Studies on Antinociceptive, Antipyretic and Anti-inflammatory Activities of

Putranjiva roxburghii Wall. in an Animal Model

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Pharmacy in Pharmaceutical Sciences

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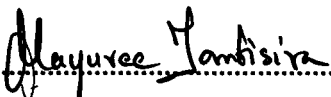
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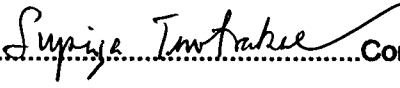
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ชื่อวิทยานิพนธ์   การศึกษาฤทธิ์แก้ปวด แก้ไข้ และแก้อักเสบของมะคำไก่ใน
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บทคัดย่อ

ทำการทดสอบผลการระงับปวด โดยการเหนี่ยวนำให้เกิดความปวดด้วยกรดอะเซติก ความร้อนและฟอร์มาลินในหนูถีบจักร และผลต่อการลดไข้ โดยการเหนี่ยวนำให้เกิดไข้ด้วยยีสต์ในหนูขาวของสารสกัดด้วยอีเทอร์และสารสกัดเอทานอลจากใบมะคำไก่ ผลต่อการตอบสนองต่อการอักเสบซึ่งเกิดจากการเหนี่ยวนำด้วยคาร์ราจีนินในหนูขาว น้ำมันสลอดเหนี่ยวนำให้เกิดการบวมที่หูของหนูถีบจักรและที่ทวารของหนูขาว และการอักเสบเรื้อรังที่เหนี่ยวนำด้วยน้ำมันสลอดเป็นโพรงอากาศที่หลังของหนูขาว พบว่าทั้งสารสกัดด้วยอีเทอร์และสารสกัดเอทานอล (100, 200 และ 400 มก./กก. ทางปาก) สามารถลดจำนวนของการบิดและยืดของลำตัวเมื่อถูกกระตุ้นด้วยกรดอะเซติกในหนูถีบจักร โดยขึ้นกับขนาดยา สารสกัดทั้งสองไม่มีผลต่อการทดสอบด้วยความร้อนในหนูถีบจักร ที่ขนาดยา 400 มก/กก. สารสกัดลดการเลียในช่วงเฟสหลังเท่านั้นของการทดสอบด้วยฟอร์มาลินในหนูถีบจักร และลดไข้ซึ่งเกิดจากการเหนี่ยวนำด้วยยีสต์ในหนูขาว สารสกัดมีฤทธิ์ยับยั้งการอักเสบปานกลางที่เกิดจากการเหนี่ยวนำด้วยคาร์ราจีนินในหนูขาว ทั้งสารสกัดด้วยอีเทอร์และสารสกัดเอทานอล (1.25, 2.5 และ 5.0 มก./หู) ยับยั้งน้ำมันสลอดเหนี่ยวนำให้เกิดการบวมที่หูของหนูถีบจักรขึ้นกับขนาดยา สารสกัดยับยั้งน้ำมันสลอดเหนี่ยวนำให้เกิดการบวมที่ทวารของหนูขาวเฉพาะที่ขนาดสูง 800 มก./กก. สำหรับการอักเสบเรื้อรังสารสกัดทั้งสองลดของเหลวในโพรงอากาศที่หลังของหนูขาวเฉพาะที่ขนาดสูง 800 มก./กก. จากผลการทดลองนี้ชี้แนะว่า สารสกัดอีเทอร์และสารสกัดเอทานอลจากใบมะคำไก่มีฤทธิ์ระงับปวด ลดไข้และด้านการอักเสบ และฤทธิ์ด้านการอักเสบอาจเนื่องจากการยับยั้งสารสื่อกลางของการอักเสบ การเคลื่อนย้ายของเซลล์ และการไหลออกของของเหลวพลาสมาที่บริเวณเนื้อเยื่อที่มีการอักเสบ

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ABSTRACT

The effects of the ether and ethanol extracts from the leaves of *Putranjiva roxburghii* Wall. (*P. roxburghii*) was assessed on nociceptive response using writhing, hot plate and formalin tests in mice and the antipyretic activity in yeast-induced fever in rats. Anti-inflammatory activity using carrageenin-induced paw edema in rats, croton oil-induced ear edema in mice, croton oil-induced anus edema and chronic inflammation induced in air pouches of rats, were also investigated. It was found that both of the ether extract and ethanol extract (100, 200 and 400 mg/kg, p.o.) dose dependently produced significant analgesic activity in acetic acid-induced writhing in mice. Neither the ether extract nor ethanol extract had significant effects on antinociceptive response in the hot plate test in mice. At the dose of 400 mg/kg, the extracts significantly suppressed the licking activity only in the late phase of formalin test in mice and decreased fever induced by yeast in rats. The extracts possessed moderate inhibitory activity of inflammation in carrageenin-induced paw edema in rats. Either the ether extract or ethanol extract inhibited croton oil-induced ear edema after topically applied of mice's ear in dose dependent manner (1.25, 2.5 and 5.0 mg/ear). The extracts suppressed croton oil-induced anus edema only at the high dose of 800 mg/kg after application of croton oil onto the recto-anus of rats. In chronic inflammation, both of the extracts also significantly decreased the fluid in air pouches of rats only at the high dose of 800 mg/kg. These results suggested that the ether and ethanol extracts of *P. roxburghii* possess an analgesic, antipyretic and anti–inflammatory action, which seem to be involved in the suppression of some peripheral mediators, cell migration and/or plasma exudation.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	=	Arachidonic acid
ATL	=	Aspirin-triggered lipoxin
BBB	=	Blood-brain barrier
CNS	=	Central nervous system
$CHCl_3$	=	Chloroform
COX-1	=	Cyclooxygenase 1
COX-2	=	Cyclooxygenase 2
CysLTs	=	Cysteinyl-leukotrienes (LTC_4, LTD_4, and LTE_4)
$cPLA_2$	=	Cytosolic phospholipase A_2
etc.	=	et cetera
FLAP	=	Five-Lipoxygenase-Activating Protein
GI	=	Gastrointestinal
g	=	Gram
5-HPETE	=	5-hydroperoxy eicosatetraenoic acid
IFN	=	Interferon
IL	=	Interleukin
IL-1β	=	Interleukins 1β
IL-6	=	Interleukins 6
IL-1	=	Interleukin 1
i.p.	=	Intraperitoneal
kg	=	Kilogram
LT	=	Leukotrienes
LTB_4	=	Leukotriene B_4
LOX	=	Lipoxygenases
LPS	=	Lipopolysaccharide
LXA_4	=	Lipoxin A_4
LXB	=	Lipoxin B_4
5-LO	=	5-lipoxygenase
°C	=	Degree Celsius
μL	=	Microliter

LIST OF ABBREVIATIONS AND SYMBOLS

μg	=	Microgram
ED_{50}	=	Median effective dose
MeOH	=	Methanol
LD_{50}	=	Median lethal dose
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mm	=	millimeter
NSAIDs	=	Nonsteroidal anti-inflammatory drugs
No.	=	Number
%	=	Percentage
/	=	Per
p.o.	=	*Per os*
PLA_2	=	Phospholipase A_2
PGs	=	Prostaglandins
PGD_2	=	Prostaglandin D_2
PGE_2	=	Prostaglandin E_2
PGI_2	=	Prostaglandin I_2
PMNs	=	Polymorphonuclear leukocytes
sec	=	Second
5-HT	=	Serotonin
s.c.	=	Subcutaneously
S.M.E.	=	Standard error of the Mean
Tb	=	Temperature
TXA_2	=	Thromboxane A_2
TNF	=	Tumor necrosis factor
w/v	=	Weight by volume
w/w	=	Weight by weight

CHAPTER 1

INTRODUCTION

Drypetes roxburghii Wall. or *Putranjiva roxburghii* Wall. (*P. roxburghii*) is known in Thai as Ma Kham Kai or Pra Kham Kai in the family Euphorbiaceae (Figure 1). It is widely grown in Nepal, Bangladesh, India, Indo-China, Myanmar, Sri Lanka and Thailand (Phuphathanaphong and Chayamarit, 2008). It is a tree of up to 15 m high, with drooping leaves dioecious; bark thin, grey; young shoots pubescent, twigs glabrescent. The leaves are distichous; petiole 0.5-1 cm long, pubescent; blade oblong, oblong-elliptic or elliptic, 4-14 by 2-5 cm subcoriaceous, glabrous, base obliquely cuneate, margin crenate or serrate, slightly undulate, apex acuminate, acute or obtuse, dark green above, light green underneath; nerves very thin, irregularly arranged, veins and veinlets reticulate. The flowers are yellowish green. (Phuphathanaphong and Chayamarit, 2008). It has been reported to contain two new triterpenoids namely putranjivanonol (a hydroxy ketone), and putranjic acid (a hydroxy acid), isolated from the trunk bark of *P. roxburghii* Wall. (Garg and Mitra, 1968). The isolation of friedelin, putranjivadione, friedelanol and a new triterpenoid roxburgholone, $C_{30}H_{50}O_2$, from the bark of *P. roxburghii* has been described (Sengupta and Mukherjee, 1968). By a combination of chemical and physical methods, it was shown that putranjivadione is friedelane-3, 7-dione. The acidic fraction of the alcoholic extract of the leaf of *P. roxburghii* Wall. yielded a new triterpene acid, roxburghonic acid (Garg and Mitra, 1971a). A new biflavonoid named putraflavone was isolated from the alcoholic extract of the leaf of *P. roxburghii* (Garg and Mitra, 1971b).

Some activities of *P. roxburghii* have been reported, the leaf extract of *P. roxburghii* significantly induced mitosis-disruptive chromosomal changes in mouse bone marrow cells (Awasthy et al., 2000). Pollen extract of *P. roxburghii* exhibited cross-reactivity with *Ricinus communis* (Singh et al., 1997). *P. roxburghii* has been used in Thai folklore medicine for a long time, its leaf and stem bark are traditionally used for the treatment of fever, whereas the whole plant is used for the treatment of hemorrhoid, tuberculosis and fever (นันทวัน และ อรนุช, 2542) but no pharmacological studies in vivo have previously been conducted on antinociceptive, antipyretic and anti-inflammatory actions of this plant. In the present study, a crude ether and ethanol extracts from the

leaves of *P. roxburghii* Wall. are evaluated for antinociceptive, antipyretic and anti-inflammatory activities in animal models.
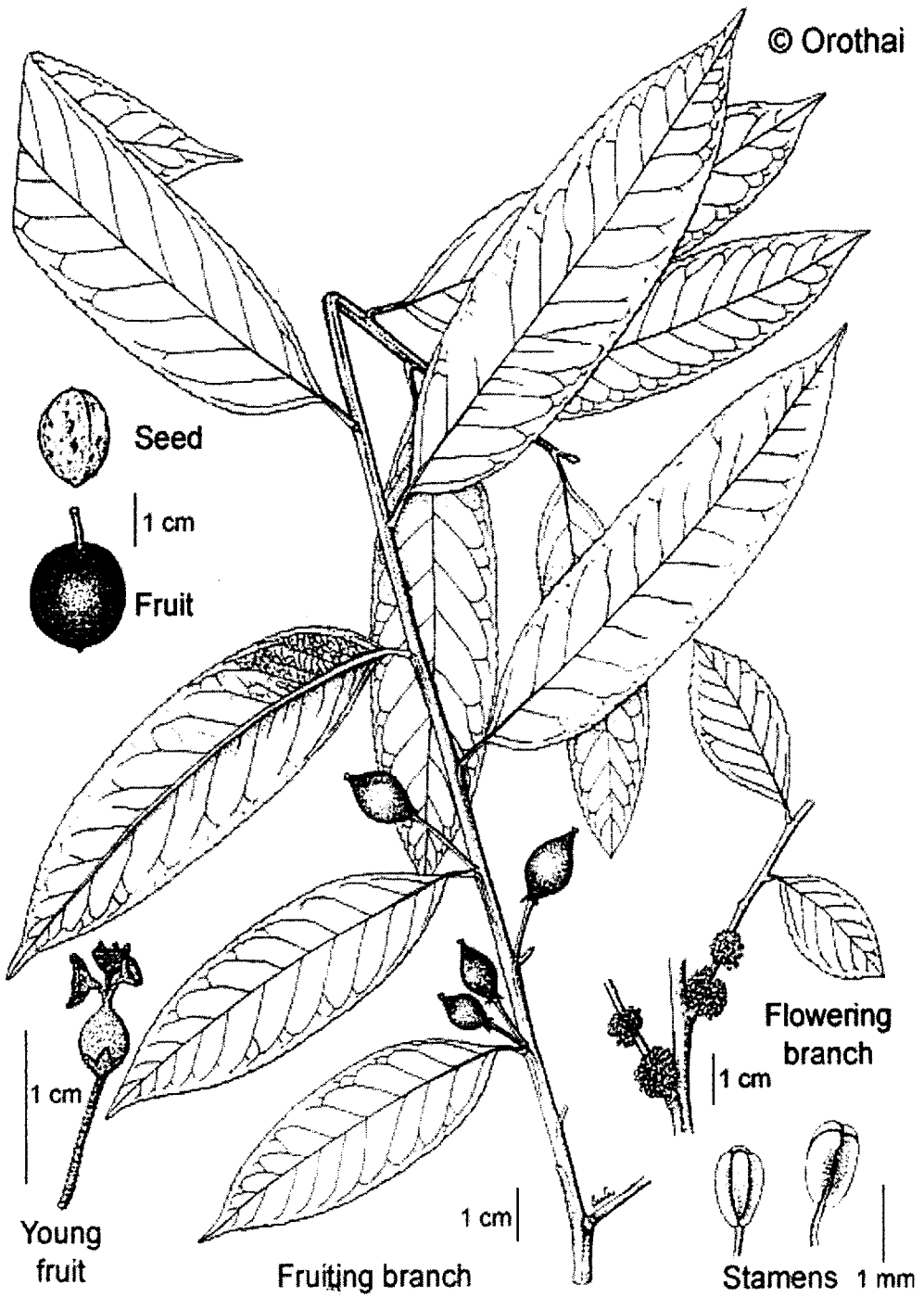
Figure 1. *Putranjiva roxburghii* Wall. (Phuphathanaphong and Chayamarit, 2008).

Review of Literatures

1. *P. roxburghii* Wall.

In Thai folklore medicine, its leaf and stem bark are traditionally used for the treatment of fever, whereas the whole plant is used for the treatment of hemorrhoid, tuberculosis and fever (นันทวัน และ อรนุช, 2542).

The leaf extract of *P. roxburghii* significantly induced mitosis-disruptive chromosomal changes in mouse bone marrow cells (Awasthy et al., 2000).

The oil of *P. roxburghii* exhibited the greatest toxicity. The oil was found to be fungicidal and thermostable at its minimum inhibitory concentration (MIC) of 400 ppm. The oil was characterized by the determination of its various physico-chemical properties. Thus, the oil of *P. roxburghii* showed potential as a preservative for peanut seeds against spoilage by fungi and insects during storage (Tripathi and Kumar, 2007).

Chemistry
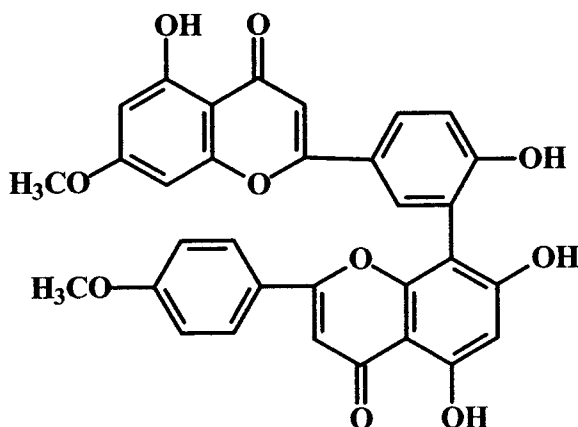
Figure 2. Chemical structure of putraflavone (Garg and Mitra, 1971).
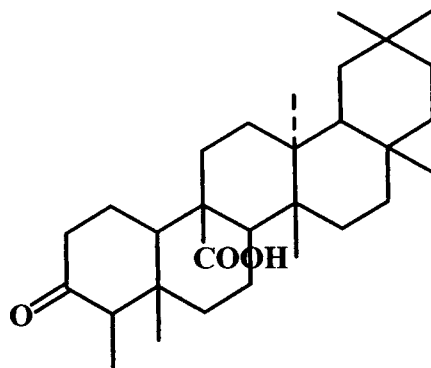
Figure 3. Chemical structure of roxburghonic acid (Garg and Mitra, 1971).

A new bioflavonoid (Figure 2), named putraflavone, was isolated from the acidic fraction of the alcoholic extract of the leaf of *P. roxburghii* Wall. Putraflavone is indicated to be the biflavonoid, $C_{32}H_{22}O_{10}$ built up of partially methylated units of apigenin. The pale yellow compound gave olive green colour with ferric chloride and orange red with Mg-HCl (Garg and Mitra, 1971).

Two new triterpenoids namely putranjivanonol (a hydroxy ketone), and putranjic acid (a hydroxy acid), have been isolated from the trunk bark of *P. roxburghii* Wall. along with friedelin and putranjivadione and a sterol (Garg and Mitra, 1968).

The acidic fraction of the alcoholic extract of the leaf of *P. roxburghii* Wall. yielded a new triterpene acid, roxburghonic acid, $C_{30}H_{48}O_3$ (Figure 3), which has been shown by physico-chemical methods to be 3-keto-friedelan-25-oic acid. Roxburghonic acid (Garg and Mitra, 1971).

The isolation of friedelin (Figure 4), putranjivadione (Figure 5), friedelanol and a new triterpenoid roxburgholone, $C_{30}H_{50}O_2$, from the bark of *P. roxburghii* has been described. Roxburgholone has been shown to be 3α-hydroxyfriedelan-7-one. The hydroxyketone obtained by sodium borohydride reduction of putranjivadione has been shown to be 3β-hydroxyfriedelan-7-one (Sengupta and Mukherjee, 1968).
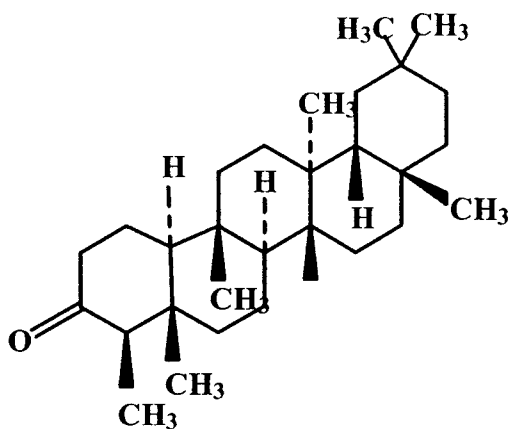
Figure 4. Chemical structure of friedelin (Merck, 1996).

Friedelin has been identified. (Merck, 1996). Calophyllolide, isolated from the nut (Rastogi et al, 1998), its ED_{50} was 140 mg/kg orally and showed anti-inflammatory and anti-arthritic activities in formaldehyde-induced arthritis and adjuvant arthritis in rats. LD_{50} was 2.5 g/kg p.o. It was devoid of ulcerogenic activity up to twice the ED_{50} dose.
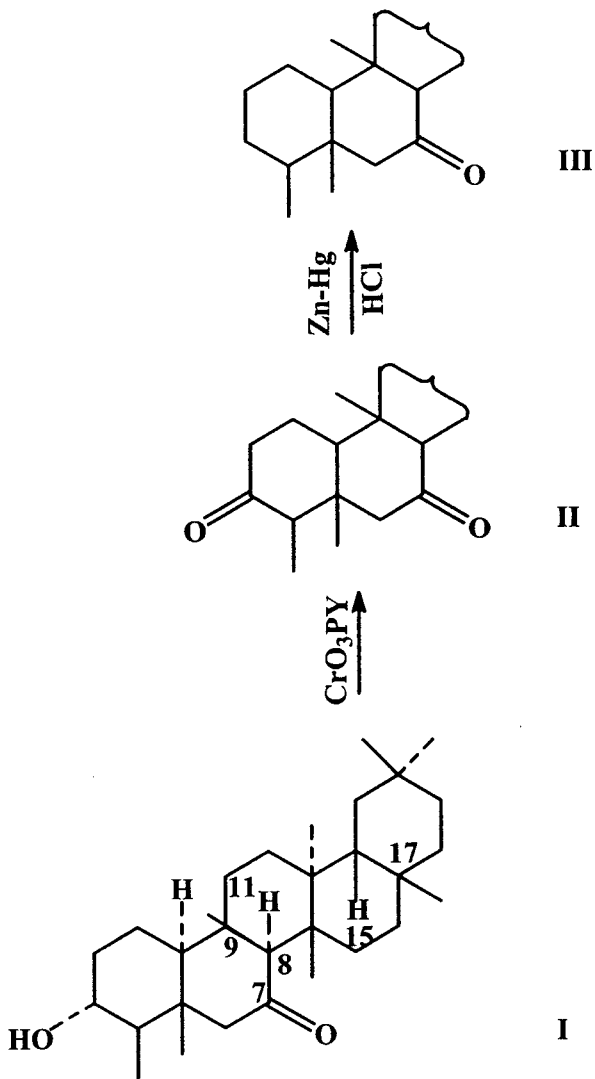
Figure 5. Chemical structure of putranjivanonol (I), putranjivadione (II) and putranjivone (III) (Garg and Mitra, 1971).

2. Diseases

2.1 Pain

Pain is classified into three types: physiological pain (acute pain), inflammatory pain, and neuropathic pain (Figure 6). Physiological pain has a high-threshold, is well localized and transient, and has a stimulus-response relationship. Its fundamental role is to operate a protective system, warning of contact with potentially damaging stimuli. Noxious stimuli required to elicit this pain are sufficiently different from those that produce innocuous sensations. This is due to highly specialized peripheral sensory pathways: the fine myelinated A and unmyelinated C primary afferent fibers for noxious stimuli and the thick myelinated A fibers for innocuous stimuli (Figure 6). Under physiological conditions, the innocuous stimulus does not induce pain, but rather relieve pain (original gate control theory). Tissue injury or inflammation involves the production and release of a number of chemical mediators including bradykinin, serotonin and PGs, which can originate locally or from cells that infiltrate the site of inflammation (Figure 7). A mixture of these agents known as inflammatory 'soup' contributes to changes in vascular permeability, resulting in erythema and edema. The inflammatory 'soup' also sensitizes peripheral nociceptors by initiating a cascade of events that change ionic conductances of the nociceptor peripheral terminal (Figure 6). Nociceptive projection neurons in the spinal cord transmit information to a number of regions of the brainstem and dienchephalon, including the thalamus, periaquaductal gray, parabrachial region, and bulbar reticular formation as well as to limbic structures via ascending pathways. Noxious inputs to thalamus are further project to primary and secondary somatosensory cortex (Figure 7) (Craig and Dostrovsky, 1999). Neuropathic pain is a condition that can result from trauma to the spinal cord and peripheral nerves or be accompanied with post-herpetic neuralgia and reflex sympathetic dystrophy. Under such pathological conditions, C fibers become hypersensitive and induce spontaneous pain and hyperalgesia to noxious stimuli. Innocuous tactile stimuli mediated by large A fibers generate pain called allodynia. These pain sensations outlast their biological usefulness. Therefore, there is considerable interest in the neurochemical mechanisms of hyperalgesia and allodynia in relation to inflammation and neuropathic pain (Dray et al., 1994; Woolf, 1994). As summarized in Figure 6, various animal models have been developed to elucidate the mechanisms of these painful states and to evaluate analgesics for exploring effective treatment. Now, these animal models have been often employed for behavioral analyses of knockout mice

in combination with the evaluation of pro- and antinociceptive agents. To help the understanding of the results introduced in this article, several animal models are briefly explained.
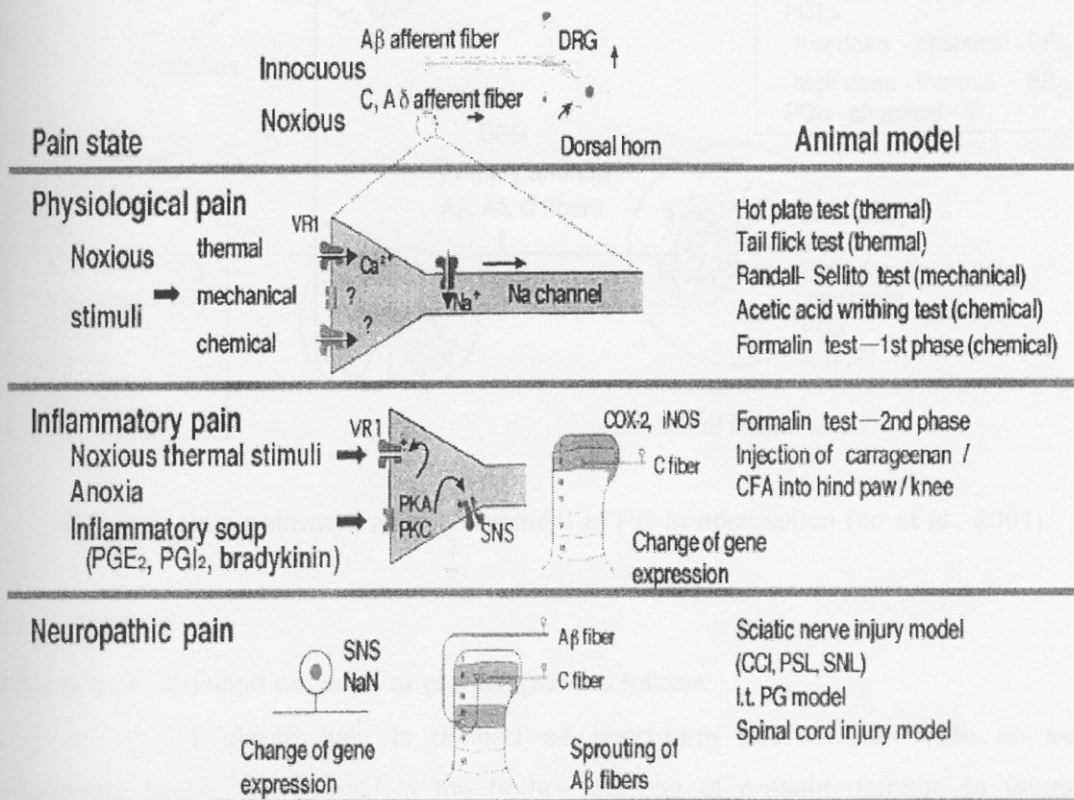
Figure 6. Three pain states and animal models (Ito et al., 2001).
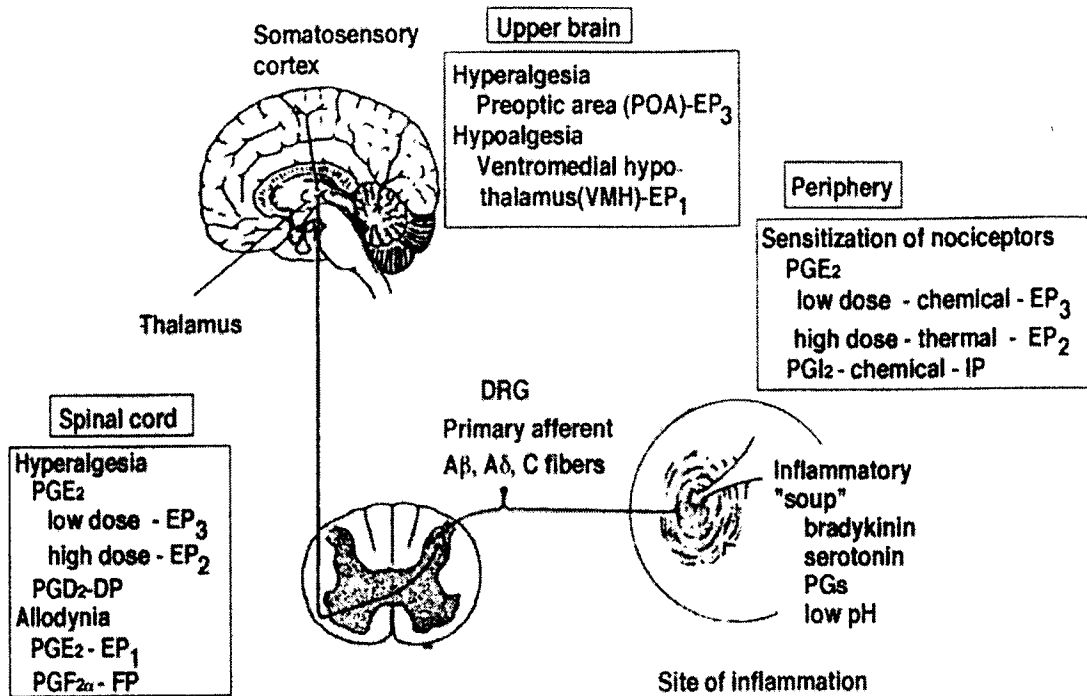
Figure 7. Pain pathways and involvement of PG in nociception (Ito et al., 2001).

Types of Pain

Pain can be classified as acute or chronic pain as follows.

1. Acute pain is defined as short-term pain or pain with an easily identifiable cause. Acute pain is the body's warning of present damage to tissue or disease. It is often fast and sharp followed by aching pain. Acute pain is centralized in one area before becoming somewhat spread out. This type of pain responds well to medications.

2. Chronic pain was originally defined as pain that has lasted 6 months or longer. It is now defined as pain that persists longer than the normal course of time associated with a particular type of injury. This constant or intermittent pain has often outlived its purpose, as it does not help the body to prevent injury. It is often more difficult to treat than acute pain. Expert care is generally necessary to treat any pain that has become chronic and coordinated treatment from an interdisciplinary health care team, including medical physicians, physical therapists, and psychologists or psychiatrists, often beneficial. An anterior cingulectomy, neurosurgery that disconnects the anterior cingulate gyrus, can be used in extreme cases to treat chronic pain. Post-surgery, the patient will still feel the sensation of pain, but not the accompanying emotion. There have been some

theories that in proper treating of acute pain can lead to chronic pain (Dahl and Moiniche, 2004). The experience of physiological pain can be grouped according to the source and related nociceptors (pain detecting neurons).

3. Neuropathic pain originates from the damage of peripheral or central nerves and neurons. It is certainly the form of pain most difficult to treat. It is often accompanied by intense spontaneous pain and by pain evoked by even modest stimulation. Inflammatory and neuropathic pain may outlast the duration of the primary cause of pain. They can thus turn into chronic pain syndromes, in which plastic changes in nociceptive processing have occurred that are no longer readily reversible by pharmacological treatment (Hanns, 2007).

Drugs used for pain

Morphine

Morphine is an extremely powerful opiate analgesic drug and is the principal active agent in opium. Like other opiates, morphine acts directly on the central nervous system (CNS) to relieve pain, and at synapses of the actuate nucleus, in particular. Side effects include impairment of mental performance, euphoria, drowsiness, lethargy, and blurred vision. It also decreases hunger, inhibits the cough reflex, and produces constipation. Morphine is highly addictive when compared to other substances. Tolerance and physical and psychological dependences develop quickly.

Administration: Morphine may be given parent rally as subcutaneous, intravenous, or epidural injections. When injected, particularly intravenously, morphine produces an intense contraction sensation in the muscles and thus produces a powerful 'rush'. The military sometimes issues morphine loaded in an auto injector.

Orally, it comes as an elixir, concentrated solution, powder (for compounding) or in tablet form. Morphine is rarely supplied in suppository form. Due to its poor oral bioavailability, oral morphine is only one-sixth to one-third of the potency of potential morphine. Morphine is available in extended-release capsules for chronic administration, as well as immediate-release formulations.

Morphine is used legally:

1. Analgesic in hospital settings for

    - Pain after surgery

    - Pain associated with trauma

2. In the relief of severe chronic pain

    - Cancer pain

    - Pain from kidney stones

3. As an adjunct to general anesthesia

4. In epidural anesthesia

5. For palliative care (i.e. to alleviate pain without curing the underlying reason

    for it)

6. As an antitussive for severe cough

7. As an antidiarrheal in chronic conditions (e.g., for diarrhea associated with

    AIDS)

Contraindications

- Acute pancreatitis (this may be a result of morphine use as well)

- Renal failure (due to accumulation of the morphine-6-glucuronide metabolite)

Mechanism of action

        Morphine (Figure 8) is an upload receptor agonist-Its main effect is binding to and activating the μ-upload receptors in the central nervous system. Activation of these receptors is associated with analgesia, sedation, euphoria, physical dependence and respiratory depression. Morphine is also a K-upload receptor agonist, with this action associated with spinal analgesia and mitosis.
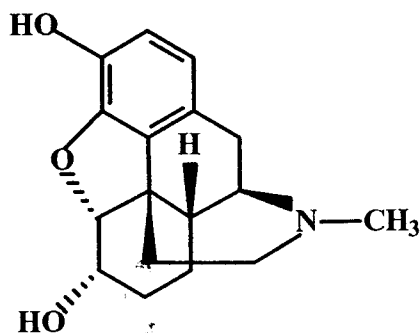
Figure 8. Structure of morphine

2.2 Fever

Fever is a regulated rise in body temperature (Tb) resulting from contact with infectious or inflammatory stimuli. Although there are considerable data indicating that moderate fevers are beneficial to the infected host, above a certain temperature (e.g., 41°C) the elevation in Tb, by itself, may be harmful (Kluger et al., 1975). Many cytokines are capable of modulating fever and are important for the orchestration of both systemic and local inflammatory responses. Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α have been implicated as key mediators of fever using several animal models. IL-1β, IL-6, IL-8, macrophage inflammatory protein-1β, interferon (IFN)-γ, and others have been characterized as endogenous pyrogens, or fever inducers (Kluger, 1991). Many hormones are also endogenous antipyretics; that is, they reduce fever. These include melanocyte-stimulating hormone, arginine vasopressin, and glucocorticoids. There is evidence that TNF-α may be an endogenous pyrogen under certain circumstances and an endogenous antipyretic under other conditions.

The action of pro-inflammatory cytokines on the CNS is, therefore, mainly modulated by the local production of intermediate molecules such as prostaglandins (PGs), which, in turn, activate hypothalamic centers responsible for thermoregulation, or by the cytokine-induced release of leptin from adipocytes. Leptin can freely cross the blood-brain barrier and directly stimulate hypothalamic thermoregulatory centers (Bishai et al., 1987). Nevertheless, prostanoids do increase in the brain of febrile cats (Sirko et al., 1989) and inhibitors of cyclooxygenase 2 (COX-2), one of the enzymes responsible for PGE$_2$ formation, are efficacious in attenuating LPS-induced fever (McCann et al., 2005).

Pathophysiology of fever

The febrile response is a complex physiologic reaction to disease involving a cytokine-mediated rise in body temperature, generation of acute-phase reactants, and activation of numerous physiologic, endocrinology, and immunologic systems (Mackowiak et al., 1997). The temperature of the body is dependent on maintaining a balance between the production and dissipation of heat. Under normal circumstances, heat is generated internally during metabolic processes or when external environmental temperatures exceed those of the body. Heat can also be produced by increased skeletal muscle activity, such as shivering. Heat loss occurs predominantly from the skin via

evaporative losses and also, to some extent, via the lungs. Much like other fundamental aspects of human biology, core body temperature is regulated closely by intricate control mechanisms, involving a complex interplay of autonomic, endocrine, and behavioral responses. The hypothalamus is central to this process, functioning as a thermostat, controlling thermoregulatory mechanisms that balance heat production with heat loss. Integral to the process is the heat-sensitive receptors located in the peptic area of the anterior hypothalamus. These receptors, which are sensitive to elevations in blood temperature, increase signal output as the temperature rises above a fixed thermal set-point (37.1°C average) and decrease output when the temperature drops below the set-point. Similar receptors are in the skin, spinal cord, and abdomen, sending impulses to the hypothalamus via the spinal cord.

With core body temperature elevations, the sympathetic system is inhibited, leading to vasodilatation of skin vessels and stimulation of the sweat glands to facilitate evaporative loss. This process prevails until the body temperature matches the thermal set point, when heat production matches heat loss. Similarly, when body temperature is below the thermal set point, a variety of responses is initiated to conserve and increase production of heat. They include activation of the sympathetic nervous system to induce vasoconstriction of skin blood vessels; inhibition of sweating; activation of the shivering center in the posterior hypothalamus, thereby increasing muscle heat production and secretion of neurotransmitters which increase cell metabolism and, consequently, heat production. The hypothalamus also affects behavioral influences in humans, with individuals changing clothes and/or seeking appropriate shelter to maintain body temperature. In vivo experiments with rabbits revealed that thermoregulation requires an intact sympathetic nervous system and can be modulated by various adrenoceptor antagonists (Bencsics et al., 1995; Ohashi and Saigusa, 1997).

Thermoregulatory substances

The stereotypic nature of the febrile response, regardless of the inciting event, has led to an improved understanding of the interactions between the immune and central nervous systems that cause fever. Fever is considered a hallmark of immune system activation, resulting in a regulated rise in body temperature. The regulation of this phenomenon is accomplished by the actions of two types of endogenous immune regulatory proteins called cytokines, some functioning as pyrogens and others acting as antipyretics (Figure 9) (Shalini and Donna, 2006).

A number of exogenous substances can evoke fever in animal models. Lipopolysaccharide (LPS), a cell wall product derived from gram-negative bacteria, has been the most extensively studied exogenous pyrogen, although others such as superantigens, peptidoglycans, muramyldipeptides (derived from gram-positive and gram negative bacteria), and viral products have also been investigated. When injected systemically into experimental animals, exogenous pyrogens have been shown to induce the production of pro-inflammatory cytokines, such as interleukins 1β (IL-1β) and 6 (IL-6), interferon (IF)-α, and tumor necrosis factor (TNF), which enter the hypothalamic circulation and stimulate release of local prostaglandins, resetting the hypothalamic thermal set point. The action of these pyrogenic cytokines may be opposed by other cytokines (IL-10) and substances such as arginine, vasopressin, melanocyte-stimulating hormone, and glucocorticoids), all of which have antipyretic properties, thus limiting the magnitude and duration of fever. TNF has been shown to have pyrogenic and antipyretic properties, depending upon the experimental conditions. Ultimately, it is the sum of the interactions of pyrogenic and antipyretic cytokines that is responsible for the height and duration of a febrile response.

Cytokines are thought to exert their effect on the brain via direct and indirect mechanisms (Sternberg, 1997). Peripherally produced cytokines reach the central nervous system (CNS) directly by crossing at leaky areas in the blood-brain barrier (BBB) via circumventricular vascular organs known as organum vasculosum laminae terminalis, which are networks of enlarged capillaries surrounding the hypothalamic regulatory centers (Watkins et al., 1995). In disease states such as bacterial infections, the BBB can be compromised further, leading to an influx of cytokines from the periphery and accounting for several of the neurologic manifestations associated with sickness behavior, including fever (Elmquist et al., 1997). Cytokines are also produced locally within the CNS

(Breder et al., 1988). This local production of cytokines in the CNS may account for the hyperpyrexia of CNS hemorrhage.

Evidence suggests that cytokines produced peripherally or centrally are involved directly in the complex autonomic febrile response (Sternberg, 1997; Watkins et al., 1995; Elmquist et al., 1997; Breder et al., 1988; Woiciechowsky et al., 1998). In the periphery, IL-1 and TNF induce an increased production of IL-6, the principal endogenous pyrogen (Luheshi et al., 1997). Among the cytokines measurable in plasma during LPS-induced fever, circulating levels of IL-6 have shown the best correlation with fever (Roth et al., 1993). Large amounts of IL-6 have been found to be present in all febrile diseases, and IL-6 induced by IL-1 or the combination of IL-1 plus TNF, likely accounts for the clinical fever. Mice without the gene for IL-6 do not develop fever during bacterial infection (Kozak et al., 1998) whereas IL-10 knockout mice develop exacerbated fever that correlates with enhanced plasma levels of IL-6 (Leon et al., 1999). Thus, endogenous IL-6 functions as a pyrogen, whereas IL-10 inhibits the production of IL-6, functioning as an endogenous antipyretic.

Although it is not fully understood, it is proposed that pro-inflammatory cytokines stimulate the central production of the inducible enzyme cyclooxygenase (COX) 2 and subsequently, the production of prostaglandins of the E series (Li et al., 2001a). These prostaglandins activate thermoregulatory neurons of the anterior hypothalamic area to elevate body temperature. Studies have shown that peripherally produced cytokines can also communicate with the brain indirectly in several ways, including the stimulation of terminal fibers of the autonomic nervous system. The vagal route is one of the best-known ways by which cytokines can influence the brain. Noradrenalin is the principal neurotransmitter, although several others, such as acetylcholine, endorphins, enkephalins, substance P, somatostatin, and vasoactive intestinal peptide, have also been implicated (Vizi, 1998).
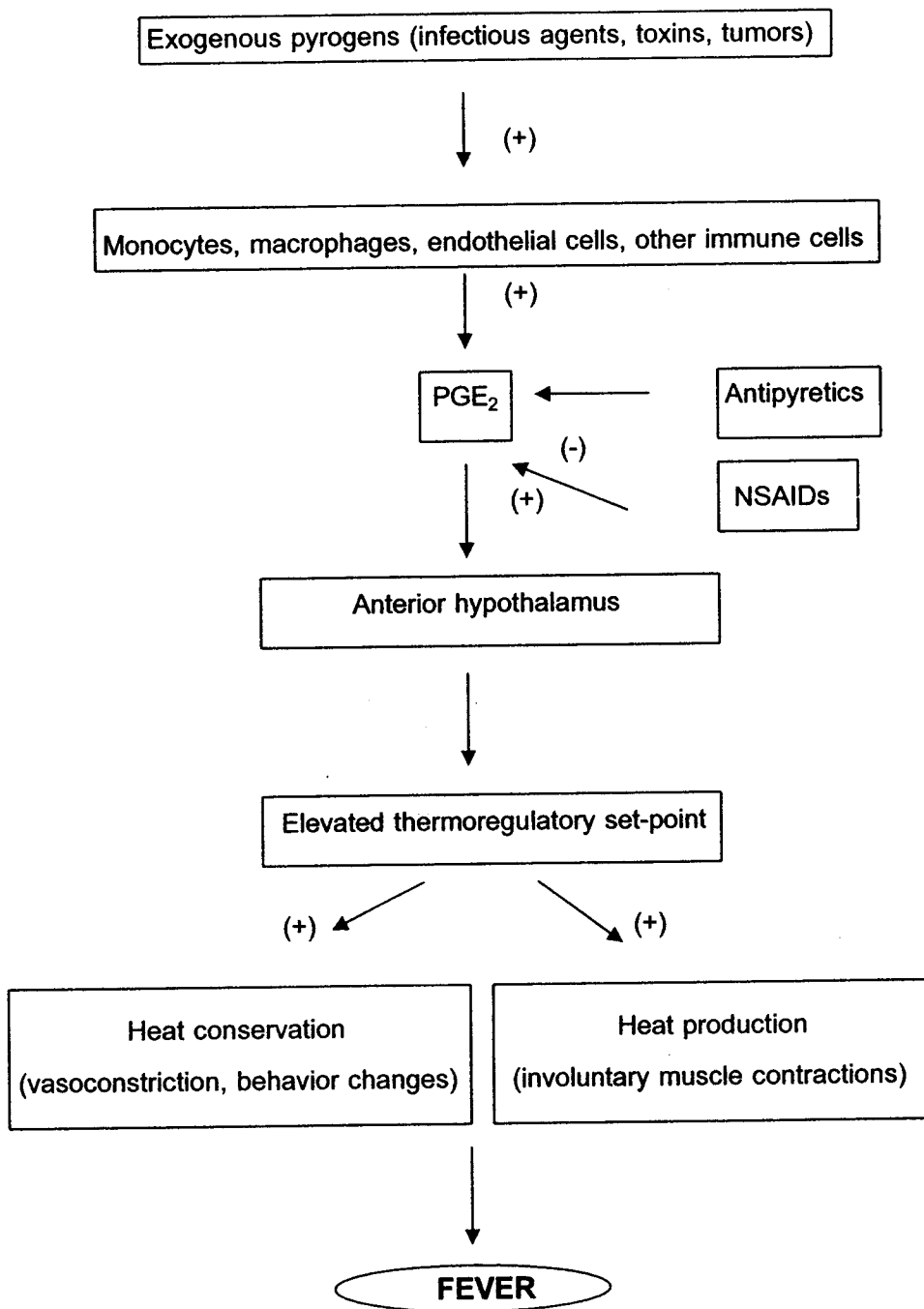
Figure 9. Pathophysiologic mechanism of fever (Shalini and Donna, 2006). Abbreviations: NSAIDs = nonsteroidal anti-inflammatory drugs; PGE_2=prostaglandin E_2.

Drugs used for fever
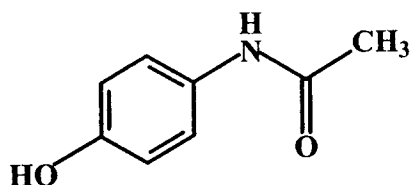
Figure 10. Structure of paracetamol (acetaminophen)

Paracetamol

Paracetamol (Figure 10) is a common analgesic and antipyretic drug that is used for the relief of fever, headaches, and other minor aches and pains. Paracetamol is also useful in managing more severe pain, allowing lower dosages of additional non-steroidal anti-inflammatory drugs (NSAIDs) or opioid analgesics to be used, thereby minimizing overall side-effects. It is a major ingredient in numerous cold and flu medications, as well as many prescription analgesics. It is considered safe for human use in recommended doses, but because of its wide availability, deliberate or accidental overdoses are fairly common.

Mechanism of action

Paracetamol has long been suspected of having a similar mechanism of action to aspirin because of the similarity in structure. That is, it has been assumed that paracetamol acts by reducing production of prostaglandins, which are involved in the pain and fever processes, by inhibiting the cyclooxygenase (COX) enzyme as aspirin does.

However, there are important differences between the effects of aspirin and those of paracetamol. Prostaglandins participate in the inflammatory response which is why aspirin has been known to trigger symptoms in asthmatics, but paracetamol has no appreciable anti-inflammatory action and hence does not have this side-effect. Furthermore, the COX enzyme also produces thromboxanes, which aid in blood clotting. Aspirin reduces blood clotting, but paracetamol does not. Finally, aspirin and the other NSAIDs commonly have detrimental effects on the stomach lining, where prostaglandins serve a protective role, but paracetamol is safe. Indeed, while aspirin acts as an irreversible inhibitor of COX and directly blocks the enzyme's active site, paracetamol

indirectly blocks COX, and this blockade is ineffective in the presence of peroxides (Boutaud et al., 2002). This might explain why paracetamol is effective in the central nervous system and in endothelial cells but not in platelets and immune cells which have high levels of peroxides.

In 2002, it was reported that paracetamol selectively blocks a variant of the COX enzyme that was different from known variants COX-1 and COX-2 (Swierkosz et al., 2002). This enzyme, which is only expressed in the brain and the spinal cord, is now referred to as COX-3. Its exact mechanism of action is still poorly understood, but future research may provide further insight into how it works.

A single study has shown that administration of paracetamol increases the bioavailability of serotonin (5-HT) in rats (Pina et al., 1996) but the mechanism is unknown and untested in humans. In 2006, it was shown that paracetamol is converted to N-arachidonoylphenolamine, a compound already known (AM404) as an endogenous cannabinoid. Such as, it activates the CB1 cannabinoid receptor; a CB (1) receptor antagonist completely blocks the analgesic action of paracetamol (Bertolini et al., 2006).

2.3 Inflammation

Inflammation is a local reaction of the vascular and supporting elements of a tissue to injury resulting in the formation of a protein-rich exudates; it is a protective response of the nonspecific immune system that serves to localize neutralize, or to destroy an injurious agent in preparation for the process of healing. The cardinal signs of inflammation are rubor (redness), calor (heat), dolor (pain), tumor (swelling), and functio laesa (loss of function). Cause of inflammation includes physical agents, chemical agents, immunological reactions, and infection by pathogenic organism (Wilson, 2003). Inflammation is divided into acute and chronic patterns. The characteristics of acute inflammation are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. Chronic inflammation includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and chronic lung diseases (Collins et al., 2001).

Type of inflammation

1. Acute inflammation

Acute inflammation is characterized by the rapid influx of Polymorphonuclear leukocytes (PMNs) into affected tissues. Tissue injury, microbes, and surgical trauma all activate the local biosynthesis of arachidonate-derived prostaglandins and leukotriene B_4 (LTB_4) through pathways involving cyclooxygenases (COX-1 and COX-2) and lipoxygenases (LOX). Initially, prostaglandins and LTB_4 control permeability, blood flow, and vascular dilation needed for leukocyte adhesion, diapedesis, and recruitment (Figure 11) (Levy et al., 2001).

2. Chronic inflammation

Chronic inflammation may progress from acute inflammation if the injurious agent persists, but more often than not, the response is chronic from the outset. In contrast to the largely vascular changes of acute inflammation, chronic inflammation is characterized by infiltration of damaged tissue by mononuclear cells such as macrophages, lymphocytes, and plasma cells, together with tissue destruction and attempts at repair. The macrophage is the key player of the chronic inflammatory response. This is due to the great number of bioactive products it releases. These mediators form part of the body's powerful defense against invasion and injury. The downside, however, is that persistent or pathological macrophage activation can result in continued tissue damage. This underlies a variety of disease processes from rheumatoid arthritis to atherosclerosis (Mairi et al., 2004).

Skin trauma
- Host invasion
- Surgical trauma
- Barrier loss

Bacteria

Macrophage

Neutrophil apoptosis

LTB₄

PMN recruitment

Lipoxins/ATLs
- Limit PMN infiltration
- Stimulate nonphlogistic phagocytosis of apoptotic neutrophils

LXB₄

Lipoxins

PMNs

LXA₄

Prostaglandins and LTB₄
- Regulate blood flow
- Vascular permeability
- Vascular dilation

Platelets

Postcapillary venule

Class switching

| Initiation | Prostaglandins | Leukotrienes | Lipoxins | Resolvins and protectins | Resolution |

Time

Figure 11. Temporal changes in lipid mediators in the progression of acute inflammation. Host invasion, surgical trauma, and barrier loss all initially stimulate prostaglandins and initiate early events in acute inflammation. LTB₄ is a potent chemo attractant that recruits PMNs, which move by diapedesis from postcapillary venules into the site of inflammation. During the progression of inflammation, intraluminal platelet-leukocyte transcellular interactions generate LXA₄ and LXB₄, which are 'stop signals' that reduce the further recruitment of PMNs. As exudates form and pustules are walled off, PGE₂ and PGD₂ signal termination by activating the expression of 15-LOX in neutrophils. This temporal dissociation and production of lipoxins from arachidonic acid is referred to as 'class switching' of eicosanoids from prostaglandins and leukotrienes to lipoxins, and accompanies a phenotypic shift in the neutrophils present within exudates. Next, new families of lipid mediators-resolvins and protectins-are generated from the omega-3 PUFAs eicosapentaenoic acid and docosahexaenoic acid. Aspirin jump-starts resolution by acetylating COX-2 and subsequently generating aspirin-triggered 15-epi-lipoxins as well as aspirin triggered epimers of both resolvins and protectins. As homeostasis is resumed, apoptotic neutrophils increase the expression of CC-chemokine receptor 5, which binds chemokines and helps clear the inflammatory milieu. The apoptotic

neutrophils then undergo nonphlogistic phagocytosis by macrophages. Abbreviations: ATL, aspirin-triggered lipoxin; COX, cyclooxygenase; LOX, lipoxygenase; LTB_4, leukotriene B_4; LXA_4, lipoxin A_4; LXB_4, lipoxin B_4; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PMN, polymorphonuclear leukocyte (Yacoubian and Serhan, 2007).

Arachidonic acid metabolism in inflammation

Arachidonic acid released from phospholipids by the action of the enzyme phospholipase A_2 can be metabolized by either cyclooxygenase 1 and 2 or by 5-lipoxygenase. Cyclooxygenases generate prostanoids like prostaglandin I_2 (PGI_2) and PGE_2 that are involved in physiological processes including platelet aggregation and regulation of gastrointestinal and renal blood flow. However, both PGI_2 and PGE_2 are potent vasodilator agents that contribute to the vascular signs of the inflammatory reactions. 5-lipoxygenase forms leukotrienes (LT) such as LTB_4, a potent chemotaxin, and LTC_4, LTD_4, LTE_4, which are potent bronchoconstrictor agents.

Prostaglandin H syntheses, This synthetic pathway begins with the generation of arachidonic acid from membrane phospholipids by the enzyme phospholipase A. Arachidonic acid then provides the substrate upon which a series of enzymatically catalyzed reactions occur. The enzyme 12-lipoxygenase converts arachidonic acid to the unstable intermediates 12-hydroperoxyeicosatetraenoic acid and 12-hydroxyeicosatetraenoic acid, which are further metabolized by 5-lipoxygenase, resulting in the formation of various leukotrienes. Conversely, metabolism of arachidonic acid by the enzyme prostaglandin (PG) H synthase results in the formation of prostaglandins. The cyclooxygenase activity of PGH synthase converts arachidonic acid to PGG_2, which is then metabolized by the peroxidase activity of this enzyme to PGH_2. The modification of PGH_2 by specific synthases results in the production of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2 (TXA_2) (Smith, 1992). These substances are important mediators of a variety of cellular functions. PGH synthase, also referred to as cyclooxygenase (COX), exists in two forms, termed COX-1 and COX-2 (Williams and DuBois, 1996). COX-1 is a constitutive isoform expressed on the endoplasmic reticular membrane of all cells, including gastric, kidney, and vascular cells, as well as in platelets (Morita et al., 1995), and it results in the formation of a variety of prostaglandins that mediate normal cellular functions. For example, maintenance of renal blood flow, gastric mucosal protection, and platelet activation and aggregation are all affected by COX-1-

catalyzed prostaglandin synthesis (Figure 9) (Smith, 1992). COX-2 is expressed constitutively in micro vascular endothelial cells, in which it is a source of PGI_2 under normal conditions (McAdam et al., 1999); this COX-2-derived PGI_2 may have an atheroprotective effect in laboratory animals (Egan et al., 2004). Low levels of COX-2 mRNA and protein have been identified in human platelets; however, COX-2 is not routinely expressed in most normally functioning cells. Rather, it is induced in the inflammatory state by growth factors, cytokines, and other inflammatory stimuli and results in the synthesis of prostaglandins that mediate the inflammatory response (Xie et al., 1991; Kujubu et al., 1991).

Prostanoids like PGI_2 and PGE_2 are powerful vasodilators in their own right and synergize with other autacoids like histamine and bradykinin. This combined dilator action on precapillary arterioles that contributes to the redness and increased blood flow in areas of acute inflammation. These prostanoids also potentiate the effect on vascular permeability by bradykinin and histamine, contributing to plasma exudation and the formation of inflammatory edema. PGI_2 and PGE_2 do not cause overt pain, but produce hyperalgesia by sensitizing afferent C fibers. Moreover, PGE_2 acts on neurones in the thermoregulatory network of the hypothalamus, causing increase in body temperature. There is general agreement that this prostaglandin mediates fever induced by endogenous pyrogens like interleukin 1 (IL-1) (Vane and Botting, 1995).

Especially PGI_2 and PGE_2, have important cytoprotective effects on the gastrointestinal (GI) mucosa. Different mechanisms contribute to this protective action: inhibition of secretion of both acid and pepsin, stimulation of mucus formation and bicarbonate secretion, and improved mucosal blood flow. Further, PGE_2 contributes to normal renal physiology by regulating the vascular tone and the normal blood flow (Katori and Majima, 2000), while PGI_2 is involved in vascular homeostasis and normal platelet functions (Vane and Botting, 1995).

Through a 5-hydroperoxy intermediate, 5-LOX enzyme produces the leukotrienes (LT), so called for their occurrence in leukocytes and a characteristic conjugated triene structure. The first compound to be formed is LTA_4, an unstable metabolite containing an epoxide moiety. Derived from LTA_4 are LTB_4 by enzymatic hydrolysis and LTC_4 by addition of the tripeptide glutathione catalyzed by glutathione-S-transferase. From LTC_4, LTD_4 and LTE_4 are produced. The three compounds form the group of the cysteinyl-leukotrienes (Penrose et al., 1999).

The biological effects of both prostanoids and leukotrienes are consistent with the role of mediators of the main phenomena of inflammation such as vascular changes, increase in body temperature, and leukocyte migration. Importantly, during inflammatory processes, these mediators are synthesized and released by resident and migrated cells in concentrations sufficient to cause their biological effects.

LTB_4 is a powerful chemotactic agent for both neutrophils and macrophages. On neutrophils, it also causes cell degranulation with release of lysosomal enzymes and upregulation of the membrane adhesion molecules. On macrophages and lymphocytes, it stimulates the release of pro inflammatory cytokines. The cysteinyl-leukotrienes (LTC_4, LTD_4, and LTE_4) are important mediators of bronchial asthma, causing constriction of bronchial tissue and edema mediated by augmented venular permeability. LTE_4 is less potent than LTC_4 and LTD_4, but its effect is much longer lasting. This allergic pulmonary inflammation results from the activation of cells with a full $5-LOX/LTC_4$ synthase pathway, including monocytes, eosinophils, basophils, and mast cells (Penrose et al., 1999).

Cyclooxygenase pathway (Figure 12)

The normal process begins with arachidonic acid, a dietary unsaturated fatty acid obtained from animal fats. This acid is converted by the enzyme cyclooxygenase to synthesize different prostaglandins. The prostaglandins go on to stimulate many other regulatory functions and reactionary responses in the body. Researches (Tannenbaum et al., 1996; Vane, 1996; Emery, 1996) have shown that there are two types of cyclooxygenase, denoted COX-1 and COX-2. Each type of cyclooxygenase leads itself to producing different types of prostaglandins.

Different mechanisms stimulate the two types of cyclooxygenase (COX). COX-1 is stimulated continuously by normal body physiology. The COX-1 enzyme is constitutive, meaning that its concentration in the body remains stable. It is present in most tissues and converts arachidonic acid into prostaglandins. These prostaglandins in turn stimulate normal body functions, such as stomach mucus production and kidney water excretion, as well as platelet formation. The location of the COX-1 enzyme dictates the function of the prostaglandins releases (Vane, 1996). For example, COX-1 in the stomach wall produces prostaglandins that stimulate mucous production. In contrast, the COX-2 enzyme is induced. It is not normally present in cells but its expression can be

increased dramatically by the action of macrophages, the scavenger cells of the immune system (Tannenbaum et al., 1996). COX-2's most important role is in inflammation. COX-2 is involved in producing prostaglandins for an inflammatory response. COX-2 is induced in various cells by cytokines, hormones, and mitogens and accounts for PG production in the course of inflammation. The increase in PG biosynthesis in inflamed tissue is accompanied by a rapid enhancement of COX-2 gene expression, which may prolong and enhance the biosynthesis of PGs (Ito et al., 2001). COX-1 is stimulated continually, and COX-2 is stimulated only as a part of an immune response.

Since both COX-1 and COX-2 are constitutively expressed in the brain and enhanced PG synthesis has been reported in various pathological processes, there has been considerable interest in the role of PGs that are synthesized in the CNS COX-1 was distributed in neurons throughout the brain, but it was more prominent in the forebrain, where PGs may be involved in complex integrative functions including modulation of sensory processing (Breder et al., 1992). COX-2 was first identified in rat CNS as one of mRNAs induced by electrical seizures (Yamagata et al., 1993). The expression of COX-2 in the normal CNS was restricted to parts of cortex, hippocampus, hypothalamus, amygdala, and brainstem involved in the processing of nociceptive sensory input and in the generation of coordinated autonomic, endocrine, and behavioral response (Yamagata et al., 1993; Breder et al., 1995).

Lipoxygenase pathway

LTs are lipid messengers that play central role in immune responses and tissue homeostasis (Funk, 2001). Biosynthesis of LTs from AA was initially described in polymorphonuclear leukocytes and monocytes. First described in 1937 as the slow reacting substances of anaphylaxis (SRS-A), these lipid mediators are now known as the cysteinyl LTs (CysLTs), LTC_4, LTD_4 and LTE_4 (Lewis et al., 1990). Synthesis of LTs can be divided in two pathways: one to create CysLTs and another to create LTB_4 (Figure 13). Some of important properties of 5-LO pathway proteins are summarized. 5-LO is the key enzyme in LT biosynthesis and is located in the nucleus in some cell types and in the cytosol of others (Peters-Golden and Brock, 2001). 5-LO is 72- to 80-kd monomeric soluble protein containing one nonheme iron believed necessary for catalysis (Rouzer et al., 1985). This enzyme possesses an NH2-terminal domain that binds to calcium iron and is essential for nuclear membrane translocation (Chen and Funk, 2001). Active

mainly in myeloid cells, such as monocytes, macrophages, B lymphocytes, granulocytes, mast cells, and DC, 5-LO requires Ca^{2+} and is stimulated by ATP, phosphatidylcholine, lipids, and hydroperoxides (Radmark, 2002). Following cellular activation, 5-LO translocates to the nuclear membrane where it is able to interact with an 18-kd membrane-associated protein referred to as five-lipoxygenase-activating protein (FLAP). FLAP is an AA-binding protein whose function is to optimally present substrate to 5-LO (Mancini et al., 1993). The first evidence that the 18-kd protein is absolutely required for cellular5-LO activity comes from transfection studies in human osteosarcoma cell lines (Dixon et al., 1990). In this system, expression of 5-LO alone resulted in no detectable cellular 5-LO activity following challenge with calcium ionophore A-23187. LT synthesis only occurred when 5-LO and FLAP were coexpressed in these cells. FLAP is expressed on T cells (Jakobsson et al., 1992), macrophages, and DC (Harizi et al., 2003), but not on erythrocytes or endothelial cells.

Cellular activation by immune complexes, bacterial peptides, and other stimuli elicit a sequence of events that include cytosolic phospholipase A_2 (cPLA$_2$) and 5-LO translocation to the nuclear envelope to produce 5- hydroperoxy eicosatetraenoic acid (5-HPETE) from AA. Subsequently, 5-HPETE is dehydrated to yield the epoxide LTA$_4$, a pivotal intermediate in the biosynthesis of inflammatory and anaphylactic mediators. LTA$_4$ undergoes transformation by one or more of three possible fates depending on the cellular context: hydrolysis, conjugation with glutathione, or transcellular metabolism to generate bioactive lipid mediators (Gronert, 1999). In neutrophils and monocytes, LTA$_4$ is converted predominately to the chemoattractant LTB$_4$ by LTA$_4$ hydrolase (Radmark et al., 1984), but in human eosinophils, mast cells, and basophils, LTA$_4$ is conjugated with reduced glutathione by LTC$_4$ synthase to form the first of the CysLTs, LTC$_4$ (Yoshimoto et al.,1988). After carrier-mediated cellular export, sequential cleavage of the glutathionyl side chain of LTC$_4$ generates the extracellular metabolites LTD$_4$ and LTE$_4$. LTB$_4$ may be degraded by microsomal ω-oxidation and peroxisomal β-oxidation in myeloid cells and hepatocytes. Degradation is accompanied by loss of biological activity. It is important to note that degradative enzymes are increased by the transcription factor peroxisome proliferator-activated receptor α (PPARα) and this nuclear hormone receptor is in turn activated by binding with LTB$_4$ (Devchand et al., 1996). This feedback loop limits the duration of action of LTB$_4$.
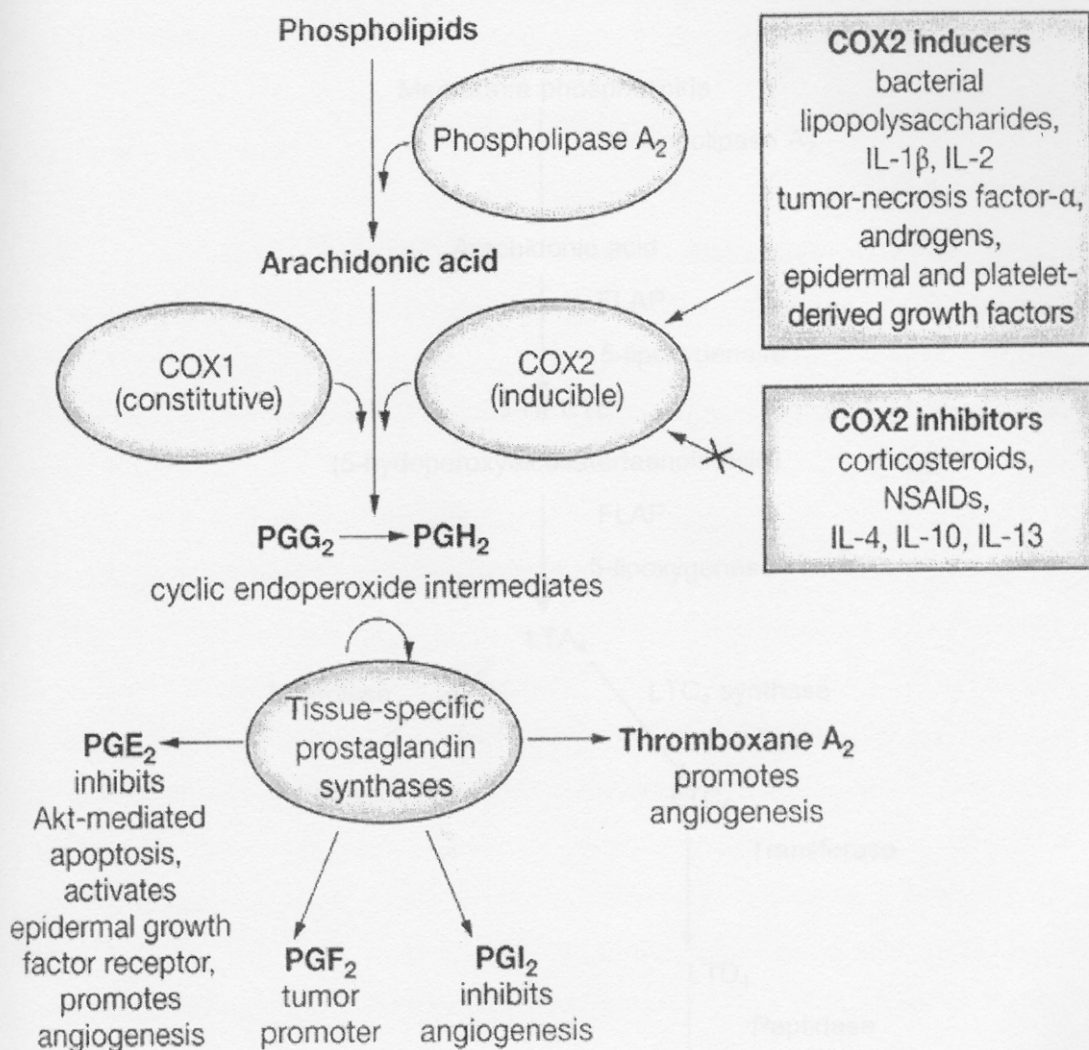
Figure 12. Arachidonate cascade. PLA_2, phospholipase A_2; COX, cyclooxygenase and Prostaglandin synthesis (Zha et al., 2004).

Membrane phospholipids

Phospholipase A_2

Arachidonic acid

FLAP

5-lipoxygenase

5-HPETE

(5-hydoperoxyeicosatertaenoic acid)

FLAP

5-lipoxygenase

LTA_4

Hydrolase

LTC_4 synthase

LTB_4

LTC_4

Transferase

LTD_4

Peptidase

LTE_4
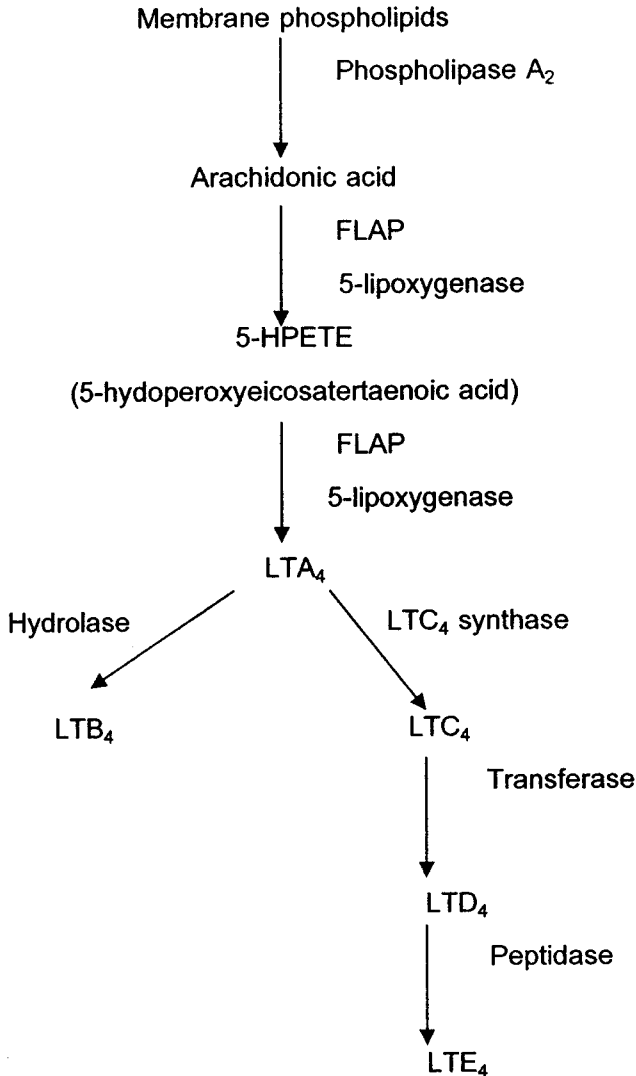
Figure 13. Pathways of LT biosynthesis. Arachidonic acid is liberated from membrane phospholipids by the action of cytosolic phospholipase A_2 (cPLA$_2$) and presented to 5-LO enzyme by FLAP. 5-LO enzyme converts arachidonic acid to an unstable intermediate termed 5-hydroperoxyeicosatetraenoic acid (5-HPETE). Subsequently, 5-HPETE is dehydrated to yield the epoxide LTA_4, a pivotal intermediate in the biosynthesis of inflammatory and anaphylactic mediators. Enzymatic hydrolysis of LTA_4 by LTA_4 hydrolase results in the formation of LTB_4. Alternatively, LTC_4 synthase catalyzes the conjunction of LTA_4 with glutathione to form LTC_4, which may be converted to LTD_4 and LTE_4 by the actions of the transferase and peptidase enzymes, respectively (Harizi and Gualde, 2004).

Drugs used of for inflammation

Anti-inflammatory drugs

Anti-inflammatory drugs are used to treat rheumatoid disorders, and also in other inflammatory diseases and injuries. Their anti-inflammatory activity is due to their ability to inhibit the cyclooxygenase activity of prostaglandin synthase, an enzyme which mediates the production of prostaglandins from arachadonic acid. These drugs were developed as an alternative to the corticosteroids and their analogues, which have many side effects.

Non-Steroidal Anti-inflammatory Drugs (NSAIDS)

NSAIDs are used to treat pain, fever, and inflammation. Traditional NSAIDs block COX-1 and COX-2 enzymes that the body uses to manufacture substances called prostaglandins. Since COX-1 prostaglandins are stomach-protective, blocking this enzyme is associated with gastrointestinal toxicity, a known side effect of these drugs. Newer NSAIDs, called COX-2 inhibitors block primarily COX-2 prostaglandins associated with pain, fever, and inflammation, and are less risky to the stomach. However, this is not proven, and some COX-2 inhibitors have been taken off the market due to excess risk of heart attacks attributable to their use. Drugs in this family are as follows.

Aspirin

Aspirin (Figure 14) or acetylsalicylic acid is a drug in the family of salicylates, often used as an analgesic (against minor pains and aches), antipyretic (against fever), and anti-inflammatory. It has also an anticoagulant (blood-thinning) effect and is used in long-term low-doses to prevent heart attacks.

Low-dose long-term aspirin irreversibly blocks the formation of thromboxane A_2 in platelets, producing an inhibitory effect on platelet aggregation, and this blood-thinning property makes it useful for reducing the incidence of heart attacks. Aspirin produced for this purpose often comes in 75 or 81 mg dispersible tablets and is sometimes called "junior aspirin" or "Baby aspirin." High doses of aspirin are also given immediately after an acute heart attack. These doses may also inhibit the synthesis of prothrombin and may therefore produce a second and different anticoagulant effect.

Several hundred fatal overdoses of aspirin occur annually, but the vast majority of its uses are beneficial. Its primary undesirable side effects, especially in stronger doses, are gastrointestinal distress (including ulcers and stomach bleeding) and tinnitus. Another side effect, due to its anticoagulant properties, is increased bleeding in menstruating women. Since there appears to be a connection between aspirin and Reye's syndrome, aspirin is no longer used to control flu-like symptoms in minors (Macdonald, 2002).
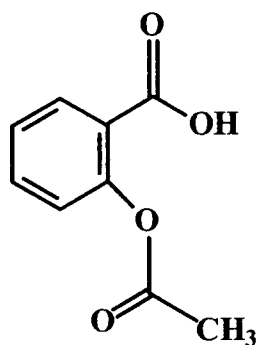
Figure 14. Structure of aspirin

Mechanism of action

The pharmacological effects of aspirin are mediated primarily through its interference with prostaglandin biosynthesis (Antman et al., 2005). Aspirin's inhibitory effect on prostaglandin biosynthesis is mediated by its covalent modification of COX through the irreversible acetylation of a specific serine moiety (Kujubu et al., 1991; Roth and Majerus, 1975). Aspirin-induced acetylation of serine 529 of COX-1 results in a conformational change in the active site of the enzyme, thus preventing it from binding arachidonic acid. Conversely, aspirin-induced acetylation of serine 516 of COX-2 alters the function of the enzyme so that it metabolizes arachidonic acid to 15-*R*-hydroxyeicosatetraenoic acid (Smith and DeWitt, 1995). In the presence of aspirin, both COX-1 and COX-2 become incapable of converting arachidonic acid to PGH_2, a necessary precursor of prostaglandin biosynthesis. The inhibition of COX-2 likely accounts for the beneficial anti-inflammatory effects of aspirin, whereas inhibition of COX-

1 accounts for its antithrombotic effect. Inhibition of COX-1 also interferes with production of the homeostatic prostanoids, thereby potentially resulting in serious toxicities, including renal failure, gastric mucosal ulceration, and impaired hemostasis. Aspirin does not inhibit the activity of lipoxygenase; thus, it does not affect the generation of leukotrienes.

Physiologically, aspirin is a relatively weak inhibitor of platelets because it blocks only thromboxane-dependent platelet activation and aggregation. Several other stimuli, including shear force, increased plasma catecholamines, thrombin, and ADP, can activate platelets despite aspirin therapy and likely contribute to thrombosis in acute coronary syndromes (Folts et al., 1999).

Indomethacin
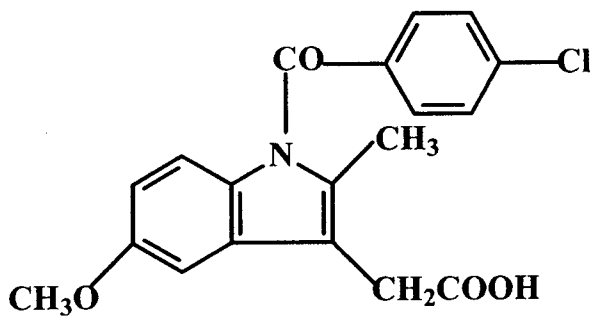
Figure 15. Structure of indomethacin

Indomethacin (INN) or Indomethacin (USAN and former BAN) is a non-steroidal anti-inflammatory drug commonly used to reduce fever, pain, stiffness, and swelling. It works by inhibiting the production of prostaglandins, molecules known to cause these symptoms. It is marketed under many trade names, including Indocin, Indocid, Indochron E-R, and Indocin-SR.

Mechanism of action

Indomethacin (Figure 15) is a nonselective inhibitor of cyclooxygenase (COX) 1 and 2, enzymes that participate in prostaglandin synthesis from arachidonic acid. Prostaglandins are hormone-like molecules normally found in the body, where they have a wide variety of effects, some of which lead to pain, fever, and inflammation.

Prostaglandins also cause uterine contractions in pregnant women. Indomethacin is an effective tocolytic agent, able to delay premature labor by reducing uterine contractions through inhibition of PG synthesis in the uterus and possibly through calcium channel blockade.

Indomethacin has two additional modes of actions with clinical importance:

- It inhibits motility of polymorphonuclear leucocytes, similar to colchicines.
- It uncouples oxidative phosphorylation in cartilaginous (and hepatic) mitochondria, like salicylates.

These additional effects account as well for the analgesic and the anti-inflammatory properties. Indomethacin readily crosses the placenta, and can reduce fetal urine production to treat polyhydramnios. It does so by reducing renal blood flow and increasing renal vascular resistance, possibly by enhancing the effects of vasopressin on the fetal kidneys.

2.4 Hemorrhoids

Hemorrhoids are mentioned in ancient medical writings of every culture, including Babylonian, Hindu, Greek, Egyptian, and Hebrew. The word "hemorrhoid" is derived from the Greek "haema" = blood, and "rhoos" = flowing, and was originally used by Hippocrates to describe the flow of blood from the veins of the anus.

Hemorrhoids are normal anatomic features of the human anal canal, forming pads that bulge into the lumen. The anorectal area has a mucosal lining, a framework composed of blood vessels, smooth muscle and supporting tissues, as well as an anchoring connective tissue system that secures the hemorrhoidal tissues to the internal sphincters. This hemorrhoidal system is reported to cushion the anal canal during defecation (Abcarian et al., 1994).

The anal canal sub mucosa is discontinuous and composed of three complexes located at the right posterior, right anterior and left lateral anal wall (Cocchiara,

1991; Pfenninger, 1995; Hodes, 1996). These three areas are often referred to as the anal canal cushions. Their distribution is important because the discontinuous arrangement allows the anal canal to distend, when signaled by the pressure receptors, during a bowel movement. In addition, these cushions have a rich vascular system, both arterial and venous (Pfenninger, 1995).

The dentate, pectinate or anorectal line is the point where squamous epithelium of the outer anal skin transitions to a mucosal glandular epithelium. This is significant to note because pain fibers generally cease at the dentate line. As a result, hemorrhoidal disorders above the dentate line rarely cause discomfort and those below the line usually cause pain (Pfenninger, 1995; Hodes, 1996).

Also important to the anatomy of the anorectal area are the encircling muscles. The internal and external sphincters control the passage of fecal material into and out of the anal canal. The involuntary muscle responsible for the control of defecation is the internal sphincter. Feces are passed into the canal via autonomic stimulation. On the other hand, the external sphincter is under voluntary control and remains closed to prohibit the passage of fecal materials out of the rectum (Hodes, 1996; Wuest and Gossel, 1992).

Hemorrhoid disease is present when there are alterations to this normal anatomy, causing blood vessels that lie beneath the anal mucosa and perianal skin to dilate. In addition, the muscular and connective tissue supports may become damaged, resulting in the downward displacement of hemorrhoidal cushions. These events then lead to symptomatology and possible complications of hemorrhoids.

Anatomy of hemorrhoids

The arteries supplying blood to the anal canal descend into the canal from the rectum above and form a rich network of arteries that communicate with each other around the anal canal. Because of this rich network of arteries, hemorrhoidal blood vessels have a ready supply of arterial blood. This explains why bleeding from hemorrhoids is bright red (arterial blood) rather than dark red (venous blood), and why bleeding from hemorrhoids occasionally can be severe. The blood vessels that supply the hemorrhoidal vessels pass through the supporting tissue of the hemorrhoidal cushions. The anal veins drain blood away from the anal canal and the hemorrhoids. These veins drain in two directions. The first direction is upwards into the rectum, and the second is

downwards beneath the skin surrounding the anus. The dentate line is a line within the anal canal that denotes the transition from anal skin (anoderm) to the lining of the rectum.

Classification of Hemorrhoids

Hemorrhoids are classified by their point of origin: internal, external, and internal- external.

Internal Hemorrhoids

Internal hemorrhoids originate above the pectinate or dentate line in the anal canal and are covered by a mucous membrane. The internal hemorrhoids become symptomatic when the anal lining and connective tissues become loose and weakened and the vascular system becomes engorged. A grading system has been defined for internal hemorrhoids to assist with the selection of a treatment plan. The grade of the hemorrhoid depends on the extent of descent into the anal canal and out of the anus. First degree internal hemorrhoids are enlarged; however, they never prolapse and rarely produce symptomatology. Second degree internal hemorrhoids prolapse with defecation but they return to their original position spontaneously. Neither first nor second degree hemorrhoids can be visualized during an external examination but require the use of a proctoscope. On the other hand, third degree internal hemorrhoids prolapse with each bowel movement and even with physical exertion. These specific hemorrhoids require manual replacement back to their point of origin. Fourth degree internal hemorrhoids are permanently prolapsed despite attempts to reintroduce them to their original position (Abcarian et al., 1994; Cocchiara, 1991; Pfenninger, 1995; Hodes, 1996). They are especially at risk for thrombosis, severe pain and bleeding.

Internal hemorrhoids are classified by history and not by physical examination. They are graded as follows:

Grade I internal hemorrhoids project into the anal canal with minimal bleeding or may be asymptomatic, but they do not prolapse.

Grade II hemorrhoids protrude beyond the anal verge with straining or defecating and reduce spontaneously when straining ceases.

Grade III hemorrhoids protrude either spontaneously or with straining and will require manual reduction.

Grade IV hemorrhoids chronically prolepses and, if they are reducible,

they fall out again. Irreducible, strangulated hemorrhoids are a surgical emergency.

External Hemorrhoids

External hemorrhoids are swollen areas of skin and blood vessels around the anus (below the dentate line) (Hodes, 1996).They are lined with squamous epithelium that is highly innervated and sensitive. These particular hemorrhoids are also at risk for thrombosis.

Internal-External: When internal and external hemorrhoids occur simultaneously, they are referred to as mixed hemorrhoids (Pfenninger, 1995; Hodes, 1996).

Epidemiology/Etiology

Hemorrhoid disease is a common disorder of the anorectal area. It has been estimated that hemorrhoids affect over 10 million individuals in the United States (Abcarian et al., 1994; Hodes, 1996). Reports suggest that one-half of all Americans over the age of 50 have suffered from hemorrhoids (Hodes, 1996; Wuest and Gossel, 1992; Sause, 1995). The exact cause of hemorrhoids has not been determined. However, several factors appear to contribute to their etiology. Patients with a low dietary fiber intake may be at risk for developing hemorrhoids (Abcarian et al., 1994; Hodes, 1996; Wuest and Gossel, 1992). Typically, these patients' stool is hard with very little bulk or moisture, thereby increasing the risk of straining during a bowel movement. These results in elevated pressure in the rectal vascular system which could lead to hemorrhoidal disorders (Wuest and Gossel, 1992). Pregnancy is another factor linked to hemorrhoids. Due to the in utero placement of the fetus, pregnant women have increased pressure in the anorectal area which may result in internal hemorrhoids (Abcarian et al., 1994; Hodes, 1996; Wuest and Gossel, 1992). In addition, labor may aggravate the condition, prolonging symptoms after the birth process.

Another population at risk for hemorrhoids is those individuals whose occupations require heavy lifting or prolonged standing (Cocchiara, 1991; Wuest and Gossel, 1992). Heavy lifting may put additional strain on both the muscles and venous system in the anorectal area, making hemorrhoidal development a possibility. Prolonged standing may cause blood to pool in the rectal vascular system, especially since there are no venous valves in this area, thus predisposing workers to hemorrhoidal problems.

Decreased physical activity may also contribute to hemorrhoid development. Less active individuals may experience a decline in muscle tone of the anorectal area, thereby making the patient prone to weak hemorrhoidal cushions. Other patients at risk for the development of hemorrhoids are those who overuse stimulant laxatives. These individuals are at risk of chronically straining the anal sphincters during defecation, which may result in symptoms (Wuest and Gossel, 1992).

Signs and Symptoms

Signs and symptoms of hemorrhoid disease are the result of vascular congestion that occurs when veins become engorged secondary to obstructed blood flow. In addition, collagen and elastin that support the anorectal region weaken, causing hemorrhoidal cushions to lose their support and become displaced.

Bleeding: One of the most common symptoms of hemorrhoids is bleeding from the rectal area before, during and after defecation. The anal mucosa becomes eroded, releasing a bright red blood usually evident to the patient via blood-tinted toilet bowl water, stool or toilet tissue. The blood may result from external or internal hemorrhoids (Abcarian et al., 1994; Cocchiara, 1991; Hodes, 1996).

Itching: Itching may result secondary to mild inflammation usually associated with prolapsed internal hemorrhoids that secrete a mucous discharge. Further irritation and itching can occur when dyes and perfumes in fabrics and toilet tissue come into contact with the already sensitive area.

Inflammation and Swelling: Inflammation and swelling may result from trauma imposed on the anorectal area due to engorged hemorrhoidal tissues. Inflamed tissues may cause pain, especially to a patient with external hemorrhoids. Standing for prolonged periods of time or defecation may intensify the pain. As expected, those with uncomplicated internal hemorrhoids should not feel pain secondary to an absence of pain receptors above the dentate line.

Seepage: Seepage of fecal matter or mucus may occur if persistent swelling or inflammation in the anorectal area prevents the anal sphincter from closing properly.

Protrusion and Prolapsed: A frequent sign of uncomplicated internal or external hemorrhoids is protrusion, a projection of tissue outside the anal canal. The protrusion may be evident following a bowel movement, prolonged standing or physical

exertion. Unless the protrusion becomes thromboses, infected, or ulcerated, it is painless. A prolapsed hemorrhoid is an internal hemorrhoid that protrudes beyond the dentate line and is seen at the anal orifice (Hodes, 1996).

Thrombosis: When hemorrhoids remain persistently prolapsed, patients are at risk for thrombosis. This is a lump or clot that may cause severe pain in the rectal area for approximately a week (Cocchiara, 1991; Hodes, 1996). The lump slowly disappears; however, a skin tag remains evident. If, by chance, the thrombus hemorrhoid does not heal, then gangrene as well as ulceration may ensue, leading to further complications of the anorectal area.

Drugs used for hemorrhoid

Anti-hemorrhoid drugs are medicines that reduce the swelling and relieve the discomfort of hemorrhoids (swellings in the area around the anus).

Daflon 500 mg is a unique micronized purified flavonoid fraction (MPFF) containing 90 % diosmin and 10 % flavonoids expressed as hesperidin. Indications, treatment of venous disease, i.e., chronic venous disease and hemorrhoidal disease (HD). The efficacy of Daflon 500 is accounted for by its specific action on the principal elements of venous disease. Daflon 500 is phlebotonic: It reinforces venous tone by prolonging the activity of parietal noradrenalin. Thus, Daflon 500 decreases venous capacitance, venous distensibility and venous emptying time. Daflon 500 protects the microcirculation by fighting the microcirculation-damaging process; it combats venous inflammation by decreasing leukocyte activation, and as a consequence, by inhibiting the release of inflammatory mediators, principally free radicals and prostaglandins. Thus, Daflon 500 normalizes capillary permeability and strengthens capillary resistance. Daflon 500 acts on the lymphatic system: It improves lymphatic drainage by increasing lymph flow and lymph oncotic pressure. This action on the lymphatic system associated with a phlebotonic and vasculoprotective effect, explains the activity of Daflon 500 on chronic venous insufficiency associated edema.

Siduol consists of dl-alpha-tocopheryl calcium succinate (vitamin E) 25 mg, lysozyme chloride 10 mg, rutin 100 mg, vitamin K1 12 mg, pluronic 20 mg. Indications, for treatment of internal and external hemorrhoids, anal fissures and associates subjective symptoms

Objective: To evaluate the antinociceptive, antipyretic and anti-inflammatory activities of the ether and ethanol extracts from the leaves of *P. roxburghii* Wall. in animal models.

CHAPTER 2

MATERIALS AND METHODS

Plant material

The air dried leaf of *P. roxburghii* Wall. (Euphorbiaceae) was collected and purchased from herbal drugstores in Songkhla Province, Thailand. The plant material was identified by direct comparison with herbarium specimen at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Voucher specimens (number: SS 2549-001) of the plant material were deposited in the same place. The air dried leaf was pulverized and stored in air tight, light protect container.

Preparation of the extracts from the leaves of *Putranjiva roxburghii* Wall. (Garg and Mitra, 1971)

The air dried leaves of *P. roxburghii* (1.2 kg) were macerated with 95% ethanol at room temperature, filtered and evaporated to give a syrupy mass. The marc was remacerated with ethanol four times, filtered and evaporated. All syrup masses were mixed to give 350 g ethanol extract (29% yield). The ethanol extract was further exhaustively extracted with ether and the ethereal solution thus obtained was separated into acidic and neutral fractions by washing with 0.5% NaOH solution. The alkali soluble fraction on acidification extracts with ether and evaporated to give a syrupy mass of 84 g ether extract (24% yield). These ethanol extract and ether extract were dissolved in cosolvent (propylene glycol: tween 80: water 4:1:4) (Reanmongkol et al., 2002) and used as the test extracts. All doses were expressed in terms of mg of crude extract/ kg body weight. The ether extracts containing flavonoids after evaporation of the solvent. The crude mixture of flavonoids and other compounds was subjected to silica gel column chromatography. The elution was commenced with $CHCl_3$-MeOH and $CHCl_3$ to give putraflavone and roxburghonic acid and then collected and analyzed by TLC. The ether extract compounds were examined by TLC using with several solvent systems of different polarities (2%, 5%, 10%, 50% MeOH-$CHCl_3$ and 100% $CHCl_3$) as the mobile phase. The

flavonoid type components were visualized first under UV-light and then by spraying the TLC plate with spraying agent and heat.

Animals

Animals used in this study were male Swiss albino mice weighing 25-35 g and male Wistar rats weighing 150-200 g. The rats were handled for 5-10 min daily for several days before experiments. All animals were obtained from the Animal House, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were housed for at least one week in the laboratory animal room prior to testing. Food and water were given *ad libitum* unless otherwise specified. All experimental protocols were approved by the Animal Ethic Committee, Prince of Songkla University, Thailand (No 0521.11/166). When the experiments were finished, all of experimental animals were terminated by cervical dislocation.

Chemicals and Instruments

The following drugs were used: morphine sulfate, Brewer's yeast, carrageenin, croton oil (Sigma Chem. Co., St. Louis, U.S.A), aspirin, tween 80 (Srichand United Dispensary Co., Ltd., Bangkok, Thailand), sodium chloride (Carlo Erba, Germany), acetic acid (AR grade, J.T, Baker Inc., Phillipsburg U.S.A), propylene glycol, indomethacin (Vidhyasom Co., Ltd., Bangkok, Thailand), diethyl ether, chloroform, ethanol (Labscan Asia Co., Ltd), acetone (Fluka Biochemika).

Plethysmometer (Ugo Basile, Italy), Hot plate analgesia meter (Harvard Apparatus Ltd., U.K.) and Digital thermometer (SK-1250 MC, Sato Keiryoki Mfg. Co., Ltd., Japan) were used.

Test agents administration

Group	Name group	Details
1	Cosolvent	propylene glycol:tween 80:distilled water = 4:1:4
2, 3	Standard drug	Aspirin: 200 mg/kg
		Indomethacin: 1.00 mg/ear
		Morphine sulfate: 10 mg/kg
4,5,6	Ether extract	100, 200, 400 mg/kg, respectively.
7,8,9	Ethanol extract	100, 200, 400 mg/kg, respectively.

Methods

Acute toxicity

The ether and ethanol extracts of *P. roxburghii* at the dose of 2 g/kg (OECD Test Guideline 401,1987) was orally administered to a group of both male and female mice (each sex consists of 10 mice). Behavior parameters were observed during a period of 8 hours and 7 days after the extracts administration such as convulsion, hyperactivity, sedation, grooming, and loss of righting reflex, increased or decreased respiration. Food and water were given *ad libitum*.

Antinociceptive activities

1. Writhing test

Writhing behavior was tested, in which 0.6% acetic acid solution (10 ml/kg body weight) was injected intraperitoneally and the number of writhings and stretchings was counted over a 20 min period as previously reported (Koster et al., 1959; Hendershot & Forsaith, 1959). The ether and ethanol extracts of *P. roxburghii* (100, 200 and 400 mg/kg), a reference analgesic drug aspirin (200 mg/kg), or cosolvent were orally administered 30 min before acetic acid. Ten mice were used for each group in this test:

Group 1: Control group : Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)
Group 2: Aspirin (200 mg/kg, p.o.)
Group 3: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)
Group 4: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)
Group 5: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)
Group 6: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)
Group 7: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)
Group 8: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

30 min ──────── Number of writhes (20 min) ──────►

Drug (p.o.)   0.6% Acetic acid
                (10 ml/kg, i.p.)

2. Hot plate test

The hot plate test was carried out according to the method described by Woolfe & MacDonald (1944). Mice were placed on a hot plate maintained at 55 °C ± 1 °C. Latency of nociceptive response such as licking of a hind limb or jumping was measured. Starting thirty minutes after oral administration of the test agents except morphine (15 min after administration), the nociceptive response was measured every 15 min over a 60 min period. Morphine sulfate was injected subcutaneously. The cut-off time was 45 sec. Only the mice that showed nociceptive responses within 15 sec were used for the experiment. Ten mice were used for each group in this test:

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Aspirin (200 mg/kg, p.o.)

Group 3: Morphine sulfate (10 mg/kg, s.c.)

Group 4: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 6: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 9: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Latency of nociceptive response (sec.)

```
   ◄──────── 30 min ────────►
           ◄── 15 min ──►      │        │        │        │
   ────────────────────────────────────────────────────────
   ▲                 ▲
   │                 │          0       15       30       45      60 min
   │                 │
 Drug (p.o.)   Morphine sulphate
               (10 mg/kg, s.c)
```

3. Formalin test

Thirty minutes after oral administration of the ether and ethanol extracts of *P. roxburghii* (100, 200 and 400 mg/kg), aspirin (200 mg/kg) or cosolvent except morphine (15 min after administration), 20 µl of 2.5% formalin in saline was injected subcutaneously to a hind paw of the mice. Morphine sulfate was injected subcutaneously. The time spent for licking the injected paw was recorded and the data were expressed as total licking time in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection (Hunskaar et *al.*, 1985). Ten mice were used for each group in this test:

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Aspirin (200 mg/kg, p.o.)

Group 3: Morphine sulfate (10 mg/kg, s.c.)

Group 4: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 6: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 9: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Early phase | Late phase

30 min | 0-5 min | 15-30 min

15 min | 15 min

Drug (p.o.) Morphine sulphate (10 mg/kg, s.c) 2.5% Formalin (20 µl, s.c.)

Antipyretic activity

Yeast-induced fever

Antipyretic activity of drug was measured by slightly modifying the method described by Adams et al. (1968). Male Wistar rats were fasted overnight with water *ad libitum* before the experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension (10 ml/kg) into the animals' dorsum region. Seventeen hours after the injection, the rectal temperature of each rat was measured using a digital thermometer (SK-1250 MC, Sato Keiryoki Mfg. Co., Ltd., Japan). Only rats that showed an increase in temperature of at least 0.7 °C were used for the experiments. The ether and ethanol extracts of *P. roxburghii* (100, 200 and 400 mg/kg), aspirin (200 mg/kg, p.o.) or cosolvent vehicle were administered orally and the temperature was measured at 1, 2, 3, 4 and 5 hr after drug administration. Six rats were used for each group in this test:

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Aspirin (200 mg/kg, p.o.)

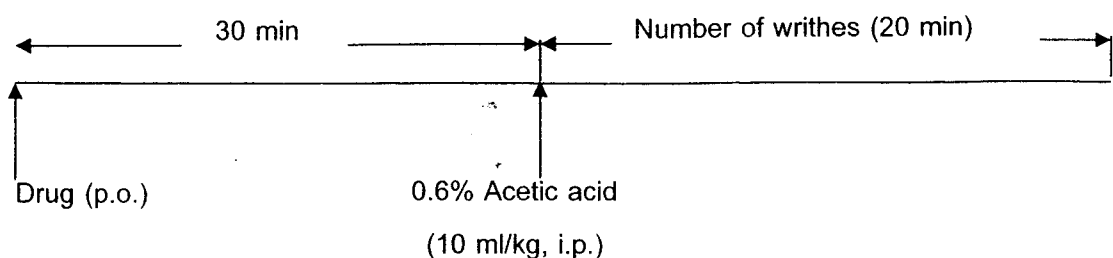Group 3: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 4: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 6: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Anti-inflammatory activities

1. Carrageenin-induced paw edema

According to the method described by Winter et al. (1962), the initial right hind paw volume of the rats was measured using a plethysmometer (Ugo Basile, Italy) and then 0.1 ml of 1% (w/v) carrageenin was subcutaneously injected into the subplantar region of the right hind paw. The volume of right hind paw was measured at 1, 2, 3, 4, and 5 h after carrageenin injection and the edema volume were determined. Cosolvent, the ether and ethanol extracts of *P. roxburghii* (100, 200 and 400 mg/kg), or aspirin (200 mg/kg, p.o.) was orally administered 30 min before carrageenin injection. Six rats were used for each group in this test:

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Aspirin (200 mg/kg, p.o.)

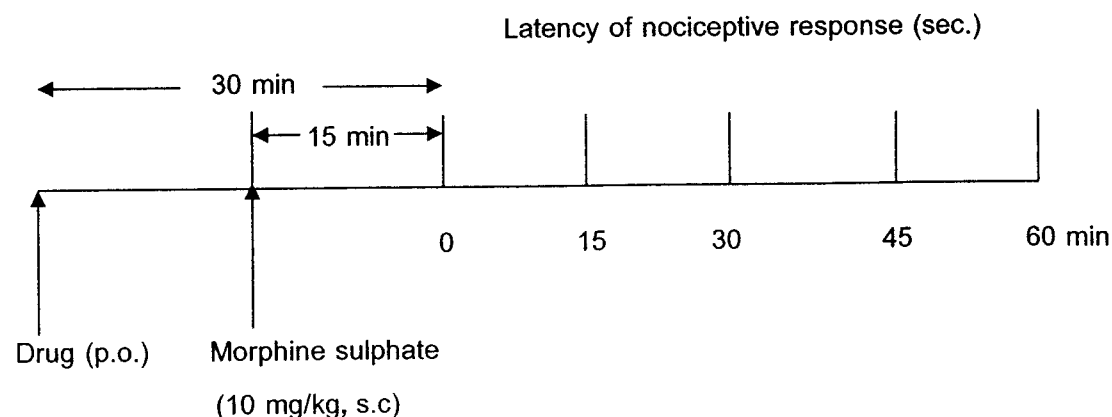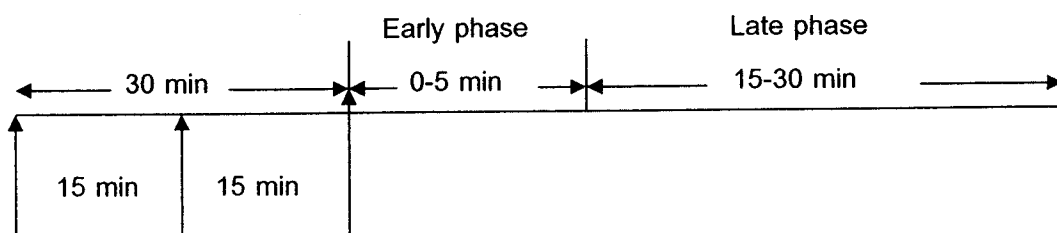Group 3: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 4: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 6: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)
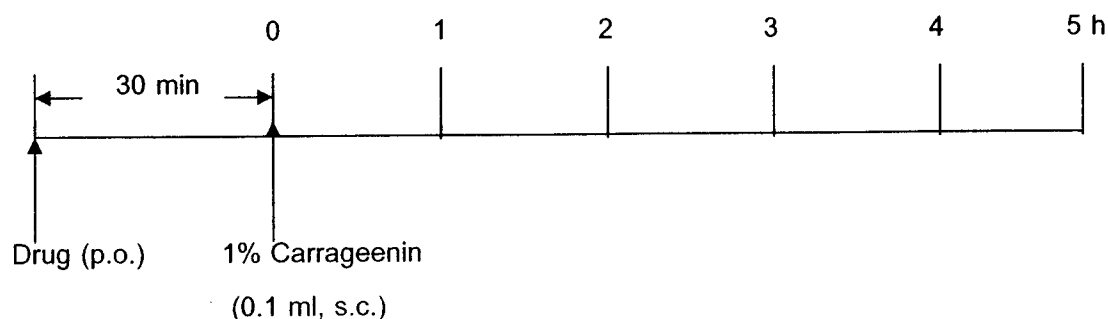
2. Croton oil-induced mouse ear edema

Edema was induced according to the method described by Tubaro et al. (1985). Cutaneous inflammation was induced by application of 10 µl of an acetone solution containing the irritant (5% croton oil) to the inner surface of the right ear of the mice. The left ear received an equal volume of acetone. The ether and ethanol extracts of *P. roxburghii* (1.25, 2.5 and 5.0 mg/ear) were applied topically to the right ear about 60 min before the croton oil. The left ear received the vehicle. As a reference, indomethacin (1 mg/ear) was used. Four hours after the application of the irritant agent, the mice were sacrificed and a plug (7-mm Ø) was removed from both the treated and untreated ears. The edematous response was measured as the weight difference between the two plugs.

Group 1: Control group: acetone

Group 2: Indomethacin (1.00 mg/ear)

Group 3: Ether extract of *P. roxburghii* leaves (1.25 mg/ear)

Group 4: Ether extract of *P. roxburghii* leaves (2.5 mg/ear)

Group 5: Ether extract of *P. roxburghii* leaves (5 mg/ear.)

Group 6: Ethanol extract of *P. roxburghii* leaves (1.25 mg/ear.)

Group 7: Ethanol extract of *P. roxburghii* leaves (2.5 mg/ear.)

Group 8: Ethanol extract of *P. roxburghii* leaves (5 mg/ear.)

Drug (10 µl),
Topically applied
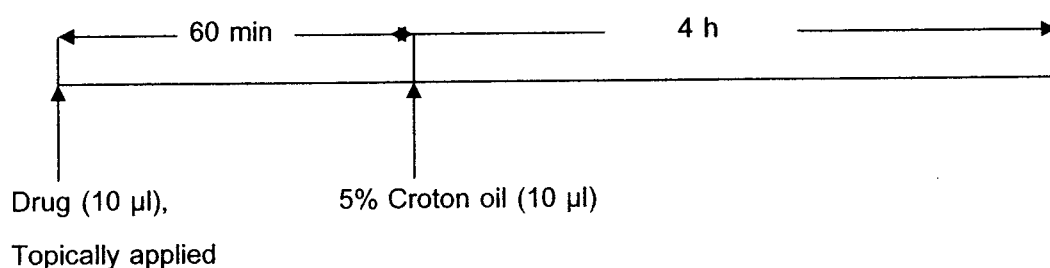
3. Croton oil-induced anus edema

A hemorrhoid model was prepared by means of application of croton oil onto the recto-anus of rats. Cotton swab soaked with the inducer, which consisted of 6% croton oil in diethyl ether, was inserted into the anus. The following condition was found to be optimal for preparing the model: cotton swab containing 0.2 ml of the inducer solution was applied to the anus of rats for 10 sec. Cosolvent, the ether and ethanol extracts of *P. roxburghii* (100, 200, 400 and 800 mg/kg), or aspirin was orally administered after croton oil onto the recto-anus of rats (1 hour), the croton oil-induced anus edema of rats was measured by slightly modifying the method described by (Nishiki et al., 1988). The rats were orally administered once daily throughout the experimental period of 3 days. On the 4 days, rats were sacrificed by cervical dislocation. The size of rats anus was measurement by vernier caliper.

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Aspirin (200 mg/kg, p.o.)

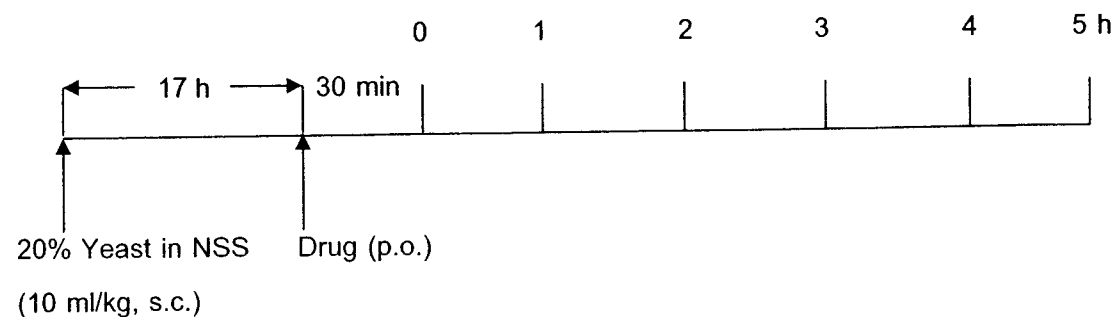Group 3: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 4: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 6: Ether extract of *P. roxburghii* leaves (800 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 9: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 10: Ethanol extract of *P. roxburghii* leaves (800 mg/kg, p.o.)

1 h ——————— 3 days

6% Croton oil   Drug (p.o.) daily, 3 days
(0.2 ml) (10 sec)
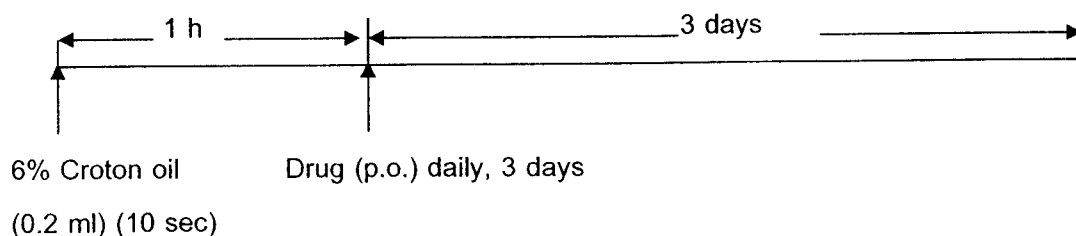
4. Chronic inflammation induced in air pouches of rats

To produce a chronic inflammatory process, 0.5 ml of 1% croton oil was injected into an air pouch formed on the back of adult rats by subcutaneous injection of 25 ml of air. The rats were then orally administrated (p.o., n=6) daily for 7 days with the ether and ethanol extracts of *P. roxburghii* (100, 200 , 400 and 800 mg/kg), cosolvent vehicle, or indomethacin (1.5 mg/kg). After 8 days the animals were sacrificed by cervical dislocation and the volume of exudates in the air pouch was measured (Selye, 1953).

Group 1: Control group: Cosolvent (propylene glycol : tween 80 : distilled water = 4:1:4)

Group 2: Indomethacin (1.5 mg/kg, p.o.)

Group 3: Ether extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 4: Ether extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 5: Ether extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 6: Ether extract of *P. roxburghii* leaves (800 mg/kg, p.o.)

Group 7: Ethanol extract of *P. roxburghii* leaves (100 mg/kg, p.o.)

Group 8: Ethanol extract of *P. roxburghii* leaves (200 mg/kg, p.o.)

Group 9: Ethanol extract of *P. roxburghii* leaves (400 mg/kg, p.o.)

Group 10: Ethanol extract of *P. roxburghii* leaves (800 mg/kg, p.o.)

30 min   7 days

1% Croton oil   Drug (p.o.) daily, 7 days

(0.5 ml)

Statistical analysis

Data were expressed as mean ± SEM and were analyzed statistically by one-way ANOVA procedures, followed by using Bonferroni's test. A difference was considered at $p<0.05$.

CHAPTER 3

RESULTS

The extracts from the leaves of *P. roxburghii* Wall.

The air dried leaves of *P. roxburghii* (1.2 Kg) are macerated with 95% ethanol at room temperature, then filtered and evaporated to give a syrupy mass. The marc was remacerated with ethanol four times, filtered and evaporated. All syrup masses were mixed to give 350 g of ethanol extract (29% yield). The ethanol extract was exhaustively extracted with ether and evaporated to give a syrupy mass 84 g of ether extract (24% yields). The ethanol and ether extracts gave orange red colour with shinoda test Mg-HCl (Malec and Pomilio, 2003). The percent yield of the extracts from dried leaves of *P. roxburghii* was shown in Table 1.

Table 1. The weight and percent yield of the extracts from dried leaves of *P. roxburghii* (1.2 kg).

Extract	Weight (g)	% yield
Ether extract	84	24
Ethanol extract	350	29

Thin-layer Chromatography (TLC) of the extracts from *P. roxburghii* Wall. leaves

The TLC chromatogram of the ether extract in various solvent systems under alcoholic-H_2SO_4 and heat detection were shown as the following (Figure 16).

(A) 2% MeOH/CHCl₃   (B) 5% MeOH/CHCl₃   (C) 10%MeOH/CHCl₃

(D) 50% MeOH/CHCl₃   (E) 100% CHCl₃
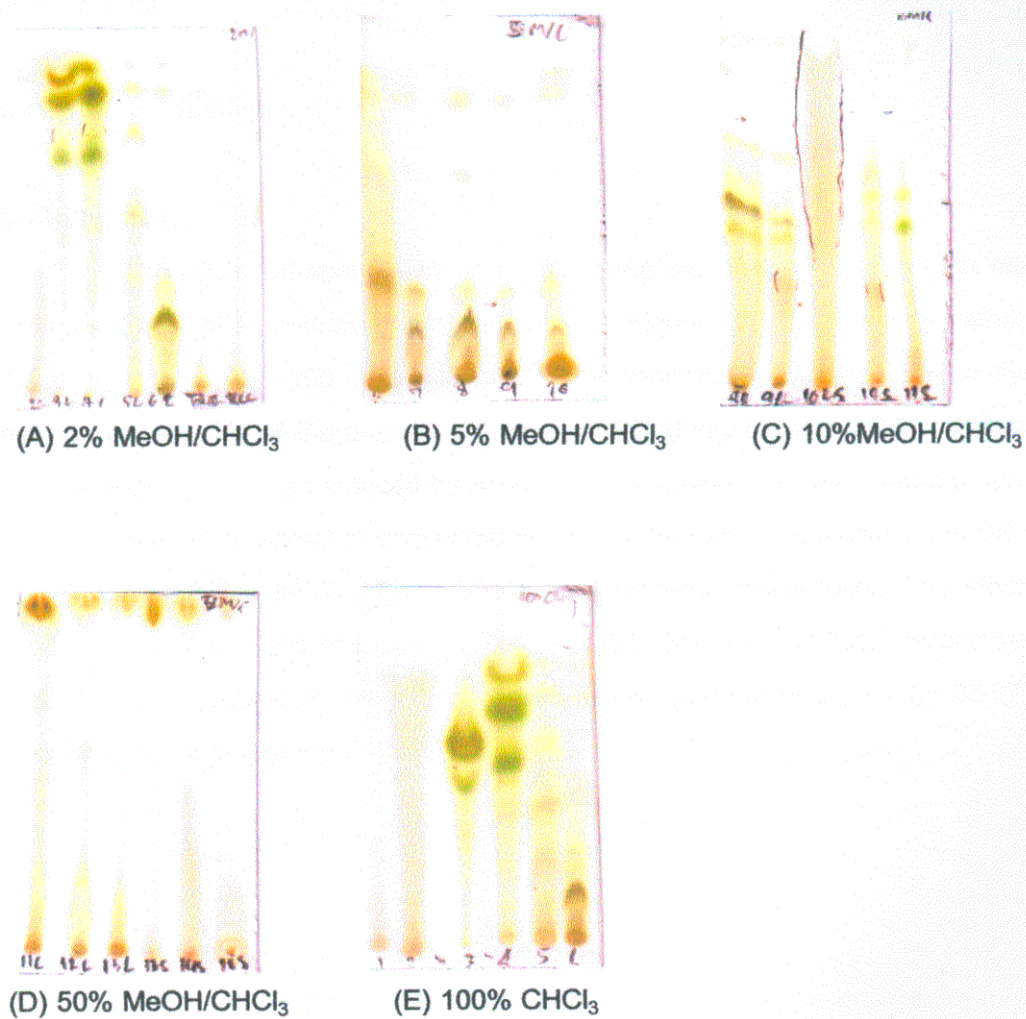
Figure 16. The TLC chromatogram of the ether extract in various solvent systems
(A) 2% MeOH/CHCl₃, (B) 5% MeOH/CHCl₃, (C) 10% MeOH/CHCl₃,
(D) 50% MeOH/CHCl₃ and (E) 100% CHCl₃.

Acute toxicity

The ether and ethanol extracts of *P. roxburghii* leaves which were orally administered at the dose 2 g/kg to mice (10 males and 10 females) did not change in the behavioral responses during the observation period. No mortality was observed up to 7 days of monitoring.

Antinociceptive Activities

1.   Writhing test

The results of acetic acid-induced writhing responses in mice of the ether and ethanol extracts of *P. roxburghii* are presented in Figure 17. The ether and ethanol extracts at doses of 100, 200 and 400 mg/kg dependently suppressed the writhing response in mice. Aspirin at the dose of 200 mg/kg elicited significant ($p<0.01$) inhibitory effect on the writhing response induced by acetic acid, compared with the control group.

The ether extract at concentration of 100, 200 and 400 mg/kg exhibited marked writhing inhibition by 42.01%, 51.52%, and 60.62%, respectively; The ethanol extract also inhibited writhing response by 24.58%, 34.13%, and 63.96%, respectively. Aspirin (200 mg/kg), reference drug, suppressed acetic acid-induced pain by 68.97%; compared with the control group (Table 2).

Table 2. Effect of the ether extract, ethanol extract of *P. roxburghii* leaves and

aspirin on the number of writhing induced by 0.6% acetic acid in mice.

Drug	Dose (mg/kg, p.o.)	Number of writhing	Inhibition (%)
Cosolvent	-	38.09±1.3	0
Aspirin	200	11.82±1.4*	68.97
Ether extract	100	22.09±1.5*	42.01
	200	18.45±1.4*	51.52
	400	15.00±2.4*	60.62
Ethanol extract	100	28.73±2.1*	24.58
	200	25.09±1.9*	34.13
	400	13.73±1.6*	63.96

Each value represents as mean ± SEM (n=10).

The ether and ethanol extracts of *P roxburghii* were orally administered thirty minutes before 0.6% acetic acid injection. Number of writhings and stretchings was counted over a 20 min period after injection of 0.6% acetic acid.

* $p < 0.01$, significantly different, compared with the control (cosolvent) group (Bonferroni's test).
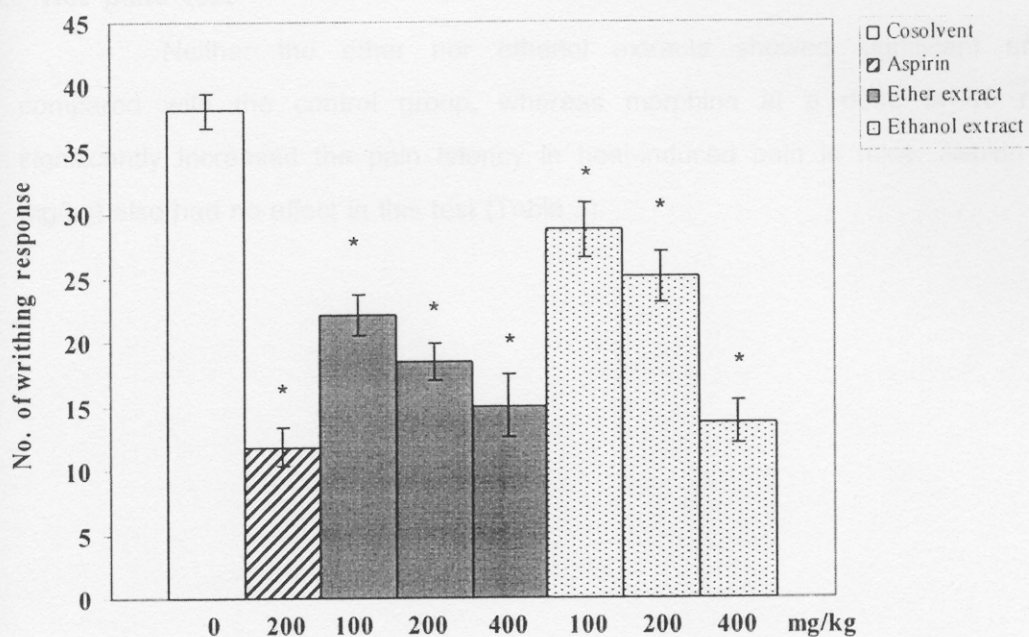
Figure 17. Effect of the ether extract, ethanol extracts of *P. roxburghii* leaves and aspirin the number of writhing induced by 0.6% acetic acid in mice. *$p<0.01$, significantly different, compared with the control (cosolvent) group (Bonferroni's test).

2. Hot plate test

Neither the ether nor ethanol extracts showed significant effects, compared with the control group, whereas morphine at a dose of 10 mg/kg significantly increased the pain latency in heat-induced pain in mice. Aspirin (200 mg/kg) also had no effect in this test (Table 3).

Table 3. Effect of the ether and ethanol extracts of *P. roxburghii* leaves, aspirin and morphine on nociceptive response induced by heat in mice.

Drug	Dose (mg/kg, p.o.)	Latency of nociceptive response (sec)				
		0 min	15 min	30 min	45 min	60 min
Cosolvent	-	19.94±0.7	17.66±0.8	19.93±1.0	25.22±0.9	19.10±1.7
Aspirin	200	23.05±1.0	21.11±0.9	21.14±0.5	20.21±0.7	21.55±0.8
Morphine sulphate (s.c)	10	39.03±1.7*	43.18±1.4*	45.27±0.1*	45.14±0.1*	45.20±0.4*
Ether extract	100	18.71±1.0	17.98±1.0	21.03±1.1	22.76±0.6	21.43±0.8
	200	18.94±0.8	18.32±1.0	21.70±0.9	20.25±1.0	20.41±0.5
	400	20.86±0.6	19.46±1.0	23.90±0.9	20.10±0.8	20.14±1.2
Ethanol extract	100	18.24±0.9	15.57±0.8	16.70±1.0	15.87±1.4	18.06±0.6
	200	18.66±0.8	15.72±0.9	17.44±0.9	18.11±1.2	18.69±1.5
	400	20.84±1.0	18.79±0.9	21.70±1.7	20.52±2.1	21.06±1.7

Each value represents as mean ± SEM (n=10).

Latency of nociceptive response was initially measured after oral administration of ether and ethanol extracts of *P. roxburghii*, aspirin or morphine injection (s.c.). The nociceptive response was measured every 15 min over a 60 min period.

* $p<0.01$, significantly different, compared with the control group (Bonferroni's test).

3. Formalin test

In the early phase, no significant inhibition of licking activity was observed in mice after oral administration of ether and ethanol extracts of *P. roxburghii* at the doses of 100, 200 and 400 mg/kg and aspirin (200 mg/kg), compared with the control group.

In the late phase, the ether and ethanol extracts of *P. roxburghii* at the dose 400 mg/kg markedly decreased the licking time in mice. Aspirin at a dose of 200 mg/kg could also intensively inhibit the licking response by 58.19% in this phase. Morphine (10 mg/kg) reduced the licking time in both early and late phases of the formalin test in mice (Figure 18).

The percentage inhibition of licking activity was 57.67% and 50.31% observed during late phase respectively at 400 mg/kg ether and ethanol extracts of *P. roxburghii*, compared with control group (Table 4).

Table 4. Effect of the ether extract and ethanol extract of *P. roxburghii* leaves, aspirin and morphine on nociceptive response by formalin-induced paw licking in mice.

Drug	Dose (mg/kg, p.o.)	Licking of the hind paw (sec)			
		Early phase (0-5 min)	Inhibition (%)	Late phase (15-30 min)	Inhibition (%)
Cosolvent	-	78.93±4.1	0	74.80±11.9	0
Aspirin	200	74.69±5.7	5.38	31.28±4.6*	58.19
Morphine Sulphate (s.c)	10	33.59±2.7*	57.45	3.56±1.2*	95.25
Ether extract	100	82.24±9.1	-4.19	59.84±9.3	20.00
	200	86.44±6.0	-9.57	55.40±6.5	25.94
	400	73.33±5.6	7.1	31.71±4.5*	57.67
Ethanol extract	100	74.59±3.3	5.5	51.15±2.9	31.62
	200	68.55±2.2	13.16	49.05±3.7	34.43
	400	68.76±2.4	12.89	37.17±1.3*	50.31

Each value represents as mean ± SEM (n=10).

Thirty minutes after test drug administration (p.o.), 2.5 % formalin was injected s.c. to mice hind paw.

Fifteen minutes after morphine administration (s.c.), 2.5 % formalin was injected s.c. to mice hind paw.

* $p < 0.01$, significantly different, compared with the control group (Bonferroni's test).
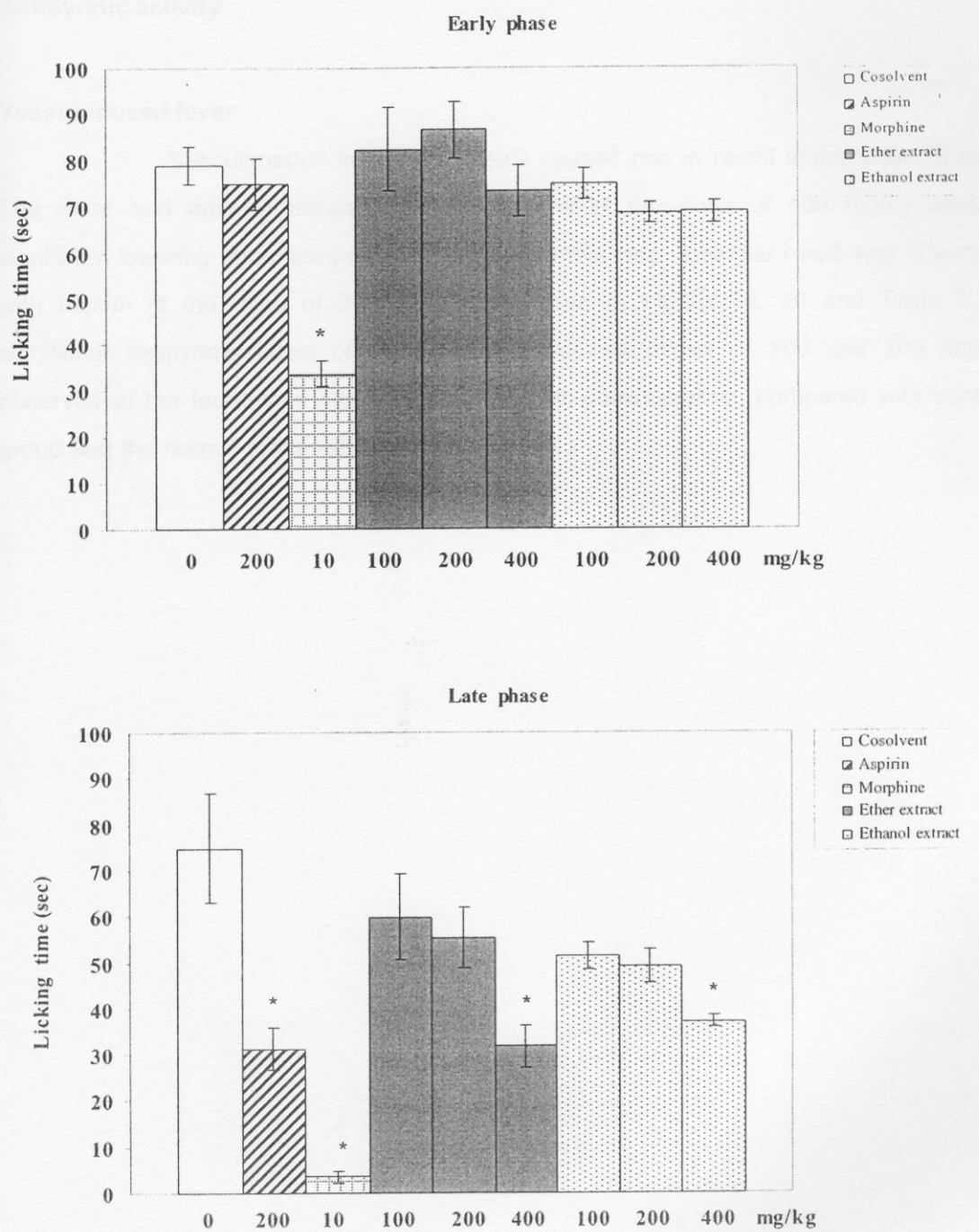
Figure 18. Effect of the ether and ethanol extracts of *P. roxburghii* leaves, aspirin and morphine on nociceptive response by formalin-induced paw licking in mice. *p<0.01, significantly different compared with the control group (Bonferroni's test).

Antipyretic activity

Yeast-induced fever

Subcutaneous injection of yeast caused rise in rectal temperature of rats. The ether and ethanol extracts of *P. roxburghii* at the dose of 400 mg/kg caused significant lowering rectal temperature in hyperthermia rats. A similar result was observed with aspirin at the dose of 200 mg/kg as shown in Figure 19, 20 and Table 5. A significant antipyretic effect of the ethanol extract at doses of 100 and 200 mg/kg observed at the fourth and fifth hour after extract administration, compared with control group and the normal temperature of rats at 36 °C.

Table 5. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and aspirin on brewer's yeast-induced fever in rats.

Drug	Dose (mg/kg, p.o.)	Average rectal temperature (°C)					
		0	1 h	2 h	3 h	4 h	5 h
Cosolvent	-	37.2±0.2	37.1±0.1	36.5±0.1	36.4±0.1	36.5±0.1	36.8±0.2
Aspirin	200	37.3±0.3	35.7±0.2**	35.4±0.1**	35.3± 0.2**	35.1±0.1**	35.0±0.5**
Ether extract	100	37.8±0.1	36.9±0.1	36.7±0.1	36.4±0.1	35.9±0.2	35.9±0.4
	200	37.5±0.2	36.4±0.1	36.4±0.2	36.0±0.2	35.8±0.2	35.9±0.2
	400	37.1±0.2	36.2±0.1*	35.6±0.7**	35.6±0.1*	35.3±0.2**	35.2±0.2**
Ethanol extract	100	37.2±0.3	36.6±0.2	36.4±0.5	36.0±0.1	35.9±0.1	35.9±0.2
	200	37.0±0.1	36.3±0.1	35.9±0.3	35.7±0.1	35.8±0.2	35.8±0.3
	400	37.3±0.1	35.8±0.4**	35.4±0.2**	35.4±0.2**	35.3±0.1**	35.3±0.1**

Each value represents as mean ± SEM (n=6).

Rectal temperature was measured after yeast injection 17 h.

* $p < 0.05$, ** $p < 0.01$, significantly different, compared with the control group (Bonferroni's test).

Figure 19. Effect of the ether extract of *P. roxburghii* leaves and aspirin on brewer's yeast-induced fever in rats.

*$p<0.05$, ** $p<0.01$, significantly different, compared with the control group (Bonferroni's test).
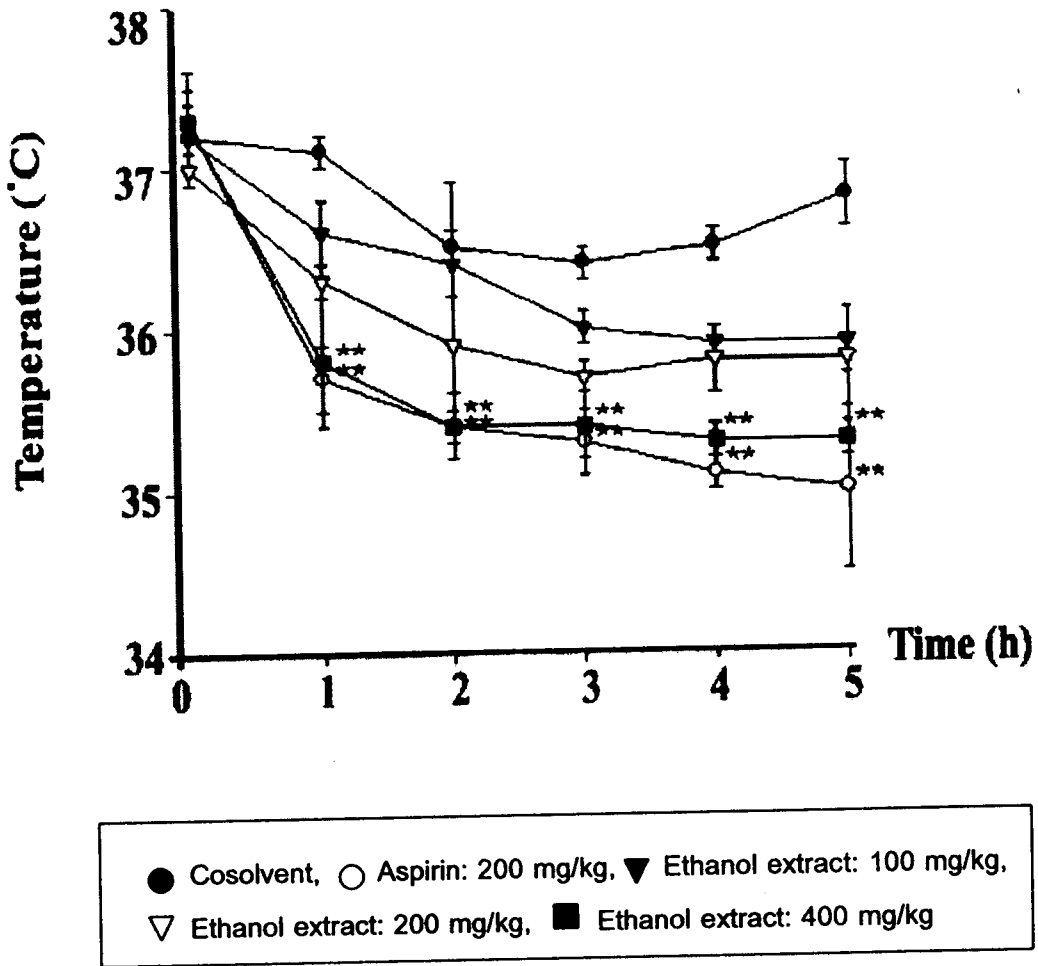
Figure 20. Effect of the ethanol extracts of *P. roxburghii* leaves and aspirin on brewer's yeast-induced fever in rats.

*$p<0.05$, ** $p<0.01$, significantly different, compared with the control group (Bonferroni's test).

Anti-inflammatory activity

1. Carrageenin-induced paw edema

The effect of third solvent fractions of ethanol and ether extracts of the dried leaves of *P. roxburghii* on carrageenin-induced acute inflammation, measured by the increased paw volume of the rats at different time periods (0, 1, 2, 3, 4, 5 h) is presented in Table 6.

The inhibitory activity on carrageenin-induced hind paw edema in rats caused by oral administration of *P. roxburghii* extracts, at various assessment times after carrageenin injection is shown in Figure 21 and 22. Aspirin at the dose of 200 mg/kg exhibited significantly edema inhibition. The ethanol extract at the dose of 400 mg/kg also possessed significant inhibitory effect on carrageenin-induced paw edema at all recorded times. A significant anti-edema effect of the ethanol extract at the dose of 200 mg/kg was observed at the second, third, fourth and fifth hour after carrageenin injection. The ethanol extract at the dose of 100 mg/kg significantly inhibited on carrageenin-induced paw edema at the fourth and fifth hour. The present study shows that ether extract from the leaves of *P. roxburghii* at the dose of 400 mg/kg significantly inhibited the edema formation at the third, fourth and fifth hour after carrageenin injection. A marked inhibition of edema formation was at the fourth hour with the doses of 100 and 200 mg/kg.

Table 6. Effect of the ether extract and ethanol extract of *P. roxburghii* leaves and
aspirin on carrageenin-induced paw edema in rats.

Drug	Dose (mg/kg, p.o.)	Paw volume (ml)					
		0	1 h	2 h	3 h	4 h	5 h
Cosolvent	-	5.8±0.3	7.0±0.3	7.5±0.2	8.9±0.4	8.8±0.1	8.6±0.2
Aspirin	200	5.2±0.2	5.8±0.3*	6.0±0.2**	6.7±0.2**	6.8±0.5**	6.4±0.2**
Ether extract	100	6.1±0.2	6.6±0.2	6.9±0.2	7.6±0.2	8.0±0.4	7.9±0.3
	200	6.1±0.4	6.7±0.1	7.1±0.2	7.2±0.3	7.9±0.4	7.4±0.3
	400	5.6±0.4	6.1±0.1	6.4±0.1	6.9±0.2*	7.0±0.2**	6.8±0.1**
Ethanol extract	100	5.5±0.4	6.1±0.2	6.5±0.3	7.2±0.2	7.9±0.2	7.4±0.6
	200	5.4±0.4	6.0±0.1	6.1±0.1**	6.8±0.1**	7.2±0.2*	7.1±0.2*
	400	5.3±0.1	5.9±0.1*	6.1±0.1**	6.7±0.2**	6.6±0.1**	6.5±0.1**

Each value represents as mean ± SEM (n=6).

Oral administration of ether and ethanol extracts of *P. roxburghii* thirty minutes before 1%
carrageenin (s.c.) injection, the volume of right hind paw was measured at 1, 2, 3, 4, and
5 h after 1% carrageenin injection.

* $p<0.05$, ** $p<0.01$, significantly different compared with the control group (Bonferroni's
test).
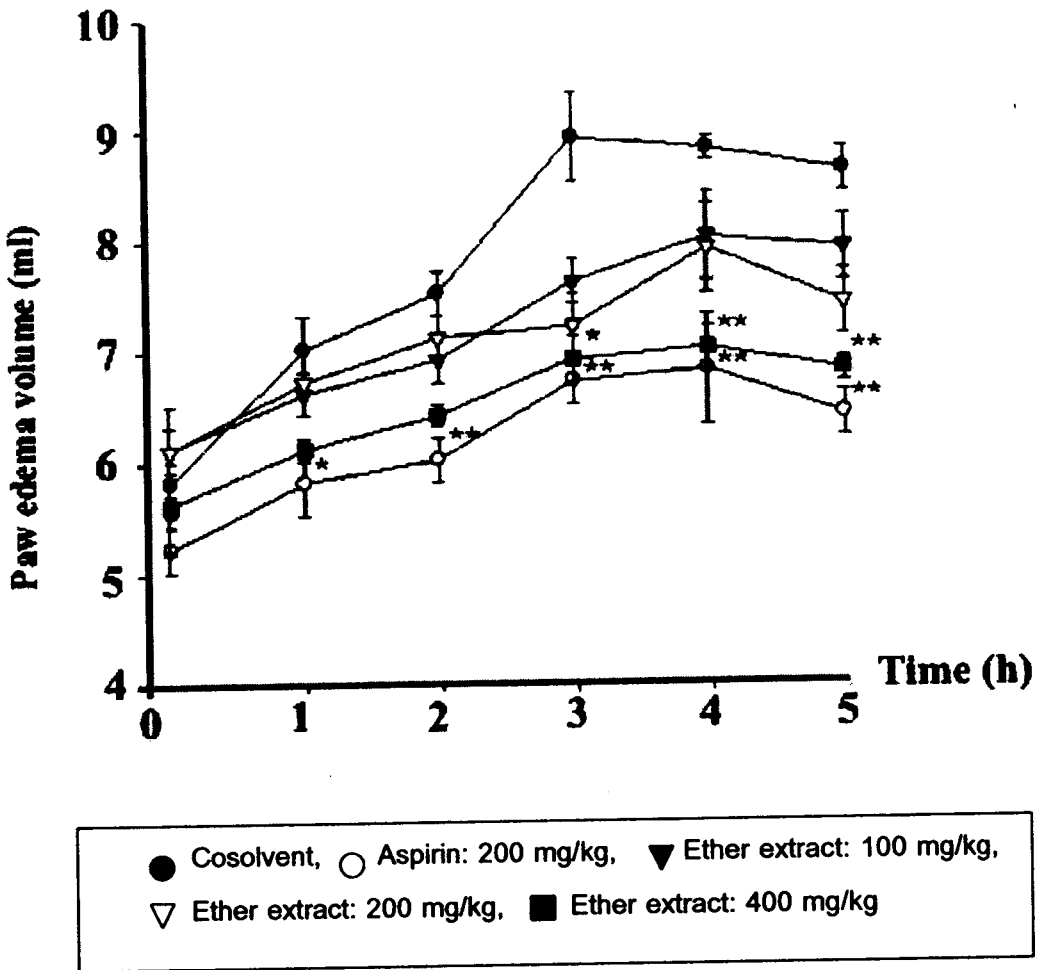
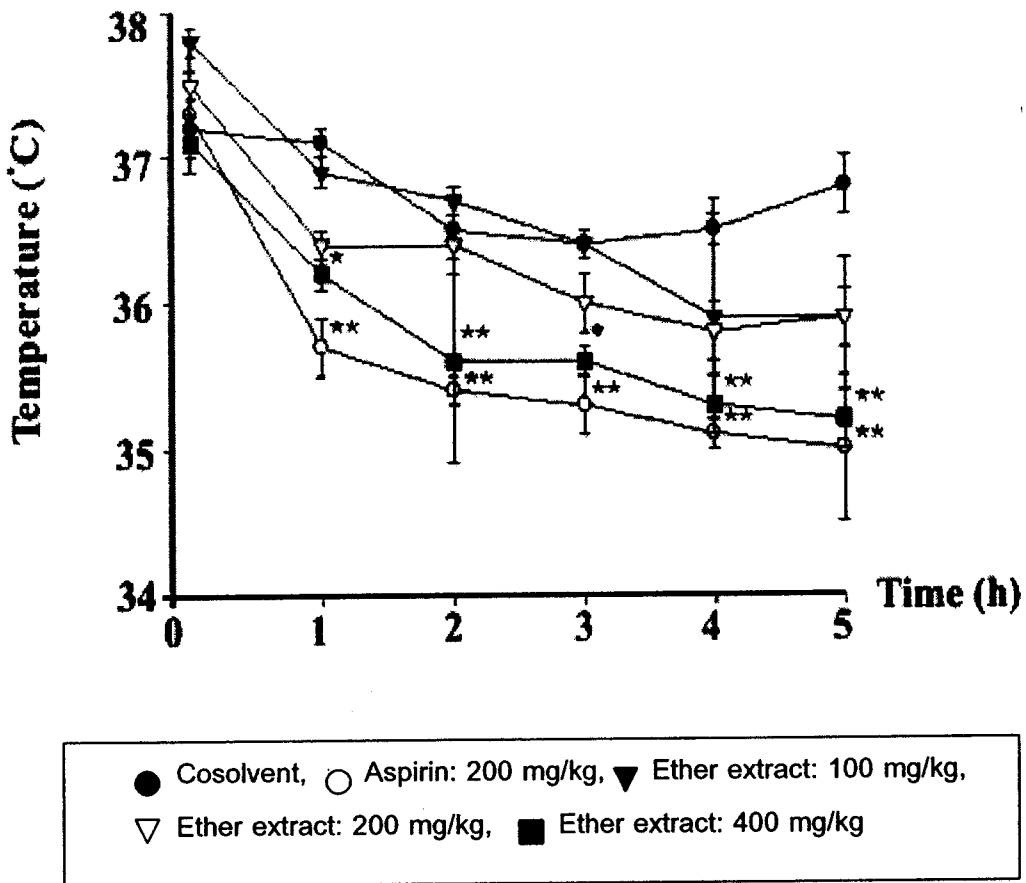Figure 21. Effect of the ether extract of *P. roxburghii* leaves and aspirin on

carrageenin-induced paw edema in rats.

*$p<0.05$, ** $p<0.01$, significantly different compared with the control group

(Bonferroni's test).
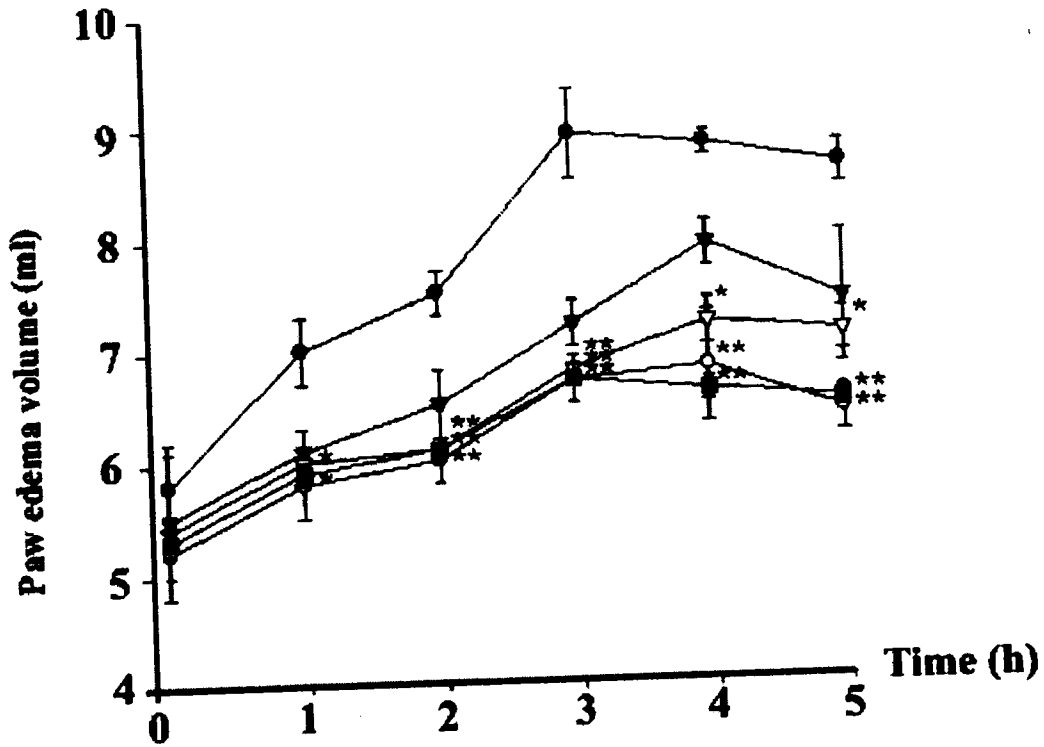
Figure 22. Effect of the ethanol extracts of *P. roxburghii* leaves and aspirin on carrageenin-induced paw edema in rats.

*$p<0.05$, ** $p<0.01$, significantly different compared with the control group (Bonferroni's test).

## 2. Croton oil-induced mouse ear edema.

The results demonstrated topical anti-inflammatory activity of the ether and ethanol extracts of *P. roxburghii*. The results of croton oil-induced mouse ear edema were presented in Table 7 and Figure 23, topical application of the ether extract (5.0 mg/ear) significantly inhibited ear edema in mice by 42 % and the ethanol extract (2.5, 5.0 mg/ear) significantly inhibited the ear edema formation by 28% and 51%, respectively, compared with control group.

Table 7. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and indomethacin on croton oil-induced mouse ear edema.

Drug	Dose (mg/ear)	Weight (mg)	Inhibition (%)
Control	-	13.84±0.44	0
Indomethacin	1.00	6.07±0.38*	57
Ether extract	1.25	12.68±0.94	9
	2.50	11.26±0.90	19
	5.00	8.08±0.54*	42
Ethanol extract	1.25	12.61±0.63	9
	2.50	10.01±0.40*	28
	5.00	6.80±0.53*	51

Each value represents as mean ± SEM (n=10).

Test extract or drugs were topically applied 60 min before croton oil application.

* $p < 0.01$, significantly different, compared with the control group (Bonferroni's test).

16
14
12
Weight (mg)
10
8
6
4
2
0

□ Control
□ Indomethacin
▣ Ether extract
□ Ethanol extract

0   1   1.25   2.5   5   1.25   2.5   5   mg/ear
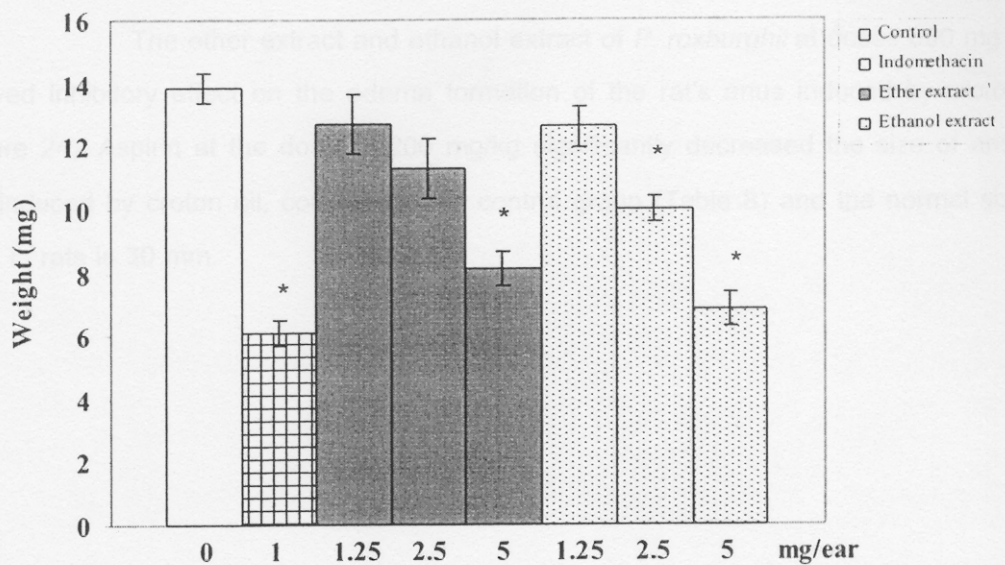
Figure 23. Effect of the ether extract and ethanol extract of *P. roxburghii* leaves and aspirin on croton oil-induced mouse ear edema.

*p<0.01, significantly different, compared with the control group (Bonferroni's test).

3. Croton oil-induced anus edema

The ether extract and ethanol extract of *P. roxburghii* at doses 800 mg /kg showed inhibitory effect on the edema formation of the rat's anus induced by croton oil (Figure 24). Aspirin at the dose of 200 mg/kg significantly decreased the size of anus in rats induced by croton oil, compared with control group (Table 8) and the normal size of anus in rats is 30 mm.

Table 8. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and aspirin on
croton oil-induced anus edema in rats.

Drug	Dose (mg/kg, p.o.)	Size of anus (mm)	% Inhibition
Cosolvent	-	46.50±1.0	0
Aspirin	200	39.50±0.7*	15.0
Ether extract	100	45.17±1.5	2.8
	200	44.67±1.3	3.9
	400	43.20±1.3	7.0
	800	36.50±0.9*	21.5
Ethanol extract	100	46.67±1.5	-0.3
	200	44.17±1.6	5.0
	400	42.00±1.2	9.6
	800	36.00±1.0*	22.5

Each value represents as mean ± SEM (n=6).

Cotton swab soaked with the inducer, which consisted of 6% croton oil in diethyl ether, was inserted into the anus, orally administered of ether and ethanol extracts of *P. roxburghii* after croton oil application 1 hour. The rats were orally administered once daily throughout the experimental period of 3 days. The sizes of anus were measured with vernier caliper.

* $p<0.01$, significantly different compared with the control group (Bonferroni's test).
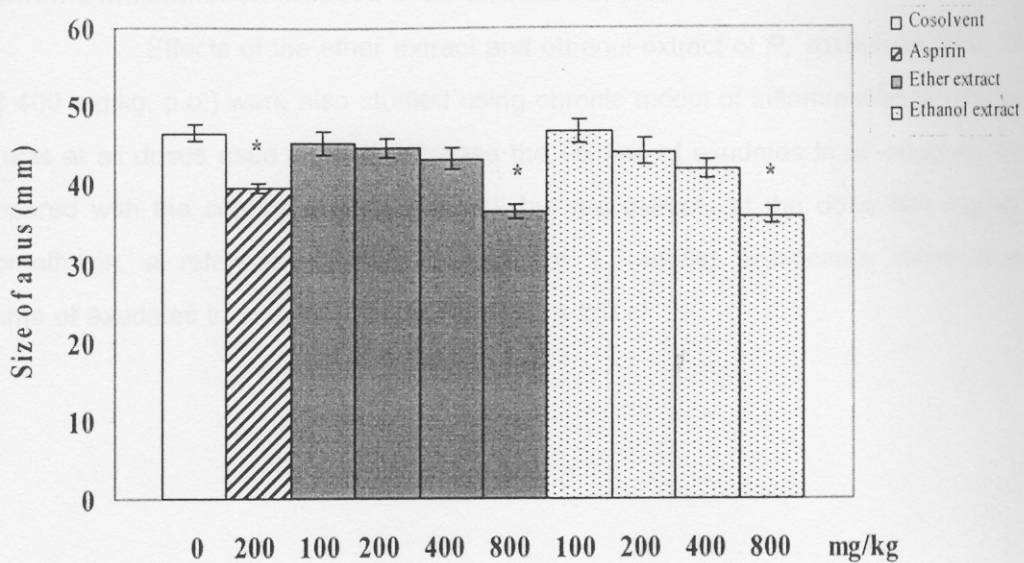
Figure 24. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and aspirin on croton oil-induced anus edema in rats.

*p<0.01, significantly different compared with the control group (Bonferroni's test).

4. Chronic inflammation induced in air pouches of rats

Effects of the ether extract and ethanol extract of *P. roxburghii* (100, 200 And 400 mg/kg, p.o.) were also studied using chronic model of inflammation. *P. roxburghii* extracts at all doses used did not decrease the volume of exudates in air-pouches of rats compared with the control group (Table 9) but the extracts at the dose 800 mg/kg and indomethacin, a reference drug at a dose of 1.5 mg/kg significantly decreased the volume of exudates in air-pouches of rats (Figure 25).

Table 9. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and indomethacin on chronic inflammation induced in air pouches of rats.

Drug	Dose (mg/kg)	Fluid in pouch (ml)	% Inhibition
Cosolvent	-	6.13±0.1	0
Indomethacin	1.5	3.45±0.1**	43.7
Ether extract	100	5.88±0.7	4.0
	200	5.86±0.1	4.4
	400	5.83±0.1	4.8
	800	5.06±0.2*	17.4
Ethanol extract	100	5.93±0.2	3.2
	200	5.90±0.1	3.7
	400	5.83±0.1	4.8
	800	4.90±0.1*	20.0

Each value represents as mean ± SEM (n=6).

The volume of exudates in the air pouch after 0.5 ml croton oil injection into an air pouch formed on the back of rats.

*$p<0.05$, ** $p<0.01$, significantly different compared with the control group (Bonferroni's test).
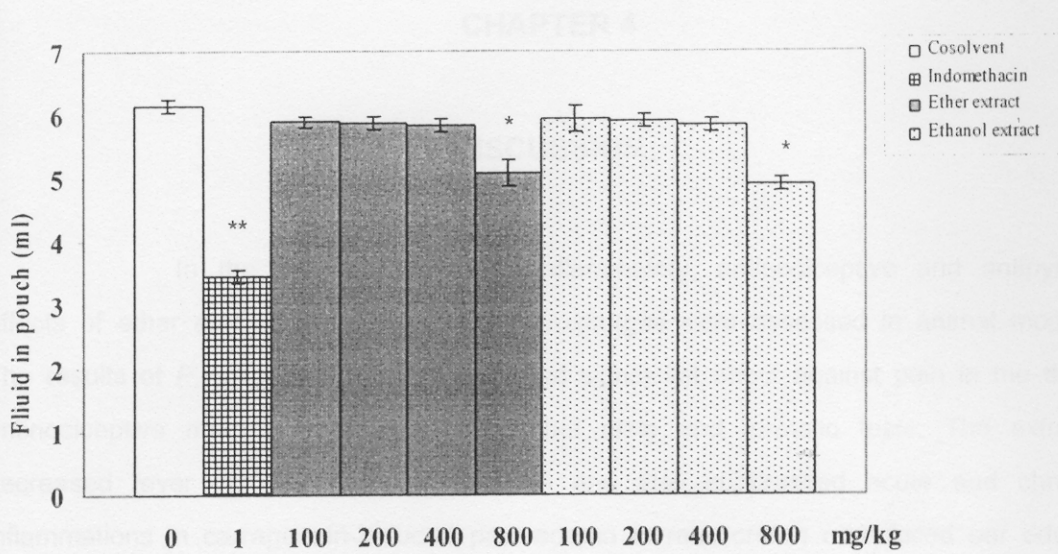
Figure 25. Effect of the ether and ethanol extracts of *P. roxburghii* leaves and
indomethacin on chronic inflammation induced in air pouches of rats.
*p<0.05, **p<0.01, significantly different compared with the control group
(Bonferroni's test).

CHAPTER 4

DISCUSSION

In the present study, anti-inflammatory, antinociceptive and antipyretic effects of ether and ethanol extracts of *P. roxburghii* were assessed in animal models. The results of *P. roxburghii* extracts exhibited significant effect against pain in the three antinociceptive models in mice: writhing, hot plate and formalin tests. The extracts decreased fever induced by yeast in rats and also suppressed acute and chronic inflammations in carrageenin-induced paw edema in rats, croton oil-induced ear edema and anus edema in mice, and granuloma pouch in rats.

In acute toxicity, no motality was observed in mice. Thus *P. roxburghii* extracts are relatively safe.

The pain in abdominal writhes induced by acetic acid is an analgesic screening test (Gupta and Verma, 1991), the writhing test allows us to identify central and peripheral analgesic compounds involuntary muscle twitches of the abdomen may be of interest because of their similarity with some of those known in visceral disorders (Le Bars et al., 2001b). In acetic acid-induced abdominal writhing which is the visceral pain model, the inflammatory response releases arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis plays a role in the nociceptive mechanism (Franzotti et al., 2002), since acetic acid indirectly induced the release of endogenous mediators stimulated the nociceptive neurons that were sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) (Sa'nchez-Mateo et al., 2006). In the present study, the *P. roxburghii* extracts significantly exhibited analgesic effect and this effect may be due to inhibition of the synthesis of the arachidonic acid metabolites. The ether extract and ethanol extract of *P. roxburghii* exhibited protective action against nociception in writhing test similarly to aspirin. These results suggest that the ether extract and the ethanol extract possess antinociceptive activities.

The hot plate test has been found to be suitable for evaluation of centrally acting analgesics (Gupta et al., 2005). In the present study, morphine, centrally acting analgesic drug, produced an inhibitory effect on the nociceptive response in this test, while the ether extract and the ethanol extract of *P. roxburghii* did not show

antinociceptive effects in this test. Hence, suggested that the apparent antinociceptive action of the *P. roxburghii* extracts may be mediated through peripheral but not central mechanism.

Formalin injection into the mice hind paw results in biphasic pain-related behaviors (such as licking, flinching and biting of the injured paw) that seems to involve two distinct mechanisms. The first phase (acute pain) appears immediately following formalin injection lasting only few minutes and is believed to be driven by primary afferent nociceptor activity (Shibata et al., 1989). The formalin test was conducted to confirm and study the possible analgesic mechanism of action of the ether extract and ethanol extract of *P. roxburghii*. The test consists of two distinct phases those possibly reflecting different types of pain mechanisms. The first phase starts immediately after injection of formalin and lasts about 5 min. This is due to direct chemical peripheral stimulation of nociceptors that seems to be caused predominantly by C fiber activation (Heapy et al., 1987). In this phase, first response is evoked by the direct formalin stimulation of the nerve endings followed by substance P release which may play a role through cooperation with bradykinin in this phase. The second phase (tonic pain) starts approximately 15-20 min after formalin injection and lasts for 20-40 min. Inflammation in the late phase is due to the release of peripheral mediators like serotonin, histamine, bradykinin, prostaglandins and at least to some degree, the sensitization of central nociceptive neurons (Tjolsen et al., 1992). In this second phase various mediators operate in a sequence to produce an inflammatory response and has been correlated with the elevated production of prostaglandin (PG), induction of cyclo-oxygenase (COX) and release of nitric oxide (NO) (Hama and Menzaghi, 2001; Le Bars et al., 2001b). The formalin test is sensitive to non-steroidal anti-inflammatory drugs and other mild analgesics. It is now well accepted that the anti-nociceptive efficacy of NSAIDs not only depends upon the inhibition of PG synthesis at the site of injury but also on the prevention of a nociception-induced PG, especially PGE_2 release in the spinal cord (Ferreira et al., 1973). Drugs that act primarily on the central nervous system inhibit both phases, while peripherally acting drugs inhibit the late phase (Hunsnkaar and Hole, 1987). Central analgesic drugs such as morphine inhibited both phases equally, while peripherally acting drugs such as NSAIDs aspirin suppressed mainly in the late phase. Our results showed that the ether and ethanol extracts of *P. roxburghii* exerted significant inhibitory effect on nociceptive response of the late phase but not in the early phase. These results suggested that the inhibitory effect of

the extracts from *P. roxburghii* on nociceptive response of the late phase may due to involve in the suppression of the release of peripheral mediators e.g., serotonin, histamine, bradykinin, nitric oxide and/or prostaglandins.

Increased body temperature and pain are known as the main symptoms of the body against an inflammatory stimulation. Hence, a drug possessing anti-inflammatory activity may also exhibit antipyretic and analgesic properties. Antipyretic activity is commonly mentioned as a characteristic of drugs or compounds which have an inhibitory effect on prostaglandin biosynthesis (Vane, 1987). Fever may be result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states. The yeast induced fever in rats was employed to investigate the antipyretic activity of *P. roxburghii* extracts. Doses of the ether and ethanol extracts of the plant in the effective antinociceptive dose range were used. It was found that the ether and ethanol extracts at dose of 400 mg/kg showed a significant decrease in rectal temperature similar to aspirin. The ether extract of *P. roxburghii* at the doses of 100, 200 and 400 mg/kg showed significant decrease in yeast-induced fever. Antipyretics are drugs, which reduced the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained (Burke et al., 2006). This result seems to support the view that the ether and ethanol extracts of *P. roxburghii* may have some influence on prostaglandin biosynthesis, because prostaglandin is believed to be a regulator of body temperature (Milton, 1982).

Inflammation is a local reaction of the vascular and supporting elements of a tissue to injury resulting in the formation of a protein-rich exudates; it is a protective response of the nonspecific immune system that serves to localize neutralize, or to destroy an injurious agent in preparation for the process of healing. The cardinal signs of inflammation are rubor (redness), calor (heat), dolor (pain), tumor (swelling), and functio laesa (loss of function). Cause of inflammation includes physical agents, chemical agents, immunological reactions, and infection by pathogenic organism (Wilson et al, 2003). Inflammation is divided into acute and chronic patterns. The characteristics of acute inflammation are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously (Collins et al., 2001).

Carrageenin-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the anti-edematous effect of natural products, since the relative potency estimates obtained from most drugs tend to reflect clinical experience (Winter et al., 1962). The local injection of carrageenin-induced inflammatory process in the rat involves was divided into three phase by several mediators released in ordinate sequence (Di Rosa, 1972). An initial phase, during the first 1.5 h, is caused by the release of histamine and serotonin, a second phase is mediated by bradykinin from 1.5 to 2.5 h and finally, a third phase, the mediator of which is suspected to be prostaglandins occurs from 2.5 to 6 h after carrageenin injection. This third phase appears to be the most interesting compared with the two earlier phases. Thus, the maximal vascular response as determined with leukocyte migration to the inflamed area, also reaches its maximum level in this third phase (Vinegar et al., 1969). It is well established that prostaglandins, by virtue of their activity as modulators of inflammatory responses, have a major role in the inflammatory mechanism. The carrageenin-induced hind paw edema in rat is known to be sensitive to cyclooxygenase inhibitors, but not to lipoxygenase inhibitors, and has been used to evaluate the effect of non-steroidal anti-inflammatory agents which primarily inhibit the cyclooxygenase involved in prostaglandins synthesis. It has been demonstrated that the suppression of carrageenin-induced hind paw edema after the third hour correlates reasonably with therapeutic doses of most clinically effective anti-inflammatory agents (Di Rosa, 1972). The inflammation induced by carrageenin involves cell migration, plasma exudation and production of mediators, such as NO, prostaglandin E_2 (PGE$_2$), IL-1β, IL-6 and TNF-α (Salvemini et al., 1996; Loram et al., 2007). Oral pretreatment of animal with *P. roxburghii* extracts resulted in a significant inhibition of carrageenin-evoked hind paw edema in second and third phase. These results suggested that the anti-inflammatory activity of the ether and ethanol extracts may involve in the inhibition of bradykinin and/or prostaglandin synthesis, cell migration and/or plasma exudation.

The croton oil-induced ear edema test has a good predictive value for screening topical anti-inflammatory agents, which has certain advantages for natural product testing. First, the response is local, and involves the skin of the ear, thus, the topical application avoids drug metabolism and excretion. Secondly, this model uses very small amounts of drugs (Jacobs et al., 1985). The mechanism of croton oil-induced inflammation involves an increase in phospholipase A2 activity (Kondoh et al., 1985;

McColl et al., 1986), which in turn leads to the release of arachidonic acid and subsequent biosynthesis of leukotrienes and prostaglandins (Ashendel and Boutwell, 1979; Furstenberger and Marks, 1980). Arachidonic acid metabolites have been firmly shown to act as mediators of the inflammatory response via cyclooxygenase and lipooxygenase activity, and have therefore been a target for the development of therapeutic agents (Inoue et al., 1989). In the present study, the ether and ethanol extracts of *P. roxburghii* significantly inhibited the mice ear edema induced by croton oil. As the important mediators involved in croton oil-induced mouse ear edema are prostaglandins, histamine and serotonin, and the lipoxygenase pathway also an important role (Chen et al., 1994). It is possible that the extracts may inhibit some of these mediators.

The ether and ethanol extracts of *P. roxburghii* decreased the size of anus only at the high dose of 800 mg/kg in croton oil-induced anus edema in rats. The characteristics of acute inflammation are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils (Collins et al., 2001), suggested that the ether and ethanol extracts of *P. roxburghii* decreased edema and inflammatory mediators involved e.g., prostaglandins, histamine, serotonin and the emigration of leukocytes to the inflamed area.

Granuloma pouch technique was modified using croton oil as irritant. An aseptic inflammation resulting in large volume of hemorrhaged exudates is elicited which resembles the subacute type of inflammation. Instead of croton oil can be used as an irritant. Therefore, croton oil-induced granuloma pouch offer a model for exudative type of inflammation. Though, the chemical mediators of this type of response are unknown (Warren, 1972), protein synthesis is necessary for the formation of granuloma. This includes a diverse leukocyte infiltrate of macrophages, neutrophils, eosinophils, and mast cells often in association with lymphocytes (Levy et al., 2001). In the control animal, the pouch was filled by a large amount of hemorrhagic exudates, which accumulated under considerable pressure and hastened the absorption of air. Indomethacin inhibited the inflammatory response under these conditions. Our results showed that the ether and ethanol extracts of *P. roxburghii* at doses of 100, 200 and 400 mg/kg did not decrease the volume of fluid exudates on chronic inflammation, but the extracts decreased the volume of inflammatory exudates induced in chronic inflammation induced in air pouches of rats only at the high dose of 800 mg/kg. These results suggested that *P. roxburghii*

extracts had potential inhibitory action on exudate formation and decreased the volume of inflammatory exudates and leukocyte infiltrate of macrophages, neutrophils, eosinophils, and mast cells often in association with lymphocytes.

The ether extract was subjected to column chromatography using chloroform: methanol, then collected and subjected to shinoda test for flavonoids which was followed by TLC using chloroform: methanol (2%, 5%, 10%, 50% and 100% Chloroform), solvent system. The visualization of spots was carried out by spraying with $FeCl_3$: K_3FeCN_6 (1% aqueous solution 1:1) (Markham, 1975). The ether extract is given positive response for the flavonoids by producing pink color to shinoda test. TLC using the same solvent system and spraying reagent as mentioned earlier confirmed the presence of flavonoids by producing a green color spot (Bhujbal et al., 2008). The developed on the TLC, the spots showed pink colorization by spraying 10% H_2SO_4 solution and heating which indicated the presence of triterpenoids in the extract (Kim et al., 2005).

The phytochemical study of *P. roxburghii* has been reported. The acidic fraction of the alcoholic extracts of the leaf of *P. roxburghii* yielded a new triterpene acid, roxburghonic acid (Garg and Mitra, 1971a) and a new biflavonoid named putraflavone was isolated from the alcoholic extract of the leaf of *P. roxburghii* (Garg and Mitra, 1971b). Thus, these compounds may involve in the activity of *P. roxburghii* extracts. In the present study, *P. roxburghii* extracts possess antipyretic and anti-inflammatory activities on anus edema which support the traditional uses for the treatment of fever and hemorrhoid in Thai folklore medicine (นันทวัน และ อรนุช, 2542).

Based on these results, we concluded that the ether and ethanol extracts of *P. roxburghii* leaves possessed antinociceptive, antipyretic and anti-inflammatory activities. These results suggested that the anti-inflammatory action of *P. roxburghii* extracts seem to be involved in the suppression of some peripheral mediators, cell migration and/or plasma exudation. However, further studies are needed to clarify the mechanism of action of *P. roxburghii* extracts.

Summary

1. The ether and ethanol extracts of *P. roxburghii* at doses of 100, 200 and 400 mg/kg significantly decreased the number of writhings and stretchings induced by acetic acid in mice.

2. Neither the ether nor ethanol extracts had significant effect in the hot plate test.

3. Both ether and ethanol extracts significantly suppressed licking activity only in the late phase at the dose of 400 mg/kg in mice.

4. Either the ether or ethanol extracts decreased fever induced by yeast in rats at the same dose range.

5. The extracts possessed moderate inhibitory activity of inflammation in carrageenin-induced paw edema in rats.

6. The ether extract (5.0 mg/ear) and the ethanol extract (2.5, 5.0 mg/ear) significantly inhibited the mouse ear edema induced by croton oil.

7. The ether and ethanol extracts only at the high dose of 800 mg/kg exhibited anti-inflammatory activity in the croton oil-induced anus edema in rats.

8. The ether and ethanol extracts of *P. roxburghii* only at the high dose of 800 mg/kg decreased the volume of inflammatory exudates in chronic inflammation induced in air pouch of rats.

9. These results suggested that the anti-inflammatory action of *P. roxburghii* extracts seem to be involved in the suppression of some peripheral mediators, cell migration and/or plasma exudation.

REFERENCES

นันทวัน บุญยะประภัศร และ อรนุช โชคชัยเจริญพร 2542. สมุนไพร...ไม้พื้นบ้าน เล่ม 3, บริษัท
ประชาชน จำกัด, กรุงเทพฯ, หน้า 262-273.

Abcarian, H., Williams, J.A., Christiansen, J., 1994. Benign anorectal disease: definition,
characterization and analysis of treatment. Am J Gastroenterol 89, 182-193.

Adams, S.S., Hebborn, P., Nicholson, J.S., 1968. Some aspects of the pharmacology of
ibufenac, a non-steroidal anti-inflammatory agent. J Pharm Pharmacol 20, 305-
312.

Antman, E.M., DeMets, D., Loscalzo, J., 2005. Cyclooxygenase inhibition and
cardiovascular risk. Circulation 112, 759-770.

Ashendel, C.L., Boutwell, R.K., 1979. Prostaglandin E and F levels in mouse epidermis
are increased by tumor-promoting phorbol esters. Biochem Biophys Res Commun
90, 623-627.

Awasthy, K.S., Chaurasia, O.P., Sinha, S.P., 2000. Cytogenetic toxicity of leaf extract of
Putranjiva roxburghii, a medicinal plant. J Toxicol Sci 25, 177-180.

Bencsics, A., Elenkov, I.J., Vizi, E.J., 1995. Alpha 2-, alpha 2A-, alpha 2B/2C
adrenoceptor subtype antagonists prevent lipopolysaccharide-induced fever
response in rabbits. Brain Res 705, 302-306.

Bhujbal, S.S., Chitlange, S.S., Suralkar, A., Shinde, D.B., Patil, M.J., 2008. Anti-
Inflammatory activity of an isolated flavonoid fraction from Celosia argentea Linn.
Journal of Medicinal Plants Research (JMPR) 2, 052-054.

Bishai, I., Dinarello, C.A., Coceani, F., 1987. Prostaglandin formation in feline cerebral
microvessels: effect of endotoxin and interleukin-1. Can J Physiol Pharmacol 65,
2225-2230.

Boutaud, O., Aronoff , D.M., Richardson, J.H., Marnett, L.J., Oates, J.A, 2002. Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H_2 synthases. Proc Natl Acad Sci USA 99, 7130-7135.

Breder, C.D., Dewitt, D., Kraig, R.P., 1995. Characterization of inducible cyclooxygenase in rat brain. J Comp Neurol 335, 296-315.

Breder, C.D., Dinarello, C.A., Saper, C.B., 1988. Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240, 321-324.

Breder, C.D., Smith, W.L., Raz, A., Masferrer, J., Seibert, K., Needleman, P., Saper, C.B., 1992. Distribution and characterization of cyclooxygenase immunoreactivity in the ovine brain. J Comp Neurol 322, 409-438.

Burke, A., Smyth, E., Fitzgerald, G.A., 2006. Analgesic-Antipyretic agents; pharmacotherapy of Gout. In: Brunton, L.L., Lazo, J.S., Parker, K.L., Goodman and Gilman Pharmacological Basis of therapeutics. 11th ed., McGraw-Hill: New York   671-715.

Chen, X.S., Funk, C.D., 2001. The N-terminal "beta-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. J Biol Chem 276, 811-818.

Chen, X.S., Sheller, J.R., Johnson, E.N., C.D., 1994. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gent. Nature 372, 179-182.

Cocchiara, J.L., 1991. Hemorrhoids, a practical approach to an aggravating problem. Postgrad Med 89, 149-152.

Collins, T., 2001. In: Cotran, R.S., Kumar, V., Collins, T., (Eds.), Robbins Pathologic Basis of Disease, Harcourt India Pvt. Ltd., New Delhi, 50-88

Craig, A.D., Dostrovsky, J.O., 1999. Medulla to thalamus. In: Wall, P.D., Melzack, R., (Eds.), The Textbook of Pain, fourth ed. Churchill Livingstone, London, 183-214.

Dahl, J.B., Moiniche, S., 2004. Pre-emptive analgesia. Br Med Bull 71, 13-27.

Devchand, P.R., Keller, H., Peters, J.M., Vazquez, M., Gonzalez, F.J., Wahli, W., 1996. The PPARalpha-leukotriene B4 pathway to inflammation control. Nature 384, 39-43.

Di Rosa, M., 1972. Biological properties of carrageenin. J Pharm Pharmacol 24, 89-102.

Dixon, R.A., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., Miller, D.K., 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature 343, 282-284.

Dray, A., Urban, L., Dickenson, A., 1994. Pharmacology of chronic pain. Trends Pharmacol Sci 15, 190-197.

Egan, K.M., Lawson, J.A., Fries, S., Koller, B., Rader, D.J., Smyth, E.M., FitzGerald, G.A., 2004. COX-2-derived prostacyclin confers atheroprotection on female mice. Science 306, 1954-1957.

Elmquist, J.K., Scammell, T.E., Saper, C.B., 1997. Mechanisms of CNS response to systemic immune challenge: the febrile response. Trends Neurosci 20, 565-570.

Emery, P., 1996. Clinical implications of selective cyclooxygenase-2 inhibition. Scand J Rheumatol 25, 23-28.

Ferreira, S.H., Moncada, S., Vane, J.R., 1973. Prostaglandins and the mechanism of analgesia produced by aspirin-like drugs. Br J Pharmacol 49, 86-97.

Folts, J.D., Schafer, A.L., Loscalzo, J., Willerson, J.T., Muller J.E., 1999. A perspective on the potential problems with aspirin as an antithrombotic agent: A comparison of studies in an animal model with clinical trials. J Am Coll Cardiol 33, 295-303.

Franzotti, E.M., Santos, C.V.F., Rodrigues, H.M.S.L., Mourao, R.H.V., Andrade, M.R., Antoniolli, A.R., 2002. Anti-inflammatory, analgesic and acute toxicity of *Sida cardiafolia* L. J Ethnopharmacol 72, 273-278.

Funk, C.D., 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871-1875.

Furstenberger, G., Marks, F., 1980. Early prostaglandin E synthesis is an obligatory event in the induction of cell proliferation in mouse epidermis *in vivo* by phorbol ester TPA. Biochem Biophys Res Commun 92, 749-756.

Garg, H.S., Mitra, C.R., 1968. *Putranjiva roxburghii* wall.-II Triterpenes of the trunk bark. Phytochemistry 7, 2053-2055.

Garg, H.S., Mitra, C.R., 1971a. Roxburghonic acid-a friedelane triterpenoid keto acid of the leaf of *putranjiva roxburghii*. Phytochemistry 10, 865-869.

Garg, H.S., Mitra, C.R., 1971b. Putraflavone, a new biflavonoid from *Putranjiva roxburghii*. Phytochemistry 10, 2787-2791.

Gronert, K., Clish, C.B., Romano, M., Serhan, C.N., 1999. Transcellular regulation of eicosanoid biosynthesis. Methods Mol Biol 120, 119-144.

Gupta, M., Mazumder, U.K., Sambath Kumar, R., Gomathi, P., Rajeshwar, Y., Kakoti, B.B.,Tamil, S.V., 2005. Anti-inflammatory, analgesic and antipyretic effects of methanol extract from Bauhinia racemosa stem bark in animal models. J Ethnopharmacol 98, 267-273.

Gupta, M.M., Verma, R.K., 1991. Lipid constituents of *Cissus quadrangularis*. Phytochemistry 30, 875-878.

Hama, A., Menzaghi, F., 2001. Antagonist of nicotinic acetylcholine receptors (nAChR) enhances formalin-induced nociception in rats: tonic role of nAChRs in the control of pain following injury. Brain Res 888, 102-106.

Hanns, U., Zeilhofer, A., 2007. Commentary Prostanoids in nociception and pain. Biochem Pharmacol 73, 165-174.

Harizi, H., Gualde, N., 2004. 5-Lipoxygenase Pathway, Dendritic Cells, and Adaptive immunity. J Biomed Biotechnol 2, 99-105.

Harizi, H., Juzan, M., Moreau, J.F., Gualde, N., 2003. Prostaglandins inhibit 5-lipoxygenase-activating protein expression and leukotriene B_4 production from dendritic cells via an IL-10-dependent mechanism. J Immunol 170, 139-146.

Heapy, C.G., Jamieson, A., Russell, N.J.W., 1987. Afferent C-fiber and A-delta activity in models of inflammation. Br J Pharmacol 90, 164.

Hendershot, L.C., Forsaith, J., 1959. Antagonism of the frequency of phenylquinone-induced writhing in the mouse by weak analgesics. J Pharmacol Exp Ther 125, 237-240.

Hodes, B., 1996. Hemorrhoidal Products. In: Handbook of Nonprescription Drugs, 11th ed. American Pharmaceutical Association; Washington, D.C, 261-271.

Hunskaar, S., Fasmer, O.B., Hole, K., 1985. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Meth 14, 69-76.

Husnkaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103-119.

Ito, S., Okuda-Ashitaka, E., Minami, T., 2001. Central and peripheral roles of prostaglandins in pain and their interactions with novel neuropeptides nociceptin and nocistatin. Neurosci Res 41, 299-332.

Jacobs, R.S., Culver, P., Langdom, R., O'Brien, T., White, S., 1985. Some pharmacological observations on marine natural products. Tetrahedron 41, 981-983.

Jakobsson, P.J., Steinhilber, D., Odlander, B., Radmark, O., Claesson, H.E., Samuelsson ,B., 1992. On the expression and regulation of 5-lipoxygenase in human lymphocytes. Proc Natl Acad Sci USA 89, 3521-3525.

Kim, D.H., Han, K.M., Chung, I.S., Kim, D.K., Kim, S.H., Kwon, B.M., Jeong, T.S., Park, M.H., Ahn, E.M., Baek, N.I., 2005. Triterpenoids from the Flower of *Campsis grandiflora* K. Schum as Human Acyl-CoA: Cholesterol Acyltransferase Inhibitors. Arch Pharm Res 28, 550-556.

Kluger, M.J., 1991. Fever: role of pyrogens and cryogens. Physiol. Rev 71, 93-127,

Kluger, M.J., Ringler, D.H., Anver, M.R., 1975. Fever and survival. Science 188, 166-168,

Kondoh, H., Sato, Y., Kanoh, H., 1985. Arachidonic acid metabolism in cultured mouse keratinocytes. J Investig Dermatol 85, 64-69.

Koster, R., Anderson, M., Beer, E.J., 1959. Acetic acid for analgesic screening. Fed Proc 18, 412.

Kozak, W., Kluger, M.J., Soszynski, D., Conn, C.A., Rudolph, K., Leon, L.R., Zheng, H., 1998. IL6 and IL1 in fever: studies using cytokine-deficient (knockout) mice. Ann NY Acad Sci 29, 85633-85647

Kujubu, D.A., Fletcher, B.S., Varnum, B.C., Lim, R.W., Herschman, H.R., 1991. TIS10, a phobol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/ cyclooxygenase homologue. J Biol Chem 266, 12866-12872.

Le Bars, D., Gozariu, M., Cadden, S.W., 2001a. Animal models of nociception. Pharmacol Rev 53, 597-652.

Le Bars, D., Gozariu, M., Cadden, S.W., 2001b. Animal models of nociception. Pharmacol Rev 53, 628-651.

Leon, L.R., Kozak, W., Rudolph, K., Kluger, M.J., 1999. An antipyretic role for interleukin-10 in LPS fever in mice. Am J Psychiatry 276, 81-89.

Levy, B.D., Clish, C.B., Schmidt, B., Gronert, K., Serhan, C.N., 2001. Lipid mediator class switching during acute inflammation: signals in resolution. Nat Immunol 2, 612-619.

Lewis, R.A., Austen, K.F., Soberman, R.J., 1990. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N Engl J Med 323, 645-655.

Li, S., Ballou, L.R., Morham, S.G., 2001. Cyclooxygenase-2 mediates the febrile response of mice to interleukin-1 beta. Brain Res 910, 163-173.

Loram, L.C., Fuller, A., Fick, L.G., Cartmell, T., Poole, S., Mitchell, D., 2007. Cytokine profiles during carrageenin-induced inflammatory hyperalgesia in rat muscle and hind paw. J. Pain 8, 127-136.

Luheshi, G.N., Stefferl, A., Turnbull, A., 1997. Febrile response to tissue inflammation involves both peripheral and brain IL-1 and TNF-alpha in the rat. Am J Physiol Regul Integr Comp Physiol 272, 862-868.

Macdonald, S., 2002. "Aspirin use to be banned in under 16 year olds". Br Med J 325, 988.

Mackowiak, P.A., Bartlett, J.G., Borden, E.C., 1997. Concepts of fever: recent advances and lingering dogma. Clin Infect Dis 25, 119-138.

Mairi, M., Georgina, L.H., Emad, M., 2004. El-Omar Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. Am J Physiol Gastrointest Liver Physiol 286, 515-520.

Malec, L.S., Pomilio, A.B., 2003. Herbivory effects on the chemical constituents of *Bromus pictus*. Molecular medicinal chemistry 1, 30-38.

Mancini, J.A, Abramovitz, M., Cox, M.E, Wong, E., Charleson, S., Perrier, H., Wang, Z., Prasit, P., Vickers, P.J., 1993. 5-lipoxygenase-activating protein is an arachidonate binding protein. FEBS Lett 318, 277-281.

Markham, K., 1975. Isolation techniques for flavonoids. Academic press, New York 21-27.

McAdam, B.F., Catella-Lawson, F., Mardini, I.A., Kapoor, S., Lawson, J.A., FitzGerald, G. A., 1999. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 96, 272-277.

McCann, M.E., Rickes, E.L., Hora, D.F., Cunningham, P.K., Zhang, D., Brideau, C., Black, W.C., Hickey, G.J., 2005. In vitro effects and in vivo efficacy of a novel cyclooxygenase-2 inhibitor in cats with lipopolysaccharide-induced pyrexia. Am J Vet Res 66, 1278-1284.

McColl, S.R., Hurst, N.P., Cleland, L.G., 1986. Modulation by phorbol myristate acetate of arachidonic acid release and leukotriene synthesis by human polymorphonuclear leukocytes stimulated with A23187. Biochem Biophys Res Commun 141, 399-404.

Merck., 1996. The Merck Index. 12 th ed. Merck & Co. Inc. Whitehouse Station, NJ, USA. ISBN No. 0911910-12-3.

Milton, A.S., 1982. Prostaglandins and fever. Trends Pharmacol Sci 40, 490-492.

Nishiki, K., Nishinaga, K., Kudoh, D., Iwai, K., 1988. Croton oil-induced hemorrhoid model in rat: comparison of anti-inflammatory activity of diflucortolone valerate with other glucocortricoids. Nippon Yakurigaku Zasshi 92, 215-222. (Abstract)

OECD, 1987. OECD Guideline for Testing of Chemicals, No 401: Acute Oral Toxicity. Organization for Economic Co-operation and Development, Paris.

Ohashi, K., Saigusa, T., 1997. Sympathetic nervous responses during cytokine-induced fever in conscious rabbits. Pflugers Arch 433, 691-698.

Penrose, J.F., Austen, K.F., Lam, B.K., 1999. Leukotrienes: biosynthetic pathways, release, and receptor-mediated actions with relevance to disease states. In: Gallin J.I., Snyderman R., editors. Inflammation: basic principles and clinical correlates. Philadelphia: Lippincott Williams & Wilkins, 361-372.

Peters-Golden, M., Brock, T.G., 2001. Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. FEBS Lett 487, 323-326.

Pfenninger, J.L., 1995. Nonsurgical treatment options for internal hemorrhoids. Am Fam Physician 52, 821-834.

Phuphathanaphong, L., Chayamarit, K., 2008. Flora of Thailand Euphorbiaceae. The National Herbarium Nederland (NHN).
Available
at:http://www.nationaalherbarium.nl/Thaieuph/ThDspecies/ThDrypetes.htm
(Accessed: July 27, 2008).

Pina, L.A., Sandrini, M., Vitale, G., 1996. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. Eur J Pharmacol 308, 31-40.

Radmark, O., 2002. Arachidonate 5-lipoxygenase. Prostaglandins Other Lipid Mediat. 68-69, 211-234.

Radmark, O., Shimizu, T., Jornvall, H., Samuelsson, B., 1984. Leukotriene A4 hydrolase in human leukocytes. Purification and properties. J Biol Chem 259, 12339-12345.

Rastogi, R.P., Mehrotra. B.N., 1998. Compendium of Indian Medicinal Plants. Vol.5. Central Drug Research Institute, Lucknow & Publications and Information Directorate, New Dehli. ISBN No. 81-85042-14-4.

Reanmongkol, W., Subhadhirasakul, S., Pairat, C., Poungsawai, C., Choochare, W., 2002. Antinociceptive activity of *Dyera costulata* extract in experimental animals. Songklanakarin J Sci Technol 24, 227-234.

Roth, J., Conn, C.A., Kluger, M.J., 1993. Kinetics of systemic and intrahypothalamic IL-6 and tumor necrosis factor during endotoxin fever in the guinea pig. Am J Psychiatry 265, 653-658.

Roth, G.J., Majerus, P.W., 1975. The mechanism of the effect of aspirin on human platelets: Acetylation of a particulate fraction protein. J Clin Invest 56, 624-632.

Rouzer, C.A., Shimizu, T., Samuelsson, B., 1985. On the nature of the 5-lipoxygenase reaction in human leukocytes: characterization of a membrane associated stimulatory factor. Proc Natl Acad Sci USA. 82, 7505-7509.

Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., Currie, M.G., 1996. Nitric oxide: a key mediator in the early and late phase of carrageenin-induced rat paw inflammation. Br J Pharmacol 118, 829-838

Sa´nchez-Mateo, C.C., Bonkanka, C.X., Hern'andez-P'erez, M., Rabanal, R.M., 2006. Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. fil. J Ethnopharmacol 107, 1-6.

Sause, R.B., 1995. Self-treatment of hemorrhoids. US Pharm 20, 32-40.

Selye, H., 1953. Use of granuloma pouch technique in the study of antiphologistic corticoids. Proc Soc Exp Biol Med 82, 328-333.

Sengupta, P., Mukherjee, J., 1968. Terpenoids and related compounds-XI[1] The structure of roxburgholone, a new triterpenoid constituent of *Putranjiva roxburghii*. Tetrahedron 24, 6259-6264.

Shalini, D., Donna, S.Z., 2006. Pathophysiology and Management of Fever. (8 screen). Available at:http://www.SupportiveOncology.net/ (Accessed: August 30, 2006).

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

Sirko, S., Bishai, I., Coceani, F., 1989. Prostaglandin formation in the hypothalamus in vivo: effect of pyrogens. Am J Physiol 256, 616-624.

Smith, W.L., 1992. Prostanoid biosynthesis and the mechanism of action. Am J Physiol, 263,118-191.

Smith, W.L., DeWitt, D.L., 1995. Biochemistry of prostaglandin endoperoxidase H synthase-1 and-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. Semin Nephrol 15, 179-194.

Sternberg, E.M., 1997. Neural-immune interactions in health and disease. J Clin Invest 100, 2641-2647.

Swierkosz, T.A., Jordan, L., McBride, M., McGough, K., Devlin, J., Botting, R.M., 2002. Actions of paracetamol on cyclooxygenases in tissue and cell homogenates of mouse and rabbit. Med Sci Monit 8, 496-503.

Tannenbaum, H., Davis, P., Russell, A.S., Atkinson, M.H., Maksymowych, W., Huang, S.H., Bellm M., Hawker, G.A., Juby, A., Vanner, S., Sibley, J., 1996. An evidence-based approach to prescribing NSAIDs in musculoskeletal disease: a Canadian consensus. J Can Med Assoc 155, 77-88.

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.

Tripathi, N.N., Kumar, N., 2007. *Putranjiva roxburghii* oil-A potential herbal preservative for peanuts during storage. J Stored Prod Res 43, 435-442.

Tubaro, A., Dri, P., Delbello, G., Zilli, C., Logia, R.D., 1985. The croton oil ear test revisited. Agents and Actions 17, 47-49.

Vane, J.R., 1987. The evolution of non-steroidal anti-inflammatory drugs and their mechanisms of action. Drugs 33, 18-27.

Vane J.R., 1996. Mechanism of action of NSAIDs. Br J Rheumatol 35, 1-3.

Vane, J.R., Botting, R.M., 1995. New insights into the mode of action of anti-inflammatory drugs. Inflamm Res 44, 1-10.

Vineger, R., Scheiber, W., Hugo, R., 1969. Biphasic development of carrageenin edema in rats. J Pharmacol Exp Ther 166, 96-103,

Vizi, E.S., 1998. Receptor-mediated local fine-tuning by noradrenergic innervation of neuroendocrine and immune systems. Ann NY Acad Sci 851, 388-396.

Warren, K.S., 1972. In: inflammation. Lepaw I.H., Waed P.A., New York. Academic Press, 203.

Watkins, L.R., Maier, S.F., Goehler, L.E., 1995. Cytokine-to-brain communication: a review and analysis of alternative mechanisms. Life Sci 57, 1011-1026.

Williams, C.S., DuBois, R.N., 1996. Prostaglandin endoperoxide synthase: Why two isoforms. Am J Physiol, 270, 393-400.

Wilson, L.M., 2003. Clinical Concepts of Disease Processes. In: Price, S.A., Wilson, L.M., (Eds.), Pathophysiology, Mosby St. Louis, Missouri, 44-61.

Winter, C.A., Risley, E.A., Nuss, G.W., 1962. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc Soc Exp Biol Med 111, 544-547.

Woiciechowsky, C., Asadullah, K., Nestler, D., Eberhardt, B., Platzer, C., Schöning, B., Glöckner, F., Lanksch, W.R., Volk, H.D., Döcke, W.D., 1998. Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury. Nat Med 4, 808-813.

Woolfe, G., MacDonald, A.D., 1944. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). J Pharmacology and Exp Therapeutics 80, 300-330.

Wuest, J.R., Gossel, T.A., 1992. Continuing Education. Advising consumers on OTC hemorrhoidal remedies: an update. Massachusetts State Pharmaceutical Association Newsletter 9, 25-29

Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L., Simmons, D.L., 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA, 88, 2692-2696.

Yacoubian, S., Serhan, C.N., 2007. New endogenous anti-inflammatory and proresolving lipid mediators: implications for rheumatic diseases. Nat Clin Pract Rheumatol 3, 570-579.

Yamagata, K., Andreasson, K.I., Kaufmann, W.E., Barnes, C.A., Worley, P.F., 1993. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. Neuron 11, 371-386.

Yoshimoto, T., Soberman, R.J., Spur, B., Austen, K.F., 1988. Properties of highly purified leukotriene C4 synthase of guinea pig lung. J Clin Invest 81, 866-871.

Zha, V., Yegnasubramanian, W., Nelson, W., Isaacs, A., Marzo, D., 2004. Cyclooxygenases in cancer: progress and perspective. Cancer Lett 215, 1-20.

APPENDIX-A

ANTINOCICEPTIVE, ANTIPYRETIC AND ANTI-INFLAMMATORY EFFECTS OF THE ETHANOL EXTRACT FROM *PUTRANJIVA ROXBURGHII* WALL LEAVES IN EXPERIMENTAL ANIMALS

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Introduction

Drypetes roxburghii Wall or *Putranjiva roxburghii* Wall (*P. roxburghii*) is known in Thai as Ma Kham Kai or Pra Kham Kai in the family Euphorbiaceae. It has been reported triterpenoids namely putranjivanonol (a hydroxy ketone), putranjic acid (a hydroxy acid) (Garg & Mitra, 1968), friedelin, putranjivadione, friedelanol and roxburgholone, isolated from the bark of *P. roxburghii* (Sengupta & Mukherjee, 1968). The alcoholic extract of the leaf of *P. roxburghii* Wall, yielded a new triterpene acid, roxburghonic acid, and new biflavonoid named putraflavone (Garg & Mitra, 1971).

Some pharmacological activities of *P. roxburghii* have been reported. The leaf extract of *P. roxburghii* significantly induced mitosis-disruptive chromosomal changes in bone marrow cells in rats (Awasthy et al., 2000). In Thai folklore medicine, its leaf and stem bark are traditionally used for the treatment of fever, whole of plant used for the treatment of hemorrhoid, tuberculosis and fever. (Boonyaprapat & Chokechaicharoenporn, 1999). Although *P. roxburghii* has been used for a long time but no pharmacological studies in *vivo* have previously been conducted on antinociceptive, antipyretic and anti-inflammatory action of this plant. In the present study, a crude ethanolic extract from the leaves of *P. roxburghii* Wall was evaluated for antinociceptive, antipyretic and anti-inflammatory activities in animal models.

Objective

To evaluate the antinociceptive, antipyretic and anti-inflammatory activities of the ethanolic extract from the leaves of *Putranjiva roxburghii* Wall in animal models.

Methodology

Plant material

The air dried leaf of *P. roxburghii* Wall. (Euphorbiaceae) was collected and purchased from herbal drugstores in Songkhla, Thailand. The plant material was identified by direct comparison with herbarium speciment at Department of Pharmacognosy and Pharmace Prince of Songkla University. Voucher specimens of the plant material was deposited in the same place. The air dried leaf was pulverized and stored in air tight container.

Preparation of the alcoholic extract from the leaves of P. roxburghii Wall.

The air dried leaves of *P. roxburghii* were macerated with 95% ethanol at room temperature, then filtered and evaporated to give a syrupy mass. This ethanol extract was dissolved in cosolvent (propylene glycol: tween 80: water 4:1:4) and used as the test extract . All doses were expressed in terms of mg of crude extract/ kg body weight.

Animals

Animals used in this study were male Swiss albino mice weighing 25-35 g and male Wister rats weighing 150-200 g. The rats were handled for 5-10 min daily for several days before experiments. All animals were obtained from the Animal House, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were housed for at least one week in the laboratory animal room prior to testing. Food and water were given *ad libitum* unless otherwise specified. All experimental protocols were approved by the Institutional Committee for Ethicals use of animals, Prince of Songkla University, Thailand.

Antinociceptive Activities

1. Writhing test

Writhing behavior was tested, in which 0.6% acetic acid solution (10 ml/kg body weight) was injected intraperitoneally and the number of writhings and stretchings was counted over a 20 min period as previously reported (Koster et al., 1959; Hendershot & Forsaith, 1959). The ethanol extract of *P. roxburghii* (100, 200 and 400 mg/kg), a reference analgesic drug aspirin (200 mg/kg), or cosolvent was orally administered 30 min before acetic acid. Ten mice were used for each group in this test.

2. Hot plate test

The hot plate test was carried out according to the method described by Woolfe and MacDonald (1944). Mice were placed on a hot plate maintained at 55 °C $\pm$ 1 °C. Latency of nociceptive response such as licking of a hind limb or jumping was measured. Starting thirty minutes after oral administration of the test agents except morphine (15 min after administration), the nociceptive response was measured every 15 min over a 60 min period. Morphine sulfate was injected subcutaneously. The cut–off time was 45 sec. Only the mice that showed nociceptive responses within 15 sec were used for the experiment. Ten mice were used for each group in this test.

3. Formalin test

Thirty minutes after oral administration of the *P. roxburghii* extract (100, 200 and 400 mg/kg), aspirin (200 mg/kg) or cosolvent except morphine (15 min after administration), 20 µl of 2.5% formalin in saline was injected subcutaneously to a hindpaw of the mice. Morphine sulfate was injected subcutaneously. The time spent for licking the injected paw was recorded and the data were expressed as total licking time in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection (Hunskaar *et al.*, 1985). Ten mice were used for each group in this test.

Antipyretic activity
Yeast-induced fever

Antipyretic activity of drug was measured by slightly modifying the method described by Adams et al. (1968). Male Wistar rats were fasted overnight with water *ad libitum* before the experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension (10 ml/kg) into the animals' dorsum region. Seventeen hours after the injection, the rectal temperature of each rat was measured using a digital thermometer (SK-1250 MC, Sato Keiryoki Mfg. Co., Ltd., Japan). Only rats that showed an increase in temperature of at least 0.7 °C were used for the experiments. The *P. roxburghii* extract (100, 200 and 400 mg/kg, p.o.), aspirin (200 mg/kg, p.o.) or cosolvent vehicle was administered orally and the temperature was measured at 1, 2, 3, 4 and 5 hr after drug administration. Six rats were used for each group in this test.

Anti-inflammatory activity
Carrageenin-induced paw edema

According to the method described by Winnter et al. (1962), the initial right hind paw volume of the rats was measured using a plethysmometer (Ugo Basile) and then 0.1 ml of 1% (w/v) carrageenin was subcutaneously injected into the subplantar region of the right hind paw. The volume of right hind paw was measured at 1, 2, 3, 4, and 5 hr after carrageenin injection, and the edema volume was determined. Cosolvent, *P. roxburghii* extract (100, 200 and 400 mg/kg, p.o.), or aspirin (200 mg/kg, p.o.) was orally administered 30 min before carrageenin injection. Six rats were used for each group in this test.

Statistical analysis
Data were expressed as mean ± SEM and were analyzed statistically by oneway ANOVA procedures, followed by using Bonferroni's test. A difference was considered at $p < 0.05$

Result

Antinociceptive activities
1. Writhing test

The results of acetic acid-induced writhing responses in mice indicate the analgesic activity of the ethanol extract of *P. roxburghii* are presented in Fig 1. The ethanol extract dose (100, 200 and 400 mg/kg) dependently suppressed the writhing response in mice. Aspirin at the dose of 200 mg/kg body weight elicited significant ($p < 0.05$) inhibitory effect on the writhing response induced by acetic acid when compared with control.

2. Hot plate test

Morphine at a dose of 10 mg/kg significantly increased the pain latency, whereas the ethanol extract of *P. roxburghii* (100, 200 and 400 mg/kg, p.o.) did not show significant effects compared to the control group. (Table 1.)

3. Formalin test

In the early phase, there were no significant inhibition of licking activity at the doses of 100, 200 and 400 mg/kg of *P. roxburghii* extract and aspirin compared to the control group.

In the late phase aspirin at a dose of 200 mg/kg body weight could intensively inhibit the licking response. *P. roxburghii* extract at the dose of 400 mg/kg body weight markedly decreased the licking time in this phase. Morphine (10 mg/kg) reduced the licking time in both early and late phases of the formalin test in mice (Fig 2).

Antipyretic activity
Yeast-induced fever

Subcutaneous injection of yeast caused rise in rectal temperature of rats. *P. roxburghii* extract at the dose of 400 mg/kg significantly reduced the rectal temperature of the animals similarly to aspirin (Table 2). A significant antipyretic effect of *P. roxburghii* extract at doses of 100 and 200 mg/kg observed at the fourth and fifth hour after extract administration ($P < 0.05$).

Anti-inflammatory activity
Carrageenin-induced paw edema

The inhibitory activity on carrageenin-induced hind paw edema in rats caused by the oral administration of *P. roxburghii* extract, at various assessment times after carrageenin injection is shown in Table 3. Aspirin at the dose of 200 mg/kg body weight exhibited significant edema inhibition. *P. roxburghii* extract at the dose of 400 mg/kg body weight also possessed significant inhibitory effect on carrageenin-induced paw edema at all recorded times. A significant anti-edema effect of *P. roxburghii* extract at the dose of 200 mg/kg was observed at the second, third, fourth and fifth hour after carrageenin injection. *P. roxburghii* extract at the dose of 100 mg/kg significantly inhibit effect on carrageenin-induced paw edema at the fourth and fifth hour ($P < 0.05$).
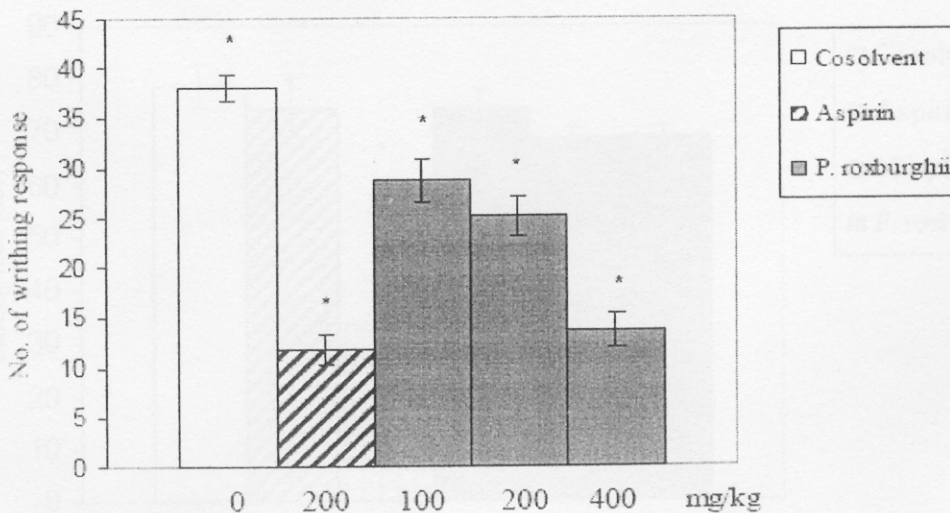

Fig 1 Analagesic effects of the ethanol extract of *P. roxburghii* Wall leaves and aspirin on acetic acid-induced writhing response in mice. Each value represents as mean $\pm$ SEM (n=10). * $p < 0.05$ as compared with the control (cosolvent) group (Bonferroni's test).

Table 1 Effect of the ethanol extract of *P. roxburghii* Wall leaves, aspirin and morphine on nociceptive response induced by heat in mice.

Drug	Dose (mg/kg.p.o.)	Latency of nociceptive response (sec)				
		0 min	15 min	30 min	45 min	60 min
Cosolvent 19.1±1.7	-		19.94±0.7	17.66±0.8	19.93±1.0	25.22±0.9
Aspirin 21.5±0.8	200		23.05±1.0	21.11±0.9	21.14±0.5	20.21±0.7
Morphine 45.2±0.4* Sulphate (s.c.)	10		39.03±1.7*	43.18±1.4*	45.27±0.1*	45.14±0.0*
P. roxburghii 18.0±1.6	100		18.24±0.9	15.57±0.8	16.70±1.0	15.87±1.4
18.6±1.5	200		18.66±0.8	15.72±0.9	17.44±0.9	18.11±1.2
21.0±1.7	400		20.84±1.0	18.79±0.9	21.70±1.7	20.52± 2.1

Each value represents as mean ± SEM (n=10)
* $p<0.05$, significantly different compared with the control (cosolvent) group (Bonferroni's test).
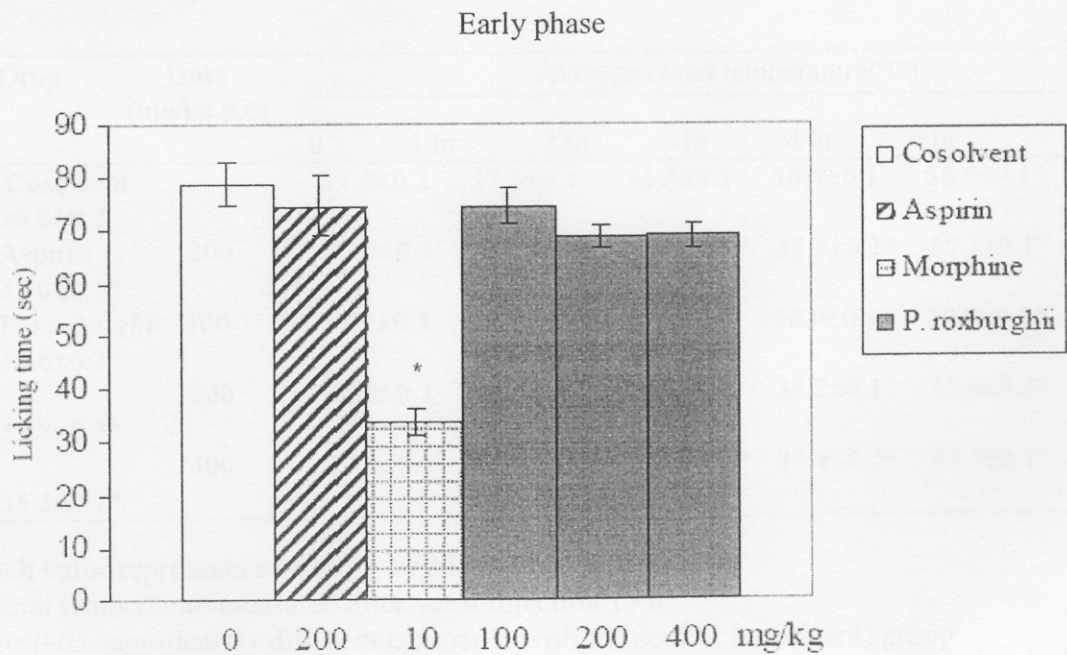
Early phase

Fig 2 Effects of the ethanol extract of *P. roxburghii* Wall leaves, aspirin and morphine on nociceptive response by formalin-induced paw licking in the early phase and late phase in mice. Each value represents as mean ± SEM (n=10) * $p<0.05$ as compared with the control (cosolvent) group (Bonferroni's test).
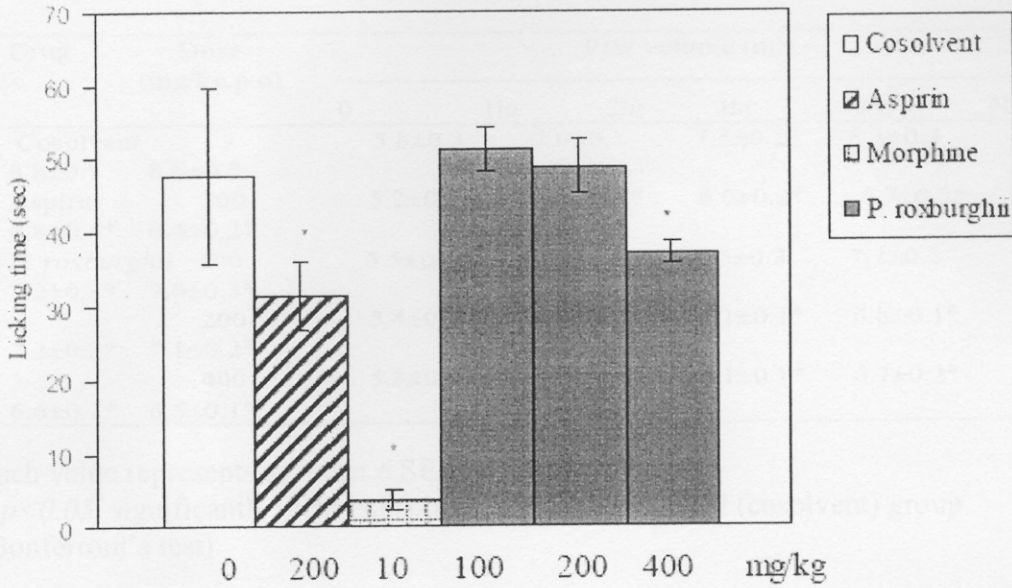
Late phase

Fig 2 (Continued)

Table 2 Effect of the ethanol extract of *P. roxburghii* Wall leaves and aspirin on brewer's yeast-induced fever in rats.

Drug	Dose (mg/kg.p.o)	Average rectal temperature(°C)					
		0	1 hr	2 hr	3 hr	4 hr	5 hr
Cosolvent 36.8±0.2	-	37.2±0.2	37.1±0.1	36.5±0.1	36.4±0.1	36.5±0.1	
Aspirin 35.0±0.1*	200	37.3±0.3	35.7±0.2*	35.4±0.1*	35.3±0.2*	35.1±0.1*	
P. roxburghii 35.6±0.2*	100	37.2±0.3	36.6±0.2	36.4±0.2	36.0±0.1	35.7±0.1*	
35.8±0.3*	200	37.0±0.1	36.3±0.1	35.9±0.1	35.7±0.1	35.6±0.2*	
35.3±0.1*	400	37.3±0.1	35.8±0.4*	35.4±0.1*	35.4±0.2*	35.3±0.1*	

Each value represents as mean ± SEM (n=6)
Rectal temperature measured after yeast injection 17 h
* $p<0.05$, significantly different compared with the control (cosolvent) group (Bonferroni's test).

Table 3 Effect of the ethanol extract of *P. roxburghii* Wall leaves and aspirin on carrageenin-induced paw edema in rats.

Drug	Dose (mg/kg.p.o)	Paw volume (ml)					
		0	1hr	2hr	3hr	4hr	5hr
Cosolvent	-	5.8±0.3	7.0±0.3	7.5±0.2	8.3±0.4	8.8±0.1	8.6±0.2
Aspirin	200	5.2±0.2	5.8±0.3*	6.0±0.2*	6.7±0.2*	6.8±0.4*	6.4±0.2*
P. roxburghii	100	5.5±0.3	6.1±0.2	6.5±0.3	7.2±0.2	7.2±0.3*	7.0±0.3*
	200	5.4±0.1	6.0±0.1	6.1±0.1*	6.8±0.1*	7.2±0.2*	7.1±0.2*
	400	5.3±0.1	5.9±0.1*	6.1±0.1*	6.7±0.2*	6.6±0.1*	6.5±0.1*

Each value represents as mean ± SEM (n=6)
* $p<0.05$, significantly different compared with the control (cosolvent) group (Bonferroni's test).

Discussion

In acetic acid-induced abdominal writhing which is the visceral pain model, the processor releases arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis plays a role in the nociceptive mechanism (Franzotti et al., 2002). However, it is known that constriction induced by acetic acid was considered to be a non-selective anti-nociceptive model, since acetic acid indirectly induced the release of endogenous mediators stimulated the nociceptive neurons that were sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) (Sa´nchez-Mateo et al., 2006). In the present study, the *P. roxburghii* extract significantly exhibited analgesic effect and this effect may be due to inhibition of the synthesis of the arachidonic acid metabolite. The ethanol extract of *P. roxburghii* exhibited protective action against nociception in writing test similarly to aspirin. These results suggest that the ethanol extract apparently possess antinociceptive activities.

The hot plate test has been found to be suitable for evaluation of centrally acting analgesics (Gupta et al., 2005). In the present study. Morphine, centrally acting analgesic drug, produced an inhibitory effect on the nociceptive response in this test, while the ethanol extract of *P. roxburghii* did not show antinociceptive effects in this test. Hence, but may be it is suggested that the apparent antinociceptive action of the *P. roxburghii* extract may be mediated through peripheral but not central mechanism.

In the formalin test, there is a distinctive biphasic nociceptive response termed as early and late phases. Drugs that act primarily on the central nervous system inhibit both phases, while peripherally acting drugs inhibit the late phase (Hunsnkaar & Hole, 1987). Inflammation in the late phase is due to the release of peripheral mediators like serotonin, histamine, bradykinin, prostaglandins and at least to some degree, the sensitization of central nociceptive neurons. The peripheral inflammatory processes are involved in the late phase and are blocked by non

steroidal anti-inflammatory drugs (NSAIDs) while the first phase seems to be unaffected (Tjolsen et al., 1992). Central analgesic drugs, such as morphine, inhibited equally in both phases, while peripherally acting drugs, such as NSIDs, aspirin suppressed mainly in the late phase. Our results showed that the *P. roxburghii* extract exerted significant inhibitory effect on nociceptive response of the late phase but not in early phase. Inhibition of only the late phase of the formalin test is a typical characteristic of cyclooxygenase inhibitors, suggesting a peripheral analgesic activity of *P. roxburghii* extract.

The *P. roxburghii* extract significantly inhibited the writhing and licking activity of the late phase in formalin test response in mice, as compared to the reference peripheral acting drugs such as aspirin which block prostaglandin *via* cyclooxygenase in peripheral tissues, could probably be due to the blockade of the effect or the release of endogenous substances that excite pain nerve endings.

Antipyretic activity is commonly mentioned as a characteristic of drugs or compounds which have an inhibitory effect on prostaglandin biosynthesis (Vane, 1987). Fever may be result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease state. The yeast induced fever in rats was employed to investigate the antipyretic activity of *P. roxburghii* extract. It was found that *P. roxburghii* extract at dose 400 mg/kg showed a significant decrease in rectal temperature similar to aspirin. Antipyretic are drugs, which reduced the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained (Goodman & Gilman, 1996). This result seems to support the view that *P. roxburghii* extract may have some influence on prostaglandin biosynthesis, because prostaglandin is believed to be a regulator of body temperature (Milton, 1982).

Carrageenin-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the anti-edematous effect of natural products. The carrageenin-induced hind paw edema in rat is know to be sensitive to cyclooxygenase inhibitors, but not to lipoxygenase inhibitors. (Di Rosa, 1972). Oral pretreatment of animal with *P. roxburghii* extract resulted in a significant inhibition of carrageenin-evoked hind paw edema. Therefore, it is suggested that the action mechanism of *P. roxburghii* extract may be related to prostaglandin synthesis inhibition. The mechanism of action of *P. roxburghii* extract is similar to aspirin, namely inhibition of prostaglandin synthesis.

Conclusion

Based on these results, we concluded that the ethanol extract of possessed antinociceptive, antipyretic and anti-inflammatory activities. Which its mechanism of action seems to be involved in inhibition of prostaglandin synthesis.

Acknowledgments

References

Adams, S. S., Hebborn, P., & Nicholson, J. S. (1968). Some aspects of the pharmacology of ibufenac, a non-steroidal anti-inflammatory agent. *Journal of Pharmacy and Pharmacology, 20*, 305-312.

Awasthy, K. S., Chaurasia, O. P., Sinha, S. P., (2000). Cytogenetic toxicity of leaf extract of *Putranjiva roxburghii*, a medicinal plant. *Journal of Toxicological Sciences, 25*, 177-80.

Boonyaprapat, N., & Chokechaicharoenporn, O. (1999). *Herbs Local Plants 3*. Bangkok: Prachachon.

Di Rosa, M. (1972). Biological properties of carrageenan. *Journal of Pharmacy and Pharmacology, 24*(2), 89-102.

Franzotti, E. M., Santos, C. V. F., Rodrigues, H. M. S. L., Mourao, R. H. V., Andrade, M., R., & Antoniolli, A. R. (2002). Anti-inflammatory, analgesic and acute toxicity of *Sida cardiafolia* L. *Journal of Ethnopharmacology, 72*, 273-278.

Garg, H. S., & Mitra, C. R. (1968). *Putranjiva roxburghii* all.-II Triterpenes of the trunkBark. *Phytochemistry, 7*, 2053-2055.

Garg, H. S., & Mitra, C. R. (1971). Putraflavone, a new biflavonoid from *Putranjiva roxburghii*. *Phytochemistry, 10*, 2787-2791.

Goodman & Gilman. (1996). *The pharmacological basis of therapeutics* (9th ed.) New York: McGraw-Hill.

Gupta, M., Mazumder, U. K., Sambath, K. R., Gomathi, P., Rajeshwar, Y., Kakoti, B. B., & Tamil, S. V. (2005). Anti-inflammatory, analgesic and antipyretic effects of methanol extract from *Bauhinia racemosa* stem bark in animal models. *Journal of Ethnopharmacology, 98*, 267-273.

Hendershot, L. C., & Forsaith, J. (1959). Antagonism of the frequency of phenylquinone-induced writhing in the mouse by weak analgesics. *The Journal of Pharmacology and Experimental Therapeutics, 125*, 237-240.

Hunskaar, S., Fasmer, O. B., & Hole, K. (1985). Formalin test in mice, a useful technique for evaluating mild analgesics. *The Journal of Neuroscience, 14*, 69-76.

Husnkaar, S., & Hole, K. (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain, 30*, 103-119.

Koster, R., Anderson, M., & de Beer, E. J. (1959). Acetic acid for analgesic screening. Federation. Proceedinges, 18, 412.

Milton, A. S. (1982). Prostaglandins and fever. *Trends in Pharmacological Sciences, 40*, 490-492.

Sa'nchez-Mateo, C. C., Bonkanka, C. X., Hern'andez-P'erez, M., & Rabanal, R. M. (2006). Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L.fil. *Journal of Ethnopharmacology, 107*, 1-6.

Sengupta, P., & Mukherjee, J. (1968). Terpenoids and related compounds-XI The structure of roxburgholone, a new triterpenoid constituent of *Putranjiva roxburghi* . *Tetrahedron, 24*, 6259-6264.

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.

Vane, J. R. (1987). The evolution of non-steroidal anti-inflammatory drugs and their mechanisms of action. *Drugs, 33*, 18-27.

Winter, C. A., Risley, E. A., & Nuss, G. W. (1962). Carrageenin–induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. In *Proceedings of the Society for Experimental Biology and Medicine* (pp. 111, 544-547). n.p.

Woolfe, G., & MacDonald, A. D. (1944). The evaluation of the analgesic action of pethidine hydrochloride (Demerol). *The Journal of Pharmacology and Experimental Therapeutics, 80*, 300-330.

APPENDIX-B

สำนักวิจัยและพัฒนา
มหาวิทยาลัยสงขลานครินทร์
อ.หาดใหญ่ จ.สงขลา 90110

Ref.10/51

หนังสือรับรอง

โครงการวิจัย เรื่อง   การศึกษาฤทธิ์แก้ปวด แก้ไข้ และแก้อักเสบของสารสกัดมะคำไก่ในสัตว์ทดลอง
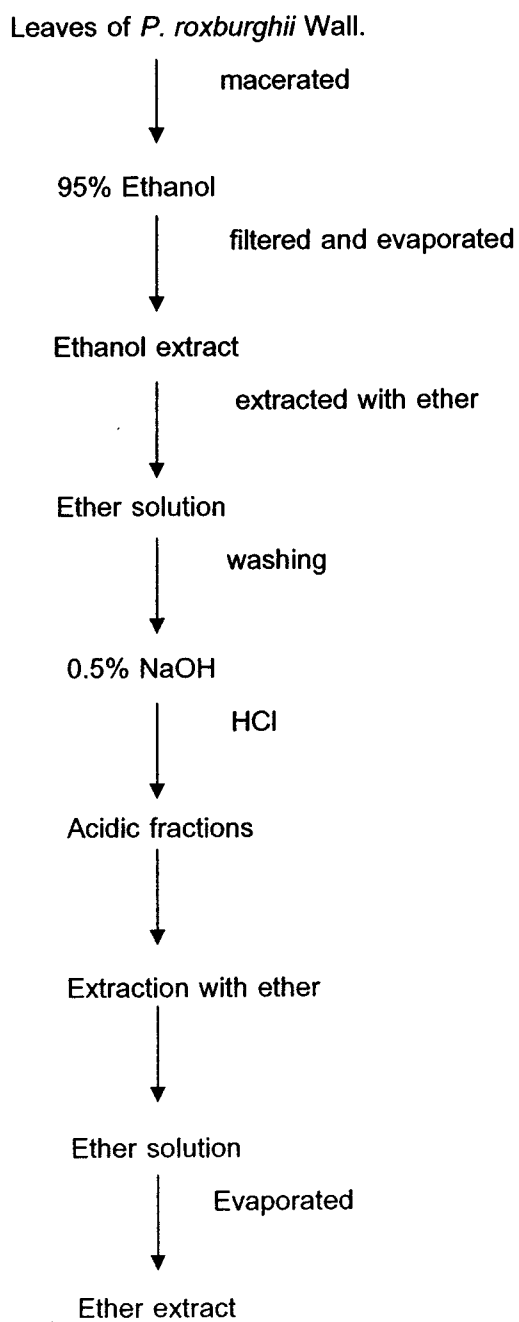
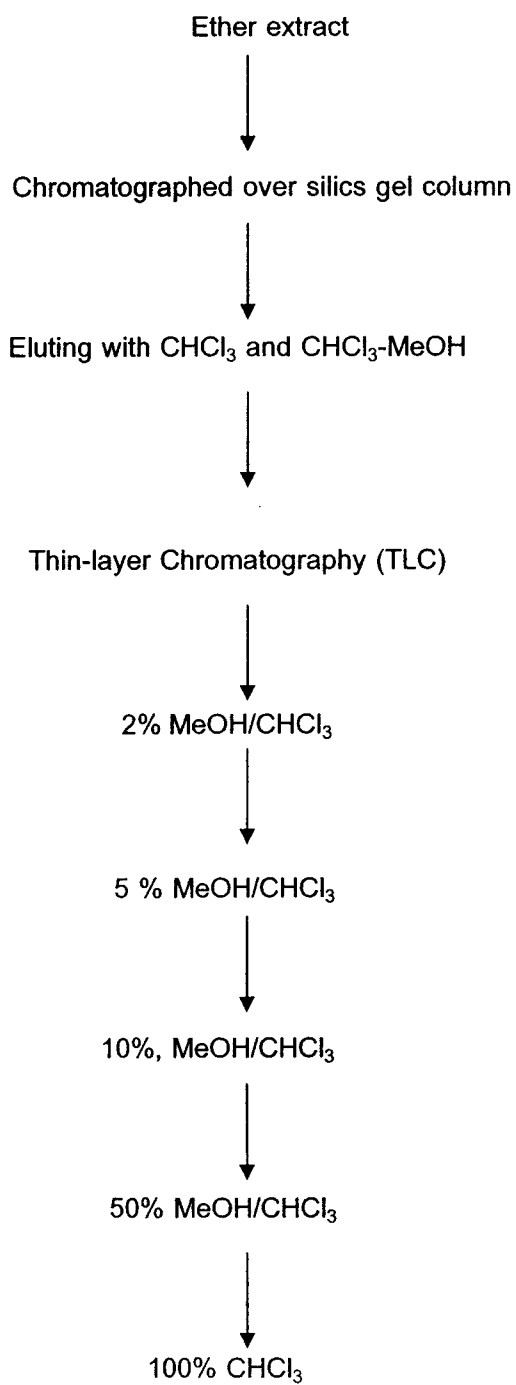**หัวหน้าโครงการวิจัย**   รศ.ดร.วันทนา เหรียญมงคล

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง มหาวิทยาลัยสงขลานครินทร์

    ให้ไว้ ณ วันที่ 29 เมษายน 2551

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มหาวิทยาลัยสงขลานครินทร์

APPENDIX-C

Extraction procedure

Leaves of *P. roxburghii* Wall.

| macerated

↓

95% Ethanol

| filtered and evaporated

↓

Ethanol extract

| extracted with ether

↓

Ether solution

| washing

↓

0.5% NaOH

| HCl

↓

Acidic fractions

|

↓

Extraction with ether

|

↓

Ether solution

| Evaporated

↓

Ether extract

Isolation of the ether extract

Ether extract

↓

Chromatographed over silics gel column

↓

Eluting with $CHCl_3$ and $CHCl_3$-MeOH

↓

Thin-layer Chromatography (TLC)

↓

2% MeOH/$CHCl_3$

↓

5 % MeOH/$CHCl_3$

↓

10%, MeOH/$CHCl_3$

↓

50% MeOH/$CHCl_3$

↓

100% $CHCl_3$

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Thaksin University	2001

List of Publication and Proceeding

Noppapan, T., Reanmongkol, W., Subhadhirasakul, S., 2008. Antinociceptive, antipyretic and anti-inflammatory effects of the ethanol extract from *Putranjiva roxburghii* Wall. leaves in experimental animals. 9th National Grad Research Conference, Graduate School, Burapha University, Thailand, pp. 1-10.