ฤทธิ์ระงับปวดของสิ่งสกัดด้วยเอทานอลจากกาวผึ้งไทย

นางรัตนา ช้อนทอง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# ANTINOCICEPTIVE ACTIVITY OF THE ETHANOLIC EXTRACT FROM THAI PROPOLIS

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for the Degree of Master of Science Program in Pharmacology
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| Thesis Title | Antinociceptive activity of the ethanolic extract from Thai propolis |
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รัตนา ช้อนทอง : ฤทธิ์ระงับปวดของสิ่งสกัดด้วยเอทานอลจากกาวผึ้งไทย. (ANTINOCICEPTIVE ACTIVITY OF THE ETHANOLIC EXTRACT FROM THAI PROPOLIS) อ. ที่ปรึกษา: ร.ท.หญิง ผศ.ดร. ภัสราภา โตวิวัฒน์, อ.ที่ปรึกษาร่วม: รศ.ดร. นิจศิริ เรืองรังษี, 111 หน้า.

โพรพอลิส (กาวผึ้ง) เป็นสารจำพวกเรซินซึ่งประกอบด้วยสารผสมเชิงซ้อนของเมแทบอไลต์ทุติยภูมิที่ผลิตโดยผึ้ง มีการนำกาวผึ้งมาใช้ในการรักษาโรคหลายชนิดในประเทศต่างๆหลายประเทศ ในการทดลองครั้งนี้มุ่งศึกษาฤทธิ์ระงับปวดของสิ่งสกัดจากกาวผึ้งไทยขนาดต่างๆกัน ในหนูถีบจักร โดยทดลองวางหนูบนแผ่นร้อนและจับเวลาที่หนูถีบจักรสามารถทนอยู่บนแผ่นร้อนได้ (hot-plate test) ทั้งก่อนและหลังฉีดน้ำมันข้าวโพด น้ำเกลือ มอร์ฟีน (10 มก./กก.) อินโดเมทาซิน (150 มก./กก.) หรือสิ่งสกัดจากกาวผึ้งไทย ขนาด 12.5, 25, 50, 100, 200, 400, และ 800 มก./น้ำหนักตัว 1 กิโลกรัม เข้าทางช่องท้อง ที่เวลา 15, 30, 45, 60, 90, 120, และ 240 นาทีโดยเวลาสูงสุดที่อนุญาตให้หนูถีบจักรอยู่บนแผ่นความร้อนเท่ากับ 45 วินาที และนำเวลาสูงสุดที่หนูถีบจักรสามารถทนต่อความร้อนได้มาคำนวณเปอร์เซ็นต์สูงสุดที่หนูถีบจักรสามารถทนต่อความร้อนได้ (%MPE) เพื่อนำมาคำนวณหาพื้นที่ใต้กราฟระหว่าง %MPE และเวลา (area of analgesia) จากการทดลองพบว่า สิ่งสกัดตั้งแต่ 50 มก./กก.ขึ้นไปสามารถทำให้หนูถีบจักรทนต่อความร้อนได้นานขึ้นอย่างมีนัยสำคัญทางสถิติ และหนูถีบจักรจะสามารถทนต่อความร้อนได้เพิ่มขึ้นตามขนาดของสิ่งสกัดที่สูงขึ้น โดยฤทธิ์ระงับปวดของสิ่งสกัด ( 200 มก./กก.) ถูกยับยั้งด้วยนาล็อกโซนและนาเทร็กโซน แต่ไม่ถูกยับยั้งด้วย NMDA (0.38 มก./กก.) แสดงว่าสิ่งสกัดจากกาวผึ้งไทยน่าจะออกฤทธิ์ผ่านวิถีของ opioid ในการทดลองที่ทำให้หนูเกิดความเจ็บปวดด้วยความร้อนโดยการส่องไฟที่หางหนูถีบจักร (tail-flick test) และจับเวลาที่หนูถีบจักรสามารถทนต่อความร้อนจนกระทั่งกระดกหางหนี (เวลาสูงสุดที่อนุญาตให้ส่องไฟที่หางหนูเท่ากับ 4 วินาที) พบว่า สิ่งสกัดตั้งแต่ 25 มก./กก.ขึ้นไปสามารถเพิ่มเวลาที่หนูทนต่อความร้อนโดยไม่กระดกหางหนีได้นานขึ้นเมื่อเทียบกับกลุ่มควบคุม ในการทดสอบ 7 ครั้งภายใน 4 ชั่วโมงหลังให้สารทดสอบ

ในการทดสอบที่ทำให้หนูถีบจักรเกิดความเจ็บปวดด้วยการฉีด formalin เข้าใต้ผิวหนังที่บริเวณอุ้งเท้าหลัง หลังฉีดน้ำมันข้าวโพด น้ำเกลือ มอร์ฟีน (10 มก./กก.) อินโดเมทาซิน (150 มก./กก.) หรือสิ่งสกัดจากกาวผึ้งไทย ขนาด 50, 100, 200, 400, และ 800 มก./น้ำหนักตัว 1 กิโลกรัม เข้าทางช่องท้องและนับจำนวนครั้งที่หนูเลียอุ้งเท้าที่ได้รับการกระตุ้น แบ่งเป็น 2 ช่วงเวลาในนาทีที่ 0-5 (ระยะที่ 1) และ นาทีที่ 25-30 (ระยะที่ 2) หลังฉีด formalin พบว่า สิ่งสกัดในขนาด 200 มก./กก. ทำให้หนูเลียอุ้งเท้าหลังลดลงในระยะที่ 1 เมื่อเทียบกับกลุ่มควบคุม และ สิ่งสกัด 200 มก./กก.ขึ้นไปสามารถลดจำนวนการเลียอุ้งเท้าหลังตามขนาดของสิ่งสกัดที่สูงขึ้นอย่างมีนัยสำคัญทางสถิติในระยะที่ 2 โดยสิ่งสกัดในขนาด 200-800 มก./กก. ไม่ทำให้หนูถีบจักรสูญเสียการทรงตัวเมื่อเปรียบเทียบกับกลุ่มควบคุมเมื่อทดสอบด้วย rota-rod จากผลการทดลองทั้งหมดสรุปได้ว่า สิ่งสกัดจากกาวผึ้งไทยมีฤทธิ์ระงับปวด และฤทธิ์ระงับปวดจะเพิ่มขึ้นตามขนาดของสิ่งสกัดที่เพิ่มขึ้น โดยไม่มีผลต่อการทรงตัวของสัตว์ทดลอง และกลไกการออกฤทธิ์น่าจะเกี่ยวข้องกับตัวรับของ opioid

สาขาวิชา เภสัชวิทยา..... ลายมือชื่อนิสิต .....

ปีการศึกษา 2549..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

## 4689137820 : MAJOR PHARMACOLOGY

KEY WORD: THAI PROPOLIS / HOT-PLATE / TAIL-FLICK / FORMALIN TEST

RATTANA CHONTHONG: ANTINOCICEPTIVE EFFECT OF THE ETHANOLIC EXTRACT FROM THAI PROPOLIS. THESIS ADVISOR: ASSIST PROF. LT. PASARAPA TOWIWAT, PH.D., THESIS CO- ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, PH.D., 111 pp.

Propolis (bee glue) is a resinous substance which contains a complex mixture of secondary metabolites, produced by honeybees. It has long been used in many countries for the management of several diseases. In these studies, we initially determined the analgesic property of a range of the ethanolic extract of Thai propolis (ETP) doses in the mouse hot-plate test. Hot-plate latencies (cut-off 45 sec) were determined in male ICR mice prior to the intraperitoneal (i.p.) administration of corn oil, 0.9% normal saline solution (NSS), morphine (MO: 10 mg/kg), indomethacin (IND: 150 mg/kg) or various doses of ETP (12.5, 25, 50, 100, 200, 400, 800 mg/kg). Hot-plate latencies were subsequently determined at 15, 30, 45, 60, 90, 120, 240 min. The percent maximum possible effect (%MPE) was calculated and used in determination of the area of analgesia (%MPE-min). ETP in doses of 50-800 mg/kg produced a significant dose-related analgesic response. ETP (200 mg/kg) produced analgesic response that was naloxone and naltrexone-sensitive suggesting opioid-mediated mechanism. In mouse tail-flick analgesia test, tail-flick latencies (cut-off 4 sec) were determined prior to the i.p. administration of corn oil, NSS, MO, IND or various doses of ETP (12.5-800 mg/kg) and were subsequently determined at 7 intervals over a 4-hr period. ETP in doses of 25-800 mg/kg produced a dose-dependent analgesic response.

Studies then determined the analgesic effect of ETP using the formalin-induced nociception test. After the i.p. administration of corn oil, NSS, MO, IND or various doses of ETP (50-800 mg/kg), 2.5% solution of formalin was administered into subplantar area of the right hind paw. The number of licks were subsequently determined at the first 0–5 min, and the following 25–30 min after formalin administration. ETP dose of 200 mg/kg produced analgesic response during the first phase and ETP doses of 200 mg/kg or higher produced a significant dose-related analgesic response during the second phase. ETP doses of 200-800 mg/kg i.p. failed to produce motor impairment compared to vehicle control in the rota-rod test. Taken together these results demonstrate that the ethanolic extract of Thai propolis produced analgesic effect that was dose-dependent in all analgesic testing models without altering motor performance and mechanism of actions seem to be related to the opioid receptors.

Field of study....Pharmacology..... Student's signature.....Rattana Chonthong.....

Academic...year...2006..... Advisor's signature.....Lt. Pasarapa Towiwat.....

Co-advisor's signature .....Nijsiri Ruangrungsi.....

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R. Chonthong

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| µm | = micrometer |
| µl | = microlitre |
| $\alpha$ | = alpha |
| $\beta$ | = beta |
| $\delta$ | = delta |
| °C | = degree of celcius |
| %MPE | = percentage of the maximum possible effect |
| / | = per |
| AUC | = area under the curve |
| cm | = centrimetre |
| ETP | = the ethanolic extract of Thai propolis |
| $ED_{50}$ | = fifty percent of effective dose |
| gm | = gram |
| hr | = hour |
| i.p. | = intraperitoneal |
| IND | = indomethacin |
| L | = litre |
| min | = minute |
| ml/kg | = millilitre per kilogram |
| mg/kg | = milligram per kilogram |
| MO | = morphine sulphate |
| m/sec | = metre per second |
| N | = sample size |
| NMDA | = N- methyl-D- aspartate |
| NSS | = normal saline solution |
| NAL | = naloxone |
| NALT | = naltrexone |
| sec | = second |

# CHAPTER I
# INTRODUCTION

Pain is defined by the International Association on the Study of pain (IASP) as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain is the crucial perceptions that the brain must evolve for the continued survival of the human organism as hunger and thirst. Pain is always subjective, and there are no specific tests that can quantitatively or qualitatively measure pain. The field of pain medicine and management, although still in its infancy, has seen enormous advances in the clinical arena and in the basic science. Currently, it is still early in the so-called decade of pain (2000-2010). The specific mechanisms contributing to an individual's pain are often unknown. Pain is a subjective experience, hard to define exactly, even though we all know what we mean by it. Typically, it is a direct response to an untoward event associated with tissue damage, such as injury, inflammation (Daniel *et al*., 2001).

Pain normally is evoked only by relatively high intensities of stimuli that have the potential to cause injury. Such pain is an example of nociception, or the detection of a potentially harmful stimulus, and is initiated by the activation of the peripheral endings of nociceptive neurons. The stimulus intensity required to activate nociceptors and evoke pain in uninjured tissue is below the intensity required to produce actual tissue damage, serves as a warning to prevent injury from occurring. When tissue injury has occurred, with the concomitant production of endogenous inflammatory agent, a state of inflammatory pain hypersensitivity may be induced, in which the stimulus response relationship is radically altered (Ropper, Brown, and Phill, 2005).

At present, analgesic drugs are widely used, such as NSAIDs account for about $100million annually of U.S. drug expenditures, while opioids and adjunctive medication therapies are associated with lower expenditures. However, clinically significant gastrointestinal events (e.g., gastrointestinal hemorrhage) attributed to NSAIDs may cause as many as 15,000 or more deaths in the United States. The severe chronic pain

e.g., advanced cancers, need to continue using strong narcotics to induce quality of life and the adverse effects can not be avoided. Therefore, the new drug with specific mechanism with pain relief is needed to be discovered.

There are many researches reported about the antinociceptive effect of propolis in various countries. de Campos et al. (de Campos, Paulino, da Silva, Scremin, and Calixto, 1998) reported that the ethanolic extract of Cuba propolis induced analgesic effect in hot-plate test. The ethanolic extract of Brazilian propolis was ineffective when assessed in the tail-flick and hot-plate assays but it produced anti-inflammatory effect in Randall-Selitto test (Ledon, Casaco, Gonzalez, Merino, Gonzalez, and Tolon, 1997). The ethanolic extract of Bulgarian propolis inhibited inflammatory effect in acetic acid, formalin, and capsaicin induced inflammatory tests (Paulino, Dantas, Bankova, Longhi, Scremin, and de Castro, 2003). The analgesic effect of the propolis varies with the sources (Castaldo, and Capasso, 2002). Thailand is in the tropical zone like South America and has a lot of species of plants that also grow in Cuba and Brazil. These studies are therefore designed to examine in various animal models the antinociceptive property of the ethanolic extract of Thai propolis.

## PURPOSE OF THE STUDY

To evaluate the antinociceptive effect of the ethanolic extract of Thai propolis compared with reference drugs and to investigate the possible mechanism involved.

## HYPOTHESIS

The ethanolic extract of Thai propolis has antinociceptive activity in nociceptive tests including tail-flick test, hot-plate test, and formalin test.

## EXPECTED BENEFIT AND APPLICATION

Knowledge from the studies of mechanism and antinociceptivie activity of the ethanolic extract of Thai propolis may lead to the development of a new analgesic drug from natural sources of Thailand that have never been used for any purposes.

# CHAPTER II
# REVIEW OF RELATED LITERATURES

## BACKGROUND AND RATIONALE

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The word 'unpleasant' comprises the whole rage of disagreeable feelings from being merely inconvenienced to misery, anguish, anxiety, depression and desperation, to the ultimate cure of suicide. The general principle that the best treatment of the symptom is removal of its cause applies. But this is often impossible to remove the cause of pain such as postsurgical or advanced cancer (Herfindal and Gourley, 2005).

## PAIN PROCESSING

The four physiological processes involved with pain are transduction, transmission, modulation, and perception. Mechanical, thermal, and chemical stimuli are ***transduced*** in primary afferent neurons. The "electrical" pain signals (produces via ion current/potential) are ***transmitted*** to the central nervous system. Along the way to the brain, pain signals may be ***modulated*** at various different points. Finally, in the brain, pain ***perception*** takes place (Ropper et al., 2005).

### 1. Transduction

Transduction is the reception of the noxious impulse at nociceptors from tissue insult. Two phenomena occur via the nociceptors. The first is receptor activation or transduction, in which chemical, thermal, or mechanical energy is translated to an electrochemical nerve impulse in the primary afferent nerve of nociceptors. The process typically begins with nerve depolarization. For pain caused by mechanical, thermal, chemical or physical stimulation of most tissues (e.g., skin, muscle, fascia, joints, and bone) initiation of nerve depolarization and transmission of nociceptive information begin at the nociceptors, i.e., transduction occurs primarily at the nociceptors (Haines, 2006).

More recent evidence indicates the three categories of cutaneous receptors can be identified: mechanoreceptors and thermoreceptors respond at a low threshold of stimulation, and impulses are transmitted by the way of large nerve fibers to the posterior columns of the spinal cord and thence via the medial lemniscus. Nociceptors (noxious receptors) have a high response threshold of heat and pressure, transmission is by the way of small myelinated fibers (A delta fibers, diameter 1–5 μm) and unmyelinated C fibers (diameter 0.5-1 μm). So the free nerve endings of the A-delta and C fibers are called nociceptors, as illustrated in the figure 1.

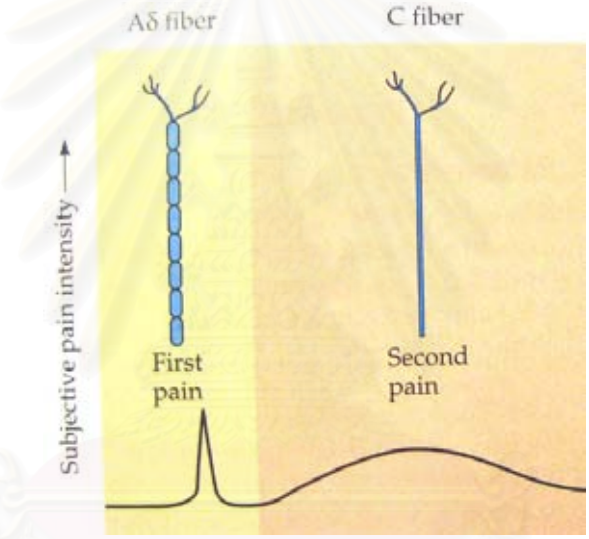


**Figure 1** A-delta and C fibers (Purves *et al*., 2004)

When tissue injury initiates nociception, numerous changes and interactions that may depolarize or sensitize the nerve endings occur, as illustrated in figure 3. Once the process begins, less intense noxious stimulation is needed to maintain depolarization of the phenomenon. Many substances that may initiate, facilitate, or inhibit the response to the painful stimulus are found in close proximity to the nerve endings (Lipman, 2004).

Substance P is a polypeptide that facilitates nociception. It is synthesized in the dorsal root ganglia and can be found in the nerve endings. When tissue injury occurs, antidromic (backward) stimulation of peripheral nerves releases substance P into the area of injury, as illustrated in the figure 2. This sensitizes the nerve endings causing edema. Tissue injury also results in norepinephrine release from sympathatic nerve

endings: that also sensitizes or depolarizes the nerve endings. The tissue damage itself can facilitate production and release of various chemical compounds including prostaglandins, bradykinin, histamine, and serotonin. These proinflammatory mediators further sensitize the nociceptors and cause local tissue reactions including edema and vasodilatation. Bradykinin can increase the production of prostaglandins and serotonin can increase the release of substance P from the nerve endings. When nociception occurs in response to injury, this chain of events perpetuates and accentuates the response (Rang, *et al*., 2003).

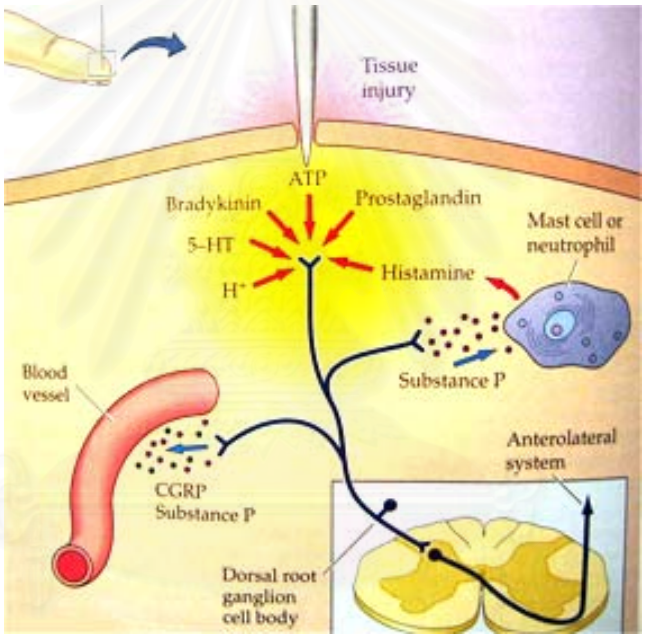


**Figure 2** Pain receptor activation (Purves *et al*., 2004)



2. Transmission

The second event is transmission of the impulse as coded electrochemical information to structures in the CNS. Transmission occurs initially in the spinal cord, where neurons relay messages form nociceptors to the brain. Noxious (nociceptive) impulses are transmitted from the peripheral site of injury toward the spinal cord along the two types of primary afferent nociceptive neurons, A-delta and C fibers. The cell body of nociceptors is located in the dorsal root ganglion, and most of their axons terminate in the dorsal horn of the spinal cord. Some afferents project to the spinal cord

through a ventral root as well, and both roots are thought to be important for pain transmission (Ropper, 2005).

C fibers respond to stronger, duller, slower impulses than A-delta fibers that transmit sharp impulses more rapidly. Nociceptive impulses pass through the dorsal root ganglia and proceed into the dorsal horn of the spinal cord. When the noxious stimulation is of sufficient intensity, these impulses excite secondary neurons and the nociceptive impulses ascend toward higher centers in the brain. Nerve endings in the periphery are fairly specialized in their response to changes caused by tissue stimulation, but there is further specialization of the nerve system as the neurons travel to the cord and on to the brain.

Nociceptive and non-nociceptive neurons terminate in different portions of the dorsal horn of the spinal cord. Nociceptive information arrives primarily in the superficial laminae and lamina V of the dorsal horn, whereas non-nociceptive information travels to laminae IV and VI. The neurochemistry of the different laminae of dorsal horn varies depending on function. Only laminae I, II, and V contain Substance P, glutamate, and receptors for both of these substances. Even more intriguing is the presence of inhibitory substances and receptors that can suppress the transmission of nociceptive information (Haines, 2006).

Within the dorsal horn of the spinal cord, presynaptic nerves terminate. These fibers contain Substance P and glutamate. Receptors for substance P, and the excitatory amino acids glutamate i.e., neurokinin-one ($NK_1$) and non-NMDA (N-Methyl D-Aspartate) receptors, respectively, are located on the post-synaptic nerve membrane. Both receptors are excitatory and play a role in the transmission of the nociceptive information to the secondary neuron. Substance P tends to increase the excitability of secondary neurons while glutamate tends to cause depolarization.

The presence of substance P permits lower concentrations of glutamate to depolarize the secondary neurons. Glutamate also acts on interneuron in the area through the NMDA receptor, and this can activate a less direct pathway to excite the

secondary neurons. The NMDA receptor is important when prolonged activation of this system occurs such as in chronic pain states, especially neuropathic pain.

The dorsal horn of the spinal cord is an important site for pharmacologic intervention. Capsaicin, the enzyme found in all hot peppers, and related compounds can activate vanilloid receptors in both skin and spinal cord, depleting substance P and other excitatory substances from the nerve endings (Rang et al., 2006).

Other important events occur within the spinal cord. Sensory input activates pathway to transmit nociceptive information to the level of consciousness, and the same nociceptive information can activate both motor and autonomic responses through reflex pathways. Nociceptive information can activate a motor response. Somatic (e.g., skin, fascia, muscle, bone) or visceral (internal organs, e.g., bowel, bladder) nociceptive input to the spinal cord can activate the motor neurons and cause increased muscle tone (rigidity) or muscle spasm (Lipman, 2004).

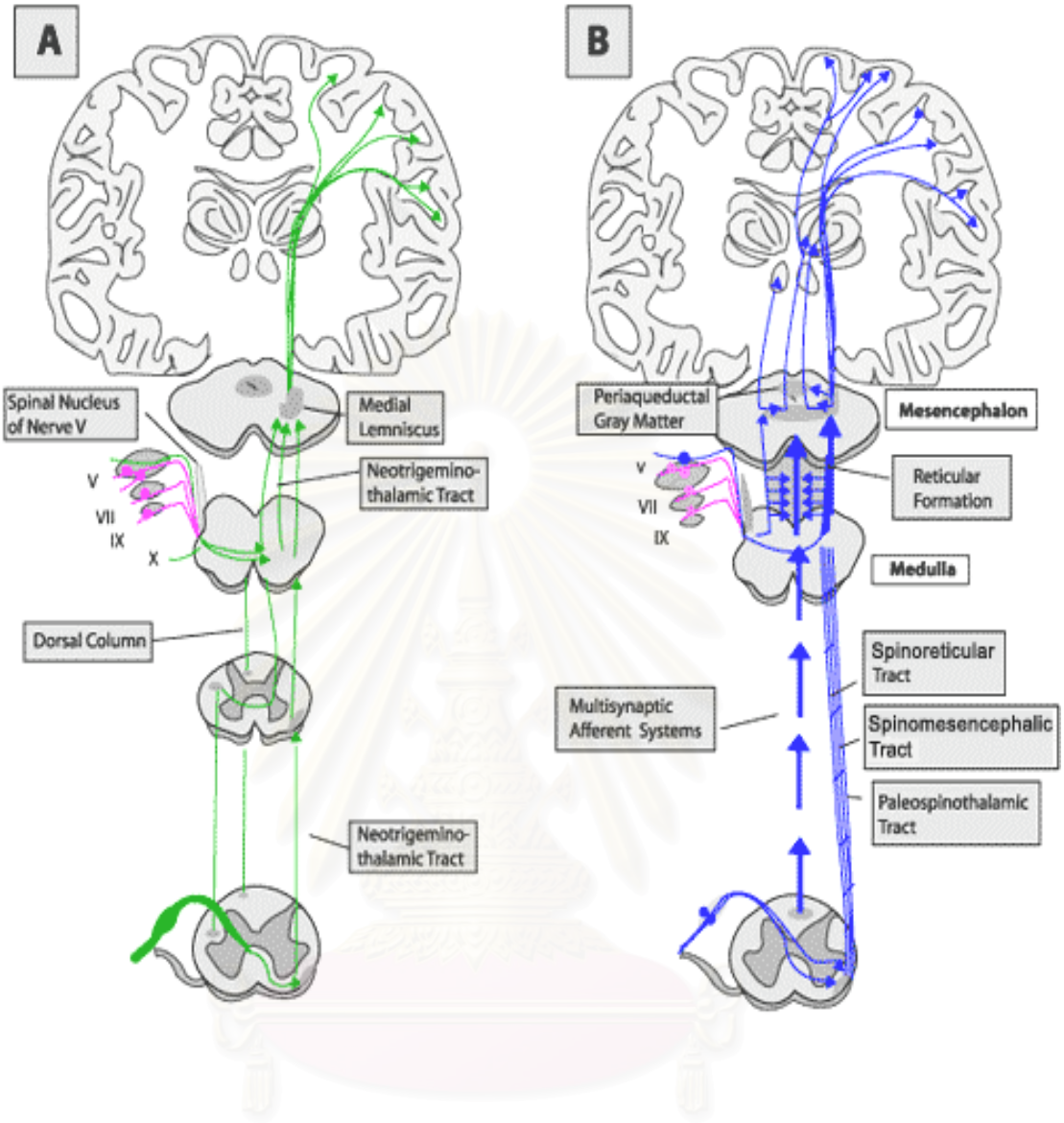


**Figure 3** Ascending pathways (Purves *et al*., 2004)

The process of transmission involves the ascending pathways. The three major groupings of ascending nociceptive pathways are the spinothalamic tract, the spinobulber projections (spinoreticular tract and spinomesencephalic tract), and the spinohypothalamic tract. Additionally, three indirect ascending pathways that are intregrated and relayed via multiple central nervous system areas toward the brain are the post-synaptic dorsal column system, the spinocervicothalamic tract pathway, and the spinoparabranchial pathway, as illustrated in the figure 3.

Although there seem to be other ways that pain signals can arrive at the brain, it has been proposed that there are two major pain systems (medial and lateral), both feeding the thalamus as a relay center to the brain. The medial pain system relays pain signals to the medial (paleo-) thalamus. The medial thalamus is not highly somatotypically organized and is believed to project in a net-like fashion to large areas of the cortex (especially the insula and anterior cortex). It is believed to be mainly involved in the motional and affective component of pain. The lateral pain system relay pain signals to the lateral (neo-) thalamus. The lateral thalamus is somatotopically highly organized and is believed to project mainly to the primary and secondary somatosensory areas of the cerebral cortex, as illustrated in the figure 4. It seems to be predominantly involved with the processing and transmission of the discriminative components of nociceptive-type information (i.e., letting the brain know the exact "coordinates" of where the pain is coming from in the body). This is highly complex and possibly somewhat inaccurate, and primary and secondary somatosensory areas probably involved somewhat in contributing to or sharping the effective aspects of pain; however, these concepts may be useful conceptually (Ropper et al., 2005).

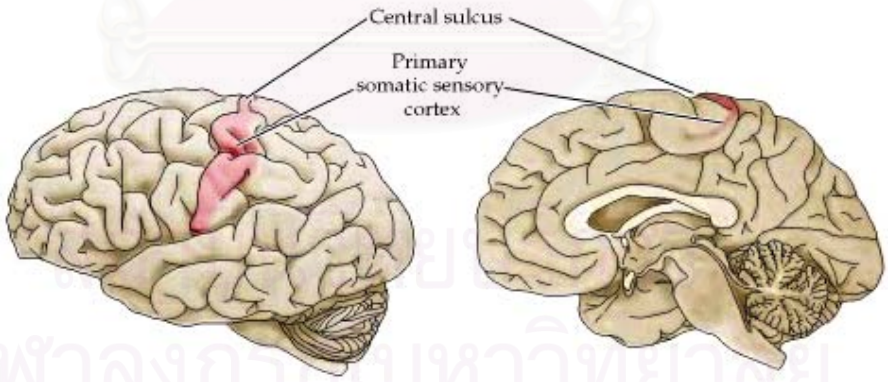


**Figure 4** Pain area of cortex (Purves *et al*., 2004)

### 3. Pain perception

From the level of the spinal cord, nociceptive information travels to several levels of the brain and eventually to the cerebral cortex. Technically, only when nociceptive information reaches the level of consciousness is the input intellectually perceived as “pain”. Several subcortical sites, including the periaqueductal gray, the thalamus, the amygdala, and the celebellum, may also play a role in contributing to pain perception. Noxious heat and noxious cold stimuli activate four cortical sites: the region of the central sulcus, the region of the lateral operculum, the insula, and the anterior cingulated cortex (Lipman, 2004).

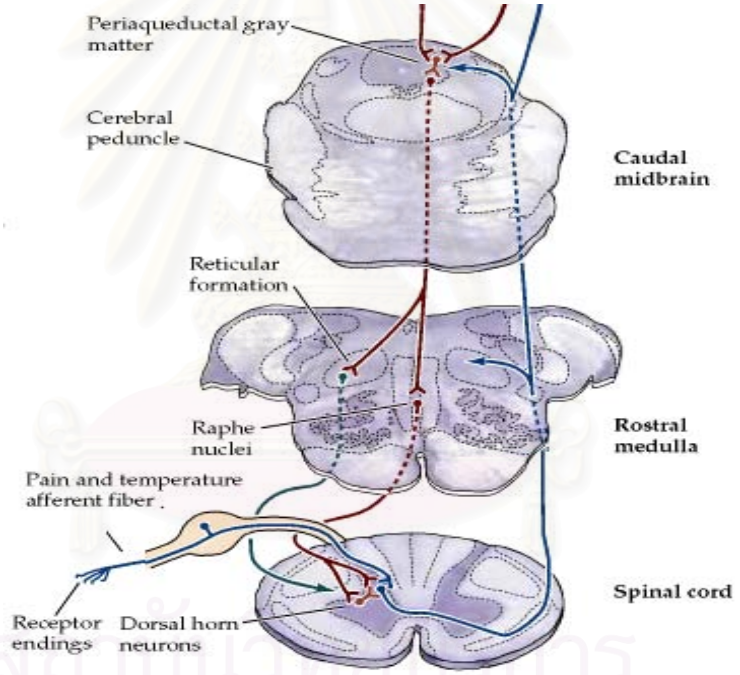


**Figure 5** Descending pathway (Purves *et al.*, 2004)

### 4. Pain modulation

#### 4.1 Descending inhibitory controls

Modulation of analgesia can probably occur at numerous areas of the nervous system, but a major role seems to involve the CNS descending (inhibitory) pathways. A key part of this descending system is the periaqueductal gray (PAG) area of the

midbrain, a small area of gray matter surrounding the central canal. The PAG receives inputs from many other brain regions, including hypothalamus, cortex, and thalamus, and it is through to represent the mechanism whereby cortical and other inputs act to control the nociceptive 'gate' in dorsal horn, as illustrated in the figure 5.

The main neuronal pathway activated by PAG stimulation runs first to an area of medulla close to the midline, known as the nucleus raphe magnus (NRM), and thence via fibers running in the dorsolateral funiculus of the spinal cord, which form synaptic connections on dorsal horn interneurons. The major transmitter at these synapse is 5 – hydroxytryptamine (5-HT), and the interneurons in turn act to inhibit the discharge of spinothalamic neurons. The NRM itself receives an input from spinothalamic neurons, via the adjacent *nucleus reticularis paragigatocellularis* (NRPG), so this descending inhibitory system may form part of a regulatory feedback loop whereby transmission through the dorsal horn is controlled according to the amount of activity reaching the thalamus (Haines, 2006).

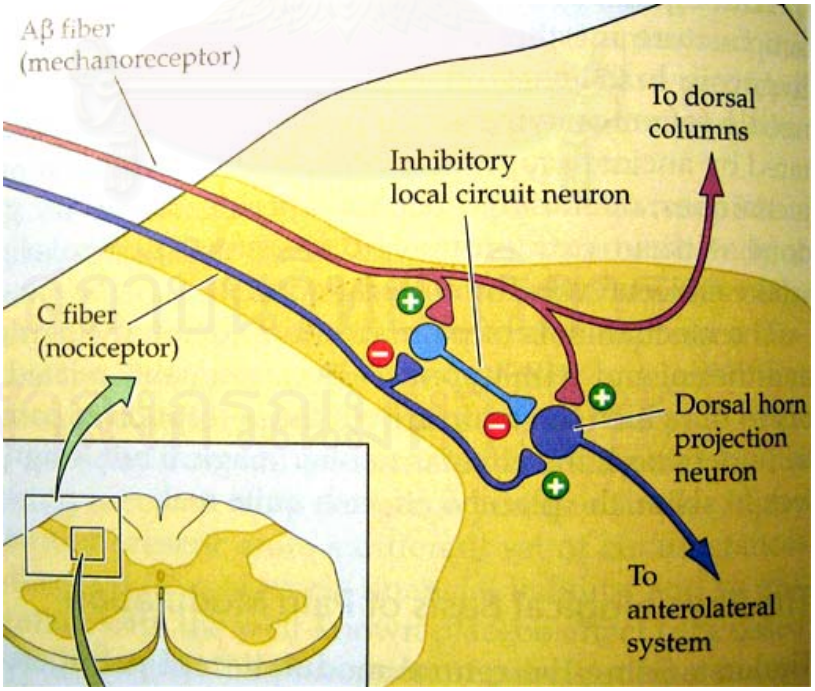


**Figure 6** Gate control theory (Purves *et al*., 2004)

### 4.2 The substantia gelatinosa and the gate control theory

Cells of lamina II of the dorsal horn (the substantia gelatinsa, SG) are mainly short inhibitory interneurons projecting to lamina I and lamina V. and they regulate transmission at the first synapse of the nociceptive pathway, between the primary afferent fibers and the spinothalamic tract transmission neurons. This gatekeeper function gave rise to the term gate control theory. The SG cells respond both to the activity of afferent fibers entering the cord (thus allowing the arrival of impulses via one group of afferent fibers to regulate the transmission of impulses via another pathway) and to the activity of descending pathways. This system regulates the passage of impulses from the peripheral afferent fibers to the thalamus via transmission neurons originating in the dorsal horn. Neurons in the substantia gelatinosa (SG) of the dorsal horn act to inhibit the transmission pathway. Inhibitory interneurons are activated by descending inhibitory neurons or by non-nociceptive afferent input. They are inhibited by nociceptive C-fiber input, so the persistent C-fiber activity facilitates excitation excitation of the transmission cells by either nociceptive or non-nociceptive inputs, as illustrated in the figure 6. This autofacillitation causes successive bursts of activity in the nociceptive afferents to become increasingly effective in activating transmission neurons. The SG is rich in both opioid peptides and opioid receptors and may be an important site of action for morphine – like drugs, and it is evident that similar 'gate' mechanisms also operate in the thalamus (Rang, 2003).

From the spinothalamic tracts, the projection fibers form synapse mainly in the ventral and medial parts of thalamus with cells having axons projecting to the somatosensory cortex. Stimulation to noxious stimuli in the periphery causes analgesia. Functional imaging studies in the conscious subjects suggest that the affective component of pain sensation involves a specific region of the cingulated cortex, distinct from the somatosensory cortex (lesions of which do not prevent the sensation of pain), though they can alter its quality.

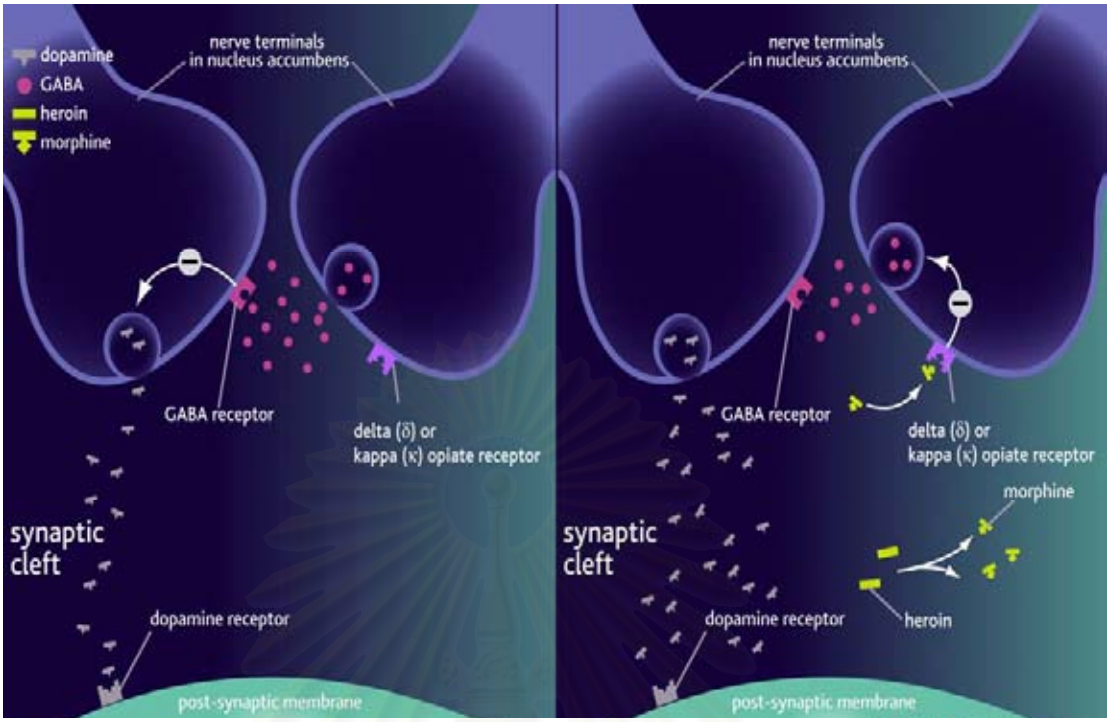


**Figure 7** Opioid receptor (adapted from CNS forum.com)

### 4.3 Modulation and interruption of central pain processing

#### 4.3.1 Opioid Receptors

Subpopulations of opioid receptors are characterized by their sensitivity to selective opioid agonists. Specific receptors in the CNS and peripheral tissues are responsible for modulating the effects of opioids and they are subdivided into four types: the mu ($\mu$), delta ( $\delta$), kappa ($\kappa$), and epsilon ($\varepsilon$) receptors. Sigma ($\sigma$) receptors were once considered part of the class of opioid receptors, but are now classified as a distinct receptor type. The $\mu$- and $\kappa$-receptors both produce analgesia, whereas the $\mu$-receptor is responsible for the habituating and withdrawal effects of the opioids. Mu-receptors, located primarily in pain-modulating areas of the CNS, induce central analgesia and respiratory depression. Kappa-receptors are responsible for analgesia at the levels of the spinal cord and the brain and are found in greatest concentration in the cerebral cortex and in the substantia gelatinosa of the dorsal horn. Because they are thought to produce analgesia without inducing opioid habituation, there is great interest

in the development of κ-specific receptor agonists. Although experimental κ-agonists such as spiradoline have shown low dependence and abuse liability, they are not ideal analgesics because of their psychotomimetic (hallucinogenic) and dysphoric effects. New evidence suggests that sex differences may exist with regard to receptor sensitivity. Delta-Receptors are located in the limbic area of the brain and in the spinal cord and may play a role in the euphoria that selected opioids produce. Evidence also exists that implicates them in analgesia at the spinal cord level. Some researchers consider ζ-receptors to be a subpopulation of the μ-receptors, or as mediators of μ-receptors. The function of ε-receptors has not yet been elucidated, whereas σ-receptor although not true opioid receptors, are believed to produce the psychotomimetic and dysphoric effects of some opioid agonists and partial agonists such as butonphanol and pentozocine (Rang, 2003).

Endogenous opioids known as endorphins, enkephalins, and dynorphins are found in varying concentrations in the CNS. Their roles are not completely understood, but dynorphins and enkephalins appear to be responsible for intrinsic regulation of pain perception within the medulla, while endorphins and enkephalins probably serve this function within the substantia gelatinosa. Each of endogenous opioids has greater preference for a particular receptor type: β- endorphin and enkephalins are potent at μ- and δ-receptors, while the κ-receptors is the target site for the dynorphins, as illustrated in the figure 7.

The site of action of opioids depends upon the method of administration. Systemically injected are ingested opioids produce high brain opioid concentrations with relatively low spinal concentrations. At the spinal level, opioids are thought to inhibit pain signals carried by the A-delta and C fibers at their synapses in the substatia gelatinosa (Rang, 2003).

Opioids exert at least part of their analgesic action by inhibiting subatance P release in the central and peripheral nervous systems. They also interfere with the actions of prostaglandins at the peripheral sites, particularly μ-receptor specific opioids

which inhibit $PGE_2$ hyperalgesia in a dose-dependent fashion. It is speculated that opioids produce analgesia by causing adenosine release, because methylxanthines such as caffeine can antagonize the effects of morphine.

Opioids may exert their inhibitory actions via hyperpolarization of neurons through altered conductance of potassium or calcium. However, evidence exists that they also cause in vitro excitatory actions at the nerve terminals (Katzung, 2004).

Naloxone and naltrexone are the narcotic antagonist. They work by blocking the opioid receptors in the brain and therefore blocking the effects of heroin and other opioids. Naloxone is the short-acting opioid receptor antagonist, used to rapidly reverse the effects of opioid intoxication or overdose. Naltexone , the long-acting opioid receptor antagonist, has also been shown to reduce craving and consumption for some patients who are alcohol dependent. They act as illustrated in the figure 8.

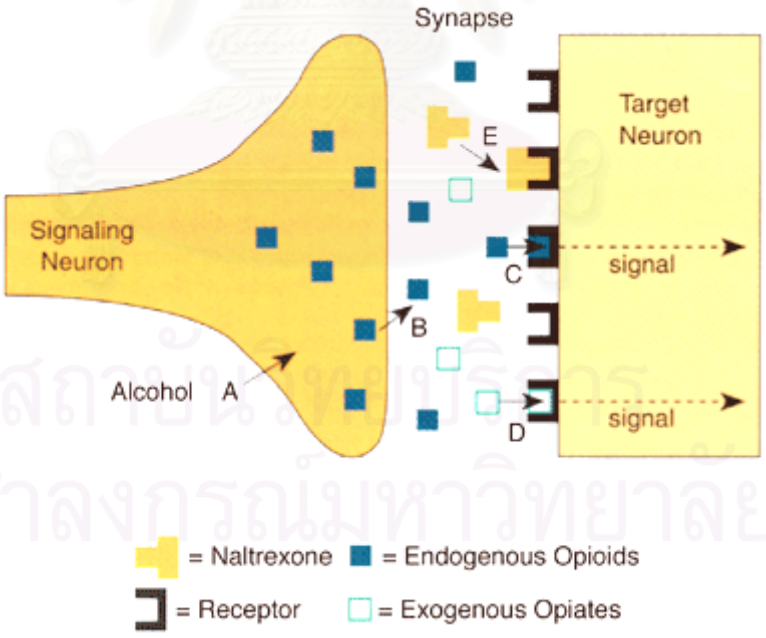


**Figure 8** Opioid receptor activation of a nerve and blockage of the signal by naltrexone (Purves *et al*., 2004)

### 4.3.2 Other pain-responsive receptors

Table 1 Lists of the receptors that are involved in modulation of pain pathways. The adrenergic agonists norepinephrine and clonidine, an $\alpha_2$ agonist, produce significant analgesia in human when administered into the spinal fluid, highlighting the role of adrenergic modulation of pain. Although it can produce peripheral hyperalgesia by enhancing prostacyclin production, norepinephine acts centrally on the dorsal horn via descending impulses from the brain to inhibit pain. The antinociceptive actions of both clonidine and norepinephrine can be reversed in a dose-dependent manner with adrenergic antagonists such as yohimbine (Lipman, 2004).

Serotonin receptors are found along the spinothalamic tract. Serotonin appears to reduce pain centrally by modulating descending impulses from the brain. This forms the basis ofr treatment of neuropathic pain syndromes with antidepressants that block presynaptic reuptake of serotonin. However, noradrenergic systems are likely also involved in this phenomenon, because selective serotonin reuptake inhibitors do not appear to be as effective in treating neurogenic pain as the tricyclic antidepressants (TCAs), which block reuptake of both serotonin and norepinephrine.

Cholinergic binding sites have been discovered in the dorsal horn. Application of the muscarinic agonist acetylcholine produces analgesia, which can be reversed by atropine. Such antinociceptive effects are not reduced by opioid antaginists.

Gamma-aminobutyric acid (GABA) receptors (GABAergic receptors) are divided into 2 types: $GABA_A$ receptors are sensitive to muscimol and $GABA_B$ receptors to baclofen. Of known GABAergic compounds, only baclofen has been shown to produce analgesia, although nonspecific GABA agonists such as clonazepam may also be useful for some painful conditions. $GABA_B$ agonists inhibit firing of the nociceptors, particularly the C fibres. Unlike opioids, baclofen does not inhibit substance P release in the spinal column (Ropper et al., 2005).

**Table 1** Receptors that are involved in modulation of pain pathway.

| Receptor | Subtypes | Agonist | Action | Location | Antagonist |
|---|---|---|---|---|---|
| Opioid | $\mu$, $\delta$, $\kappa$ | morphine | Analgesia | Brain and spinal cord | naloxone |
| Adrenergic | $\alpha_1$ | | Reduction in sympathetic nervous system output | dorsal column | prazosin |
| | $\alpha_2$ | clonidine | | dorsal column | yohimbine |
| | $\alpha$ and $\beta$ | norepinephrine | | dorsal column | yohimbine |
| Serotonergic Type I | | tricyclic antidepressants | | spinothalamic tract | cypro-heptadine |
| Cholinergic | acetylcholine | antinociception | | dorsal horn | atropine |
| GABAergic | A | | Inhibits firing of nociceptors | peripheral | |
| | B | baclofen | | dorsal horn | |



## ANIMAL MODELS FOR NOCICEPTIVE TESTING

Sensory systems have the role of informing the brain about the state of the external environments and the internal milieu of the organism. Pain is a perception, and as such, it is one of the outputs of the system in more highly evolved animal, the nociceptive system. Pain constitutes an alarm that ultimately has the role of helping to project the organism: it both triggers reactions and induces learned avoidance

behaviors, which may decrease whatever is causing the pain and, as a result, may limit the (potentially) damaging consequences (Daniel *et al*., 2001).

Because the goal of the animal models is the understanding of acute pain in human. The absence of verbal communication in animal is undoubtedly an obstacle to the evaluation of pain. There are circumstances during which there can be little doubt that an animal is feeling pain, notable when it is responding to stimuli through vocal responses such as squealing or groaning. On the other hand, it is far more difficult to certify that at a given moment, an animal feels no pain because it is presenting no typical physical signs or overt behaviors.

Experimental studies of nociception on conscious animals are often designated "behavioral studies", it mean is simply and implicitly that all responses, including simple withdrawal reflexes, are part of an animal's behavioral repertoire. The behavioral tests that are used to study nociception, nociceptive tests, constitute "input-output" system. The tests must specify the characteristics of the input (the stimulus applied by the scientist) and the output (the reaction of the animal). So 'input' is the stimulus (electrical, thermal, mechanical, or chemical) and 'output' is the response (Daniel *et al*., 2001).

**Animal models of nociception**

**1. Use of short-duration stimuli ("phasic pain")**

These tests are the most commonly used. In general they a) involve a short period of stimulation; b) have somatic rather than visceral sites of stimulation; c) involve measuring thresholds with the result that they generate no information whatsoever regarding responses to frankly nociceptive stimuli; d) usually involve measuring the response time to a stimulus of increasing intensity with the explicit or implicit assumption that this reaction time is related to the threshold; e) involve stimulation of minimal surface areas, with two important exceptions: the hot plate and the electrified grid, where the four paws and tail of the animal are stimulated simultaneously; and f) can be classified by the nature of the stimulus, be it thermal, mechanical, or electrical.

### 1.1 Tests based on the use of thermal stimuli

In tests involving thermal stimuli, it is always the skin that is stimulated. These tests do not involve visceral or musculoskeletal tissues. However, it is important not to forget that radiant heat also stimulates thermoreceptors and that, consequently, the application of a ramped thermal stimulus will result in an organized and unalterable sequence of activation, namely thermoreceptors, then thermoreceptors plus nociceptors, then nociceptors alone, and finally (possibly) nociceptors plus "paradoxical cold" receptors. In practice, the animal withdraws itself quickly from the stimulus, and therefore only the first part of this scenario takes place. The source of nociceptive stimulation can be distant from its target (e.g., radiant heat from a lamp) or can be in direct contact with the skin. Radiant heat constitutes a relatively selective stimulus for nociceptors and has an advantage over the other modes of thermal stimulation in that it produces no tactile stimulus (Daniel *et al*., 2001).

#### 1.1.1 The tail-flick test

There are two variants of the tail-flick test. One consists of applying radiant heat to a small surface of the tail. The other involves immersing the tail in water at a predetermined temperature. Although apparently similar, the two alternatives are actually quite different at a physical level: the cutaneous temperature varies with in square root of time in the first case and more rapidly in the second. In addition, the stimulated surface areas can be different.

**- The tail- flick test using radiant heat:**

The tail-flick test with radiant heat is an extremely simplified. Version of the method used on human subjects by Hardy et al. (1940). Indeed, Hardy and his colleagues eventually used the technique in the rat (Hardy, 1953; Hardy et al., 1957). The application of thermal radiation to the tail of an animal provokes the withdrawal of the tail by a brief vigorous movement (D'Amour and Smith, 1941; Smith et al., 1943). It is the reaction time of this movement that is recorded (often referred to as "tail-flick latency"). This is achieved by starting a timer at the

same time as the application of the heat source. By using a rheostat, the intensity of current through the filament and therefore of radiant heat emission can be controlled in such a way that one can empirically predetermine the time until the withdrawal of the tail. A photoelectric cell stops the timer and switches off the lamp at the moment the tail is withdrawn. A lengthening of the reaction time is interpreted as an analgesic action. It is advisable not to prolong the exposure to radiant heat beyond 10 to 20 sec, otherwise the skin may be burned. The advantages of this method are its simplicity and the small interanimal variability in reaction time measurements under a given set of controlled conditions. The reaction time of the tail movement varies with the intensity (power) of the source of radiant heat: when it is more intense, the temperature slope is steeper and, consequently, the reaction time is shorter. The test is more sensitive to morphine when the distal part of the tail is stimulated than when a more proximal part is stimulated, with the middle part giving an intermediate effect .To this day, no one has found a satisfactory explanation for these observations. All that can be said is that the tail of the rat is a complex structure, the movement of which is effected by between 8 and 14 muscles , and the conical form of which could influence how much of it [and what type(s) of receptors] are affected by thermal stimulation. It is also possible that heat reaches the nociceptors more rapidly at the tip of the tail where the skin is thinner. The tail-flick is a spinal reflex. As with all reflexes, it is subject to control by supraspinal structures.

**- The tail- flick test using immersion of the tail:**

The use of immersion of the tail is apparently a variant of the test described above. The most obvious difference is that the area of stimulation is far greater. Immersion of an animal's tail in hot water provokes an abrupt movement of the tail and sometimes the recoiling of the whole body. This test is actually quite different from the previous one in sofar as immersion of the tail in a hot liquid increases its temperature very quickly and in a more or less linear fashion, which is not the case with radiant heat. The main interest in this

response, which arguably has not been exploited sufficiently, lies in the possibility of applying different temperatures. Thus, lower temperatures can be used to seek evidence for the effects of minor analgesics (Sewell and Spencer, 1976; Luttinger, 1985). This also applies to using a bath in which the temperature increases slowly (Farré *et al*., 1989).

**1.1.2 The paw withdrawal test**

In principle, this test is entirely comparable to the test of D'Amour and Smith (1941) but offers the advantage that it does not involve the preeminent organ of thermoregulation in rats and mice, i.e., the tail (Hargreaves et al., 1988; Yeomans and Proudfit, 1994). With the aim of studying hyperalgesic phenomena resulting from inflammation (Hargreaves et al., 1988) had an inspired idea for supplementing the model of Randall and Selitto (Randall and Selitto, 1957) radiant heat was applied to a paw that had already been inflamed by a subcutaneous injection of carrageenin. For this purpose, inflammation can also be produced by exposure to ultraviolet rays (Perkins *et al*., 1993). One advantage in these tests is that heat is applied (to the plantar surface of the foot) of a freely moving animal. However, there is a disadvantage in that the position of the leg becomes a factor since the background level of activity in the flexors varies with the position of the animal. The withdrawal of the hind paw in the anesthetized rat and came to the following conclusions: when the heating slope is steep (6.5°C/s), the paw withdrawal reaction time is short and the skin surface temperature reaches a high level, suggesting Aδ fibers are activated; when the heating is slow (1°C/s), the reaction time is longer and skin temperature increases less, activating only C fibers (Yeomans and Proudfit, 1994; Yeomans *et al*., 1996). Morphine is far more active in the second than in the first of these tests (Lu *et al*., 1997).

**1.1.3 The hot-plate test**

This test consists of introducing a rat or mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid (Woolfe and MacDonald, 1944; Eddy and Leimbach, 1953; O'Callaghan and

Holzman, 1975). A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses. As far as analgesic substances are concerned, the paw-licking behavior is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50°C or less (Ankier, 1974) or if the temperature is increased in a progressive and linear fashion, e.g., from 43 to 52°C at 2.5°C/min (Hunskaar *et al*., 1985). The behavior is relatively stereotyped in the mouse but is more complex in the rat, which sniffs, licks its forepaws, licks its hind paws, straightens up, and stamps its feet, starts and stops washing itself, among other things. These behaviors have been labeled "chaotic defensive movements" (Knoll *et al*., 1955). Espejo and Mir identified and described 12 different behaviors. Because so many of these behaviors exist, observation of them is difficult (Espejo and Mir 1993). Furthermore, this test is very susceptible to learning phenomena, which result in a progressive shortening of the jumping reaction time accompanied by the disappearance of the licking behavior (Knoll *et al*., 1955).

Thus, the animal may lick the paws and then jump during the first test but will jump almost immediately, certainly with a much shorter reaction time, during subsequent Similarly, even putting the animals on an unheated plate just once to watch the test leads in subsequent tests to a diminution in the reaction time under standard conditions with a constant noxious temperature. In the final analysis, it should be noted that this test consists of stimulating the four limbs and even the tail of the animal simultaneously (Knoll et al., 1955). Such heterotopic stimuli involving large body areas undoubtedly trigger diffuse inhibitory controls that are likely to disturb the observed responses.

### 1.1.4 Tests using cold stimuli

Cold is very rarely used to test acute pain. On the other hand, it is more common to test cold allodynia in animal models of neuropathies. The techniques are directly inspired by those that use heat by contact: immersion of the tail or a limb (Pizziketti *et al*.,

1985), or placing the animal on a cold surface (Bennett and Xie, 1988; Jasmin *et al*., 1998).

**1.2 Tests based on the use of mechanical stimuli**

The preferred sites for applying nociceptive mechanical stimuli are the hind paw and the tail. Tests using constant pressure have been abandoned progressively for those applying gradually increasing pressures. In the course of such a test, a pressure of increasing intensity is applied to a punctiform area on the hind paw or, far less commonly, on the tail. In practice, the paw or tail is jammed between a plane surface and a blunt point mounted on top of a system of cogwheels with a cursor that can be displaced along the length of a graduated beam (Green *et al*., 1951). These devices permit the application of increasing measurable pressures and the interruption of the test when the threshold is reached. The measured parameter is the threshold (weight in grams) for the appearance of a given behavior. When the pressure increases, one can see successively the reflex withdrawal of the paw, a more complex movement whereby the animal tries to release its trapped limb, then a sort of struggle, and finally a vocal reaction. If the first of these reactions is undoubtedly a proper spinal reflex, the last two clearly involve supraspinal structures. This type of mechanical stimulation has a certain number of disadvantages (Fennessy and Lee, 1975): a) it is sometimes difficult to measure the intensity of the stimulus with precision; b) repetition of the mechanical stimulus can produce a diminution or conversely an increase in the sensitivity of the stimulated part of the body, in the latter case, this carries the risk that the tissues may be altered by inflammatory reactions that could call into question the validity of repeated tests; c) the necessity of applying relatively high pressures, which explains the weak sensitivity of the method and the relatively small number of substances that have been shown to be active by this test; and d) a non-negligible level of variability of the responses. With the aim of improving the sensitivity of the test, Randall and Selitto proposed comparing thresholds observed with a healthy paw and with an inflamed paw (Randall and Selitto, 1957).

The inflammation was induced beforehand by a subcutaneous injection into the area to be stimulated of substances such as croton oil, beer yeast, or carrageenan, the last of these being the most commonly used today. Even though it was found that the sensitivity of the method was improved, it was to the detriment of its specificity because, a priori, two different pharmacological effects, analgesic and anti-inflammatory, could be confused. It is therefore quite difficult to state that there has been analgesic or even "analgesic" activity. However, a comparison in the same animal of responses triggered from a healthy and an inflamed paw allows this problem to be overcome: nonsteroidal anti-inflammatory drugs (NSAIDs) are inactive on the former but do increase the (lowered) vocalization threshold when pressure is applied to the latter (Winter and Flataker, 1965b). One can increase the discrimination between different analgesic substances with this test by reducing the rate at which the pressure applied to the paw is increased and by increasing the time limit for subjecting the animal to the stimulus, the cutoff time (Chipkin *et al.*, 1983).

**1.3 Tests based on the use of electrical stimuli**

When such gradually increasing intensities of electrical stimuli are apply,one can observe successively a reflex movement of the tail, vocalization at the time of stimulation, and then vocalization continuing beyond the period of stimulation ("vocalization after-discharge"). These responses are organized on a hierarchical basis; they depend on the different levels of integration of the nociceptive signal in the central nervous system: the spinal cord, the brainstem, and the thalamus. The last of these can reflect affective and motivational aspects of pain behavior (Borszcz, 1995). The sensitivity to morphine of the thresholds for these three responses increases with the levels themselves: reflex < vocalization during stimulation < prolonged vocalization (Paalzow and Paalzow, 1975).This differential effect on the different behavioral responses suggests different sites of action that are organized hierarchically.

**2. Use of long-duration stimuli ("tonic pain")**

Basically, these tests involve using an irritant, algogenic chemical agent as the nociceptive stimulus. They differ from the vast majority of other tests in that they abandon the principle of determining the nociceptive threshold and involve a quantitative approach to the behavior observed after the application of a stimulus with a potency that is going to vary with time.

They can be thought of as a kind of model for tonic pain. However, they are not models for chronic pain because their duration is only in the order of some tens of minutes. The main types of behavioral test based on such stimuli use intradermal or intraperitoneal injections. The use of intra-arterial or intradental bradykinin is less common. Although intracapsular (jaw) injections of algogenic substances have also been used recently in pharmacological studies of pain in nonbehavioral models in which the animals are anesthetized (Broton and Sessle, 1988) In addition, there are behavioral tests that use the intracapsular administration of urate crystals, Freund's adjuvant, or carrageenin, but these are related to models of chronic inflammatory pain (Okuda *et al*., 1984). In this section, we also consider tests based on the stimulation of hollow organs. These animal models of visceral pain can be split into two categories on the basis of stimulus type: those involving the administration of algogenic agents, and those involving distension of hollow organs. In the latter case, one can add a subcategory of distension following induced inflammation of the hollow organ.

**2.1 Intradermal injections**

The most commonly used substance for intradermal injections is formalin (the "formalin test"). The term 'formalin' usually means a 37% solution of formaldehyde. Less commonly used are hypertonic saline (Hwang and Wilcox, 1986), ethylene diamine tetra-acetic acid (Teiger, 1976), Freund's adjuvant (Ladarola *et al*., 1988), capsaicin (Sakurada *et al*., 1992), and bee sting (Larivière and Melzack, 1996). Other substances have been tested but with less success (Wheeler-Aceto *et al*., 1990).

A 0.5 to 15% solution of formalin injected into the dorsal surface of the rat forepaw provokes a painful behavior that can be assessed on a four-level scale related to posture: 0, normal posture; 1, with the injected paw remaining on the ground but not supporting the animal; 2, with the injected paw clearly raised; and 3, with the injected paw being licked, nibbled, or shaken (Dubuisson and Dennis, 1977). The response is given a mark, and the results are expressed either continuously per unit of time or at regular time intervals when several animals are observed sequentially (Abbott *et al*., 1999). This method has also been used in the mouse, cat, and monkey (Dubuisson and Dennis, 1977; Alreja *et al*., 1984; Hunskaar *et al*., 1985; Murray *et al*., 1988; Tjølsen *et al*., 1992).

The measured parameter can also be the number of licks or twitches of the paw per unit of time, the cumulative time spent biting/licking the paw, or even a measure of the overall agitation of the animal obtained by a strain gauge coupled to the cage. Such specific behaviors resulting from an injection of formalin can be captured automatically by a camera attached to a computer; in this way, the effects of a pharmacological substance on such motor activity can be identified, analyzed, and uncoupled from antinociceptive effects (Jourdan *et al*., 1997). In the rat and the mouse, intraplantar injections of formalin produce a biphasic behavioral reaction. This behavior consists of an initial phase, occurring about 3 min after the injection, and then after a quiescent period, a second phase between the 20th and 30th minutes. The intensities of these behaviors are dependent on the concentration of formalin that is administered (Rosland *et al*., 1990).

The first phase results essentially from the direct stimulation of nociceptors, whereas the second involves a period of sensitization during which inflammatory phenomena occur. The central or peripheral origin of this second phase has been the subject of debate. For some, the second phase results from central processes triggered by the neuronal activation during the first phase. However, this hypothesis seems unlikely not only because formalin provokes biphasic activity in afferent fibers, but even more so because the blocking of the first phase by substances with rapid actions (e.g.,

subcutaneous lidocaine or intravenous remifentanil) does not suppress the second phase (Dallel *et al.*, 1995). Thus, the second phase cannot be interpreted as a consequence of the first; it clearly also originates from peripheral mechanisms. Opioid analgesics seem to be antinociceptive for both phases, although the second is more sensitive to these substances. In contrast, NSAIDs such as indomethacin seem to suppress only the second phase (Hunskaar and Hole, 1987), especially when the formalin is injected in high concentrations (Yashpal and Coderre, 1998).

**2.2 Intraperitoneal injections of irritant agents (the “writhing test”)**

The intraperitoneal administration of agents that irritate serous membranes provokes a very stereotyped behavior in the mouse and the rat which is characterized by abdominal contractions, movements of the body as a whole (particularly of the hind paws), twisting of dorsoabdominal muscles, and a reduction in motor activity and motor incoordination. The test is sometimes called the abdominal contortion test, the abdominal constriction response, or the stretching test, but more commonly it is known as the "writhing test". Generally the measurements are of the occurrence per unit of time of abdominal cramps resulting from the injection of the algogenic agent.

These behaviors are considered to be reflexes (Hammond, 1989) and to be evidence of visceral pain (Vyklicky, 1979); however, it would probably be wiser to call it peritoneovisceral pain. Indeed, given the well established fact that the parietal peritoneum receives a somatic innervations, it is possible that the pain may not be visceral at all. However, the pain is probably similar to that resulting from peritonitis. Indeed, these tests work not only for all major and minor analgesics, but equally for numerous other substances, including some that have no analgesic action, e.g., adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics. (Hendershot and Forsaith, 1959; Pearl *et al.*, 1968). Thus, a positive result with this test does not necessarily mean there is analgesic activity. Nevertheless, because all analgesics inhibit abdominal cramps, this method is useful for sifting molecules whose pharmacodynamic properties are unknown. The specificity can be

improved by undertaking a preliminary Roto-rod test to detect and eliminate molecules that alter the motor performance of the animal (Pearl *et al*., 1969b).

Although the writhing test has a poor specificity, it is sensitive and, after a fashion, predictive, as shown by the correlation between $ED_{50}$ values obtained in rats using this test and analgesic doses in humans. Intraperitoneal injections of algogenic substances have also been used in nonbehavioral models of nociception, i.e., models in which the animal is anesthetized. For example, changes in mean arterial blood pressure and intragastric pressure have been used as indicators of nociceptive responses to intraperitoneal bradykinin in anesthetized rats (Holzer-Petsche, 1992).

**2.3 Stimulation of hollow organs**

In addition to such tests of peritoneal or visceral nociception, other tests involve injecting algogenic substances directly into hollow organs and, as such, may be regarded as models for true visceral pain. For example, administration of formalin into the rat colon can produce a complex biphasic type of "pain behavior" involving an initial phase of body stretching and contraction of either the flanks or the whole body and a second phase that predominantly involves abdominal licking and nibbling. Similarly, a number of models have been developed for bladder pain, whereby reflexes and/or more complex behaviors have been observed following intravesical administration of capsaicin, capsaicin-like substances (Craft *et al*., 1993), more recently, a model for inflammatory uterine pain was developed, whereby intrauterine injections of mustard oil produced complex behavior patterns in rats (Wesselmann *et al*., 1998).

Models of visceral nociception have also used mechanical stimulation of parts of the genitourinary system in conscious animals, although such stimuli are more common in tests including anesthetized animals. It is possible to record a number of responses to intense mechanical stimulation of hollow viscera in anesthetized animals, and these have formed the basis of a number of tests. For example cardiovascular responses can be produced in anesthetized rats by colorectal distension with or without inflammation; the cardiovascular response involved a decrease in systemic arterial blood pressure, which

is in contrast to the increases in blood pressure evoked by visceral distension in awake animals. Clearly, the cardiovascular responses to visceral distension are preparation-dependent, and as a result, it may be even more important than in other models of pain to establish a good normal baseline response before the administration of drugs being tested. Finally, other responses to visceral distension have been monitored in models involving anesthetized preparations, notably changes in intragastric pressure during duodenal distension (Daniel *et al*., 2001).

## TREATMENT

### 1. PHARMACOTHERAPY

#### 1.1 Opioid analgesics

Opioid analgesics (narcotics), the most powerful analgesics, are the mainstay for treatment of severe acute pain and chronic pain due to cancer and other serious disorders. Opioids are preferred because they are so effective in controlling pain. The use of opioids to treat chronic pain not due to cancer is becoming more acceptable but is still relatively uncommon. Opioids are not appropriate for everyone (Howard and Huda, 2006). Opioids are all chemically related to morphine, a natural substance extracted from poppies, although some opioids are extracted from other plants and other opioids are produced in a laboratory. Opioids have many side effects. People who take opioids for acute pain often become drowsy. For some people, this drowsiness is welcome, but for others, it is not. Most people who take opioids become tolerant of this effect and do not continue to feel drowsy. Some people who continue to feel drowsy are given stimulant drugs, such as, to keep them awake and alert. Opioids may also cause confusion, especially in older people. Opioids often cause constipation and retention of urine, especially in older people. Stimulant laxatives, such as sienna, help prevent or relieve the constipation. Increasing intake of fluids can also help. Sometimes people with pain feel nauseated, and opioids can increase the nausea (McEvoy, 2003).

Doctors carefully weigh the benefits and side effects when they consider these drugs for the treatment of chronic pain. With repeated use of opioids over time, some people need higher doses because the body adapts to and thus responds less well to the drug; this phenomenon is called tolerance. For other people, the same dose remains effective for a long time. People who take opioids for a long time usually become dependent on them; that is, they experience withdrawal symptoms if the drug is stopped. When opioids are stopped after long-term use, the dose must be gradually tapered to minimize the development of such symptoms. Dependence is not the same as addiction, which is the disruptive behavior or activity associated with obtaining and using the drug. Although addiction is possible, it appears to be rare among people who take opioids to control pain. Too often, exaggerated concern about the addiction potential of opioids leads to undertreatment of pain and needless suffering. People with severe pain should not avoid opioids, and adequate doses should be taken as needed. When possible, opioids are taken by mouth. Opioids are given by injection when people cannot take them by mouth. For people who are helped by an opioid but cannot tolerate its side effects, an opioid can be administered directly into the space around the spinal cord through a pump, thus providing high concentrations of the drug to the brain.

One opioid, fentanyl, is available as a skin patch. It provides pain relief for up to 72 hours. Different opioid analgesics have different advantages and disadvantages. Morphine, the prototype of these drugs, can be taken by mouth (orally) or by injection. There are two oral forms: sustained-release and immediate-release. Different sustained-release forms provide relief for 8 to 24 hours. These drugs are widely used to treat chronic pain. The immediate-release form provides short-lived relief, usually for less than 3 hours. In injected forms, 2 to 6 times less is required than in oral forms, because when morphine is taken by mouth, much of the drug is chemically altered (metabolized) by the liver before it reaches the bloodstream. Usually, the difference in the amount needed for the different routes does not change the effects of the drug. Pain relief with injected forms is quicker than that with oral forms, but relief does not last as long.

Opioids are essential to the management of acute pain. For example, opioid analgesics are usually prescribed after surgery. They are most effective when taken every few hours, before pain becomes severe. The dose may be increased, or another drug (such as a nonsteroidal anti-inflammatory drug) may be added if the pain temporarily worsens, if the person needs to exercise (movement can be more painful), or if the wound dressing is about to be changed. When the pain eases, doctors reduce the dose and prescribe nonopioid analgesics, such as acetaminophen (Katzung, 2004).

**1.2 Nonopioid analgesic: NSAIDs and Acetaminophen**

**NSAIDs:** Most nonopioid analgesics are classified as nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs are used to treat mild to moderate pain and may be combined with opioids to treat moderate to severe pain. NSAIDs not only relieve pain, but they also reduce the inflammation that often accompanies and worsens pain. NSAIDs tend to irritate the stomach's lining and cause digestive upset (such as heartburn, indigestion, nausea, bloating, diarrhea, and stomach pain), peptic ulcers, and bleeding in the digestive tract. Nonsteroidal anti-inflammatory drugs (NSAIDs) work in two ways: They reduce the sensation of pain, and they reduce the inflammation that often accompanies and worsens pain. NSAIDs produce these effects because they reduce the production of hormone-like substances called prostaglandins. Different prostaglandins have different functions, such as sensitizing pain receptors to mechanical and chemical stimulation and causing blood vessels to dilate (Herfindal and Gourley, 2005). Most NSAIDs reduce prostaglandin production by blocking both cyclooxygenase (COX) enzymes (COX-1 and COX-2), which are crucial to the formation of prostaglandins.

A new group of NSAIDs, the coxibs (COX-2 inhibitors), tend to block only COX-2. Prostaglandins that are formed through the action of the COX-2 enzymes are released in response to an injury—burn, break, sprain, strain, or invasion by a microorganism. The result is inflammation, which is a protective response: The blood supply to the injured area increases, bringing in fluids and white blood cells to wall off the damaged tissue

and remove any invading microorganisms. Prostaglandins that are formed through the action of COX-1 enzymes help protect the digestive tract from stomach acid and play a crucial role in blood clotting. All NSAIDs, even the coxibs, reduce the production of these prostaglandins. Consequently, NSAIDs may irritate the stomach's lining and cause digestive upset, peptic ulcers, and bleeding in the digestive tract. Other NSAIDs block two enzymes: COX-1, which is involved in the production of the prostaglandins that protect the stomach and play a crucial role in blood clotting; and COX-2, which is involved in the production of the prostaglandins that promote inflammation. Coxibs tend to block only COX-2 enzymes. Thus, coxibs are as effective as other NSAIDs in the treatment of pain and inflammation. But coxibs are less likely to damage the stomach; to cause nausea, bloating, heartburn, bleeding, and peptic ulcers; and to interfere with clotting than are other NSAIDs. Because of these differences, coxibs may be useful for people who cannot tolerate other NSAIDs and for people who are at high risk of complications from use of other NSAIDs. Such people include older people, people who are taking anticoagulants, those who have a history of ulcers, and those who must take an analgesic for a long time. However, one of the coxibs, rofecoxib (withdrawn from the market), appears to increase the risk of heart attack and stroke after long-term use (Herfindal and Gourley, 2005).

**Acetaminophen**: This drug is roughly comparable to aspirin in its potential to relieve pain and lower a fever. But unlike NSAIDs, acetaminophen has virtually no useful anti-inflammatory activity, does not affect the blood's ability to clot, and has almost no adverse effects on the stomach. How acetaminophen works is not clearly understood. Acetaminophen is taken by mouth or suppository, and its effects generally last 4 to 6 hours. High doses can lead to liver damage, which may be irreversible. People with a liver disorder should use lower doses than usually taken. Whether lower doses taken for a long time can harm the liver is less certain. People who consume large amounts of alcohol are probably at highest risk of liver damage from overuse of acetaminophen. People who are taking acetaminophen and stop eating because of a bad cold,

influenza, or another reason may be more vulnerable to liver damage. Taking high doses for a long time may lead to kidney damage (McEvoy, 2003).

**1.3 Analgesic adjuvants and other medications**

Adjuvant analgesics are drugs that are not usually used for pain relief but may relieve pain in certain circumstances and that, when used to relieve pain, are usually used with other analgesics or nondrug pain treatments. The adjuvant analgesics most commonly used for pain are antidepressants (such as amitriptyline and desipramine) anticonvulsants (such as gabapentin, carbamazepine, and phenytoin), and oral and topical local anesthetics. Antidepressants can potentially relieve pain in people who do not have depression. There is some evidence that tricyclic antidepressants are more effective for this purpose than other antidepressants, but selective serotonin reuptake inhibitor (SSRI) antidepressants (such as fluoxetine) are tolerated better. People may respond to one antidepressant and not others. Anticonvulsants may be used to relieve neuropathic pain. Gabapentin is used most often, but many others, including phenytoin, carbamazepine, clonazepam, divalproex, lamotrigine, topiramate, and oxcarbazepine, may be tried. Anticonvulsants, such as divalproex, can also prevent migraine headaches.

Mexiletine, a local anesthetic taken by mouth to treat abnormal heart rhythms, is sometimes used to treat neuropathic pain. Local anesthetics are more commonly placed directly on or near a sore area to help reduce pain. For example, doctors may inject a local anesthetic, such as lidocaine, into the skin to control pain due to an injury or even due to neuropathic pain syndromes. Local anesthetics are also used in nerve blocks. For example, a sympathetic nerve block involves injecting a local anesthetic into a group of nerves near the spine, in the neck for pain in the upper body or in the lower back for pain in the lower body. Occasionally, pain related to nerve injury can be treated by injecting a caustic substance, such as phenol, into a nerve to destroy it, by freezing the nerve (in cryotherapy), or by burning the nerve with a radiofrequency probe. These techniques may be used to treat facial pain due to trigeminal neuralgia. Topical

anesthetics, such as lidocaine applied as a lotion, ointment, or skin patch, can be used to control pain due to some conditions. These anesthetics are usually used for a short period of time. For example, an anesthetic mouthwash can be used to relieve a sore throat. However, some people with chronic pain benefit from using topical anesthetics for a long time. For example, a lidocaine patch can be used to relieve postherpetic neuralgia. A cream containing capsaicin, a substance found in hot peppers, sometimes helps reduce the pain caused by such disorders as herpes zoster and osteoarthritis. It is most often used by people with localized pain due to arthritis. This cream must be applied several times a day (McEvoy, 2003).

## 2. NONPHARMACOLOGIC THERAPY

### 2.1 Surgery

Cordotomy is a method of severing the sympathetic chains that emanate from the spinal cord. Indications of such intervention are short life expectancy and specific unilateral of focal pain. In percutaneous cordotomy, a lesion is produced in the spinothalamic tract, most often at level of the first or second cervical vertebrae. This method has virtually replaced open cordotomy, in which a quadrant of the spinal cord is almost completely severed at the cervical or thoracic level pain relief by either technique is transient, rarely lasting more than 2 years, the advantage of cordotomy includes analgesia without significant loss of motor function or touch sensation (Herfindal and Gourley, 2005).

### 2.2 Neuroablative blocks and neurolysis

Chemical destruction of nerves (neurolysis) is used at spinal nerve roots and is a relatively simple and painless procedure, which can be done with minimal equipment. It is shorter-acting than cordotomy, but unlike this procedure, can be done in the elderly and those with poor general health. Agents used include absolute alcohol and phenol (Herfindal and Gourley, 2005).

### 2.3 Central and peripheral nervous system stimulators

Various types of central and peripheral nervous system stimulators are used for neurogenic, neurophatic, and ischemic pain syndromes. Dorsal column stimulators (DGS) operate on a principle similar to that of TENS, because both produce analgesia by inducing partial depolarization of neurons. DCS consists of an electrode placed on the epidural space and attached to a programmable continuous-pulse pacemaker implanted into a subcutaneous pocket in the abdomen. A sensory thalamic stimulator (STS) consists of an electrode placed into the thalamus of the brain. DCS and STS are used in cases of intractable neurogenic pain unresponsive to medications or other therapies. Peripheral nerve stimulators are implantable devices that are most successful in pain syndromes caused by injury to a peripheral nerve. Newer stimulators are taking the form of thermal, vibrotactile, and magnetic stimulators, although these methods have not evolved sufficiently for widespread use in pain management (Herfindal and Gourley, 2005).

## 3. PHYSICAL THERAPY

In addition to drugs, many other treatments can help relieve pain. Applying cold or warm compresses directly to a painful area often helps. Ultrasonography that provides deep heat (diathermy) may relieve the pain of osteoarthritis and muscle strain.

## 4. ALTERNATIVE THERAPIES

### 4.1 Acupuncture

Some people benefit from transcutaneous electrical nerve stimulation (TENS). A gentle electric current is applied through electrodes placed on the skin's surface. TENS produces a tingling sensation without increasing muscle tension. It can be applied continuously or several times a day for 20 minutes to several hours. The timing and length of stimulation vary because each person responds differently. Often, people are taught to use the TENS device, so that they can use it as needed. TENS may be useful for chronic pain. Acupuncture involves inserting tiny needles into specific areas of the

body. The mechanisms by which acupuncture works are poorly understood, and some experts still doubt the technique's effectiveness. Some people find substantial relief with acupuncture, at least for a time. Biofeedback and other cognitive techniques (such as relaxation training, hypnosis, and distraction techniques) can help people control, reduce, or cope with pain by changing the way they focus their attention. In one distraction technique, people may learn to visualize themselves in a calm, comforting place (such as in a hammock or on a beach) when they feel pain. The importance of psychologic support for people in pain should not be underestimated. Friends and family members should be aware that people in pain suffer, need support, and may develop depression and anxiety, which may require psychologic counseling (Herfindal and Gourley, 2005).

### 4.2 Herbal medications and dietary supplements

A variety of herbal medications have been used to treat pain arising from different regions of the body. Many of these herbal medications contain volatile oils such as camphor and other compounds such as sesquiterpene lactones and flavonoids. Feverfew (*Tanacetum parthenium*) contains all of these chemical entries and has been used for headache and rheumatic diseases (e.g., arthritis). It is thought to impede platelet aggregration and prostaglandin synthesis as well as release of histamines and other inflammatory mediators. Comfrey (*Symphytum officinale*), marigold (*Calendula officinalis*), peppermint (*Mentha piperita*), and primrose (*Primula elatior*) are a few examples of other herbals used for relief of muscle and neurogenic pain syndromes, it is difficult to find controlled clinical trials of these agents in humans, although some have undergone animal trials to identify their active components and a pharmacology of these component. Opium poppy (*Papaver somniferum*) is used a nuture analgesic in other parts of the world and gave rise to modern-day opioids such as morphine. Marijuana (*Cannabis sativa*) is currently under investigation for analgesic properties using methods to evaluate the effects of the cannabinoid receptors in mammals, but definitive trials are not yet documented.

Glucosamine and chondroitin sulfates are dietary supplements under investigation in clinical trials for relief of symptoms of osteoarthritis in weight-bearing joints such as the knee. Both are components of glucosaminoglycans. Glucosamine is also required for biosynthesis of glycoproteins, proteoglycans, and hyaluronate, all structural components of joint connective tissue. Limited studies comparing their use to NSAIDs are available, but more extensive trails are as yet unpublished. The National Institutes of Health is currently investigating these agents (Herfindal and Gourley, 2005).

## REVIEW OF PROPOLIS

**Figure 9** Propolis collection

**Figure 10** Propolis

Propolis, a natural resinous substance collected by honeybees (*Apis mellifera*) from buds and exudates of plants. Propolis (bee glue) is a sticky darkcoloured material that honeybees collect from living plants, mix with wax and use in construction and adaptation of their nests. The term 'propolis' was used in Ancient Greece: *pro* (for, in front of, e.g., at the entrance to) and *polis* (city or community); a substance that is for or in defence of the city or hive (Castaldo and Capasso, 2002. Bees apply propolis in a thin layer on the internal walls of their hive or other cavity they inhabit. It is used to block holes and cracks, to repair combs, to strengthen the thin borders of the comb, and for making the entrance of the hive weathertight or easier to defend. Propolis also is used as an "embalming" substance to cover hive invaders which bees have killed but cannot transport out of the hive (Burdock, 1998). Bees make use of the mechanical properties of propolis and of its biological action: bee glue contains the putrefaction of the

“embalmed” intruders, it is responsible for the lower incidence of bacteria and molds within the hive than in the atmosphere outside. The action against micro-organisms is an essential characteristic of propolis and it has been used by human beings since ancient times for its pharmaceutical properties. Propolis possesses antibacterial, antifungal and antiviral properties and many other beneficial biological activities: antiinflammatory, antiulcer, local anaesthetic, hepatoprotective, antitumor, immunostimulating, etc. (Burdock, 1998). For this reason propolis is widely used as a popular remedy in folk medicine, in apitherapy, as a constituent of “biocosmetics”, “health food” and for numerous further purposes. These properties of propolis have attracted the attention of scientists since the late 60’s. During the last 40 years, many investigations have been published on the chemical composition, biological activity, pharmacology and therapeutical uses of propolis. The first comprehensive review was published by Ghisalberti (Ghisalberti, 1978).

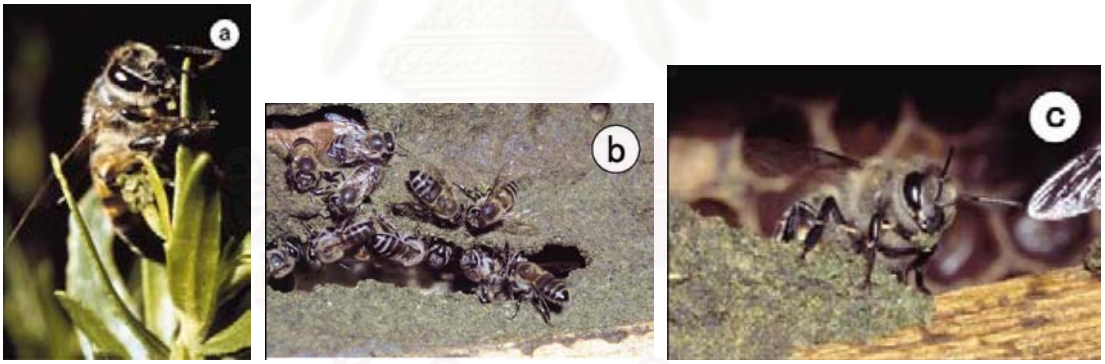


**Figure 11** Honeybees collecting the leaf bud to bring them back to their nest as Propolis.

Twenty years later, there is considerable information on the chemistry and biological activity of propolis but the situation with its application in therapy has barely changed. The main problem is the striking variability of its chemical composition depending on the site of collection, because in different ecosystems different plant exudates and secretions could serve as a source of propolis. This is a great problem especially for samples originating from tropical regions. The chemical standardization of propolis based on its “active principles” has not been realized. Chemical studies

conducted with propolis extracts revealed the existence of a very complex mixture of different naturally-occurring constituents with more than 300 constituents identified to date, such as phenolic acid, terpenes, cinamic acid, caffeic acid, several esters, and also flavonoids. Propolis composition varies with the season and the geographic region; such extraordinary variability among samples from different sources leads to variation of the pharmacological properties of propolis. The high biodiversity of propolis has been discussed in a recent review (Marcucci, 1995). In temperate zones, the main constituents are flavonoids, while in tropical zones other classes of bioactive components have been described, such as aromatic acid derivatives, specific terpenoids and prenylated p coumaric acids and acetophenones. Propolis exhibits a variety of biological activities including bactericidal, antiviral, fungicidal, anti-tumoural, anti-oxidant, and anti-inflammatory properties (Marcucci, 1995). Recently, Russo et al. have shown that propolis can induce a relaxant effect in the guinea pig isolated trachea through the interaction of several mechanisms of action, such as nitric oxide, vasoactive intestinal peptide, and potassium channels modulators (Russo *et al*., 2002) We have previously demonstrated the anti-hyperalgesic action of an ethanol extract acetic acid, kaolin, or zymosan models of nociception and also that this extract significantly inhibited capsaicin induced pain and reverted the hyperalgesia induced by bradykinin (Khayyal *et al*., 1993). The biological activity of propolis is associated mainly with phenolic compounds such flavonoids and derivatives of hydroxycinnamic acids. Three derivatives of p-coumaric acid isolated from a Brazilian sample presented a relaxant effect on smooth muscle isolated from guinea pig trachea (Paulino *et al*., 1998). In a previous study reported the chemical characterization of a standard ethanolic extract of a Bulgarian sample, named Et-Blg, that presented a high content of flavonoids and showed a strong inhibitory activity against *Staphylococcus aureus, Candida albicans,* and *Trypanosoma cruzi* (Castaldo and Capasso, 2002). Other report demonstrated the potential anti-hyperalgesic and anti-inflammatory properties and also the in vitro relaxant action in the guinea pig trachea of Et-Blg (Ledon, 1997).

Propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including

organic debris. Constituents of propolis are mainly polyphenols. The major polyphenols are flavonoids accompanied by pinocembrin, galangin, pinobanksin, phenolic acid (esters caffeic acid phenethyl ester; CAPE), phenolic aldehydes, ketones, etc. Nowadays, it is still used for the treatment of various diseases, and in products like 'health foods', 'biocosmetics', etc., because of its versatile biological activities. Tropical propolis samples, and especially Brazilian ones, have shown significant differences in their chemical composition to propolis from temperate zone. For this reason, Brazilian bee glue has recently become a subject of increasing interest for scientists. It was found that propolis from different regions of Brazil display different chemical composition, depending on the local flora at the site of collection. Park *et al*. have specified 12 types of Brazilian propolis according to its geographical origin, chemical composition and source plant. The most popular and well studied Brazilian propolis is the so-called green or Alecrim propolis, which originates from *Baccharis dracunculifolia* (Asteraceae). Till now, no chemical data have been published on red propolis from Brazil. In Brazil, red propolis is collected in the North regions. Red colored propolis is reported to be typical for Cuba, where its plant source was identified as *Clusia nemorosa* (Clusiaceae), and for Venezuela, where bees collect it from *Clusia scrobiculata* .The first papers to analyze propolis based on chemical evidence appeared in the 70's: Lavie in France and Popravko in Russia analyzed propolis flavonoid composition and compared it to poplar and birch bud exudates, respectively.

Many other publications followed and now it is generally accepted and chemically demonstrated that in temperate zones the bud exudates of Populus species and their hybrids are the main source of bee glue. This is true for Europe, North America, and the non-tropical regions of Asia. Even in New Zealand, introduced poplar species are the source plants (Marcucci, 1995). In Russia however, and especially in its northern parts, birch buds (*Betula verrucosa*) supply bees with the worthy glue. In tropical regions there are no poplars and birches, and bees have to find new plant sources of bee glue.

Knowledge of propolis plant sources is important to beekeepers to be sure that their bees have the proper plants in their flight range. It is known that colonies suffer when they cannot collect propolis, bees are even said to use “propolis substituents” like paints, asphalt and mineral oils which could severely threaten pharmaceutical uses of bee glue. In the last few years propolis from tropic regions and especially from Brazil has become the subject of increasing interest. This has led to the identification of many new compounds in propolis, some of them possessing remarkable biological activity.

**Chemical Composition**

**1. Flavonoids**

Among many different groups of natural products, flavonoids, are a group of chemical entities of benzo-pyrone derivatives widely distributed in the Plant Kingdom. They are mainly classified as chalcones, flavan-3-ols, flavanones, flavones and flavonols, isoflavones, and biflavonoids. They have relatively simple chemical structures, but more than 4,000 derivatives have been reported from nature, indicating their chemical diversities.

Flavonoids, also known as nature’s tender drugs, possess various biological/pharmacological activities including anticancer, antimicrobial, antiviral, antiinflammatory, immunomodulatory, and antithrombotic activities. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been utilized in Chinese medicine and the cosmetic industry as a form of crude plant extracts. Many investigations have proven that varieties of flavonoid molecules possess anti inflammatory activity on various animal models of inflammation. Especially, some flavonoids were found to inhibit chronic inflammation of several experimental animal models. There have been several proposed cellular action mechanisms explaining in *vivo* anti-inflammatory activity of flavonoids. They possess antioxidative and radical scavenging activities. They could regulate cellular activities of the inflammation-related cells: mast cells, macrophages, lymphocytes, and neutrophils. For instance, some flavonoids inhibit histamine release from mast cells and others inhibit T-cell proliferation.

These properties of flavonoids have been recently summarized (Marquez et al., 2004). In addition, certain flavonoids modulate the enzyme activities of arachidonic acid (AA) metabolizing enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX), and lipoxygenase (LOX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS). An inhibition of these enzymes by flavonoids reduces the production of AA, prostaglandins(PG), leukotrienes (LT), and NO, crucial mediators of inflammation. Thus, the inhibition of these enzymes exerted by flavonoids is definitely one of the important cellular mechanisms of anti-inflammation.

Furthermore, in recent years, many lines of evidence support the idea that certain flavonoids are the modulators of gene expression, especially the modulators of proinflammatory gene expression, thus leading to the attenuation of the inflammatory response. At present, it is not known to what extent these proinflammatory gene expressions contribute to the inflammatory response. However, it is evident that flavonoids show anti-inflammatory activity, at least in part, by the suppression of these proinflammatory gene expressions.

**2. Prenylated p-coumaric acids and acetophenones**

Another class of phenolics newly found in Brazilian bee-glue, are prenylated p-coumaric acids and their derivatives with cyclized prenyl residues: Acetophenone derivatives containing a modified prenyl-substituent, also have been isolated from Brazilian propolis. Prenylated p-coumaric acids and ace-tophenones are secondary metabolites,typical for South American Baccharisspecies.

**3. Lignans and other phenolics**

Recent investigations of tropical propolis from countries other than Brazil resulted in the identification of a series of lignans, whose plant sources still remain unknown. In propolis from the Canary Islands, thirteen lignans of the furofuran type were found by GC-MS, most of them were only tentatively identified.

**4. Di- and triterpenes**

Diterpenes seem to be another important class of Brazilian propolis constituents. New diterpenic acids with valuable biological activities have been identified: an antitumor clerodane derivative, the cytotoxic substances and its E-isomer and antibacterial labdane type acids.

**5. Volatile compounds**

Volatile compounds are found in low concentrations in propolis, but their aroma and significant biological activity make them of importance for the character-isation of propolis. In propolis volatiles, a number of new propolis components were identified, mainly mono- and sesquiter-penoids. The volatiles from tropical regions contained some sesquiter-penoids that were not found in samples from the temperate zone, e.g. ledol , spatuleno, germacren. Prenylated acetophenones were found in volatiles from Brazilian samples, together with other aromatic compounds new for propolis (Burdock, 1998).

**6. Hydrocarbons**

Propolis waxes from Brazilian samples were recently analyzed. The hydrocarbons and monoesters found were similar to those of European samples. The composition of propolis wax is similar to that of comb wax, which suggests that propolis waxes are secreted by bees, rather than originating from plants (Marcucci, 1995).

**7. Mineral elements**

In recent studies on the mineral elements of propolis, Ca, Mg, K, Na, Fe and Zn were determined in Macedonian samples, and Fe, Mn, Zn and Cu in ethanolic extracts of Cuban samples. Both studies were carried out using atomic absorption spectroscopy (Marcucci, 1995).

**Biological activity of the components**

It is important to note that most of the latest investigations on new propolis constituents are connected to their biological activity. Some of the prenylated p-coumaricacids possess antibacterial and cyto-toxic activities. Caffeoylquinic acid

derivatives showed immunomodulatory and hepatoprotective action. The furofuran lignans were shown to inhibit the growth of some bacteria. Diterpenic acids isolated from Brazilian propolis showed cytotoxic and antibacterial activity. The limited number of “chemical types” of propolis and the biological activities related to the chemistry (Burdock, 1998).

The antiinflammatory effect of an ointment containing propolis extract (3%-7%) was examined using carrageenan-induced hind paw edema in rats. Treatment with the ointment inhibited the edema moderately, and the inhibition was significant at 5% and 7%. Additionally, the effect of the ointment on chemotaxis of human polymorphonuclear leukocytes (PMNs) was investigated using the agarose plate method. Migration of PMNs toward zymosan-treated serum was inhibited in the presence of 5% propolis ointment. These results demonstrate that topical application of propolis extract is effective in inhibiting carrageenan-induced rat hind paw edema, and its inhibitory effect on the chemotaxis of PMNs may also contribute to the antiinflammatory effect. Phamacological activities of a standard ethanol extract from Brazilian green propolis, typified as BRP1, were evaluated in mouse models of pain and inflammation. Intraperitoneal injection (i.p.) of propolis inhibited acetic acid-induced abdominal constrictions, and in the formalin test, for the neurogenic and inflammatory phases.

The extract was ineffective when assessed in the hot-plate assay. In serotonin-induced paw edema, propolis led to a maximal inhibition (MI) of 51.6 % after 120 min when administered i.p. and of 36 % after 15 min by the oral route. When the inflammatory agent was complete Freund's adjuvant, inhibition of paw edema was also observed after administration of the extract by both. The ethanolic extract of red propolis extract induced the formation of granular layer in the mouse tail test used as a model of psoriasis. Propolis 50 mg/kg i.g. showed anti-inflammatory activity in the cotton-pellet granuloma assay in rats, in croton oil-induced edema in mice at a dose of 25% (2.5 microL), and in the peritoneal capillary permeability test in mice at a dose of 10 mg/kg. The extract (25 mg/kg i.g.) showed analgesic effect in the model of acetic acid-

induced writhings, whereas 40 mg/kg was effective in the hot plate test in mice (de Campos *et al*., 1998).

Use of products containing propolis has resulted in extensive dermal contact and it is now increasingly being used a dietary supplement. Unlike many 'natural' remedies, there is a substantive database on the biological activity and toxicity of propolis indicating it may have many antibiotic, antifungal, antiviral and anti-tumor properties, among other attributes. Although reports of allergic reactions are not uncommon, propolis is relatively non-toxic, with a no-effect level (NOEL) in a 90-mouse study of 1400 mg/kg body weight/day (Burdock, 1998).

# CHAPTER III
# METERIALS AND METHODS

## ANIMALS

Male ICR mice (18-25 gm) used obtained from National Laboratory Animal Center, Salaya, Mahidol University, Nakornprathom and served as experimented subjects. They were housed in the animal facillty, Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard condition of temperature (25°C), 12 hr/12 hr light/dark cycles and had accessed to standard pellet diet (C.P. Company, Thailand) and tap water *ad libitum*. The animals were acclimated to the facility for at least a week before starting the experiments. At the end of each experiment, animals were sacrificed with diethylether. The number of animals used in each treatment was typically six to ten per group. The study protocols had been approved by the Ethical Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

## PREPARATION OF THE ETHANOLIC EXTRACT THAI PROPOLIS

1. The propolis sample was collected from Amphur Phang, Chiangmai District, Thailand. All propolis used in this study were collected during April – May 2004.
2. 500 grams of propolis was cut into the pieces and extracted with 95% ethanol, 1:10 (w/v) for 24 hr at room temperature.
3. The whole extract (dark-brown solution) was filtered and transferred into 1 L conical flask. The filtrate was evaporated to dryness using water bath at 50°C.
4. The yield was ground to resin and was kept in tight container at $\leq$ 2-8°C until the time of animal treatment.

A result from 500 grams of crude propolis, crushed and extracted with 95% ethanol was filtered. The filtrate was evaporated resulting in the resin of 100 grams. Therefore, crude propolis contained approximately 10% w/w yield of evaporated propolis resin. The resin appeared dark-brown color.

The dark-brown resin of the extract was dissolved in corn oil, which had no effect *per se* on animal and *in vitro* tests, and the suspension of the extract was used for the pharmacological study. The doses employed are express in terms of dried resin (mg/kg body weight).

## DRUGS

The reference drugs were dissolved or suspended in 0.9% sodium chloride solution (The Government Pharmaceutical Organization, Thailand), except the propolis extract was dissolved in corn oil, and given intraperitoneally to the animals. The control animals were given an equivalent volume of vehicle in the same route. Morphine sulphate (10 mg/kg, Thai FDA), Indomethacin (150 mg/kg, Sigma Chemical Co., USA) were used as standard analgesic drugs. Naloxone (1 mg/kg, Sigma Chemical Co., USA), Naltrexone (5 mg/kg, Sigma Chemical Co., USA), NMDA (0.38 mg/kg, Sigma Chemical Co., USA), and 0.37%formaldehyde (2.5%formalin in 0.9% sodium chloride solution 20 µl/mouse, Sigma Chemical Co., USA).

## EXPERIMENTAL METHODS

### Hot - plate Analgesic Testing

The male ICR mice weighing 18-25 g were used. Analgesic testing was determined using the hot-plate method. The surface of the hot plate (measuring 28 x 28 cm was set at 50 °C ± 0.5° C and was surrounded by a clear Plexiglas wall cylinder , 20 cm in diameter and 30 cm in height to confine the animal to the heated surface during testing. On the day of testing, animals were randomly assigned to one of eight treatment groups and underwent 3 pre-drug baseline trials on the hot-plate spaced 5-10 min apart. Only those animals which had a pretreatment hot-plate latency time of less than

45 sec were utilized in these studies. Mice were then administered various doses of treatments and retested. Each mouse was placed on the hot-plate from an elevation of 5 cm and the latency to the licking of a rear paw or a vigorous jumping up from the surface of the metal plate was used as the end point and recorded with a stop watch. If this behavior was not observed within 45 sec the animals was removed from the hot-plate, given a score of 45 for its paw-lick latency and returned to its cage (the maximum time allowed for an animal to remain on the surface of the plate during testing was 45 sec). The average of the last two trials served as the baseline per-drug paw-lick latency.

Immediately, after the third baseline trial on the hot-plate, the drug administration took place with intraperitoneal (i.p.) vehicle (10 ml/kg) or test drugs, morphine sulphate (10 mg/kg), indomethacin (150 mg/kg) or various doses of the ethanolic extract of Thai propolis ( ETP; 12.5, 25, 50, 100, 200, 400,and 800 mg/kg). All animals were placed on the hot-plate for 7 subsequent trials at 15, 30, 45, 60, 90,120, and 240 min after injection. Thus, $ED_{50}$ were computed and dose- time response curve were generated.

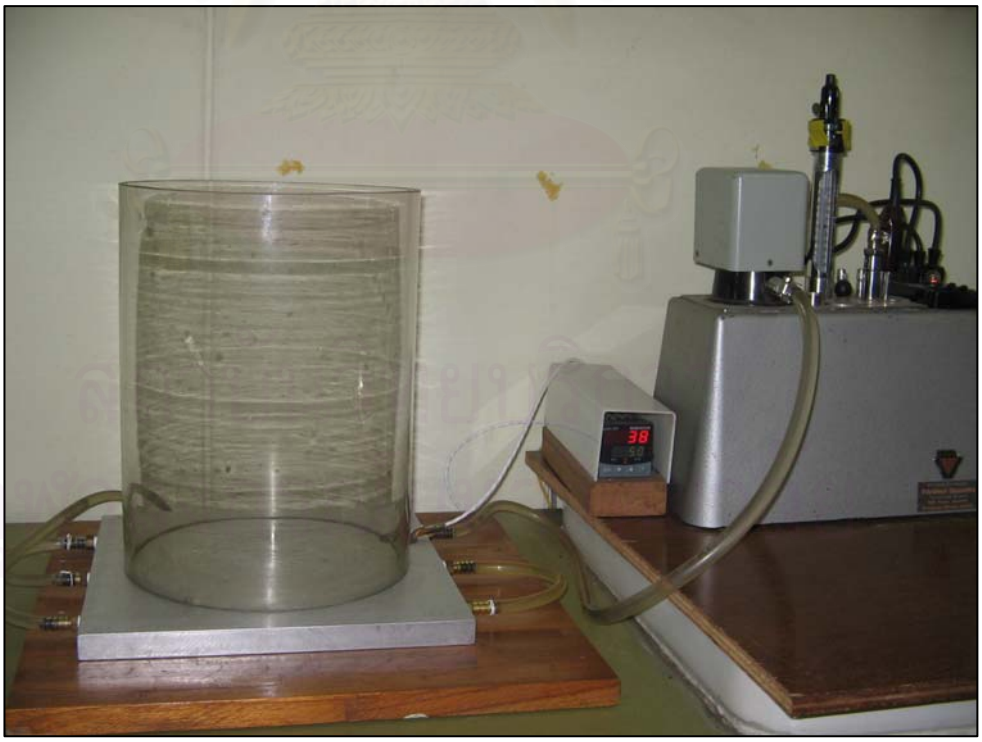

**Figure 12** Hot-plate Analgesiometer

**Tail – flick Analgesic Testing**

These studies employed the tail-flick assay described by D'Amour and Smith (1941), with minor modifications. Male ICR mice weighing 18-25 g were used (n = 10 per group). Mice were placed in individual Plexiglas restrainers with an opening to allow the tail to protrude. Each tail rested in a shallow groove housing a light sensitive sensor. A beam of radiant heat (24-V,high amperage 150- watt light bulb situated 8 cm above the tail) was aimed at the middle of the marked dorsal portion of the distal part of each subject's tail that has been blackened length 1 cm with a black ink maker pen in order to absorb the maximum amount of heat and for uniform heat absorption (about 4 cm from the tip) The device (Harvard Tail-flick Analgesia meter) automatically recorded (in 0.1 sec) the latency between the onset of the light beam stimulus and the response to heat, at which point the light beam was terminated. The maximum duration of each test was set at 4.0 sec to minimize the potential for thermal injury. The stimulus intensity was set so that the baseline tail-flick latencies were approximately 1.0 – 1.5 sec (intensity 3.7 A).The intensity was not changed for any animal within any given experiment. Animals failing to respond within 1.5 sec were excluded from testing. On the day of testing, all animals were test 3 predrug tail-flick baseline conducted at 10-15 min intervals. The score from the third trial served as the baseline measure for each subjects.

Immediately, after the third baseline trial on the tail-flick test, the drug administration took place with either intraperritoneal (i.p) vehicle or test drugs morphine sulphate (10 mg/kg), indomethacin (150 mg/kg) and ETP (12.5, 25, 50, 100, 200, 400, and 800 mg/kg).Tail-flick latencies were recorded at 15, 30,45, 60, 90,120, and 240 min after injection. Thus, dose-and time response curve were generated. Analgesic will prolong the reaction time significantly and the doses required to induce this effect in 50% of the animals (Effective Dose-50, $ED_{50}$) can be computed.


**Figure 13** Tail-flick Analgesiometer

**Formalin test**

Male ICR mice weighing 18-25 grams (N=10 per group) were used in this experiment using the model described by Hunskaar and Hole (1987). On the day of testing, all animals were pretreat with either i.p. vehicle or test drugs morphine sulphate (10 mg/kg), indomethacin (150 mg/kg) and ETP (50, 100, 200, 400, and 800 mg/kg), and 30 min later the plantar surface of the right hind paw in each mouse was injected with 20µl of 2.5% formalin and was placed into a clear plastic cage for observation. The number of licks was monitored at the first 0 – 5 min (first phase, neurogenic), and the following 25 – 30 min after formalin injection (second phase, inflammatory). The number of licks is indicated by mice raised the injected-paw for licking or biting. The percentage of inhibition of the first and second phase were analyzed.

**Analysis of the analgesic mechanism of action of ETP**

The possible participation of the opioid system in the antinociceptive effect of ETP was investigated. To analyze this mechanism we also used the model of mouse hot-plate test. Animals were pretreated naloxone 1 mg/kg i.p. (Pieretti *et al*., 1999; Perrot *et al*., 2001), or naltrexone 5 mg/kg, or NMDA 0.38 mg/kg i.p (Davis and Inturrisi, 1999) 30 min before ETP (200 mg/kg i.p.) administration.

**Rota-rod test**

To rule out the possibility of motor impairment from intraperitoneal administration of the propolis extract, mice were tested for their ability to perform a rota-rod test after administration various doses of ETP (n=6) compared to vehicle controls intraperitoneally. Mice were placed on a horizontal rod (3.5 cm diameter) rotating at a speed of 16.5 rpm (Dunham and Miya, 1957). The mice capable remaining on the top for 60 sec or more, in three successive trails were selected for the study (Skyba *et al*., 2002; Chattopadhyay *et al*., 2003). Each group of the animals was then placed on the rod at an interval of 30, 60, 120, and 240 min after injection. The results are expressd as the time (seconds) in which animals remained on the rota-rod. The cut off time used was 60 sec (Pearl *et al*., 1969).

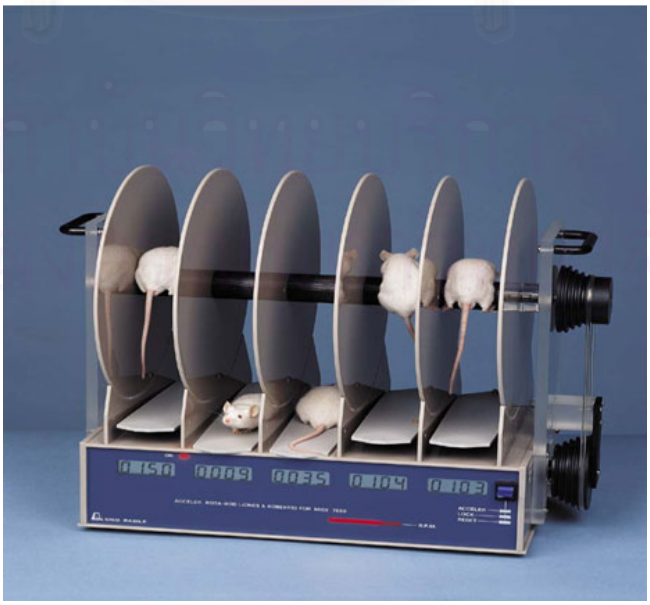

**Figure 14** Rota-rod Apparatus

## DATA TREATMENT AND STATISTICAL ANALYSE

Statistical analyses were performed on the dose-response curves by analysis of variance (ANOVA) and, where appropriate, were followed by Tukey's *Post Hoc* testing (SPSS version 13.0 for windows). The time-course of hot-plate latency, tail flick latency are expressed as the mean percent maximum possible effect (%MPE) accordy to the following formula:

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

Note: cut-off time for hot-plate test = 45 seconds

cut-off time for tail-flick test = 4 seconds

Dose-effect curves for the hot plate and tail-flick assays were derived by computing the area under the corresponding 0-240 min time-course-%MPE curves; area were calculated using the trapezoidal rule (Tallarida and Murray, 1987). The minimum level of statistical significance was set at $p < 0.05$.

Formalin test, the number of licks was expressed as the percent inhibition of unit of time (first phase and second phase) compared to control, accordy to the following formula:

$$\%\ \text{inhibition} = \frac{\text{the number of licks (control) - the number of licks (sample)}}{\text{the number of licks (control)}} \times 100$$

# CHAPTER IV
# RESULTS

## MOUSE HOT-PLATE TEST

To demonstrated the validity of the hot-plate analgesic testing following intraperitoneal (i.p.) drug administration, mice received morphine sulphate (MO; 10 mg/kg) i.p. and were tested during the subsequent 240 min period. As expected MO significantly ($p$<0.01) increased hot-plate latency producing an area of analgesia of 13397.19±410.89 %MPE-min compared with that of normal saline solution (NSS) (-1195.09±1394.85 %MPE-min; Figure 15). The i.p. administration of indomethacin (IND; 150 mg/kg) also influenced the hot-plate latency and area of analgesia (4929.64± 354.10 %MPE-min) when compared to NSS ($p$<0.01; Figure 16).

Initial studies utilizing the hot-plate test in mice to examine the efficacy of ETP in producing analgesia. Mice were injected i.p. corn oil or various doses of ETP (12.5, 25, 50, 100, 200, 400, 800 mg/kg). ETP doses of 50 mg/kg or higher significantly ($p$<0.01, $p$<0.01, $p$<0.01, $p$<0.01, $p$<0.01, respectively) increased hot-plate latency when compared to the vehicle group. Additionally, ETP doses of 50 mg/kg and higher also significantly ($p$<0.05, $p$<0.01, $p$<0.01, $p$<0.01, $p$<0.01, respectively) increased hot-plate latency when compared to the lowest dose of ETP used (Figure 17). MO showed the highest analgesic response compared to all test groups. ETP dose of 50 mg/kg produced analgesic response similar to IND (Figure 18).

When the log dose of ETP was plotted versus the area of analgesia, a significant linear correlation was observed. When all seven doses of ETP (12.5, 25, 50, 100, 200, 400, 800 mg/kg) were plotted a significant linear correlation coefficient ($r^2$) equal to 0.69 was observed, while the plotting of only five doses (12.5, 25, 50, 100, and 200 mg/kg) revealed a significant linear correlation coefficient of 0.98 (Figure 19 & 20). $ED_{50}$ was calculated from the log dose probit line and was equal to 48.56(9.05-260.56) mg/kg (Figure 21). The analgesic peak effect of ETP was reached within 120 min after i.p.

administration in all ETP doses tested and individual time courses of the response are shown in Figure 22.

In order to investigate any role of the opioid receptor in ETP actions, mice were then administered i.p. NSS or naloxone (NAL; 1 mg/kg), a short-acting opioid receptor antagonist, ETP 200 mg/kg or the combination of naloxone and ETP 200 mg/kg. Naloxone alone failed to produce significant responses when compared to vehicle control. ETP dose of 200 mg/kg produced significant ($p<0.01$) response when compared to vehicle control. The inclusion of naloxone with ETP significantly ($p<0.05$) attenuated the analgesic response due to ETP indicating that opioid receptors are involved in the analgesic response produced by ETP (Figure 23). Additionally, naltrexone (NALT; 5 mg/kg), a long-acting opioid receptor antagonist, ETP 200 mg/kg or the combination of naltrexone and ETP 200 mg/kg were administered i.p.. Naltrexone alone failed to produce significant responses when compared to vehicle control. ETP dose of 200 mg/kg produced significant responses when compared to vehicle control. The inclusion of naltrexone with ETP significantly ($p<0.01$) attenuated the analgesic response due to ETP indicating that opioid receptors are most likely involved in the analgesic response produced by ETP (Figure 24).

To further explore the mechanism of ETP in this analgesic testing model, mice were then administered i.p. NSS, N-methyl D-aspartic acid (NMDA; 0.38 mg/kg), ETP 200 mg/kg or combination of NMDA and ETP 200 mg/kg. NMDA alone failed to produce significant analgesic responses when compared to vehicle control. ETP 200 mg/kg produced significant ($p<0.01$) analgesic responses when compared to vehicle control. The inclusion of NMDA did not produce significant responses when compared to ETP alone (Figure 25).

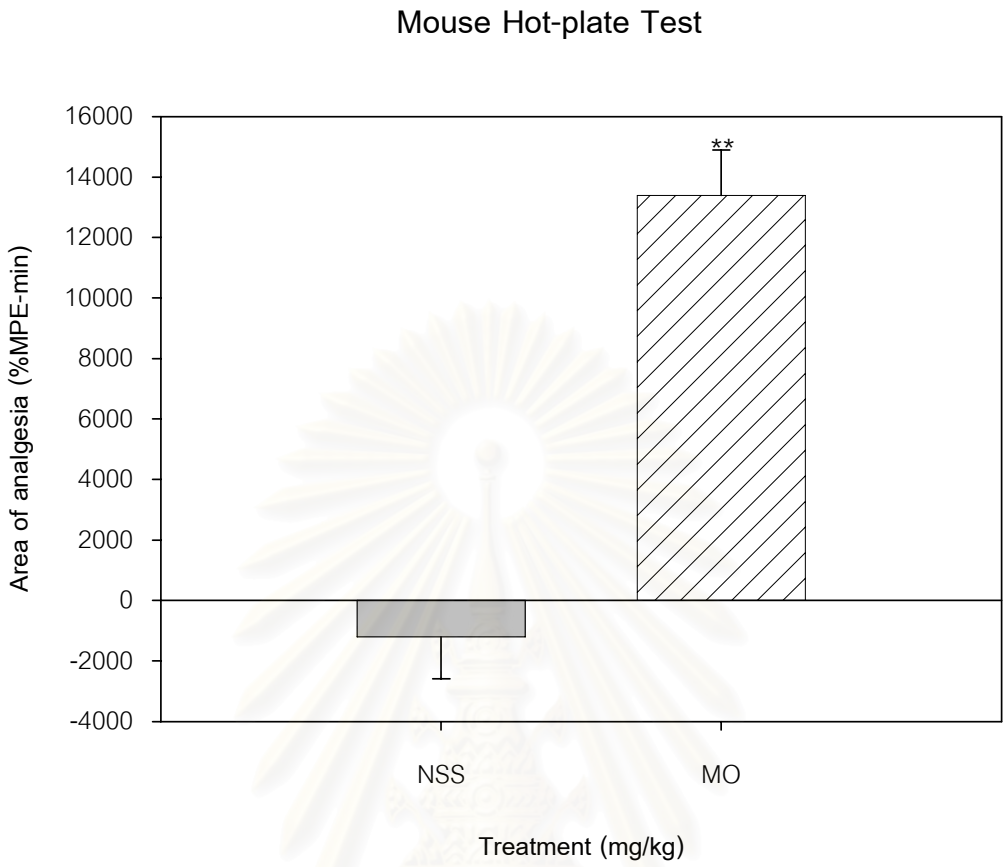


**Figure 15** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

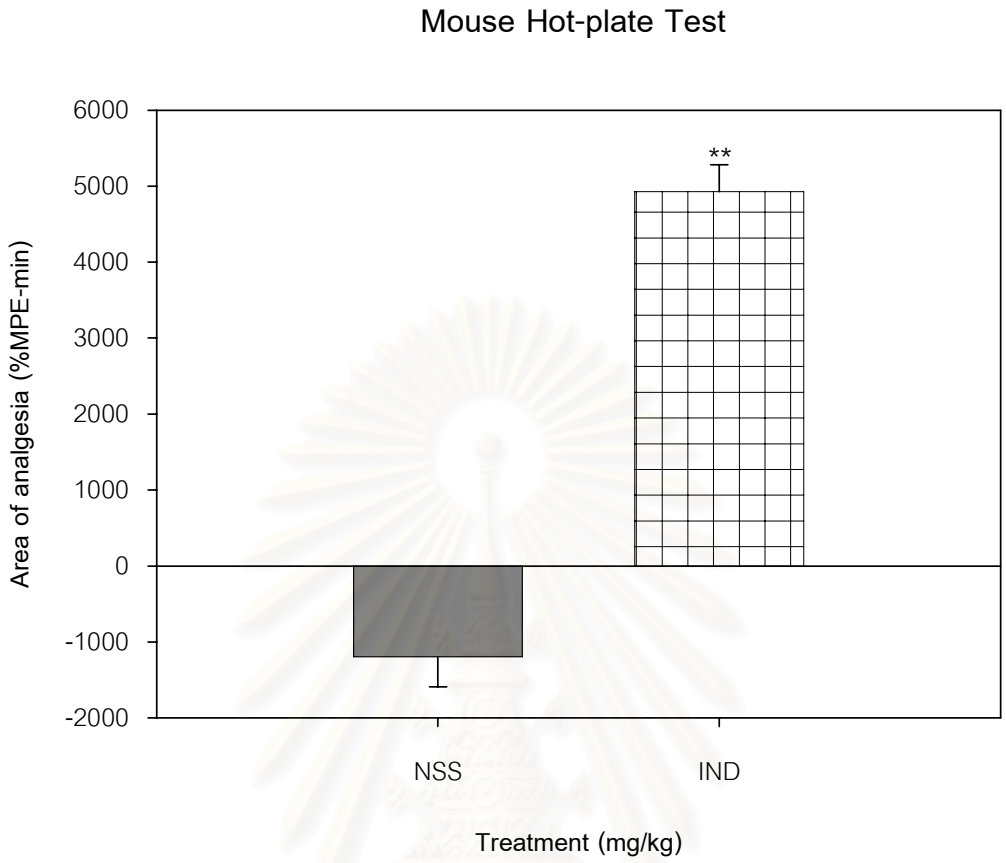


**Figure 16** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and indomethacin (IND; 150 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

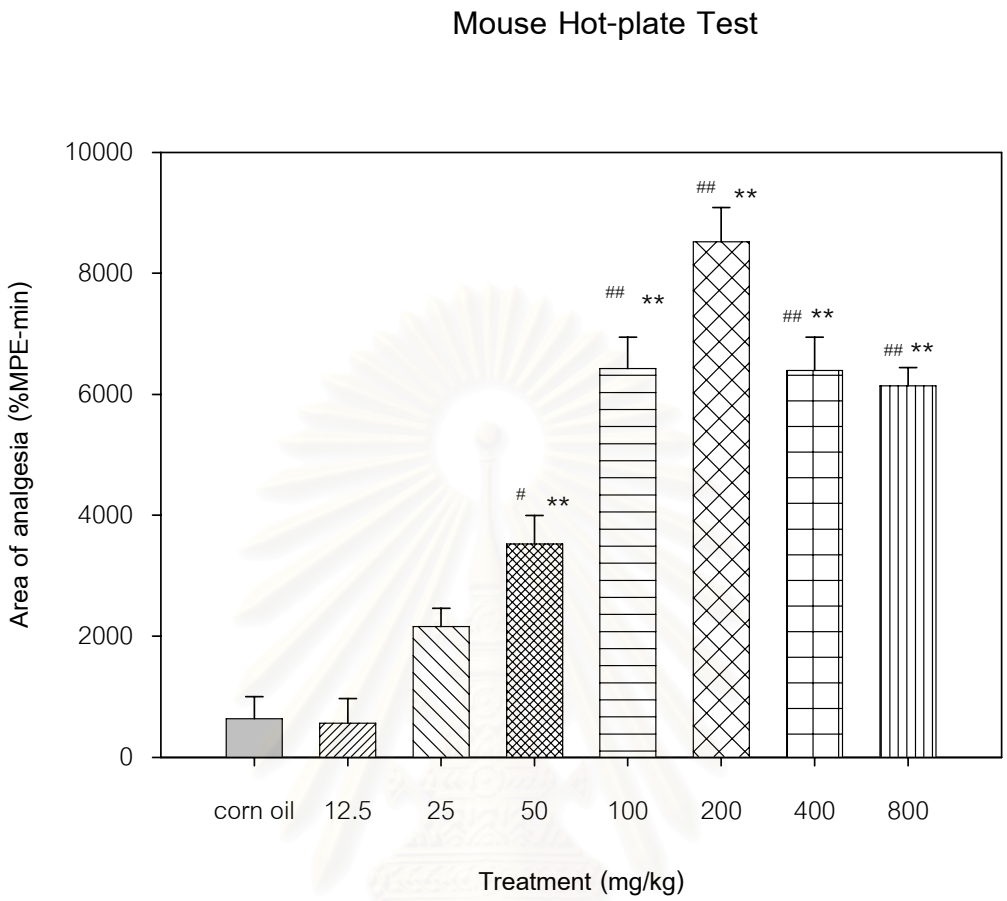


**Figure 17** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of corn oil and various doses of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to corn oil. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ significantly different compared to ETP 12.5 mg/kg.

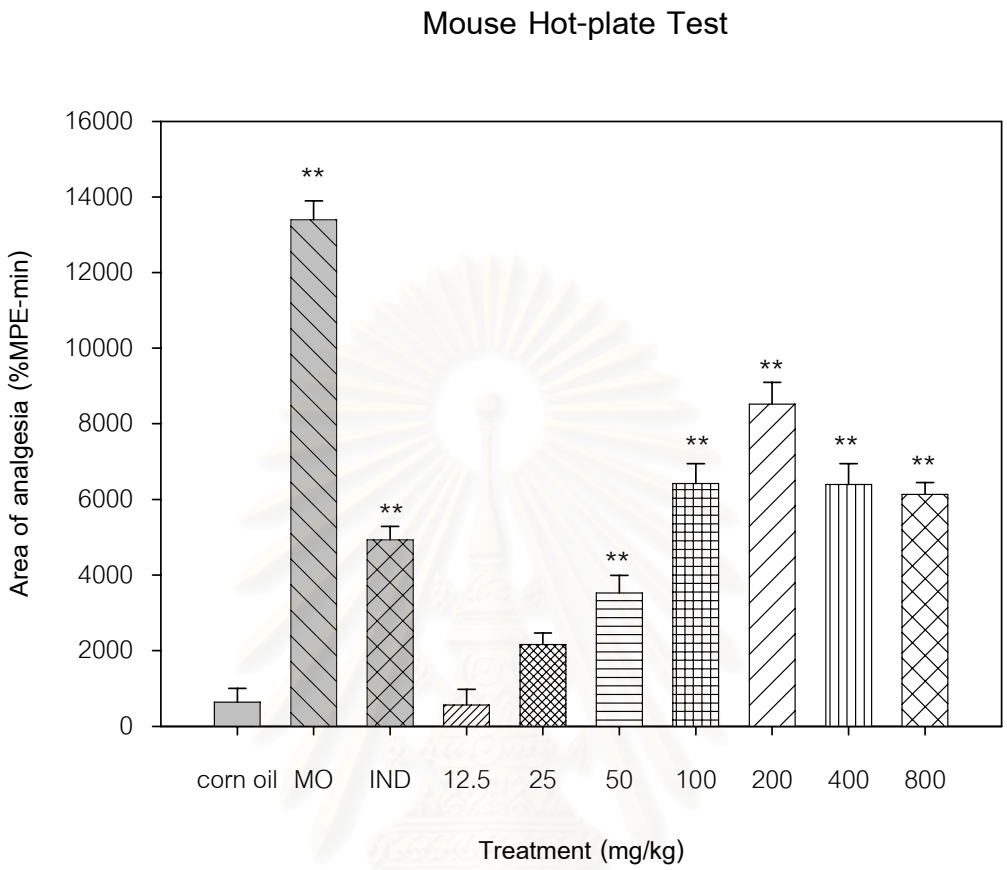


**Figure 18** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of corn oil, morphine sulfate (MO; 10 mg/kg), indomethacin (IND; 150 mg/kg), and various doses of the ethanolic extract of Thai propolis (ETP; 12.5-800 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to corn oil.

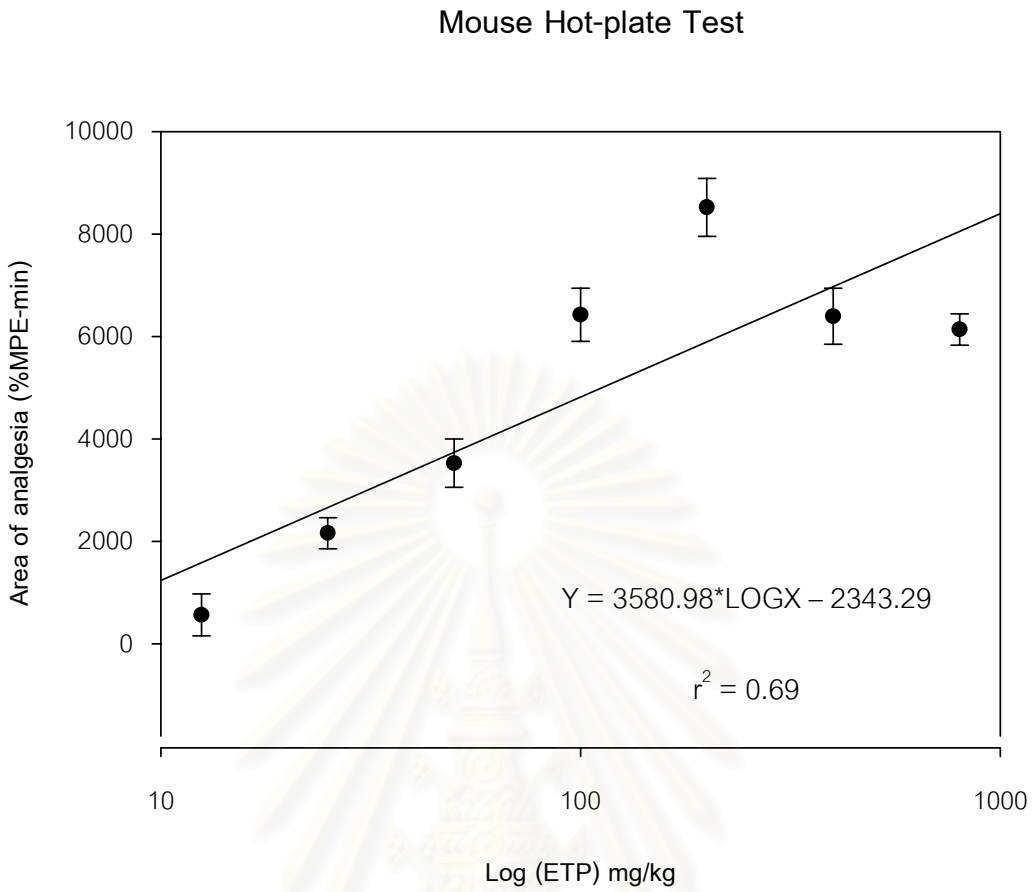


**Figure 19** Linear regression of area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. The regression equation was Y = 3580.98*LOGX – 2343.29, $r^2$ = 0.69.

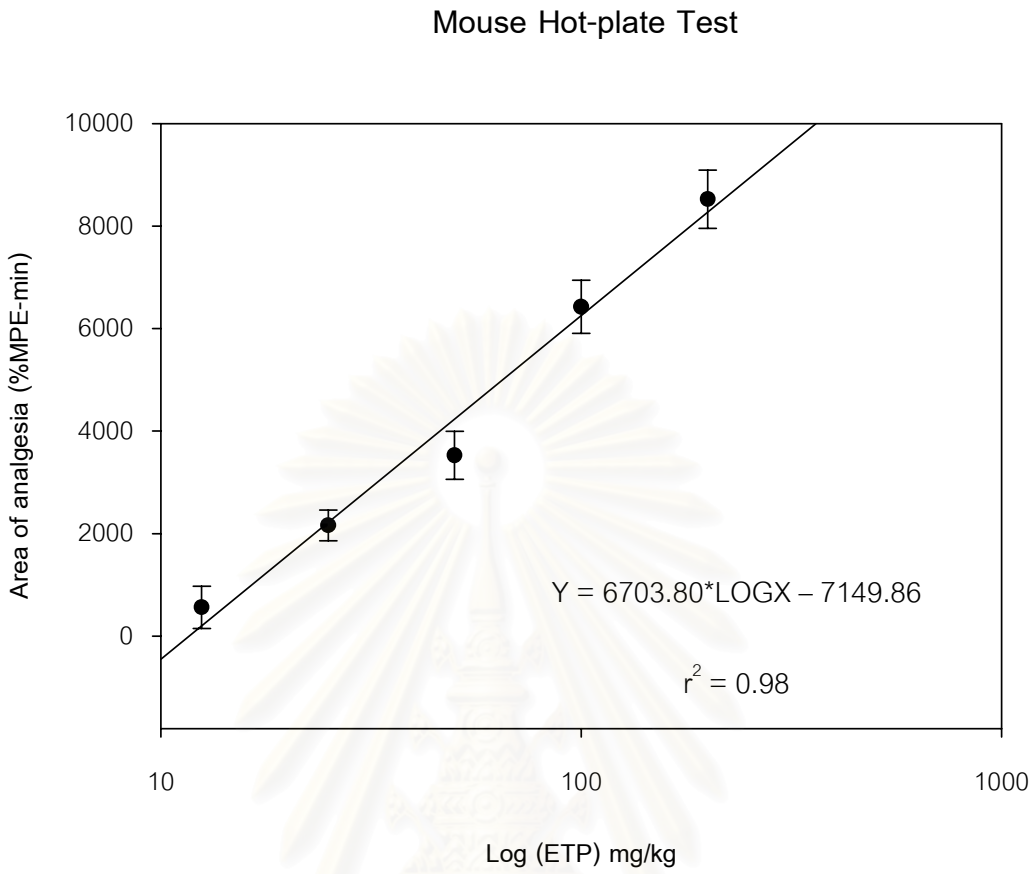


**Figure 20** Linear regression of area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of the ethanolic extract of Thai propolis (ETP; 12.5 - 200 mg/kg). N= 10 for all groups. The regression equation was Y = 6703.80*LOGX – 7149.86, $r^2$ = 0.98.

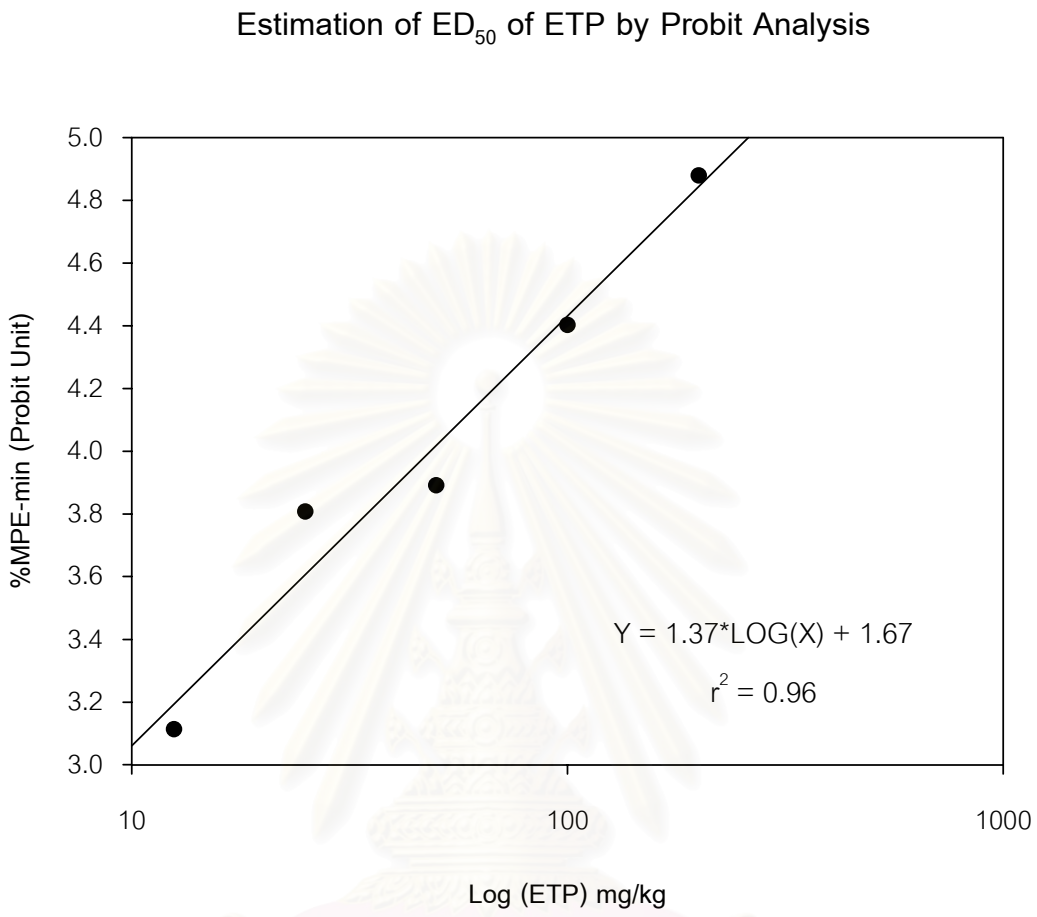


**Figure 21** Linear regression of %MPE-min (Probit unit) at 120 minutes after intraperitoneal administration of various doses of ETP (12.5-200 mg/kg) using hot-plate test. N= 10 for all groups. The $ED_{50}$ was calculated from the log dose probit line as Y = 1.37*LOG(X) + 1.67, $r^2$ = 0.96 and equal to 48.56(9.05 - 260.56) mg/kg.

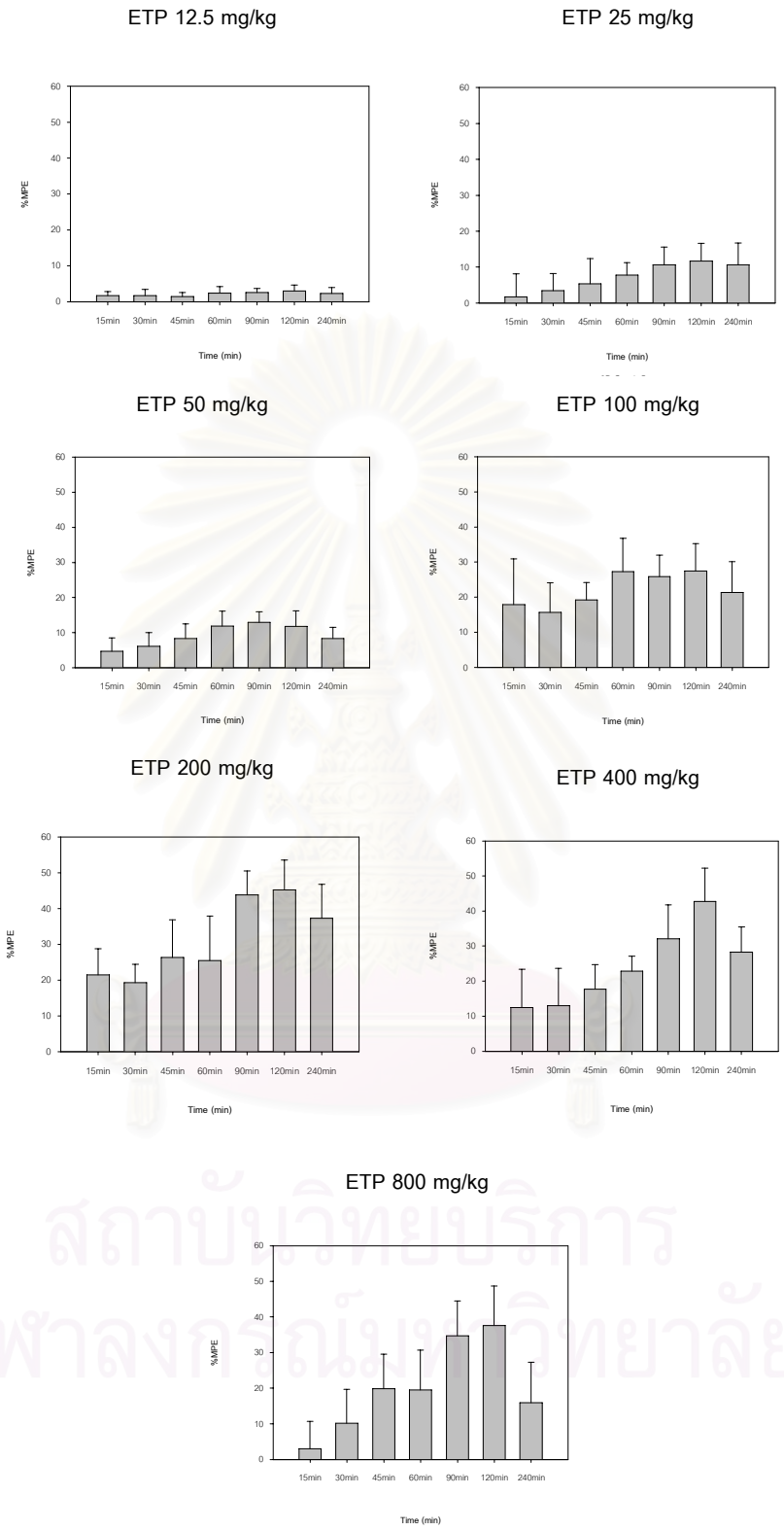


**Figure 22** Individual time courses of the response (%MPE versus time (min)) after intraperitoneal administration of various doses of the ethanolic extract of Thai propolis (ETP; 12.5-800 mg/kg). N= 10 for all groups.

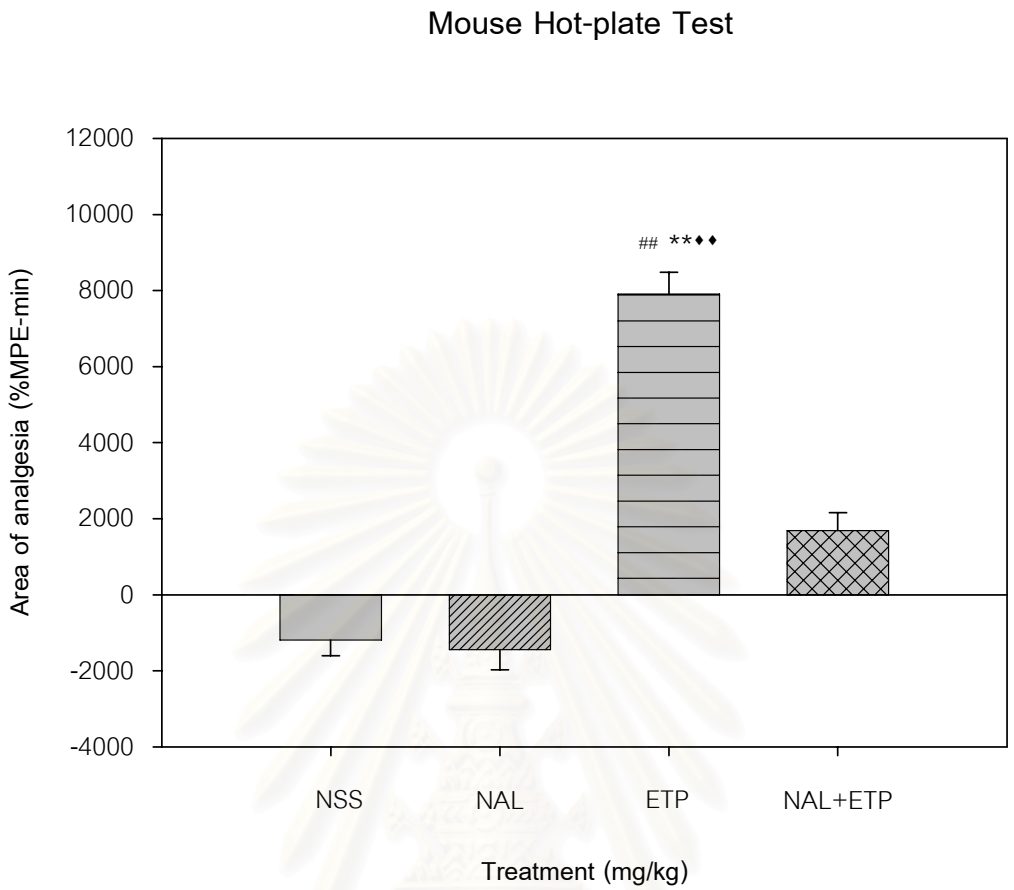


**Figure 23** Area of analgesia (%MPE-min) from 0 - 240 min after intraperitoneal administration of 0.9% normal saline solution (NSS), naloxone (NAL; 1 mg/kg), and the ethanolic extract of Thai propolis (ETP; 200 mg/kg), and the combination of naloxone and the ethanolic extract of Thai propolis (1/200 mg/kg). N= 10 for all groups. ** $p <$ 0.01 significantly different compared to NSS; ## $p < 0.01$ significantly different compared to NAL; ♦♦$p < 0.01$ significantly different compared to NAL+ETP.

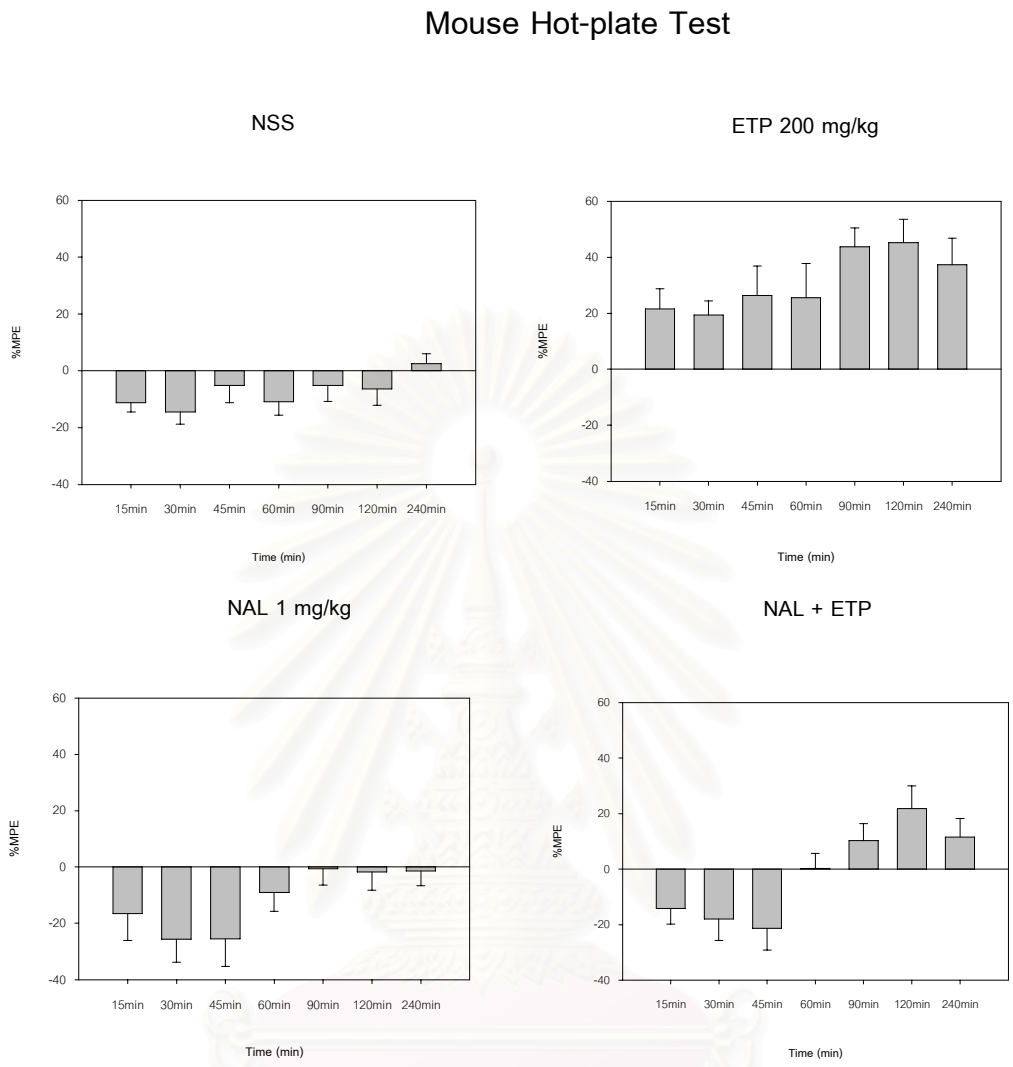


**Figure 24** Individual time courses of the response (%MPE versus time (min)) after intraperitoneal administration of 0.9% normal saline solution (NSS), the ethanolic extract of Thai propolis (ETP; 200 mg/kg), naloxone (NAL; 1 mg/kg), and the combination of naloxone and the ethanolic extract of Thai propolis (1/200 mg/kg). N= 10 for all groups.

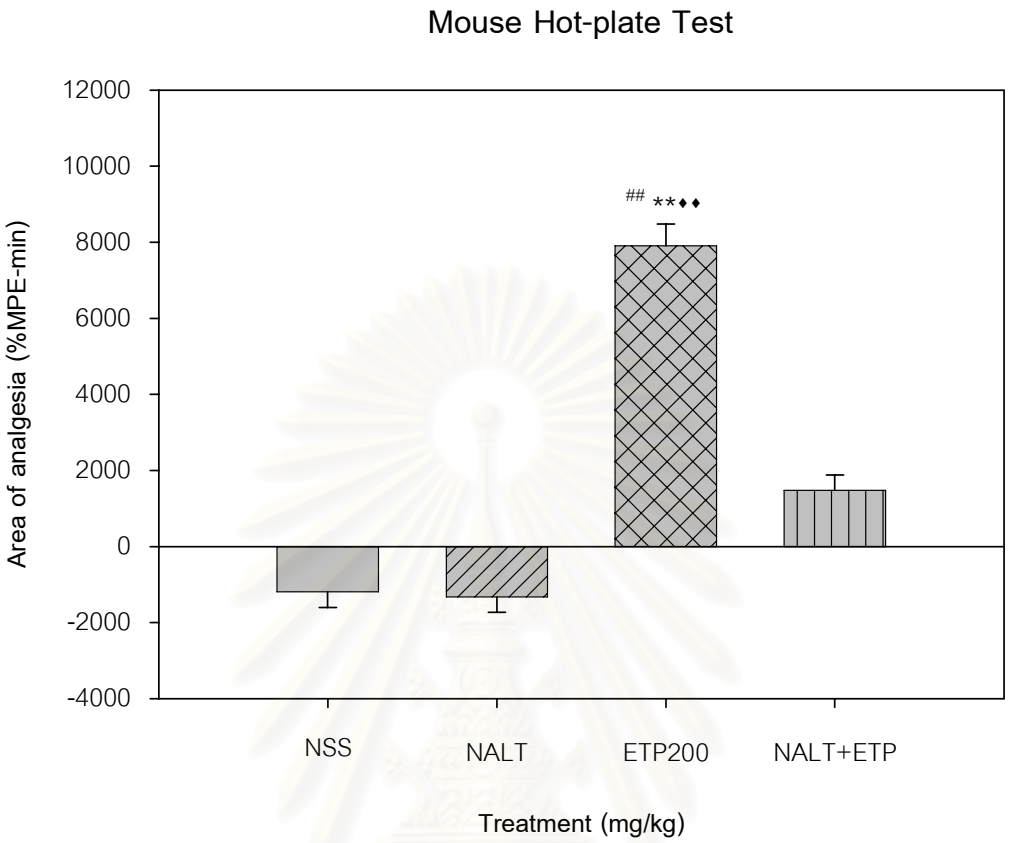


**Figure 25** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), naltrexone (NALT; 5 mg/kg), the ethanolic extract of Thai propolis (ETP; 200 mg/kg), and the combination of naltrexone and the ethanolic extract of Thai propolis (5/200 mg/kg). N= 10 for all groups. ** $p <$ 0.01 significantly different compared to NSS; ## $p <$ 0.01 significantly different compared to NALT; ♦♦$p <$ 0.01 significantly different compared to NALT+ETP.

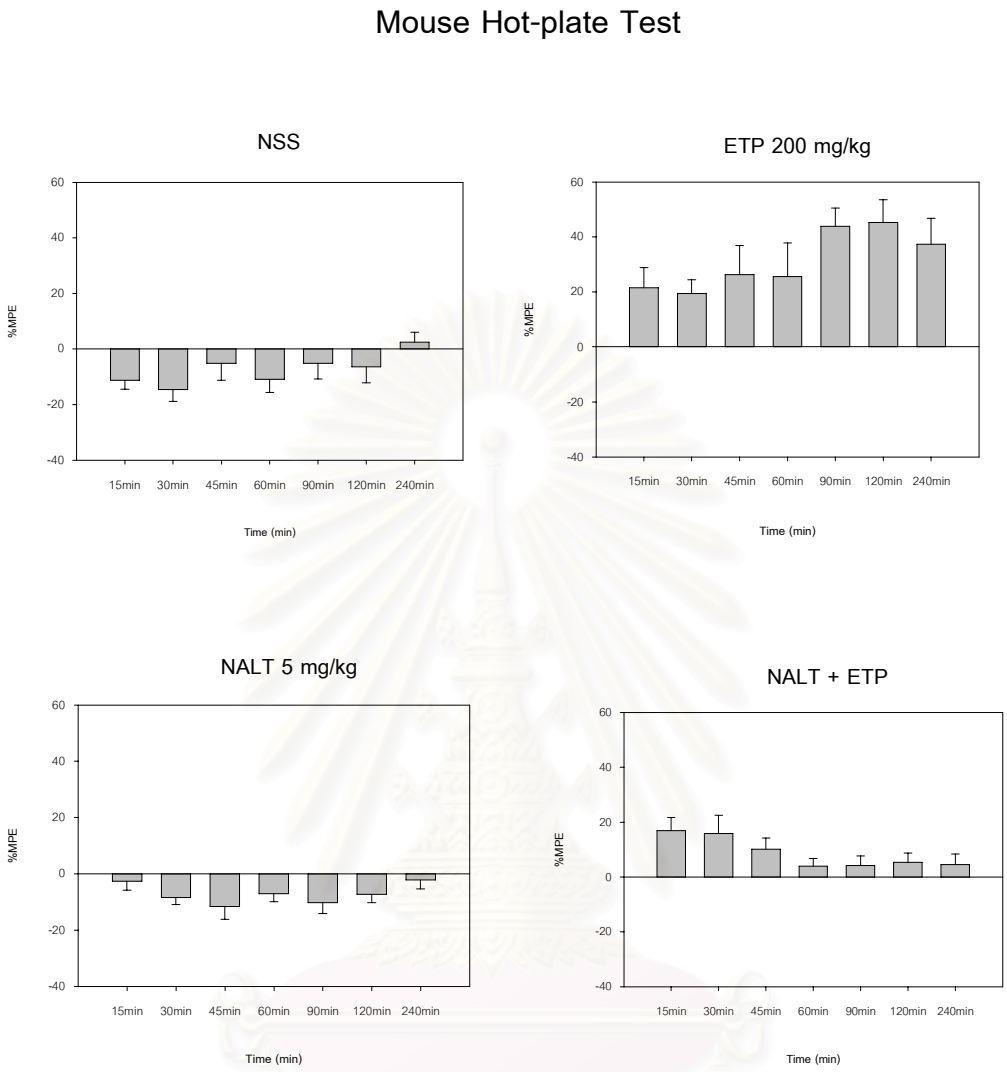


**Figure 26** Individual time courses of the response (%MPE versus time (min)) after intraperitoneal administration of 0.9% normal saline solution (NSS), the ethanolic extract of Thai propolis (ETP; 200 mg/kg), naltrexone (NALT; 5 mg/kg), and the combination of naltrexone and the ethanolic extract of Thai propolis (5/200 mg/kg). N= 10 for all groups.

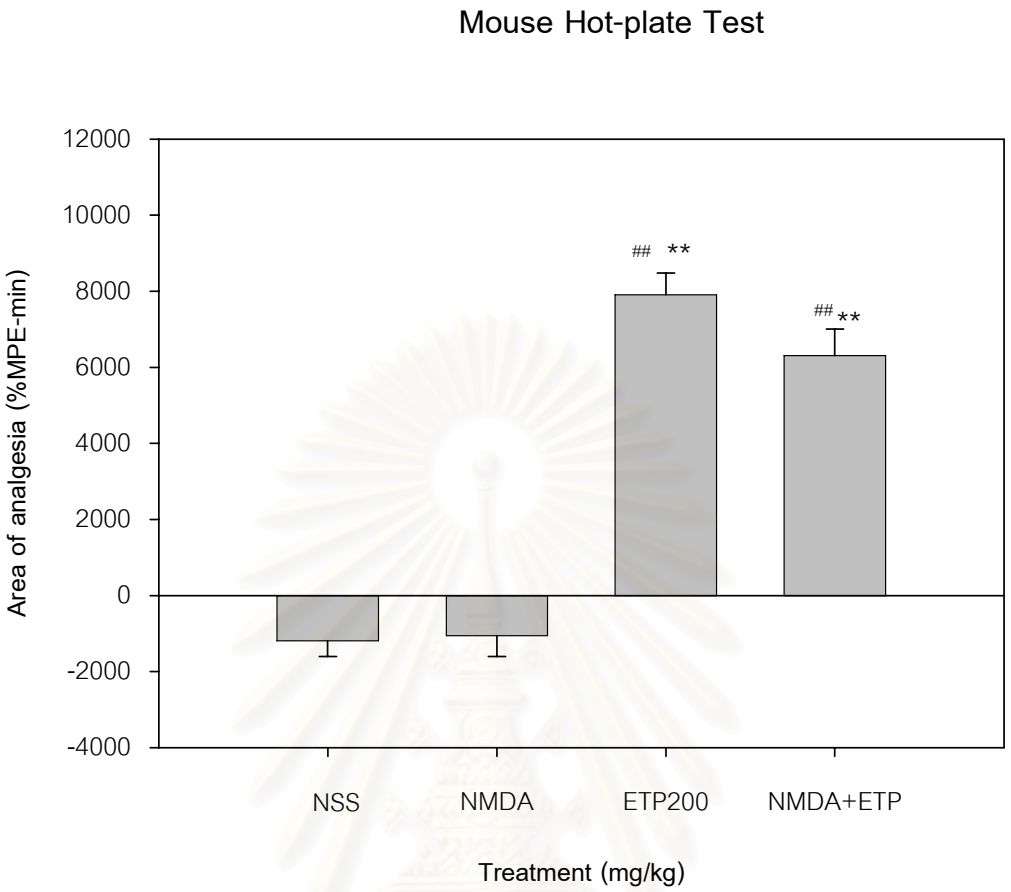


**Figure 27** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), N-methyl D-aspartate (NMDA; 0.38 mg/kg), the ethanolic extract of Thai propolis (ETP; 200 mg/kg), and the combination of N-methyl D-aspartate and the ethanolic extract of Thai propolis (0.38/200 mg/kg). N= 10 for all groups.

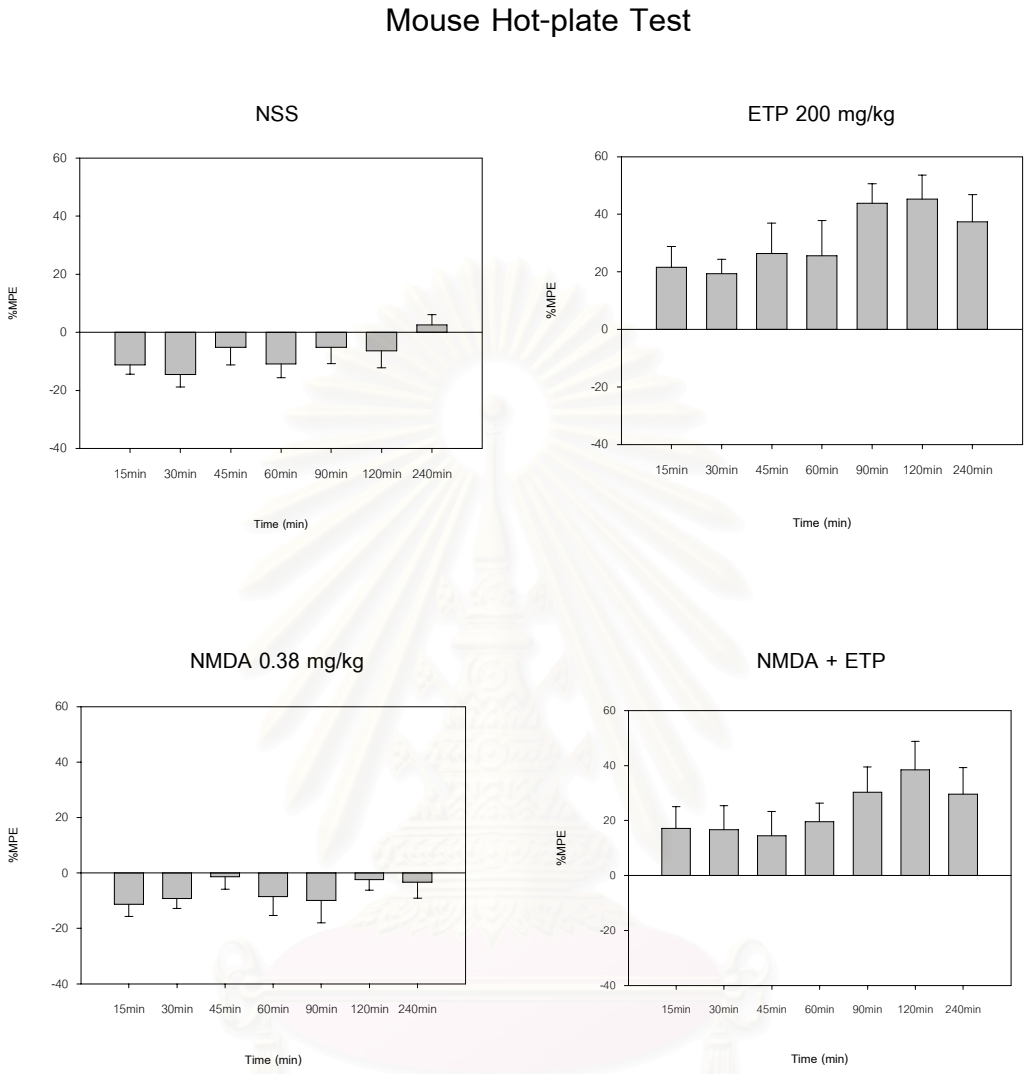


**Figure 28** Individual time courses of the response (%MPE versus time (min)) after intraperitoneal administration of 0.9% normal saline solution (NSS), the ethanolic extract of Thai propolis (ETP; 200 mg/kg), N-methyl D-aspartate (NMDA; 0.38 mg/kg), and the combination of N-methyl D-aspartate and the ethanolic extract of Thai propolis (0.38/200 mg/kg). N= 10 for all groups.

## MOUSE TAIL- FLICK TEST

To demonstrated the validity of the mouse tail-flick analgesic testing following intraperitoneal (i.p.) drug administration, mice received morphine sulfate (MO; 10 mg/kg) i.p. and were tested during the subsequent 240 min period. As expected MO significantly ($p<0.01$) increased tail-flick latency producing an area of analgesia of 24361.93 ± 573.18 %MPE-min compared with that of normal saline solution (NSS) which produced an area of analgesia of 87.85 ± 219.82 %MPE-min (Figure 26). The i.p. administration of indomethacin (IND; 150 mg/kg), a nonsteroidal anti-inflammatory drug (NSAIDs), also influenced the tail- flick latency and area of analgesia (5132.59 ± 302.38 %MPE-min) when compared to NSS ($p<0.01$; Figure 27).

Studies then utilized the mouse tail-flick method to examine the efficacy of the ethanolic extract of Thai propolis (ETP) in producing analgesia. Mice were then administered corn oil or various doses of ETP (12.5, 25, 50, 100, 200, 400, 800 mg/kg) i.p.. ETP doses of 25 mg/kg or higher produced significant ($p<0.01$) analgesic response compared to corn oil (Figure 28). Additionally, ETP doses of 100 mg/kg and higher also significantly ($p<0.01$) increased tail-flick latency when compared to the lowest dose of ETP used (Figure 29). ETP doses of 200 mg/kg or higher produced significant ($p<0.01$, $p<0.05$, $p<0.05$, respectively) analgesic response when compared to IND, while ETP doses of 50 mg/kg produced analgesic response similar to IND (Figure 29). MO showed the highest analgesic response compared to all test groups.

When the log of the ETP doses were plotted versus the area of analgesia, a significant linear correlation ($r^2= 0.69$) was observed while the plotting of only five doses (12.5, 25, 50, 100, and 200 mg/kg) revealed a significant linear correlation coefficient of 0.98 (Figure 30 & 31). $ED_{50}$ was calculated from the log dose probit line and was equal to 40.36 (3.43-475.19) mg/kg (Figure 32). The analgesic peak effect of ETP was reached within 120 min after i.p. administration in all ETP doses tested and individual time courses of the responses are shown in Figure 33.

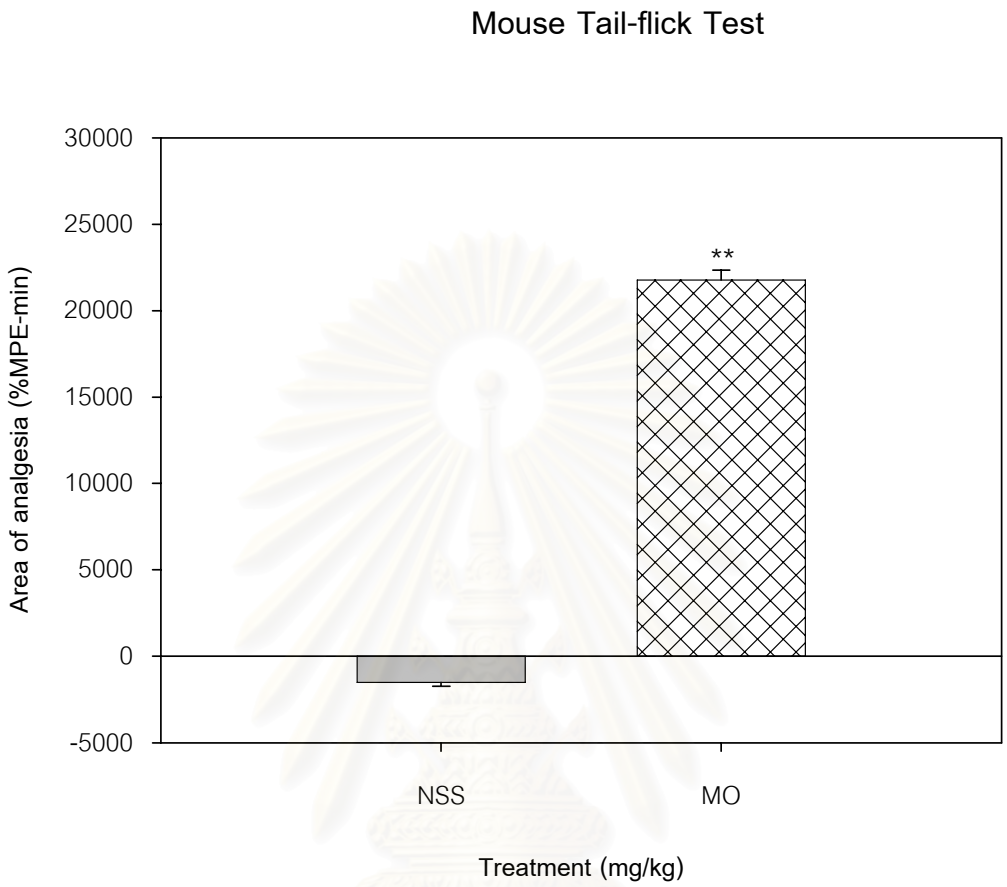


**Figure 29** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

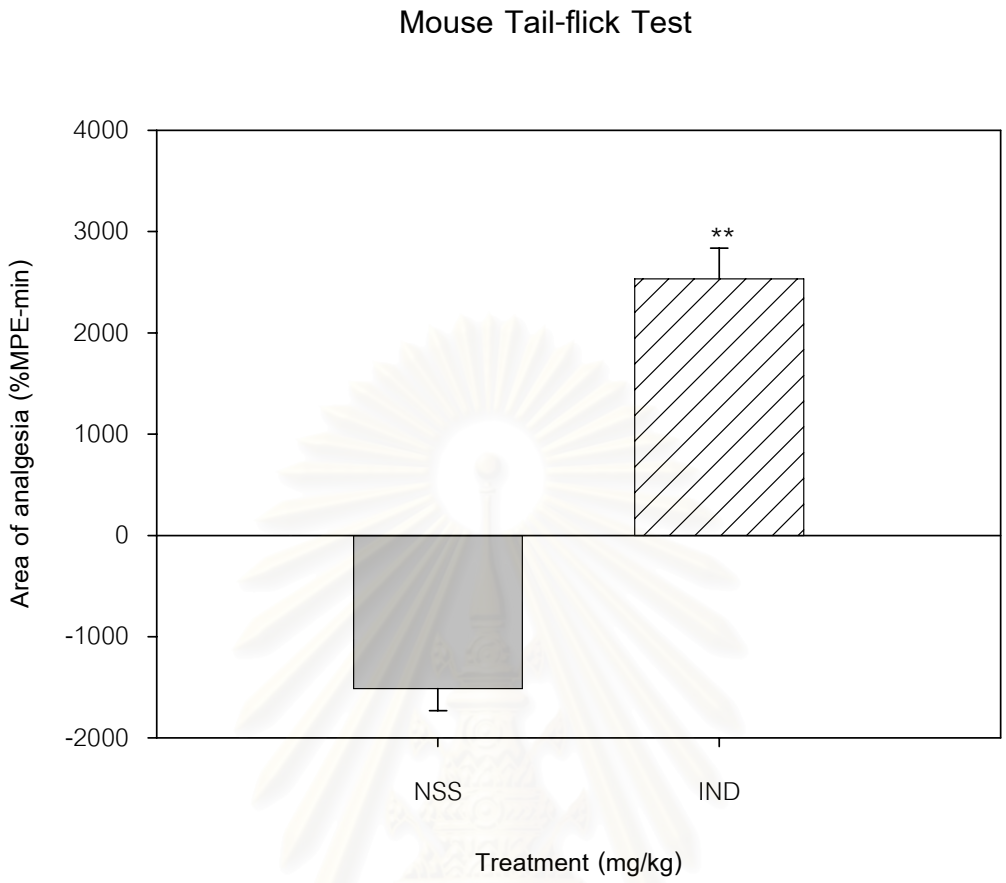


**Figure 30** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and indomethacin (IND; 150 mg/kg). N= 10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

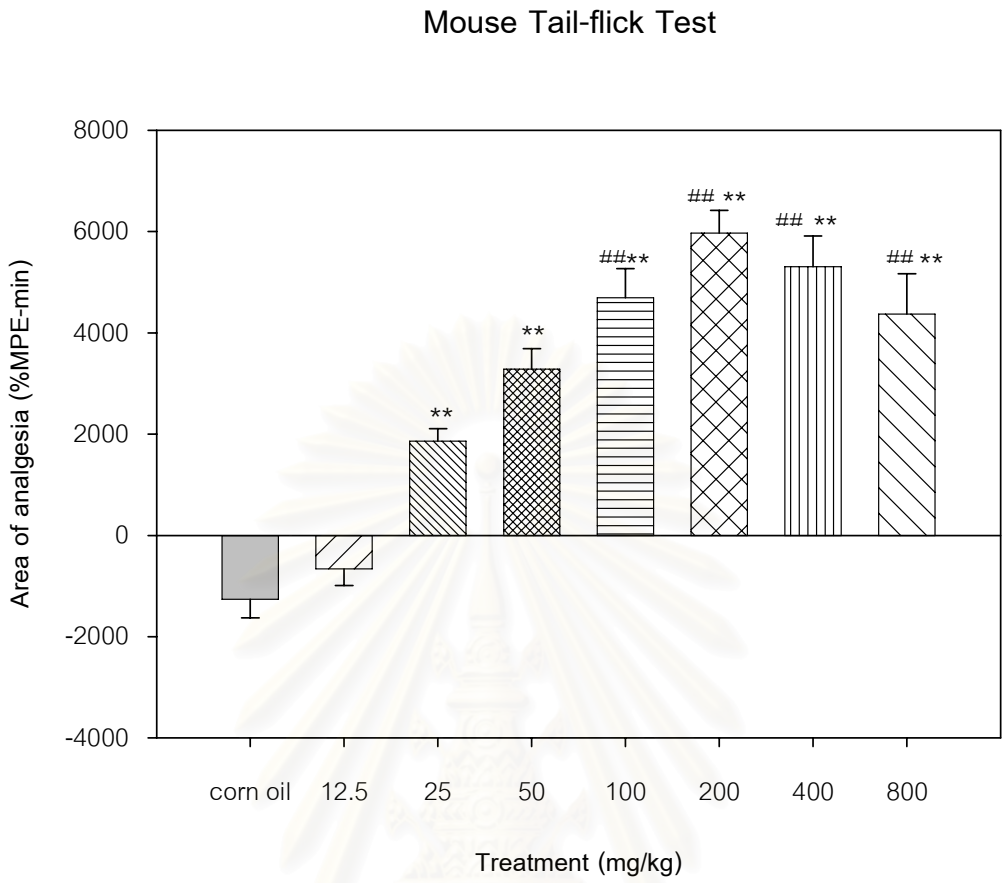


**Figure 31** Area of analgesia (%MPE-min) from 0 – 240 minutes after intraperitoneal administration of corn oil and various doses of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. $^{**}p < 0.01$ significantly different compared to corn oil, $^{\#\#}p < 0.01$ significantly different compared to ETP 12.5 mg/kg.

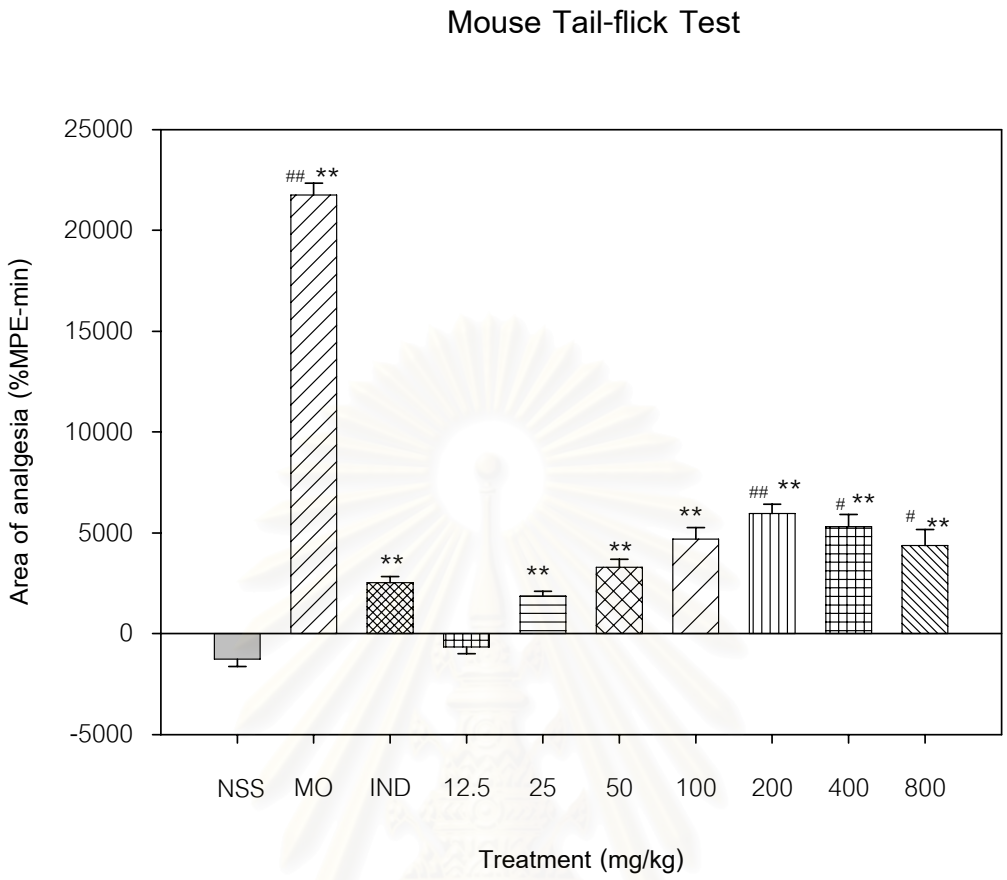


**Figure 32** Area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of corn oil, morphine sulphate (MO; 10 mg/kg), Indomethacin (IND; 150 mg/kg), and various doses of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. $^{**}p < 0.01$ significantly different compared to corn oil; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different compared to indomethacin.

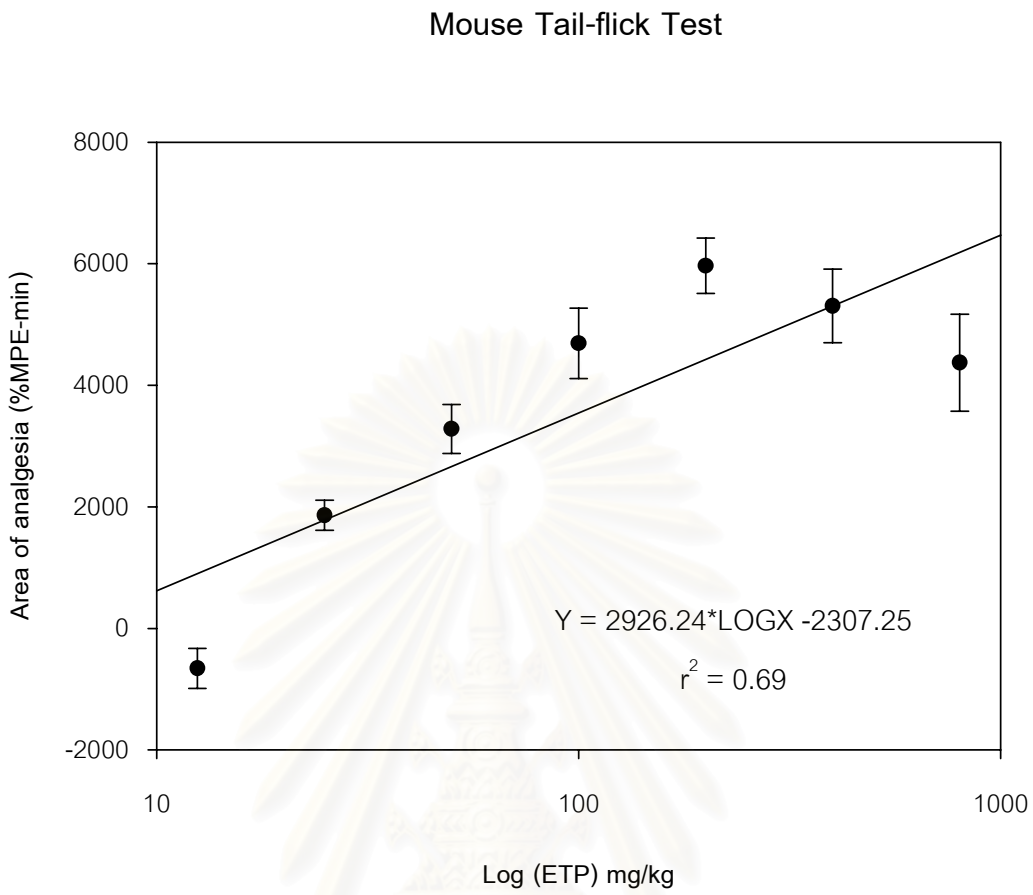


**Figure 33** Linear regression of area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. The regression equation was Y = 2926.24*LOGX -2307.25, $r^2$ = 0.69.

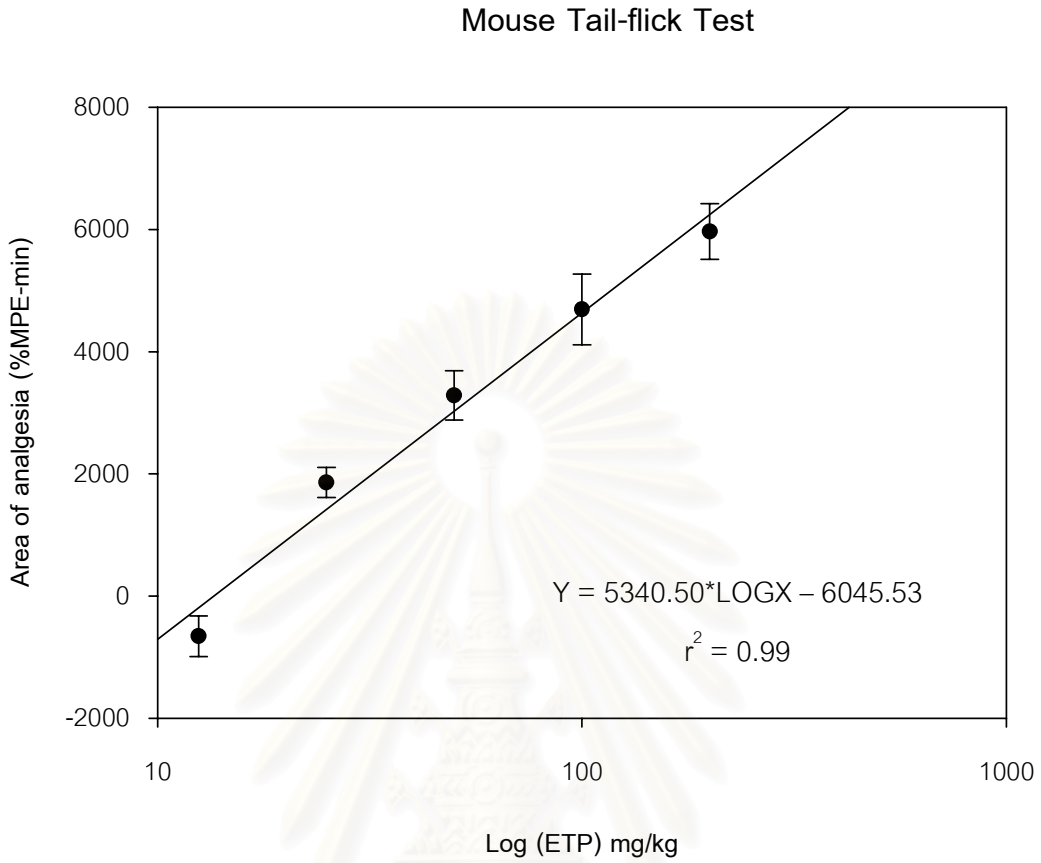


**Figure 34** Linear regression of area of analgesia (%MPE-min) from 0 - 240 minutes after intraperitoneal administration of the ethanolic extract of Thai propolis (ETP; 12.5 - 200 mg/kg). N= 10 for all groups. The regression equation was Y = 5340.50*LOGX – 6045.53, $r^2$ = 0.99.

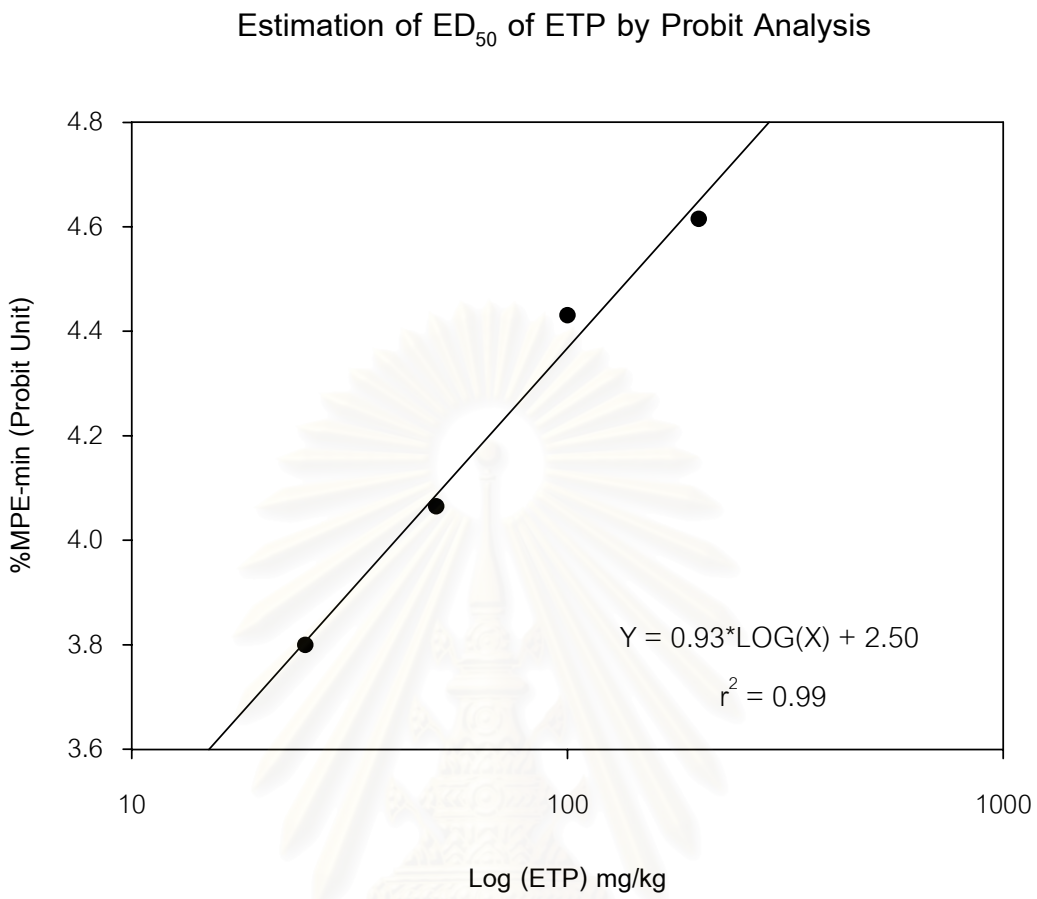


**Figure 35** Linear regression of %MPE (Probit unit) at 120 minutes after intraperitoneal administration of various doses of the ethanolic extract of Thai propolis (ETP; 12.5-200 mg/kg) using tail-flick test. N= 10 for all groups. The $ED_{50}$ was calculated from the log dose probit line as Y = 0.93*LOG(X) + 2.50, $r^2$ = 0.99 and equal to 40.36 (3.43 - 475.19) mg/kg.

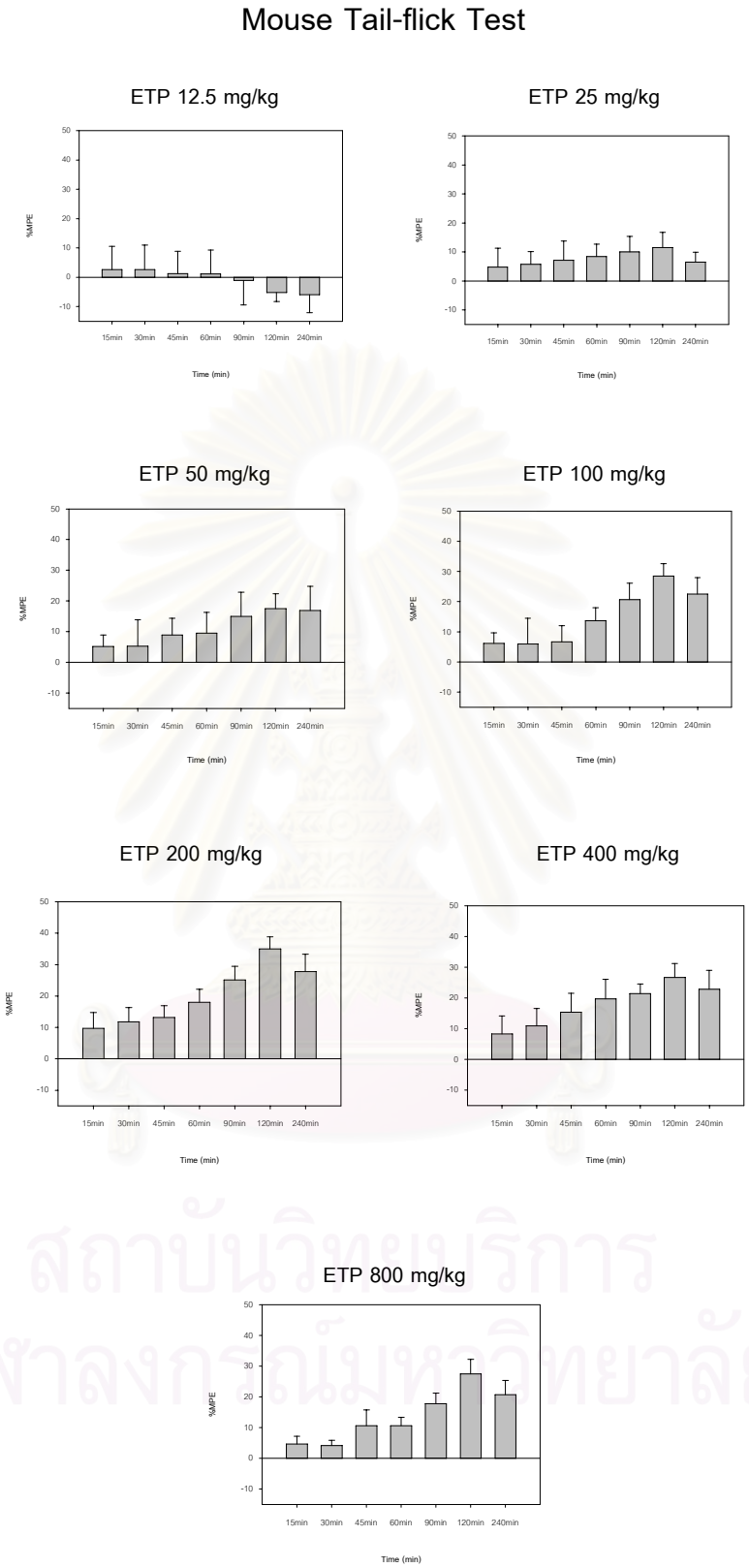


**Figure 36** Individual time courses of the response (%MPE versus time (min)) after intraperitoneal administration of various doses of the ethanolic extract of Thai propolis (ETP; 12.5-800 mg/kg). N= 10 for all groups.

## MOUSE FORMALIN TEST

The studies were then conducted utilizing the formalin-induced nociception technique which the number of licks were indicated by mice raised the injected-paw for licking or biting, were measured. The animal was observed simultaneously from 0 to 30 min following formalin injection. The number of licks per unit of time was monitored during the first 0–5 min, the neurogenic phase and the following 25–30 min, the inflammatory phase. During the first phase, MO (10 mg/kg) significantly ($p<0.01$) decreased the number of licks and 56.01 %inhibition when compared with that of NSS (15±2.9 vs. 34.1± 2.37 time; Figure 34). MO also significantly ($p<0.05$) decreased the number of licks and 64.92% inhibition when compared to NSS (11.4±4.77 vs. 32.5±3.99 time) during the second phase (Figure 34). The i.p. administration of IND (150 mg/kg) decreased the number of licks and 32.55, 74.46%inhibition during both first and second phases significantly ($p<0.05$, $p<0.01$, respectively) when compared to NSS (Figure 35).

In order to examine the efficacy of ETP in producing analgesia when the animals were stimulated by chemical stimuli, mice were injected i.p. corn oil or various doses of ETP (50, 100, 200, 400, 800 mg/kg). ETP dose of 200 mg/kg significantly ($p<0.05$) decreased the number of licks and 21.30 %inhibition when compared to the vehicle group during the first phase (Figure 36). For the second phase, ETP doses of 200 mg/kg or higher significantly ($p<0.05$, $p<0.05$, $p<0.01$, respectively) decreased the number of licks compared to the vehicle group (Figure 37). Additionally, the percent of inhibition second phase of ETP 200, 400, and 800 mg/kg were 57.11, 61.84, and 84.21%inhibition, respectively.

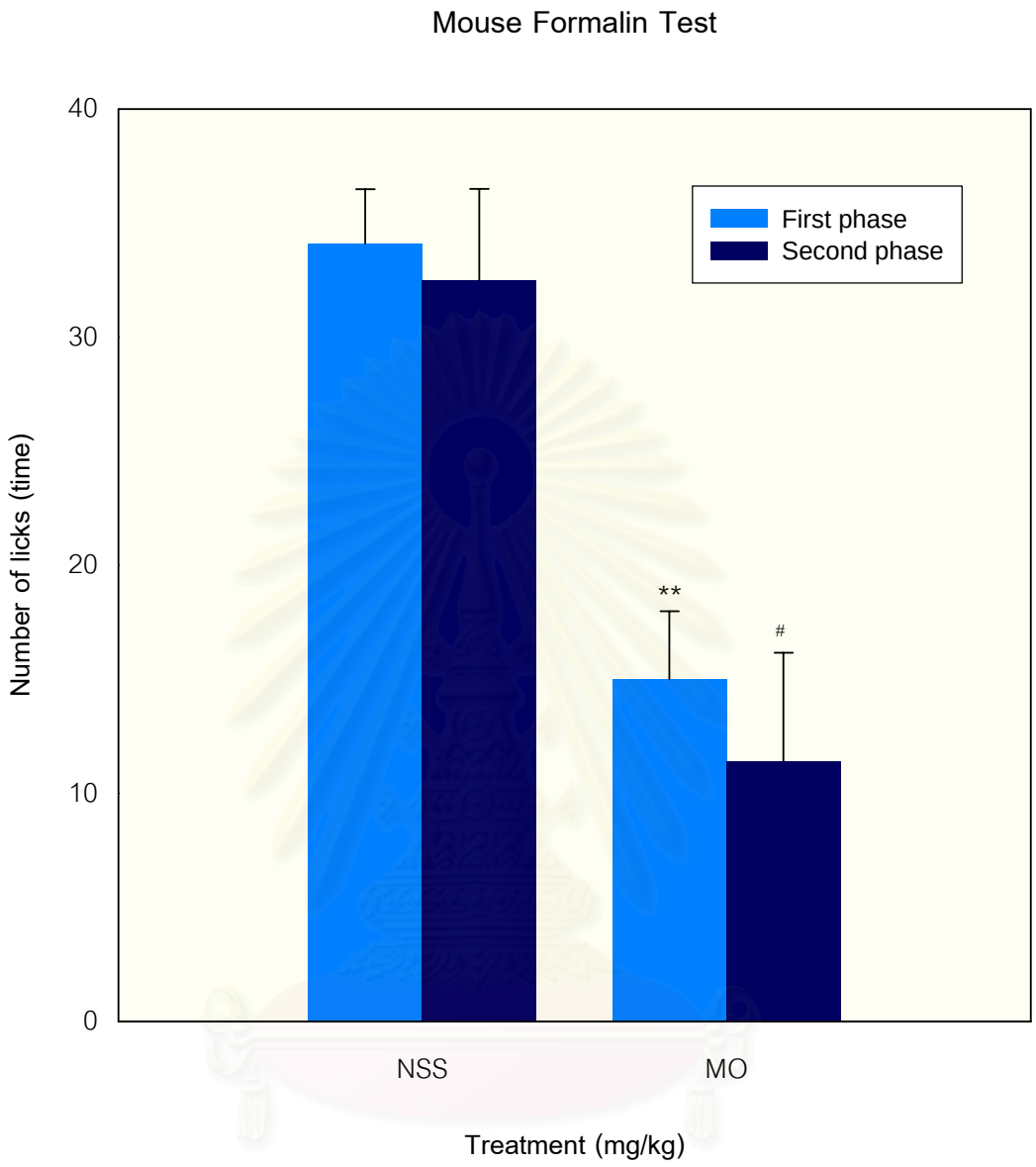


**Figure 37** Number of licks of the first phase (0-5 min) and the second phase (25-30 min) after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). N= 10 for all groups. $^{**}p < 0.01$ significantly different compared to NSS (first phase); # $p < 0.05$ significantly different compared to NSS (second phase).

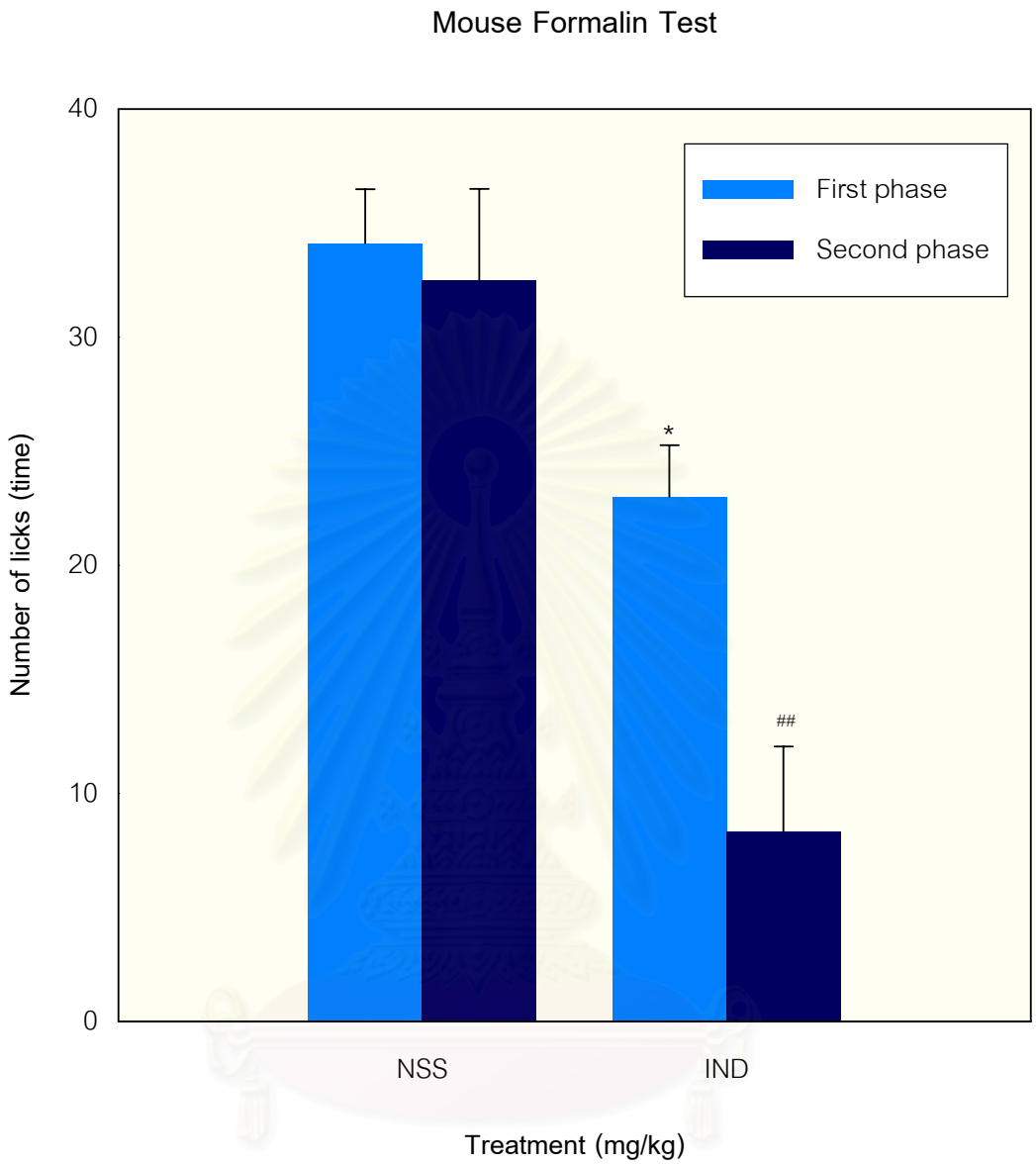


**Figure 38** Number of licks of the first phase (0-5 min) and the second phase (25-30 min) after intraperitoneal administration of 0.9% normal saline solution (NSS) and indomethacin (IND; 150 mg/kg). N= 10 for all groups. $^{*}p < 0.05$, significantly different compared to NSS (first phase); $^{\#\#}p < 0.01$ significantly different compared to NSS (second phase).

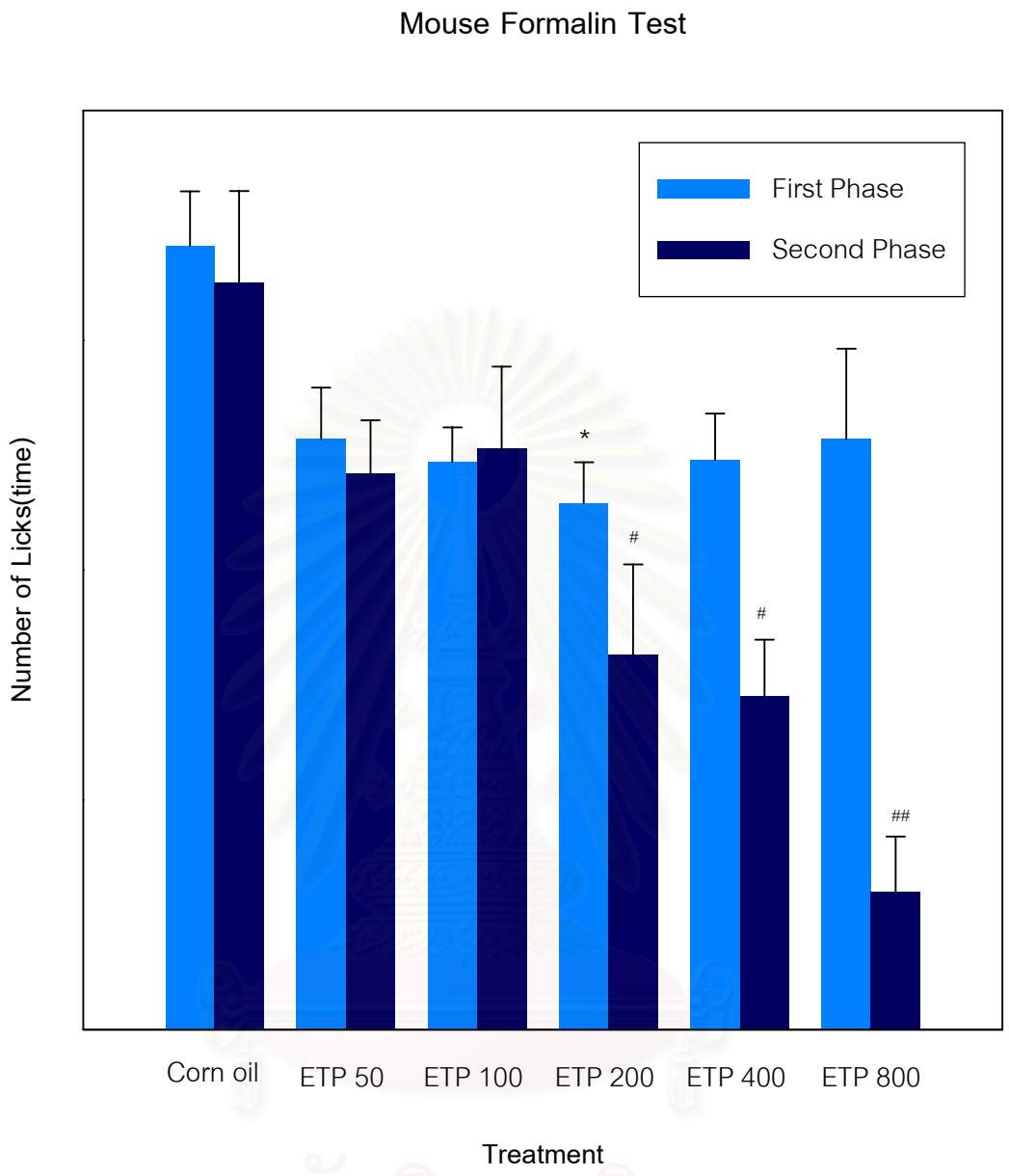


**Figure 39** Number of licks of the first phase (0-5 min) and the second phase (25-30 min) after intraperitoneal administration of corn oil and various doses of the ethanolic extract of Thai propolis (ETP; 12.5 - 800 mg/kg). N= 10 for all groups. $^{*}p < 0.05$ significantly different compared to corn oil (first phase); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different compared to corn oil (second phase).

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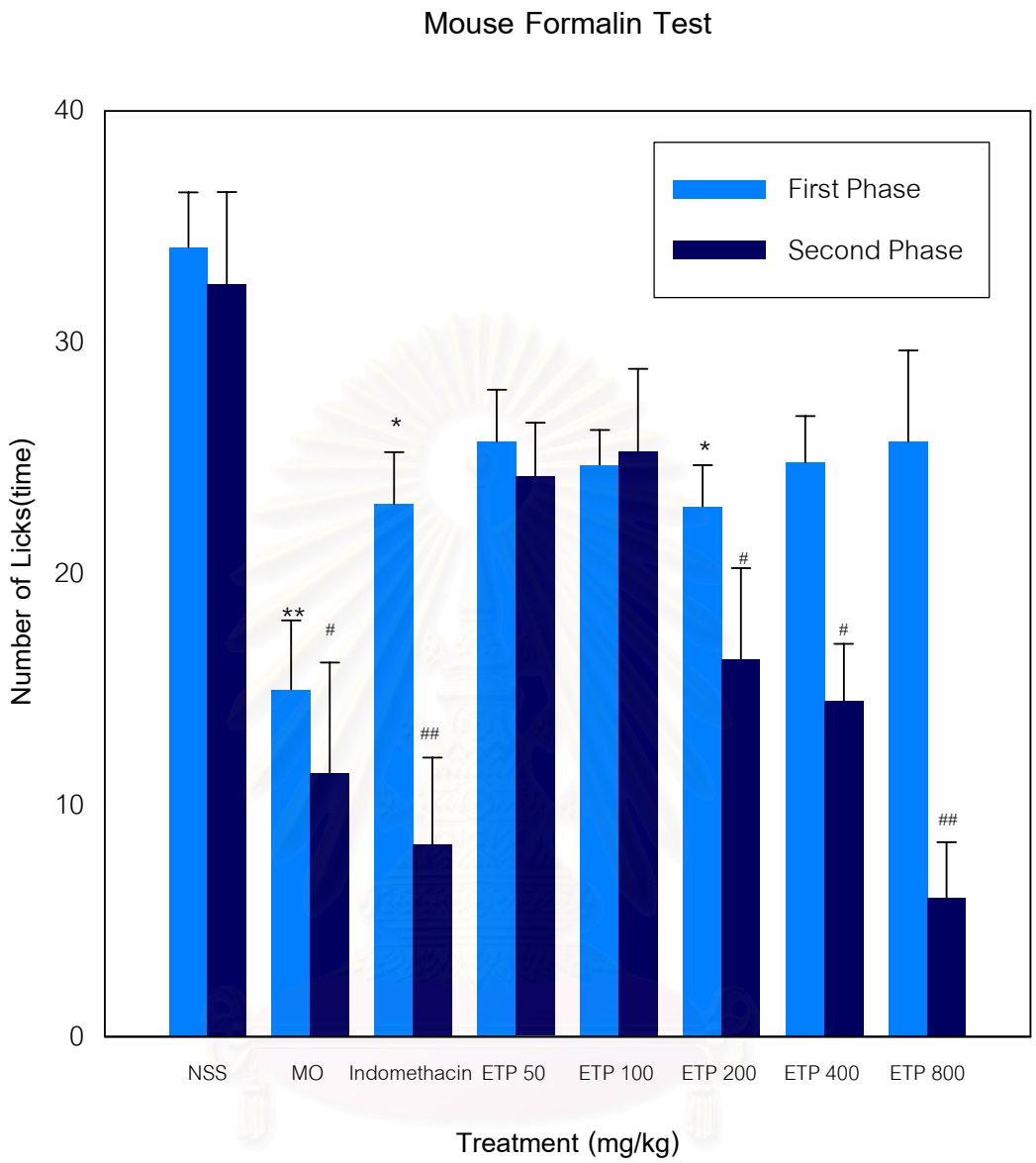


**Figure 40** Number of licks of the first phase (0-5 min) and the second phase (25-30 min) after intraperitoneal administration of 0.9% normal saline solution (NSS), morphine sulphate (MO; 10 mg/kg), indomethacin (IND; 150 mg/kg) and various doses of the ethanolic extract of Thai propolis (ETP; 50-800 mg/kg). N= 10 for all groups. $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different compared to NSS (first phase); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different compared to NSS (second phase).

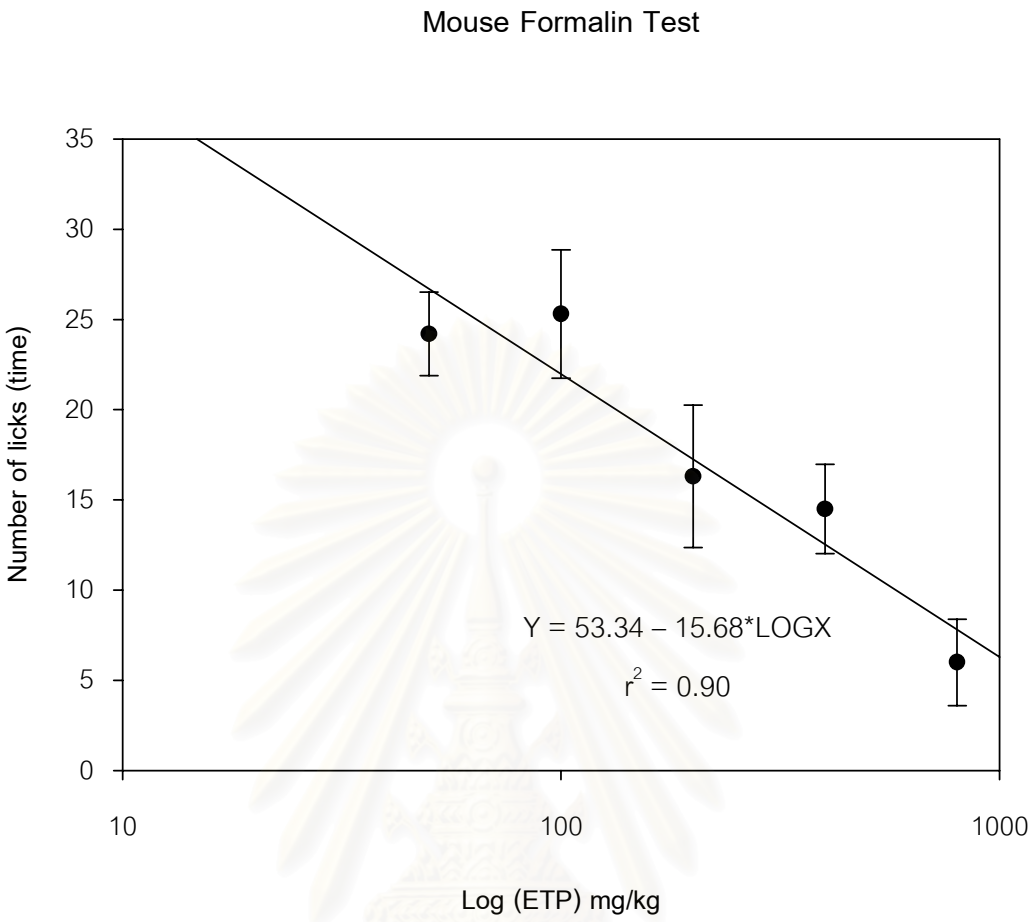


**Figure 41** Linear regression of number of licks (time) from the second phase after intraperitoneal administration of ethanolic extract of Thai propolis (ETP; 50-800 mg/kg). N= 10 for all groups. The regression equation was Y = 53.34 – 15.68*LOGX, $r^2$ = 0.90.

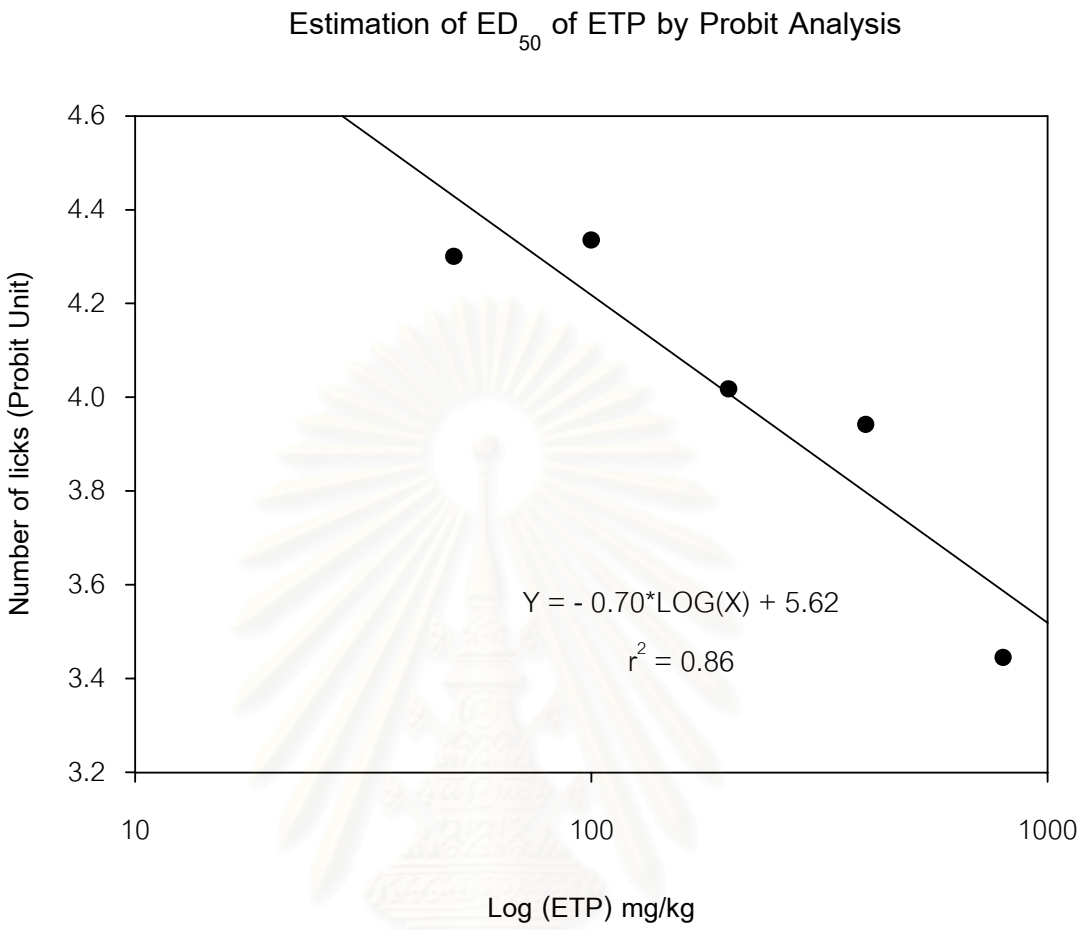


**Figure 42** Linear regression of number of licks (Probit unit) of the second phase after intraperitoneal administration of various doses of the ethanolic extract of Thai propolis (ETP; 50-800 mg/kg) using formalin test. N= 10 for all groups. The $ED_{50}$ was calculated from the log dose probit line as Y = - 0.70*LOG(X)+ 5.62, $r^2$ = 0.86 and equal to 206.20 (7.69 – 5531.68) mg/kg.

ROTA-ROD TEST

In order to measure the effect of ETP on motor performance, mice were then treated i.p. with various doses of ETP (200, 400, 800 mg/kg) and tested on the rota-rod for 60 seconds. The rota-rod performance of mice was observed at 30, 60, 120, and 240 minutes after ETP administration. Data showed that all doses of ETP tested did not significantly produce motor impairment (Figure 43).

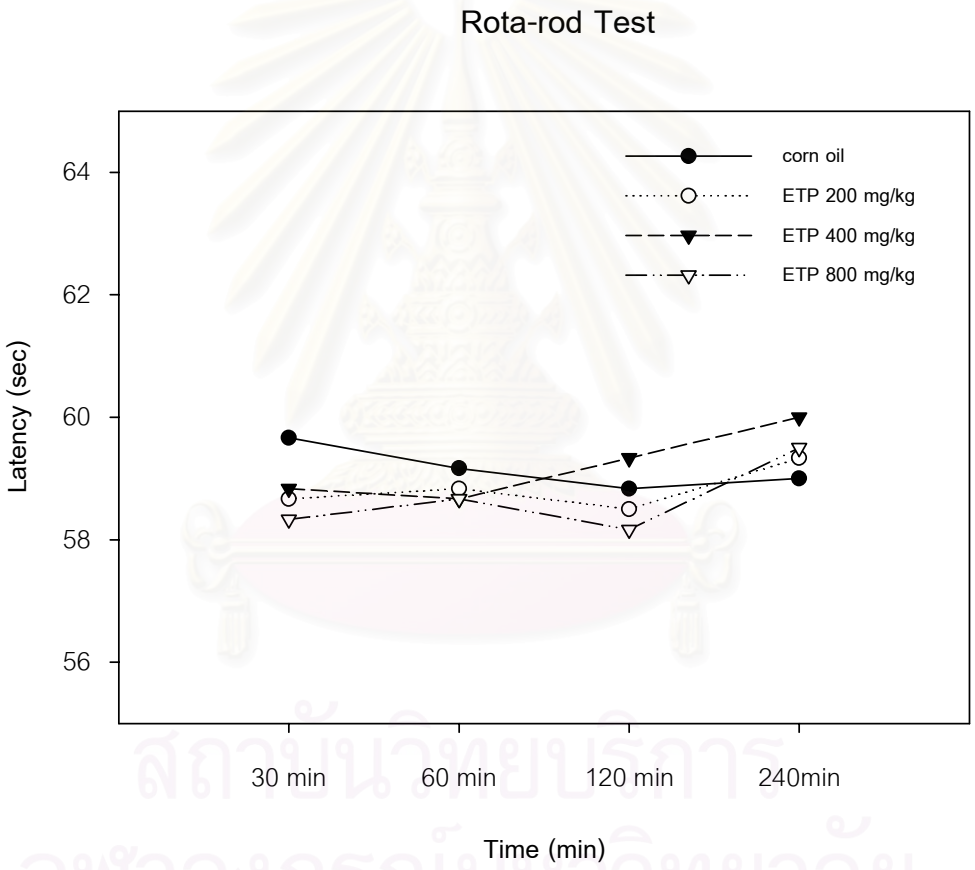


**Figure 43** Rota -rod latency of corn oil and various doses of the ethanolic extract of Thai propolis (ETP; 200-800 mg/kg) on rota -rod at 30, 60, 120, and 240 minutes after intraperitoneal administration.

# CHAPTER V
# DISCUSSION AND CONCLUSION

These studies have demonstrated the antinociceptive effect of the ethanolic extract of Thai propolis (ETP) assessed in thermal (tail-flick and hot-plate test), and chemical (formalin test) models in mice. Initial attempts to investigate the analgesic effect of ETP utilized the standard mouse hot-plate test, the supraspinal level nociceptive testing model. ETP doses of 50-800 mg/kg demonstrated a dose-response relationship with the mouse hot-plate technique. The linear regression equation for all doses of ETP was [Response = 3580.9831*LOG(X) – 2343.2904], $r^2$ = 0.69 and [Response = 6703.7994*LOG(X) – 7149.8577], $r^2$ = 0.98 when excluding the two highest of ETP (Figure 19 & 20).ETP doses of 12.5-25 mg/kg had no analgesic effect in this model (Figure 17). The analgesic action of ETP was observed during 240 min period (Figure 18). Morphine (MO) as a reference standard had shown potent analgesic effect but indomethacin (IND) had little influence on the response in this animal model. ETP doses of 50-800 mg/kg appeared to produce similar analgesic response compared to IND (Figure 18). The ED50 was equal to 48.56 mg/kg (Figure 19). It can be concluded that ETP produced milder analgesic effect in the supraspinal level compared to morphine.

NAL and NALT, opioid receptor antagonists, were utilized to investigate the involvement of opioid receptor in the analgesic effects of ETP (Figure 23). The results from both NAL and NALT showed the involvement of opioid receptors in analgesia produced by ETP (Figure 24). Since NMDA coadministration did not appear to attenuate the analgesic response of ETP, suggested no involvement of NMDA receptor in ETP analgesia (Figure 25).

Studies were then undertaken to investigate the effectiveness of ETP utilizing the mouse tail-flick test, the spinal level nociceptive testing model. MO administered i.p. produced significant analgesic response as expected (Figure26). IND also produced

analgesia but at a lesser extent compared to MO in this animal model (Figure 27). The lowest dose of ETP (12.5 mg/kg) had no analgesic effect in this model (Figure 28). ETP at doses of 25-800 mg/kg administered i.p. produced a dose-related analgesic response and ETP dose of 50 mg/kg appeared to produce analgesic effect similar to IND (Figure 29). The linear regression equation for all doses of ETP was [Response = 2926.2380*LOG(X) – 2307.2472], $r^2$ = 0.69 and [Response = 5340.4974*LOG(X) – 6045.5298], $r^2$ = 0.99 when excluding the two highest doses of ETP (Figure 30 & 31). ETP doses of 200-800 mg/kg produced significant analgesic response compared to IND, a potent cyclooxygenase inhibitor. ETP also produced milder analgesic effect in the spinal level compared to morphine.

The formalin test was chosen to measure ETP effect against chemical stimuli. Intraperitoneal administration of MO and IND at dose tested produce significant analgesic response compared to NSS treated controls utilizing this method (Figure 34 & 35). ETP dose of 200 mg/kg reduced the number of licks in the first phase of nociception (neurogenic phase) similar to IND, and ETP dose of 800 mg/kg reduced the number of licks in second phase of nociception (inflammatory phase) similar to IND. ETP doses of 200-400 mg/kg reduced the number of licks in the second phase similar to MO (Figure 37). ETP (200-800mg/kg) eliticed a dose-dependent inhibition of the number of licks during the second phase, with a maximal reduction of appoximately 84.21 % of the control group.

ETP has demonstrated analgesic response in all testing models suggesting that ETP could produce analgesia via both spinal and supraspinal mechanisms. The hot-plate analgesia testing seemed to be more sensitive for evaluation central analgesic effect of ETP as ETP had shown higher analgesic response compared to NSS treated group.

In order to evaluate the posible non-specific muscle realxant or sedative effects of ETP, mice were treated on the rota-rod. Animals were treated with various doses of ETP (200, 400, 800 mg/kg) i.p. 30 min before being tested. ETP at all doses tested failed

to significantly alter the rota-rod performance compared to NSS treated controls. Therefore, the antinociception caused by ETP is probalbly unrelated with impairment of motor response, as at doses in which the extract produced pronounced antinociception it had no significant effect on the motor function of animals, as demonstrated by the complete lack of effect when the rota-rod apparatus was used for testing.

The chemical composition of ETP was analyzed utilizing GC/MS method. The major constituents are flavonoids: Dihydrochrysin (Galangin flavanone; 21.16%), Galangin (17.81%), Chrysin (19.61%), Chrysin derivative (13.51%), Tectochrysin (13.64%), Pinostrobin chalcone (7.79%), 3, 4-Dimethoxycinnamic acid (4.22%), and 4-Hydroxy-methyl-benzaldehyde (2.26%). The components of propolis such as some flavonoids and phenylethyl caffeate were reported to be capable of scavenging free radicals, and phenylethyl caffeate was the most effective in modulating eicosanoid production by mouse macrophages (Burdock, 1998). Another report demonstrated *in vivo* anti-inflammatory and analgesic activities of Bulgarian propolis (Et-Blg) with the synthesis and / or liberation of inflammatory mediators and the high content of phenolic components in the extract. Using smooth muscle preparations from guinea pig trachea, Et-Blg was able to block the depolarization induced by KCl (similarly to nicardipine) and to inhibit the contraction induced by histamine, capsaicin, and carbachol. Since KCl and histamine mediate the opening of calcium channels and carbachol, the intracellular release of this ion. The observed effect of the propolis extract was associated with the control of calcium mobilization (Paulino *et al*., 2003). The previous study reported that the ethanol extract of a Brazilian sample and isolated compounds induced a relaxant effect in this model (Rossi, 2002). Duangjai (2006) reported the anti-inflammatory effect of the ethanolic extract of Thai propolis (EEP) in carrageenan-induced rat paw edema. The maximum inhibition of paw edema occurred at the concentration of 300 mg/ml when given intraperitoneally.

In conclusion, the current study has demonstrated that as reported for many naturally-occurring substances isolated from plants, the ethanol fraction obtained from

Thai propolis exerts a pronounced antinociception when assessed in chemical model of nociception which represents an inflammatory process or chronic pain and a mild antinociception when assessed in thermal models of nociception which represents acute pain in rodents.

These analgesic effects being due, at least in part, to the presence of flavonoids and phenolic acids. The precise mechanisms involved in their action are, at this moment, not completely understood; it is most likely involved with the opioid pathway.

## FUTURE RESEARCH

In these studies there was evidence that ethanolic extract of Thai propolis was capable of significantly produced analgesic and anti-inflammatory response, most likely via an opioid mechanism, and supports the potential use of the extract.

The future research could comprise of several objectives as listed below

(1) To identify and remove possible toxic substances from the extract.

(2) To investigate the antipyretic effect of various doses of ETP.

(3) To investigate the anesthetic effect of various doses of ETP.

(4) To investigate the potential use of ETP in combination with other analgesic or nonsteroidal anti-inflammatory drugs.

(5) To investigate other routes of administration that might be more appropriate for the use of ETP and possibly enhance the analgesic effect of ETP.

(6) To better understand the mechanism of ETP that is involved in producing its analgesic and anti-inflammatory effects.

(7) To observe other opioid antagonist to better characterize the mechanism of ETP effects.

(8) To test side effects and toxic effects of ETP at high doses.

These and other studies may provide important clues to help understand the mechanisms underlying the analgesic effect of ETP and further support the use of such compounds in a clinical setting.

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# APPENDICES

# APPENDIX A

**Table 2** %MPE-Time in Mouse Hot-plate Test from 0-240 min after intraperitoneal administration of the various doses of the ethanolic extract of Thai propolis (ETP; 12.5–800 mg/kg). N=10 for all groups. Data presented as mean±S.E.M.

| ETP (mg/kg) | 15 min | 30 min | 45 min | 60 min | 90 min | 120 min | 240 min | Area of analgesia |
|---|---|---|---|---|---|---|---|---|
| 12.5 | 1.716±4.134 | 1.679±3.709 | 1.446±5.144 | 2.449±3.814 | 2.598±5.117 | 2.960±1.714 | 2.260±2.742 | 526.886±409.539 |
| 25 | 1.711±6.384 | 3.397±4.832 | 5.333±7.003 | 7.773±3.426 | 10.635±4.936 | 11.650±4.907 | 10.616±6.016 | 2012.378±301.223 |
| 50 | 4.668±3.786 | 6.117±3.866 | 8.310±4.217 | 11.887±4.246 | 12.917±2.988 | 11.785±4.387 | 28.366±3.181 | 3346.389±469.260 |
| 100 | 17.883±13.022 | 25.696±8.389 | 29.255±4.926 | 27.294±9.508 | 25.910±6.094 | 27.469±7.826 | 31.335±8.801 | 6061.460±517.421 |
| 200 | 21.503±7.267 | 19.344±5.030 | 26.35±10.531 | 25.507±12.372 | 43.763±6.744 | 45.153±8.413 | 37.347±9.466 | 7909.366±568.486 |
| 400 | 12.478±10.850 | 13.022±10.691 | 17.731±6.974 | 22.860±4.2192 | 37.576±11.098 | 32.110±9.604 | 28.279±7.190 | 5869.021±546.896 |
| 800 | 3.005±7.678 | 10.126±9.595 | 19.825±9.711 | 19.552±11.110 | 34.722±9.802 | 42.723±9.505 | 15.947±11.273 | 5650.857±304.426 |

**Table 3** %MPE-Time in Mouse Hot-plate Test 0-240 min of NSS, ETP (200 mg/kg), NAL (1 mg/kg), NALT (5 mg/kg), NMDA (0.38 mg/kg),and combination with ETP, intraperitoneally data present as mean %MPE±S.E.M. N=10 per group.

| Treatment | 15 min | 30 min | 45 min | 60 min | 90 min | 120 min | 240 min | Area of analgesia (%MPE-min) |
|---|---|---|---|---|---|---|---|---|
| ETP | 21.503±7.267 | 19.344±5.030 | 26.35±10.531 | 25.507±12.372 | 43.763±6.744 | 45.153±8.413 | 37.347±9.466 | 7909.366±568.486 |
| NSS | -11.231±3.227 | -14.525±4.257 | -5.159±6.077 | -10.942±4.671 | -5.181±5.599 | -6.405±5.803 | 2.512±3.567 | -1195.098±410.896 |
| NAL | -16.593±9.503 | -25.649±8.145 | -25.518±9.738 | -9.037±10.661 | -0.637±7.725 | -1.773±8.428 | -1.421±8.147 | -1448.1595±521.232 |
| NAL+ETP | -14.127±5.692 | -18.012±7.772 | -21.336±7.838 | 0.153±5.563 | 10.321±6.011 | 21.812±8.221 | 11.52±6.697 | 1693.5535±463.547 |
| NALT | -2.598±3.121 | -8.339±2.497 | -11.564±4.534 | -6.973±2.906 | -10.226±3.817 | -7.218±3.003 | -2.127±3.195 | -1326.9935±398.854 |
| NALT+ETP | 16.876±4.782 | 15.823±6.653 | 10.119±4.172 | 3.908±2.911 | 4.217±3.452 | 5.325±3.426 | 4.636±3.816 | 1475.207±402.552 |
| NMDA | -11.307±4.400 | -9.202±3.593 | -1.417±4.431 | -8.519±6.850 | -9.891±8.128 | -2.339±3.846 | -3.37±5.808 | -1056.4485±541.156 |
| NMDA+ETP | 17.194±13.853 | 16.706±12.701 | 14.47±11.823 | 19.558±14.842 | 30.364±14.144 | 38.452±10.368 | 29.578±9.656 | 6310.009±699.807 |

**Table 4** %MPE-Time in Mouse Tail-flick Test 0-240 min of ETP dose 12.5–800 mg/kg i.p. data present as mean %MPE±S.E.M. N=10 per group.

| Dose ETP (mg/kg) | 15 min | 30 min | 45 min | 60 min | 90 min | 120 min | 240 min | Area of analgesia (%MPE-min) |
|---|---|---|---|---|---|---|---|---|
| 12.5 | 2.63±7.94 | 2.6±11.34 | 1.21±7.64 | 1.11±8.12 | -1.13±8.28 | -5.15±3.15 | -5.98±6.10 | -641.56±329.65 |
| 25 | 4.79±6.53 | 5.70±10.44 | 7.12±6.61 | 9.97±5.44 | 11.50±5.28 | 8.45±4.22 | 6.54±13.41 | 1698.63±247.00 |
| 50 | 5.14±3.70 | 5.31±8.58 | 8.88±5.43 | 9.56±6.70 | 14.99±7.82 | 17.48±4.88 | 16.91±7.87 | 3070.49±404.17 |
| 100 | 6.20±3.43 | 5.91±8.54 | 6.67±5.35 | 13.65±4.36 | 20.64±5.47 | 28.43±4.17 | 22.47±12.49 | 4399.52±577.92 |
| 200 | 9.71±5.07 | 11.79±4.53 | 13.11±3.82 | 17.94±4.17 | 25.05±4.36 | 34.98±3.79 | 27.77±10.50 | 5613.30±454.37 |
| 400 | 8.25±12.92 | 10.93±5.56 | 15.34±6.11 | 19.70±6.36 | 18.38±3.19 | 26.67±4.50 | 29.86±12.09 | 5046.98±603.96 |
| 800 | 4.68±2.46 | 10.67±5.10 | 4.19±1.68 | 10.62±2.69 | 17.77±3.51 | 27.48±4.82 | 20.76±14.65 | 4122.97±795.96 |

**Table 5** %MPE-Time in Mouse Formalin Test 0-240 min of ETP dose 50–800 mg/kg i.p. data present as mean %MPE±S.E.M. N=10 per group.

| Dose ETP (mg/kg) | First Phase | Second Phase |
|---|---|---|
| 50 | 25.7±2.2 | 24.2±2.3 |
| 100 | 24.7±1.5 | 25.3±3.5 |
| 200 | 22.9±1.7 | 16.3±3.9 |
| 400 | 24.8±2.0 | 14.5±2.4 |
| 800 | 25.7±3.9 | 6.0±2.3 |

**Table 6** Effect of ETP on motor impairment in rota-rod test. N= 6 per group.

| Group | Time after ETP injection (mean±S.E.M) | | | |
|---|---|---|---|---|
| | 30 min | 60 min | 120 min | 240min |
| Corn oil | 59.66±0.81 | 59.16±1.60 | 58.83±1.32 | 59.00±1.54 |
| ETP 200 mg/kg | 58.66±1.63 | 58.83±1.32 | 58.50±1.76 | 59.33±1.632 |
| ETP 400 mg/kg | 58.83±1.32 | 58.66±1.632 | 59.33±1.21 | 60.00±0 |
| ETP 800 mg/kg | 58.33±2.065 | 58.66±1.21 | 58.16±1.60 | 59.50±1.22 |

# APPENDIX B

## Chemical constituents in ETP by GC/MS

**Figure 44** Chromatogram of ETP from GC/MS

The Ethanolic Extract of Thai Propolis 0.1 gm in methanol 1 ml, inject 1 µl to GC/MS

(Trace GC Finnigan, Polaris Q MS)

Column BPX5 30m x 0.25 mm x 0.25 µm

Column temp 150°C ramp 10°C/min to 250°C hold 35 min

Injector temp 300°C

Carrier gas He flow rate 1 ml/min

MS scan 40.00-650 m/z

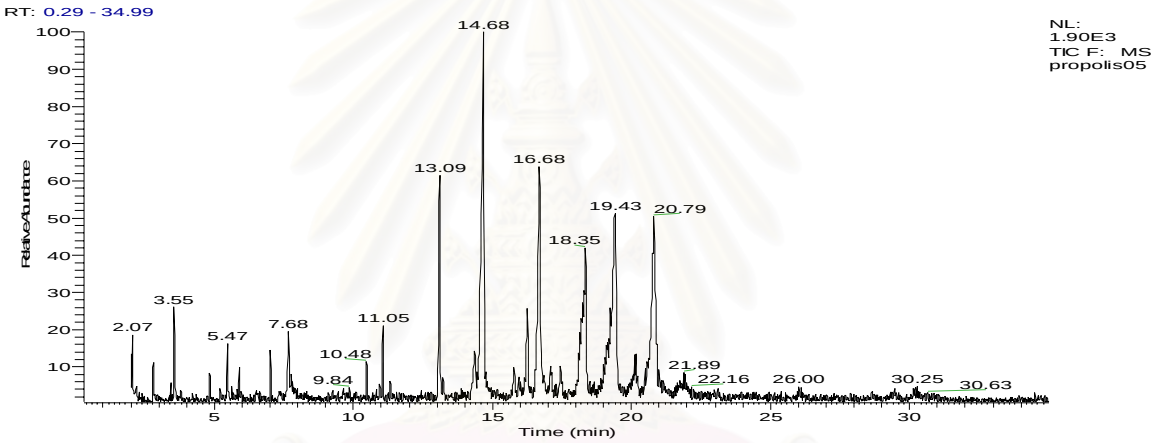


**Table 7** Chemical constituents of ETP from GC/MS

| RT | Chemical composition | Peak Area | Area % |
|---|---|---|---|
| 3.56 | 4-Hydroxymethylbenzaldehyde | 1317 | 2.26 |
| 7.67 | 3,4-Dimethoxycinnamic acid | 2454 | 4.22 |
| 13.08 | Pinostrobin chalcone | 4530 | 7.79 |
| 14.66 | Dihydrochrysin (Galangin flavanone) | 12313 | 21.16 |
| 16.68 | Tectochrysin | 7935 | 13.64 |
| 18.33 | Chrysin derivative | 7859 | 13.51 |
| 19.40 | Chrysin | 11407 | 19.61 |
| 20.79 | Galangin | 10365 | 17.81 |

**Figure 45** Mass spectrum of ETP at Rt 3.56

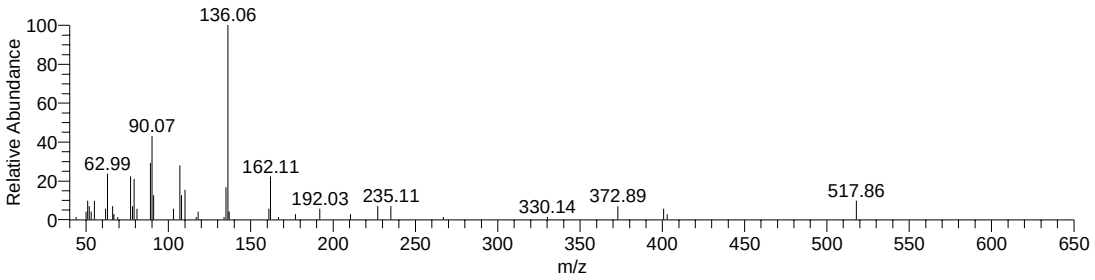


**Table 8** Chemical constituents of ETP at Rt 3.56

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 3.56 | Benzene, 1-(bromomethyl)-3-nitro- | 481 | replib | C7H6BrNO2 |
| 3.56 | 2-Hydroxy-3-methylbenzaldehyde | 547 | mainlib | C8H8O2 |
| 3.56 | Benzaldehyde, 2-hydroxy-6-methyl- | 557 | mainlib | C8H8O2 |

**Figure 46** Mass spectrum of ETP at Rt 7.67

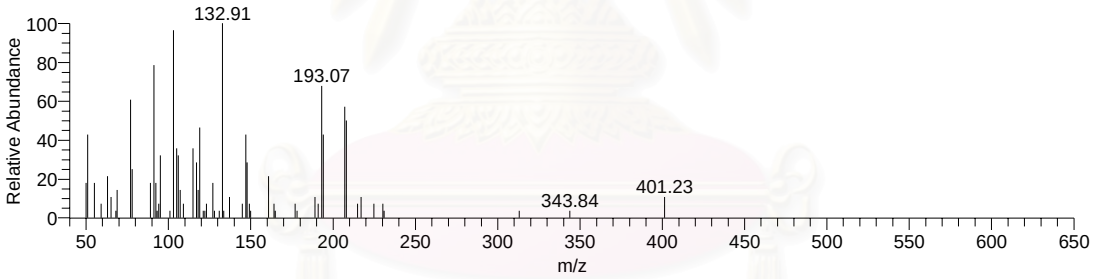


**Table 9** Chemical constituents of ETP at Rt 3.56

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 7.67 | trans-2,3-Methylenedioxy-b-methyl-b-nitrostyrene | 382 | mainlib | C10H9NO4 |
| 7.67 | trans-3-Methoxy-b-methyl-b-nirostyrene | 394 | mainlib | C10H11NO3 |
| 7.67 | Benzaldehyde, 4-(1-methylethyl)- | 425 | replib | C10H12O |

**Figure 47** Mass spectrum of ETP at Rt 13.08

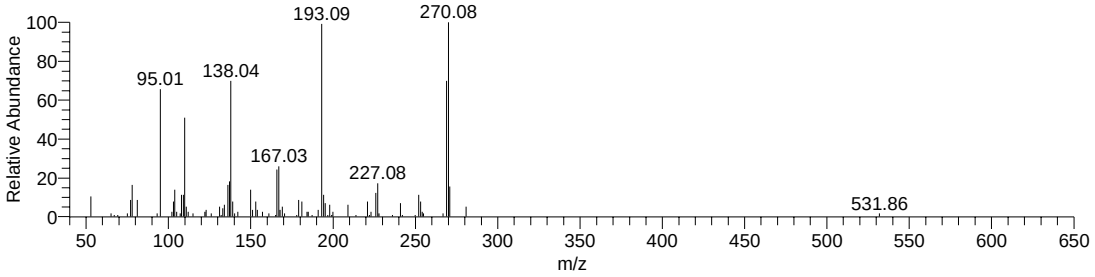


**Table 10** Chemical constituents of ETP at Rt 13.08

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 13.08 | 1H-Benzimidazole, 1,2-diphenyl- | 439 | mainlib | C19H14N2 |
| 13.08 | Physcion-10,10'-bianthrone | 489 | mainlib | C32H26O8 |
| 13.08 | 2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)- | 758 | mainlib | C16H14O4 |

**Figure 48** Mass spectrum of ETP at Rt 255.09

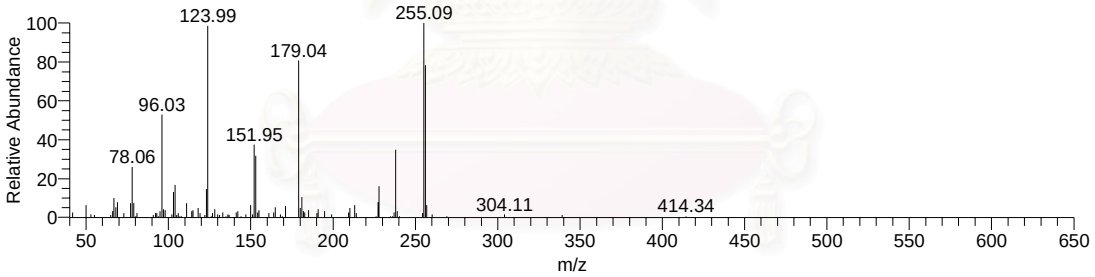


**Table 11** Chemical constituents of ETP at Rt 255.09

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 14.66 | 2-Methylaminomethyl-5-nitrobenzophenone | 334 | replib | C14H12N2O3 |
| 14.66 | Indolo[2,3-a]quinolizin-2-ol, 1,2,3,4,6,7,12,12b-octahydro-2-methyl-, trans- | 345 | mainlib | C16H20N2O |
| 14.66 | 4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)- | 725 | mainlib | C15H12O4 |

**Figure 49** Mass spectrum of ETP at Rt 16.68

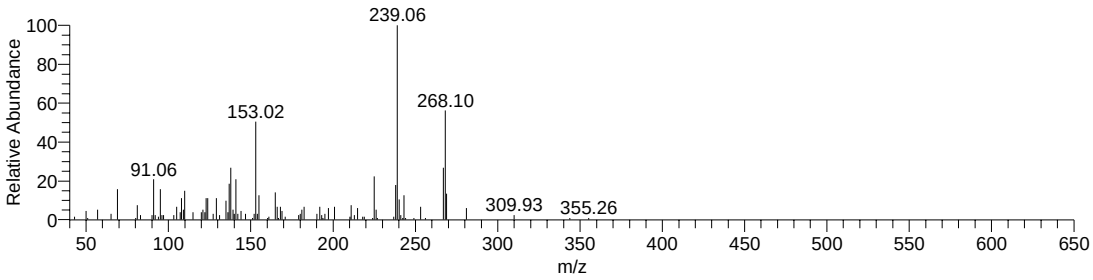


**Table 12** Chemical constituents of ETP at Rt 16.68

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 16.68 | p-Anisaldehyde, azine | 349 | mainlib | C16H16N2O2 |
| 16.68 | 4H-1-Benzopyran-4-one, 3-hydroxy-7-methoxy-2-phenyl- | 388 | replib | C16H12O4 |
| 16.68 | 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl- | 635 | mainlib | C16H12O4 |

**Figure 50** Mass spectrum of ETP at Rt 18.33

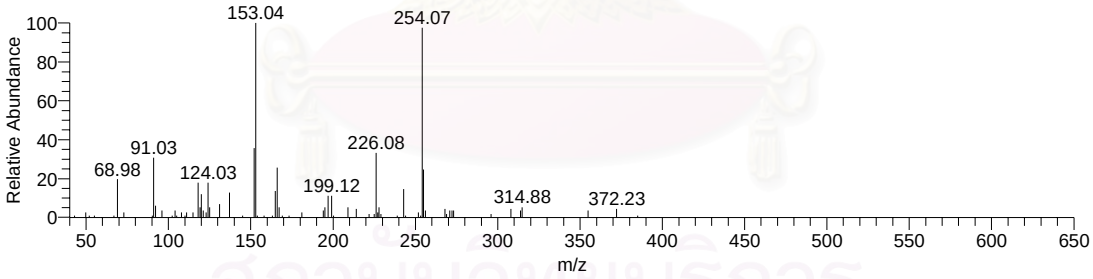


**Table 13** Chemical constituents of ETP at Rt 18.33

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 18.33 | Chrysin | 450 | replib | C15H10O4 |
| 18.33 | Chrysin | 486 | replib | C15H10O4 |
| 18.33 | [1,1'-Biphenyl]-4-carboxylic acid, 2',4'-dimethyl-, ethyl ester | 488 | mainlib | C17H18O2 |

**Figure 51** Mass spectrum of ETP at Rt 19.40

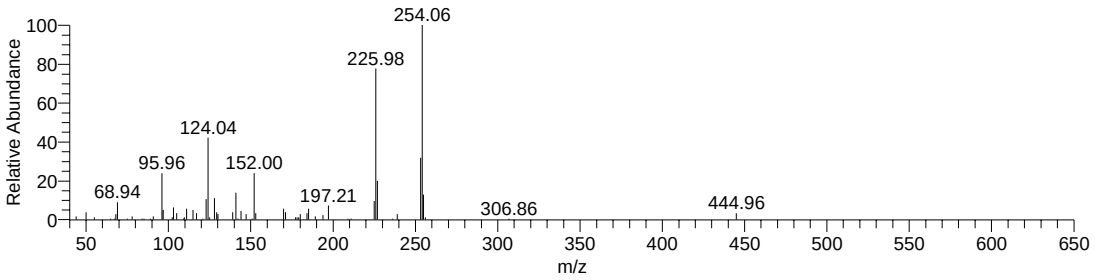


**Table 14** Chemical constituents of ETP at Rt 19.40

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 19.40 | 4H-1-Benzopyran-4-one, 5,7-dihydroxy-3-phenyl- | 518 | mainlib | C15H10O4 |
| 19.40 | Chrysin | 726 | replib | C15H10O4 |
| 19.40 | Chrysin | 734 | mainlib | C15H10O4 |

**Figure 42** Mass spectrum of ETP at Rt 20.79

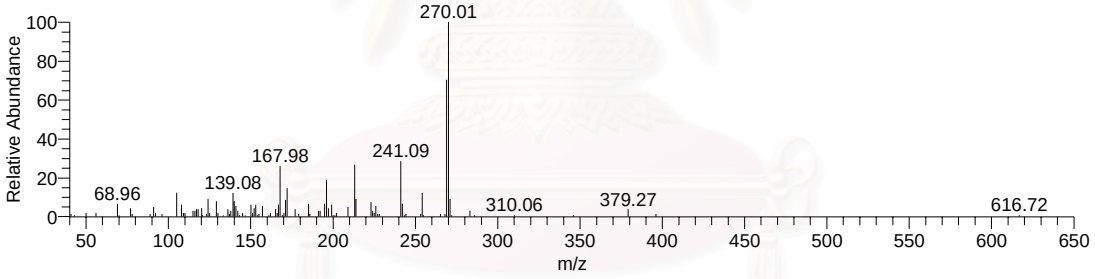


**Table 15** Chemical constituents of ETP at Rt 20.79

| RT | Name | SI | Library | Formula |
|---|---|---|---|---|
| 20.79 | 9,10-Anthracenedione, 1,5-dihydroxy-3-(hydroxymethyl)- | 401 | mainlib | C15H10O5 |
| 20.79 | 9,10-Anthracenedione, 1,8-dihydroxy-3-(hydroxymethyl)- | 470 | mainlib | C15H10O5 |
| 20.79 | 4H-1-Benzopyran-4-one, 3,5,7-trihydroxy-2-phenyl- | 605 | mainlib | C15H10O5 |

# VITAE

Mrs. Rattana Chonthong was born in January 18, 1972 in Lampang, Thailand. She graduated with a Bachelor of Nurse in 1995 from Chieangmai University. After graduation, she worked in Onco-gyn unit of Maharacha Nakorn Chieangmai Hospital for 5th years. Now she currently is working in Med-Surg unit of Srivichai 3 Hospital, Samuthsakorn, Thailand.