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ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF THE ETHANOLIC EXTRACT OF *CURCUMA* AFF. *AMADA*

Miss Apicha Jabsuwan

A Thesis Submitted in Partial Fulfillment of the Requirements
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อภิชา จาบสุวรรณ : ฤทธิ์ระงับปวดและฤทธิ์ต้านการอักเสบของสิ่งสกัดเอทานอลของว่านรางจืด (ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF THE ETHANOLIC EXTRACT OF *CURCUMA* AFF. *AMADA*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ภัสราภา โตวิวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สุชาดา สุขหร่อง, สมพร สวัสดิสรรพ์, 132 หน้า.

ว่านรางจืด (*Curcuma* aff. *amada*) เป็นสมุนไพรไทยที่มีสรรพคุณแก้พิษ ล้างพิษ และต้านการอักเสบ อย่างไรก็ตามยังไม่มีข้อมูลทางวิทยาศาสตร์ที่สนับสนุนฤทธิ์ระงับปวดและต้านการอักเสบของสมุนไพรชนิดนี้ ในการตรวจสอบฤทธิ์ระงับปวดและฤทธิ์ต้านการอักเสบของสิ่งสกัดเอทานอลของว่านรางจืด และการตรวจสอบกลไกการออกฤทธิ์ ทำการประเมินผลฤทธิ์ระงับปวดของสิ่งสกัดเอทานอลของว่านรางจืดในหนูเม้าส์ด้วยวิธีเหนี่ยวนำให้หนูเม้าส์เกิดความเจ็บปวดด้วยแผ่นความร้อน เหนี่ยวนำให้หนูเม้าส์บิดงอลำตัวด้วยกรดอะซิติก และการทดสอบด้วยฟอร์มาลิน ศึกษาฤทธิ์ต้านการอักเสบของสิ่งสกัดเอทานอลของว่านรางจืดด้วยการประเมินการบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยคาราจีแนน การบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยพีจีอี 2 การบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยกรดอะราคิโดนิก และการเกิดแกรนูโลมาจากการเหนี่ยวนำด้วยก้อนลำสี ในการตรวจสอบกลไกการออกฤทธิ์ต้านการอักเสบนั้น เนื้อเยื่ออุ้งเท้าหนูจะถูกตรวจสอบทางจุลกายวิภาคศาสตร์ การได้รับสิ่งสกัดเอทานอลของว่านรางจืดขนาด 100 และ 200 มก./กก. โดยการป้อน จะเพิ่มระยะเวลาที่หนูทนอยู่บนแผ่นความร้อนได้อย่างมีนัยสำคัญทางสถิติ ($p<0.01$และ $p<0.001$ ตามลำดับ) ซึ่งฤทธิ์ระงับปวดนี้ถูกยับยั้งได้ด้วยนาลอกโซน แสดงให้เห็นว่ามีความเกี่ยวข้องกับตัวรับโอปิออยด์ สิ่งสกัดเอทานอลของว่านรางจืดขนาด 12.5, 25, 50, 100 และ 200 มก./กก. สามารถลดจำนวนครั้งของการบิดงอลำตัวของหนูจากการเหนี่ยวนำด้วยกรดอะซิติกได้อย่างมีนัยสำคัญทางสถิติ และสามารถยับยั้งการเลียอุ้งเท้าหนูจากการเหนี่ยวนำด้วยฟอร์มาลินในทั้งสองช่วงเวลาได้อย่างมีนัยสำคัญทางสถิติ สิ่งสกัดเอทานอลของว่านรางจืดขนาด 12.5, 25, 50, 100 และ 200 มก./กก. สามารถระงับการบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยคาราจีแนนที่ชั่วโมงที่ 4, 5 และ 6 หลังฉีดคาราจีแนนได้อย่างมีนัยสำคัญทางสถิติ และมีฤทธิ์ต่อต้านการบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยพีจีอี 2 อย่างมีนัยสำคัญทางสถิติ สิ่งสกัดเอทานอลของว่านรางจืดไม่สามารถยับยั้งการบวมของอุ้งเท้าหนูจากการเหนี่ยวนำด้วยกรดอะราคิโดนิกได้ การศึกษาทางจุลกายวิภาคศาสตร์แสดงให้เห็นว่า สิ่งสกัดเอทานอลของว่านรางจืดทุกขนาดสามารถลดการแทรกซึมของเซลล์เม็ดเลือดขาวนิวโทรฟิลจากการเหนี่ยวนำด้วยคาราจีแนนได้อย่างมีนัยสำคัญทางสถิติ สิ่งสกัดเอทานอลของว่านรางจืดขนาด 200 มก./กก.ยังสามารถยับยั้งการเกิดแกรนูโลมาจากการเหนี่ยวนำด้วยก้อนลำสีในหนูเม้าส์ได้อย่างมีนัยสำคัญทางสถิติ จากผลการทดสอบทั้งหมดแสดงว่า สิ่งสกัดเอทานอลของว่านรางจืดมีฤทธิ์ระงับปวดทั้งในระบบประสาทส่วนกลางและระบบประสาทส่วนปลาย และมีฤทธิ์ต้านการอักเสบต่อการอักเสบเฉียบพลันและการอักเสบเรื้อรังโดยไม่มีผลพิษเฉียบพลันที่ชัดเจน กลไกการออกฤทธิ์ระงับปวดดูเหมือนว่ามีความเกี่ยวข้องบางส่วนกับตัวรับโอปิออยด์ กลไกต้านการอักเสบอาจเกิดจากการรบกวนการหลั่งของพีจีหรือการยับยั้งผลของพีจีอี 2 ของสิ่งสกัดเอทานอลของว่านรางจืด ข้อมูลที่ได้จากการศึกษาเหล่านี้สนับสนุนการใช้สิ่งสกัดนี้ทางเภสัชพฤกษศาสตร์วิทยาสำหรับการรักษาอาการปวดและความผิดปกติของการอักเสบ อย่างไรก็ตามควรมีการตรวจสอบข้อมูลด้านความปลอดภัยของสิ่งสกัดนี้ต่อไป

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต ....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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APICHA JABSUWAN: ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF THE ETHANOLIC EXTRACT OF *CURCUMA* AFF. *AMADA*. ADVISOR: ASST. PROF. FLG. PASARAPA TOWIWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. POL.CAPT. SUCHADA SUKRONG, Ph.D., PROF. SOMPORN SWASDISON, D.D.S.,Ph.D., 132 pp.

Curcuma aff. *amada* is used for treatment of poisoning, detoxification and anti-inflammation in Thai folk medicine. However, there is no scientific evidence supporting the potential antinociceptive and anti-inflammatory activities of this plant. To investigate the antinociceptive and anti-inflammatory effects of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE), and to examine the mechanisms of actions underlying these effects. The antinociceptive effect of CAE was assessed in mice using hot-plate, acetic acid-induced writhing and formalin tests. The anti-inflammatory effect of CAE was investigated by evaluation of carrageenan-induced paw edema, PGE_2-induced paw edema, arachidonic acid-induced paw edema, and cotton pellet-induced granuloma formation. To investigate the mechanism of anti-inflammatory action, paw tissues were examined histologically. Oral administration of CAE (100 and 200 mg/kg) significantly ($p<0.01$ and $p<0.001$, respectively) increased hot-plate latencies and this effect was reversed by naloxone, indicating involvement of opioid receptors. CAE (12.5, 25, 50, 100 and 200 mg/kg) significantly reduced acetic acid-induced writhing and caused significant inhibition of formalin-induced paw licking in both phases. In anti-inflammatory tests, CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg significantly suppressed carrageenan-induced paw edema at 4, 5 and 6 h after carrageenan injection and showed significant activity against PGE_2-induced paw edema. CAE failed to inhibit paw edema induced by arachidonic acid. Histological studies showed that all doses of CAE decreased infiltration of neutrophils induced by carrageenan. CAE at 200 mg/kg also inhibited cotton pellet-induced granuloma formation in mice. Taken together, these results show that CAE possesses both central and peripheral antinociceptive activities and has anti-inflammatory effects against acute and chronic inflammation with no obvious acute toxic effects. The analgesic mechanism of action seems to be partly related to opioid receptors. The mechanism of anti-inflammatory effect may be due to the interference of CAE on the liberation of PGs or inhibition of PGE_2 effects. These data support the ethnopharmacological use of this extract for treatment of pain and inflammatory disorders. However, further evaluation of the safety profile of the extract is needed.

Field of Study: Pharmacology	Student's Signature ....
Academic Year: 2014	Advisor's Signature ....
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LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
κ	=	kappa
δ	=	delta
µL	=	microliter
/	=	per
%	=	percent
% MPE	=	percentage of the maximum possible effect
℃	=	degree celsius
AA	=	arachidonic acid
AUC	=	area under the curves (area of analgesia)
ADH	=	Anti-diuretic hormone
cm	=	centimeter
cAMP	=	cyclic adenosine monophosphate
CAE	=	the ethanolic extract of *Curcuma* aff. *amada* rhizome
CNS	=	central nervous system
Co.	=	company
COX	=	cyclooxygenase
CR	=	corticosteroid receptor
DRG	=	dorsal root ganglia
e.g.	=	example gratia
et al.	=	et alli (and other)
GI	=	gastrointestinal
FRAP	=	ferric-reducing antioxidant power assay

g	=	gram
h	=	hour
IASP	=	International Association for the Study of Pain
i.e.	=	id est
IL	=	interleukin
IND	=	indomethacin
i.p.	=	intraperitoneal
LOX	=	lipoxygenase
LT	=	leukotriene
LTA_4	=	leukotriene A_4
LTB_4	=	leukotriene B_4
LTC_4	=	leukotriene C_4
LTD_4	=	leukotriene D_4
m	=	meter
mg/kg	=	milligram per kilogram
mg/mL	=	milligram per milliliter
min	=	minute
mL	=	milliliter
mL/kg	=	milliliter per kilogram
MO	=	morphine sulphate
mm	=	millimeter
m/s	=	meter per second
N	=	sample size
NAL	=	naloxone
NF-**K**B	=	nuclear factor kappa B
NMDA	=	N-methyl-D-aspartate
NO	=	nitric oxide

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NSAIDs	=	non-steroidal anti-inflammatory drugs
NSS	=	normal saline solution
PAF	=	platelet activating factor
PAG	=	periaqueductal gray
PG	=	prostaglandin
PGD_2	=	prostaglandin D_2
PGE_2	=	prostaglandin E_2
$PGF_{2\alpha}$	=	prostaglandin F_2 alpha
PGI_2	=	prostacyclin
PLA_2	=	phospholipase A_2
p.o.	=	per os
ROS	=	reactive oxygen species
rpm	=	revolutions per minute
s	=	second
TNF-α	=	tumor necrosis factor-alpha
TX	=	thromboxane
TXA_2	=	thromboxane A_2
vs	=	versus
w/v	=	weight by volume
w/w	=	weight by weight

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Chapter I

INTRODUCTION

Background and Rationale

Pain is a major health problem in the general population and lead to extensive medical consultation. Pain is an unpleasant experience that prevails throughout time. However, pain can become a disease when it occurs or persists in the absence of tissue damage or following appropriate healing of injured tissues. This pain has considerable negative impact on quality of life, and has profound economic impact on the family and society. Pain relief with drugs is impossible to achieve or there is a need to interrupt their use because of side effects. For this reason, several studies have attempted to find effective analgesic agents obtained from traditional plant-derived medicines with high efficacy and minor adverse effects or less toxicity (Calixto et al., 2000).

Inflammation is a complex biological response for maintaining homeostasis in the body. It is a response of the immunological defense system to microbial infections, burns, allergens, mechanical or chemical injuries and other noxious stimuli. Inflammation is a complex series of cascade reactions, including enzyme activation, release of chemical mediators, effusion of fluids, cell migration, and tissue damage and repair (Vane & Botting, 1995). Inflammation is involved in the pathogenesis of diseases such as gout, rheumatoid arthritis, pulmonary emphysema, diabetes, neurodegenerative, cardiovascular, cancer and other life-threatening diseases (Lawrence, Willoughby, & Gilroy, 2002). Therefore, inflammatory-associated diseases are major cause of morbidity of people worldwide.

Analgesic and anti-inflammatory drugs are widely used for the clinical treatment of pain and inflammatory diseases. These drugs have serious side effects

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and adverse effects. An acute overdose of acetaminophen commonly used for the management of perioperative pain, causes mild to moderate hepatotoxicity and has been associated with renal tubular necrosis, hypoglycemic coma and thrombocytopenia (Gürsoy et al., 1996). Non-steroidal anti-inflammatory drug (NSAIDs) are associated with upper gastrointestinal bleeding, ulcers, small intestinal perforation and hepatotoxicity (Lanas et al., 2006). Common side effects of opioids administration include sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance, and respiratory depression (Porreca & Ossipov, 2009). Corticosteroids have been used for various indications in clinical setting for considerable time because of their potent immunosuppressive and anti-inflammatory properties. Corticosteroid withdrawal can induce mental disturbances (i.e., agitation, anxiety, depression and psychotic reactions) and acute adrenal insufficiency (Buchman, 2001; Patel et al., 2001) The findings for new pharmacologically active agents obtained from medicinal plants have led to the discovery of many clinically useful drugs that play a major role in the treatment of human diseases (Harvey, 2008). Therefore, analgesic and anti-inflammatory compounds with better safety profiles is needed for the treatment of pain and inflammation.

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Many different pharmacological actions of plants in the genus *Curcuma* have been shown in *in vitro* and *in vivo* models. For example, *Curcuma longa* (Turmeric) has been reported to have antioxidant, hepatoprotective, anticarcinogenic and cardioprotective effects (Çıkrıkçı, Mozioglu, & Yılmaz, 2008) and *Curcuma amada* Roxb. (Mango ginger) has antioxidant, antibacterial, antifungal, anti-inflammatory, platelet aggregation inhibitory, cytotoxicity, antiallergic, hypotriglyceridemic, biopesticide, enterokinase inhibitory, antituberculosis, CNS depressant, analgesic, aphrodisiac, and antihypercholesterolemic properties (Policegoudra, Aradhya, & Singh, 2011). Amadaldehyde, a pure compound isolated from a chloroform extract of mango ginger, has bactericidal, antioxidant, cytotoxicity and platelet aggregation

inhibitory activities (Policegoudra, Rehna, Rao, & Aradhya, 2010). Most activities from plants in the genus *Curcuma* result from the presence of curcumin and its derivatives in the plant extract.

Curcuma aff. *amada* (family Zingiberaceae) is known in Thai as "Wan Rang Chuet". A specific species name for *Curcuma* aff. *amada* plant has yet to be assigned; however, it appears to have an affinity similar to *Curcuma amada*. The rhizome of *Curcuma* aff. *amada* is used for treatment of poisoning, detoxification and inflammation in Thai traditional medicine. The decoction of its rhizome is used to treat abscess and poisoning from insecticides and ethyl alcohol (Kanathum, 2008). Aqueous and ethanolic extracts of *Curcuma* aff. *amada* were shown to have antioxidant activity in the Ferric reducing antioxidant power (FRAP) assay and to have natural phenolic components (Suwanchaikasem, Phadungcharoen, & Sukrong, 2013). Several studies have indicated that reactive oxygen species scavenging and anti-inflammatory activities of medicinal plants are influenced by natural phenolic components with antioxidant effects (Chang et al., 2001). However, the active compounds responsible for the biological activities of *Curcuma* aff. *amada* rhizome extract (CAE) have not been determined and there is no scientific evidence supporting the anti-inflammatory activity and other pharmacological properties of *Curcuma* aff. *amada*.

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Purpose of the study

To investigate the antinociceptive and anti-inflammatory effects of the ethanolic extract of *Curcuma* aff. *amada* rhizome, and to examine the mechanisms of actions underlying these effects.

Hypothesis

The ethanolic extract of *Curcuma* aff. *amada* rhizome has antinociceptive and anti-inflammatory effects in various animal models.

Research design

Experimental Research

Expected benefit and application

The results obtained from this study provide scientific evidence of antinociceptive and anti-inflammatory effects of the ethanolic extract of *Curcuma* aff. *amada* rhizome and support ethnopharmacological use of this extract for the treatment of pain and inflammatory disorders.

Key words

Curcuma aff. *amada*

Antinociceptive

Anti-inflammatory

Hot-plate test

Acetic acid-induced writhing

Formalin test

Carrageenan-induced mouse paw edema

Prostaglandin E_2-induced mouse paw edema

Arachidonic acid-induced mouse paw edema

Cotton pellet-induced granuloma formation

Histological examination

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CHAPTER II

LITERATURE REVIEWS

Pain

Pain is a physiological consequence of tissue injury that serves a vital protective function. It is an extremely complex process that involves the interaction of an array of neurotransmitters and neuromodulators at all level of the neuraxis. In 1996 the International Association for the Study of Pain (IASP) defined pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey & Bogduk, 1994).

Nociceptors

Nociceptive receptors are free nerve endings found throughout the body (i.e. skin, viscera, muscle, blood vessels and joint singular capsules), that detect signals from damaged tissue and also indirectly respond to chemicals released from the damage. The cell bodies of nociceptors are located in the trigeminal ganglia for the face, and the dorsal root ganglia for the body, and have both the peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively. Nociceptors are activated by intense thermal, mechanical or chemical stimuli when stimulus intensities reach the noxious range, and generate impulses along afferent nerves to the central nervous system (CNS) (Dubin & Patapoutian, 2010).

The classification of the nociceptor base on two main nerve fiber types which is the terminal end (Figure 1).

The first class of nociceptor includes small-diameter, unmyelinated "C" fibers bundles in fascicles surrounded by Schwann cells and support conduction velocities

of 0.5-2 m/s that conduct the nerve impulse slowly, which transmit pain that is poorly-localized, dull and aching. C-fibers terminate in superficial laminae I and II in the dorsal horn of spinal cord. The C-fibers respond to chemical, thermal and mechanical stimuli (Steeds, 2013).

The second class of nociceptor includes large-diameter, myelinated "Aδ" fibers that support conduction velocities of 5-15 m/s, and carry impulses rapidly, which transmit well-localized sharp pain. A-delta fibers terminate in superficial laminae I and V in the dorsal horn of spinal cord. These fibers respond to mechanical and mechanothermal stimuli (Steeds, 2013).

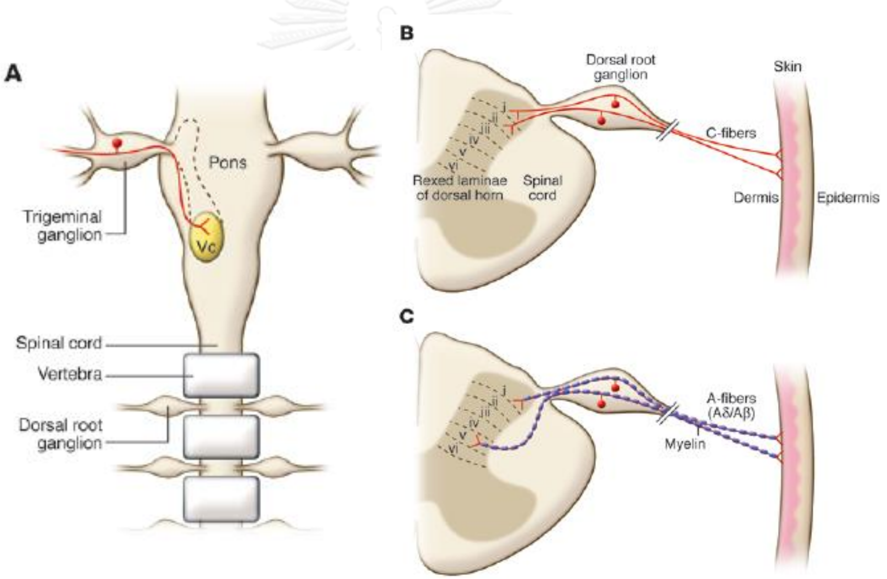

Figure 1 Nociceptive receptors or nociceptors.

(A) Somatosensory neurons are located in trigeminal and dorsal root ganglia located along-side the spinal column and medulla. Afferent neurons project centrally to the brain stem (Vc) and dorsal horn of the spinal cord and peripherally to the skin and other organ. (B) The nociceptor is unmyelinated with small diameter axons (C-fibers, red). Their peripheral afferent innervates the skin and central process projects to superficial laminae I and II of the dorsal horn. (C) A-fiber nociceptors are myelinated and usually have conduction velocities in the A-delta range (red). A-fiber nociceptor projects to superficial laminae I and V of the dorsal horn (Dubin & Patapoutian, 2010).

Classification of pain

Pain can be categorized base on duration of pain (i.e., acute vs. chronic pain) and underlying pathophysiology pain (i.e., nociceptive and neuropathic and psychogenic) (Figure 2).

Acute and Chronic Pain

Acute pain comes on suddenly and has a limited duration. It's frequently caused by damage to tissues such as needle prick, burns and surgical interventions. The local injury does not overwhelm the body's reparative mechanisms called healing process, which occur without medical intervention. Therefore, medical interventions may be useful to reduce pain and to speed up the healing process. Treatment approaches include nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and common analgesic drugs (opioids and non-opioids) can adequately control the pain. This type of pain usually takes a few days or a few weeks. However, period of acute pain not exceed 4-6 weeks (Loeser & Melzack, 1999). If acute pain is not effectively treated it may develop into a chronic pain condition.

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Chronic pain is associated with prolonged tissue damage or injuries to the peripheral and CNS including low back pain, pelvic and abdominal pain, rheumatoid arthritis, osteoarthritis, fibromyalgia, migraine and cancer, which also involve complex changes in nociceptive pathways (Dray, 1995). The local injury may exceed the body's capability for healing, which the intensity of the pain is out of proportion to the original injury or tissue damage. The chronic pain is unmitigated, it is likely that stress, environmental and affective factors may be increased on the original damaged tissue and contribute on the intensity and existence of the pain. Some investigators use duration of ≥6 months to designate pain as chronic. All types of chronic pain lead patient to seek for medical consultation (Loeser & Melzack, 1999).

Nociceptive pain

Nociceptive pain caused by tissues damage which stimulated specific sensory receptors or nociceptors in somatic or visceral structures, and thus serves as an "alarm" and protective system against noxious stimuli (Serra, 1999).

Neuropathic Pain

Neuropathic pain is a complex pain state or caused by a primary lesion or dysfunction in the nervous system (Backonja, 2003). The pain is due to an abnormal responsiveness or function of the peripheral or CNS (or both), in which heightened gain or sensitivity of the sensory apparatus amplifies symptoms. Underlying causes include infections, trauma, surgery, chemotherapy, neurotoxins, nerve compression, metabolic abnormalities, inflammatory and tumor infiltration (Dworlzin et al., 2003). Neuropathic pain is also characterized by evoked pains (Dubin & Patapoutian, 2010):

Allodynia is painful response to a normally innocuous stimulus (i.e., the friction of clothing rubbing against the skin).

Hyperalgesia (an increased sensation of pain in response to a normally painful stimulus) is pain of abnormal severity following a noxious stimulus or sensitization occurs when intense and repeated stimuli from tissue damage. This results in a lowered threshold for activation of primary afferent nociceptors, which leads to innocuous stimuli causing severe pain. For example, patients with pharyngitis, swallowing can be extremely painful (Dubin & Patapoutian, 2010).

Psychogenic or Idiopathic Pain

Psychogenic or Idiopathic or functional pain is caused by depression, anxiety, fear of death and irregularities in the functioning of the CNS (Woolf, 2004).

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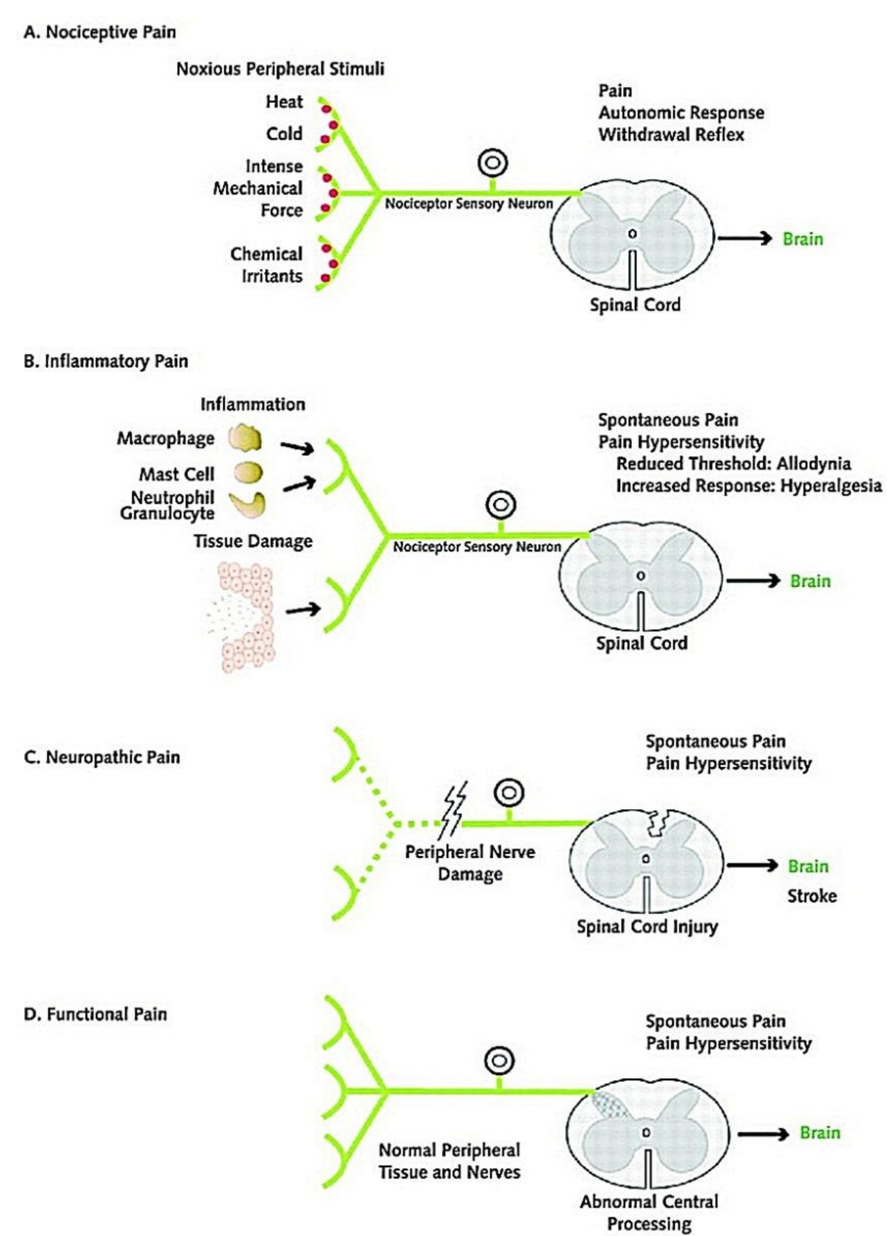

Figure 2 Pain can be broadly divided into four classes.

(A) Nociceptive pain represents the sensation associated with the detection of potentially tissue-damaging noxious stimuli and is protective. (B) Inflammatory pain is associated with tissue damage and the infiltration of immune cells and can promote repair by causing pain hypersensitivity until healing occurs. (C) Neuropathic pain is a disease state caused by damage to the nervous system (D) Functional pain caused by abnormal CNS (dysfunctional) (Woolf, 2004).

Peripheral sensitization

In general, somatosensation begins with activation of peripheral afferent fibers. These fibers are part of the peripheral with cell bodies located in the dorsal horn. Nociceptors have the capacity to respond to intense heat, cold, mechanical, and chemicals stimuli. A partial list of these chemicals includes histamine, bradykinin, prostaglandins, adenosine triphosphatase, neuropeptides, chemokine, cytokine and growth factor. These cellular mediators act to sensitize nociceptors to further neural input. This produce changes in the number and location of ion channels. As a result, the threshold for depolarization is lowered and in ectopic discharges. Consequently, the response of nociceptors is increased, a phenomenon known as peripheral sensitization (Beydoun & Backonja, 2003; Pasero, 2004).

Central sensitization

Central sensitization begins with the abnormal hyperexcitability of central nociceptor neurons, a cascade of events in the dorsal horn of the spinal cord that are triggered by release of transmitters from nociceptor central terminals, leading to alterations in synaptic receptor density, kinetics, threshold and stimulation, and thus dramatically increasing transmission of pain. The receptors involved in these changes are the glutamate-activated *N*-methyl-D-aspartate (NMDA) receptor and glutamate metabotropic receptors. Glutamate also plays a major role in the process of sensitization causes an increase in intracellular calcium levels (Woolf, 2004). These changes, lead to a series of biochemical reaction in dorsal horn neuron. The threshold for activation is lowered and the response to stimuli is increased. These changes result in a phenomenon known as “wind-up,” an increased excitability and sensitivity of spinal cord neuron (Beydoun & Backonja, 2003; Pasero, 2004).

Pain pathways

The sequence of events by which a stimulus is perceived involves four processes; an overview of these processes is presented below.

1. Transduction

Transduction is the cellular process whereby the noxious stimuli are changed into the electrical energy (action potential) necessary to transmit pain. Transduction begins in the peripheral terminals of primary afferent neurons called nociceptors are express molecules such as the transient receptor potential cation channel subtype V1 (TRPV1) to detect thermal and voltage-gated sodium channels to detect chemical and mechanical noxious stimuli. Tissue damage can sensitize the nociceptors, causing the release of a number of substances (e.g., histamine, bradykinin, substance P and prostaglandin). This damage facilitates the transmission of pain that elicits an action potential in nocicptor fibers and enter the spinal cord (Pasero, 2004).

2. Transmission

Transmission of the pain impulse along the primary afferent neurons (nociceptor fibers) begin when transduction is complete. Two types of nociceptor fibers, C-fibers and A-delta fibers transmit pain from the site of transduction to the spinal cord. C-fibers transmit pain that is poorly-localized, dull and aching. A-delta fibers transmit pain that is well-localized sharp pain. These fibers synapse in the dorsal horn of the spinal cord. Synaptic transmission are mediated by chemical neurotransmittes released from central sensory nerve ending. Many neurotransmitter systems have been implicated in this process, such as adenosine, gamma-aminobutyric acid and other nociceptive substances (i.e., glutamate, calcitonin gene-related peptide and cholecystokinin-like substances). The neural signals transmit from the spinal cord to the higher processing centers in the CNS via multiple

ascending pathways including the spinothalamic and spinoreticular tracts, which carry pain messages to the thalamus and branches to the brainstem nuclei, respectively. The thalamus is a key relay station for the transmission of nociceptive information to the cerebral cortex. Neurons in several regions of the cerebral cortex respond selectively to nociceptive input (DeLeo, 2006; Pasero, 2004).

3. Modulation

Modulation, the process of inhibition and alteration of pain transmission occurs at several locations within the CNS. The modulation are involved with the descending pathway because they originate in the brain stem and descend to the dorsal horn of the spinal cord. Descending inhibitory pathways release substances such as endogenous opioids, norepinephrine (NE) and serotonin (5HT), which can inhibit the transmission of noxious stimuli and produce analgesia (Basbaum, Bautista, Scherrer, & Julius, 2009). This modulation is important for the attenuation of acute pain, and the facilitatory aspect has been implicated in the establishment and maintenance of chronic pain state.

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4. Perception

Perception of pain are the outcome of the pain transduction, transmission and modulation, and are influenced by behavioral and emotional factors. The perception of pain is thought to occur in multiple area of the brain including the somatosensory cortex, insular cortices, frontal lobes and limbic system are involved. Although major defining components of pain are attributed to process that take place in specific areas of the brain. For example, the somatosensory cortex appears to be mainly involved in sensory-discriminative aspects of pain, which allows the person to identify the type, intensity of the painful stimulus. The insula has been involved in affective-emotional response to the noxious stimulus and in affective

aspects of pain-related learning and memory. The anterior cingulate cortex is related to pain unpleasantness and may serve the integration of general affect, cognition and response selection (Schnitzler & Ploner, 2000).

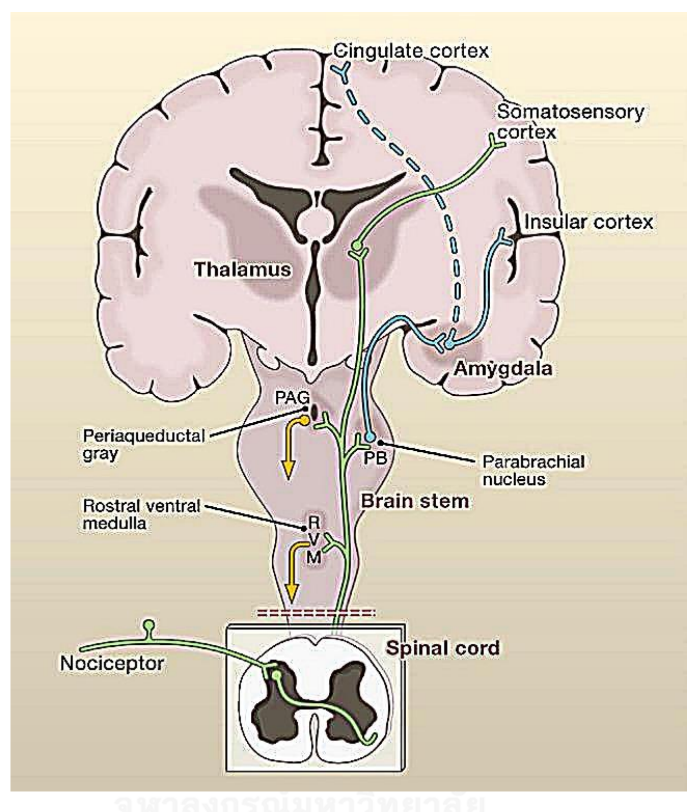

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Figure 3 Anatomy of the pain pathway.

The primary afferent neuron carry noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information to the somatosensory cortex via thalamus, providing information about the location and intensity of the painful stimulus. Other projection neurons engage the cingulate and insular cortices via connections in the brain stem (parabrachial nucleus) and amygdala, contributing to the affective component of the pain experience. This ascending pathway also accesses neurons of the rostral ventral medulla and midbrain periaqueductral gray to engage descending feedback systems that regulate the output from the spinal cord (Basbaum et al., 2009).

Pharmacological management of pain

1. Nonopioid Analgesics (Acetaminophen and Non-steroidal Anti-inflammatory Drug (NSAIDs))

Acetaminophen, also known as paracetamol, has analgesic and antipyretic actions but with very weak anti-inflammatory activity. It is often selected as initial therapy for mild to moderate pain, which considered first line in several pain situations such as headache, low back pain and osteoarthritis. The mechanism of actions of acetaminophen has not been clearly elucidated. It inhibits prostaglandin synthesis in the CNS by unknown mechanism and blocks pain impulses in the periphery. It is generally accepted that acetaminophen has a weak anti-inflammatory action, a reduction in tissue swelling after dental surgery have been reported (Botting, 2000). However, acetaminophen has unacceptable serious adverse effects including mild to moderate hepatotoxicity, renal tubular necrosis, hypoglycemic coma and thrombocytopenia (Gürsoy et al., 1996).

NSAIDs such as aspirin, naproxen, ibuprofen, and indomethacin have analgesic, antipyretic and anti-inflammatory actions. These drugs are used for short-term treatment of inflammatory pain in wide variety of disorders including bone pain and musculoskeletal injuries. They are particularly useful in treating patients with chronic disease include the relief of pain accompanying dysmenorrhea, and that associated with neoplastic bone metastases. The analgesic action of NSAIDs is due to inhibition of cyclooxygenase (COX) enzyme. More recent research indicates that NSAIDs reduce the production of prostaglandin (PG) that contributed to both peripheral and central sensitization in inflammatory pain, which results in reduced nociceptors sensitization. Initially, PGs contribute to hyperalgesia by sensitizing nociceptive sensory nerve endings to other mediators (such as histamine and bradykinin). Peripherally, peripheral inflammation induces a substantial increase in COX-2 and prostaglandin synthase expression in the CNS. Centrally, PGs are recognized to have direct actions

at the level of the spinal cord by enhancing nociception, notably the terminals of sensory neurons in the dorsal horn. Both COX-1 and COX-2 are expressed constitutively in dorsal root ganglia and spinal dorsal and ventral gray matter, but inhibition of COX-2 and not COX-1 reduces hyperalgesia (Green & Harris, 1993; Griffin & Woolf, 2008). NSAIDs also modulate pain intensity by suppressing prostanoid formation in the spinal cord and other regions of the CNS (Samad et al., 2001).

These therapeutic agents demonstrated a flat-dose response curve, with higher doses producing no greater efficacy than moderate dose but resulting in increased adverse effects including gastrointestinal (GI) irritation, gastroduodenal ulcer, hepatic dysfunction, renal insufficiency, platelet inhibition, and sodium retention. Newer NSAIDs such as celecoxib, rofecoxib, parecoxib and valdecoxib have been purposefully developed to inhibit COX-2 selectively with the intent to reduce the risk of gastrointestinal bleeding caused primarily by COX-1 inhibition. However, the selective COX-2 inhibitor has been questioned due to the increased risk of myocardial infarction and stroke noted in several trials. Consequently, rofecoxib, valdecoxib and parecoxib were removed from the market after confirmed by case-control studies of increased risk of heart attacks and stroke due to loss of the protective effects of COX-2 enzyme on the cardiovascular system. It has been suggested that the selective COX-2 inhibitor depresses prostanoid formation in the vasculature, significantly reduce levels of prostacyclin (PGI_2), an inhibitor of platelet aggregation, and do not affect thromboxane. Inhibition of these mediators, particularly of PGI_2, increases the probability of thrombotic events hypertension, and heart failure particularly in patients at elevated cardiovascular risk (Green & Harris, 1993; Yu et al., 2012).

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2. Opioid Analgesics

Opioid analgesics have long been accepted as appropriate treatment for acute pain and moderate to severe pain, whether due to trauma, surgery, cancer or non-cancer conditions. For chronic pain, their use was once highly controversial; however, use of opioids in chronic pain is now gaining acceptance as the mainstay of therapy pain due to cancer (Portenoy & Foley, 1986). The many subsequent pharmacological studies, now confirmed by receptor cloning, show that there are three types of opioid receptor, termed mu, delta and kappa (all of them are typical G-protein-coupled receptors). The action of opioid involves with a G-protein-coupled mechanism that blocks the release of pain-propagating neurotransmitter in the brain and spinal cord. The opioids are classified by their activity at the receptor site and duration of action (Cichewicz, 2004).

Opioid agonists

Pure agonists (e.g. morphine, methadone, pethidine and fentanyl) have affinity for mu receptors and generally lower affinity for delta and kappa sites. Especially, morphine is considered the reference standard for all drugs classified as highly potent analgesics, which the specific mechanism of action of opioid-induced analgesia is due to effects at spinal and multiple supraspinal sites as well as potential peripheral sites. As a result of its many site of action within the CNS, morphine not only alleviates pain perception but also blocks pain processing. It also suppresses the autonomic reactions aroused by pain impulses in the brain stem (Main & Spanswick, 2000).

Partial agonist (e.g. buprenorphine) binds with the mu receptor and competes 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

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level of analgesia may be reduced. They show only limited activity (Green & Harris, 1993).

Opioid agonist-antagonist

The opioid agonist-antagonist (e.g. pentazocine, butorphanol, nalbuphine, nalorphine, bremazocine and dezocine) 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. In general, partial agonist drugs should be reserved for patients who are unable to tolerate a pure agonist (Stoelting & Hillier, 2012).

Opioid antagonist

Pure antagonists (e.g. naloxone and naltrexone) have a high affinity for the mu receptor and less affinity for the delta and kappa receptors. These drugs are used to reverse respiratory depression post-operatively, to treat opioid poisoning and to prevent relapse in detoxified opioid addicts. Naloxone is a short-acting opioid antagonist administered parenterally, with a short duration of action (30 to 45 minutes) that prompts the need for supplemental doses or a continuous infusion to prevent a recurrence of respiratory depression (Barber, 1997).

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Side effects

The side effects common to all opioids include drowsiness, sedation, hallucinations, constipation, nausea, vomiting, urinary retention, hypertension, cardiovascular stimulation (tachycardia, bradycardia, cardiac dysrhythmias) and respiratory depression and withdrawal in the newborn of an opioid-dependent mother. In terms of medication management, the most frequent are sedation, nausea, and constipation. Sedation and nausea are common when initiating therapy

and when increasing doses. Nausea can be prevented with antiemetic drugs. Therefore, respiratory depression is a serious adverse effect, and usually occurs after acute administration in opioid-naïve patients (Barber, 1997).

Inflammation

Inflammation is a complex process that is part of the body's response to harmful stimuli such as infection, chemical, trauma, foreign bodies and immune reaction due to hypersensitivity. Inflammation is caused by release of multiple chemical mediators from tissue damage and migrating cells. The reaction of inflammation mainly consists of two types; the vascular changes and the response of various cell types such as lymphocytes and fibroblasts cells found in connective tissue (Nathan, 2002). Specific symptoms of inflammation include pain, swelling, redness, heat and loss of function. The category of inflammation is based according to the time course and cellular components involved.

Acute inflammation

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Acute inflammation is rapid in onset and of short duration, lasting from a few minutes to as long as a few days. It is characterized by the release of chemical mediators, fluid and plasma protein exudation from the local microvasculature and neutrophils accumulation in the focus of injury site, followed by activation to eliminate the offending agent (Ialenti, Ianaro, Moncada, & Di Rosa, 1992).

Causes of acute inflammation

1) Physical agents, e.g. heat, cold, trauma, ultraviolet light, radiation.
2) Irritant and corrosive chemical substances, e.g. acids, alkalis.
3) Microbial infections, e.g. pyrogenic bacteria.
4) Tissue necrosis, e.g. ischemia resulting in a myocardial infarction.

Chronic inflammation

Chronic inflammation is inflammation of prolonged duration (weeks to months to years) in which active inflammation, tissue injury, and healing proceed simultaneously. Chronic inflammation is characterized by the infiltration of mononuclear cells including lymphocytes, macrophage and plasma cells. Tissue destruction, largely directed by the inflammatory cells and attempts at healing by connective tissue replacement of damaged tissue, involving vascular proliferation and scarring (Robbins, Kumar, Abbas, & Aster, 2012).

Causes of chronic inflammation

1) Persistent microbial infections, most characteristically by a selected set of microorganisms that resistant to phagocytosis or intracellular killing mechanisms, e.g. tuberculosis, leprosy.
2) Foreign bodies, which can be endogenous (e.g. bone, adipose tissue, uric acid crystals) or exogenous material such as inhaled particulate silica, which can induce a chronic inflammatory response in the lung.
3) Autoimmune diseases, e.g. Hashimoto’s thyroiditis, rheumatoid arthritis, multiple sclerosis.
4) Primary granulomatous diseases, e.g. Crohn’s disease, sarcoidosis.

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Classical signs of acute inflammation

The clinical signs of this phenomenon are redness, heat, swelling, pain and loss of function. The acute response has two major components that are produced by rapid vascular response and cellular events that are characteristic of acute inflammation.

Vascular response

The vascular reactions consist of alterations in vessel caliber resulting in increased blood flow (vasodilation) and structural changes that permit plasma protein to leave the circulation (increased vascular permeability).

Vasodiation

The changes begins relatively quickly after injury, which these process is mediated by both neurogenic and chemical mediator system and usually resolves within second to minutes. The chemical mediator including nitric oxide (NO), histamine and other soluble agents acts to increase blood flow and expansion of the capillary bed. This condition is known as hyperemia, and the increased blood flow is responsible for redness and warmth at site of tissue injury.

Increased vascular permeability

The arteriolar vasodilation and augmented blood flow increase intravascular hydrostatic pressure and the movement of fluid from capillaries. This fluid, called a transudate, is essentially an ultrafiltrate of blood plasma and contains little protein. However, transudation is soon eclipsed by increasing vascular permeability that allows the movement of protein-rich fluid (called an exudate). The loss of exudate into the perivascular space reduces the intravascular osmotic pressure and increase the osmotic pressure of the interstitial fluid. The result is outflow of water and ions into the extravascular tissues, which this fluid accumulation is called edema (Kumar, Abbas, & Fausto, 2005; Larsen & Henson, 1983).

Cellular events

The sequence of events in the extravasation of leukocytes from the vascular lumen to the extravascular space is response to inflammation includes recruitment of leukocytes to injury site, phagocytosis and intracellular killing.

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Recruitment of leukocytes to site of injury

1) Margination to the plasmatic zone, a process of leukocyte accumulation at periphery vessels, is assisted by the slowing of the blood leukocytes flow near the vessel wall in the plasmatic zone.

2) Rolling of leukocytes tumble on the endothelial surface, due to the repeated formation and destruction of transient adhesions with the endothelium.

3) Adhesions of leukocytes eventually firmly stick to the vascular endothelium, due to the interaction of paired molecules on the leukocyte and endothelial cell surface. The endothelial adhesion molecules include ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) bids to the specific intergrins, which are transmembrane heterodimeric glycoproteins that also function as cell receptors for extracellular matrix.

4) Transmigration of leukocytes that pass between the endothelial cell junctions by amoeboid movement through the vessel wall into tissue space (Robbins et al., 2012).

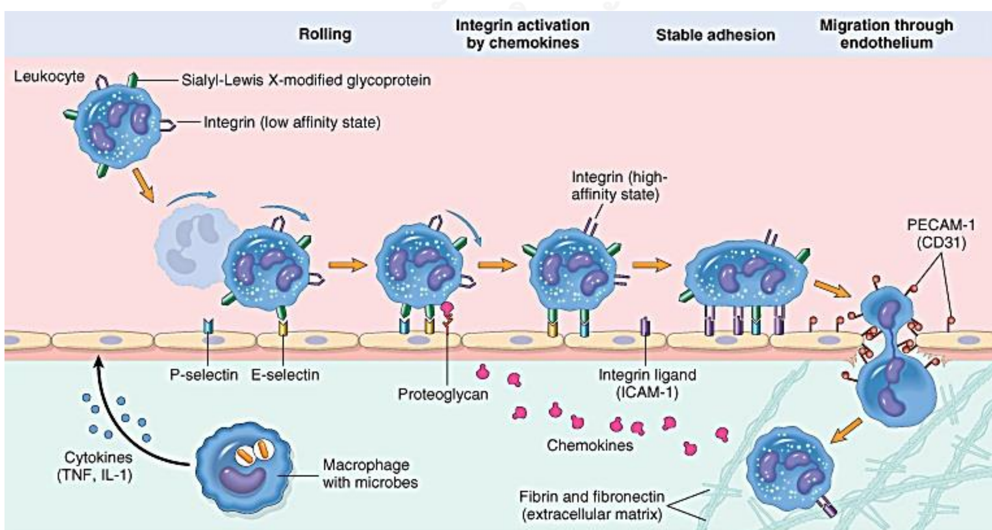

Figure 4 Sequence of events in leukocyte emigration in inflammation (Robbins et al., 2012).

Phagocytosis and intracellular killing

Phagocytosis and the elaboration of degradative enzymes are two major benefits of having recruited leukocytes at the site of inflammation. Phagocytosis consists of three distinct but interrelated steps

1) Recognition and attachment of leukocytes to most microorganisms is facilitated by serum protein generically called opsonins; it's bind specific molecules on microbial surfaces and in turn facilitates binding with specific opsonin receptor on leukocytes.

2) Engulfment, pseudopods are extended around the object, eventually forming a phagocytic vacuole. The membrane of the vacuole then fuses with the membrane of lysosomal granule, resulting in discharge of the granule's contents into the phagolysosome and degranulation of the leukocytes.

3) Killing and degradation of the ingested material, which microbial killing is accomplished largely by reactive oxygen species (Kumar et al., 2005).

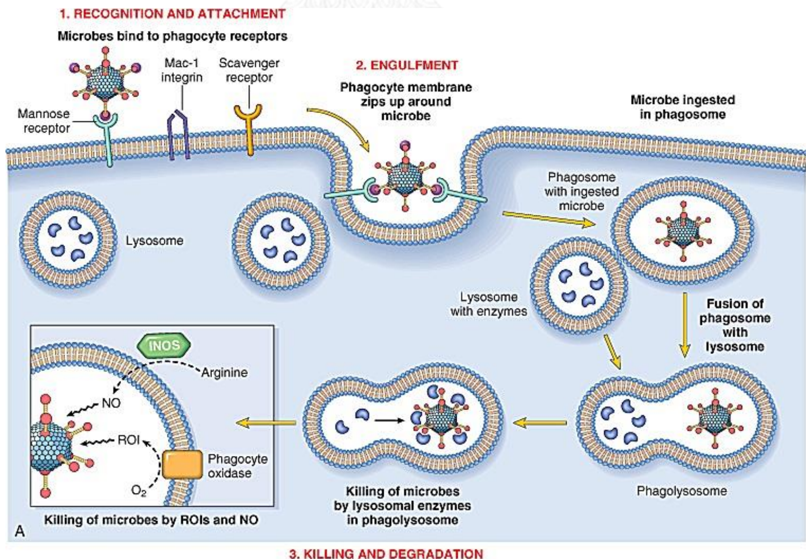

Figure 5 The process of phagocytosis of a particle (e.g. bacterium) involves attachment and binding to receptors on the leukocyte surface, followed by engulfment, fusion of the phagocytic vacuole with granules and degranulation by production of reactive oxygen intermediates within phagocytic vesicles (Kumar et al., 2005).

Chemical mediators of inflammation

A variety of substances are released upon damage to cells. The inflammatory mediator can be thought of as a chemical messenger that will act on blood vessel and/or cell to contribute to an inflammatory response. The mediators of the inflammatory response can be classified in several ways.

Vasoactive: Histamine and serotonin

The two major vasoactive amines including histamine and serotonin have important actions on blood vessels. They are stored as preformed molecules in cells and are therefore among the first mediators to be released during inflammation.

Histamine is stored in mast cells and basophils and is released mostly from mast cells. The release of histamine causes arteriolar dilation and rapidly increases vascular permeability by inducing venular endothelial contraction. Histamine receptors are widely expressed by cells of the innate and adaptive immunity. At least 4 types of histamine receptors have been characterized; its vasoactive effects are mediated via binding to H_1 receptors on microvascular endothelial cells. Therefore, histamine modulates the synthesis of adhesion and costimulatory molecules and cytokines with important functional consequences (Giustizieri et al., 2004).

Serotonin (5-Hydroxytryptamine; 5-HT) is released from platelets and mast cells during injury or inflammation. Serotonin is a preformed vasoactive mediator found within platelet granules that is released during platelet aggregation. It is responsible for dilating blood vessels and produced mild and transient pain by induces a direct sensitization of nociceptors via 5-HT_1 and 5-HT_2 receptors (Dray, Urban, & Dickenson, 1994).

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Plasma proteins

Many of the effects of inflammation are mediated by three interrelated plasma-derived factors including kinin system, clotting system, and complement system, all linked by the initial activation of Hageman factor. Hageman factor (also known as factor XII of the intrinsic coagulation cascade) is generated within the plasma and is activated by exposure to negatively charged surfaces such as basement membrane, proteolytic enzymes, foreign material and activated platelet (at site of endothelial injury). This key factor triggers activation of addition plasma protease systems that are important in inflammation (Robbins et al., 2012).

Kinin system

Kinins are potent inflammatory agents formed in plasma and tissue by the action of serine protease kallikreins on specific plasma glycoproteins, termed kininogens. The kallikreins, an intermediate in the kinin casecade with chemotactic activity, is also a potent activator of Hageman factor and thus allows amplification of the entire pathway. Kinin system activation leads ultimately to the formation of bradykinin from its circulating precursor, high-molecular weight kininogen. Bradykinin and related peptide regulate multiple physiologic processes including blood pressure, contraction and relaxation of smooth muscle, plasma extravasation, cell migration, inflammatory cell activation and inflammatory-mediated pain responses. However, kinins are rapidly degraded to inactive products by kininases and have short-lived functions (Proud & Kaplan, 1988; Robbins et al., 2012).

Clotting system

The resultant factor XIIa causes thrombin activation, which in turn cleaves circulating soluble fibrinogen to generate an insoluble fibrin. Factor Xa, an intermediate in the clotting cascade, causes increase in vascular permeability and

leukocyte emigration. Thrombin participates in inflammation by enhancing leukocyte adhesion to endothelium and by generating fibrinopeptides that increase vascular permeability and are chemotactic for leukocytes. While activated Hageman factor is inducing clotting, it is concurrently activating the fibrinolytic system. The resulting product, plasmin also cleaves the complement C3 component to C3a, causes vasodilation and increase vascular permeability (Robbins et al., 2012).

Complement system

The complement system has an important role in both immunity and inflammation. The most critical step in the elaboration of the biologic functions of complement is the activation of the third component, C3. The various other complement-derived factors generated along the way affect a variety of phenomena in acute inflammation:

- Vascular effects, C3a and C5 causes increase vascular permeability and vasodilation by inducing mast cells to release their histamine. C5a also activates the lipoxygenase pathway of arachidonic acid metabolism in neutrophils and monocytes.
- Leukocyte activation, adhesion, and chemotaxis. C5a activates leukocyte, thereby increasing adhesion to endothelium. It is also a potent chemotactic agent for neutrophils, monocytes, basophils and eosinophils.
- Phagocytosis. C3b and C3bi act as opsonins, augmenting phagocytosis by cells bearing C3b receptors (neutrophils and macrophage) (Carroll, 2004).

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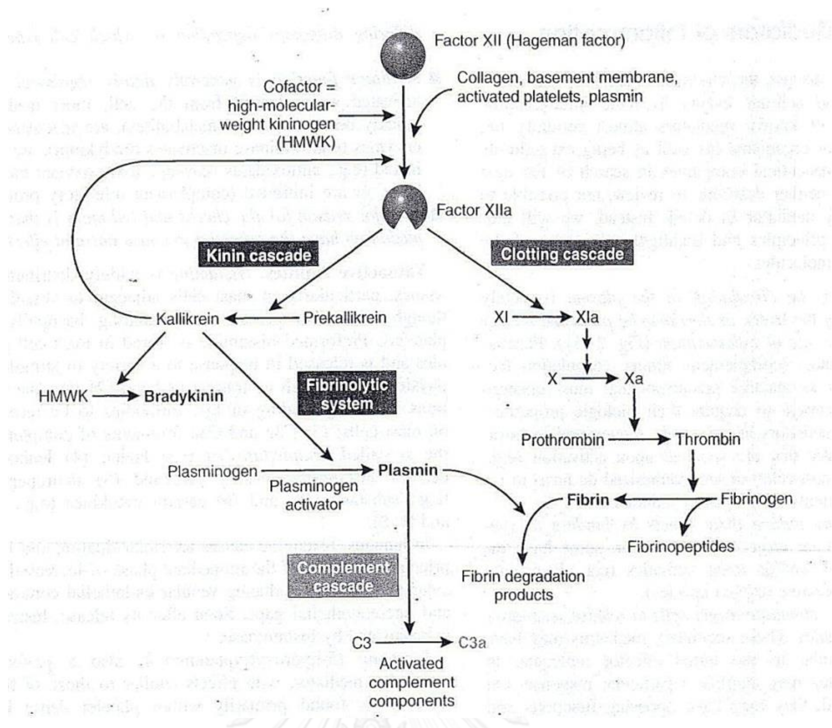

Figure 6 Intercorrelationships between the plasma mediator systems triggered by activation of factor XII (Hageman factor) (Robbins et al., 2012).

Platelet activation factors (PAF)

Another potent inflammatory mediator derived from membrane phospholipids is platelet-activating factor (PAF), synthesized by virtually all activated inflammatory cells, injured tissue cells and endothelial cells. PAF is acetyl glycerol ether phosphocholine; it is generated from the membrane phospholipids of neutrophils, monocytes, basophils, endothelium and platelets by the action of phospholipase A_2 (PLA_2). PAF acts directly on target cells via a specific G-protein couple receptor. During inflammation, PAF stimulates neutrophils, macrophages, platelet, endothelial cells and vascular smooth muscle cells. It induces platelet aggregation and degranulation and leading to the release of serotonin, thereby causing changes in vascular permeability. It also stimulates the synthesis of arachidonic acid derivatives, particularly eicosanoids (Morrison & Ryan, 1987).

Arachidonic acid (AA) metabolites: Prostaglandins, Leukotrienes and Lipoxins

Arachidonic acid (AA) is a 20 carbon polyunsaturated fatty acid produced primarily from dietary linoleic acid and present in the body mainly in its esterified form as a component of cell membrane phospholipids. AA is released from these phospholipid thorough the action of PLA_2 that have been activated by chemical, mechanical, physical stimuli or by inflammatory mediators. PLA_2 in many cells, cytosolic PLA_2 ($cPLA_2$) is a principle lipolytic enzyme providing AA for eicosanoid biosynthesis. AA is oxidatively metabolized by two major groups of enzymes, cyclooxygenase (COX) and lipoxygenase (LOX) and is thereby the precursor for the prostanoids (PGs) and leukotrienes (LTs), respectively (Needleman, Jakschik, Morrison, & Lefkowith, 1986).

Cyclooxygenase pathways

There are COX enzyme isoforms include COX-1 and COX-2. These enzymes originate from different genes and have distinct tissue-specific expressions and biologic functions. COX-1 is constitutively expressed in most tissues and believed to responsible for the production of prostanoids in homeostasis, such as gastric epithelial cytoprotection and renal blood flow maintenance. COX-2 expression is inducibly expressed only at marginal levels under condition in many tissues and is upregulated in response to a variety of stimuli, pro-inflammatory, hormones and growth factors as well as associated with inflammation and disease states. Its pivotal role in the production of prostanoids during inflammation and in cancerous states provided the rationale for the development of COX-2 selective NSAIDs (Smyth, Grosser, Wang, Yu, & FitzGerald, 2009).

The prostanoids syntheses; first, 2 molecules of oxygen are insert into AA, cyclizing it to produce PGG_2. Next, an endoperoxidase reaction reduces PGG_2 to PGH_2. Terminal PG synthases then convert PGH_2 into prostanoids: PGD_2 by hematopoietic

PGD synthase; PGI_2 by PGI synthase; PGE_2 by microsomal PGE synthase (mPGES)-1, mPGES-2, or cytosolic PGES; PGF_{2a} by PGF synthase; and thromboxane A_2 (TXA_2) by thromboxane synthase. The prostanoids act on specific G-protein-coupled receptors including two PGD_2 receptors (DP1 and DP2), one PGI_2 receptor (IP), four PGE2 receptor subtypes (EP1, EP2, EP3, and EP4), one PGF_{2a} receptor (FP) and one TXA_2 receptor (TP) (Chen, Yang, & Grosser, 2013).

The prostaglandins E_2 (PGE_2) are also involved in the pathogenesis of pain and fever in inflammation. PGE_2 is thought to be the principal proinflammatory prostanoid and contributes to pain hypersensitivity by modulating multiple sites in the nociceptive pathways, including peripheral and central sensitization. It acts by markedly increased vasodilation and vascular permeability.

The prostaglandins I_2 (PGI_2) are involved in vasodilation and are potent inhibitor of platelet aggregation, which elevates cAMP levels in platelets, vascular smooth muscle cells and this biochemical action appears to mediate the antiaggregatory effects on platelets.

The thromboxane A_2 (TXA_2) is produced by activated platelets, granulocytes and mast cells. It is a potent inducer of platelet aggregation and vasoconstriction. On the other studies, lack of thromboxane synthase but possesses prostacyclin synthase leads to the formation of PGI_2 (Robbins et al., 2012).

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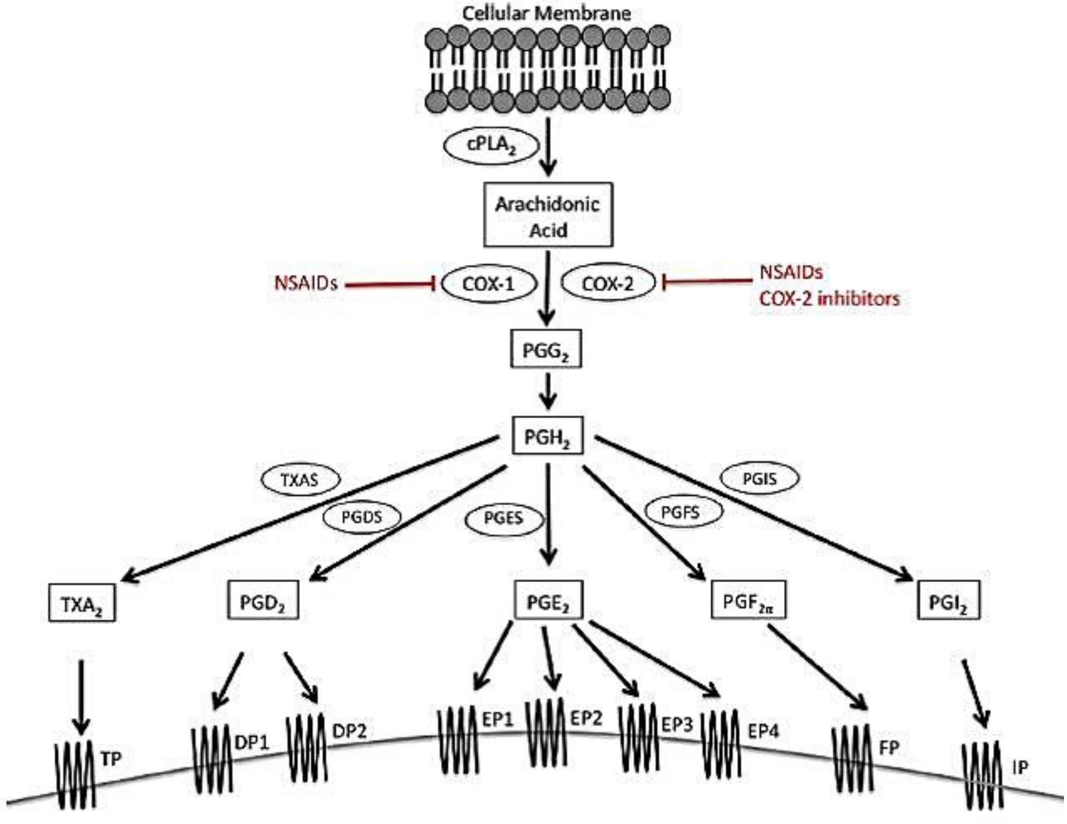

Figure 7 Prostaglandin biosynthesis: the release of arachidonic acid (AA) from membrane phospholipids by $cPLA_2$, cytosolic phospholipase A2; cyclooxygenase (COX)-1 or COX-2 converts AA to prostaglandin (PG) G_2 and next, an endoperoxidase reaction reduces PGG_2 to PGH_2. Thromboxane (TX) A_2, PGD_2, PGE_2, PGF_{2a}, and PGI_2 are then generated by specific PG synthase enzymes and signal by the indicated G protein-coupled receptors. Nonsteriodal anti-inflammatory drugs (NSAIDs) block COX-1 and COX-2 (Fanning & Boyce, 2013).

Lipoxygenase pathways

Arachidonic acid is first oxidized to 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE) by 5-Lipoxygenase (5-LOX) and then dehydrated to leukotriene A_4 (LTA_4), which is expressed by mast cells, macrophages, and neutrophils. LTA_4 in turn gives rise to LTB_4 by enzymatic hydrolysis or to LTC_4 by addition of glutathione. LTB_4 is a potent chemotactic agent and causes aggregation of neutrophils. LTC_4 and its subsequent metabolites, LTD_4 and LTE_4, cause vasoconstriction, bronchospasm and increased vascular permeability (Fanning & Boyce, 2013).

Lipoxins, the third class of proinflammatory products of AA, are also synthesized by platelets and neutrophils within the lumen using transcellular pathways. Platelet alone cannot form lipoxins A_4 and lipoxins B_4 but they can form the metabolites from intermediate LTA_4 derived from adjacent neutrophils. The lipoxins inhibit leukocyte recruitment and the cellular components of inflammation (Robbins et al., 2012).

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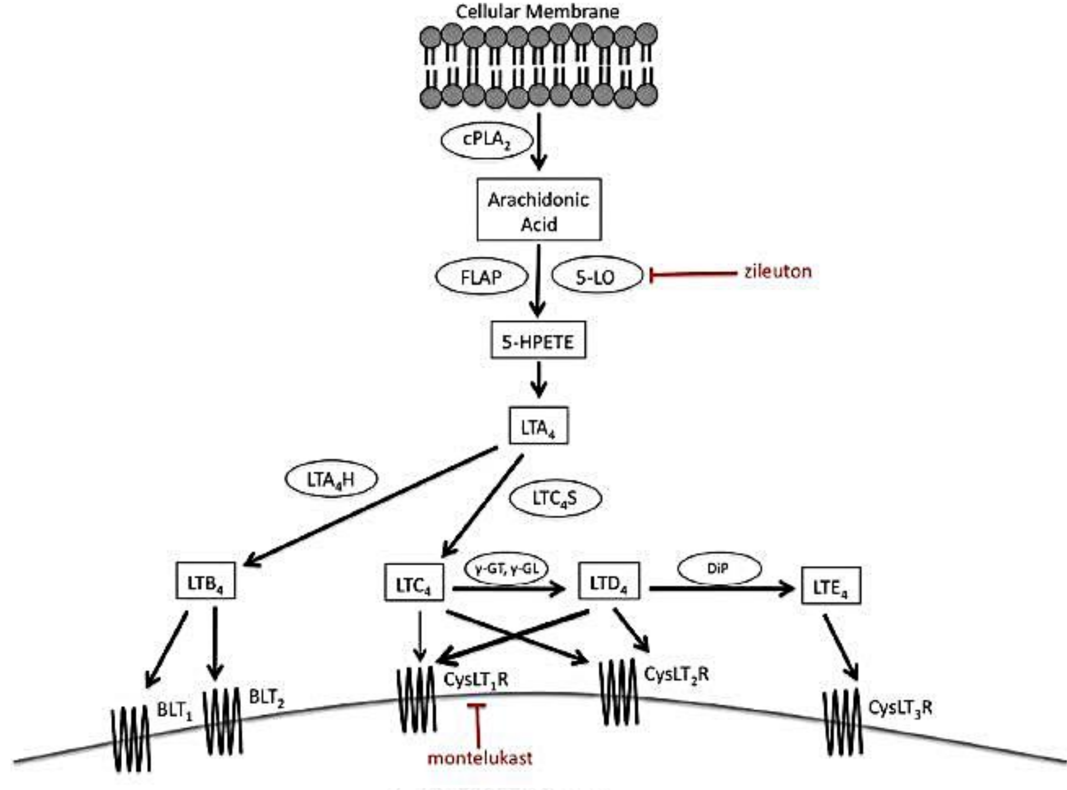

Figure 8 Leukotriene biosynthesis; $cPLA_2$, cytosolic phospholipase A_2; 5-Lipoxygenase (5-LOX) and 5-LOX activating protein (FLAP) metabolize arachidonic acid to leukotriene (LT) A_4, which can be converted to LTB_4 (by LTA_4H) or the cysteinyl leukotrienes (cys-LTs; by leukotriene C_4 synthase [LTC_4S]), where it is converted to LTD_4 and then to LTE_4, the most stable cys-LTs then bind to the indicated the G protein-coupled receptors; 5-HPETE, 5-hydroxyperoxyeicosatetraenoic acid; BLT, leukotriene B receptor; DiP, dipeptidase; g-GL, g-glutamyl leukotrienase; g-GT, g-glutamyl transpeptidase. Zileuton is lipoxygynase inhibitor, inhibit lipoxygynase enzyme (Fanning & Boyce, 2013).

Cytokines

Cytokines are polypeptide products of many cell types that produced at site of tissue injury that regulate inflammatory responses, ranging from initial changes in vascular permeability to resolution and restoration of tissue integrity. Cytokines may be roughly grouped into five classes based on their actions or target cells.

- Cytokines involved in innate immunity that is the primary response to injurious stimuli. These include two major inflammatory cytokines, TNF-α and IL-1.
- Cytokines that regulate lymphocyte function such as activation, growth and differentiation (e.g. IL-2 which stimulates proliferation and transforming growth factor β, which inhibits lymphocyte growth).
- Cytokines that activate inflammatory cells (in particular, macrophages) during cell-mediated immune responses, such as interferon-μ (IFN-μ) and IL-12.
- Cytokines that stimulate hematopoiesis, including granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-3
- Chemokines that have chemotactic activity for various leukocytes.

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Pro-inflammatory cytokine such as TNF-α and IL-1 are produced by activated macrophages and other cell, which are central to the development and amplification of inflammatory responses. These cytokine activate signaling pathways in endothelial cells that regulate the expression of these adhesion molecules to initiate the capture of circulating leukocytes and release cytokines, chemokines and reactive oxygen species (ROS) (Bradley, 2008; Robbins et al., 2012).

Nitric oxide (NO)

The nitric oxide (NO) is derived from L-arginine by the action of specific neuronal and non-neuronal forms of NO synthase. NO-mediated signaling plays

various physiological processes, including immune defense and inflammatory response including smooth muscle relaxation and antagonism of platelet adhesion and it serves as an endogenous regulator of leukocyte recruitment (Kumar et al., 2005).

Oxygen-derived free radicals

Oxygen-derived free radicals are synthesized via the NADPH oxidase pathway and are released from neutrophils and macrophage after stimulation by chemotactic agent, immune complexes, or phagocytic activity. The reaction oxygen species (ROS) includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicle (OH^-) can increase chemokine, cytokine and adhesion molecule expression, thus amplifying the cascade of inflammatory mediators (Robbins et al., 2012).

Cells of inflammation

Neutrophils

The polymorphonuclear cells (PMN) or neutrophils are the major cellular participants in acute inflammation. It displays granulated cytoplasm and a nucleus with two to four lobes. Neutrophils are stored in the bone marrow, circulate in the blood and rapidly accumulate at site of tissue injury. They are activated in response to phagocytic stimuli, cytokine, chemotactic agent and Ag-Ab complexes, which bind to specific receptors on the neutrophil surface membrane. The neutrophil phagocytosed invading microbes and necrotic tissue by ROS, an event that corresponds to the resolution phase of acute inflammation.

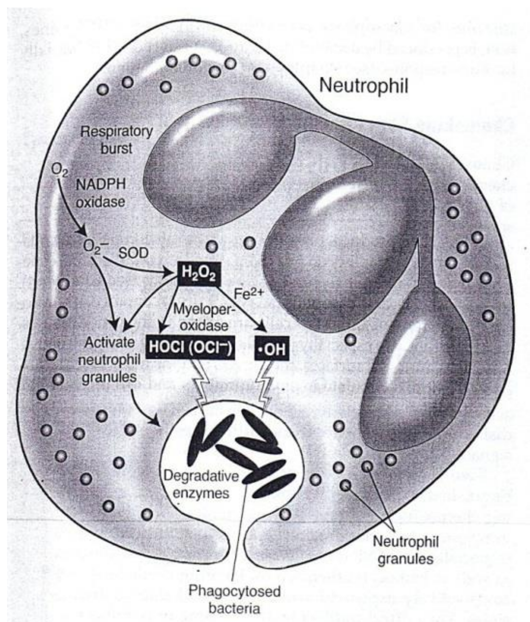

Figure 9 Generation of reactive oxygen species (ROS) in neutrophils as results of phagocytosis of bacteria (Robbins et al., 2012).

Monocytes/Macrophages

Monocytes/Macrophages are important in acute and chronic inflammation. Circulating monocytes have a single-lobed, likely kidney-shaped, nucleus. They are originates in the bone marrow and can exit the circulation to migrate into tissue and become resident macrophages. The half-life of circulating monocytes is about one day; under the influence of chemotactic factors and adhesion molecules, they begin to emigrate at a site of injury within the first 24 to 48 hours after onset of acute inflammation, as described previously. Monocytes/Macrophage produces potent histamine, serotonin, as well as AA metabolites, PAF and inflammatory cytokines. These cells are especially important for maintaining chronic inflammation. After activation, macrophages secrete a wide variety of biologically active products, which can result in the tissue injury and fibrosis characteristic of chronic inflammation.

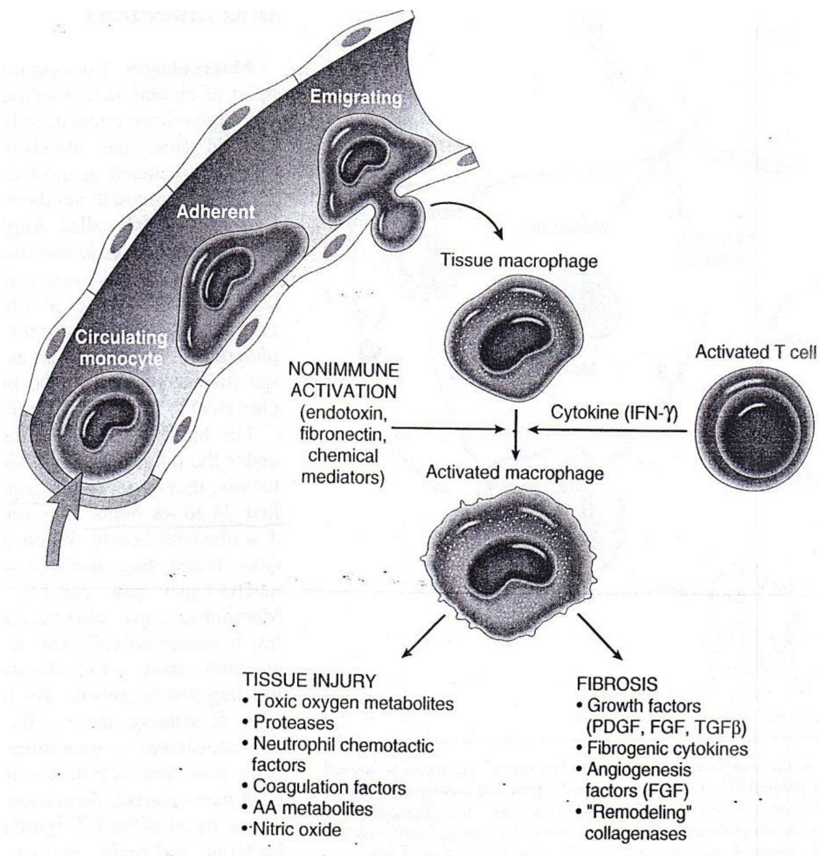

Figure 10 Maturation of circulating monocytes into activated macrophages (Robbins et al., 2012).

Granulomatous inflammation

Granulomatous inflammation is a distinctive pattern of chronic inflammation. The principal cells involved in granulomatous inflammation are macrophage, epithelioid cells and multinucleated giant cells. Granuloma may also develop in response to relatively inert foreign bodies (e.g. breast implant, suture splinter) forming so called foreign body granulomas. Notably, granuloma formation does not always lead to eradication of the cause agent, which is frequently resistant to killing or degradation and also in the development of necrosis and fibrosis. However, the formation of granulomas effectively “walls off” the offending agent and is therefore a useful defense mechanism (Adams, 1976; Sheffield, 1990).

Pharmacological management of inflammation

Corticosteroids

Corticosteroids (glucocorticoids) are one of the therapeutic mainstays in the treatment of a large number of inflammatory, allergy and immunologically mediated diseases. Glucocorticoids bind to a corticosteroid receptor (CR) and the complex translocates to the nucleus where it bind to a glucocorticoid-responsive element (GRE) that blocks several inflammatory pathways. This complex also physically increases the transcription of a number of anti-inflammatory genes and interacts with NF-κB to block its transcriptional activity; this action inhibits the production of pro-inflammatory mediators, including IL-1β and TNF-α. Furthermore, glucocorticoids also cause induction and activation of polypeptide lipocortin-1, which is an anti-inflammatory protein that physically interacts with and inhibits phospholipase A_2, that synthesis of eicosanoids. Moreover, glucocorticoids can induction of MAPK phosphatase 1 and repression of transcription COX-2 (Rhen & Cidlowski, 2005). Probably the most important physiological and clinical effect of corticosteroids administration in humans is their ability to inhibit the recruitment of neutrophils and monocytes-macrophages to an inflammatory site. The serious adverse effects generally follow prolonged administration of glucocorticoids are Cushing's syndrome, immunosuppression, tendon rupture, peptic ulcer and glaucoma. Glucocorticoids also have damaging effects on bone in adults. Osteoporosis and an increased risk of fractures are the main side effects of glucocorticoids therapy (Parrillo & Fauci, 1979) .

Nonsteroidal Anti-inflammatory drugs (NSAIDs)

Aspirin (acetylsalicylic acid) is the first NSAlDs that was introduced in 1899. It has been used for more than 100 years by individuals around the world for its analgesic, anti-inflammatory, antipyretic and platelet-inhibitory properties. Aspirin

inhibits the biosynthesis of prostaglandins, which an irreversible acetylation and consequent inactivation of COX; thus, aspirin inactivates COX permanently. Platelets are devoid of the ability to produce additional cyclooxygenase; thus, thromboxane synthesis is arrested. Aspirin has various side effects that can be life-threatening. Their effects on the enzyme COX-1 such as gastritis and peptic ulcer disease are frequent side effects. Other adverse effects include hepatitis, erythema, anemia, hepatotoxicity, interstitial nephritis, toxic epidermal necrolysis (TEN), and Stevens-Johnson syndrome (Hart & Huskisson, 1984; Simon & Namazy, 2003).

NSAIDs are widely used for the treatment of inflammatory diseases. These drugs inhibit the COX enzyme, which catalyzes the conversion of AA to PGs and thromboxane. Prostaglandin release appears to induce blood vessels to the permeability effects of other mediators and the NSAIDs appear in most cases to remove this induction. Prostaglandins, especially PGE_2 are capable of producing feature of inflammation. Initially, the inflammatory prostaglandins are probably generated in the injured tissues but could be augmented by the influx into the tissues of polymorphonuclear leucocytes (PMNs) which produce prostaglandins during phagocytosis. The dominant migratory cell in chronic inflammatory lesions is the macrophage, which also produces prostaglandins. There is a reasonably good correlation between an NSAID's potency in reducing experimentally induced edema in laboratory animals (Hart & Huskisson, 1984).

The majority of NSAIDs are weakly acidic, highly bound to plasma proteins (albumin), and lipophilic such as diclofenac, etodolac, indomethacin and ketololac. Indomethacin is a nonselective COX inhibitor introduced in 1963. This drug is a more potent inhibitor of the cyclooxygenases than is aspirin, but patient intolerance generally limits its use to short-term dosing. Oral administration of indomethacin has excellent bioavailability. Peak concentrations occur 1–2 h after dosing. Indomethacin is 90% bound to plasma proteins and tissues. The concentration of the drug in the

cerebrospinal fluid is low, but its concentration in synovial fluid is equal to that in plasma within 5 h of administration. Complaints associated with gastrointestinal irritation are common side effects, including diarrhea, and ulcerative lesions are a contraindication to indomethacin use (Frost, 2014).

Cyclooxygenase-2 selective

COX-2 inhibitors (celecoxib, rofecoxib, and valdecoxib) were approved for use in the United States and Europe, but both rofecoxib and valdecoxib have now been withdrawn from the market due to their adverse event profile. In an animal model of acute inflammation (injection of carrageenan into the paw), edema was produced that was associated with marked accumulation of COX-2 mRNA and thromboxane. A selective inhibitor of COX-2 inhibited edema at the inflammatory site and was analgesic but had no effect on prostaglandins production in the stomach and did not cause gastric toxicity. The development of COX-2 inhibitors was originally thought to offer a solution to NSAIDs' problem, but these agents have been associated with an increased risk of cardio-renal effect (Frost, 2014).

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Animal models

Nociceptive Activity Testing

Experiments on pain using human subjects are practically challenging and ethically self-limiting, and thus laboratory animal models of pain are widely used. Numerous animal behavioral models have been developed and designed as a means to investigate mechanisms underlying nociceptive. Therefore, to study pain transmission, identify a potential target for analgesic therapy and characterize the analgesic profile of novel compounds for alleviating pain, an array of experimental animal pain models has been developed mainly in rat and mouse, reflecting all types of pain. Depending on the model, pain measurements can encompass

spontaneous pain behaviors as well as pain evoked by various modalities. Several modalities of noxious stimulation are commonly used induced pain (e.g., thermal, mechanical, electrical, chemical); typical parameters that are measured include pain threshold, pain tolerance, and ratings of suprathreshold noxious stimuli (Joshi & Honore, 2006; Stanley & Paice, 1997). A few of the most commonly used methods will be explained, which may indicate important aspect of investigations of pain in animals.

Hot-Plate test

Woolfe and MacDonald originally described the hot-plate test in 1944, which it is specifically used for the screening of centrally acting analgesic (Woolfe & MacDonald, 1944). These test measures a complex response to thermal stimuli induced hyperalgesia that specific to centrally-mediated nociception, that presumably involves both supraspinal and spinal mechanisms (Khan, Saeed, Khan, Khan, & Ashraf, 2011). The paws of mice and rats are very sensitive to heat which the behavioral responses including jumping, withdrawal of the paw and licking hind paw were considered to be supraspinally integrated responses. Administration of centrally acting analgesics were known to activate the release of endogenous peptide by periaqueductal gray matter (PAG), which are carry to the spinal cord to inhibit the pain transmission within the dorsal horn, causes prolongation of the latency time of response (Le Bars, Gozariu, & Cadden, 2001; Woolfe & MacDonald, 1944).

Originally the test measured nociceptive responses of mice placed on the hot-plate at temperatures varying from 55 to 70°C. Later Eddy et al. modified this test to use a constant temperature of about 55±0.5°C. Subsequently most researchers have used this temperature. Therefore, there are variations of this test, which employ different temperatures in order to determine nociception. The hot-plate at temperatures 50°C also known as “cold” plate, utilized in some study, is

used often to determine the analgesic activity of weaker analgesics such as nonsteroidal anti-inflammatory drugs. The hot-plate maintained at a temperature of 52°C, or “warm” plate, measures the analgesic activity of mildly potent analgesic such as codeine and morphine. The 55°C hot-plate technique easily identifies and quantifies potent analgesic compounds including fentanyl, morphine and other opioid analgesics. However, at this temperature non-narotic analgesics have no or only weak antinociceptive effects (Tjolsen, Hole, & Hunskaar, 1994).

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. A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times including jumping and paw licking. As for analgesic substances are concerned, the paw licking behavior is affected only by opioids, whereas, the jumping reaction time is increased equally by weak powerful analgesics such as paracetamol or acetylsalicylic acid, especially when the temperature of the plate is 50°C or less. The sensitivity and specificity of this test can be increased by measuring the reaction time of the first evoked behavior regardless of whether it is jumping or paw licking. 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 paw licking behavior. Thus, the animal may lick the paws and then jump during the first test but will jump almost immediately certainly with a much shorter reaction time during subsequent tests. Similarly, even putting the animals on an unheated plate just once to watch the test leads in subsequent tests to a reduction in the reaction time under standard conditions with a constant noxious temperature (Le Bars et al., 2001).

Writhing test

The writhing model represents a chemical nociceptive test. This test is most commonly used for measure peripheral analgesic activity and sometime called the abdominal constriction response, or stretching test. Pain is induced by intraperitoneal injection of irritating agent such as phenylquinone, acetic acid, acetylcholine, potassium chloride, bradykinin, adrenaline, tryptamine adenosine triphosphate, and oxytocin into the peritoneal cavity induces a syndrome called writhing. The writhes respond consists of contractions of the abdominal, and elongation passing caudally along the abdominal wall, sometimes accompanied by twisting and turning of the trunk and followed by extension of the hind limbs. These behaviors are considered to be reflexes and involved with visceral pain. However, the pain is probably similar to that resulting from peritonitis (Le Bars et al., 2001).

The mechanism of the irritating agent is clearly unknown, however several mediators have been proposed. Previous study demonstrated the considerable increase of PGE_2 level in the peritoneal fluid of rats 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. An examination of cells collected by washing the peritoneum revealed a large decrease between 15 and 30 min after injection of the irritant and 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 drugs, non-narcotic analgesics and some monoamino-oxidase inhibitors and antioxidants) prevented prostaglandin release (Deraedt, Jouquey, Delevallée, & Flahaut, 1980).

The advantage of this method is simple to perform, is sensitive and that it allows for the evidence to be obtained for effects produced by weak analgesics.

However, 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, muscle relaxants, that can be improved by undertaking a preliminary the roto-rod test to detect and eliminate molecules that alter the motor performance of the animal (Pearl, Stander, & McKean, 1969).

Formalin test

The formalin test is a valid and reliable model of nociception and sensitive for various classes of centrally analgesic agents (Dubuisson & Dennis, 1978). The noxious stimulus is an injection of dilute formalin under the subcutaneously into the plantar surface of the hind paw. The response is the amount of time the animals spent licking the injected paw (Wheeler-Aceto & Cowan, 1991). This test may allow the researchers to dissociate between non-inflammatory and inflammatory pain which is a rough classification of analgesics as well as to identify mechanism of analgesics between central and peripheral component. The injections of formalin have been reported to produce a distinct biphasic nociceptive response can be identified. The first phase (0-5 min) was defined after the formalin injection that results from a direct stimulation of nociceptors. Bradykinin, substance P and glutamate are thought to participate in this phase, which is believed to be non-inflammatory pain. In contrast, prostaglandins do not play an important role during in early phase (Hunskaar & Hole, 1987; Shibata, Ohkubo, Takahashi, & Inoki, 1989). The second phase (15-30 min) after injection of formalin is thought to be an inflammatory response with associated inflammatory pain, which is believed to be involved several inflammatory mediators including histamine, serotonin, bradykinin and prostaglandins. In general, centrally acting drugs such as morphine inhibited both phase. Peripherally acting drugs such as nonsteroidal anti-inflammatory drugs and corticosteroids inhibited only the second phase (Hunskaar & Hole, 1987). Previous

study proposed that the early phase resulted from C-fiber activation, while the late phase appeared to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Tjolsen, Berge, Hunskaar, Rosland, & Hole, 1992). These results indicated that the first and second phase responses induced by formalin have distinct characteristic properties.

Anti-inflammatory activity testing

Animals have been used as models to study inflammation and autoimmunity for more than 80 years, it may be concluded that the animal models will also be reflective of the state of knowledge regarding such diseases. Nevertheless, animal models of rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis have been successfully used to enhance the understanding of the human disease and have made significant contributions to the development of powerful new therapies (Webb, 2014). Pharmacological methods for testing acute and sub-acute inflammation including UV-erythema in guinea pigs, oxazolone-induced ear edema in mice and rats, croton-oil edema in mice and rats, paw edema in mice and rats, pleurisy tests and granuloma pouch technique (Vogel, 2007).

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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 and mice after injection of a phlogistic agent such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin or carrageenan. 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 infected

paw is measured before and after application of the phlogistic agent and the paw volume of the treated animals is compared to controls (Vogel, 2007).

Carrageenan-induced paw edema is the well-researched and highly reproducible model and it therefore frequently chosen for the screening of acute anti-inflammatory action of natural compounds (Morris, 2003). The test is based upon the ability of the drug to inhibit the acute edema produced in the hind paw of the mice after injection of carrageenan. This model has been described as a biphasic event, in which various mediators operate in sequence to produce inflammatory response. The early phase (0-2 h after carrageenan injection) is mainly mediated by histamine, serotonin, bradykinin and kinins. The late phase (3-6 h after injection of carrageenan) has been involved with the prostaglandins appear to be the most important mediators in the late phase (Crunkhorn & Meacock, 1971; Rosa, 1972; Rosa & Willoughby, 1971). And more recently has been attributed to the involvement of COX, free radicals and NO in the hind paw exudate (Oka et al., 2007). The edema at 3 h after the administration of carrageenan was considered to reach the highest response. The paw edema responds to anti-inflammatory drugs that causes a reduction in inflammatory responses (Andrade, Cardoso, Carvalho, & Bastos, 2007; Kale, Misar, Dave, Joshi, & Mujumdar, 2007).

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Cotton-pellet induced granuloma

The cotton pellet-induced granuloma formation test is one of the most commonly used for the evaluation of chronic anti-inflammatory activity of the drugs. The method has been described first by Meier et al. (1950). The subcutaneous implantation of cotton pellet directly triggers an acute inflammatory response. The acute response is insufficient to eliminate the pro-inflammatory agents, leads to the chronic inflammatory reaction. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils, mononuclear cells and undifferentiated

connective tissue can be observed besides the fluid infiltration. After several days, the amount of newly formed connective tissue or granuloma can be measured by weighing the dried pellets after removal. The increased weight of dried cotton pellet has been shown to correlate with the amount of granulomatous tissue (Swingle & Shideman, 1972).

Histological examination

Histology is the biological science concerned with the microscopic structures of cells, tissues, and organs of human, animals, plants, and fungi, in relation to their function. After the removal of a tissue sample, a series of process must take place to ensure the final microscope slides are of a diagnostic quality. An overview of the histological processes is presented below.

Fixation

The process of fixation of biological specimen is the important step in the processing of the histology examination and aim to stop any autolytic and bacterial decomposition. Fixation stabilizes protiens, rendering the cell and changes the tissue receptiveness to further processing. The most commonly used fixative is a formalin; however, other fixative (e.g. Bouin, Carnoy, Paraformaldehyde, potassium dichromate, chromic acid) are necessary depends on the planned subsequent protocol. The choice of the fixative determines the results, since every fixative has assets and drawbacks for the preservation of particular histological structure and especially the binding site for differential staining, e.g. in immunohistology.

Decalcification

When small foci of calcium are present in the tissue section, cutting a quality section may be difficult. The block may be removed from the chunk after rough

cutting of the tissue. The time exposure to the decal will vary depending on the tissue.

Dehydration

The process of dehydration is the removal of water and aqueous fixative from the tissue components. Several dehydrating reagents are hydrophilic, possessing strong polar groups that interact with the molecules in the tissue. Dehydration should be accomplished slowly, if the concentration gradient between the fluid inside and outside the tissue is excessive, diffusion current cross the cell membranes during fluid exchange, increasing the possibility of cell distortion. Excessive dehydration causes the tissue to become hard, brittle and shrunken. The specimens are always processed through a graded series of reagents of increasing concentration. Many dehydrating agents are ethanol, ethanol acetone, methanol, isopropyl, glycol and denatured alcohols. If the dehydrant of choice is ethanol, the tissue is first immersed in 70% ethanol in water, followed by 95% and 100% solution.

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Clearing

The clearing reagent acts as an intermediary between the dehydration and infiltration solution. The process is the removal of dehydrating solutions, making the tissue components receptive to the infiltrating medium. The criteria for choosing a suitable agent are rapid of removal of dehydrating agent, ease of removal by melted paraffin, minimal tissue damage and flammability. Most clearing agents are flammable liquids, which the boiling point of the clearing agent gives an indication of its speed of replacement by melted paraffin.

Clearing agents suitable for routine histology laboratories is xylene, a flammable, liquid with a characteristic petroleum or aromatic odor, miscible with

most organic solvents and paraffin. Over-exposure during processing will cause over-hardening.

Embedding

Embedding of histological material is performing in order to provide a mechanical stable and hard entity, which can be sectioned to various coherent thin sections. The section should enable a spatial reconstruction of the original sample after differentiating analyses. The most common material for tissue embedding is paraffin (wax embedding), which form a matrix preventing tissue structure distortion during section.

Sectioning

Whole mount samples can only be investigated from their outer surface. Analyses inside the samples need dissection with preferably minor destruction of the structural context. In general, penetration of staining chemicals is enhanced afterwards, and microscopic and biophysical inspection is improved as the spatial resolution corresponds to the thickness of the section. Biological samples, which are embedded in paraffin or resin, can generally be sectioned by a special device, a microtome. Some microtomes are equipped with a motor-driven support, both for controlling the section thickness as well as the force of the blade. Several types of microtomes are available, each optimized for variably embedded samples: Paraffin embedded samples can be sectioned by sled microtomes or rotary microtomes equipped with metal knives. The blades are either manufactured of especially hardened steel for sections between 5 and 30 μm, for semi thin sections (2–5 μm) and ultra-thin sections (about 40–100 nm).

Staining

Multiple histological staining methods allow identifying cellular structures, tissue components, and organ systems, also enzymatic processes and involved macromolecules. It is even possible to clearly differentiate between organic and inorganic material as well as decaying from living material by selective marking of minerals and molecules. For any staining it is necessary that the structures that should be stained are free of any embedding media. This means that for standard-histology the paraffin has to be removed by special procedures so that the staining chemicals can react with the tissues.

The hematoxylin and eosin stain (H&E) is the most widely used stain. Hematoxylin is extracted from the heartwood or logwood of the tree *Haematoxylon campechianum*, can be prepared in numerous ways and has a widespread applicability to tissue from different site. Essentially, the Hematoxylin component stains the cell nuclei blue-black, with a well intranuclear detail. Eosin is the most suitable to combine with the Hematoxylin to demonstrate the general histological of tissue, which the eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange and red.

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Mounting

For further histological processing, the sections must be mounted, most often on glass slides or on grids coated with a special synthetic film. The major difficulty is the choice of the best possible sticking procedures in order to prevent both, a floating resp. a loss of the sections during the staining protocol and undesired background staining by the glue. While before often unreliable “home-made” coating procedures, e.g., by chromic sulphate or polylysine, were used, now various types of pretreated glass slides are commercially available, they have been optimize for selected histological procedures (Redi, 2010).

Curcuma* aff. *amada

Figure *11*_Curcuma_ aff. *amada*

An inflorescence (A); flowers (B); rhizomes (C) (Suwanchaikasem et al., 2013).

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Family Zingiberaceae

Vernacular names Thailand: Wan Rang Chuet

Distribution

Curcuma is a large genus comprising more than 80 species of rhizomatous perennial herbs and herbs distributed throughout tropical of Asia, Australia and extend to Africa. The plant of the genus represents of the most popular medicinal herbs (Purseglove, Brown, Green, & Robbins, 1981).

Uses

The rhizome of *Curcuma* aff. *amada* has been used for treatment of poisoning, detoxification and anti-inflammation in Thai traditional medicine. The decoction of its rhizome is used to treat abscess and poisoning from insecticides and ethyl alcohol (Kanathum, 2008).

Description

Curcuma aff. *amada* is a shrub with underground rhizome belonging to the family Zingiberaceae. The medicinal plant is characterized as 0.81.2 m tall; petiole 12-30 cm; leaves basal; leaf blade elliptic or elliptic oblong, 35-85 x 13-21 cm, glabrous, base attenuate, oblique, apex caudate. Inflorescences terminal on pseudostems; peduncle 15-22 cm; spike cylindric, 14-20 x 5-8 cm; fertile bracts pale green, ovate, 3.5-4.5 x 2-3.2 cm, apex obtuse; coma bract whitish, ovate or elliptic oblong, apex acute, red and pink. Calyx clavate, apex irregularly 3-toothed; corolla tube pale yellow, funnelform, 2.5-3.2 cm; lobesyellowish, oblong, apex mucronate; lateral staminoides pale yellow, obovate, 2 x 11 cm, apex 2 cleft; labellum pale yellow band at center, ovate, 2 x 1.6 cm, apex convex, emarginated; rhizomes many branched, white or yellowish inside, aromatic; roots tuberous at tip (Kanathum, 2008).

Pharmacological Activities

Antioxidant Activity

The aqueous and ethanolic extract of *Curcuma* aff. *amada* were shown to have antioxidant activity in the Ferric reducing antioxidant power (FRAP) assay. (Suwanchaikasem et al., 2013).

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Chapter III

MATERIALS AND METHODS

Experimental animals

Male ICR mice weighing 18-25 g were used in hot-plate, acetic acid-induced writhing, formalin, carrageenan-induced mouse paw edema, PGE_2-induced mouse paw edema, arachidonic acid-induced mouse paw edema and rota-rod tests. Male ICR mice weighing 25-35 g were used in cotton pellet-induced granuloma formation test. The mice were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were housed in the Animal facility of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under conditions of 25±2°C, 50-60% humidity, and a 12 h light/12 h dark cycle, with standard pellet diet and water provided *ad libitum*. The mice were acclimatized for 7 days before use in the experiments. At the end of each experiment, mice were sacrificed by carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Protocol Approval No. 14-33-004).

Plant material

Rhizomes of *Curcuma* aff. *amada* were collected from Chachoengsao province and identified by Associate Professor Chaiyo Chaichantipyuth, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The voucher specimen (No.SS-0809601) was deposited in the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences,

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Chulalongkorn University. The ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE) was provided by Associate Professor Suchada Sukrong, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Preparation of plant extract

Extraction procedures for CAE are described in Suwanchaikasem et al. (2013). Briefly, the plant was ground into powder with an electric blender, and the fine powder (20 g) was extracted with 200 mL of ethanol. The ethanolic extract was macerated at room temperature for 72 h and filtered with Whatman No.1 filter paper. The filtrate obtained from the extract was dried using a vacuum evaporator at 50°C. The resulting dry ethanol extract was weighed and the percentage yield calculated as 22.61 % (w/w). The extract was then stored at 4°C until use.

Drugs and chemicals

Morphine sulfate (MO; Thai FDA), formaldehyde (Merck Chemical, Germany), acetic acid (Merck, Darmstadt, Germany), naloxone (NAL; Sigma, USA), λ-carrageenan (Sigma, USA), prostaglandin E_2 (PGE_2; Sigma, USA) and pentobarbitone sodium (Ceva Sante Animale, France) were dissolved in 0.9% sodium chloride solution (NSS; General Hospital Products Public, Thailand). CAE and indomethacin (IND; Sigma, USA) were suspended in 2% (w/v) Tween 80 (Srichansahaosoth, Thailand). Caffeic acid (Sigma, USA) was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma, USA). Arachidonic acid (AA; Sigma, USA) was dissolved in 0.2 M sodium bicarbonate buffer (pH 8.43-8.56). MO and IND were used as standard analgesic drugs. Caffeic acid and IND were used as standard anti-inflammatory agents.

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Experiment methods

Antinociceptive activity testing

Hot-plate test

A hot-plate test was conducted as described by Woolfe and Macdonald in 1944. Male ICR mice weighing 18-25 g were used in this study. Mice were placed on the hot-plate (Harvard Apparatus, USA), maintained the temperature at 55±1°C and confined with a clear Plexiglas wall cylinder with 20 cm diameter and 30 cm height. The hot-plate latency, the time from contact of the mouse with the hot plate to licking of a hind paw or jumping up from the surface, was recorded by a stop-watch.

On the day of testing, mice were randomly assigned to control and treatment groups of 10 animals each. Mice were measured on the hot-plate for three pre-drug baseline trials conducted at 5-10 min intervals. The average of the last two pre-drug baseline trials was used as pre-drug latency for each mouse. Only animals which had pretreatment hot-plate latency time < 45 s were utilized. If this behavior was not observed within 45 s, the animal was removed from the hot-plate to avoid burn and the latency was truncated as 45 s. Mice received orally administration of NSS (10 mL/kg), 2% Tween 80 (10 mL/kg), MO (2 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg). The post-drug latency was determined 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) using the following formula:

$$\% \text{ MPE} = \left(\frac{\text{(Post-drug latency) - (baseline latency)}}{\text{(Cut-off time) - (baseline latency)}} \right) \times 100$$

where cut-off time for the hot-plate test= 45 s.

The area of analgesia for the hot-plate assay was derived by calculating the area under the corresponding 0-240 min time-course-% MPE curve; area were calculated using the trapezoidal rule (Tallarida & Murray, 1987).

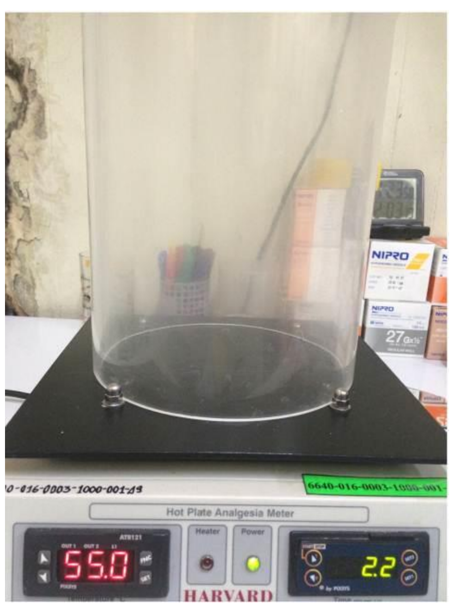

Figure 12 Hot-plate Analgesiometer (Harvard Apparatus, USA)

Analysis of the mechanism of antinociceptive action

The possible participation of the opioid receptors in the antinociceptive effect of CAE was investigated using the model of mouse hot-plate test. Mice were pretreated intraperitoneally with NAL (5 mg/kg, i.p.) 10 min before oral administration of CAE (200 mg/kg) and the hot-plate latencies were measured.

Acetic acid-induced writhing test

The acetic acid-induced writhing method was conducted as described by Koster et al. in 1959. Male ICR mice weighing 18-25 g were used. Group of 8 animals were used for control and treated mice. On the day of testing, mice were pretreated orally with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/kg) 1 h before intraperitoneal injection of 0.6% acetic acid solution (10 mL/kg). The animals were then placed individually in a glass cylinder for observation of writhe response, manifesting as abdominal constriction, pelvic rotation and subsequent stretching of at least one hind limb. The number of writhes were observed and counted in 5-min periods for 30 min after acetic acid administration. Antinociceptive activity was reported as percentage of inhibition of the writhing response compared with the vehicle control group. The percentage of inhibition of the writhing response was calculated using the following formula:

$$\text{\% Inhibition of writhes} = \frac{\text{MW (control)} - \text{MW (test)}}{\text{MW (control)}} \times 100$$

where MW = the mean number of writhes.

Figure 13 Writhing response in mouse

Formalin test

The formalin test was performed as described by Hunskaar and Hole in 1987. Male ICR mice (18-25 g) were used. Group of 8 animals were used for control and treated mice. On the day of testing, mice were orally administered with NSS (10 mL/kg), 2% Tween 80 (10 mL/kg), MO (2 mg/kg), IND (10 mg/kg) or various doses of CAE (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/kg) 1 h before subcutaneous injection of 2.5% formalin solution (20 µl) into the plantar surface of the left hind paw. Following formalin injection, the mouse was immediately placed individually in a glass cylinder for observation. The times spent for licking and biting the injected paw were recorded at 0-5 min (early phase) and 15-30 min (late phase) after formalin injection. The percentage inhibition of licking time was calculated using the following formula:

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

where ML = the mean licking time (s).

Figure 14 Formalin-induced paw licking in mouse

Acute anti-inflammatory activity

Carrageenan-induced mouse paw edema

The experiment was conducted using the procedures described by Levy (1969). Male ICR mice weighing 18-25 g were used. Group of 8 animals were used for control and treated mice. The mouse paw was marked with black ink at the level of the lateral malleolus. Mice were orally administered with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) 1 h before subcutaneous injection of 1% carrageenan solution (50 μl) into the plantar surface of the left hind paw. Paw volume was measured using a plethysmometer (Ugo Basile7150, Italy) 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:

$$\text{\% Inhibition of paw edema} = \left(\frac{(Vt\text{-}Vb)\ \text{control} - (Vt\text{-}Vb)\ \text{treated}}{(Vt\text{-}Vb)\ \text{control}} \right) \times 100$$

where Vt = paw volume after carrageenan injection at each time point (mL); and Vb = paw volume before carrageenan injection (mL).

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Figure 15 Measurement of paw volume using a plethysmometer

Analysis of the mechanism of anti-inflammatory action

The mechanism of anti-inflammatory activity of CAE was examined using the PGE_2-induced mouse paw edema model as described by Akkol et al. (2009). Male ICR mice weighing 18-25 g were used. Group of 8 animals were used for control and treated mice. On the day of testing, the mouse paw was marked with black ink at the level of lateral malleolus. Mice were orally administered 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or CAE (50 mg/kg) 1 h before subcutaneous injection of 0.01% PGE_2 solution (50 µl) into the plantar surface of the left hind paw. Paw volume was measured before and 0.5, 1, 1.5, 2, 3 and 4 h after PGE_2 injection, using a plethysmometer (Ugo Basile7150, Italy). The edema volume in the hind paw was expressed as a mean increase in paw volume relative to the control. The percentage inhibition of paw edema was calculated using the following formula:

$$\% \text{ Inhibition of paw edema} = \left(\frac{(Vt\text{-}Vb)\text{ control} - (Vt\text{-}Vb)\text{ treated}}{(Vt\text{-}Vb)\text{ control}} \right) \times 100$$

where Vt = paw volume after PGE_2 injection at each time point (mL); and Vb = paw volume before PGE_2 injection (mL).

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Arachidonic acid-induced mouse paw edema

The method used was first described by Di Martino et al. (1987). Male ICR mice weighing 18-25 g were used. Group of 8 animals were used for control and treated mice. On the day of testing, the mouse paw was marked with black ink at the level of lateral malleolus. Thereafter, mice were pretreated with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) orally or 10% DMSO (10 mL/kg) or caffeic acid (100 mL/kg) intraperitoneally. One hour later, 0.5% AA solution (50 µl) was injected subcutaneously into the plantar

surface of the left hind paw. Paw volume was measured before and 0.5, 1, 1.5, 2, 3 and 4 h after AA injection.

$$\% \text{ Inhibition of paw edema} = \left(\frac{(\text{Vt-Vb}) \text{ control} - (\text{Vt-Vb}) \text{ treated}}{(\text{Vt-Vb}) \text{ control}} \right) \times 100$$

where Vt = paw volume after AA injection at each time point (mL); and Vb = paw volume before AA injection (mL).

Histological changes associated with carrageenan-induced mouse paw edema

After induction of carrageenan-induced paw edema, mice were sacrificed and the left hind paw of each mouse was removed and fixed in 10% neutral buffered formalin solution for 1 week at room temperature. Each sample was decalcified in ferric acid, dehydrated in graded ethanol, embedded in paraffin and cut into 3 μm thick sections, the section was then stained with hematoxylin and eosin (H&E). Five representative tissue sections were randomly chosen from each group and examined histologically with a light microscope (Olympus, Japan). In each tissue section, the numbers of neutrophils were counted at a 400× magnification using the CellSense Standard Program (Olympus, Japan). The average value from five fields was used in statistical analysis (Huang et al., 2011).

Chronic anti-inflammatory activity

Cotton pellet-induced granuloma formation in mice

The method was conducted as described by Meier et al. (1950). Male ICR mice weighing 25-35 g were used for the study. Animals were randomly divided into 7 groups of 8 animals each. On the day of testing, mice were anesthetized with sodium pentobarbital (40 mg/kg) and the back skin was shaved and disinfected with 70% ethanol. An incision was then made and pre-weighed sterile cotton pellets

(10±0.5 mg) were implanted subcutaneously into the back area. Mice were treated orally with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) once daily for 7 consecutive days. On day 8, the mice were sacrificed and pellets surrounded with granulomatous tissue were carefully removed and dried to constant weight at 60°C for 16 h. The weight of granuloma was determined and the percentage inhibition of granuloma was calculated using the following formula:

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

where Wg = the mean weight of granuloma (mg).

Figure 16 Granulomatous lesion

Safety evaluation

To rule out motor impairment or sedation effect of CAE, a rota-rod test was performed as described by Dunham and Miya (1957). Male ICR mice weighing 18-25 g were used for the study. Mice were placed on a horizontal rod rotating at a speed of 16 rpm (Ugo Basile, Italy). Mice capable of remaining on the rotating rod for 60 s or more in three successive trials were used in the study. Each mouse was treated orally with 2% Tween 80 (10 mL/kg) or CAE (200 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 (s) for which the animal remained on the rota-rod.

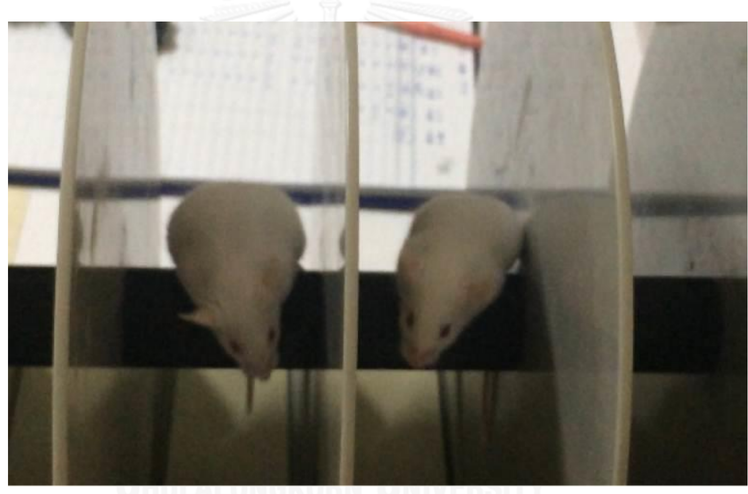

Figure 17 Rota-rod test

Acute toxicity

Animals used in the study were observed for any behavioral changes (such as diarrhea, salivation, respiratory stress) for 72 h. Morbidity or mortality was recorded for each group at the end of the observation period.

Data treatment and statistical analyses

Results are expressed as the mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) and Student t-test followed by a *post hoc* Tukey test for multiple comparisons using SPSS for Windows ver. 17.0, with $p<0.05$ indicating significance.

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CHAPTER IV

RESULTS

Hot-plate test

The hot-plate test was designed to assess potential antinociceptive effect of drugs administered. Initial study utilizing the hot-plate test in mice to examine the efficacy of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE) in producing analgesia. Mice were administered orally 2% Tween 80 or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg), NSS (10 mL/kg) and MO (2 mg/kg) and were tested during subsequent 240 min period.

MO, a centrally-acting analgesic drug, markedly increased hot-plate latency producing an area of analgesia (13,212.17±1,234.83 %MPE-min) compared to NSS. CAE at doses of 100 and 200 mg/kg significantly ($p<0.01$ and $p<0.001$ respectively) increased the hot-plate latencies producing an area of analgesia (9,625.37±1,982.30 and 14,789.71±1,572.13 %MPE-min, respectively) when compared to 2% Tween 80 (1,954.53±1,422.51 %MPE-min). CAE at doses of 100 and 200 mg/kg showed antinociceptive activity comparable to MO (Figure 18).

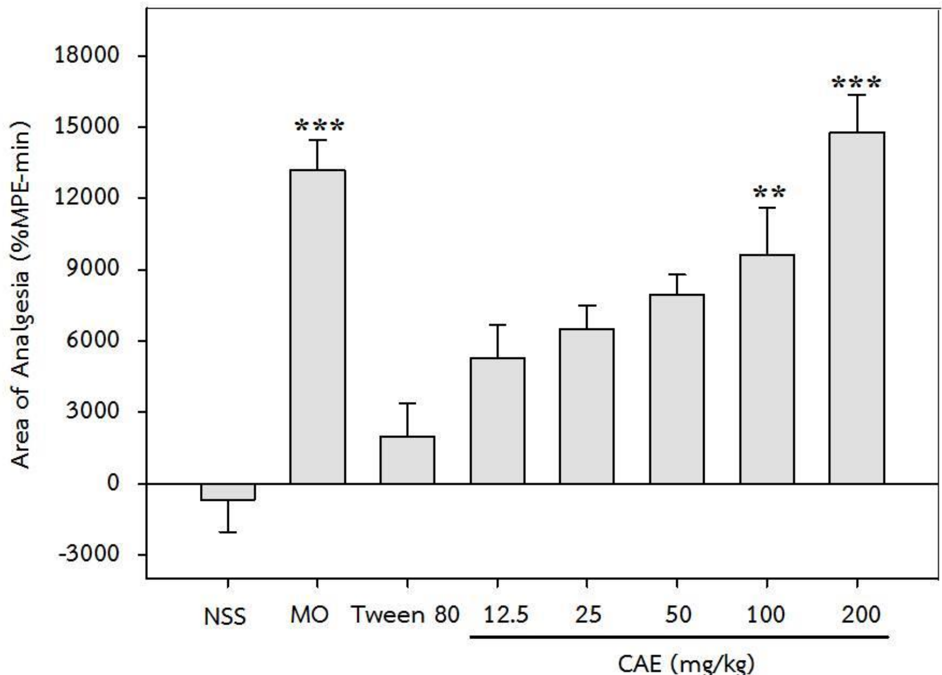

Figure 18 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of normal saline (NSS; 10 mL/kg), morphine sulfate (MO; 2 mg/kg), 2% Tween 80 (10 mL/kg), and various doses of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 12.5-200 mg/kg). N=10 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to their respective controls.

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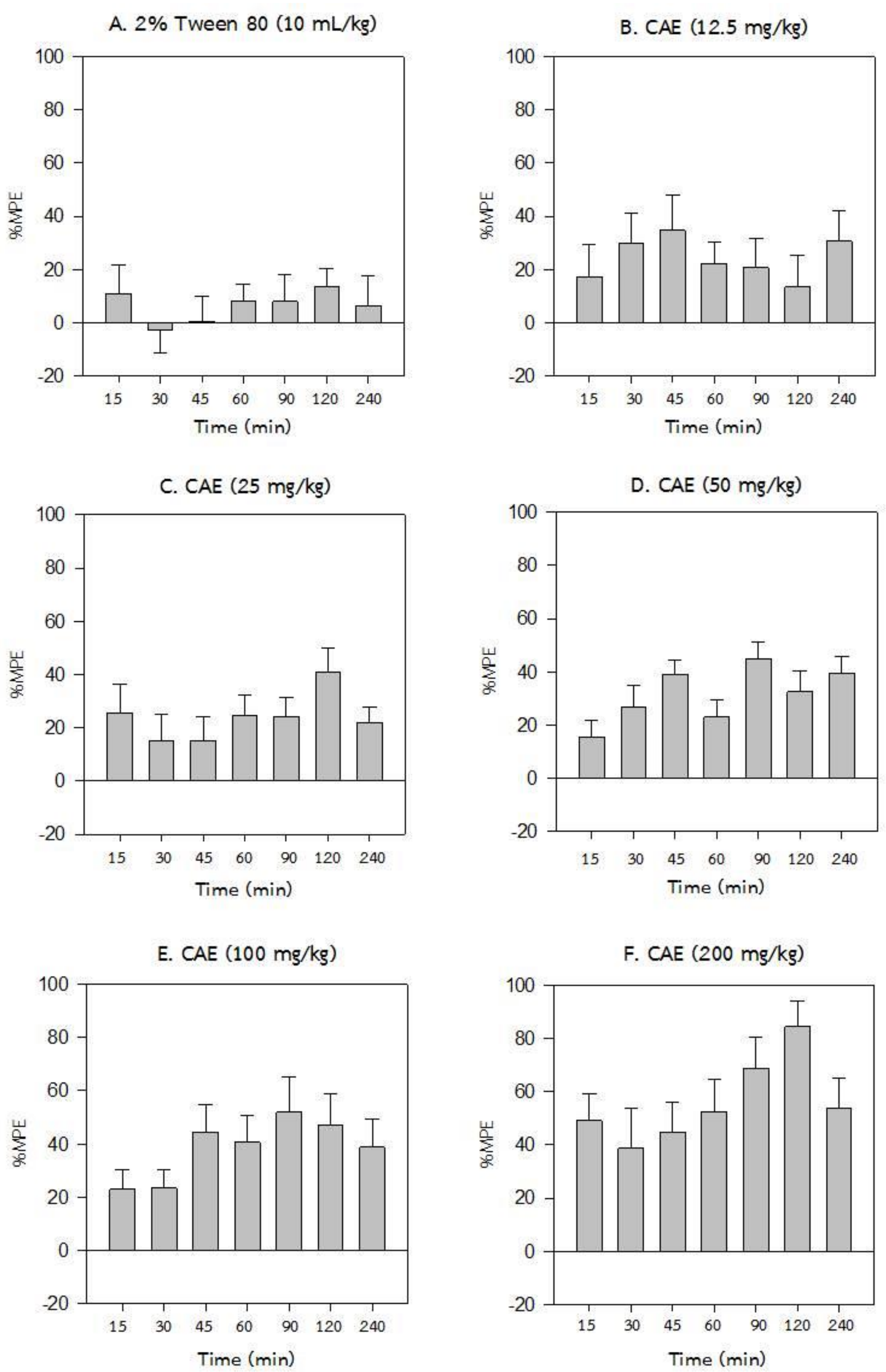

Figure 19 Individual time courses of the response (%MPE versus time (min)) after oral administration of various doses of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 12.5-200 mg/kg). N=10 for all groups.

A. 2% Tween 80 (10 mL/kg), B. CAE (12.5 mg/kg), C. CAE (25 mg/kg), D. CAE (50 mg/kg), E. CAE (100 mg/kg), F. CAE (200 mg/kg).

Analysis of the mechanism of antinociceptive action

Involvement of opioid receptor pathway

In order to investigate possible role of opioid receptors in antinociceptive action of CAE, mice were then administered NSS (10 mL/kg, i.p.), NAL (5 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), CAE (200 mg/kg, p.o.), and combination of NAL and CAE (5/200 mg/kg). NAL alone failed to produce significant response when compared to NSS. CAE at dose tested (200 mg/kg) produced significant (p<0.05) analgesic response when compared to 2% Tween 80. The inclusion of naloxone with CAE significantly (p<0.001) attenuated the analgesic response due to CAE indicating that opioid receptors are involved in the analgesic response produced by CAE (Figure 20).

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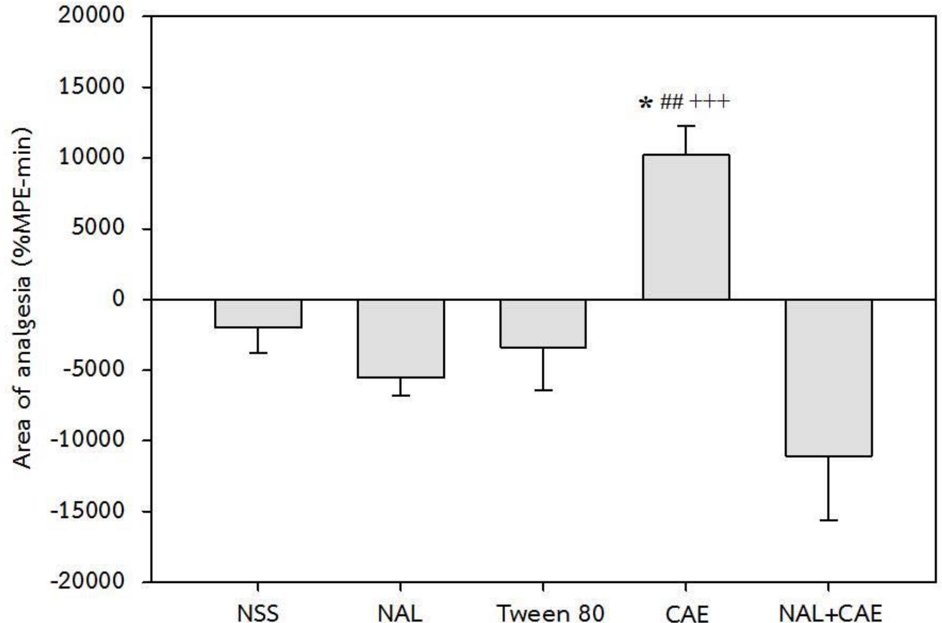

Figure 20 Area of analgesia (%MPE-min) from 0-240 minutes after administration of normal saline (NSS; 10 mL/kg, i.p.), naloxone (NAL; 5 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 200 mg/kg, p.o.), and combination of naloxone and CAE (5/200 mg/kg). N=10 for all groups.

$^{*}p<0.05$ significantly different compared to 2% Tween 80.

$^{\#\#}p<0.01$ significantly different compared to NAL.

$^{+++}p<0.001$ significantly different compared to NAL+CAE.

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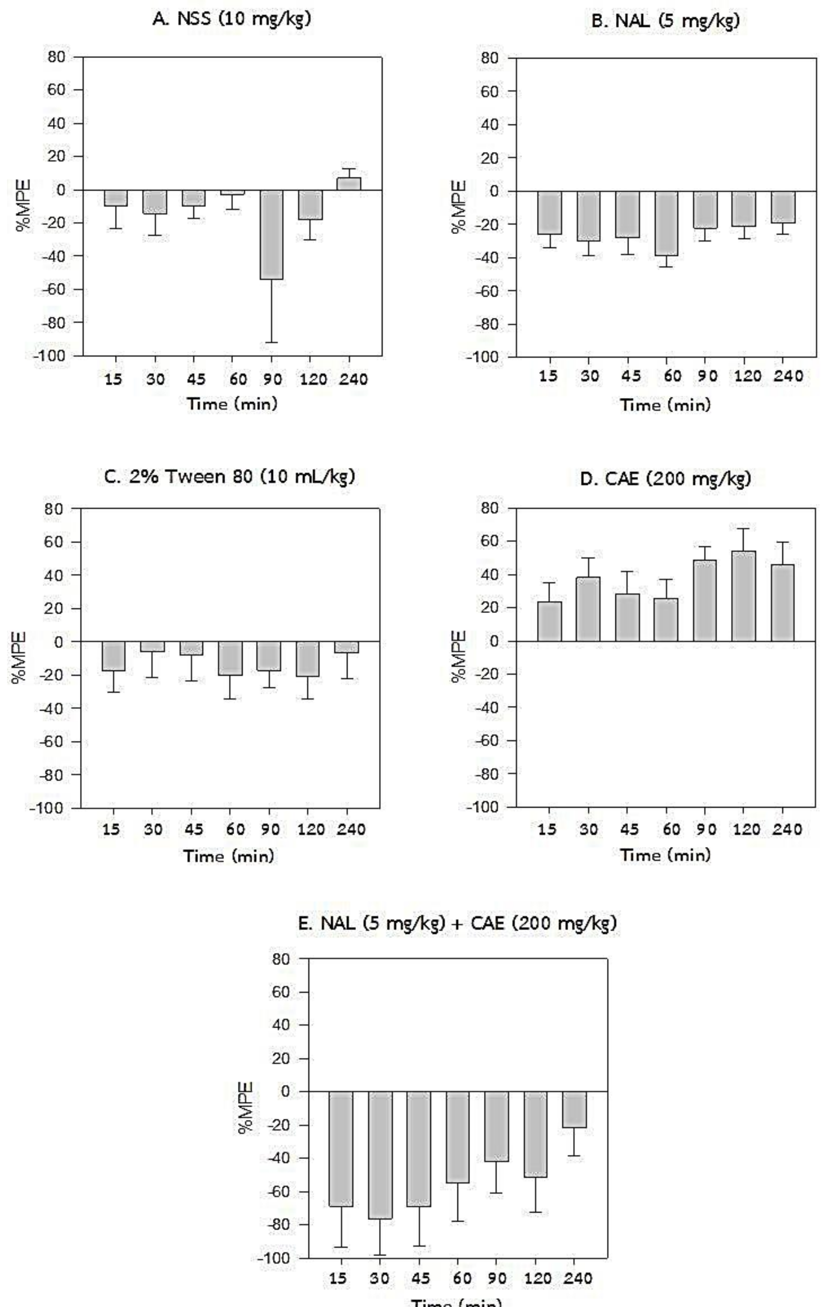

Figure 21 Individual time courses of the response (%MPE versus time (min)) after administration of normal saline (NSS; 10 ml/kg, i.p.), naloxone (NAL; 5 mg/kg, i.p.), 2% Tween 80 (10 ml/kg, p.o.), the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 200 mg/kg, p.o.), and combination of naloxone and CAE (5/200 mg/kg). N=10 for all groups.

A. NSS (10 mL/kg), B. NAL (5 mg/kg), C. 2% Tween 80 (10 mL/kg), D. CAE (200 mg/kg), E. NAL (5mg/kg) + CAE (200 mg/kg).

Acetic acid-induced writhing test

Study then utilized the acetic acid-induced writhing method to examine the analgesic efficacy of CAE. Each mouse was orally administered 2% Tween 80 (10 mL/kg), IND (10 mg/kg), and various doses of CAE (3.125-200 mg/kg).

CAE at doses of 12.5, 25, 50, 100, and 200 mg/kg significantly (p<0.01) decreased the number of writhes induced by acetic acid by 67.93%, 65.06%, 63.12%, 62.11% and 58.23%, respectively when compared to vehicle control. CAE 12.5 mg/kg showed a maximum inhibition of writhes. The reference drug, IND significantly (p<0.001) decreased the number of writhes by 75.70% when compared to the vehicle control. The antinociceptive efficacy of CAE at doses of 12.5, 25, 50, 100, and 200 mg/kg were comparable to that of IND (Figure 22).

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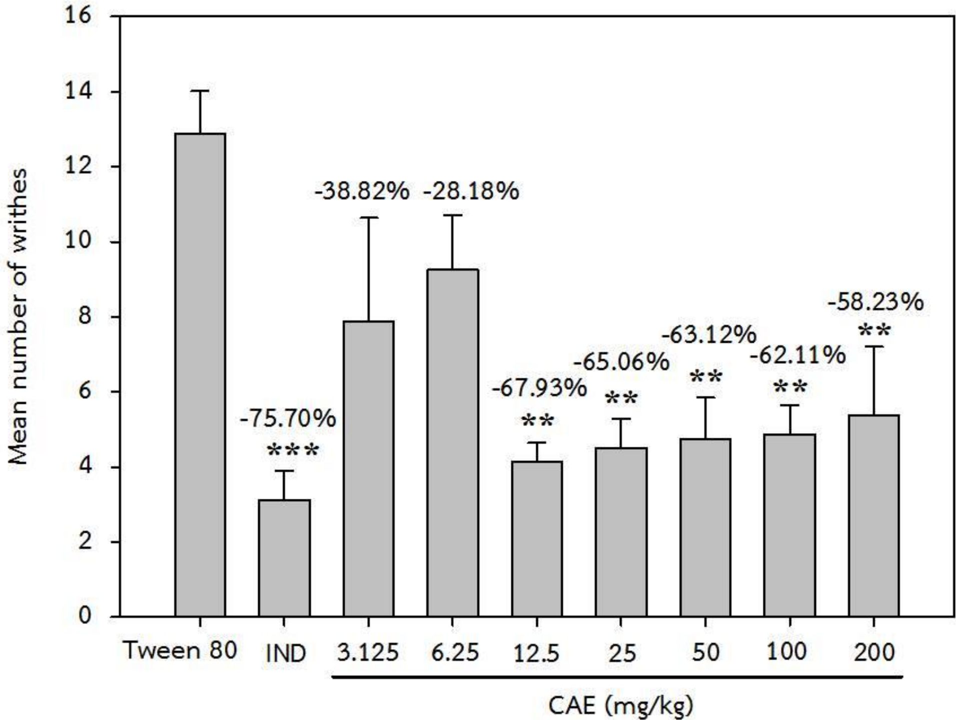

Figure 22 Mean number of writhes after oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 3.125-200 mg/kg). N = 8 for all groups.

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

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Formalin test

Study then utilized the formalin test to examine the analgesic efficacy of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE). Mice were orally administered NSS (10 mL/kg), MO (2 mg/kg), 2% Tween 80 (10 mL/kg), IND (10 mg/kg), or various doses of CAE (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/kg).

MO (2 mg/kg) significantly decreased the licking time in the early (0-5 min after formalin injection) and late (15-30 min after formalin injection) phases of the pain response by 59.14% and 67.80%, respectively, compared with NSS (p<0.001 and p<0.001, respectively) (Figure 23). IND (10 mg/kg) significantly (p<0.001) reduced the time of licking and biting of injected paws during the late phase by 72.66%. However, the pain response during the early phase appeared to be unaffected by IND. CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg caused significant inhibition of early phase of formalin-induced nociception by 45.16%, 49.66%, 39.73%, 40.93% and 55.76%, respectively, compared to 2% Tween 80 (p<0.01, p<0.001, p<0.01, p<0.01, p<0.001, respectively) and late phase of formalin-induced nociception by 77.12%, 78.94%, 64.33%, 57.61% and 87.94%, respectively, compared to 2% Tween 80 (p<0.001, p<0.001, p<0.001, p<0.01, p<0.001, respectively). CAE at doses of 200 mg/kg showed a maximum inhibition of formalin-induced nociception of both phases. The inhibitory effects of CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg on formalin-induced nociception in both phases were comparable to that of MO and in the late phase were comparable to that of IND (Table 1).

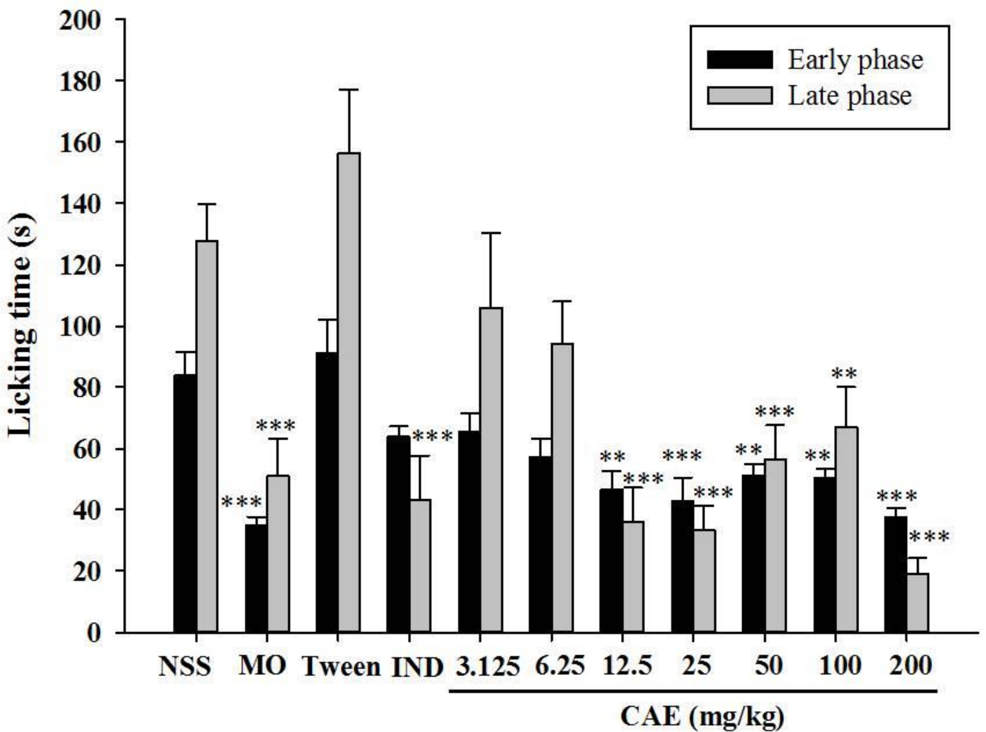

Figure 23 Time spent on paw licking after oral administration of normal saline (NSS; 10 mL/kg), morphine sulfate (MO; 2 mg/kg), 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 3.125-200 mg/kg). N=8 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to their respective controls.

Table 1 Time spent on paw licking after oral administration of normal saline (NSS; 10 mL/kg), morphine sulfate (MO; 2 mg/kg), 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 3.125-200 mg/kg). N=8 for all groups. Each value represents mean ± S.E.M. Inhibition of paw licking is reported as percentage compared to vehicle control.

Treatment	Dose (mg/kg)	Licking time (s) (% inhibition)	
		Early phase	Late phase
NSS		83.90±7.81	127.58±12.27
MO	2	34.74±2.92*** (-59.14%)	50.95±12.17*** (-67.80%)
2% Tween 80		85.03±10.55	158.24±20.63
IND	10	63.77±3.37 (-25.00%)	43.26±14.36*** (-72.66%)
CAE	3.125	65.58±6.08 (-22.87%)	106.06±24.46 (-32.98%)
	6.25	57.32±5.98 (-32.59%)	94.21±13.83 (-40.47%)
	12.5	46.63±5.97** (-45.16%)	36.21±11.06*** (-77.12%)
	25	42.80±7.65*** (-49.66%)	33.33±8.08*** (-78.94%)
	50	51.25±3.73** (-39.73%)	56.44±11.18*** (-64.33%)
	100	50.23±3.13** (-40.93%)	67.08±12.99*** (-57.61%)
	200	37.62±3.01*** (-55.76%)	19.08±5.12*** (-87.94%)

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to their respective controls.

Acute anti-inflammatory activity

Carrageenan-induced mouse paw edema

Study then utilized the carrageenan-induced mouse paw edema test to examine the acute anti-inflammatory efficacy of CAE. Each mouse was orally administered 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg), or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg).

Carrageenan injection in the control group induced localized paw edema that was evident 1 h after injection and increased progressively in severity up to 6 h after injection. All doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) significantly decreased paw edema induced by carrageenan at 4, 5 and 6 h (CAE 12.5 mg/kg: $p<0.05$, $p<0.01$, $p<0.01$, respectively; CAE 25 mg/kg: $p<0.05$, $p<0.001$, $p<0.001$, respectively; CAE 50 mg/kg: $p<0.01$, $p<0.001$, $p<0.001$, respectively; CAE 100 mg/kg: $p<0.01$, $p<0.01$, $p<0.01$, respectively; CAE 200 mg/kg: $p<0.01$, $p<0.001$, $p<0.001$, respectively). CAE 12.5 mg/kg showed a maximum inhibition of paw edema of 69.17% at 5 h. CAE 25 mg/kg showed a maximum inhibition of paw edema of 75.76% at 6 h. CAE 50 mg/kg showed a maximum inhibition of paw edema of 80.45% at 5 h. CAE 100 mg/kg showed a maximum inhibition of paw edema of 65.41% at 4 h. CAE 200 mg/kg showed a maximum inhibition of paw edema of 82% at 6 h. Thus, the percentage inhibition of paw edema of CAE at all doses tested appeared to be sustainable for 4-6 h after carrageenan injection. No significant effects of all doses of CAE on carrageenan-induced paw edema were observed at 1, 2 and 3 h. Compared to the control group, IND at 10 mg/kg caused a significant reduction of hind paw edema at 4, 5 and 6 h ($p<0.01$, $p<0.001$, and $p<0.001$, respectively) and showed a maximum inhibition of 84.21% at 6 h after carrageenan injection. In general, CAE at all doses tested appeared to have a comparable inhibitory activity on carrageenan-induced paw edema compared to IND (Table 2).

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Table 2 Changes in edema volume (mL) from 1 to 6 h after carrageenan injection following oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg). N=8 for all groups. Data are shown as the mean ± S.E.M. Inhibition of paw edema is reported as percentage compared to 2% Tween 80.

Treatment (mg/kg)	Paw edema ± S.E.M. (% inhibition)					
	1 h	2 h	3 h	4 h	5 h	6 h
Tween 80	0.09250±0.01191	0.10250±0.01849	0.12750±0.01810	0.16000±0.02276	0.16625±0.02236	0.16500±0.02087
IND 10 mg/kg	0.05000±0.01615 (-45.95%)	0.07625±0.01802 (-25.61%)	0.08125±0.02108 (-36.27%)	0.04750±0.01790** (-70.31%)	0.02625±0.01375*** (-84.21%)	0.03000±0.01035*** (-81.82%)
CAE 12.5 mg/kg	0.06750±0.01980 (-27.03%)	0.08250±0.01130 (-19.51%)	0.07250±0.02085 (-43.14%)	0.05875±0.02287* (-63.28%)	0.05125±0.01274** (-69.17%)	0.05500±0.01722** (-66.67%)
CAE 25 mg/kg	0.06875±0.01608 (-25.68%)	0.07625±0.01569 (-25.61%)	0.12750±0.01264 (-0.00%)	0.06375±0.01558* (-60.16%)	0.04250±0.02266*** (-74.44%)	0.04000±0.01476*** (-75.76%)
CAE 50 mg/kg	0.05875±0.01517 (-36.49%)	0.07750±0.02664 (-24.39%)	0.09250±0.02596 (-27.45%)	0.03375±0.02291** (-78.91%)	0.03250±0.02374*** (-80.45%)	0.03750±0.02194*** (-77.27%)
CAE 100 mg/kg	0.08125±0.01329 (-12.16%)	0.07875±0.02091 (-23.17%)	0.08125±0.02524 (-36.27%)	0.05250±0.02024** (-67.19%)	0.05750±0.01897** (-65.41%)	0.06000±0.02171** (-63.64%)
CAE 200 mg/kg	0.05500±0.01669 (-40.54%)	0.07125±0.02496 (-30.49%)	0.07750±0.02161 (-39.22%)	0.05625±0.01936** (-64.84%)	0.03625±0.01362*** (-78.20%)	0.02625±0.00844*** (-84.09%)

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

Analysis of the anti-inflammatory mechanism of action

Prostaglandin E_2-induced mouse paw edema

The mechanism of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE) in producing anti-inflammation was examined utilizing the PGE_2-induced paw edema test. Mice were orally administered 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or CAE (50 mg/kg) one hour before injection of PGE_2 solution 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 times 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 75.81% at 4 h. CAE at 50 mg/kg significantly (p<0.001) decreased paw edema at all times tested after PGE_2 administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 65.32% at 4 h (Table 3).

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Table 3 Changes in edema volume (mL) from 0.5 to 4 h after PGE_2 solution injection following oral administration of Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 50 mg/kg). N=8 for all groups. Data are shown as the mean ± S.E.M. Inhibition of paw edema is reported as percentage compared to 2% Tween 80.

Treatment (mg/kg)	Paw edema ± S.E.M. (% inhibition)					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
Tween 80	0.18250±0.00559	0.17375±0.00653	0.16500±0.00598	0.16375±0.00375	0.16000±0.00535	0.15500±0.00567
IND 10 mg/kg	0.07375±0.00324*** (-59.59%)	0.06625±0.00263*** (-61.87%)	0.05750±0.00526*** (-65.15%)	0.04500±0.00267*** (-72.52%)	0.04500±0.00378*** (-71.88%)	0.03750±0.00648*** (-75.81%)
CAE 50 mg/kg	0.09375±0.00420*** (-48.63%)	0.07875±0.00611*** (-54.68%)	0.06750±0.00313*** (-59.09%)	0.06750±0.00313*** (-58.78%)	0.05878±0.00398*** (-63.62%)	0.05375±0.00324*** (-65.32%)

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

Arachidonic acid-induced mouse paw edema

The mechanism of CAE in producing anti-inflammation was also examined utilizing the AA-induced paw edema test. Mice were orally administered DMSO (10 mL/kg), caffeic acid (100 mg/kg), 2% Tween 80 (10 mL/kg), IND (10 mg/kg), or various doses of CAE (12.5-200 mg/kg) one hour before injection of AA solution into the plantar surface of the left hind paw.

To demonstrate the AA-induced paw edema test, caffeic acid (a lipoxygenase inhibitor) was used as a positive control. As expected caffeic acid at 100 mg/kg significantly ($p<0.05$) inhibited paw edema at 1.5, 3 and 4 h after AA administration compared to DMSO and showed a maximum inhibition of paw edema of 77.27% at 4 h. IND (a cyclooxygenase inhibitor) was used as a negative control. As expected IND did not decrease paw edema at all times tested (0.5, 1, 1.5, 2, 3 and 4 h) after AA administration compared to 2% Tween 80. No significant effects of all doses of CAE on AA-induced mouse paw edema were observed at all times tested (Table 4).

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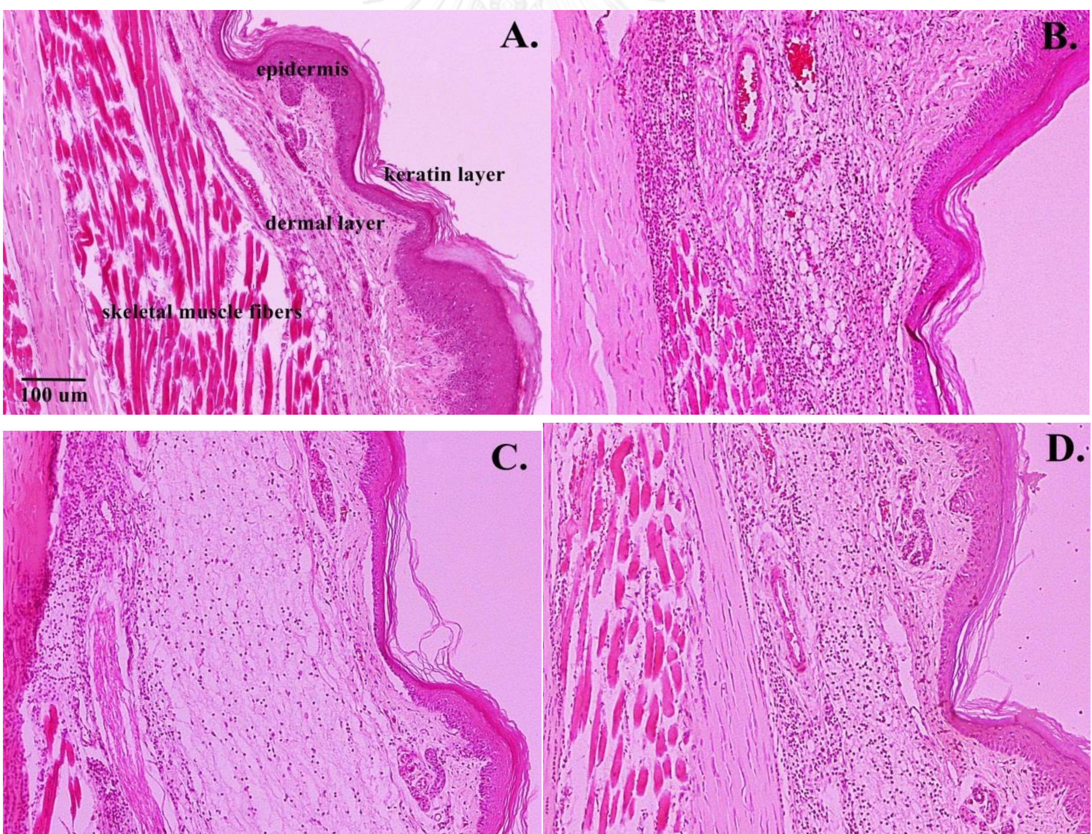
Table 4 Changes in edema volume (mL) from 0.5 to 4 h after arachidonic acid injection following administration of dimethyl sulfoxide (DMSO; 10 mL/kg, i.p.), caffeic acid (100 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), indomethacin (IND; 10 mg/kg, p.o.) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg, p.o.). N=8 for all groups. Data are shown as mean ± S.E.M. Inhibiton of paw edema is reported as percentage compared to their respective controls.

Treatment (mg/kg)	**Paw edema ± S.E.M. (% inhibition)**					
	0.5 h	**1 h**	**1.5 h**	**2 h**	**3 h**	**4 h**
DMSO	0.17000±0.03951	0.15125±0.02158	0.14625±0.01742	0.13000±0.01973	0.12375±0.01569	0.12250±0.01333
caffeic acid	0.10125±0.01517 (-43.75%)	0.08250±0.01333 (-51.82%)	0.06500±0.01350* (-63.12%)	0.07375±0.01295 (-54.62%)	0.05375±0.00706* (-66.41%)	0.03750±0.00977** (-77.27%)
Tween 80	0.18000±0.03822	0.17125±0.03125	0.17625±0.03746	0.16250±0.03379	0.16000±0.03571	0.16500±0.05137
IND 10	0.11375±0.01133 (-36.81%)	0.11125±0.01060 (-35.04%)	0.10500±0.01052 (-40.43%)	0.10625±0.00730 (-34.62%)	0.08750±0.01191 (-45.31%)	0.09250±0.01359 (-43.94%)
CAE 12.5	0.13250±0.01820 (-26.39%)	0.15250±0.01934 (-10.95%)	0.12250±0.01709 (-30.50%)	0.09625±0.02044 (-40.77%)	0.11750±.01623 (-26.56%)	0.08125±.01288 (-50.76%)
CAE 25	0.17875±0.02676 (-0.69%)	0.15375±0.03370 (-10.22%)	0.16375±0.03240 (-7.09%)	0.12250±0.03034 (-24.62%)	0.11625±0.03443 (-27.34%)	0.08250±0.02313 (-50.00%)
CAE 50	0.15625±0.02121 (-13.19%)	0.14125±0.01608 (-17.52%)	0.13875±0.01481 (-21.28%)	0.12000±0.01150 (-26.15%)	0.10250±0.01666 (-35.94%)	0.08875±0.02048 (-46.21%)
CAE 100	0.14250±0.01897 (-20.83%)	0.10375±0.02195 (-39.42%)	0.12875±0.01652 (-26.95%)	0.11250±0.01590 (-30.77%)	0.08875±0.02232 (-44.53%)	0.09125±0.01959 (-44.70%)
CAE 200	0.12625±0.01463 (-29.86%)	0.09875±0.01652 (-42.34%)	0.09000±0.01524 (-48.94%)	0.09625±0.02078 (-40.77%)	0.08500±0.02686 (-46.88%)	0.06500±0.01813 (-60.61%)

* $p<0.05$, ** $p<0.01$ significantly different compared to their respective controls.

Histological examination

Paw biopsies of carrageenan-treated mice showed massive infiltration of neutrophils in connective tissue. Pretreatment with IND and all doses of CAE (12.5-200 mg/kg) showed less neutrophil infiltration compared to 2% Tween 80 (Figure 24). IND significantly (p<0.001) decreased the number of neutrophils in paw biopsies as compared to 2% Tween 80. All doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) were showed significant reductions in the number of neutrophils (p<0.001, p<0.001, p<0.001, p<0.01, and p<0.001, respectively). The number of neutrophils of all doses of CAE was comparable to IND, with CAE at 50 mg/kg producing the lowest number of neutrophils in paw tissues (Figure 25).



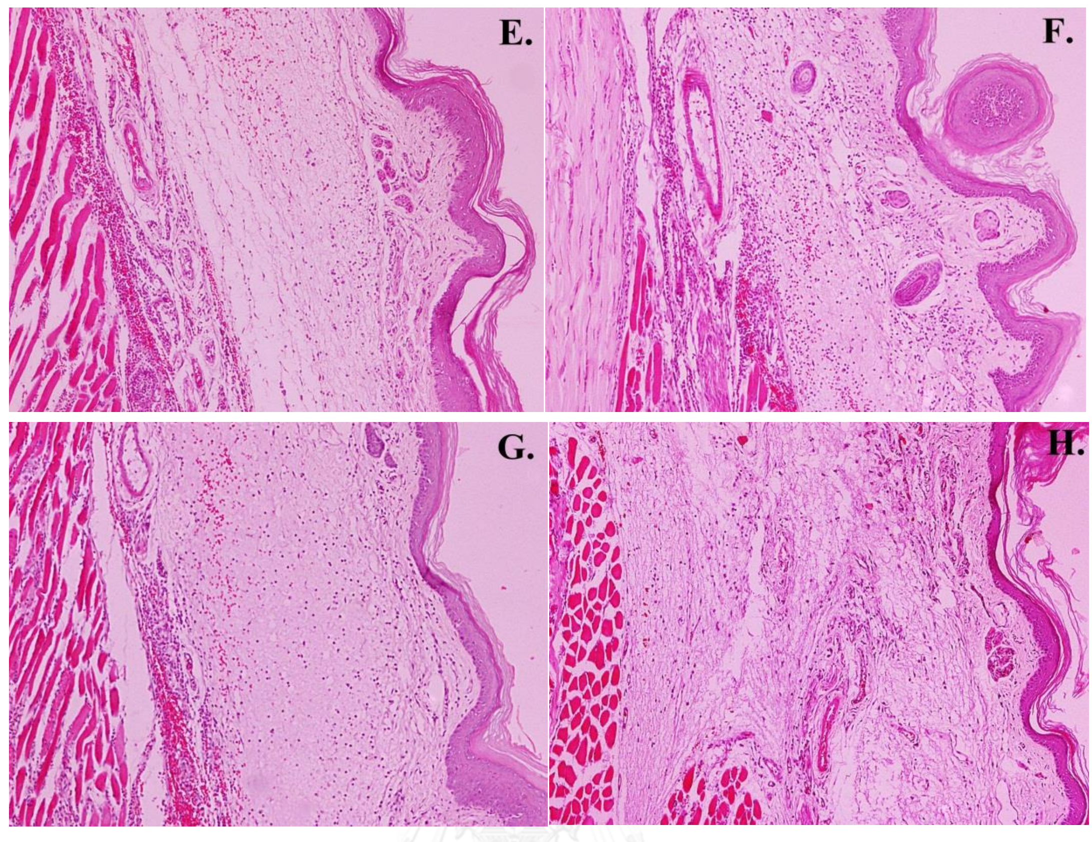


Figure 24 Representative light micrographs of H&E stained samples of mouse hind paw tissue. A: Histological structure of the epidermis and dermis of normal mouse hind paw with no obvious neutrophil infiltration, B: Carrageenan-treated mice with massive infiltration of neutrophils, C: Mice administered CAE 12.5 mg/kg before carrageenan injection, D: Mice administered CAE 25 mg/kg before carrageenan injection, E: Mice administered CAE 50 mg/kg before carrageenan injection, F: Mice administered CAE 100 mg/kg before carrageenan injection, G: Mice administered CAE 200 mg/kg before carrageenan injection, H: Mice administered IND 10 mg/kg before carrageenan injection. Scale bar = 100 µm.

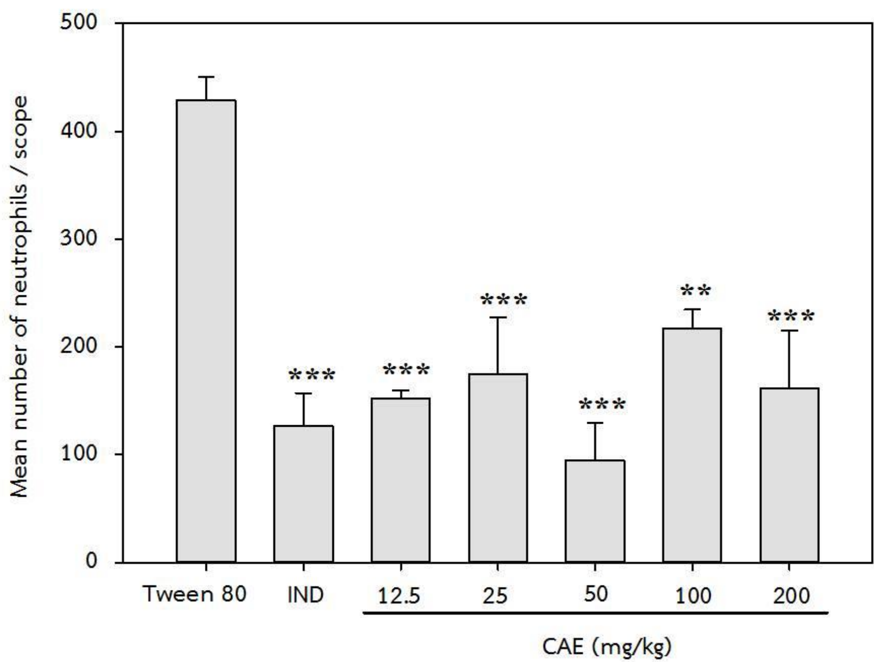


Figure 25 Number of neutrophils in each group (400×) after carrageenan injection following oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg). N=8 for all groups.

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

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Cotton pellet-induced granuloma formation in mice

Study then utilized the cotton pellet-induced granuloma formation in mouse to examine the chronic anti-inflammatory efficacy of CAE. Each mouse was orally administered 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) once daily for 7 consecutive days after cotton pellet implantation. Only CAE at 200 mg/kg significantly ($p<0.05$) inhibited cotton pellet-induced granulomatous tissue formation, compared to 2% Tween 80. The inhibitory action of CAE at 200 mg/kg was comparable to that of the standard anti-inflammatory agent, IND (Table 4).

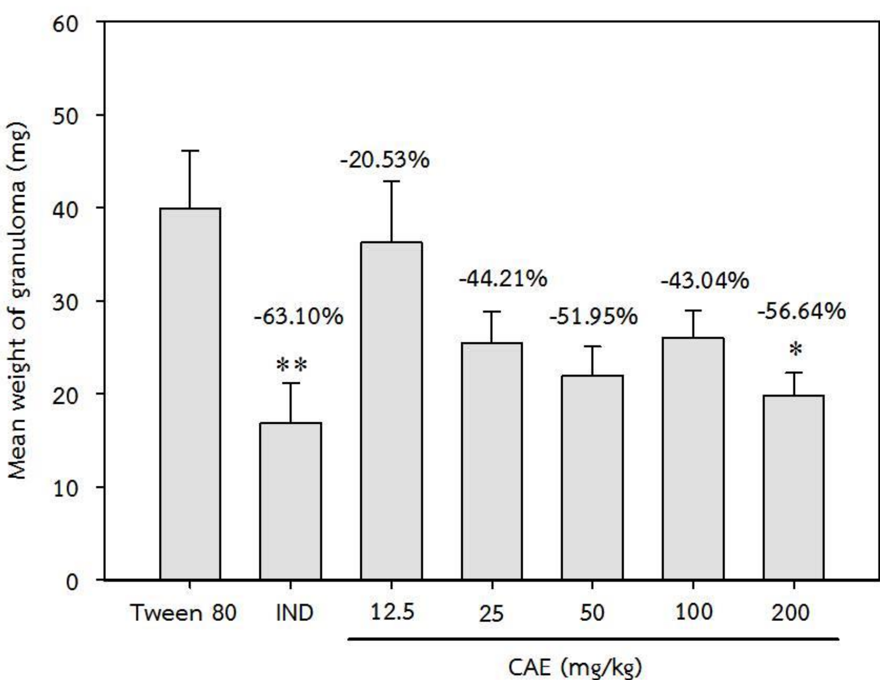

Figure 26 Effect of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg, p.o.) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg, p.o.), on the weight of granuloma in mice. N=7-8 for all groups.

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

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Table 5 Effects of oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg), and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg, p.o.) (for 7 consecutive days) on the weight of granuloma in mouse. N=7-8 for all groups. Each value represents the mean ± S.E.M.

Treatment	Dose (mg/kg)	Weight of granuloma (mg)
2% Tween 80	10	45.68857±6.16886
IND	10	16.86000±4.29426**
CAE	12.5	36.30750±6.58974
	25	25.48750±3.33902
	50	21.95375±3.12354
	100	26.02625±2.96204
	200	19.81250±2.45166*

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

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Safety evaluation

In order to determine the effect of CAE on motor response, mice were orally administered 2% Tween 80 (10 mL/kg), CAE (200 mg/kg) and tested on the rota-rod apparatus for 5 subsequent trials at 30, 60, 90, 120 and 240 min after oral administration. The results demonstrated that the highest dose of CAE (200 mg/kg) did not affect the motor response of the animals at all times tested (Figure 28).

An acute toxicity study performed to evaluate the safety of CAE resulted in no morbidity or mortality with oral administration of all doses of CAE (12.5-200 mg/kg) over an observation period of 72 h.

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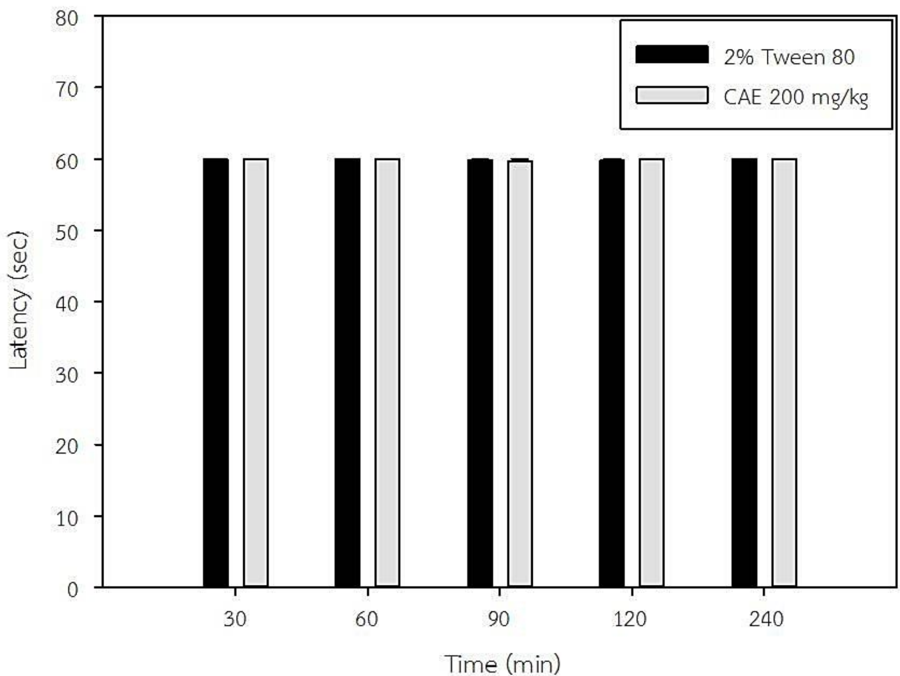

Figure 27 Rota-rod performance at 30, 60, 90, 120 and 240 minutes after oral administration of 2% Tween 80 (10 mL/kg) and the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 200 mg/kg). N = 8 for all groups.

$^{*}p<0.05$ significantly different compared to 2% Tween 80.

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CHAPTER V

DISCUSSION AND CONCLUSION

In this study, the antinociceptive and anti-inflammatory effects of CAE were investigated in several tests and animal models. The antinociceptive effects of CAE were studied using three laboratory models, that allow assessment of behavioral responses to thermal and chemically-induced pain stimuli (Le Bars et al., 2001). The hot-plate test was established for evaluation of centrally-acting analgesic drugs (Woolfe & MacDonald, 1944). This test measures complex responses to thermal stimuli induced hyperalgesia specific to centrally-mediated nociception (Khan et al., 2011). Behavioral responses including jumping or hind paw licking are considered to be supraspinal sensory integration responses and are thought to involve opioids (Le Bars et al., 2001; Woolfe & MacDonald, 1944). MO is widely used as a reference drug in this test, and we found that MO had potent analgesic activity in the hot-plate test. CAE at doses of 100 and 200 mg/kg had significant effects on hot-plate latencies compared to controls, with the highest antinociceptive effect at 200 mg/kg, indicating that CAE has a central analgesic effect. The antinociceptive action of CAE was inhibited by the specific opioid receptor antagonist, naloxone. These results suggest that CAE has opioid-like activity.

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The acetic acid-induced writhing test is usually selected to evaluate the peripheral antinociceptive effects of drugs or natural compounds (Nakamura et al., 1983). This method is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) (Sanchez-Mateo, Bonkanka, Hernandez-Perez, & Rabanal, 2006). Intraperitoneal injection of acetic acid produces a painful reaction and acute inflammation, which is considered to be visceral pain model (Nakamura et al., 1983; Sanchez-Mateo et al., 2006). Prostaglandin biosynthesis plays an important role in the nociceptive mechanism (Deraedt et al., 1980). Acetic acid is thought to increase levels of

lipoxygenase (LOX) and cyclooxygenase (COX) products (especially PGE_2) in peritoneal fluids (Ikeda, Ueno, Naraba, & Oh-ishi, 2001), and to liberate endogenous mediators such as histamine, serotonin, bradykinin and cytokines, which stimulates peripheral chemosensitive nociceptive receptors and manifests as abdominal constrictions that are accompanied by pelvic rotation and subsequent stretching of at least one hind limb (Deraedt et al., 1980; Sanchez-Mateo et al., 2006). IND, a NSAIDs, produced a significant decrease in the writhing response through inhibition of PG synthesis, resulting in peripheral analgesia. CAE at doses of 12.5-200 mg/kg showed significant activity against pain induced by acetic acid, compared to the control groups. Thus, these data indicated that CAE has an antinociceptive effect on inflammatory pain. This activity of CAE may be related to blockage of the PGE_2 effect or reduction of the release of PGE_2 that excites nociceptors, resulting in a peripheral analgesic action. Our results are consistent with the study of Mujumdar et al. (2004), in which a significant reduction in acetic acid-induced writhing was produced by the ethanolic extract of *Curcuma amada* Roxb. rhizome at doses of 50 and 100 mg/kg in mice. However, due to the lack of specificity of the writhing test, several other classes of drugs, including adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics, can also inhibit writhing (Le Bars et al., 2001). Therefore, other models are required for confirmation of the positive results in the writhing test. The formalin test was used for this purpose in the current study.

The formalin test is a valid and reliable model in pain research. Formalin causes a behavioral syndrome and most widely applicable to the study of acute and tonic pain states and the underlying physiology (Wheeler-Aceto & Cowan, 1991). Subcutaneous injections of formalin produced a distinct biphasic nociceptive response of intensive licking and biting activities. The first phase (neurogenic pain: 0-5 min after injection) is due to direct chemical stimulation of nociceptors. Nociceptive

mediators including substance P, bradykinin and glutamate are thought to participate in this phase (Hunskaar & Hole, 1987). The second phase (inflammatory pain: 20-30 min after injection) is thought to be an inflammatory response and several inflammatory mediators including histamine, serotonin, prostaglandins, NO and bradykinin are involved in this phase. Centrally-acting drugs such as MO and codeine inhibit both phases equally, whereas peripherally-acting drugs such as IND and dexamethasone inhibit the second phase selectively (Hunskaar & Hole, 1987; Shibata et al., 1989). Therefore, this test was used to determine the mechanism of action and mediators involved in the antinociceptive activity of the extract. In the current study, MO and IND were used as reference drugs. MO, a centrally-acting drug, produced a significant analgesic effect in the early and late phases, while IND, a peripherally-acting drug, suppressed nociceptive responses only in the late phase. CAE at doses of 12.5-200 mg/kg significantly reduced the time spent for paw licking in both phases, indicating a centrally acting mechanism. Thus, this analgesic activity of CAE may be due to inhibition of production and/or action of pain mediators. These results from the formalin test were in good agreement with those obtained from the hot-plate and writhing tests, thereby indicating that CAE exerts antinociceptive activity both centrally and peripherally.

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To determine the effect of CAE on motor response, a rota-rod test was performed. This test is designed to evaluate the motor coordination, skeletal muscle relaxation, convulsions and depression of the CNS produced by a test substance (Dunham & Miya, 1957). The result from the most effective dose of CAE (200 mg/kg) indicated no detectable relaxant or sedative effects. Therefore, the behavioral responses observed in the hot-plate, writhing and formalin tests were not due to motor impairment, but rather reflected a true antinociceptive effect.

To assess potential anti-inflammatory action, the effects of CAE on the acute and chronic phases of inflammation were evaluated in mouse models of

carrageenan-induced paw edema, arachidonic acid (AA)-induced paw edema and cotton pellet-induced granuloma formation. The carrageenan-induced rat paw edema test is a well-established animal model of inflammation for evaluating the acute anti-inflammatory effect of natural products (Winter et al., 1962). Carrageenan-induced mouse paw edema test was later established by Levy (1969). This test is highly sensitive to NSAIDs, and has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs (Just et al., 1998). The effect produced by carrageenan has been described as a biphasic event with involvement of multiple inflammatory mediators. The initial phase (0-2 h after injection of carrageenan) results from rapid production of pro-inflammatory agents such as histamine, serotonin and bradykinin (Rosa, 1972). The second phase of swelling (3-6 h after injection of carrageenan) is associated with release of prostaglandins and nitric oxide, produced by inducible isoforms of COX-2 and nitric oxide synthase (iNOS), respectively (Seibert et al., 1994). More recently, the second phase has also been attributed to involvement of free radicals, nitric oxide and COXs in hind paw exudates (Iwata et al., 2010). TNF-α is a mediator of carrageenan-induced inflammatory incapacitation, and is able to induce further release of kinins and leukotrienes, which play an important role in the maintenance of a long lasting nociceptive response (Kumar, Murugananthan, Nandakumar, & Talwar, 2011).

The infiltration of polymorphonuclear (PMN) cells into site of inflammation induces secretion of several pro-inflammatory mediators such as NO, PGs and cytokines (Di Rosa, Giroud, & Willoughby, 1971; Gilligan, Lovato, Erion, & Jeng, 1994). The carrageenan-induced inflammatory response has been linked to neutrophil infiltration and production of neutrophil-derived free radicals such as superoxide, hydrogen peroxide and hydroxyl radicals, as well as release of other neutrophil-derived mediators (Dawson, Sedgwick, Edwards, & Lees, 1990). Free radicals can induce biological damage and pathological events including aging, inflammation, and

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carcinogenesis (Halliwell, 2007). The inflammatory effect induced by carrageenan is associated with release of free radicals, PG, and NO for 1-6 h after administration of carrageenan. The edema effect was reaches a maximum in the third hour (Dudhgaonkar, Tandan, Bhat, Jadhav, & Kumar, 2006; Rosa & Willoughby, 1971).

IND is a COX inhibitor and contributes to the reduction of PG synthesis. This study, IND used as a positive control, significantly reduced paw edema from 4-6 h (second phase) after carrageenan administration. The results are consistent with the previous report which showed that IND caused strong inhibition of the second phase without affecting the development of the first phase (Vinegar, 1969). CAE at all doses tested (12.5-200 mg/kg) significantly decreased mouse paw edema at 4, 5, and 6 h after carrageenan injection. These results indicate that CAE exerted anti-inflammatory action during the second phase of inflammation. The mechanism underlying this activity of CAE may be mediated through inhibition of PG synthesis by COX enzyme. Previous study has shown that the ethanol extract of *Curcuma amada* Roxb. rhizome at 200 mg/kg showed significant anti-inflammatory activity in carrageenan-induced rat paw edema at 1-4 h, and this activity was dose-dependent (Mujumdar, Naik, Dandge, & Puntambekar, 2000).

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The effect of CAE on PGE_2-induced mouse paw edema was examined to determine if mechanisms other than COX inhibition were present. CAE (50 mg/kg) significantly decreased paw edema induced by PGE_2 from 0.5-4 h after PGE_2 injection, indicating that the effect of CAE was due to degradation and/or inactivation of PGE_2. The edematogenic response of PGE_2 in mouse paws is mediated by E-prostanoid $(EP)_3$ receptor activation, which involves stimulation of the protein kinase C (PKC), phospholipase C (PLC), mitogen protein-activated kinases (MAPKs) pathways, and participation of neurokinin (NK1) and vanilloid receptor (TRPV1) receptors; and is largely prevented by a selective EP_3 receptor antagonist (Claudino, Kassuya, Ferreira, & Calixto, 2006). The reduction of PGE_2-induced paw edema could explain, at least in

part, the inhibitory effects of CAE in the writhing and formalin tests. Our results in this model complement those obtained from other models involving inflammatory mediators, including the acetic acid-induced writhing and formalin licking responses.

Numerous products of the arachidonic acid (AA) cascade including the PGs, hydroxyeicosatetraenoic acids (HETEs) and LTs possess inflammatory actions. Furthermore, they have been implicated in inflammatory processes in a variety of tissues including the skin and joints. For example, PGs (PGE_2 and PGI_2) and LTs (LTC_4 and LTD_4) interact to produce edema (plasma exudation) during the inflammatory response (Ford-Hutchinson, 1985; Ford-Hutchinson & Rackman, 1983; Goetzl, Wong, & Matthay, 1986; Williams, 1979; Williams, 1983). LTB_4 promotes inflammation by induction of leukocyte accumulation, activation of leukocytes and modulation of pain through hyperalgesia (Bray, 1983; Ford-Hutchinson, 1985; Levine, Lau, Kwiat, & Goetzl, 1984). In addition, PGE_2 and PGI_2 potentiate the vascular permeability changes produced by histamine and bradykinin and these prostanoids also synergize with the complement derived peptide, C5a, and enhance the increased capillary permeability produced by LTB_4 (Goetzl et al., 1986; Williams, 1979, Williams, 1983).

The inflammatory response of AA-induced ear edema is due to formation of AA metabolites via both the cyclooxygenase and lipoxygenase pathways. AA products are capable of producing the characteristic signs of inflammation including vasodilation, hyperemia, pain, edema, and cellular infiltration (Bray, Ford-Hutchinson, & Smith, 1981; Greaves & McDonald-Gibson, 1973; Issekutz & Movat, 1982; Lewis & Austen, 1981; Peck, Piper, & Williams, 1981; Williams, 1979). AA-induced ear edema results from the rapid production of PGE_2 and LTC_4 and LTD_4 (Arner, Rule, Rudnick, & Galbraith, 1985; Chang, Oneilldavis, Lamb, Carlson, & Lewis, 1985; Humes, Opas & Bonney, 1989). These mediators are capable of promoting vasodilation and increasing vascular permeability and may act synergistically to produce edema. Very little LTB_4 is present in ears treated with AA (Humes et al., 1989). It seems that the AA

metabolites are probably produced by resident skin cell populations such as keratinocytes and vascular endothelial cells (Carlson, Chang, & Lewis, 1985). It appeared that products of the cyclooxygenase pathway, particularly PGE_2, contributed to increased blood flow through a vasodilation, but products of lipoxygenase pathway were necessary for vascular leakage and edema (Wedmore & Williams, 1981). It appears that small amounts of AA are preferentially metabolized by the cyclooxygenase pathway. Generation of chemoattractant lipoxygenase metabolites occurs only when the cyclooxygenase is overwhelmed by large amounts of AA (Young et al., 1984). AA-induced ear inflammation in mice has been reported to be sensitive for detecting the anti-inflammatory activity of lipoxygenase inhibitors (Carlson, 1985; Young, 1984). AA-induced rat paw edema was used for evaluating the *in vivo* anti-inflammatory activity of lipoxygenase inhibitor and other agents with a mechanism of action different than cyclooxygenase inhibition (Di Martino et al., 1987).

The AA-induced mouse paw edema model (Calhoun, 1987) was also used to assess the acute anti-inflammatory activity of CAE. This model is highly sensitive to inhibition of the LOX pathway and is resistant to COX inhibitors. The involvement of LOX products and mast cell mediators in the edematous response to AA render this model potentially useful for studying anti-inflammatory agents with a mechanism of action differs from that of COX inhibitors (Di Martino, Campbell, Wolff, & Hanna, 1987). As expected, IND, a COX inhibitor (negative control), could not inhibit paw edema induced by AA in this study. In contrast, caffeic acid, a LOX inhibitor (positive control), showed a LOX inhibitory property by exhibiting an anti-inflammatory effect on AA-induced paw edema as shown in the study of (Sud'Ina et al., 1993). All doses of CAE tested (12.5-200 mg/kg) were found to be ineffective in this model. CAE exerted anti-inflammatory effect through inhibition of COX pathway rather than LOX

pathway. The proposed mechanism of anti-inflammatory action of CAE is similar to that of NSAIDs which involves a decrease in PG production.

Histopathological evaluation of mouse paw tissues showed that CAE at all doses tested (12.5-200 mg/kg) significantly suppressed massive neutrophil infiltration found in control animals following carrageenan injection. CAE exhibited an anti-inflammatory effect in the late phase of carrageenan-induced paw edema by inhibition of infiltration of PMN cells and production of neutrophil-derived free radicals.

The cotton pellet induced-granuloma formation test is a common method used to investigate the transductive, exudative and proliferative components of chronic inflammation. The dried pellet weights correlates well with the amount of granulomatous tissue formation (Swingle & Shideman, 1972). The subcutaneous implantation of a cotton pellet directly triggers an acute inflammation, but is insufficient to eliminate pro-inflammatory mediators, leading to a chronic inflammatory reaction that includes infiltration of neutrophils and mononuclear cells (Bailey, Sturm, & Lopez-Ramos, 1982). Only the highest dose of CAE (200 mg/kg) effectively inhibited cotton pellet-induced granuloma formation, which indicates that CAE has anti-inflammatory activity in the chronic phase of inflammation. The study of (Mujumdar et al., 2000) showed that the ethanol extract of *Curcuma amada* Roxb. rhizome at doses of 40 and 80 mg/kg reductions in granular tissue formation dose-dependently in a cotton pellet induced-granuloma model in rat.

Curcuminoids is a significant bioactive component of several species of plants in genus Curcuma. The ratios of bisdemethoxycurcumin, demethoxycurcumin and curcumin in naturally occurring curcuminoids are about 5%, 15%, and 80%, respectively. The anti-inflammatory property of curcumin is mediated through its effects on cytokines, lipid mediators, eicosanoids, and proteolytic enzymes (Joe, 2004). Curcumin scavenges the superoxide radical, hydrogen peroxide, and NO (Alia,

2006). Previous studies demonstrated that curcumin produced a significant analgesic response in the hot-plate and acetic acid-induced writhing tests in mice and showed ant-inflammatory activity in the second phase of carrageenan-induced paw edema in rats but these effects were not dose dependent (Buadonpri et al., 2008). CAE in this study also showed antinociceptive and anti-inflammatory effects that were not dose-dependent similar to curcumin. Therefore, it is possible that curcuminoids may be responsible for the analgesic and anti-inflammatory activities of CAE.

In conclusion, this study shows that CAE had antinociceptive properties in central and peripheral models of nociception in mice, and showed anti-inflammatory properties in acute and chronic inflammation models. Further studies are required to understand mechanisms underlying these effects. Separation and isolation of active compounds in CAE are also needed. However, the results clarify the pharmacological actions of CAE and support its ethnopharmacological use and suggest the potential use of this extract for treatment of pain and inflammatory disorders.

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The future research could comprise of several objectives as listed below:

(1) To better understand the mechanism of CAE that involved in producing analgesic and anti-inflammatory effects.

(2) To better characterize the mechanism of analgesic effects of CAE using other opioid antagonists.

(3) To investigate the potential use of CAE in combination with other analgesics or anti-inflammatory drugs.

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

(5) To elucidate side effects and toxic effects of CAE at high dosage or after chronic use.

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

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REFERENCES

Adams, D. (1976). The granulomatous inflammatory response. A review. *The American journal of pathology, 84*, 164.

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

Ali, B. H., Marrif, H., Noureldayem, S. A., & Bakheit, A. O. (2006). Some biological properties of curcumin: a review. *Natural Product Communications, 1*, 509-521.

Andrade, S., Cardoso, L., Carvalho, J., & Bastos, J. (2007). Anti-inflammatory and antinociceptive activities of extract, fractions and populnoic acid from bark wood of Austroplenckia populnea. *Journal of ethnopharmacology, 109*, 464-471.

Arner, E., Rule, B., Rudnick, M., & Galbraith, W. (1985). Arachidonic acid-induced ear edema-correlation of swelling with production of lipoxygenase (LO) products. *Agents and Actions, 16*, 600-601.

Backonja, M. M. (2003). Defining neuropathic pain. *Anesthesia & Analgesia, 97*, 785-790.

Bailey, P. J., Sturm, A., & Lopez-Ramos, B. (1982). A biochemical study of the cotton pellet granuloma in the rat: Effects of dexamethasone and indomethacin. *Biochemical pharmacology, 31*, 1213-1218.

Barber, D. (1997). The physiology and pharmacology of pain: A review of opioids. *Journal of PeriAnesthesia Nursing, 12*, 95-99.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell, 139*, 267-284.

Beydoun, A., & Backonja, M. M. (2003). Mechanistic stratification of antineuralgic agents. *Journal of pain and symptom management, 25*, 18-30.

Botting, R. M. (2000). Mechanism of action of acetaminophen: is there a cyclooxygenase 3. *Clinical Infectious Diseases, 31*, 202-210.

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

Bray, M. A. (1983). The pharmacology and pathophysiology of leukotriene B_4. *British medical bulletin, 39*, 249-254.

Bray, M., Ford-Hutchinson, A., & Smith, M. (1981). Leukotriene B_4: An inflammatory mediator In *vivo*. *Prostaglandins, 22*, 213-222.

Buchman, A. L. (2001). Side effects of corticosteroid therapy. *Journal of clinical gastroenterology, 33*, 289-294.

Calhoun, W., Chang, J., & Carlson, R. P. (1987). Effect of selected antiinflammatory agents and other drugs on zymosan, arachidonic acid, PAF and carrageenan induced paw edema in the mouse. *Agents and actions, 21*, 306-309.

Calixto, J. B., Beirith, A., Ferreira, J., Santos, A. R., Filho, V. C., & Yunes, R. A. (2000). Naturally occurring antinociceptive substances from plants. *Phytotherapy research, 14*, 401-418.

Carlson, R. P., Chang, J., & Lewis, A. J. (1985). Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents. *Agents and Actions, 17*, 197-204.

Carroll, M. C. (2004). The complement system in regulation of adaptive immunity. *Nature immunology, 5*, 981-986.

Chang, J., Oneilldavis, L., Lamb, B., Carlson, R., & Lewis, A. (1985). *The utility of arachidonic acid-treated mouse ear inflammatory model as a potential screen for lipoxygenase (LO) inhibitors. Agents and Actions, 16*, 600.

Chang, S. T., Wu, J. H., Wang, S. Y., Kang, P. L., Yang, N. S., & Shyur, L. F. (2001). Antioxidant activity of extracts from Acacia confusa bark and heartwood. *Journal of Agricultural and Food Chemistry, 49*, 3420-3424.

Chen, L., Yang, G., & Grosser, T. (2013). Prostanoids and inflammatory pain. *Prostaglandins & other lipid mediators, 104*, 58-66.

Cichewicz, D. L. (2004). Synergistic interactions between cannabinoid and opioid analgesics. *Life sciences, 74*, 1317-1324.

Çıkrıkçı, S., Mozioglu, E., & Yılmaz, H. (2008). Biological activity of curcuminoids isolated from *Curcuma longa*. *Rec Nat Prod, 2*, 19-24.

Claudino, R. F., Kassuya, C. A., Ferreira, J., & Calixto, J. B. (2006). Pharmacological and molecular characterization of the mechanisms involved in prostaglandin E_2-induced mouse paw edema. *Journal of Pharmacology and Experimental Therapeutics, 318*, 611-618.

Crunkhorn, P., & Meacock, S. (1971). Mediators of the inflammation induced in the rat paw by carrageenin. *British Journal of Pharmacology, 42*, 392-402.

Dawson, J., Sedgwick, A., Edwards, J., & Lees, P. (1990). A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *International journal of tissue reactions, 13*, 171-185.

DeLeo, J. A. (2006). Basic science of pain. *The Journal of Bone & Joint Surgery, 88*, 58-62.

Deraedt, R., Jouquey, S., Delevallée, F., & Flahaut, M. (1980). Release of prostaglandins E and F in an algogenic reaction and its inhibition. *European journal of pharmacology, 61*, 17-24.

Di Rosa, M., Giroud, J., & Willoughby, D. (1971). Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *The Journal of pathology, 104*, 15-29.

Di Martino, M., Campbell Jr, G., Wolff, C., & Hanna, N. (1987). The pharmacology of arachidonic acid-induced rat paw edema. *Agents and Actions, 21*, 303-305.

Dray, A. (1995). Inflammatory mediators of pain. *British Journal of Anaesthesia, 75*, 125-131.

Dray, A., Urban, L., & Dickenson, A. (1994). Pharmacology of chronic pain. *Trends in pharmacological sciences, 15*, 190-197.

Dubin, A. E., & Patapoutian, A. (2010). Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation, 120*, 3760-3772.

Dubuisson, D., & Dennis, S. G. (1978). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain, 4*, 161-174.

Dudhgaonkar, S. P., Tandan, S. K., Bhat, A. S., Jadhav, S. H., & Kumar, D. (2006). Synergistic anti-inflammatory interaction between meloxicam and

aminoguanidine hydrochloride in carrageenan-induced acute inflammation in rats. *Life sciences, 78*, 1044-1048.

Dunham, N., & Miya, T. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *Journal of the American Pharmaceutical Association, 46*, 208-209.

Dworlzin, R. H., Allen, R., Saltorelli, M., Kenneth, E., Sthmader, M., Stein, C., Wallace, M. (2003). Advances in Neuropathic Pain. *Arch Neurol, 60*, 1524-1534.

Fanning, L. B., & Boyce, J. A. (2013). Lipid mediators and allergic diseases. *Annals of Allergy, Asthma & Immunology, 111*, 155-162.

Ford-Hutchinson, A. (1985). Leukotrienes: their formation and role as inflammatory mediators. *Federation proceedings, 44*, 25-29.

Ford-Hutchinson, A., & Rackman, A. (1983). Leukotrienes as mediators of skin inflammation. *British Journal of Dermatology, 109*, 26-29.

Frost, E. A. (2014). Comprehensive Treatment of Chronic Pain by Medical, Interventional, and Integrative Approaches. *Journal of Neurosurgical Anesthesiology, 26*, 81-82.

Gilligan, J. P., Lovato, S. J., Erion, M. D., & Jeng, A. Y. (1994). Modulation of carrageenan-induced hind paw edema by substance P. *Inflammation, 18*, 285-292.

Giustizieri, M. L., Albanesi, C., Fluhr, J., Gisondi, P., Norgauer, J., & Girolomoni, G. (2004). H1 histamine receptor mediates inflammatory responses in human keratinocytes. *Journal of allergy and clinical immunology, 114*, 1176-1182.

Goetzl, E., Wong, M., & Matthay, M. (1986). Leukotrienes in human hypersensitivity and inflammatory diseases. *Advances in Inflammation Research, 1*, 1.

Greaves, M. W., & McDonald-Gibson, W. (1973). Itch: role of prostaglandins. *British medical journal, 3*, 608-609.

Green, R., & Harris, N. (1993). Pathology and therapeutics for Pharmacists: Chapman & Hall, London.

Griffin, R. S., & Woolf, C. J. (2008). Pharmacology of analgesia. *Principles of Pharmacology. The Pathophysiologic Basis of Drug Therapy*, 240-241.

Gürsoy, M., Haznedaroğlu, I. C., Çelik, I., Sayinalp, N., Özcebe, O. I., Dündar, S. V., & Guévremont, C. (1996). Agranulocytosis, plasmacytosis, and thrombocytosis followed by a leukemoid reaction due to acute acetaminophen toxicity. *Annals of Pharmacotherapy, 30*, 762-765.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward. *Biochem journal, 401*, 1-11.

Hart, F. D., & Huskisson, E. (1984). Non-steroidal anti-inflammatory drugs. *Drugs, 27*, 232-255.

Harvey, A. L. (2008). Natural products in drug discovery. *Drug discovery today, 13*, 894-901.

Huang, M. H., Huang, S. S., Wang, B. S., Wu, C. H., Sheu, M. J., Hou, W. C., Huang, G. J. (2011). Antioxidant and anti-inflammatory properties of Cardiospermum halicacabum and its reference compounds *ex vivo* and *in vivo*. *Journal of ethnopharmacology, 133*, 743-750.

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

Ialenti, A., Ianaro, A., Moncada, S., & Di Rosa, M. (1992). Modulation of acute inflammation by endogenous nitric oxide. *European journal of pharmacology, 211*, 177-182.

Ikeda, Y., Ueno, A., Naraba, H., & Oh-ishi, S. (2001). Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. *Life sciences, 69*, 2911-2919

Issekutz, A., & Movat, H. (1982). The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. *The American journal of pathology, 107*, 300.

Joe, B., Vijaykumar, M., and Lokesh, B. R. (2004). Biological properties of curcumin-cellular and molecular mechanism of action. C*ritical Revues of Food and Science Nutrition, 44*, 97-111.

Joshi, S. K., & Honore, P. (2006). Animal models of pain for drug discovery.

Just, M. J., Recio, M. C., Giner, R. M., Cuéllar, M. J., Máñez, S., Bilia, A. R., & Ríos, J. L. (1998). Anti-Inflammatory Activity of Unusual Lupane Saponins. *Planta medica, 64*, 404-407.

Kale, M., Misar, A., Dave, V., Joshi, M., & Mujumdar, A. (2007). Anti-inflammatory activity of *Dalbergia lanceolaria* bark ethanol extract in mice and rats. *Journal of ethnopharmacology, 112*, 300-304.

Kanathum, N. (2008). Medicinal and Lucky Plant of Thailand. *Banlaesuan, Bangkok, Thailand, 258*.

Khan, H., Saeed, M., Khan, M. A., Khan, I., & Ashraf, N. (2011). Antinociceptive activity of aerial parts of Polygonatum verticillatum: attenuation of both peripheral and central pain mediators. *Phytotherapy research, 25*, 1024-1030.

Koster, R., Anderson, M., De Beer, E. J., 1959. Acetic acid for analgesic screening. *Federation Proceedings, 18*, 412-418.

Kumar, R., Murugananthan, G., Nandakumar, K., & Talwar, S. (2011). Isolation of anxiolytic principle from ethanolic root extract of *Cardiospermum halicacabum*. *Phytomedicine, 18*, 219-223.

Kumar, V., Abbas, A. K., & Fausto, N. (2005). Robbins and Cotran. *Pathologic basis of disease, 7*.

Lanas, A., García-Rodríguez, L. A., Arroyo, M. T., Gomollón, F., Feu, F., González-Pérez, A., & Santolaria, S. (2006). Risk of upper gastrointestinal ulcer bleeding associated with selective cyclooxygenase-2 inhibitors, traditional non-aspirin non-steroidal anti-inflammatory drugs, aspirin and combinations. *Gut, 55*, 1731-1738.

Larsen, G. L., & Henson, P. M. (1983). Mediators of inflammation. *Annual review of immunology, 1*, 335-359.

Lawrence, T., Willoughby, D. A., & Gilroy, D. W. (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nature Reviews Immunology, 2*, 787-795.

Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacological reviews, 53*, 597-652.

Levine, J. D., Lau, W., Kwiat, G., & Goetzl, E. J. (1984). Leukotriene B_4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. *Science, 225*, 743-745.

Levy, L. (1969). carrageenan induced paw edema in the mouse. *Life Sciences 8*, 801-808.

Lewis, R. A., & Austen, K. F. (1981). Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. *Nature, 293*, 103-108.

Loeser, J. D., & Melzack, R. (1999). Pain: an overview. *The Lancet, 353*, 1607-1609.

Main, C. J., & Spanswick, C. (2000). Pain management. *An interdisciplinary Approach. Churchill Livingstone, Edinburgh*.

Meier, R., Schuler, W., & Desaulles, P. (1950). Zur frage des mechanismus der hemmung des bindegewebswachstums durch cortisone. *Experientia, 6*, 469-471.

Merskey, H., & Bogduk, N. (1994). Classification of chronic pain, IASP Task Force on Taxonomy. *Seattle, WA: International Association for the Study of Pain.*

Morris, C. J. (2003). Carrageenan-induced paw edema in the rat and mouse. *Methods in Molecular Biology, 225*, 115-121.

Morrison, D. C., & Ryan, J. L. (1987). Mechanisms. *Annual review of medicine, 38*, 417-432.

Mujumdar, A., Naik, D., Dandge, C., & Puntambekar, H. (2000). Antiinflammatory activity of *Curcuma amada* Roxb. in albino rats. *Indian journal of Pharmacology, 32*, 375-377.

Nakamura, H., Imazu, C., Ishii, K., Yokoyama, Y., Kadokawa, T., & Shimizu, M. (1983). Site of analgesic action of zomepirac sodium, a potent non-narcotic analgesic in experimental animals. *Japanese journal of pharmacology, 33*, 875-883.

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

Needleman, P., Jakschik, B., Morrison, A., & Lefkowith, J. (1986). Arachidonic acid metabolism. *Annual review of biochemistry, 55*, 69-102.

Oka, M., Tachibana, M., Noda, K., Inoue, N., Tanaka, M., & Kuwabara, K. (2007). Relevance of anti-reactive oxygen species activity to anti-inflammatory activity

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of components of Eviprostat, a phytotherapeutic agent for benign prostatic hyperplasia. *Phytomedicine, 14*, 465-472.

Parrillo, J. E., & Fauci, A. S. (1979). Mechanisms of glucocorticoid action on immune processes. *Annual review of pharmacology and toxicology, 19*, 179-201.

Pasero, C. (2004). Pathophysiology of neuropathic pain. *Pain Management Nursing, 5*, 3-8.

Patel, L., Wales, J., Kibirige, M., Massarano, A., Couriel, J., & Clayton, P. (2001). Symptomatic adrenal insufficiency during inhaled corticosteroid treatment. *Archives of disease in childhood, 85*, 330-334.

Pearl, J., Stander, H., & McKean, D. B. (1969). Effects of analgesics and other drugs on mice in phenylquinone and rotarod tests. *Journal of Pharmacology and Experimental Therapeutics, 167*, 9-13.

Peck, M., Piper, P., & Williams, T. (1981). The effect of leukotrienes C_4 and D on the microvasculature of guinea-pig skin. *Prostaglandins, 21*, 315-321.

Policegoudra, R., Aradhya, S., & Singh, L. (2011). Mango ginger (*Curcuma amada* Roxb.): A promising spice for phytochemicals and biological activities. *Journal of biosciences, 36*, 739-748.

Policegoudra, R., Rehna, K., Rao, L. J., & Aradhya, S. (2010). Antimicrobial, antioxidant, cytotoxicity and platelet aggregation inhibitory activity of a novel molecule isolated and characterized from mango ginger (*Curcuma amada* Roxb.) rhizome. *Journal of biosciences, 35*, 231-240.

Porreca, F., & Ossipov, M. H. (2009). Nausea and vomiting side effects with opioid analgesics during treatment of chronic pain: mechanisms, implications, and management options. *Pain Medicine, 10*, 654-662.

Portenoy, R. K., & Foley, K. M. (1986). Chronic use of opioid analgesics in non-malignant pain: report of 38 cases. *Pain, 25*, 171-186.

Proud, D., & Kaplan, A. P. (1988). Kinin formation: mechanisms and role in inflammatory disorders. *Annual review of immunology, 6*, 49-83.

Purseglove, J. W., Brown, E., Green, C., & Robbins, S. (1981). *Spices, 1*.

Redi, C. (2010). Histology protocols. *European Journal of Histochemistry, 54*, 27.

Rhen, T., & Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids new mechanisms for old drugs. *New England Journal of Medicine, 353*, 1711-1723.

Robbins, S. L., Kumar, V., Abbas, A. K., & Aster, J. C. (2012). *Robbins basic pathology*: Elsevier Health Sciences.

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

Rosa, M. D., & Willoughby, D. (1971). Screens for anti-inflammatory drugs. *Journal of Pharmacy and Pharmacology, 23*, 297-298.

Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Woolf, C. J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature, 410*, 471-475.

Sanchez-Mateo, C., Bonkanka, C., Hernandez-Perez, M., & Rabanal, R. (2006). Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. *Journal of ethnopharmacology, 107*, 1-6.

Schnitzler, A., & Ploner, M. (2000). Neurophysiology and functional neuroanatomy of pain perception. *Journal of clinical neurophysiology, 17*, 592-603.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Isakson, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proceedings of the National Academy of Sciences, 91*, 12013-12017.

Serra, J. (1999). Overview of neuropathic pain syndromes. *Acta Neurologica Scandinavica, 100*, 7-11.

Sheffield, E. (1990). The granulomatous inflammatory response. *The Journal of pathology, 160*, 1-2.

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

Simon, R. A., & Namazy, J. (2003). Adverse reactions to aspirin and nonsteroidal antiinflammatory drugs (NSAIDs). *Clinical reviews in allergy & immunology, 24*, 239-251.

Smyth, E. M., Grosser, T., Wang, M., Yu, Y., & FitzGerald, G. A. (2009). Prostanoids in health and disease. *Journal of lipid research, 50*, 423-428.

Stanley, K. L., & Paice, J. A. (1997). *Animal models in pain research.* Paper presented at the Seminars in oncology nursing.

Steeds, C. E. (2013). The anatomy and physiology of pain. *Surgery (Oxford), 31*, 49-53.

Stoelting, R. K., & Hillier, S. C. (2012). *Pharmacology and physiology in anesthetic practice*: Lippincott Williams & Wilkins.

Sud'Ina, G., Mirzoeva, O., Pushkareva, M., Korshunova, G., Sumbatyan, N., & Varfolomeev, S. (1993). Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS letters, 329*, 21-24.

Suwanchaikasem, P., Phadungcharoen, T., & Sukrong, S. (2013). Authentication of the Thai medicinal plants sharing the same common name ‘Rang Chuet’: *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* aff. *amada* by combined techniques of TLC, PCR-RFLP fingerprints, and antioxidant activities. *Science Asia, 39*, 124-133.

Swingle, K., & Shideman, F. (1972). Phases of the inflammatory response to subcutaneous implantation of a cotton pellet and their modification by certain antiinflammatory agents. *Journal of Pharmacology and Experimental Therapeutics, 183*, 226-234.

Tallarida, R. J., & Murray, R. B. (1987). Manual of pharmacologic calculations with computer programs.

Tjolsen, A., Berge, O.-G., Hunskaar, S., Rosland, J. H., & Hole, K. (1992). The formalin test: an evaluation of the method. *Pain, 51*, 5-17.

Tjolsen, A., Hole, K., & Hunskaar, S. (1994). Animal models in pain research. *Handbook of Laboratory Animal Science, 2*, 137.

Vane, J., & Botting, R. (1995). New insights into the mode of action of anti-inflammatory drugs. *Inflammation Research, 44*, 1-10.

Vogel, H. (2007). *Drug discovery and evaluation: pharmacological assays, 1*: springer.

Webb, D. R. (2014). Animal models of human disease: inflammation. *Biochemical pharmacology, 87*, 121-130.

Wedmore, C. V., & Williams, T. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature, 289*, 646-650.

Wheeler-Aceto, H., & Cowan, A. (1991). Standardization of the rat paw formalin test for the evaluation of analgesics. *Psychopharmacology, 104*, 35-44.

Williams, T. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *British Journal of Pharmacology, 65*, 517-524.

Williams, T. (1983). Interactions between prostaglandins, leukotrienes and other mediators of inflammation. British medical bulletin, 39, 239-242.

Woolf, C. J. (2004). Pain: moving from symptom control toward mechanism-specific pharmacologic management. *Annals of internal medicine, 140*, 441-451.

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

Young, J. M., Spires, D. A., Bedord, C. J., Wagner, B., Ballaron, S. J., & de Young, L. M. (1984). The mouse ear inflammatory response to topical arachidonic acid. *Journal of Investigative Dermatology, 82*, 367-371.

Yu, Y., Ricciotti, E., Scalia, R., Tang, S. Y., Grant, G., Yu, Z., Puré, E. (2012). Vascular COX-2 modulates blood pressure and thrombosis in mice. *Science translational medicine, 4*, 132-154.

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APPENDIX

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Appendix A

Certificate of Project Approval by the Institutional Animal Care and Use committee, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 14-33-004	**Approval No. 14-33-004**

Protocol Title

Antinociceptive and anti-inflammatory effects of the ethanolic extract of Curcuma AFF.Amada

Principal Investigator

Pasarapa Towiwat

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
October 14, 2014	October 14, 2017

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 **PORNCHAI ROJSITTHISAK, 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

Thin-layer chromatogram of the ethanolic extract of *Curcuma* aff. *amada* rhizome

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Thin-layer chromatographic identification

The characteristic chemical pattern indicated by thin-layer chromatography profiling is useful for the primary identification of plant material. The ethanolic extract of *Curcuma* aff. *amada* rhizome (0.2 mg) was applied to a silica gel thin-layer chromatographic (TLC) plate as 8-mm-wide bands. The plates were developed in chloroform-methanol-formic acid (7:3:0.5). The chromatograms were evaluated under UV light at 254 and 365 nm to detect the target compounds. To detect flavonoid compounds, the TLC plate was sprayed with a 1% $AlCl_3$ solution and monitored under UV light at 365 nm. The TLC bioautography assay of free scavenging activity with the DPPH radical was also employed. The chromatogram was sprayed with a 0.5 nM methanolic solution of DPPH to detect antioxidant compound. The thin-layer chromatograms of the ethanolic extract of *Curcuma* aff. *amada* rhizome was prepared by Mr. Pipob Suwanchaikasem.

The chemical profiles of the ethanolic extracts are shown in Figure 28. Flavonoids, which turn yellow under UV light at 365 nm after being sprayed with an $AlCl_3$ solution, were not found in the ethanolic extract of *Curcuma* aff. *amada* rhizome.

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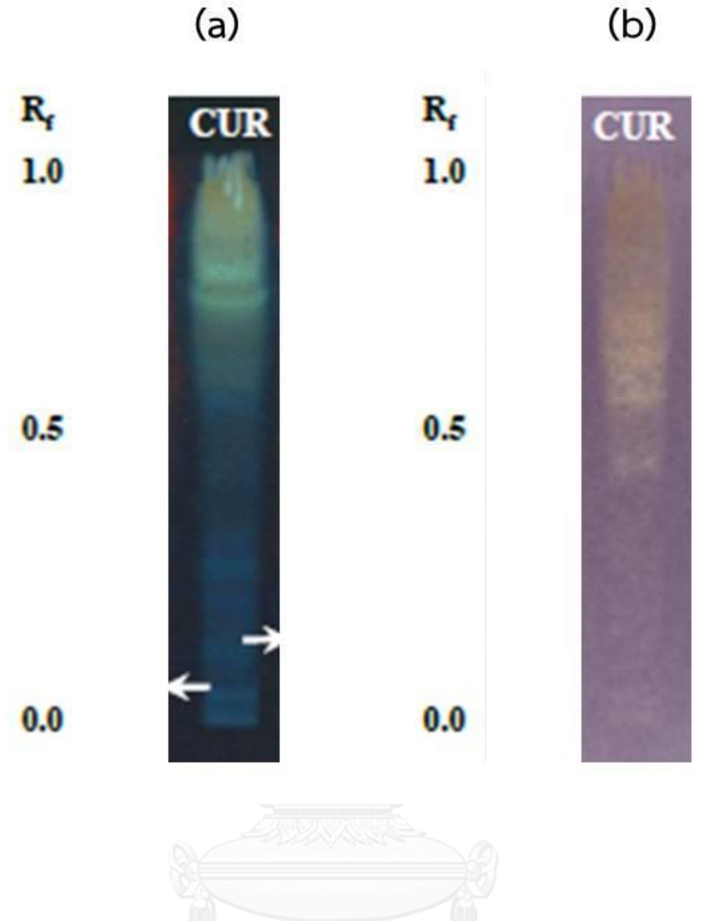

Figure 28 Thin-layer chromatograms of the ethanolic extract of *Curcuma* aff. *amada* rhizome. The plate of the ethanolic extracts were developed with chloroform-methanol-formic acid (7:3:0.5) and then viewed (a) under UV at 365 nM after being sprayed with 1% $AlCl_3$ solution or (b) under visible light after being sprayed with a 0.5 nM DPPH solution (Suwanchaikasem et al., 2013).

Appendix C

Data of Hot-plate test

Table 6 Latency (sec) in the hot-plate test from 0-240 minutes after oral administration of normal saline (NSS; 10 mL/kg), morphine sulfate (MO; 2 mg/kg), 2% Tween 80 (10 mL/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 12.5-200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
NSS	19.47±1.64	20.34±2.58	25.52±3.57	19.60±2.15	18.51±1.29	20.65±1.60	17.97±1.55	18.94±1.34
MO	17.18±1.86	32.61±2.83	31.94±3.52	30.04±3.02	9.23±2.92	37.28±1.93	31.99±2.89	33.36±1.09
Tween 80	17.83±1.71	21.12±3.05	17.80±2.28	18.35±2.40	20.10±2.17	20.67±2.15	21.42±2.25	20.19±2.71
CAE 12.5	15.87±1.65	21.44±3.32	24.87±2.80	25.71±3.59	22.65±2.58	22.60±2.91	20.14±3.34	24.31±3.34
CAE 25	13.45±1.99	20.64±3.79	18.42±2.77	19.59±1.97	20.76±2.82	20.49±2.99	26.11±3.07	20.36±2.47
CAE 50	15.10±1.47	20.34±1.27	23.66±2.12	26.36±2.17	22.37±2.53	29.14±2.24	24.62±2.65	26.35±2.59
CAE 100	13.81±1.55	20.58±2.94	21.06±2.50	26.81±3.84	25.82±3.73	29.45±4.25	28.25±3.67	25.28±3.41
CAE 200	16.75±1.86	31.02±2.74	27.69±4.10	29.12±3.24	30.70±3.67	36.83±2.56	41.42±1.92	31.92±3.15

Table 7 %MPE-min in the hot-plate test from 0-240 minutes after oral administration of normal saline (NSS; 10 mL/kg), morphine sulfate (MO; 2 mg/kg), 2% Tween 80 (10 mL/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 12.5-200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M.

Treatments (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
NSS	0.33±11.40	23.54±13.24	0.60±6.19	-8.72±9.80	1.09±9.94	-9.76±9.09	-3.71±4.54	-677.45±1361.20
MO	56.96±9.24	53.88±12.58	44.97±11.33	53.22±11.34	68.15±8.95	55.18±9.02	59.40±9.82	13212.17±1234.83
Tween 80	10.89±11.12	-2.43±8.85	0.63±9.22	8.45±6.08	8.01±10.07	13.56±6.78	6.50±11.34	1954.53±1422.51
CAE 12.5	17.21±12.25	30.14±10.90	34.74±13.04	22.41±8.18	20.70±10.83	13.48±11.85	30.73±11.24	5278.67±1401.47
CAE 25	25.61±10.90	15.19±9.74	15.09±9.21	24.45±7.72	24.08±7.11	41.10±9.05	21.92±5.70	6509.65±995.90
CAE 50	15.50±6.35	26.79±8.28	38.94±5.57	25.18±7.05	45.03±6.29	32.48±7.92	39.58±6.06	7946.58±845.58
CAE 100	22.98±7.27	23.50±6.92	44.48±10.27	40.70±10.12	51.85±13.14	47.00±11.92	38.74±10.57	9625.37±1982.30
CAE 200	49.03±10.35	38.76±15.17	44.78±11.22	52.38±12.39	68.85±11.35	84.39±9.42	53.71±11.20	14789.71±1572.13

Table 8 Latency (sec) in the hot-plate test from 0-240 minutes after oral administration of normal saline (NSS; 10 mL/kg, i.p.), naloxone (NAL; 5 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 200 mg/kg, p.o.) and combination of naloxone and CAE (5/200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	Baseline (sec)	15 min	30 min	45 min	60 min	90 min	120 min	240 min
NSS	18.98±1.71	16.91±2.90	15.92±2.69	16.63±2.34	19.14±1.84	15.03±2.29	16.18±1.78	20.93±2.14
NAL	19.08±1.65	12.97±1.90	11.90±1.98	12.76±1.78	9.93±1.03	13.73±1.99	13.94±2.20	14.45±2.02
Tween 80	17.29±2.31	14.81±1.72	18.54±1.25	17.73±2.30	14.34±1.52	14.66±1.30	14.19±1.02	17.97±1.28
CAE 200	18.56±1.44	24.38±3.20	29.22±2.91	26.56±3.29	25.93±2.45	30.98±2.65	32.28±3.84	30.35±3.65
NAL + CAE	23.19±2.59	12.70±1.70	11.17±0.94	12.68±1.29	13.65±1.80	17.67±1.68	16.18±1.41	20.79±2.59

Table 9 %MPE-min in the hot-plate test from 0-240 minutes after oral administration of normal saline (NSS; 10 mL/kg, i.p.), naloxone (NAL; 5 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 200 mg/kg, p.o.) and combination of naloxone and CAE (5/200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
NSS	-10.04±13.22	-14.40±12.82	-10.09±7.50	-2.78±9.33	-53.97±38.04	-17.94±12.36	7.12±5.31	-2000.69±1788.27
NAL	-26.00±8.23	-30.19±8.73	-28.10±9.79	-38.69±6.86	-22.22±7.59	-21.14±7.75	-19.44±6.85	-5552.59±1269.53
Tween 80	-17.12±13.07	-5.95±15.40	-7.69±16.04	-20.18±14.41	-17.16±10.71	-20.57±13.44	-6.79±15.10	-3379.55±3078.04
CAE 200	23.49±11.26	38.08±11.61	28.50±13.35	25.58±11.56	48.38±8.57	54.27±13.34	46.03±13.62	10209.78±2060.76
NAL + CAE	-68.92±24.45	-76.24±22.37	-69.35±23.83	-54.61±23.17	-41.96±19.13	-51.18±21.06	-21.82±16.81	-11077.64±4569.20

Appendix D

Data of Acetic acid-induced writhing test

Table 10 Dose-response and Time-course effects of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE; 12.5-200 mg/kg) on acetic acid-induced writhes test in mice. N= 8 all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	Number of Writhes (mean ± S.E.M.)						Total
	0-5	5-10	10-15	15-20	20-25	25-30	
Tween 80	0.75±0.37	1.38±0.60	2.50±0.53	2.75±0.53	2.25±0.56	3.25±0.31	12.88±1.14
IND	0±0	0±0	0.50±0.19	1.00±0.27	0.75±0.25	1.00±0.37	3.13±0.77 (-75.70%)
CAE 3.125	0.25±0.25	1.00±0.76	2.00±0.85	1.25±0.41	1.88±0.61	1.50±0.57	7.88±2.76 (-38.82%)
CAE 6.25	0.63±0.26	2.25±0.84	2.50±0.71	1.63±0.65	1.13±0.40	1.13±0.44	9.25±1.47 (-28.18%)
CAE 12.5	0.38±0.18	0.63±0.26	0.63±0.18	1.13±0.58	0.88±0.35	0.50±0.18	4.13±0.52 (-67.93%)
CAE 25	0.50±0.27	1.13±0.40	0.88±0.35	0.63±0.26	1.00±0.46	0.38±0.18	4.50±0.78 (-65.06%)
CAE 50	0.13±0.13	0.13±0.40	0.88±0.30	1.25±0.65	0.75±0.25	0.63±0.26	4.75±1.10 (-63.12%)
CAE 100	0.13±0.13	1.50±0.60	0.75±0.31	1.25±0.45	0.63±0.26	0.63±0.32	4.88±0.77 (-62.11%)
CAE 200	0.75±0.37	0.38±0.26	0.88±0.23	1.50±0.71	1.00±0.50	0.88±0.47	5.38±1.83 (-58.23%)

Appendix E

Data of Carrageenan-induced mouse paw edema

Table 11 Effects of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg) on paw volume in the carrageenan-induced mice paw edema. N=8 for all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	**Baseline (mL)**	**Paw edema (mL) ± S.E.M.**					
		1 h	**2 h**	**3 h**	**4 h**	**5 h**	**6 h**
Tween 80	0.2950±0.0148	0.3875±0.0118	0.3975±0.0157	0.4225±0.0261	0.4550±0.0204	0.4613±0.0264	0.4600±0.0264
IND 10	0.2638±0.0050	0.3168±0.0166	0.3663±0.0136	0.3925±0.0175	0.3975±0.0197	0.3637±0.0178	0.3425±0.0170
CAE 12.5	0.3263±0.0080	0.3938±0.0174	0.4088±0.0083	0.3988±0.0190	0.3850±0.0184	0.3775±0.0149	0.3813±0.0163
CAE 25	0.3200±0.0141	0.3888±0.0063	0.3963±0.0128	0.4475±0.0096	0.3838±0.0063	0.3625±0.0214	0.3600±0.0121
CAE 50	0.3175±0.0092	0.3763±0.0161	0.3950±0.0232	0.4100±0.0242	0.3513±0.0208	0.3500±0.0196	0.3550±0.0194
CAE 100	0.3288±0.0125	0.4100±0.0093	0.4075±0.0209	0.4100±0.0165	0.3813±0.0203	0.3863±0.0196	0.3888±0.0218
CAE 200	0.3400±0.0178	0.3950±0.0174	0.4113±0.0267	0.4175±0.0125	0.3963±0.0248	0.3763±0.0221	0.3663±0.0165

Appendix F

Data of Prostaglandins E_2-induced mouse paw edema

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Table 12 Effects of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg) on paw volume in the PGE_2-induced mice paw edema. N=8 for all groups. Data are shown as the mean ± S.E.M.

Treatments (mg/kg)	**Baseline (mL)**	**Paw edema (mL) ± S.E.M.**					
		0.5 h	**1 h**	**1.5 h**	**2 h**	**3 h**	**4 h**
Tween 80	0.2150±0.0042	0.3975±0.0053	0.3888±0.0058	0.3800±0.0042	0.3788±0.0048	0.3750±0.0046	0.3700±0.0054
IND 10	0.2275±0.0037	0.3013±0.044	0.2938±0.0032	0.2850±0.0042	0.2725±0.0045	0.2725±0.0037	0.2650±0.0060
CAE 200	0.2350±0.0115	0.3288±0.0104	0.3138±0.0118	0.3025±0.0111	0.3025±0.0115	0.2938±0.0010	0.2888±0.0093

Appendix G

Data of Arachidonic acid-induced mouse paw edema

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 13 Effects of oral administration of dimethyl sulfoxide (DMSO; 10 mL/kg, i.p.), Caffeic acid (100 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), indomethacin (IND; 10 mg/kg, p.o.) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg, p.o.) on paw volume in the AA-induced paw edema. N=8 for all groups. Data are shown as the mean ± S.E.M.

Treatment (mg/kg)	Baseline (mL)	Paw edema (mL) ± S.E.M.					
		0.5 h	1 h	1.5 h	2 h	3 h	4 h
DMSO	0.3363±0.0084	0.5063±0.0378	0.4875±0.0251	0.4825±0.0179	0.4663±0.0228	0.4600±0.0162	0.4588±0.0154
Caffeic acid	0.3563±0.0082	0.4575±0.0142	0.4388±0.0122	0.4213±0.0113	0.4300±0.0149	0.4100±0.0093	0.3938±0.0105
Tween 80	0.2913±0.0183	0.4713±0.0281	0.4625±0.0212	0.4675±0.0291	0.4538±0.0249	0.4513±0.0270	0.4563±0.0442
IND 10	0.3025±0.0156	0.4163±0.0155	0.4138±0.0164	0.4075±0.0199	0.4088±0.0181	0.3900±0.0243	0.3913±0.0129
CAE 12.5	0.3163±0.0145	0.4488±0.0261	0.4688±0.0280	0.4388±0.0262	0.4125±0.0176	0.4338±0.0215	0.3975±0.0150
CAE 25	0.3075±0.0161	0.4862±0.0215	0.4612±0.0286	0.4712±0.0274	0.4300±0.0253	0.4237±0.0311	0.3900±0.0244
CAE 50	0.3150±0.0163	0.4712±0.0114	0.4563±0.0093	0.4538±0.0145	0.4350±0.0110	0.4175±0.0082	0.4038±0.0155
CAE 100	0.3325±0.0157	0.4750±0.0100	0.4362±0.0155	0.4613±0.0159	0.4450±0.0121	0.4213±0.0192	0.4237±0.0178
CAE 200	0.3113±0.0126	0.4375±0.0239	0.4100±0.0202	0.4013±0.0203	0.4075±0.0211	0.3963±0.0273	0.3763±0.0187

Appendix H

Data of histological analysis

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Table 14 Number of neutrophils in edema paws in each scope (400×) after carrageenan injection following oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg). N=5 for all groups. Scope 3 is the hot spot area that shows maximum infiltration of neutrophil.

Scope of tissue slice	2% Tween 80 (10 ml/kg)				
	N=1	N=2	N=3	N=4	N=5
Scope 1	332	256	342	169	292
Scope 2	576	324	502	443	318
Scope 3	653	548	569	716	521
Scope 4	408	365	534	520	502
Scope 5	380	292	406	364	387
Average	469.80	357.00	471.20	442.40	404.00
	IND 10 mg/kg				
Scope 1	6	82	105	66	81
Scope 2	16	135	197	169	103
Scope 3	22	214	237	251	357
Scope 4	7	171	204	197	97
Scope 5	5	56	138	180	75
Average	11.20	131.60	176.20	172.60	142.60
	CAE 12.5 mg/kg				
Scope 1	111	71	165	143	114
Scope 2	138	85	183	170	181
Scope 3	175	241	216	183	286
Scope 4	133	176	101	182	157
Scope 5	125	92	88	130	145
Average	136.40	133.00	150.60	161.60	176.60

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Scope of tissue slice	CAE 25 mg/kg				
	N=1	N=2	N=3	N=4	N=5
Scope 1	7	261	87	180	107
Scope 2	63	390	133	243	173
Scope 3	68	407	153	366	220
Scope 4	22	357	117	170	164
Scope 5	13	311	76	149	139
Average	34.60	345.20	113.20	221.60	160.60
	CAE 50 mg/kg				
Scope 1	49	81	26	15	129
Scope 2	64	106	44	44	198
Scope 3	73	350	79	89	208
Scope 4	16	209	22	15	198
Scope 5	13	199	11	12	115
Average	43.00	189.00	36.40	35.00	169.60
	CAE 100 mg/kg				
Scope 1	183	117	163	102	193
Scope 2	249	187	325	136	208
Scope 3	276	368	418	267	289
Scope 4	183	206	346	241	176
Scope 5	152	138	176	197	137
Average	208.60	203.20	285.60	188.60	200.60
	CAE 200 mg/kg				
Scope 1	110	242	120	4	147
Scope 2	172	303	133	5	183
Scope 3	186	478	320	10	210
Scope 4	49	407	264	9	185
Scope 5	31	225	134	7	112
Average	109.60	331.00	194.20	7.00	167.40

Table 15 Mean number of neutrophils in edema paws after carrageenan injection following oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg, and various doses of the ethanolic extract of *Curcuma* aff. *amda* rhizome (CAE; 12.5-200 mg/kg). N=5 for all groups. Data are shown as the mean ± S.E.M.

Treatment	Dose (mg/kg)	Number of neutrophils
2% Tween 80	10	428.88±21.72
IND	10	126.84±30.14
CAE	12.5	151.64±8.07
	25	175.04±52.38
	50	94.60±34.74
	100	217.32±17.38
	200	161.84±53.10

VITA

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