438
IND 10	0.1125	0.0925	0.0763	0.0550	0.0788	0.0838
TLE 500	0.1138	0.1850	0.1912	0.1400	0.1538	0.1500
TLE 1000	0.1188	0.1800	0.1850	0.1388	0.1438	0.1525
TLE 1500	0.1188	0.1850	0.1950	0.1463	0.1625	0.1575
TLE 2000	0.1175	0.1475	0.1363	0.1100	0.1163	0.1275
TLE 2500	0.1200	0.1613	0.1637	0.1263	0.1387	0.1450

Appendix H

Data of Prostaglandin E_2-induced-paw edema test

Table 25 Effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (SW; 10 ml/kg) and rosmarinic acid (RA 100 mg/kg) on paw volume in the PGE_2-induced paw edema in mice. N=8 for all groups.

Treatment(mg/kg)	Paw edema (ml)±S.E.M.					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
2% Tween 80	0.1675	0.1563	0.1588	0.1575	0.1538	0.1338
IND 10	0.0810	0.0700	0.0650	0.0613	0.0613	0.0575
SW	0.1375	0.1425	0.1200	0.0913	0.0987	0.1175
RA 100	0.0788	0.0613	0.0550	0.0513	0.0425	0.0525

Table 26 Effect of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), and *T. laurifolia* leaf extract (TLE 2000 mg/kg) on paw volume in the PGE_2-induced paw edema in mice. N=8 for all groups.

Treatment(mg/kg)	Paw edema (ml)±S.E.M.					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
2% Tween 80	0.1063	0.0750	0.1025	0.0963	0.1125	0.1175
IND 10	0.0700	0.0375	0.0288	0.0250	0.0438	0.0400
TLE 2000	0.0675	0.0350	0.0338	0.0225	0.0325	0.0350

Appendix I

Data of Cotton pellet-induced granuloma formation

Table 27 The weight of granuloma after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), sterile water (10 ml/kg), and rosmarinic acid (RA; 50-150 mg/kg). N= 8 for all groups.

Treatment(mg/kg)	Cotton pellet (mg)±S.E.M.
2% Tween 80	86.90±7.1073
IND 10	45.94±7.3702
SW	95.48±10.1653
RA 50	71.40±9.8947
RA 100	58.31±5.2141
RA 150	71.40±9.8947

BIOGRAPHY

NAME	Miss Wanvisa Boonyarikpunchai
DATE OF BIRTH	August 26th, 1986
PLACE OF BIRTH	Chonburi, Thailand
INSTITUTION ATTENDED	Bachelor of Science in Pharmacy from Huachiewchalermprakert University, 2009
ADDRESS	47/3 Moo. 8 Maerumphueng Bangsaphan Prachuabkirikhan 77140