ฤทธิ์ในการเป็นยาชาเฉพาะที่ของสารสกัดเพลลิโทรีนจากลูกชะพลู

นางสาว ปิยลักษณ์ ทรัพย์ประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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LOCAL ANESTHETIC EFFECT OF PELLITORINE, AN ACTIVE COMPOUND ISOLATED FROM CHA-PLU FRUIT (*Piper sarmentosum* Roxb.)

Miss Piyaluk Sapprasert

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ปิยลักษณ์ ทรัพย์ประเสริฐ : ฤทธิ์ในการเป็นยาชาเฉพาะที่ของสารสกัดเพลลิโทรีนจากลูกชะพลู
LOCAL ANESTHETIC EFFECT OF PELLITORINE, AN ACTIVE COMPOUND ISOLATED FROM CHA-PLU FRUIT (*Piper sarmentosum* Roxb.) อาจารย์ที่ปรึกษา : รศ.ดร. บุญยงค์ ตันติสิระ, อาจารย์ที่ปรึกษาร่วม : รศ.ดร. มยุรี ตันติสิระ , 77 หน้า. ISBN 974-17-4029-8

การวิจัยนี้มีจุดมุ่งหมายเพื่อศึกษาฤทธิ์ในการเป็นยาชาเฉพาะที่ของสารสกัดจากลูกชะพลู ซึ่งมีเพลลิโทรีนเป็นส่วนประกอบหลัก โดยได้ทำการศึกษาบนเส้นประสาทไซติกของกบที่ตัดออกจากตัว รวมถึงการศึกษาโดยวัดการสะบัดหางหนีในหนูถีบจักร และวัดการกระตุกของผิวหนังในหนูตะเภา

สารทดสอบซึ่งละลายในไฮดรอกซีโพรพิลเบตาไซโคลเดรกตรินขนาดความเข้มข้น 0.1 โมลาร์ ในสารละลายริงเกอร์ ที่หยดลงไปบนเส้นประสาทไซติกของกบ พบว่าเพลลิโทรีนขนาดความเข้มข้นตั้งแต่ 10 - 25 มิลลิโมลาร์ สามารถออกฤทธิ์ยับยั้งการส่งผ่านกระแสประสาทชนิดผันกลับได้อันเกิดจากการกระตุ้นเส้นประสาทไซติกของกบที่ตัดออกจากตัวด้วยขนาดของกระแสไฟฟ้าที่สูงกว่าขนาดที่ทำให้เกิดการตอบสนองสูงสุด และพบว่าการออกฤทธิ์นี้จะเพิ่มขึ้นตามขนาดของความเข้มข้นที่เพิ่มขึ้น การสกัดกั้นการส่งผ่านกระแสประสาทอย่างสมบูรณ์จากการกระตุ้นด้วยไฟฟ้า โดยเพลลิโทรีนที่ขนาดความเข้มข้น 25 มิลลิโมลาร์และ ลิโดเคนที่ขนาดความเข้มข้น 30 มิลลิโมลาร์ ใช้ระยะเวลาทั้งสิ้น 73.75 $\pm$ 2.26 นาที และ 7.50 $\pm$ 0.94 นาที ตามลำดับ การศึกษาต่อมาด้วยอนาจีเซียมิเตอร์ โดยวัดการสะบัดหางหนีของหนูถีบจักรซึ่งได้รับสารทดสอบที่บริเวณโคนหาง พบว่าความเข้มข้นของเพลลิโทรีนและลิโดเคนที่ทำให้เกิดอาการชา ในสัตว์ทดลองจำนวนครึ่งหนึ่ง (EC_{50}) เท่ากับ 16 และ 12 มิลลิโมลาร์ ตามลำดับ การตรวจสอบต่อมาโดยการวัดการกระตุกของผิวหนังในหนูตะเภา ที่ได้รับสารทดสอบเข้าใต้ผิวหนังบริเวณหลังของหนูตะเภา ในชั้นอินตราเดอร์มอล แล้ววัดการตอบสนองต่อความรู้สึกเจ็บปวด เมื่อถูกจิ้มด้วยเข็ม พบว่าเพลลิโทรีนขนาดความเข้มข้นตั้งแต่ 3 - 14 มิลลิโมลาร์ มีผลเหมือนกับลิโดเคนที่ขนาดความเข้มข้นตั้งแต่ 4 - 16 มิลลิโมลาร์ โดยสามารถระงับความรู้สึกได้นานประมาณ 30-50 นาที

จากผลการทดลองสามารถสรุปได้ว่า เพลลิโทรีนสามารถออกฤทธิ์ในการเป็นยาชาเฉพาะที่ได้เช่นเดียวกับลิโดเคนโดยการทดสอบทั้งในกายและนอกกาย ซึ่งพบว่าอัตราการยับยั้งการส่งผ่านกระแสประสาทโดยเพลลิโทรีนและลิโดเคนบนเส้นประสาทไซติกของกบนั้น จะเพิ่มขึ้นเมื่อใช้สารละลายริงเกอร์ซึ่งมีการพร่องของโซเดียมในการหล่อเลี้ยงเส้นประสาท ทำให้สามารถสันนิษฐานได้ว่าสารทั้งสองชนิดนี้ อาจจะมีกลไกการออกฤทธิ์ที่คล้ายคลึงกัน การศึกษาถึงผลของเพลลิโทรีนต่อโซเดียมแชนแนล เป็นสิ่งที่น่าจะนำมาศึกษาต่อไป เพื่อหากลไกการออกฤทธิ์ในการเป็นยาชาเฉพาะที่ รวมถึงการทดสอบทางพิษวิทยา ที่อาจนำมาซึ่งการค้นพบยาชาเฉพาะที่ ที่มีที่มาจากธรรมชาติ

ภาควิชาสหสาขาสรีรวิทยา ลายมือชื่อนิสิต.....
สาขาวิชาสรีรวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD: PELLITORINE / LOCAL ANESTHETIC / TAIL FLICK TEST / SCIATIC NERVE / SKIN TWITCH TEST

PIYALUK SAPPRASERT: LOCAL ANESTHETIC EFFECT OF PELLITORINE, AN ACTIVE COMPOUND ISOLATED FROM CHA-PLU FRUIT (*Piper sarmentosum* Roxb.) THESIS ADVISOR: ASSOC.PROF.BOONYONG TANTISIRA, Ph.D., CO-ADVISOR: ASSOC.PROF.MAYUREE TANTISIRA, Ph.D., 77 pp. ISBN 974-17-4029-8.

The present study aimed to investigate local anesthetic effect of a semi-purified compound, containing mainly pellitorine, isolated from *Piper sarmentosum* Roxb. on isolated frog sciatic nerves. In addition, *in vivo* effect of the test compound was further investigated in mouse tail flick test and twitch - response test in guinea pig's skin.

The test compound was dissolved in 0.1 M hydroxypropyl-β-cyclodextrin in Ringer's solution and being applied onto frog sciatic nerve. It was found that pellitorine in the concentration of 10 - 25 mM exerted a concentration-dependent manner and reversible blockade of supramaximally electrical-evoked action potential of isolated frog sciatic nerves. Complete block electrical-evoked action potential exerted by 25 mM of pellitorine and 30 mM of lidocaine was achieved at 73.75 ± 2.26 min and 7.50 ± 0.94 min respectively. Further investigation in mouse tail flick test, using analgesia meter, revealed *in vivo* local anesthetic effect of test substances which was injected into mouse's tail base. The EC_{50} of pellitorine and lidocaine in this model were found to be 16 and 12 mM, respectively. This observation was subsequently confirmed by twitch response in which the test substances were infiltrated intradermally on the back of guinea pig and pricking was used to probe for response to pain. Apparently, pellitorine (3 - 14 mM) as well as lidocaine (4 – 16 mM), elicited analgesic effect with the duration of sensory block of about 30 – 50 min.

Based on the results obtained it can be concluded that pellitorine possesses *in vitro* as well as *in vivo* local anesthetic effects. The finding that the degrees of conduction block elicited by pellitorine or lidocaine on frog sciatic nerve were markedly increased in sodium deficient Ringer's solution, suggesting the similar mode of action of theses two compounds. Further investigation on the effect of pellitorine on sodium channel is required to elucidate the mechanism of local anesthetic observed. In addition toxicity which may arise should also be evaluated and that may lead to a discovery of a local anesthetic from natural source.

Inter-Departmental Program in Physiology	Student's signature Peeluk Sapprasert
Field of study Physiology	Advisor's signature Boonyong Tantisira
Academic year 2003	Co-advisor's signature Mayuree Tantisira

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List of Abbreviations

α	=	alpha
β	=	Beta
γ	=	gamma
δ	=	delta
θ	=	Theta
μl	=	microlitre
%	=	Percent
^{o}C	=	degree celcius
A	=	ampere
AUC	=	area under the curve
a.m.	=	ante meridian (before noon)
BCD	=	β-cyclodextrin
CNS	=	Central nervous system
EC_{50}	=	median effective concentration
e.g.	=	Exampli gratia (for example)
et al.	=	et alii (and other)
g	=	gram
^{1}H-NMR	=	Protron Nuclear Magnetic Resonance
J	=	joule
HPBCD	=	Hydroxypropyl-β-cyclodextrin
M	=	Molar
min	=	minute

List of Abbreviations (Continued)

ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
m-osmole	=	milliosmole
%MPE	=	percentage of the maximum possible effect
Na^+	=	Sodium ion
NSS	=	Normal saline solution
PEG400	=	Polyethylene glycol 400
p.m.	=	post meridian (after noon)
S.E.M.	=	standard error of the mean
TLC	=	Thin-Layer Chromatography
UV	=	Ultra Violet

Chapter I

Introduction

If one assumes that early humans had basically the same anatomy and behavior as their twentieth century counterparts, with perhaps a few external changes, it seems equally safe to assume that experiencing pain has always been a reality for humans, and seeking relief from that pain has always been a natural response (Prithvi, 1996).

Modern-day observation of primitive societies provides some clues about prehistoric mankind's reaction to pain. In these primitive societies, religion, magic, and medical treatment are inseparable for the treatment of ailments. Primitive peoples seem to be psychologically prepared for the effectiveness of magic as a healing force, even when modern medicine is introduced into their society. These and other observations indicate that in prehistoric times treatment of both the physical and spiritual aspects of pain and illness might have included ritual activity, medicinal plants, physical manipulation, and the application of heat, cold, or friction (Prithvi, 1996).

Development of pain management methods

Compression anesthesia

During the sixteenth century, the French surgeon Ambroise Parè was searching for a way to reduce the pain of surgery. He reported that when a firm ligation was made above the seat of the operation, bleeding would be better controlled, and pain would be greatly diminished (Liljestrand, 1971).

Refrigeration anesthesia

In 1661 Thomas Bartholin reported technique of rubbing snow and ice on the site of surgical incisions to deaden the pain. Bartholin cautioned against gangrene, however, and recommended that ice be applied only for a quarter of an hour (Liljestrand, 1971).

Morphine

Knowledge of the effects of opium dates back at least to 4000 BC to the ancient Sumerians. Arabian physicians were well versed in the uses of opium: Arabian traders introduced the drug to the orient, where it was employed mainly for the control of dysenteries. In 1803, the German pharmacist Serturner isolated the opium alkaloid that he named it morphine. It is morphine that gives opium its analgesic activity. In addition to the remarkable beneficial effects of opiods, the toxic side effects and additive potential of these drugs also have been known for centuries (Prithvi, 1996).

General anesthesia

In 1844, Boston dentist, William T G. Morton made a dramatic public demonstration of ether inhalation's effectiveness as a general anesthetic that could permit surgery without pain. However, ether fell out of favor because of its irritating properties and long induction period. One year later, chloroform replaced ether as the agent of choice until it became associated with long-term liver damage and sudden death (Lyons, 1978).

Surgery

A number of surgeons throughout the world began to attack pain by a new method: the permanent interruption of the central nervous system's afferent pathways (Prithvi, 1996).

Local anesthetic

The first local anesthetic discovered was cocaine, an alkaloid contained in large amounts in the leaves of *Erythroxylon* coca, a plant growing in the Andes Mountains. The native mountain dwellers have chewed the dried leaves of the coca shrub for its fatigue-chasing and mood-elevating effects. When the scientific expeditions to South America brought the coca shrub back to Europe, where chemists set to work to isolate the active principle from the leaves. Success was close when, in 1860, Albert Niemann isolated cocaine from erythroxylin extract; he noted that it had a bitter taste and produced a peculiar effect on the tongue, making it numb and almost devoid of

sensation (de Jong, 1994). The clinical used of cocaine in ophthalmology was initiated by Karl Koller (Koller, 1884). A year later, Halted found that cocaine blocked nerve transmission when being applied to the nerve trunk, laying the foundation for neural blockade (Halted, 1885).

Great through cocaine's benefits were, indiscriminate use coupled with high toxicity and addictive potential led to a growing recognition of its hazards and shortcomings that limits its legitimate medical uses. This led to an intensive search for less toxic substitutes for cocaine. Elucidation of the chemical structure of cocaine in 1895 was major step forward and aided chemists in synthesizing new drugs with local anesthetic activity. At last, Einhorn succeeded in synthesizing procaine in 1904 so, it could say that procaine was the first synthetic local anesthetic of ester derivative. Despite some shortcomings of its own, procaine is still used extensively and remains the standard for comparison of new local anesthetics that modified from procaine (de Jong, 1994). Departing from the ester structure of cocaine and its analogs, Löfgren investigated a series of aniline derivatives. His discovery of lidocaine in 1948 properly marks a new epoch in local anesthesia (Löfgren, 1948).

Definition of Local anesthetic

Local anesthetics are drugs that block nerve conduction when being applied locally to nerve tissue. With progressive increases of its concentrations, the transmission of autonomic, somatic sensory and somatic motor impulse is interrupted, producing autonomic nervous system blockade, sensory anesthesia, and skeletal muscle paralysis in the area innervated by the affected nerve. More than that, removal of the local anesthetic is followed by spontaneous and complete return of nerve conduction, with no evidence of structural damage to nerve fibers or cells (Hardman, 1996).

Classification of local anesthetic drugs

Local anesthetic drugs can be divided into two groups

1. Esters group: The esters are primarily of the para-aminobenzoic acid type and include chlorprocaine, procaine, propoxycaine and tetracaine.

2. Amides group: The amide or anilides that include lidocaine, mepivacaine, bupivacaine and prilocaine.

Local anesthetics with an ester linkage such as procaine and amide linkage such as lidocaine differ significantly in hypersensitivity, metabolism, and duration of action. Hypersensitivity seems to occur most prominently in response to local anesthetics of the ester-type and frequently extends to chemically related compounds. Allergic reactions to the amide type are extremely rare, and substitution of such amide-type, compounds to avoid allergic responses is usually possible (de Jong, 1994).

Toxicity of local anesthetic drug

The toxicity of local anesthetic drug depends largely on the balance between their rate of absorption and destruction. Most toxic reactions occur after the accidental intravascular injection of a large dose of local anesthetic, although an excessive dose given into an appropriate anatomic location can lead to systemic toxicity (Longnecker, 1997).

Systemic toxicity

When mistaken injected a large dose of local anesthetic into an artery or vein, the anesthetic appears in the blood as a bolus, a catastrophic combination of seizures, coma, arrhythmias and cardiac arrest (Longnecker, 1997).

Central nervous system toxicity

Low plasma concentrations of local anesthetics are likely to produce numbness of the tongue and circumoral tissues, presumably reflecting delivery of drug to these highly vascular tissues. As the plasma concentrations continue to increase, local anesthetics readily cross the blood-brain barrier and produce a predictable pattern of CNS changes. Restlessness, vertigo, tinnitus, and difficultly in focusing occur initially. Further increases in the CNS concentration of local anesthetic result in slurred speech and skeletal muscle twitching. Skeletal muscle twitching is often first evident in the face and extremities and signals the imminence of tonic - clonic seizures (Stoelting, 1999).

Cardiovascular toxicity

Direct effects on the cardiovascular system depend on dose and include myocardial depression, vasodilatation, and impair cardiac conduction because local anesthetics inhibit depolarization in cardiac muscle by preventing sodium conduction through the sodium channels (Longnecker, 1997).

Metabolism of local anesthetic drugs

The ester-type local anesthetic appears to be hydrolyzed by both liver esterase and plasma esterase. On the other hand, amide-type local anesthetics are degraded by hepatic microsomes; the initial reactions involve N-dealkylation and subsequent hydrolysis. Consequently, the amide-type local anesthetics usually have a longer duration of action than the ester type (Table 1).

Classification	Potency	Onset	Duration after infiltration (mins)
Esters			
Procaine	1	Slow	45 – 60
Chloroprocaine	4	Rapid	30 – 45
Tetracaine	16	Slow	60 – 180
Amides			
Lidocaine	1	Rapid	60 – 120
Prilocaine	1	Slow	60 – 120
Bupivacaine	4	Slow	240 - 480

Table 1. Comparative pharmacology of local anesthetics (From Stoelting, 1999).

The action of local anesthetics on the cell membrane

The primary action of local anesthetic is on the cell membrane of the axon, on which it produces electrical stabilization. The large transient increase in permeability to sodium ions, necessary for propagation of the impulse, is prevented, thus the resting potential is maintained and depolarization in response to stimulation is inhibited. Initially the threshold for electrical excitation is raised, the rate of rise of the action potential reduced, and conduction slowed, eventually propagation of the impulse fails (de Jong, 1994).

Mechanism of action

Local anesthetics prevent transmission of nerve impulse by inhibiting passage of sodium ions through ion-selective sodium channels in nerve membranes (Butterworth and Strichartz, 1990). The sodium channel itself is a specific receptor for local anesthetic molecules. Occlusion of open sodium channels by local anesthetic molecules contributes little to overall inhibition of sodium permeability. Thus local anesthetics increase the

threshold for electrical excitation in the nerve, slow the propagation of the impulse, reduce the rate of rise of the action potential, and eventually block the conduction. Several theories have been postulated over the years as to the exact mechanism of local anesthetics (Prithvi, 1996).

Presently, the most popular theories are a combination of the receptor (Hille, 1977) and membrane expansion theories (Seeman, 1975) which explaining molecular nature of local anesthetics that can block sodium conductance.

1) Receptor theory: This theory suggested that local anesthetic act directly on receptors within the sodium channels. This action accounts for about 90 percent of the nerve blocking effect of amide local anesthetics (e.g. Lidocaine) acting in cationic form. Quaternary derivatives of lidocaine, being fully ionized, cannot penetrate the cell membrane, and produce nerve block only being applied to its inner surface. The work of Hille suggested that the receptor was within the sodium channel (Hille, 1977). An amide local anesthetic can gain access either via the lipophilic pathway directly across the lipid membrane or via the axoplasmic opening (Narahashi, 1970).

2) Membrane expansion theory: This theory also accounted for conduction blockade. Benzocaine and benzylalcohol are unionzed compounds possessing local anesthetic activity. The blockade they produce is reversible by increased atmospheric pressures, whereas the block produced by conventional local anesthetics is only partially reversed. Benzocaine and benzyl alcohol inhibiting sodium influx and blocking nerve conduction by expanding or altering the configuration of the nerve membrane, thus decreasing the diameter of the sodium channel (Seeman, 1975)

Sodium channels

Binding affinities of local anesthetics to the sodium ion channels are stereo specific and depend on the conformational state of the sodium channel (Lee-Son, 1992). Sodium channels exist in activated-open, inactivated-closed, and rested-closed states during various phases of the action potential. In the resting nerve membrane, sodium channels are

distributed in equilibrium between the rested-closed and inactivated-closed states. By selectively binding to sodium channels in inactivated-closed states, local anesthetic molecules stabilize these channels in this configuration and prevent their change to the rested-closed and activated-open states in response to nerve impulses. Sodium channels in the inactivated-closed state are not permeable to sodium and thus conduction of nerve impulses in the form of propagated action potentials cannot occur. It is speculated that local anesthetics bind to specific sites located on the inner portion of sodium channels (internal gate or H gate) (Figure1) as well as obstructing sodium channels near their external openings to maintain these channels in inactivated-closed states (Butterworth and Strichartz, 1990)

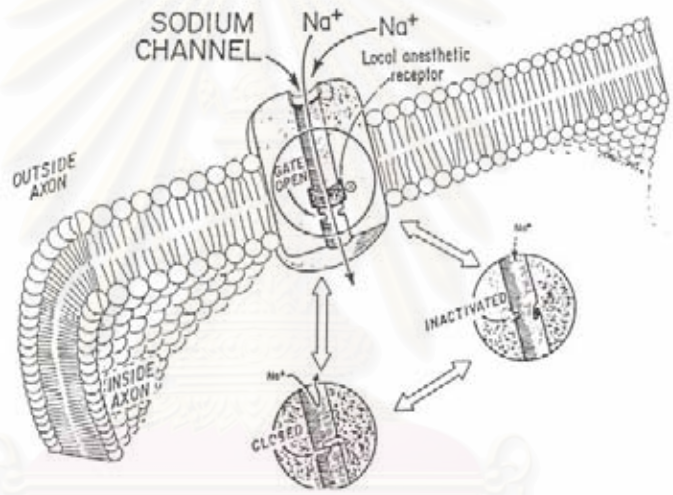


Figure 1. Diagrammatic of sodium channel in the nerve (From Savarese, 1986).

Regional Anesthesia

Regional anesthesia is classified according to the following six sites of placement of the local anesthesia solution (Stoelting, 1999):

1. Topical or surface anesthesia: Local anesthetics are used to produce topical anesthesia by applying the drug directly onto the mucous membrane of nose, mouth, tracheobronchial tree, or genitourinary tract.

2. Local infiltration: Local infiltration anesthesia involves extra vascular placement of local anesthetic in area to be anesthetized. Subcutaneous injection is an example.
3. Peripheral nerve block: Peripheral nerve block anesthesia is achieved by injection of local anesthetic solutions into tissues surrounding individual peripheral nerves or nerve plexuses.

4. IV regional anesthesia (Bier block): The injection of a local anesthetic solution into an extremity isolated from the rest of the systemic circulation by a tourniquet produces a rapid onset of anesthesia.

5. Epidural anesthesia: Epidural anesthesia solutions placed in the epidural or sacral caudal space produce epidural anesthesia by two presumed mechanisms. First, local anesthetic diffuses across the dura to act on nerve roots and the spinal cord as it does when injected directly into the lumbar subarachnoid space to produce spinal anesthesia. Second, local anesthetic also diffuses into the paravertebral area through the intervertebral foramina, producing mulitiple paravertebral nerve blocks.

6. Spinal (subarachnoid) anesthesia: Spinal anesthesia is produced by injection of local anesthetic solutions into the lumbar subarachnoid space.

	Topical anesthesia	Local infiltration	Peripheral nerve block	Intravenous regional	Epidural anesthesia	Spinal anesthesia
Procaine	No	Yes	Yes	No	No	Yes
Chloroprocaine	No	Yes	Yes	No	Yes	No
Tetracaine	Yes	No	No	No	No	Yes
Lidocaine	Yes	Yes	Yes	Yes	Yes	Yes (?)
Etiodocaine	No	Yes	Yes	No	Yes	No
Prilocaine	No	Yes	Yes	Yes	Yes	No
Mepivacaine	No	Yes	Yes	No	Yes	No
Bupivacaine	No	Yes	Yes	No	Yes	Yes
Ropivacaine	No	Yes	Yes	No	Yes	Yes
Pramaxine	Yes	No	No	No	No	No
Dyclonine	Yes	No	No	No	No	No
Hexylcaine	Yes	No	No	No	No	No
Piperocaine	Yes	No	No	No	No	No

Table 2. Use of local anesthetics to produce regional anesthesia (From Stoelting, 1999).

Amino ester Agents of local anesthetic drug

Procaine

Procaine, an ester group of local anesthetic, is rarely used at present for peripheral nerve or extradural blocks because of its low potency, slow onset, and relatively short duration of action. Although the potential for systemic toxic reactions is quite small with procaine, this agent can cause allergic-type reactions. Currently procaine is used mainly for on filtration anesthesia and differential spinal blocks in chronic pain patients (Cousins, 1998).

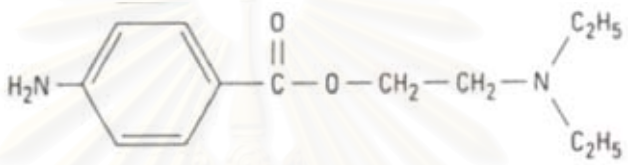


Figure 2. Molecular structure of procaine (From de Jong, 1994).

Tetracaine

Tetracaine, an ester group of local anesthetic, remains a popular drug for spinal anesthesia in the United States. Its provides a relatively rapid onset of spinal anesthesia, -about 3 to 5 minutes- a profound depth of anesthesia, and duration of 2 to 3 hours. Tetracaine is rarely used for other forms of regional anesthesia because of its extremely slow onset of action and the potential for systemic toxic reactions (Cousins, 1998).

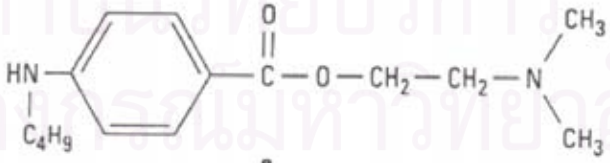


Figure 3. Molecular structure of tetracaine (From de Jong, 1994).

Amino Amide Agents of local anesthetic drug

Lidocaine

Lidocaine is an amide-linked local anesthetic of moderate potency and duration but of good penetrative power and rapid onset of action. It is effective by all routes of administration. Lidocaine is also used in ointment, jelly, and viscous and aerosol preparations for a variety of topical anesthetic procedures (Cousins, 1998). Furthermore, lidocaine has found additional application in the acute prophylaxis and treatment of cardiac arrhythmias. It stabilizes the membrane of damaged and excitable cells. Over dosage of lidocaine produces death from ventricular fibrillation. Side effects on CNS include sleepiness, dizziness, paresthesias, altered mental status, coma, and seizures (Longnecker, 1997).

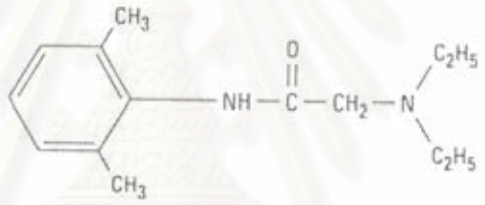


Figure 4. Molecular structure of lidocaine (From de Jong, 1994).

Mepivacaine

Mepivacaine is similar to lidocaine in terms of its anesthetic profile. It can produce a profound depth of anesthesia with a relatively onset and a moderate duration of action. This agent may be used for infiltration, peripheral nerve block, and epidural anesthesia. Differences do exist between mepivacaine and lidocaine; Mepivacaine is not effective as a topical anesthetic agent and thus is less versatile than lidocaine (Cousins, 1998).

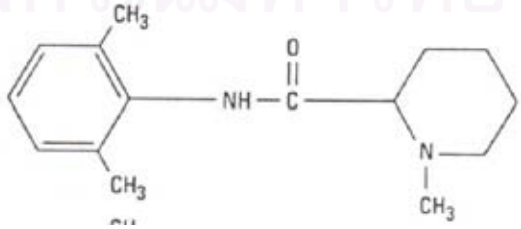


Figure 5. Molecular structure of mepivacaine (From de Jong, 1994).

Preclinical evaluation of local anesthetic activity in animal experiments

In order to exert local anesthetic activity, a compound must contact a nerve somewhat along its course, i.e. dendrites, cell body or axon. The result is a cessation of nerve impulse conduction. General considerations of local anesthetic activity are generally distinguished between conduction anesthesia, infiltration anesthesia, surface anesthesia and special pharmacological tests have been developed for each of these (Vogel, 1997).

The clinical use of local anesthetic agents requires that they are active topically, i.e. on mucous membrane or abraded skin, or by injection either subcutaneously for local action or for deposition along the axon at some site remote from the operative field to provide regional blockade of nerve conduction. The effect of drugs can be detected by showing the blockage in conduction electrically or by observing the disappearance of a reflex or in man, by observing the disappearance of sensation.

By far the largest numbers of reports have dealt with the evaluation of compounds for their local anesthetic activity. There are a wide variety of procedures that have been used in a number of different species including:

Frog

Much of the earliest work in evaluating local anesthetic activity was done using frogs (Harschfelder, 1932). Experimentation with the frog sciatic nerve is an excellent technique to study the action potentials, a principle mechanism of signal transduction which is responsible for the principle functioning of an animal or human, since 1924 until recordings from the squid giant fibers replaced it. This experiment deals with the electrical manipulation of sciatic nerve removed from frog and kept moist with frog Ringer solution. This method involving in sending a stimulating voltage through a piece of sciatic nerve that mounted in a nerve chamber and viewing the nerve reaction on an oscilloscope of electrical stimulation. Compound action potentials are then propagated in the nerve afterwards the local anesthetic was applied to the nerve. The nerve conduction block was evaluated by percent change in the height of action potential (Vogel, 1997).

Dog

Some of the first experiments done with local anesthetic agents were done in dogs. They study the effects of compounds on regional nerve transmission by direct instillation. If the concentration is sufficiently large, there is an abrupt loss of response to painful stimuli, such as pinching the skin with a hemostat or puncturing the skin with a hypodermic needle. The results of a successful administration of local anesthetic were the absence of a skin-twitch response to pinching and there was no contraction of the anal sphincter in response to stroking the quadrants of the anal margin (Dvorak and Manson, 1930).

Rabbit

Rabbit has been widely employed in the evaluation of the local anesthetic activity because it is a relatively docile animal and usually responses in a characteristic way to a painful stimulus. A number of investigators have used drug application to the rabbit cornea as a mean of evaluating topical activity on mucous membranes. The solution of the anesthetic was applied into the conjunctival sac. Effective local anesthetics extinguish the corneal reflex (blinking) elicited by any touch of the cornea.

Bieter et al in 1954 pointed out a number of advantages of the rabbit for the evaluation of an agent as a spinal anesthetic. Local anesthetic was applied on a lumbosacral junction. Evaluation in the level of anesthesia by electrically stimulated the skin and recorded the change in amplitude and rate of respiration. Pain caused the amplitude or both the amplitude and rate of respiration to increase (Bieter et al., 1954).

Guinea pig

The guinea pig has been the animal, which has been most widely employed in the evaluation of local anesthetic activity. As mentioned by Chanee and Lobstein, this is probably due to the fact that they are more sensitive than the rabbit is, so that there is a greater likelihood of detecting compounds which are only weakly active. When applied anesthetic on the cornea, effective local anesthetic extinguish the cornea reflex elicited by any attach of the cornea such as a horse hair (Chanee and Lobstein, 1944).

Probably the most widely employed test for local anesthetic activity in the guinea pig is that firstly described by Bulbring and Wajda. Two areas, with diameters of about 4 to 5 cm on the back of the guinea pig, were shaved on the day of the experiment. Saline, 0.25 ml, was injected intracutaneously and the size of the wheal was marked with ink. After observing the animal' s reaction to a pinprick in some other area of the body, pricks were administered inside the wheal at 3 to 5 second intervals in set of six every 5 minutes for 30 minutes, i.e. a total of thirty-six stimuli. The number of response failures gives an index of the degree of anesthesia (Bulbring and Wajda, 1945). The result is only a comparison of the rates of onset anesthesia. Like the results obtained with the frog's sciatic nerve, these do not indicate differences in the duration or intensity of the effect. With the corneal reflex they are moreover complicated by the failure of certain drugs to cross mucous membranes. With the twitch response test in a guinea pig' s skin, an attempt is made to assess the duration and intensity of the effects, through it ignores differences in the rate of onset and in ability to cross mucous surfaces (Mongar, 1955).

Mouse

The radiant heat method, which is use for evaluation of systemic analgesic activity, can also be used for determination of conduction anesthesia. The tail flick test was the classical method to determine the conduction anesthesia by injecting the local anesthetic into the root of the tail. The tail flick test, first described by D'Amour and Smith in 1941, measures the latency of tail flick in response to a focused thermal stimulus. It has been extensively used in mice to evaluate the antinociceptive effect of various drugs given systematically (D'Amour and Smith, 1941).

This method applied by Vogel in 1963, Group of 10 mice with weight between 18 - 22 gm. is used for each dose. Before administration of the test compound or the standard the normal reaction time is determined. The animal is placed into a restrainer with an opening for the tail at the rear wall. The tail is hold gently by the investigator. By opening the shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. For about 4 seconds the reaction of the animal is observed. Moreover, to prevent tissue

damage the heat source is terminated after 4 seconds. The mouse tries to pull the tail away. Tail flick reflex was the endpoint of this test (Vogel, 1997).

Local anesthetic activity of medicinal plants

Local anesthetic activity of medicinal plant was investigated by researchers in many countries in search for new local anesthetic drugs.

Coca (*Erythroxylon coca*)

Coca leaves were highly valued by the natives long before the Spanish conquest, the tree being known as “The Divine Plant of the Incas.” The natives chew the leaf, either as such or mixed with lime, and are thus able to travel great distances, often with heavy loads, without experiencing fatigue and without any but the most meager food rations. Cocaine was first isolated from coca leaves in 1860, but until 1884, Koller discovered its local anesthetic properties (Manuchair, 2001).

***Lavandula angustifolia* Mill**

Local anesthetic effect of the essential oil obtained from *Lavandula angustifolia* Mill was evaluated *in vivo* in the rabbit conjunctival reflex test. Treatment with a solution of essential oil of *L.angustifolia* allowed a dose-dependent increase in the number of stimuli necessary to provoke the reflex. *In vitro*, in rat phrenic nerve hemidiapharagm preparation in a dose-dependent manner, the electrically evoked contractions of rat phrenic-hemidiaphargm.,thus confirming the local anesthetic activity in the essential oil of *Lavandula angustifolia* Mill (Ghelardini et al.,1999).

Todopon Puok

The lignans of (+)-pinoresinol was isolated from Todopon Puok (*Fagraea racemosa* Jack ex Wall), a medicinal plant from Borneo, using a bioassay of the relaxation effect on norepinephrine (NE)-induced contraction in rat aortic strips. This plant extract exhibited analgesic effect on writhing symptoms in mice, which were dose-dependent, and produced local anesthetic in guinea pigs (Okuyama et al., 1995).

Turmeric

Tumeric (*Curcuma longa* Linn.) was found to exert the local anesthetic effect by blocking effect on the action potential of isolated frog sciatic nerve (Prucksunand et al., 1997).

Copaiba

Copaiba (*Copaifera jacquinii*), found mainly in tropical South America, was used as traditional medicines. The part of the plant that is often employed medicinally is the oleoresin that accumulates in cavities within the tree trunk. The copaiba resin are attributed to a group of phytochemicals but a large amount of this chemicals are caryphyllene that demonstrating dose-dependent local anesthetic properties in an *in vivo* of rabbit conjuntival reflex test (Ghelardini et al., 2001).

Cha-plu

Cha-plu (*Piper sarmentosum* Roxb.) is an edible shrub indigenous to Southeast Asian countries and has been used for medical purpose for a long time. Different parts of this plants are use for various purpose such as leaves and root are used for treatment of toothache, fungoid dermatitis on the feet, coughing and asthma by the people of Indonesia and Malaysia (Perry, 1981).

Figure 6. Photograph of Cha-plu (*Piper sarmentosum* Roxb.).

In Thailand, the plant and fruits of Cha-plu are used as an expectorant (Pongboonrod, 1976). Pongmarutai (1980) reported that Cha-plu exhibited *in vitro* activity of a reduction of blood sugar in alloxan diabetic rabbits. In 1987, a number of compounds were isolated from fruits of Cha-plu and some of them were found to exert a potent local anesthetic effect when being applied on human's lip and tongue (Likhitwitayawuid, 1987). In 1999, a semipurified compound isolated from fruits of Cha-plu was found to exert a local anesthetic activity on isolated frog sciatic nerve preparation. Since pellitorine is the principle consistent in the compound tested, thus, it is suggestive that pellitorine was responsible for the local anesthetic effect observed (Tantisira et al., 1999). Because of insolubility of the test compound in aqueous solution, it was dissolved in 70%Polyethylene glycol (PEG 400) in Frog Ringer' s solution, Later on it was toted that the solvent system interfered a blockade of electrically evoked action potential of frog sciatic nerve. Further research of local anesthetic activity in Cha-plu fruit needed more purified form of pellitorine as well as a solvent system which have no effect on the action potential of frog sciatic nerve.

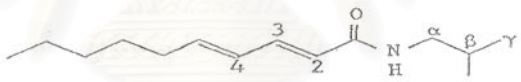


Figure 7. Molecular structure of Pellitorine (From Likhitwitayawuid, 1987).

Cyclodextrin

Living cells contain membrane-bound proteins, which can specifically recognize and bind carbohydrates. As cells are also coated with carbohydrates, this carbohydrate-protein interaction serves as a method by which cells can recognize other cells. These proteins (carbohydrate-binding proteins are called lectins) are often cell-specific, and they usually specifically bind only certain carbohydrate, so they provide a potential way by which drugs can be selectively targeted to specified cells types (JFS, 2000).

Cyclodextrins have the potential to form string complexes with certain drug molecules. However, cyclodextrins themselves have no means by which they can be recognized by specific cells. However, the attachment of appropriate carbohydrate antennae onto cyclodextrin could lead to a molecule which is not only capable of complexing drugs, but also capable of delivering this drug to cells which recognize its carbohydrate antennae (JFS, 2000).

Hydroxypropyl-β-Cyclodextrin

Hydroxypropyl-β-Cyclodextrin (HPBCD) is produced from β-Cyclodextrin (BCD) by addition of propylene oxide to some of the hydroxyl groups of BCD. The hydroxyl groups and the hydroxypropyl groups are on the exterior of the molecule that interacts with water to increase aqueous solubility of the HPBCD and the complexes directed from HPBCD. Previously 8% or 3% of HPBCD was used to solubilize Tolnafate which an antifungal drug that is not very soluble in water (Perri, 1994). Solubility of HPBCD and its complexes was greater than that of BCD. Therefore, HPBCD is used when the solubility of BCD is not sufficiently high. HPBCD has the most extensive collection of safety data with no adverse reaction reports (Coussement et al., 1990). In 1997, Khongsombat studied the effect of N (2'-propylpentanoyl)-2-pyrolidione and N-(2'-propylpentanoyl) urea on the firing rate of purkinje neurons of rat's cerebral cortex. Because of the insolubility of the test compounds in aqueous solution, HPBCD was used to dissolve them. Moreover, it was found that an application of HPBCD to the cerebellar perkinje cell did not alter the firing rate of perkinje cell (Khongsombat, 1977). Hence, in the present study HPBCD was used as a vehicle for the test compound.

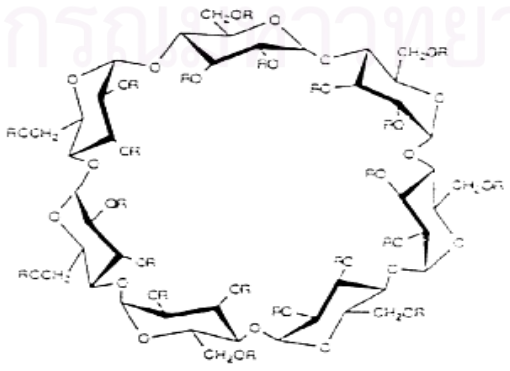


Figure 8. Molecular structure of Hydroxypropyl-β-cyclodextrin (From Aromde, 1988).

The present studies aim to investigate local anesthetic effect of pellitorine isolated from fruit of Cha-plu (*Piper sarmentosoum* Roxb.) using *in vitro* as well as *in vivo* experiments. In addition, the mechanism of local anesthetic effect of pellitorine with regards to sodium would also be investigated.

Chapter II

Materials and Methods

Experimental animals

Frogs (*Rana tigerina*), weighing 150-200 g. were used to study the nerve conduction block of pellitorine. They were obtained from local supplier in Rungsit district, Pathumtani Province.

Male ICR mice weighing 18 - 22 g.and Male guinea pigs weighing 400 – 600 g. were used in other experiments. Both of them were obtained from the National Laboratory Animal Center of Mahidol University Salaya Campus. All animals were maintained under natural light/dark cycle at control temperature (25^0C) and allowed free access to standard food (C.P.Mice Food, and C.P. Guinea pig food, Thailand) and tap water. They were acclimatized in the laboratory for at least one week before the experiments were started. All experiments were carried out between 8.00 a.m. – 6.00 p.m. Each animal was used only once. At the end of each experiment, animals were sacrificed with chloroform. Experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Equipments

1. Analog Digital Instruments (Maclab™/4, ADInstruments, Australia)

2. Macintosh Computer Model LC630 (Apple computer Inc., U.S.A.) with software programs; Scope™ V3.3 for data recording system (MacLab™, ADInstruments, Australia)

3. Bio Amplifer (ADInstruments, Australia)

4. Operating microscope (Tekagi Seiko, Japan)

5. Stage and Ocular micrometer (E LEITZ, Germany)

6. Vortex mixer (Scientific Industries Inc., U.S.A.)

7. Nerve Chamber and Drug Chamber

8. Tail flick analgesia meter (Harvard,U.S.A.)

9. Animal hair clippers (Zlingen, Germany)

10. Osmometer (Ganotec 030-DM, Germany)

11. pH meter (Beckman, U.K.)

12. UV Fluorescence analysis (CM-10, U.S.A.)

13. NMR-spectrometer (AM-250, Germany)

Chemicals

1. N-isobutyl deca-trans-4-dienamide (Pellitorine was synthesize at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.)

2. Lidocaine HCl (Sigma, U.S.A.)

3. Normal saline solution (0.9% NaCl) (General Hospital ®, Thailand)

4. Hydroxypropyl-β-cylodextrin (Aldrich, U.S.A.)

5. Chloroform (Mallinckrodt, Paris)

6. Ethanol (Mallinckrodt, Paris)

7. Petroleum ether (Mallinckrodt, Paris)

8. Silica gel (Merk, Germany)

9. Sodium hydrogen carbonate($NaHCO_3$: Riedel-de Haën, Germany)

10. Sucrose (APS Finechem, Australia)

11. Calcium chloride-2-hydrate ($CaCl_2$;Riedel-de Haë n, Germany)

12. D-Glucose monohydrate ($C_6H_{12}O_6$; Sigma, U.S.A.)

13. Potassium chloride (KCl; Riedel-de Haën, Germany)

14. Sodium chloride (NaCl; APS Finechem, Australia)

15. Sodium dihydrogen phosphate-2-hydrate (NaH_2PO_4: Riedel-de Haën, Germany)

Experimental Methods

1. Preparation of pellitorine

Dried fruits of *Piper sarmentosum* Roxb, purchased from a well recognized herbal supplier in Bangkok (Chao Grom Per), were powdered and extracted with ethanol four times under reflux. The extract was filtered and evaporated on a rotary evaporator. This extract was successively fractionated into petroleum ether, chloroform, distilled water, and dried under rotary evaporator to give petroleum ether extract, chloroform extract, and water

extract. The most active chloroform extract was subjected to silica gel column chromatography using the eluent chloroform/methanol (1%). The receive compound that evaporated until dried examined by TLC plate, which observed under UV light at wavelength of 254 nm (Likhitwitayawuid, 1987). The compound containing pellitorine as the principal compound was identified by ^{1}H-NMR spectrometer and it was kept for further study therein, Hydroxypropyl-β-cyclodextrin was used as a vehicle for pellitorine.

Identification of test compounds

Test compound, subsequently isolated, purified and identified from dried fruits of *Piper sarmentosum* Roxb, was obtained as yellowish brown powder.

^{1}H-NMR spectrum of test compound in $CDCl_3$ showed a δ 7.19(1H,dd,J=15.0,10.0, H-3), 6.12(1H,dt,J=13.1,7.0, H-5), 6.10(1H,dd,J=13.1,10.0, H-4), 5.76(1H,d,J=15.0, H-2), 5.60(1H,br,s, H-N), 3.16(2H,t,J=6.5, H-α), 2.14(2H,dd,J=6.8, 7.3,H-6),1.80(1H,m,H-β),1.42(2H,quint,J=7.1,H-7),1.30(4H,m, H-8,-9), 0.92(6H,d,J=6.7, H-γ) and 0.89(3H,t,J=6.9, H-10) which was in agreement with ^{1}H-NMR of pellitorine (Figure 9).

^{1}H-NMR spectrum of test compound in $CDCl_3$ showed a δ 10.32(1H, s, OCH), 7.32(1H, s, H-6), 6.50(1H, s, H-3), 3.98(3H, s, OCH_3), 3.95(3H, s, OCH_3) and 3.89(3H, s, OCH_3) which was in agreement with ^{1}H-NMR of asaronaldehyde (Figure 9).

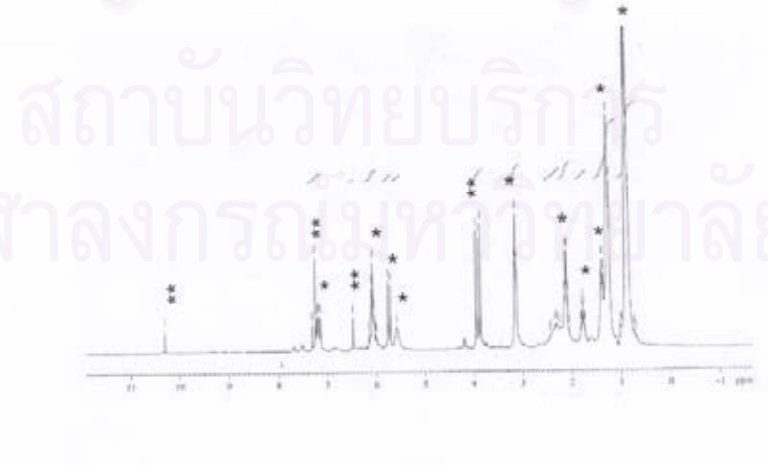


Figure 9. ^{1}H NMR spectrum of test compound was found to contain pellitorine (*) and asaronaldehyde (**).

By means of quantitative analysis by ^{1}H-NMR, the test compound was found to contain 85% of pellitorine and 15% of asaronaldehyde so; pellitorine was the principal component of test compound in combination with a minute quantity of asaronaldehyde.

2. Preparation of the test substances

The test substances, pellitorine and lidocaine, were dissolved in 0.1 M Hydroxypropyl-β-cylodextrin in normal saline solution (0.9% NaCl) and Frog Ringer's solution. Normal Frog Ringer's solution composed of the following composition served as the normal extracellular fluid: NaCl 111.22 mM: KCl 1.88 mM; $CaCl_2$ 0.82 mM; $NaHCO_3$ 2.38 mM; NaH_2PO_4 0.06 mM; Glucose 10.09 mM (pH 7.40) θ = 238 m-osmole/Kg. In sodium deficient Ringer's solution, sucrose was added in amount sufficient to maintain proper osmotic at 238 m-osmole/Kg. and ionic balance. Osmolarity of Ringer's solution was measure by osmometer (Figure 10).

Figure 10. Osmometer.

3. Evaluation of local anesthetic effect

3.1 Isolated frog sciatic nerve experiments

3.1.1 Preparation of sciatic nerve

Frogs were sacrificed by double pitting and sciatic nerve, diameter between 0.94 - 1.2 mm that measured by stage and ocular micrometer, were exposed from lateral muscles of the thigh and femoral blood vessels that run pararell to the nerve by cutting out the bony urostyle and dissect free the muscular attachments in the lower back. A thread was tied around the nerve in the urostyle area, and the nerve was cut anterior to the thread (Figure 11). Nerve branches and surrounding connective tissues were gently separated and the distal end of the nerve was tied with a thread and cut near the distal end of the tibiofibula then soaked the nerve with Ringer's solution on the petri dish. Any remaining connective tissues or nerve branches were removed and then the nerve was mounted in a nerve chamber. Care was taken to keep the isolated nerve moist throughout the experiment.

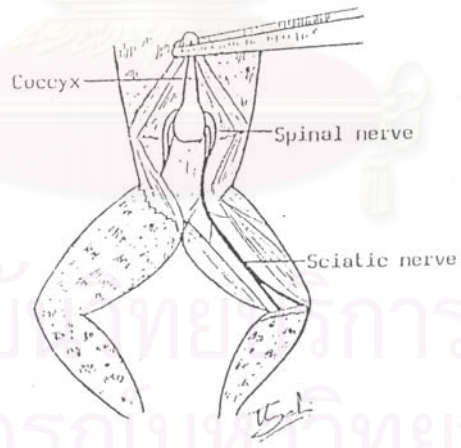


Figure 11. Preparation of frog sciatic nerve.

Local anesthetic effect of test substances was performed on 7 groups of 8 nerve trunks each. One group was used as a control (HPBCD 0.1 M in Ringer's solution). The other groups were used to determine the nerve conduction block of the test substances (15 μl of pellitorine (10, 14, 25 mM) and lidocaine (12, 16, 30 mM)).

3.1.2 Recording

The nerve was resided in the nerve chamber containing Ringer's solution. Stimulation from the stimulator in Maclab/4 (ADInstrument, Australia) was delivered at every 5 minutes through stimulating electrodes placing at the proximal end of the nerve. The action potential recorded by recording electrodes placing at the distal end of the nerve. The signals were amplified and converted from analog to digital by Bioamplifier and Maclab/4 and being recording by Macintosh computer with software "Scop V 3.3" (Figure 12). Maximal voltage was obtained by step increasing of stimulating voltage to the level that further increase did not increase the amplitude of action potential. Further increasing of maximal stimulus for 10% resulted in the supramaximal stimulus which was used to generate control action potential (mV). After that, 15 μl of the test compound was added to the drug chamber and the stimulation from stimulator was delivered at every 5 minutes until the depression of action potential was complete. The effect observed was reversible as the magnitude of response in each experiment was resumed to its control value after washing the nerve by Ringer's solution several times.

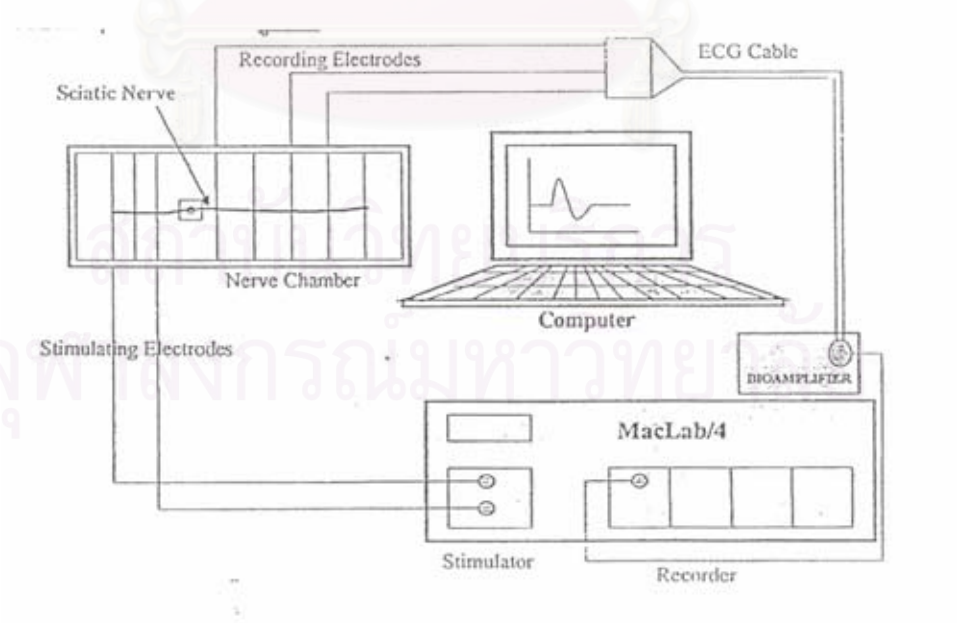


Figure 12. Diagrammatic of isolated frog sciatic nerve experiment.

3.2 Conduction anesthesia on the mouse tail

3.2.1 Experimental procedure

Male ICR mice were divided into 12 groups of 10 animals each for the determination of the EC_{50} against tail flick reflex. One group was used as a control (HPBCD 0.1 M in NSS). The other 11 groups were used for the test substances, pellitorine (10, 12, 14, 18, 25 mM) and lidocaine (4, 6, 8, 12, 18, 25 mM).

Mice were placed in individual restrainer 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 animal's tail. The analgesia meter (Figure 13) automatically recorded (in 0.1 sec) the latency between the onset of the light beam stimulus and the response to heat, at 4.0 sec to minimize thermal injury. The stimulus intensity was set so that the baseline tail flick latencies were approximately 0.8 – 1.7 sec (intensity $\cong$ 3.7A). The intensity was not changed for any animal within any given experiments. Three times of baseline trials were done before the test at 10 – 15 min intervals. The score from the third trial served as the baseline measure for each animal. The test compounds (pellitorine) and the standard (lidocaine) were injected in a volume of 0.1 ml on both sides in the area of the tail root (Vogel, 1997).The animals are submitted to the radiant heat again every10 min for 90 min after injection (0,10,20,30,40,50,60,70,80,and 90 min). The area of heating is about 1.5 cm distal to the injection site. For each individual animal the reaction time was noted.

Figure 13. Photograph of a mouse on the tail flick analgesia meter.

3.3 Infiltration anesthesia in guinea pig's wheals

3.3.1 Experimental procedure

Guinea pigs were clipping and shaving the hair on the lower back then the depilatory cream was applied on the shaved back of guinea pigs. Allow material to make contact with the skin for 15 – 20 min. using a damp cloth towel and some warm water to wash off the depilatory cream from the backs of the animals. The skin of the animals was dry with warm air blown over their backs. This produces irritation which disappears overnight (Vogel, 1997).

Male guinea pigs were divided into 7 groups of 3 animals each for the determination of degree of anesthesia. One group was used as a control (HPBCD 0.1 M in NSS). The other 6 groups were used for the test substances, pellitorine (3, 7, 10, 14 mM) and lidocaine (4, 8, 12, 16 mM). Intradermal injection of test compound was made in each animal by a sterile sharp 24 G x ½ inch needle. Each guinea pig receives one dose in the front area and another dose in the back area. Another guinea pig receives the same doses, but the solutions, which was used for the front area now used for the back area. The volumes injected (0.25 ml.) is enough to raise a wheal, which is outlined

with a marking pen. Four injections with four concentrations were used in each guinea pig (Figure 14). After that, animals were placed in restrainer cage for the test. Five minutes after the injection the sensitivity of the area is tested by pricking with 27 G x 1 inch hypodermic needle, six times lightly (no more than 1/8 inch), the skin at the site of injection and as a control, the skin as far away from it as possible. Six twitches should be recorded for this control test, but the responses at the site of the injection will indicate the degree of anesthesia. The test is repeated at five-minute intervals for a period of 55 minutes after the injection.

Figure 14. Picture of guinea pig, showing sites of injection of local anesthetic (From Thompson, 1990).

3.4 Differential nerve block by sodium deficient solutions

3.4.1 Experimental procedure

This experimental procedure was similar to the experiment in 3.1. The frog sciatic nerve was placed in the nerve chamber. Sodium deficient Ringer's solution and Normal Ringer's solution was used to dissolve the test substances. The amplitude of action potential generated by supramaximal stimuli served as a control at time zero. Then, 15 μl of the test substance was added to the drug chamber and the stimulation from stimulator was delivered at every 5 minutes until the depression of action potential

was complete. Frog sciatic nerve was divided into 4 groups of 4 subgroups for different concentration of test substances. Each subgroup comprises of 5 nerve trunks.

Group 1 was used to study the effect of pellitorine (0.8, 6, 13, 25 mM) which was dissolved in sodium deficient Ringer's solution that contains 12 mM sodium concentration.

Group 2 was used to study the effect of pellitorine (7, 10, 14, 25 mM) which was dissolved in Normal Ringer's solution that contains 114 mM sodium concentration.

Group 3 was used to study the effect of lidocaine (0.125, 0.5, 3, 6 mM) which was dissolved in sodium deficient Ringer's solution that contains 12 mM sodium concentration.

Group 4 was used to study the effect of lidocaine (6, 8, 12, 16 mM) which was dissolved in Normal Ringer's solution that contains 114 mM sodium concentration.

Calculation and statistical analysis

1. Results of times to reach complete block and percent height of action potential were expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis were performed on the difference percent height of action potential and the difference of time to reach complete block by analysis of variance (ANOVA) and followed by TUKY TEST. The minimum level of statistical significance was set at $p<0.05$.

2. Tail flick latencies are expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

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

* Cut-off time is 4 seconds

Potential of local analgesia of each treatment was expressed in terms of area of analgesia that were derived from computing the cumulative area under the corresponding 0 – 90 min time course - % MPE curves, area was calculated using the trapezoidal rule (Thompson, 1990). Statistical analysis were performed on area of analgesia by analysis of variance (ANOVA) and, where appropriate, were followed by

Newman-Keuls' post hoc testing. The minimum level of statistical significance was set at $p<0.05$.

For the determination of EC_{50} groups of 10 mice each were used to test the effect of test substances at various concentrations until at least 3 points were established from % MPE that transformed to probit unit by transformation table of Fisher and Yates (Diem and Lentrer, 1972). The linear regression method was used to fit a curve between probit unit of % MPE and concentration of test substances (log scale) by using Cricket graph program (Macintosh Computer). The 95 percent confidence interval was calculated by the method of Litfield and Wilcoxon (Litfield and Wilcoxon, 1944).

3. The number of times the needle prick fails to elicit a response during each test period is added up. This value, out of a possible 66 (Six twitches was recorded after six pricking at five-minute intervals for a period of 55 minutes), is expressed as a percentage, which indicated the degree of apparent anesthesia (Thompson, 1990).

4. The common slope from the relationship between percent reduction of action potential and the concentration of test compound in various concentration of sodium in Ringer's solution was calculated by the method of linear regression (Gardiner, 1997).

Chapter III

Results

Effect of HPBCD in Ringer's solution on isolated frog sciatic nerve experiments

In frog sciatic nerve experiment, desheating of frog sciatic nerve (diameter between 0.74 – 0.88 mm) were placed in the nerve chamber. Stimulation of the nerve produced a display as shown in Figure 15. By adjusting the voltage setting of the stimuli, maximal response, indicated by the highest amplitude of action potential observed was obtained by electrical current of 3 – 8 V. Supramaximal responses (10% more from maximal response) attained at 4 – 9 V with duration 0.1 ms and delay 1.0 ms. As shown in Figure 15, 0.1 M of HPBCD exhibited no effect on supramaximally electrical-evoked action potential of isolated frog sciatic nerves. No statistically significant difference between the amplitude of action potential before and after application of 0.1 M HPBCD in Ringer's solution was noted.

Effect of lidocaine on isolated frog sciatic nerve experiments

As shown in Figure 16 – 18 and 22, lidocaine 12, 16, 30 mM exerted a concentration-dependent manner and reversible blockade of supramaximally electrical-evoked action potential of isolated frog sciatic nerves. Complete block of electrical-evoked action potential exerted by 12, 16, 30 mM of lidocaine was achieved at 13.75$\pm$1.25, 11.87$\pm$1.87, 7.50$\pm$0.94 min respectively (Figure 24).

Effect of pellitorine on isolated frog sciatic nerve experiments

As shown in Figure 19 – 21 and 23, pellitorine 10, 14, 25 mM exerted a concentration-dependent manner and reversible blockade of supramaximally electrical-evoked action potential of isolated frog sciatic nerves. Complete block of electrical-evoked action potential exerted by 10, 14, 25 mM of pellitorine was achieved at 155.62$\pm$10.24, 137.50$\pm$8.66, 73.75$\pm$2.26 min respectively (Figure 24).

Effect of pellitorine and lidocaine in sodium deficient Ringer's solution on isolated frog sciatic nerve experiments.

As illustrated in Figure 25 - 26, it is apparent that the degree of conduction block produced by lidocaine and pellitorine is related to the external concentration of sodium. For example, in a 12 mM sodium (sodium deficient Ringer's solution) the spike can be reduced in size by 50% with a 0.07 mM concentration of lidocaine and when the sodium concentration is elevated to 114 mM (Normal Ringer's solution) the lidocaine concentration now was increased to 3.69 mM in order to give the same degree of effect (Figure 25). As illustrated in Figure 26, in a 12 mM sodium (sodium deficient Ringer's solution) the spike can be reduced in size by 50% with a 4.18 mM concentration of pellitorine and when the sodium concentration is elevated to 114 mM (normal Ringer's solution) the pellitorine concentration now was increased to 17.47 mM in order to give the same degree of effect.

Effect of 0.1 M HPBCD in mouse tail flick test.

To investigate the effect of vehicle in tail flick analgesic testing, mice received 0.1 ml of NSS and 0.1 M HPBCD on there tail base and they were tested during the subsequent 90 min period. No statistically significant difference ($p<0.05$) between tail flick latency producing an area of analgesia of 0.1 M HPBCD (-229.23 $\pm$ 156.75 %MPE-min; Figure 27) and tail flick latency producing an area of analgesia of NSS (-243.16 $\pm$ 100.358 %MPE-min; Figure 27). This indicated that HPBCD did not affect sensory blockade.

Effect of pellitorine and lidocaine in mouse tail flick test.

Initial studies utilizing the tail flick test in mice to examine the efficacy of pellitorine compared with lidocaine in producing analgesia. Mice were injected with various concentrations of pellitorine (12, 18, 25 mM) and lidocaine (12, 18, 25 mM). All concentrations of pellitorine and lidocaine significantly ($p<0.05$) increased tail-flick latency when compared to NSS and HPBCD. Additionally, pellitorine and lidocaine increased the latency of tail flick in a concentration dependent manner, 25 mM of pellitorine and lidocaine produced significant ($p<0.05$) analgesic responses when compared to the lowest concentration of pellitorine and lidocaine used (Figure 27).

When the log of the pellitorine and lidocaine concentration were plotted versus the area of analgesia a significant linear correlation was observed. All five concentration of pellitorine (10, 12, 14, 18, 25 mM) were plotted a significant linear correlation coefficient (r^2) equal to 0.934 was observed, while the plotting of six concentrations of lidocaine (4, 6, 8, 12, 18, 25 mM) revealed a significant linear correlation coefficient of 0.935 (Figure 28). The analgesic peak effect of pellitorine was reached within 40 min after administration in all concentration of pellitorine tested and individual time courses of the responses are shown in Figure 30. The EC_{50} of pellitorine and lidocaine in this model were found to be 16 (6 - 44) mM and 12 (7 - 20) mM, respectively (Figure 31). Like lidocaine, pellitorine exerted a local anesthetic in a concentration-dependent manner. In pararell to local anesthetic effect, no adverse effect was produced by pellitorine or lidocaine in the concentration given.

Effect of 0.1 M HPBCD in twitch response test of guinea pig's skin

After pricking the needle, six times lightly on the back of guinea pig, skin twitch reflex can be observed in every six pricks at five minute intervals for a period of 55 minutes. Similar to the effect of 0.1 M HPBCD after six pricks was observed along 55 minutes. This indicated that HPBCD did not affect the sensory blockade.

Effect of pellitorine and lidocaine in twitch response test of guinea pig's skin

Absence of a skin twitch was observed in the presence of pellitorine and lidocaine. All concentrations of pellitorine and lidocaine produced a significant increase ($p<0.05$) in the percent absence of skin twitch reflex compared to control group that given with 0.1M HPBCD in NSS. Duration of analgesic effect exerted by pellitorine (3 – 14 mM) and lidocaine (4 – 16 mM) was found to be 30 – 50 min. Infiltration of either pellitorine or lidocaine did not cause any observable changes in experimental animals except failure of response to pin prick (Figure. 32 – 33).

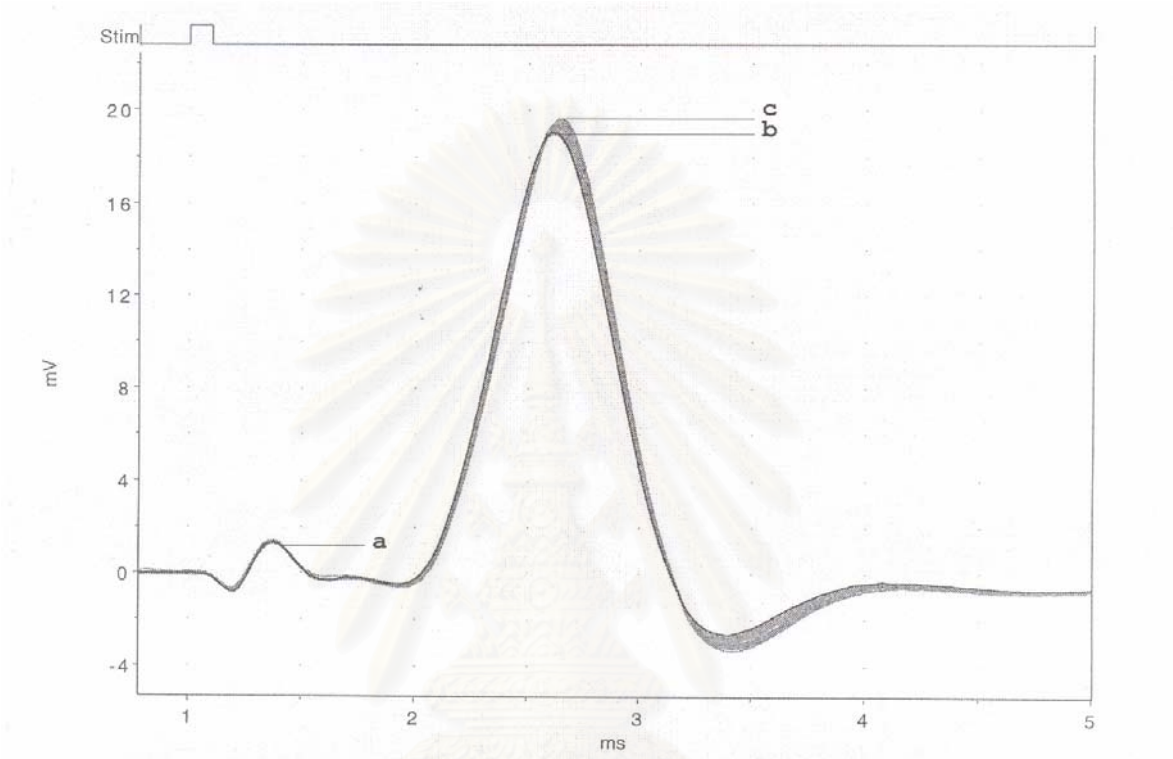


Figure 15. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (**b**) and after (**c**) an application of 0.1 M HPBCD.

a = stimulus artifacts,

b to **c** = action potential at time 0,…,40 min after an application of 0.1 M HPBCD in Ringer's solution respectively.

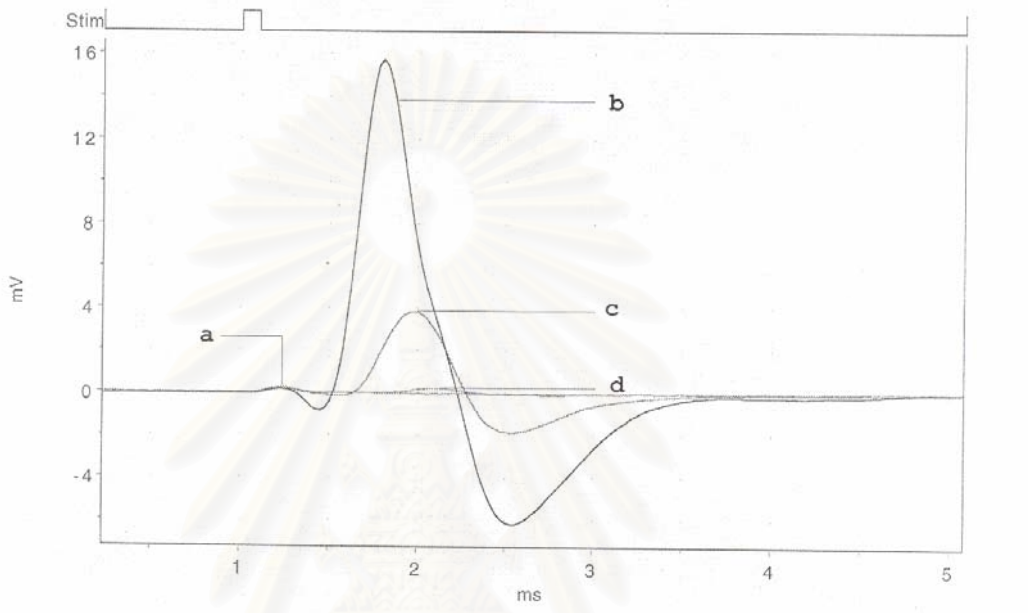


Figure 16. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (b) and after (c – d) an application of 12 mM of lidocaine.

a = stimulus artifacts,

b to **d** = action potential at time 0, 5, 10 min after an application of 12 mM of lidocaine in 0.1 M HPBCD in Ringer's solution respectively.

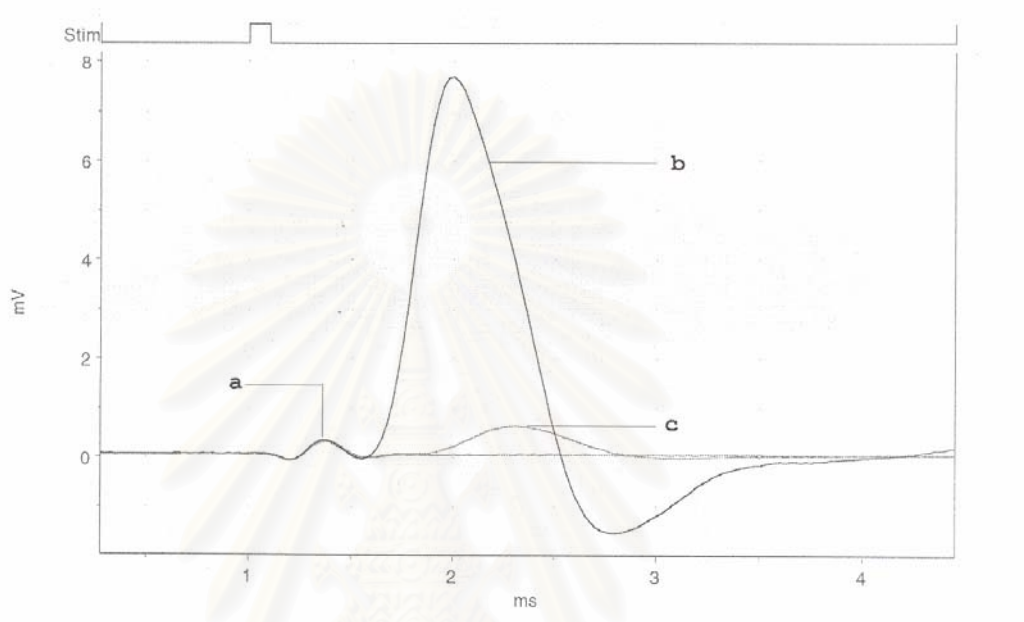


Figure 17. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (**b**) and after (**c**) an application of 16 mM of lidocaine.

a = stimulus artifacts,

b to **c** = action potential at time 0, 5 min after an application of 16 mM of lidocaine in 0.1 M HPBCD in Ringer's solution respectively.

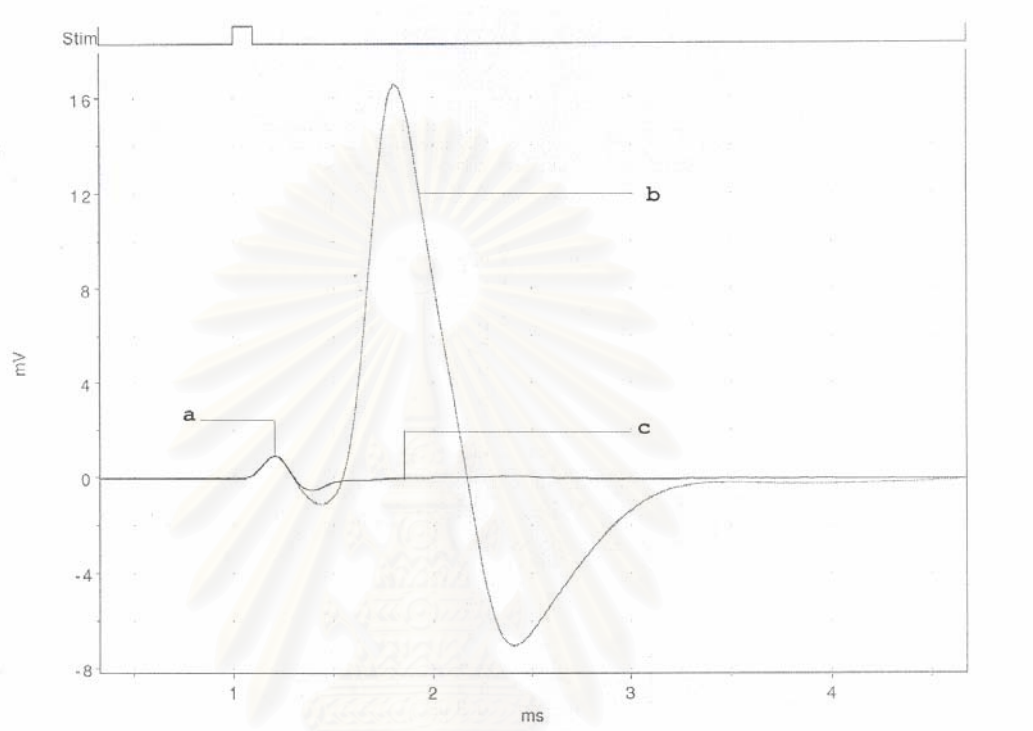


Figure 18. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (**b**) and after (**c**) an application of 30 mM of lidocaine.

a = stimulus artifacts,

b to **c** = action potential at time 0, 5 min after an application of 30 mM of lidocaine in 0.1 M HPBCD in Ringer's solution respectively.

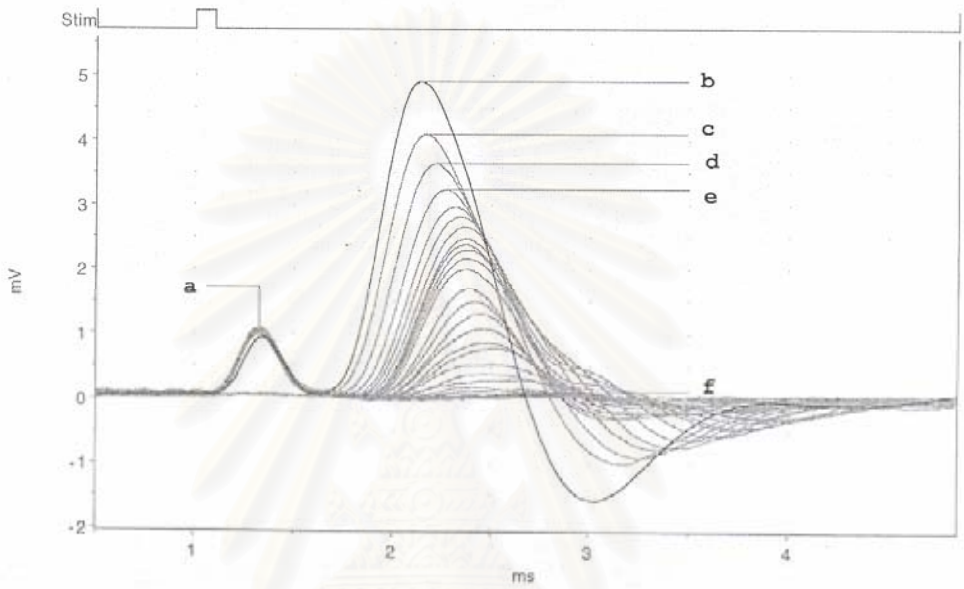


Figure 19. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (**b**) and after (**c** – **f**) an application of 10 mM of pellitorine.

a = stimulus artifacts,

b to **f** = action potential at time 0, 5, 10,15,….,125 min after an application of 10 mM of pellitorine in 0.1 M HPBCD in Ringer's solution respectively.

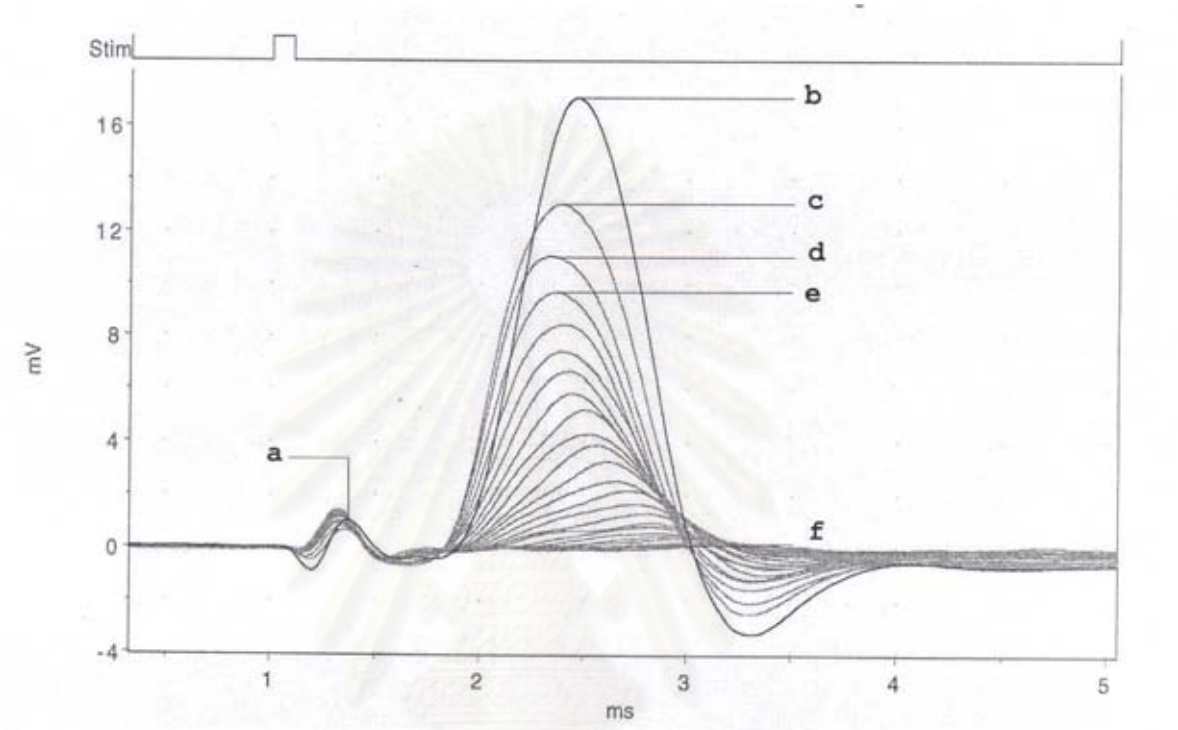


Figure 20. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (b) and after (c – f) an application of 14 mM of pellitorine.

a = stimulus artifacts,

b to **f** = action potential at time 0, 5, 10, 15,…,115 min after an application of 14 mM of pellitorine in 0.1 M HPBCD in Ringer's solution respectively.

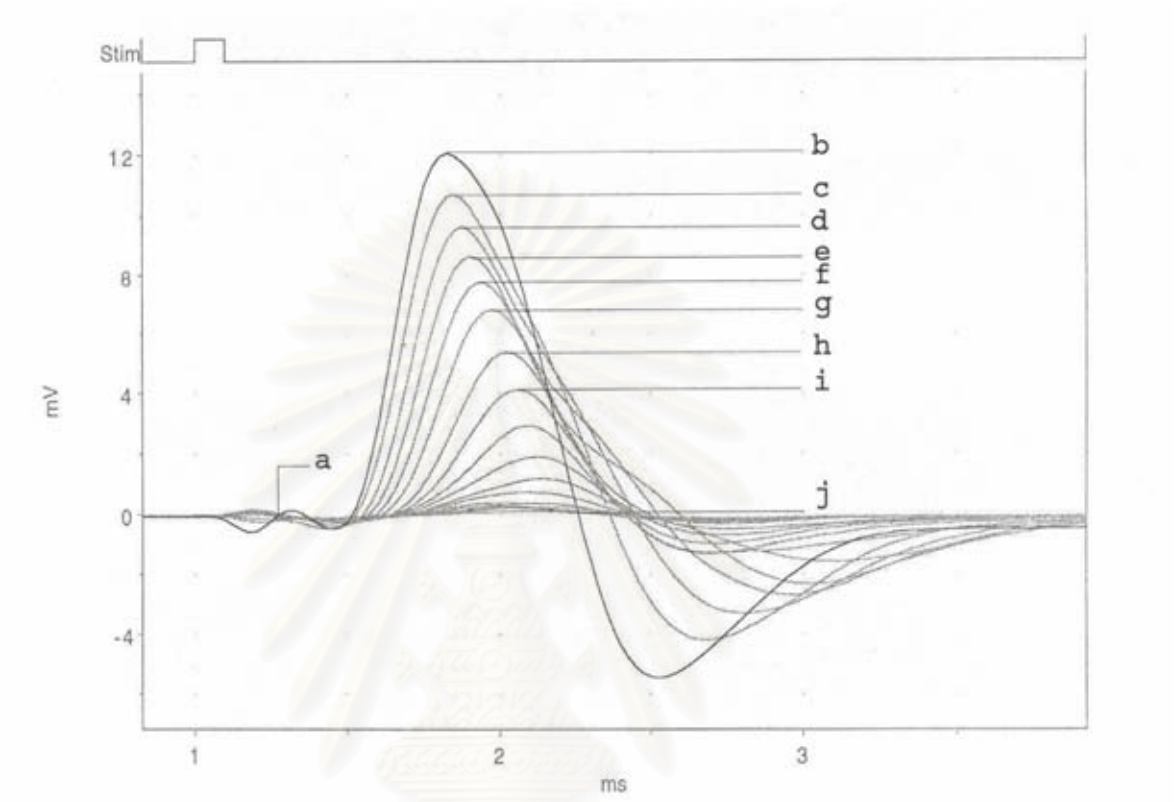


Figure 21. Display of electrically stimulated (at every 5 min) action potential of frog sciatic nerve before (**b**) and after (**c** – **j**) an application of 25 mM of pellitorine.

a = stimulus artifacts,

b to **j** = action potential at time 0, 5, 10, 15, 20, 25, 30, 35,…,70 min after an application of 25 mM of pellitorine in 0.1 M HPBCD in Ringer's solution respectively.

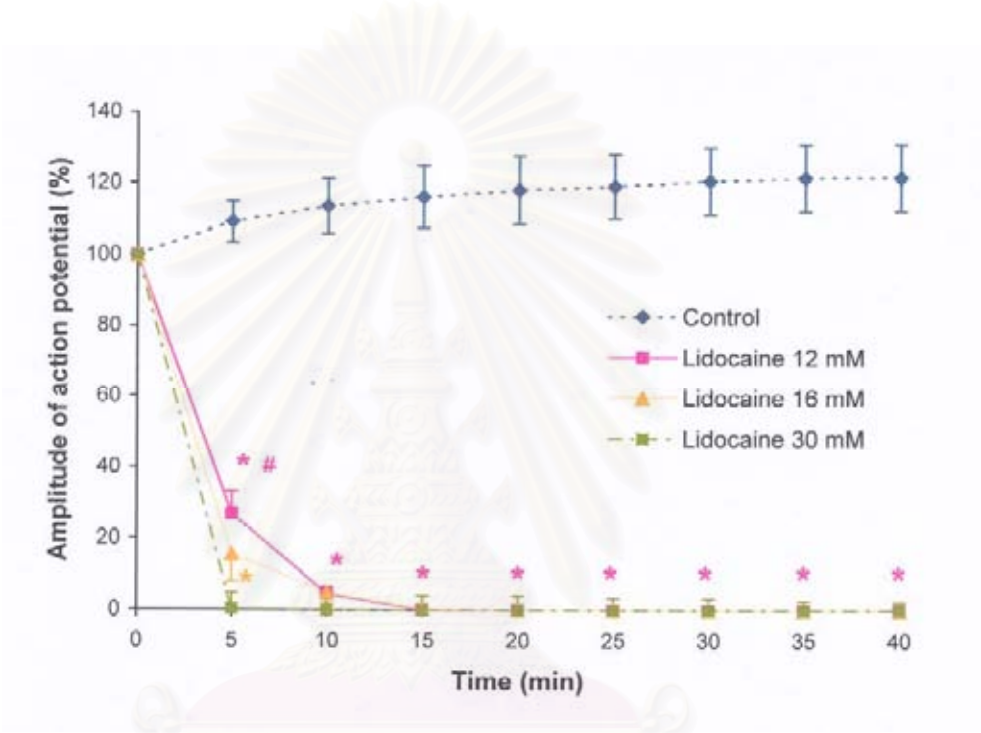


Figure 22. Conduction block of frog sciatic nerve exerted by lidocaine 12, 16, 30 mM in comparison to control group. Each point represents the mean ± S.E.M. of 8 experiments. * $p < 0.05$ significantly different compared to control group, # $p < 0.05$ significantly different compared to lidocaine 30 mM.

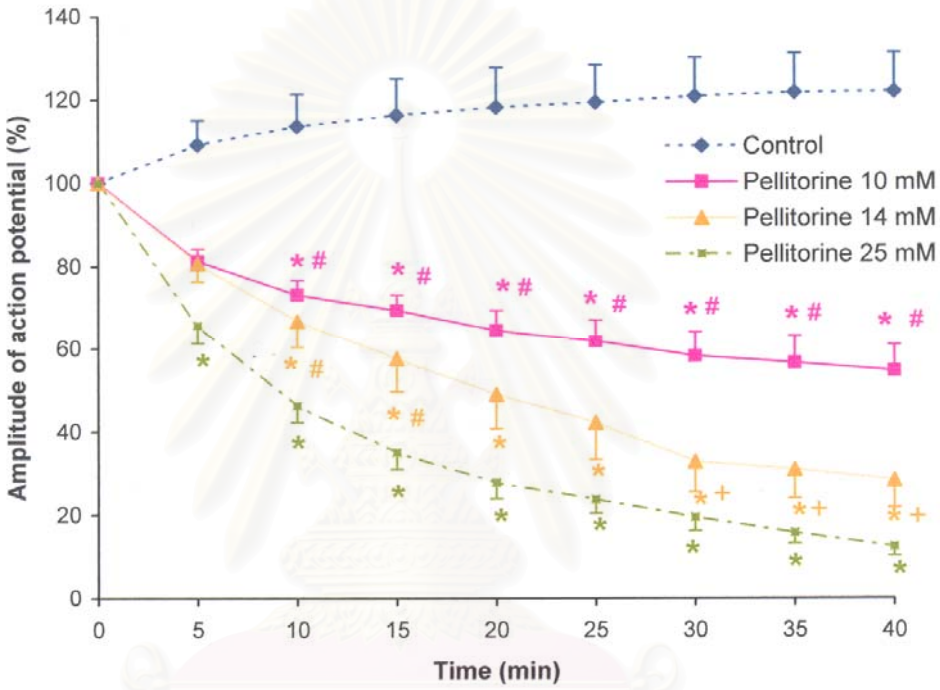


Figure 23. Conduction block of frog sciatic nerve exerted by pellitorine 10, 14, 25 mM in comparison to control group. Each point represents the mean ± S.E.M. of 8 experiments. * $p < 0.05$ significantly different compared to control group, # $p < 0.05$ significantly different compared to pellitorine 25 mM, + $p < 0.05$ significantly different compared to pellitorine 12 mM

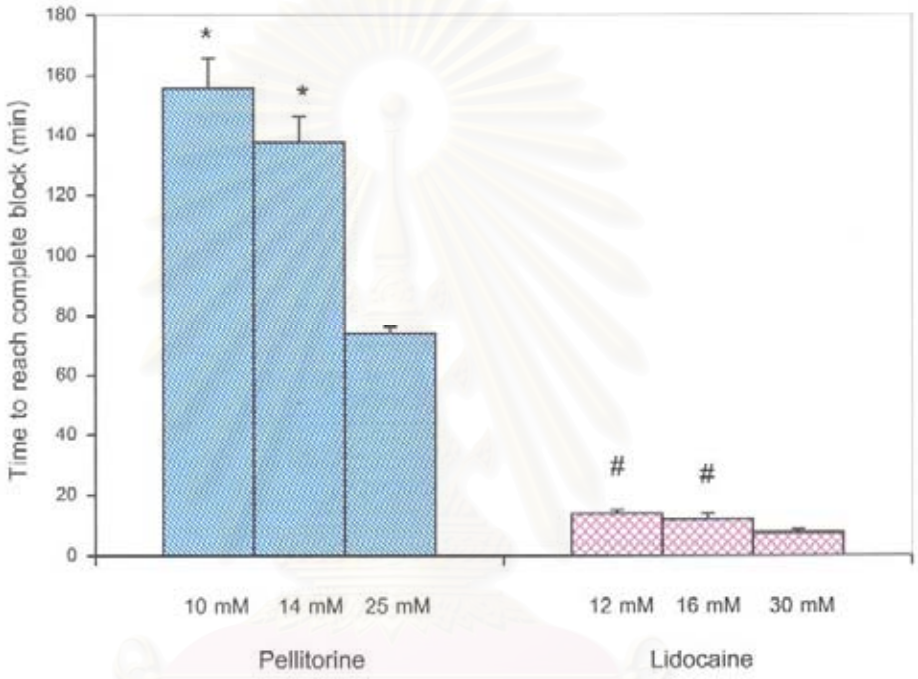


Figure 24. Time to accomplish a complete blockade of electrically-evoked action potential of frog sciatic nerve. Results are expressed as mean $\pm$ S.E.M. * $p < 0.05$ significantly different compared to pellitorine 25 mM, # $p < 0.05$ significantly different compared to lidocaine 30 mM.

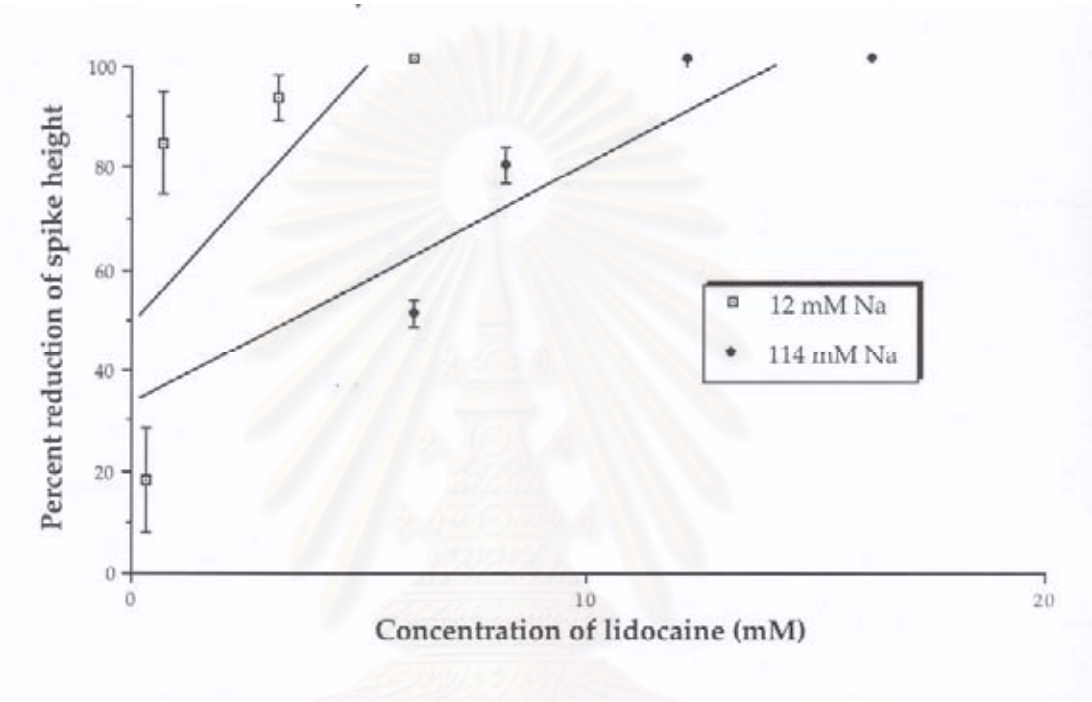


Figure 25. Concentration-response curves of lidocaine with varying concentrations of sodium in the Ringer's solution. Abscissa: concentration of lidocaine. Ordinate: degree of conduction blocks in 15 minutes. Each point is the means of 4 – 6 experiments.

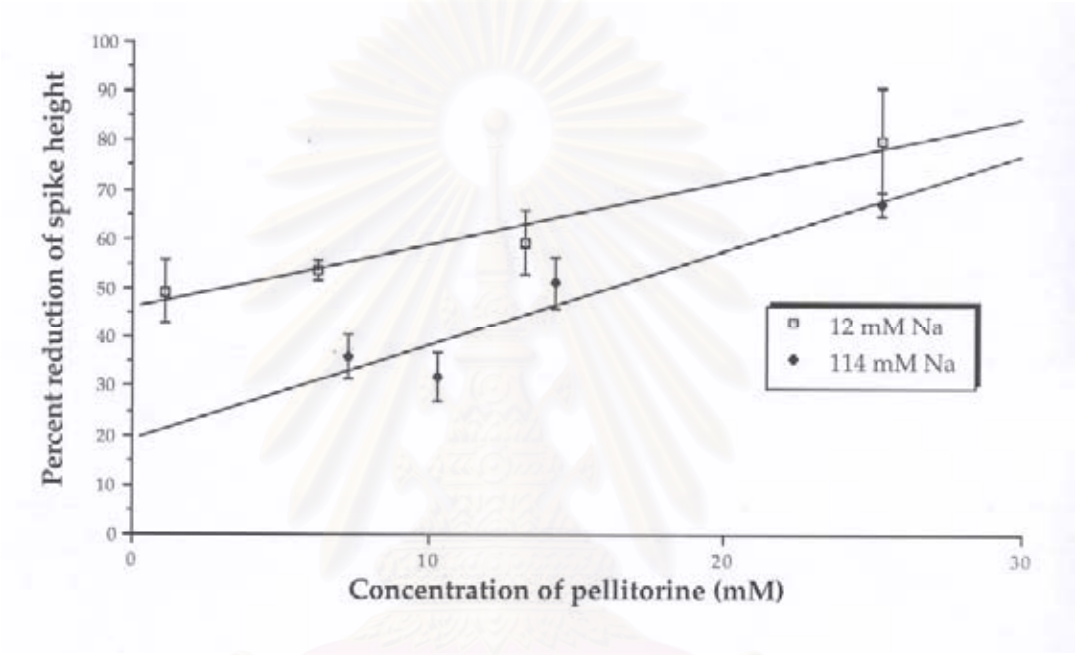


Figure 26. Concentration-response curves of pellitorine with varying concentrations of sodium in the Ringer's solution. Abscissa: concentration of pellitorine. Ordinate: degree of conduction blocks in 15 minutes. Each point is the means of 4 – 6 experiments.

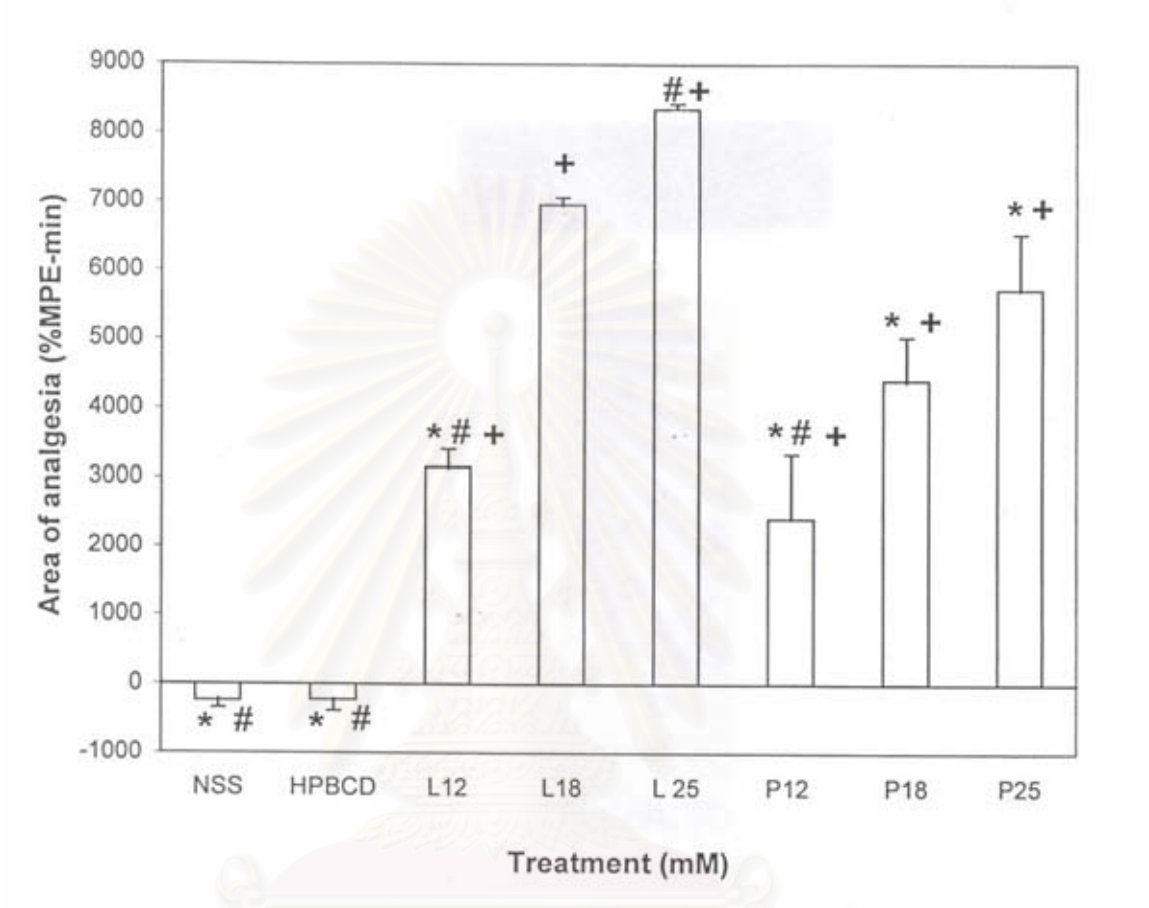


Figure 27. Comparison of area of analgesia (%MPE-min) from 0-90 minutes provided by normal saline solution (NSS), 0.1 M HPBCD in NSS, various concentrations of lidocaine (L; 12, 18, 25 mM) and various concentrations of pellitorine (P; 12, 18, 25 mM). N=10 for all groups. * $p<0.05$ significantly different compared to lidocaine (L) 25 mM, # $p<0.05$ significantly different compared to pellitorine (P) 25 mM, + $p<0.05$ significantly different compared to NSS.

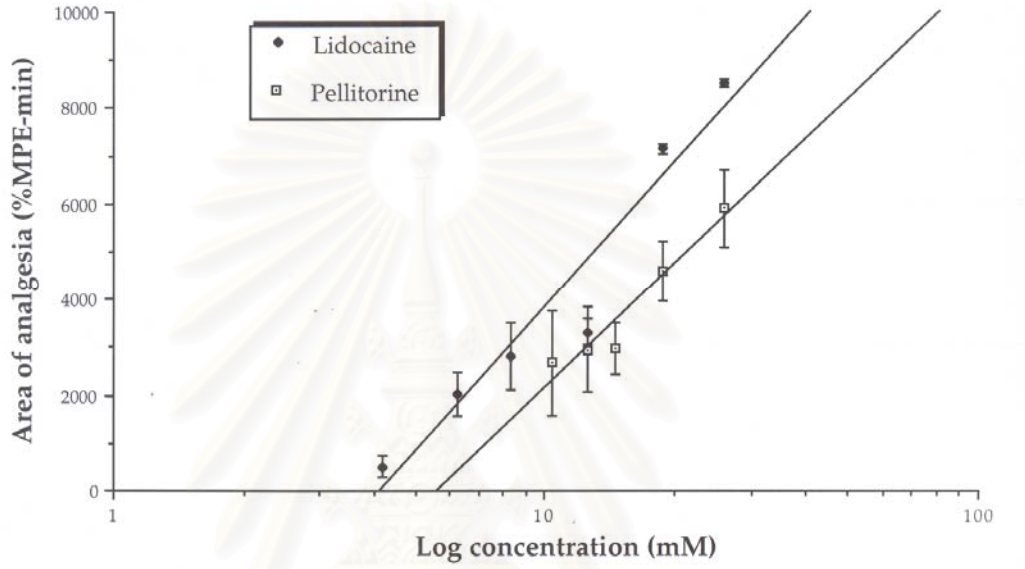


Figure 28. Linear regression of area of analgesia (%MPE-min) from 0-90 minutes after an administration of 4 – 25 mM of lidocaine and 10 – 25 mM of pellitorine on the mouse tail's base (N=10 for all groups). The regression equation of lidocaine and pellitorine was Y = -6160.5 + 1.0026 * LOG(X) r^2 = 0.935, Y = -6461.4 + 8599.2 * LOG(X) r^2 = 0.934, respectively.

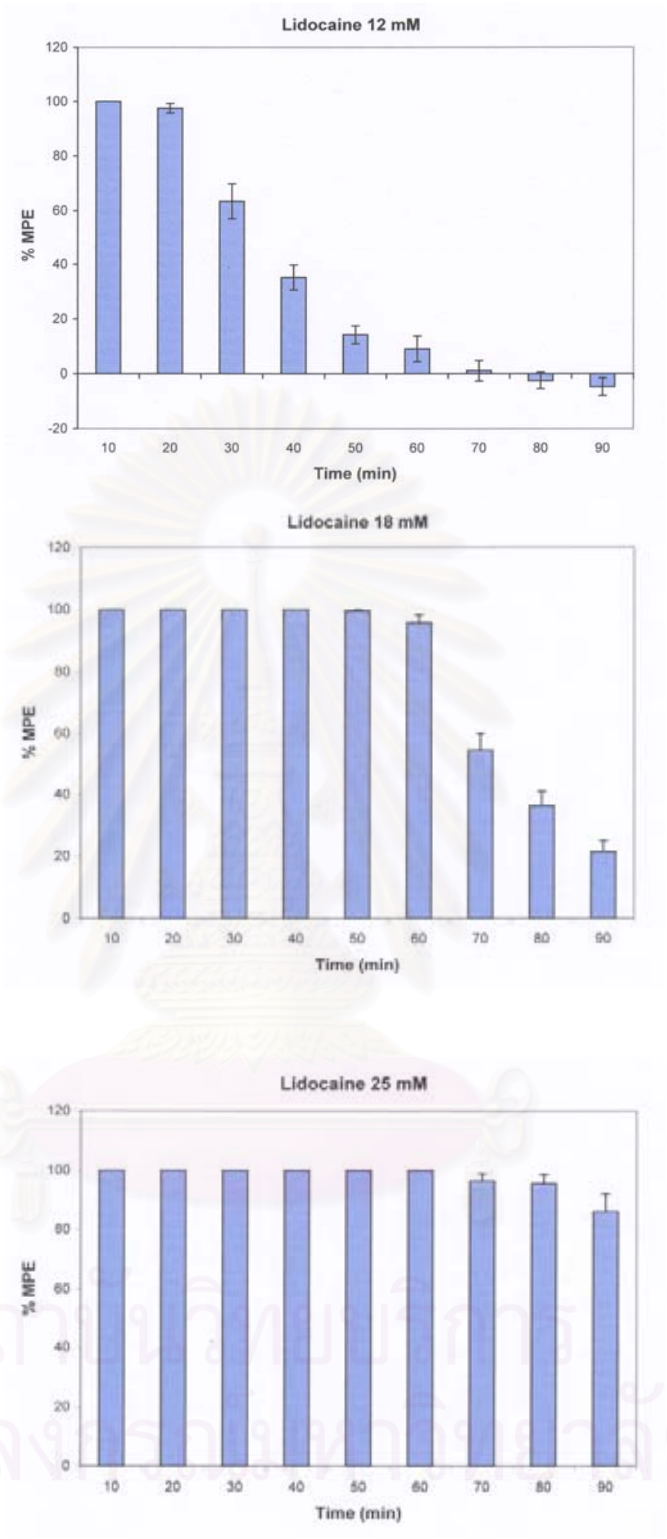


Figure 29. Individual time courses of the response (% MPE versus time (min)) after the administration of various concentrations of lidocaine (12, 18 and 25 mM) on the mouse tail's base. N=10 for all groups.

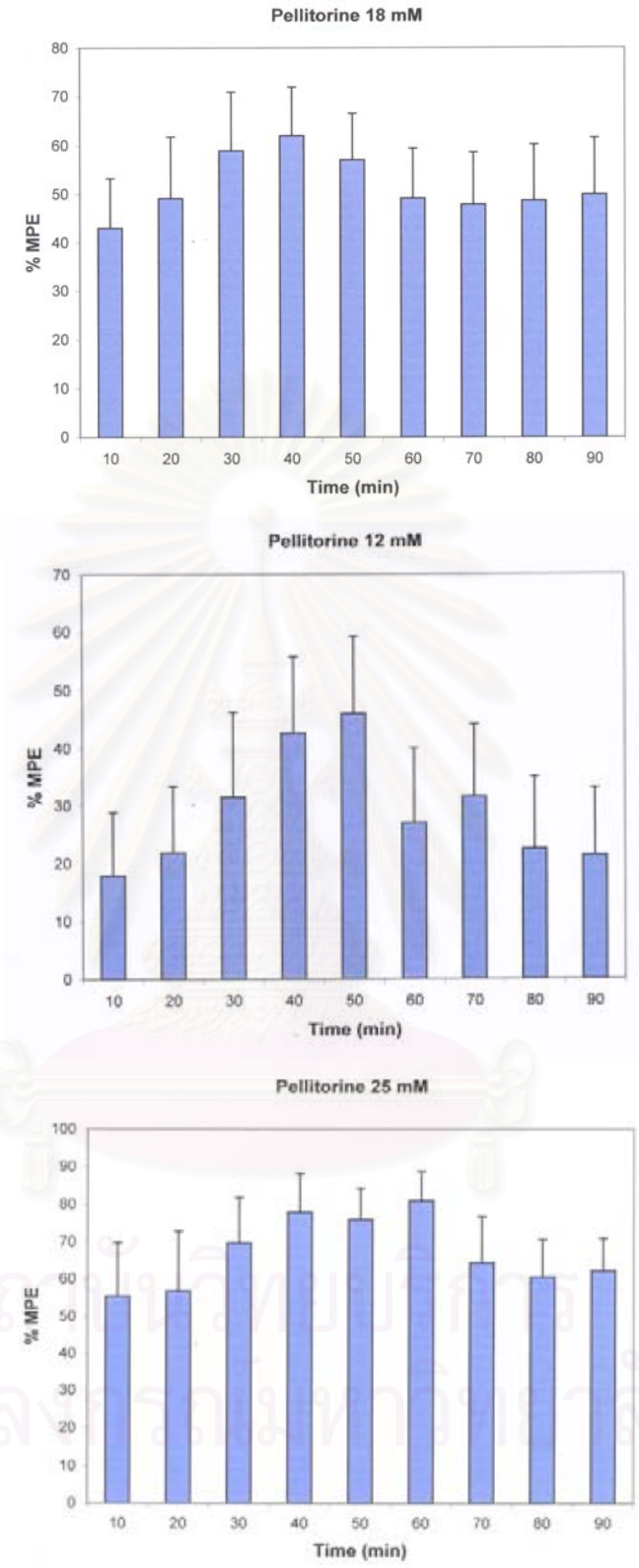
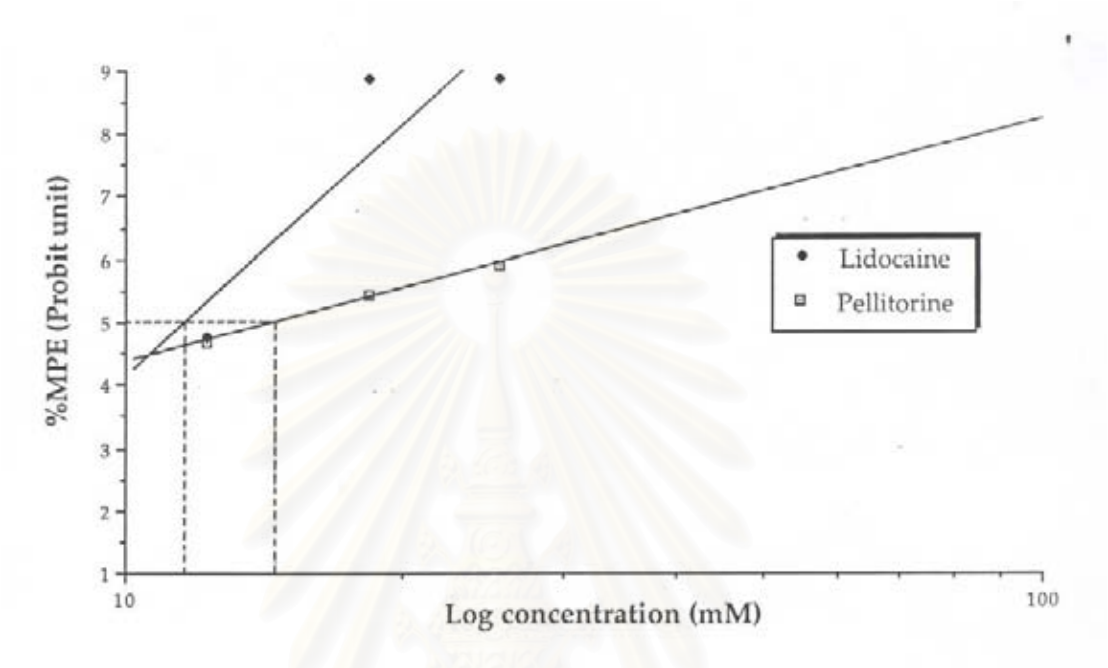


Figure 30. Individual time courses of the response (% MPE versus time (min)) after the administration of various concentrations of pellitorine (12, 18 and 25 mM) on the mouse tail's base. N=10 for all groups.



Probits (lidocaine) = -9.1476 + 13.263 * LOG(X) r^2 = 0.800

EC_{50} = 12 (7 – 20) mM

Probits (pellitorine) = 0.42436 + 3.8448 * LOG(X) r^2 = 0.994

EC_{50} = 16 (6 – 44) mM

Figure 31. Linear regression of %MPE (Probit unit) at 40 minutes after the administration of various concentration of lidocaine (4 – 25 mM) and pellitorine (10 – 25 mM) using tail flick test. N=10 for all groups. The EC_{50} was calculated from the log concentration probit line.

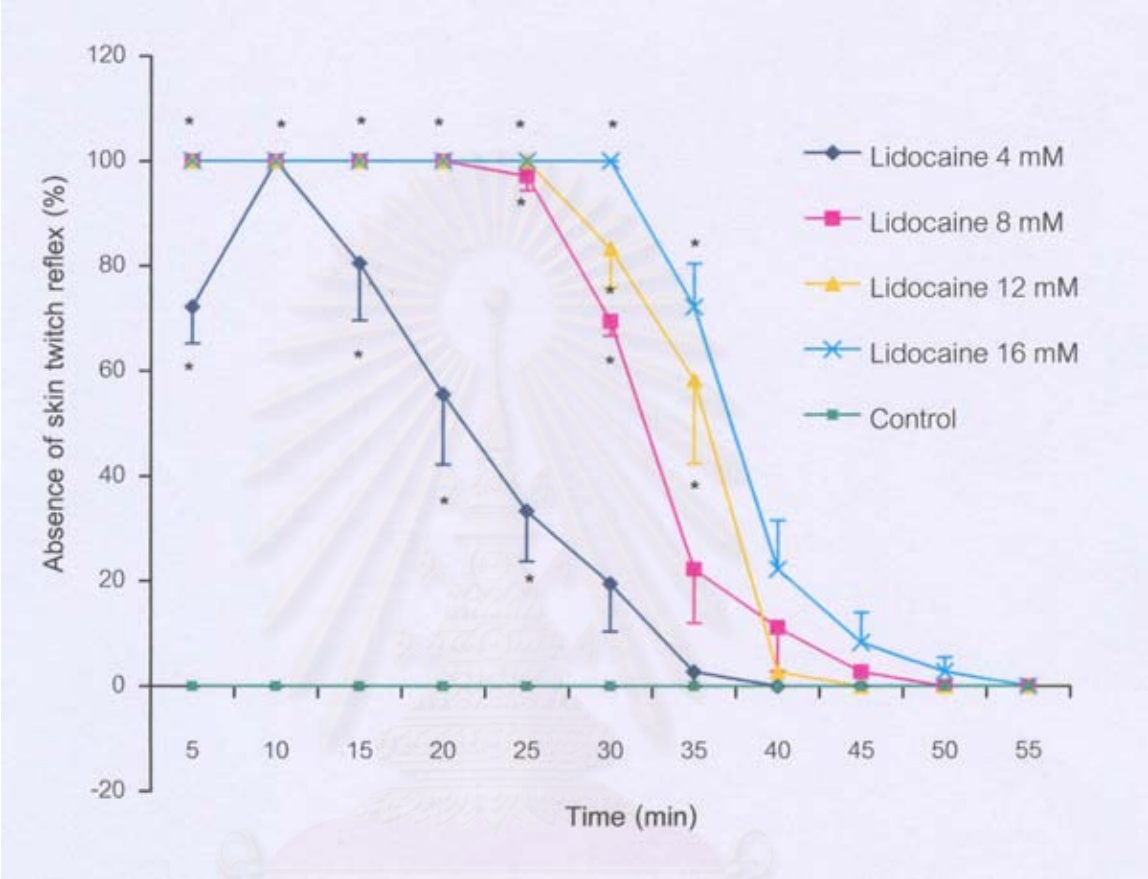


Figure 32. Time-response curves of lidocaine at concentrations of 4, 8, 12, 16 mM and control group in the response test in guinea pig's skin. Each point represents the mean ± S.E.M. of six experiments. * denoted significant difference from control, $p < 0.05$.

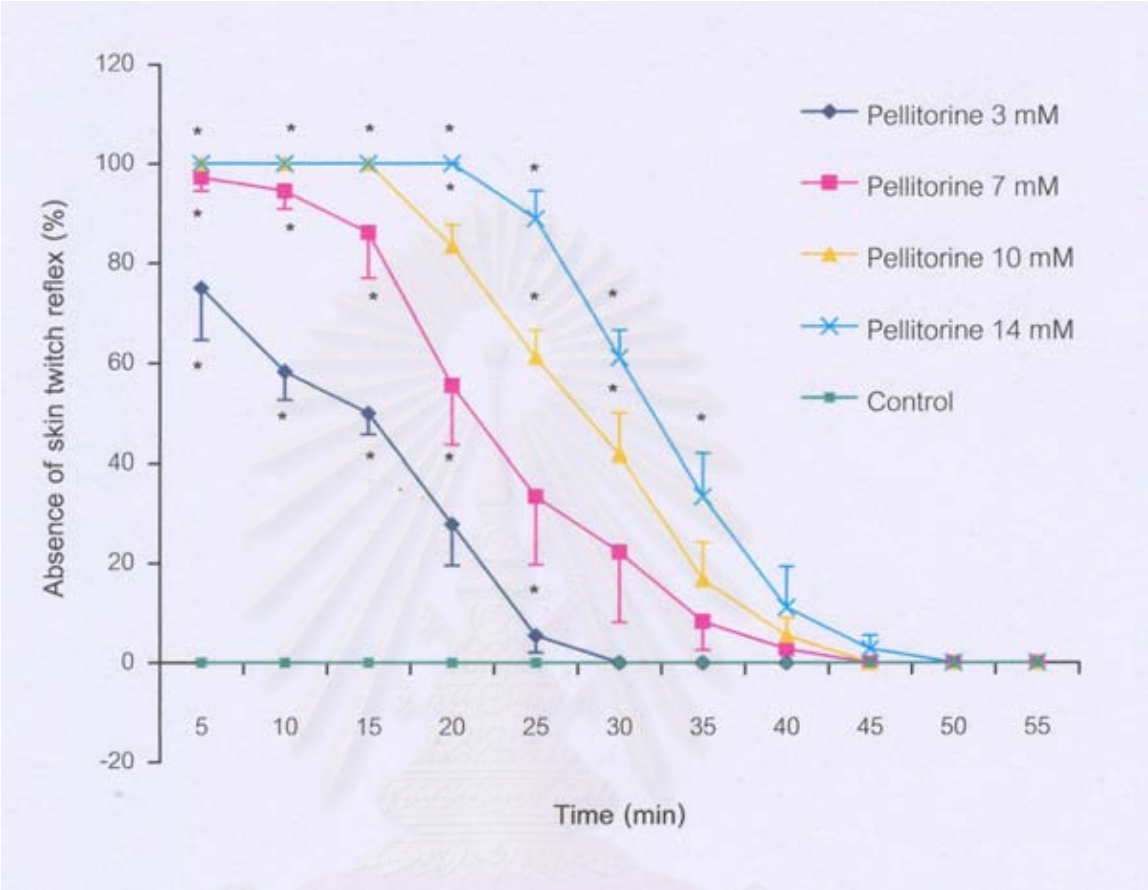


Figure 33. Time-response curves of pellitorine at concentrations of 3, 7, 10, 14 mM and control group in the response test in guinea pig's skin. Each point represents the mean ± S.E.M. of six experiments. * denoted significant difference from control, $p < 0.05$.

Chapter IV
Discussion and Conclusion

A number of *Piper* species are noted for their ethno medical properties (Pongboonrod, 1976) and different parts of *Piper sarmentosum* Roxb has been used for medical purpose for a long time (Perry, 1981). A number of compounds, isolated from fruits of Cha-Plu (*Piper sarmentosum* Roxb.) have been used for a variety of medicinal purpose, for example: pellitorine and asaronaldehyde, the former was shown to exhibit local anesthetic effect *in vitro* experiments (Likhitwitayawuid, 1987, Tantisira et al, 1999). The latter has been reported to contain antifungal activity in *in vitro* experiments (Likhitwitayawuid, 1987).

In the present study, evidence from quantitative analysis by ^{1}H-NMR spectrum of test compound, isolated from fruit of Cha-Plu (*Piper sarmentosum* Roxb.), consisted of 85% pellitorine and the remaining (15%) was asaronaldehyde. Taken into consideration that asaronaldehyde was previously reported not to posses' local anesthetic effect as did pellitorine (Likhitwitayawuid, 1987), therefore, the effects observed in the present studies should be exclusively accounted by pellitorine.

Because of aqueous insolubility of the test compound isolated from Cha-Plu, HPBCD in Ringer's solution was used as vehicle in isolated frog sciatic nerve experiment and HPBCD in NSS were chosen as a solvent to dissolve the test compound in *in vivo* experiment.

Experimentation with frog sciatic nerve was the classical preparation for studying the action potentials, since 1924. Most of the earliest works in evaluating local anesthetic activity have done using frog sciatic nerve because of its large size and length (Harschfelder, 1932).

As illustrated in isolated frog sciatic experiment, no statistical difference between the amplitude of action potential before and after application of 0.1 M HPBCD in Ringer's solution was observed. This finding indicated that HPBCD did not affect the nerve conduction. While, pellitorine exerted a local anesthetic, seen as a reversible blockade of electrically evoked action potential of frog sciatic nerve, in a concentration-dependent manner like lidocaine. Complete blockade of 10, 14, 25 mM of pellitorine and

12, 16, 30 mM of lidocaine was achieved at 155.62 ± 10.24, 137.50 ± 8.66, 73.75 ± 2.26 min and 13.75 ± 1.25, 11.87 ± 1.87, 7.50 ± 0.94 min respectively.

Accordingly, an *in vivo* local anesthetic effect of pellitorine was further illustrated by the mouse tail flick test. The effectiveness of analgesic agents in this model is highly correlated with their effectiveness in relieving pain in humans (Grumbach, 1966). The analgesic action of lidocaine and pellitorine was observed during 90 min period. Pellitorine appeared to produce longer duration of action compared to lidocaine in all concentration (Figure 29 – 30). Initially, area of analgesia produced by 12, 18, 25 mM of lidocaine and pellitorine were compared. The highest concentration of lidocaine (25 mM) produced higher analgesic response compared to all concentration of pellitorine (Figure 27). As shown in figure 31 the EC_{50} of lidocaine and pellitorine against tail flick test was 12 (7 - 20) mM and 16 (6 - 44) mM respectively. Therefore pellitorine posed comparatively weaker local anesthetic effect than lidocaine in tail flick test. Based on the result, pellitorine exerted a local anesthetic in a concentration-dependent manner like lidocaine.

In line with the obtained results, an *in vivo* local anesthetic effect of pellitorine was consequently demonstrated by twitch response test of the guinea pig's skin in which the analgesic effect of pellitorine was clearly noted. Twitch response test in guinea pig's skin has been most widely employed in the evaluation of local anesthetic activity of new compounds. Guinea pig was chosen for the initial screening because, it is easy to handle and especially good for infiltration or regional anesthesia (Thompson, 1990). As shown in figure 32 – 33, pellitorine and lidocaine demonstrated rather similar analgesic effect with the duration of 30 – 50 minutes. Therefore, in addition to being active topically (Likhitwitayawuid, 1987), it is likely that, pellitorine in 0.1 M HPBCD could be beneficial for infiltration block as well. Apparently, local anesthetic effect of pellitorine was not accompanied by serious adverse effects when being given by injection.

The data presented in frog sciatic nerve experiments indicated that the intensity of conduction block of either lidocaine or pellitorine was clearly modulated by the sodium concentration in the bathing fluid, the lower the sodium concentration, the stronger the degrees of the conduction block. For example, as seen in figure 25 – 26: in a 12 mM sodium (sodium deficient Ringer's solution) the spike can be reduced in size

by 50% with a 0.07 mM concentration of lidocaine and when the sodium concentration is elevated to 114 mM (Normal Ringer's solution) the lidocaine concentration now was increased to 3.69 mM in order to give the same degree of effect. Similarly, in a 12 mM sodium (sodium deficient Ringer's solution) the spike can be reduced in size by 50% with a 4.18 mM concentration of pellitorine and when the sodium concentration is elevated to 114 mM (normal Ringer's solution) the pellitorine concentration now was increased to 17.47 mM in order to give the same degree of effect.

As shown in table 2, which lists the local anesthetics and their primary uses (injection sites), as a rule, the actions of the many available local anesthetics are more alike than they are different. Almost any local anesthetic can be made to work for any kind of regional anesthesia (Longnecker, 1997). The choice of local anesthetic agent depends on the planned nerve block, duration of anesthesia, speed of onset and potential toxicity. In practice, a small number of drugs were used for infiltration anesthesia. The most frequently used is lidocaine; moreover, lidocaine can be used in peripheral nerve block, epidural anesthesia, spinal anesthesia, topical anesthesia and IV regional anesthesia (Prithvi, 1996). In the present study, the local anesthetic effect of pellitorine was effective in local infiltration as well as topical application (Likhitwitayawuid, 1987). Furthermore, based on the results obtained it can be concluded that pellitorine possesses *in vitro* as well as *in vivo* local anesthetic effects. The finding that the degrees of conduction block elicited by pellitorine or lidocaine on frog sciatic nerve were markedly increased in sodium deficient Ringer's solution suggests similar mode of action of theses two compounds. Further investigation on the effect of pellitorine on sodium channel is required to elucidate the mechanism of local anesthetic observed. Therefore, pellitorine could become a very versatile drug, like lidocaine. In addition toxicity which may arise should also be evaluated and that may lead to a discovery of a local anesthetic from natural source.

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Appendices

Appendix I

Data of isolated frog sciatic nerve experiments

Group	Count	Percent Height of Action Potential (%)							
		5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min
Control	8	109.14 ± 5.79	113.51 ± 7.69	116.16 ± 8.72	118.06 ± 9.53	119.17 ± 9.01	120.64 ± 9.34	121.55 ± 9.41	121.70 ± 9.42
Lidocaine 12 mM	8	26.82 ± 6.37	4.47 ± 1.94	0.05 ± 0.05	0.00 ± 0.00	0.00 + 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lidocaine 16 mM	8	15.63 ± 7.76	4.89 ± 3.21	0.33 + 57.55	0.00 + 0.00	0.00 + 0.00	0.00 + 0.00	0.00 ± 0.00	0.00 ± 0.00
Lidocaine 30 mM	8	0.36 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pellitorine 10 mM	8	81.36 ± 2.86	73.07 ± 3.56	69.37 ± 3.67	64.49 ± 4.85	61.74 ± 5.29	58.18 ± 5.88	56.54 ± 6.63	54.58 ± 6.19
Pellitorine 14 mM	8	80.74 ± 4.39	66.83 ± 6.45	57.55 ± 7.91	48.92 ± 8.24	42.17 ± 8.80	32.83 ± 7.34	30.91 ± 6.92	28.39 ± 6.65
Pellitorine 25 mM	8	65.77 ± 4.53	46.17 ± 3.90	35.02 ± 4.00	27.78 ± 3.83	23.75 ± 3.33	19.49 ± 3.20	15.79 ± 2.64	12.40 ± 2.24

Table 3. Effect of lidocaine and pellitorine on the action potential of frog sciatic nerve compared with control.

Group	Count	Diameter of nerve (mm)	Mean Time to Reach Complete Block (min)
Lidocaine 12 mM	8	0.78 ± 0.03	13.75 ± 1.25
Lidocaine 16 mM	8	0.89 ± 0.02	11.87 ± 1.87
Lidocaine 30 mM	8	0.83 ± 0.04	7.5 ± 0.94
Pellitorine 10 mM	8	0.87 ± 0.03	155.62 ± 10.24
Pellitorine 14 mM	8	0.76 ± 0.03	137.5 ± 8.66
Pellitorine 25 mM	8	0.88 ± 0.03	73.75 ± 2.26

Table 4. Mean time to reach complete block of electrically evoked action potential of frog sciatic nerve.

Group	Count	Ringer's Solution	Diameter of nerve (mm)	Percent Reduction of Action Potential in 15 min
Lidocaine 0.125 mM	4	Sodium Deficient	0.84 ± 0.03	16.53 ± 10.19
Lidocaine 0.5 mM	4	Sodium Deficient	0.75 ± 0.03	83.22 ± 10.0
Lidocaine 3 mM	5	Sodium Deficient	0.86 ± 0.03	92.18 ± 4.62
Lidocaine 6 mM	3	Sodium Deficient	0.84 ± 0.02	100 ± 0.00
Lidocaine 6 mM	5	Normal	0.78 ± 0.03	49.79 ± 2.85
Lidocaine 8 mM	5	Normal	0.87 ± 0.02	78.82 ± 3.46
Lidocaine 12 mM	5	Normal	0.83 ± 0.02	99.92 ± 0.19
Lidocaine 16 mM	5	Normal	0.86 ± 0.01	99.92 ± 0.08

Table 5. Differential nerve block with lidocaine by altering of the sodium concentration.

Group	Count	Ringer's Solution	Diameter of nerve (mm)	Percent Reduction of Action Potential in 15 min
Pellitorine 0.8 mM	5	Sodium Deficient	0.75 ± 0.03	47.65 ± 6.45
Pellitorine 6 mM	3	Sodium Deficient	0.84 ± 0.01	52.12 ± 1.99
Pellitorine 13 mM	3	Sodium Deficient	0.86 ± 0.02	57.59 ± 6.26
Pellitorine 25 mM	4	Sodium Deficient	0.86 ± 0.03	78.29 ± 10.42
Pellitorine 7 mM	5	Normal	0.82 ± 0.03	34.24 ± 4.49
Pellitorine 10 mM	5	Normal	0.80 ± 0.03	30.22 ± 5.08
Pellitorine 14 mM	5	Normal	0.78 ± 0.03	49.46 ± 5.05
Pellitorine 25 mM	5	Normal	0.87 ± 0.02	65.31 ± 2.55

Table 6. Differential nerve block with pellitorine by altering of the sodium concentration.

Appendix II

Data of mice tail flick test

Test substances	% MPE								
	10 min	20 min	30 min	40 min	50 min	60 min	70 min	80 min	90 min
Lidocaine 4 mM	26.79 ± 16.43	12.81 ± 12.56	5.50 ± 3.86	1.64 ± 1.95	0.21 ± 2.07	2.79 ± 1.52	4.15 ± 1.80	4.15 ± 1.81	3.72 ± 1.88
Lidocaine 6 mM	77.17 ± 13.19	48.77 ± 15.79	27.12 ± 13.80	22.26 ± 8.14	9.24 ± 5.02	1.21 ± 5.01	0.91 ± 4.14	2.22 ± 3.59	2.66 ± 3.09
Lidocaine 8 mM	85.71 ± 14.28	61.88 ± 14.80	43.51 ± 15.59	40.93 ± 14.85	15.61 ± 10.27	19.93 ± 8.48	0.45 ± 3.89	0.94 ± 3.16	1.38 ± 1.99
Lidocaine 12 mM	100 ± 0.00	97.51 ± 1.73	63.40 ± 6.39	35.19 ± 4.60	14.23 ± 3.23	9.07 ± 4.71	1.17 ± 3.60	2.33 ± 2.94	4.60 ± 3.19
Lidocaine 18 mM	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	99.62 ± 0.37	95.65 ± 2.64	54.24 ± 5.75	36.32 ± 4.72	21.41 ± 3.60
Lidocaine 25 mM	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	96.41 ± 2.49	95.52 ± 3.10	86.02 ± 6.03
Pellitorine 10 mM	18.26 ± 8.76	23.87 ± 12.01	24.06 ± 14.84	35.75 ± 16.61	22.69 ± 17.95	36.05 ± 12.61	35.51 ± 15.70	29.59 ± 15.51	29.48 ± 13.47
Pellitorine 12 mM	17.96 ± 10.92	21.91 ± 11.43	31.47 ± 14.84	42.60 ± 13.08	46.01 ± 13.18	26.99 ± 12.99	31.55 ± 12.47	22.49 ± 12.57	21.40 ± 11.72
Pellitorine 14 mM	31.06 ± 9.02	33.66 ± 7.53	31.95 ± 5.99	29.65 ± 6.56	30.83 ± 6.15	43.82 ± 12.72	29.98 ± 8.68	36.50 ± 10.75	28.13 ± 7.86
Pellitorine 18 mM	43.04 ± 10.26	49.14 ± 12.64	58.96 ± 11.91	62.02 ± 9.89	57.11 ± 9.38	49.19 ± 10.25	47.89 ± 10.74	48.67 ± 11.55	49.93 ± 11.64
Pellitorine 25 mM	55.41 ± 14.38	56.75 ± 16.09	69.69 ± 12.20	77.96 ± 10.20	76.04 ± 8.24	81.02 ± 7.65	64.38 ± 12.40	60.55 ± 10.18	62.26 ± 8.68

Table 7. % MPE-Time in mouse tail flick test 0-90 min of test substances. Data present as % MPE ± S.E.M. N=10 per group.

Appendix III

Data of twitch response test in guinea pig's skin

Test Substances	Animal No.	Weight (g)	Injection area	The number of times the needle prick fails to elicit a skin-twitch response											
				5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min	60 min
Lidocaine 4 mM	1	608.21	front	6\6	6\6	6\6	4\6	2\6	2\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 4 mM	2	576.24	front	4\6	6\6	6\6	5\6	4\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 4 mM	3	588.76	front	4\6	6\6	5\6	4\6	2\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 4 mM	4	578.65	back	3\6	6\6	6\6	5\6	3\6	2\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 4 mM	5	508.42	back	5\6	6\6	4\6	2\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 4 mM	6	445.92	back	4\6	6\6	2\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 8 mM	1	382.76	front	6\6	6\6	6\6	6\6	6\6	4\6	1\6	1\6	0\6	0\6	0\6	0\6
Lidocaine 8 mM	2	587.65	front	6\6	6\6	6\6	6\6	6\6	4\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 8 mM	3	508.42	front	6\6	6\6	6\6	6\6	6\6	5\6	4\6	1\6	1\6	0\6	0\6	0\6
Lidocaine 8 mM	4	392.42	back	6\6	6\6	6\6	6\6	5\6	4\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 8 mM	5	576.24	back	6\6	6\6	6\6	6\6	6\6	4\6	2\6	0\6	0\6	.0\6	0\6	0\6
Lidocaine 8 mM	6	588.76	back	6\6	6\6	6\6	6\6	6\6	4\6	1\6	0\6	0\6	0\6	0\6	0\6

0\6 indicates no anesthesia

6\6 indicates maximum anesthesia

Table 8. Analgesic response provided by lidocaine 4 and 8 mM in twitch response test of guinea pig's skin.

Test Substances	Animal No.	Weight (g)	Injection area	The number of times the needle prick fails to elicit a skin-twitch response											
				5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min	60 min
Lidocaine 12 mM	1	368.78	front	6\6	6\6	6\6	6\6	6\6	6\6	6\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 12 mM	2	608.21	front	6\6	6\6	6\6	6\6	6\6	4\6	3\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 12 mM	3	550.82	front	6\6	6\6	6\6	6\6	6\6	6\6	4\6	1\6	0\6	0\6	0\6	0\6
Lidocaine 12 mM	4	445.92	back	6\6	6\6	6\6	6\6	6\6	4\6	2\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 12 mM	5	368.78	back	6\6	6\6	6\6	6\6	6\6	6\6	6\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 12 mM	6	358.74	back	6\6	6\6	6\6	6\6	6\6	4\6	0\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 16 mM	1	578.65	front	6\6	6\6	6\6	6\6	6\6	6\6	6\6	3\6	2\6	1\6	0\6	0\6
Lidocaine 16 mM	2	588.76	front	6\6	6\6	6\6	6\6	6\6	6\6	3\6	1\6	0\6	0\6	0\6	0\6
Lidocaine 16 mM	3	445.92	front	6\6	6\6	6\6	6\6	6\6	6\6	5\6	3\6	1\6	0\6	0\6	0\6
Lidocaine 16 mM	4	508.42	back	6\6	6\6	6\6	6\6	6\6	6\6	5\6	1\6	0\6	0\6	0\6	0\6
Lidocaine 16 mM	5	358.74	back	6\6	6\6	6\6	6\6	6\6	6\6	4\6	0\6	0\6	0\6	0\6	0\6
Lidocaine 16 mM	6	392.42	back	6\6	6\6	6\6	6\6	6\6	6\6	3\6	0\6	0\6	0\6	0\6	0\6

0\6 indicates no anesthesia

6\6 indicates maximum anesthesia

Table 9. Analgesic response provided by lidocaine 12 and 16 mM in twitch response test of guinea pig's skin.

Test Substances	Animal No.	Weight (g)	Injection area	The number of times the needle prick fails to elicit a skin-twitch response											
				5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min	60 min
Pellitorine 3 mM	1	569.07	front	5\6	3\6	3\6	1\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 3 mM	2	410.25	front	6\6	3\6	3\6	1\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 3 mM	3	489.8	front	6\6	4\6	3\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 3 mM	4	447.2	back	2\6	5\6	4\6	3\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 3 mM	5	608.7	back	4\6	3\6	2\6	2\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 3 mM	6	557.4	back	4\6	3\6	3\6	3\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	1	403.56	front	6\6	6\6	6\6	4\6	4\6	4\6	2\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	2	439.7	front	5\6	5\6	6\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	3	403.4	front	6\6	6\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	4	487.6	back	6\6	6\6	6\6	6\6	5\6	4\6	1\6	1\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	5	488.75	back	6\6	5\6	4\6	2\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 7 mM	6	489.8	back	6\6	6\6	6\6	4\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6

0\6 indicates no anesthesia

6\6 indicates maximum anesthesia

Table 10. Analgesic response provided by pellitorine 3 and 7 mM in twitch response test of guinea pig's skin.

Test Substances	Animal No.	Weight (g)	Injection area	The number of times the needle prick fails to elicit a skin-twitch response											
				5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min	60 min
Pellitorine 10 mM	1	410.25	front	6\6	6\6	6\6	5\6	3\6	3\6	2\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 10 mM	2	569.07	front	6\6	6\6	6\6	6\6	5\6	3\6	2\6	1\6	0\6	0\6	0\6	0\6
Pellitorine 10 mM	3	554.43	front	6\6	6\6	6\6	5\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 10 mM	4	550.8	back	6\6	6\6	6\6	5\6	4\6	4\6	2\6	1\6	0\6	0\6	0\6	0\6
Pellitorine 10 mM	5	400.12	back	6\6	6\6	6\6	4\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 10 mM	6	599.64	back	6\6	6\6	6\6	5\6	4\6	3\6	0\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 14 mM	1	400.12	front	6\6	6\6	6\6	6\6	6\6	5\6	4\6	3\6	2\6	1\6	0\6	0\6
Pellitorine 14 mM	2	550.8	front	6\6	6\6	6\6	6\6	6\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 14 mM	3	608.7	front	6\6	6\6	6\6	6\6	5\6	4\6	1\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 14 mM	4	403.4	back	6\6	6\6	6\6	6\6	6\6	4\6	3\6	1\6	0\6	0\6	0\6	0\6
Pellitorine 14 mM	5	554.43	back	6\6	6\6	6\6	6\6	5\6	3\6	1\6	0\6	0\6	0\6	0\6	0\6
Pellitorine 14 mM	6	488.75	back	6\6	6\6	6\6	6\6	4\6	3\6	2\6	0\6	0\6	0\6	0\6	0\6

0\6 indicates no anesthesia

6\6 indicates maximum anesthesia

Table 11. Analgesic response provided by pellitorine 10 and 14 mM in twitch response test of guinea pig's skin.

Test Substances	Animal No.	Weight (g)	Injection area	The number of times the needle prick fails to elicit a skin-twitch response											
				5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min	60 min
0.1 M HPBCD in NSS	1	447.2	front	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
0.1 M HPBCD in NSS	2	608.7	front	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
0.1 M HPBCD in NSS	3	392.42	front	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
0.1 M HPBCD in NSS	4	557.4	back	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
0.1 M HPBCD in NSS	5	382.76	back	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6
0.1 M HPBCD in NSS	6	587.65	back	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6	0\6

0\6 indicates no anesthesia

6\6 indicates maximum anesthesia

Table 12. Analgesic response provided by HPBCD in NSS in twitch response test of guinea pig's skin.

Group	Absence of skin twitch reflex (%)										
	5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min	50 min	55 min
Lidocaine 4 mM	72.22 ± 7.03*	100.0 ± 0.00*	80.55 ± 10.9*	55.55 ± 13.4*	33.33 ± 9.62*	19.44 ± 9.04	2.78 ± 2.78	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lidocaine 8 mM	100.0 ± 7.03*	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	97.22 ± 2.78*	69.45 ± 2.78*	22.22 ± 10.24	11.11 ± 8.24	2.78 ± 2.78	0.00 ± 0.00	0.00 ± 0.00
Lidocaine 12 mM	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	100.00± 0.00*	100.0 ± 0.00*	83.33 ± 7.45*	58.33 ± 15.9*	2.78 ± 2.78	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lidocaine 16 mM	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	72.22 ± 8.23*	22.22 ± 9.30*	8.33 ± 5.69*	2.78 ± 2.78	0.00 ± 0.00
Pellitorine 3 mM	75.00 ± 10.3*	58.33 ± 5.69*	50.00 ± 4.30*	27.78 ± 8.24	5.56 ± 3.51	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pellitorine 7 mM	97.22 ± 2.78*	94.44 ± 3.54*	86.11 ± 9.04*	55.56 ± 11.9*	33.33 ± 13.6*	22.22 ± 14.05	8.33 ± 5.69	2.78 ± 2.78	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pellitorine 10 mM	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	83.33 ± 4.30*	61.11 ± 5.52*	41.67 ± 8.33*	16.66 ± 7.45	5.56 ± 3.51	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pellitorine 14 mM	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	100.0 ± 0.00*	88.89 ± 5.55*	61.11 ± 5.55*	33.33 ± 8.61*	11.11 ± 8.24	2.78 ± 2.78	0.00 ± 0.00	0.00 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

* denoted significant difference from control, $p < 0.05$

Table 13. Percent absence of skin twitch reflex elicited by lidocaine and pelliitorine compared with control in twitch response test of guinea pig's skin.

VITAE

Miss Piyaluk Sapprasert was born on 12th February 1977, in Samutprakarn, Thailand. She had graduated with Bachelor of Science (Physiotherapy), Mahidol University in 1999. After graduation, she studies in Chulalongkorn University for the Degree of Master of Science in Physiology since, 2000. She received the local graduate scholarship from the National Science and Technology Department Agency, NSTDA while in Chulalongkorn University. Her research on local anesthetic effect of a semi-purified compound isolated from fruit of *Piper sarmentosum* Roxb. has been presented as poster presentation in the sixth joint seminar of JSPS-NRCT Core University Exchange System on Natural Medicine in Pharmaceutical Sciences " Recent Advances in Natural Medicine Research" on December, 2003, Bangkok, Thailand. In the same year, 2003, she earned the M.S. degrees in physiology from Inter-Department, Graduate School, Chulalongkorn University.