ผลของสารสกัดใบกระท่อมด้วยเอทานอลต่อการเสพติดมอร์ฟีนในหนูเมาส์และหนูแรท

นางสาว สุภาภรณ์ อ้วนละไม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF THE ETHANOLIC EXTRACT OF *MITRAGYNA SPECIOSA* LEAVES

ON MORPHINE ADDICTION IN MICE AND RATS

Miss Supaporn Aunlamai

A Thesis Submitted in Partial Fulfillment of the Requirements

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สุภาภรณ์ อ้วนละไม: ผลของสารสกัดใบกระท่อมด้วยเอทานอลต่อการเสพติด
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OF *MITRAGYNA SPECIOSA* LEAVES ON MORPHINE ADDICTION IN
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        กระท่อมมีชื่อวิทยาศาสตร์ว่า *Mitragyna speciosa*   อยู่ในวงศ์ Rubiaceae มีการนำมาใช้โดย
ตำรับยาไทยแผนโบราณมาเป็นเวลานาน ในการรักษาอาการปวด ลดไข้ แก้ไอ แก้ท้องเสีย ลดอาการติดฝิ่น
และยังช่วยเพิ่มประสิทธิภาพและความทนต่อการทำงานภายใต้แสงแดดจัด ในปัจจุบันยังไม่มีหลักฐานที่แน่ชัด
ของฤทธิ์เสพติดของกระท่อมในสัตว์ทดลอง ดังนั้นงานวิจัยนี้จึงมุ่งศึกษาถึงผลของสารสกัดใบกระท่อมด้วยเอ
ทานอล ต่อการเคลื่อนไหว ฤทธิ์เสพติด และการเสพติดมอร์ฟีนในสัตว์ฟันแทะ โดยใช้วิธีทดสอบ 3 วิธีได้แก่
การประเมินการเคลื่อนไหว การประเมินพฤติกรรมการชอบสถานที่แบบมีเงื่อนไข และการทำให้เกิดอาการถอน
ยาด้วยตัวปิดกั้นตัวรับของฝิ่น ผลการทดลองพบว่ามอร์ฟีนขนาด 5 มก./กก. โดยการฉีดเข้าทางช่องท้อง ทำให้
เกิดการชอบสถานที่แบบมีเงื่อนไขอย่างมีนัยสำคัญทางสถิติ ในขณะที่สารสกัดใบกระท่อมด้วยเอทานอลในทุก
ขนาด (50, 100, 200 และ 400 มก. /กก. โดยการป้อน) ไม่มีผลต่อการเคลื่อนไหวและไม่ทำให้เกิดพฤติกรรม
การชอบสถานที่แบบมีเงื่อนไข และพบว่าสารสกัดใบกระท่อมด้วยเอทานอลในทุกขนาดสามารถยับยั้ง
พฤติกรรมการชอบสถานที่แบบมีเงื่อนไขจากการเหนี่ยวนำด้วยมอร์ฟีนได้ นอกจากนี้พบว่าการได้รับสารสกัด
ใบกระท่อมด้วยเอทานอลเมื่อให้แบบครั้งเดียวและแบบต่อเนื่องในทุกขนาดไม่ทำให้เกิดอาการถอนยาจากการ
ได้รับตัวปิดกั้นตัวรับของฝิ่นอย่างมีนัยสำคัญทางสถิติแต่อย่างใด แต่มอร์ฟีนทำให้เกิดอาการถอนยาได้แก่ การ
กระโดด หางตั้งตรง หางม้วนเป็นรูปตัวซี และการสะบัดตัวอย่างมีนัยสำคัญทางสถิติ จากนั้นจึงทำการ
ประเมินผลของการได้รับสารสกัดใบกระท่อมด้วยเอทานอลก่อนและหลังการเหนี่ยวนำให้เสพติดมอร์ฟีน พบว่า
การได้รับสารสกัดใบกระท่อมด้วยเอทานอลทั้งก่อนและหลังการเหนี่ยวนำให้เสพติดมอร์ฟีนในทุกขนาด
สามารถลดการกระโดดจากการได้รับนาลอกโซนอย่างมีนัยสำคัญทางสถิติ
        จากผลการศึกษาทั้งหมดสรุปได้ว่า สารสกัดใบกระท่อมด้วยเอทานอลไม่มีฤทธิ์กดหรือกระตุ้น
ระบบประสาทส่วนกลางที่ควบคุมการเคลื่อนไหว และฤทธิ์เสพติด และไม่ทำให้เกิดอาการถอนยาเมื่อให้แบบ
ครั้งเดียวและแบบต่อเนื่อง นอกจากนี้ยังสามารถยับยั้งพฤติกรรมการชอบสถานที่แบบมีเงื่อนไขจากการ
เหนี่ยวนำด้วยมอร์ฟีน และอาการถอนยาของมอร์ฟีนได้อีกด้วย ดังนั้นสารสกัดใบกระท่อมด้วยเอทานอลจึงอาจ
มีประโยชน์ในการรักษาการเสพติดมอร์ฟีนและสารเสพติดชนิดอื่นๆในกลุ่มฝิ่นได้

สาขาวิชา...........เภสัชวิทยา.............ลายมือชื่อนิสิต...
ปีการศึกษา...........2553..................ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.............
                                      ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม..............

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KEYWORDS : ADDICTION / *MITRAGYNA SPECIOSA* / MORPHINE / CONDITIONED PLACE PREFERENCE / WITHDRAWAL

SUPAPORN AUNLAMAI: EFFECTS OF THE ETHANOLIC EXTRACT OF *MITRAGYNA SPECIOSA* LEAVES ON MORPHINE ADDICTION IN MICE AND RATS.   ADVISOR: ASST. PROF. FLG. OFF. PASARAPA TOWIWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. THONGCHAI SOOKSAWATE, Ph.D., 103 pp.

Mitragyna speciosa Korth. (Rubiaceae) has been used for the treatments of pain, fever, cough, diarrhea, opioid-addiction in Thai traditional medicine for a long time and also for enhancing the labor work efficiency and tolerance under the hot sunshine atmosphere. Until now, there is no clear evidence of the rewarding effects of *M. speciosa* in animal models. The present study was aimed to determine the effects of the ethanolic extract of *M. speciosa* leaves (MS) on the locomotor activity, rewarding effect and morphine addiction in rodents. Three models including locomotor activity test, conditioned place preference test (CPP) and precipitated withdrawal with opioid antagonists were utilized in this study. The results showed that morphine (5 mg/kg i.p.) could induce significant CPP while all doses of MS (50, 100, 200 and 400 mg/kg p.o.) neither changed locomotor activity nor produced CPP. The morphine-induced CPP was suppressed by all doses of MS. In precipitated withdrawal models of acute and chronic MS treatments, all doses of MS did not show any significant withdrawal symptoms. In contrast, morphine exhibited significant withdrawal symptoms including jumping, straub tail, C-shaped tail, and wet dog shakes. Then the effects of pretreatment and post-treatment of MS on morphine withdrawal were evaluated. The results showed that both pretreatment and post-treatment with all doses of MS significantly attenuated jumping behavior precipitated by naloxone (p<0.05).

In conclusions, MS did not have psychostimulating, sedative or rewarding effects and did not produce any significant precipitated withdrawal symptoms after acute or chronic uses. Furthermore, it could suppress morphine-induced CPP and morphine withdrawal symptoms. The results suggest that MS might be helpful in the treatment of morphine and other opioid addiction.

Field of Study :   Pharmacology   Student's Signature

Academic Year :   2010   Advisor's Signature

                                             Co-advisor's Signature

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Ach	Acetylcholine
BW	Body weight
Ca^{2+}	Calcium ion
CAE/g	Catechin acid equivalent per gram
°C	Degree Celsius
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
cm	Centrimeter
CPP	Conditioned place preference
CREB	cAMP-response-element blinding protein
δ	Delta opioid receptor
DA	Dopamine
DSM	Diagnostic and Statistical Manual of Mental Disorders
DPPH	2,2-diphenyl-1-picrylhydrazyl
e.g.	Exempli gratia (for example)
etc.	Et cetera (and so on/and so forth)
FST	Forced swimming test
FACS	Flow cytometry analysis
GAE/g	Gallic acid equivalent per gram
GABA	Gramma-aminobutyric acid
GLUT	Glucose transporters
GLU	Glutamatergic
HBV	Hepatitis B
HBC	Hepatitis C
HIV	Human immunodeficiency virus
IC_{50}	The half maximal inhibitory concentration

i.e.	id est (that is)
κ	Kappa opioid receptor
MS	*Mitragyna speciosa*
μ	Mu opioid receptor
NE	Norepinephrine
NAc	Nucleus accumbens
i.p.	Intraperritoneal
K^+	Potassium channel
LAMM	Levomethadyl acetate
LC	Locus coeruleus
LD_{50}	The half maximal lethal dose
min	Minute
mg/kg	Milligram per kilogram
ml	Milliliter (s)
mg/ml	Milligram per milliliter
MICs	Minimum inhibitory concentrations
MAP	Mitogen-activated protein kinase
p.o.	Per oral
PKA	Protein kinase A
PLC	Phospholipids C
SA	Self-administration
Sec	Second
5-HT	5-hydroxytryptamine (serotonin)
S.E.M.	Standard error of means
TST	Tail suspension test
$\triangle^9$-THC	Delta-9- tetrahydrocannabinol
VTA	Ventral tegmental area
μg/ml	Microgram per milliliter

CHAPTER I

INTRODUCTION

Background and Rationale

Drug addiction and drug dependence were major health and social issues worldwide including Thailand. The Thai government has used several strategies to control it. Some of the common drugs of abuse are methamphetamine, cocaine, marijuana, heroin, and opium, etc.. Although the government has tried to control this problem by enforcing the laws, many difficulties still exist (Assanangkornchai et al., 2006). Regulatory response to illicit drug use is composed primarily of three components including enforcement, prevention, and treatment. Drug dependence is a chronic illness that is treatable if the treatment is well delivered and tailored to the needs of the particular patient. There is indeed an array of treatments that can effectively reduce drug use, help manage drug cravings, prevent relapses and restore people to productive social functioning. The treatment of drug dependence will be part of long-term, medical, psychological, and social perspectives (Baltieri et al., 2004).

Of the various substance use disorders, opioid dependence syndrome has a significant major impact on mortality and morbidity and increase health care costs. With the availability of parenterally administered opiates and the invention of the hypodermic syringe, opiate addiction and opiate withdrawal distress became major public health problems (Fernandez, 1998).

Opioid analgesics are the most effective and frequently used in patients with malignant and nonmalignant pain. Morphine which is an opiate acting through μ-opioid receptor is widely used as a potent analgesic which suppresses moderate to severe pain. Nevertheless, its use is limited due to several side effects including nausea, vomiting, sedation, euphoria, constipation, drowsiness and respiratory depression. Moreover, long term administration produced tolerance and dependence as a result of its rewarding properties (Gutstein and Akil, 2001; Haghparast et al., 2008). Heroin

(diacetylmorphine) is a highly lipophilic derivative of morphine. It acts through μ-opioid receptor same as morphine. It is more potent and rapid acting than morphine because of rapid crossing through blood-brain barrier and has a high abuse potential (Sawynok, 1986; Rook et al., 2006).

Nowadays, there is not an effective medicinal drug used to treat opioid addiction. The effective treatment needs to be combined the psychopharmacological with psychosocial treatments (Veilleux et al., 2010). Drug treatment of opioid addiction composed of detoxification and long term maintenance. The first group of medicinal drugs used for opioid addiction treatment is opioid agonist (e.g., methadone, LAAM, buprenorphine). Methadone and LAAM act through μ-opioid receptor same as morphine, therefore, they are considered highly addictive themselves and are administered under controlled conditions (Ling and Compton, 2005). They have many side effects including nausea, vomiting, constipation, sedative and respiratory depression. The second group is opioid antagonists (e.g., naloxone and naltrexone) which block opioid receptors, rendering subsequent opioid ingestion ineffective and precipitate withdrawal symptoms (Comer et al., 2006). The third group is non-opioid drug such as α_2-adrenergic agonists (e.g., clonidine and lofexidine) which are used to treat withdrawal symptoms. The side effects of these drugs are sedative and hypotension (Gowing et al., 2008b). The major concern of opioid addiction treatments at present is the recurrent of opioid addiction and many adverse effects associated with drugs used for the treatment. Many researchers have tried to find other drug which is more effective with fewer side effects and less expensive. Thus, the natural product may be an alternative choice because of its availability and lower cost.

Mitragyna speciosa Korth., called kratom in Thai, is a tropical plant found in the Southeast Asia countries including Thailand, Malaysia, Indonesia and Myanmar. In Thai traditional medicine, kratom leaves have been used for the treatment of pain, fever, wound, cough, and diarrhea. Furthermore, it has also been used to increase labor work

efficiency and tolerance under the hot sunshine atmosphere (Jansen and Prast, 1988). Additionally, it was often used for opiate-addiction treatment, self treatment of opioid withdrawal and as a replacement for opium when opium is unavailable (Jansen and Prast, 1988; Boyer et al., 2008). The major constituent of *M. speciosa* leaves, mitragynine, has been shown to act on noradrenergic and serotonergic systems and has an opioid-liked effect (Matsumoto et al., 1996b). The ethanolic extract of *M. speciosa* leaves (MS) has been shown to possess numerous pharmacological effects including antinociception, anti-inflammation in rats and suppression of ethanol withdrawal in mice (Matsumoto et al., 1996b; Kumarnsit et al., 2007; Shaik Mossadeq et al., 2009). Although *M. speciosa* leaves are a controlled substance listed in the Thailand Narcotic Act since 1943, there is no clear evidence of their rewarding effects in animal models. Thus, the aim of this study was to investigate the rewarding effects of MS and the effect of MS on morphine addiction using conditioned place preference (CPP) model in rats and precipitated withdrawal model in mice.

Objectives

To study the rewarding effects of the ethanolic extract of *Mitragyna speciosa* leaves and the effects of the extract on morphine addiction in mice and rats.

Hypothesis

The ethanolic extract of *Mitragyna speciosa* leaves has no rewarding effect, and has an ability to prevent and decrease morphine withdrawal symptoms.

Keywords

Addiction / *Mitragyna speciosa* / Morphine / Conditioned Place Preference / Withdrawal

CHAPTER II

LITERATRUE REVIEWS

Definitions of drug addiction

Drug addiction or substance dependence is a chronically relapsing brain disorder characterized by compulsion to seek and take the drug, loss of control in limiting intake, occupational and recreational activities in lieu of drug seeking or self-administration behavior, reduction in engagement in social and emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when access to the drug is prevented. Continued use induces adaptive changes in the central nervous system that lead to tolerance, physical dependence, sensitization, craving, and relapse (Koob and Le Moal, 2005; Gass and Olive, 2008).

Drugs that are addictive in humans including cocaine, amphetamine, cannabinoids, nicotine, alcohol, opioids and morphine have reward values that are they motivate actions to acquire them. If a particular action, such as seeking out a drug dealer or in laboratory animals pressing a lever directly results in a drug reward, the association between the action and the reward is strengthened. Reward pathways are strongly activated by drugs of abuse not only in humans, but also in many animal models including rodents, insects and fish. The primitive nature of reward learning and the universal ability of abuse for drug target reward systems, affords us the opportunity to model drug addiction in animals (Mohn et al., 2004).

Patients with drug dependence are typically using one or more other substances and have additional problems in the psychiatric, medical, family, social, employment or legal areas. Problems of substance dependence produce dramatic costs to all societies in terms of lost productivity, transmission of infectious diseases, family and social problems, crime, and excessive utilization of health care.

Diagnostic DSM-IV substance dependence criteria

Addiction (The World Health Organization, 1992 and American Psychiatric Association, 1994) is defined as a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three or more of the followings, to define the behavioral syndromes in which the individual uses a drug in a maladaptive manner in a 12 month period leading to significant impairment in function or distress and accompanied by evidence of tolerance and withdrawal.

Diagnostic criteria (three or more)

1) Tolerance, as defined by either of the following

a. a need for markedly increased amounts of the substance to achieve intoxication or desired effect.

b. markedly diminished effect with continued use of the same amount of the substance.

2) Withdrawal, as manifested by either of the following

a. the characteristic withdrawal syndrome for the substance.

b. the same (or closely related) substance is taken to relieve or avoid withdrawal symptoms.

3) The substance is often taken in larger amounts or over a longer period than was intended.

4) There is a persistent desire or unsuccessful efforts to cut down or control substance use.

5) A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors, driving long distances), use the substance (e.g., chain smoking), or recover from its effects.

6) Important social, occupational, or recreational activities are given up or reduced because of substance use.

7) The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.

The definitions of terms used in drug addiction are shown in Table 2.1

Table 2.1. Definitions of terms used in drug addiction (Cami and Farre, 2003)

Craving: Formerly called psychological dependence is an intense desire to reexperience the effects of a psychoactive substance. Craving is the cause of relapse after long periods of abstinence.

Physical or physiological dependence: Is an outdated term that refers to physical tolerance and the withdrawal symptoms.

Relapse: The recurrence on discontinuation of an effective medical treatment of the original condition from which the patient suffered.

Reward: Is a stimulus that the brain interprets as intrinsically positive or as something to be attained.

Sensitization: Is the increase in the expected effect of a drug after repeated administration. It is a progressive increase in an effect of a drug with repeated use or a hypersensitivity to an effect of the drug as a consequence of past exposure to the drug. Sensitization is one of the neurobiological mechanisms involved in craving and relapse.

Substance abuse: Is characterized by recurrent and clinically significant adverse consequences related to the repeated use of substances, such as failing to fulfill major role obligations, use of drugs in situations in which it is physically hazardous, occurrence of substance-related legal problems, and continued drug use despite the presence of persistent or recurrent social or interpersonal problems.

Substance dependence: For substance dependence to be diagnosed, at least three of the following must be present symptoms of tolerance, symptoms of withdrawal, the use of a substance in larger amounts or for longer periods than intended; persistent desire or unsuccessful attempts to reduce or control use or recreational activities because of drug use and continued use of a substance despite attendant health, social, or economic problems.

Withdrawal syndrome: is a cluster of symptoms that result from abstinence from the drug or from pharmacological precipitation.

The brain reward system: the mesocorticolimbic dopamine system

The brain reward system plays a central role in compulsive drug taking and addiction and is located within mesocorticolimbic dopamine systems originating in the ventral tegmental area (VTA) of midbrain and projecting to the limbic system especially, nucleus accumbens (NAc), amygdala, and the prefrontal cortex. All addictive drugs including opiate, cocaine, amphetamine, nicotine, cannabinoids, and alcohol act on this system, through different mechanisms, and activation causes euphoria (Koob, 2003).

Neurotransmitter systems involved in the reward pathway

The primary neurotransmitter of the reward pathway is dopamine. Although drugs of abuse often act through separate mechanisms and on various locations in the brain reward system, they share a final common action in that they increase dopamine levels in the brain reward system. However, neurotransmitter systems are inextricably intertwined. Thus serotonin, glutamate and GABA also modulate dopamine levels in the brain reward pathway (Torregrossa and Kalivas, 2008).

Serotonin

Even though increased dopamine in the brain reward system is generally thought to be the final common pathway for the reinforcing properties of drugs, serotonins are involved in the modulation of both drug self-administration and dopamine levels. Serotonin may be important in modulating motivational factors, or the amount of work and individual is willing to perform to obtain a drug. Serotonergic neurons project both to the NAc and VTA and appear to regulate dopamine release at the NAc (Torregrossa and Kalivas, 2008).

Glutamate

Glutamatergic projections from the prefrontal cortex, amygdala and hippocampus increase dopamine release in the NAc directly or via the VTA. Alterations in these circuits may be fundamental to addiction since glutamate not only modulates dopamine levels but is linked to reinforcing or pleasurable experiences from drugs of abuse (Torregrossa and Kalivas, 2008).

Gamma-aminobutyric acid (GABA)

GABA, another neurotransmitter involved in the modulation of dopaminergic reward system, plays a role in the mediation of effects of many drugs of abuse. GABA is an inhibitory neurotransmitter located diffusely throughout the brain. Drugs of abuse act on the GABA receptor to hyperpolarize neurons. When a neuron is hyperpolarized, it is inhibited from firing. When neurons fire, they release neurotransmitter, and since drugs of abuse inhibit these neurons, they release less GABA (Torregrossa and Kalivas, 2008).

Dopamine

Drugs of abuse have been shown to increase dopamine (DA) neurotransmitter levels in the reward pathway. DA belongs to group of neurotransmitters called cathecholamines. DA constitutes about 80% of the cathecholamines content in the brain. It is synthesized from the amino acid precursor, tyrosine, which has to be taken up through the blood brain barrier by a transporter into the dopaminergic cells. The mechanism of dopamine synthesis is shown in Figure 2.1. Role of DA are mediated motor, emotion, learning, memory, motivation, pleasure euphoria and reward. DA systems are projections originating from brain areas that synthesize this neurotransmitter give rise to three pathways: nigrostriatal, mesocorticolimbic, and tuberoinfundibular dopamine pathways. The mesocorticolimbic dopamine pathway plays an important role in rewarding effects (Vallone et al., 2000; Torregrossa and Kalivas, 2008).
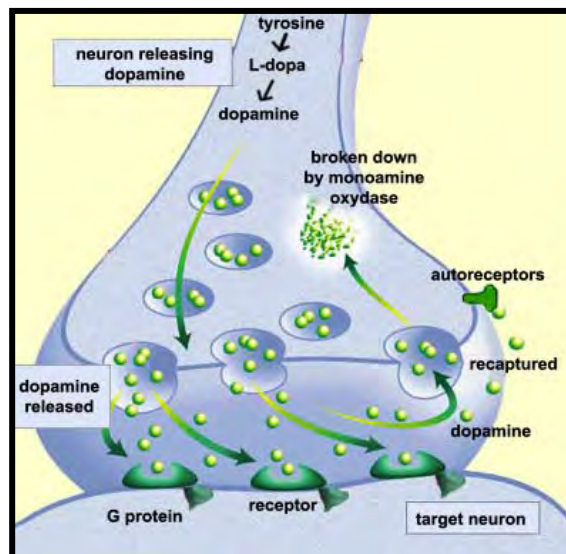
Figure 2.1. Synthesis of dopamine, the first step in the synthesis of catecholamines is the hydroxylation of tyrosine to DOPA, by tyrosine hydroxylase, which is also the rate limiting enzyme in the synthetic cascade (Youdim et al., 2006).

Mechanisms of drug addiction

Drug addiction is a complex process. Interaction of many brain nuclei with neurotransmitters or neuropeptides constitutes a neural network, which affects the intracellular signal transduction and is involved in drug addiction (Wang et al., 2005). Despite of operating through different mechanisms, drugs of abuse has a final common pathway that is the brain reward circuit. They are consists of inter-connected structures that include components of the mesocorticolimbic dopamine system which has been implicated in the control of the reward mechanism. They originate a common pathway for all drugs of abuse which is the projection from the VTA in the midbrain to the NAc, part of the basal ganglia and the prefrontal cortex, among other structures. The VTA consists of dopaminergic and GABAergic (γ-aminobutyric acid) neurons. Both neurons project to the NAc and to the prefrontal cortex. The GABAergic neurons are projection neurons and also have local connections onto dopamine cells. In addition to ordinary synaptic release of dopamine from nerve terminals, dopamine is also released from somatic and dendritic sites within the VTA. Drugs of abuse enhance the signaling of DA neurons located in the VTA that project to the NAc via the medial forebrain bundle by the action of increasing dopamine levels (Koob, 1992; Pierce and Kumaresan, 2006).

Neurobiological substrates and brain areas for the acute reinforcing effects of drugs of abuse are shown in Table 2.2

Table 2.2. Neurobiological substrates for the acute reinforcing effects of drugs of abuse (Koob and Le Moal, 2008)

Drug of abuse	Neurotransmitter	Site
Cocaine and amphetamines	Dopamine γ-aminobutyric acid	Nucleus accumbens Amygdala
Opiates	Opioid peptides Dopamine Endocannabinoids	Ventral tegmental area Nucleus accumbens
Nicotine	Nicotinic acetylcholine Dopamine γ-aminobutyric acid Opioid peptides	Nucleus accumbens Ventral tegmental area Amygdala
Δ^9-Tetrahydrocannabinol	Endocannabinoids Opioid peptides Dopamine	Nucleus accumbens Ventral tegmental area
Alcohol	Dopamine Opioid peptides γ-aminobutyric acid Endocannabinoids	Nucleus accumbens Ventral tegmental area Amygdala

Opioids

Opium is the dried latex obtained from juice of the opium poppy (*Papaver somniferum*). The constituents of opium were such as resins, sterols, triterpenoid, fatty acid, polysaccharides, and more than 25 alkaloids. The alkaloids consist of morphine 4-21%, codeine 0.7-3%, papaverine 0.5-1.3%, thebaine 0.5-2.5%, and noscapine 2-8%. Both morphine and codeine have an analgesic action. All compounds related to opium and all agonists and antagonists with morphine-like activity as well as naturally occurring and synthetic opioid peptides are opioids. Opiates are drugs derived from opium, naturally occurring alkaloids and semi-synthetic derivatives of morphine (Trescot et al., 2008).

Epidimology of opioid addiction

During recent decades, opioids addiction has become a malignant social phenomenon in world wide, causing widespread social, psychological, familial, and economic calamity. In addition, the rise of drug abuse has led to the rapid emergence of infectious diseases, including Human Immunodeficiency Virus (HIV) and Hepatitis B and C Virus (HCB and HCV). The increased incidence of infectious disease and substance abuse has led to increasing recognition of the importance of providing drug abuse treatment rather than relying only on law enforcement approaches (Mokri, 2002). Around 2000- 2001 the number of opium or heroin abusers was estimated at almost 15 million (0.2%) of the world population. In 2002 the main illegal opium producing countries were Afghanistan (76%), Myanmar (18%), Laos (2%) and Colombia (1%). In 2006, the number of current heroin users increase to 338,000, which are up from 0.06% to 0.14% in the United States. Illegally diverted prescription opioids are increasingly the primary illegal opioids. These include hydromorphone, oxycodone, codeine, meperidone, morphine and hydrocodone (Tang et al., 2006; SAMHSA, 2008).

Although opioids are useful analgesics for clinical use, major problems associated with the chronic use of opioids are tolerance and dependence. In addition, long-term use of opioids can lead to serious physical and psychological dependence,

abnormal pain sensitivity, cognitive dysfunction, hormonal changes, immune modulation, psychiatric disorders such as bipolar disorder, anxiety disorders and depression (Ballantyne and Mao, 2003; Mathers et al., 2009).

Opioid receptors

Opioids are compounds acting by binding to specific opioid receptors and mediating their action through the opioid system. The properties of opioids derive from interactions with μ-, δ- and κ-opioid receptors (Table 2.3). Endogenous opioids peptides, like the opioid alkaloids, include both natural and synthetic substance binding. These receptors are normally stimulated by endogenous peptides with enkephalins, endorphins and dynorphins, respectively. Opioid receptors and their endogenous peptide are largely distributed through the CNS and peripheral organs. This wide distribution is related to the important role that the opioid system plays in the control of several physiological responses including nociception, emotional behavior, learning and memory and regulation of reward circuits. The activation of μ- and δ-opioid receptors results in analgesia and reward. Activation of κ-opioid receptors results in analgesia and aversion. Rewarding properties of drugs may contribute to their abuse liability. (Low et al., 2000; Trescot et al., 2008).

Table 2.3. Opioid receptor subtypes (Trescot et al., 2008)

Receptor type	Endogenous opioids	Result of stimulation	Opioid receptor coupling
μ	Enkephalins	supraspinal analgesia, respiratory depression, sedation, euphoria	adenylyl cyclase inhibition, K^+ channel activation, Ca^{2+} channel inhibition, IP_3 inhibition
δ	Endorphins	spinal analgesia, sedation	adenylyl cyclase inhibition, K^+ channel activation, Ca^{2+} channel inhibition
κ	Dynorphins	spinal analgesia, dysphoria, tachycardia	adenylyl cyclase inhibition, K^+ channel activation, Ca^{2+} channel inhibition PLC activation

Classification of opioids (Gutstein and Akil, 2001)

Opioid agonist

Include natural opium alkaloids (e.g. opium, morphine, codeine and tebaine), semi-synthetic opioids (e.g. heroine, oxycodone, hydrocodone, oxymorphone and hidromorphone) and synthetic opioids (e.g. mepridine, levorphanol, methadone, sufentanil, alfentanil, fentanyl, remifentanil, propoxyphene, and levomethadyl).

Mixed opioid agonist-antagonist drugs

Nalbuphine and pentazocine have agonist activity at some receptors and antagonist activity at other receptors. It also included the partial agonists e.g. butorphanol, and buprenorphine.

Opioid antagonists

Naloxone and naltrexone are opioid antagonists and do not have agonist activity at any of the receptor sites. Antagonists block the opioid receptors, inhibit pharmacological activity of the agonist, and precipitate withdrawal in dependent patients.

Pharmacology of specific opioids

Morphine

Morphine is a strong opiate acting through μ-opioid receptor and possessing strong rewarding properties (Figure 2.2). It is a gold standard treatment with which other pain relievers are compared. Although morphine is accepted as an irreplaceable tool in pain management, it has several side effects. Adverse effects produced by morphine include among others nausea, vomiting, sedation, euphoria, miosis, drowsiness, constipation and respiratory depression. In addition, prolonged use of morphine can cause tolerance to its analgesic effect as well as psychological and physical dependence. However, the appropriate dosage and proper administration intervals allow avoiding dependence (Freye and Latasch, 2003; Ziegler, 2005).
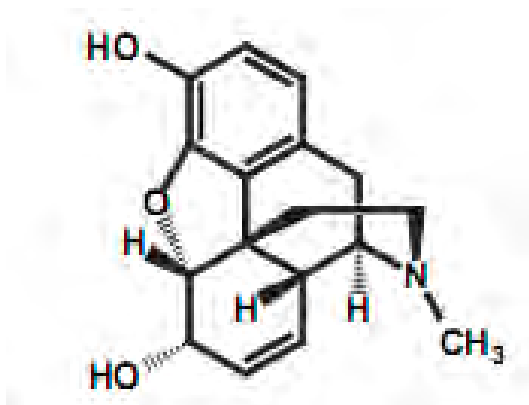
Figure 2.2 Chemical structure of morphine (Matsumoto et al., 2008)

Heroin

Heroin (diacetylmorphine) is a highly lipophilic derivative of morphine by acetylating with acetic anhydride (Figure 2.3), which is more potent and acting faster than morphine. The rapid penetration into the brain as well as the fast onset of action contributes to a high abuse potential of heroin a high affinity to μ-opioid receptors, which are responsible for its morphine-like effects. Because the effects of morphine and heroin are mediated through the same mechanism, both drugs share common therapeutic effects and there is no marked difference in side effects (Rook et al., 2006).
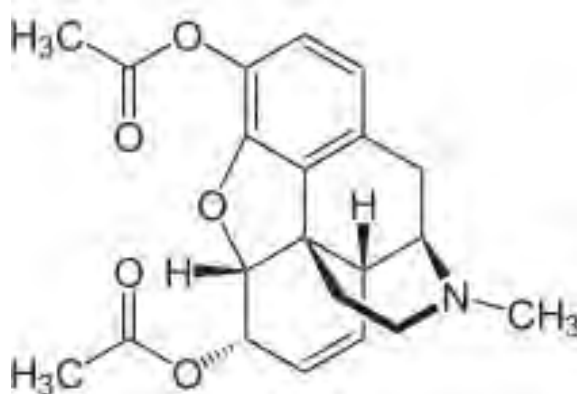
Figure 2.3 Chemical structure of heroin (Rook et al., 2006)

Mechanism of opioid dependence

Many brain regions are involved in the opioid addiction including VTA, NAc, locus ceruleus, periaqueductal gray, medial thalamus, hypothalamus, amygdala, globus pallidus, nucleus raphe magnus, gigantocellular reticular nucleus, and substantia nigra. Among these brain regions, the VTA and NAc, important components of mesocorticolimbic dopaminergic pathways, mediating reinforcement processes opioid dependence (Spanagel and Weiss, 2002; Wang et al., 2005).

Opioids have very high addictive potential and the reinforcing affects of these drugs. They are induced by activation of the mesocorticolimbic dopamine reward pathway (Koob, 1992; Xi and Stein, 2002). This reward pathway plays a critical role in addiction of any abused substance or addictive behavior. The rewarding and analgesic effects of opioids are mediated mostly by activation of the μ-opioid receptors, which widely distributed in the CNS and are predominantly located on GABAergic cells in the VTA and NAc (Haberstock-Debic et al., 2003; Xi and Stein, 2002). This activation occurs indirectly via opiate inhibition of inhibitory GABAergic interneurons in the VTA leading to a decrease in GABA release (Solecki et al., 2005), which disinhibits dopaminergic neurons resulting in enhanced extracellular levels dopamine release both in the dorsal and ventral striatum, NAc, amygdala as well as in the prefrontal cortex and orbitofrontal cortex. Opiates also directly affect NAc neurons, independent of dopamine, via activation of opioid receptors expressed by these neurons. Dopaminergic neuronal lesions of the NA or the VTA have been shown to either reduce or eliminate opiate reinforced behavior. Opiate administration into the VTA leads to an increase in dopamine release and promotes further self-administration (Koob, 2003). The proposed sites of action of the various drugs of abuse in these circuits are shown in Figure 2.4.
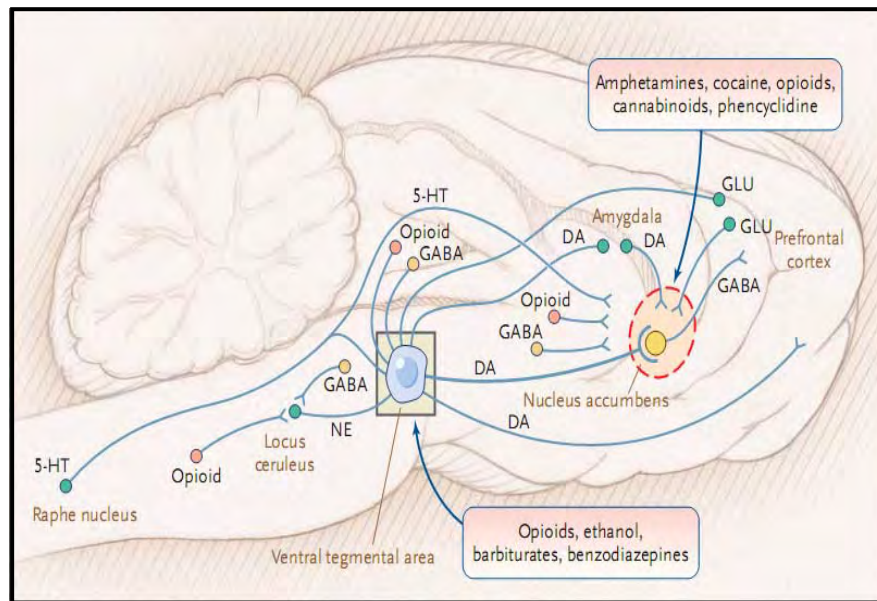
Figure 2.4 The figure shows the proposed sites of action of the various drug of abuse in these circuits. The mesocorticolimbic dopamine systems projections from cell bodies of the VTA to the NAc, amygdala, and prefrontal cortex; glutamatergic (GLU) projections from the prefrontal cortex to the NAc and the VTA; and projections from the GABA neurons of the NAc to the prefrontal cortex. Opioid interneurons modulate the GABA-inhibitory action on the VTA and influence the firing of norepinephrine (NE) neurons in the locus ceruleus. Serotonergic (5-HT) projections from the raphe nucleus extend to the VTA and the NAc (Cami and Farre, 2003).

Molecular neurobiology of opioids addiction

Opioid receptors belong to the superfamily of seven transmembrane domain receptors and produce their cellular effects via coupling with toxin-sensitive GTP-binding proteins Gi/Go. Acute administration of opiates inhibits neurotransmitter release by several mechanisms. Activation of opioid receptors leads to inhibition of cAMP production and voltage gated Ca^{2+} channels, as well as the stimulation of inwardly rectifying K^{+} channels and activation of the MAP kinase pathway. At cellular level, all these actions induce inhibition of neuronal activity and a reduction in neurotransmitter release (Figure 2.5). Chronic treatment with opiates leads to adaptations that result in changed regulation of neurotransmitter release (Figure 2.5). Opioids addiction has been

reported to cause adaptations in many intracellular second messenger pathways. One of the best established adaptations is the upregulation of the cAMP pathway after chronic administration of opiates or other drugs of abuse and opiates withdrawal. In addition, it is known that chronic opiate treatment increases levels of cAMP-dependence kinase (PKA) and the activity of PKA is increased by binding of cAMP. Activated PKA has an important role in phosphorylation of CREB (cAMP-respone-element blinding protein). CREB is a transcription factor, which has recently been reported to be involed in the expression of opiate dependence. The phosphorylation of CREB produces an increase in its activity, which leads to an increase in the c-Fos protein expression. Fos protein, as a transcription factor has been used as a marker for neuronal activation in CNS. Fos protein expression was increased and distributed in spinal cord during opiates withdrawal. An adaptation of drug addiction occurs in other brain region including VTA, NAc, the dorsal raphe nucleus and the periqueductal grey metter (Nestler, 2001; Williams et al., 2001).
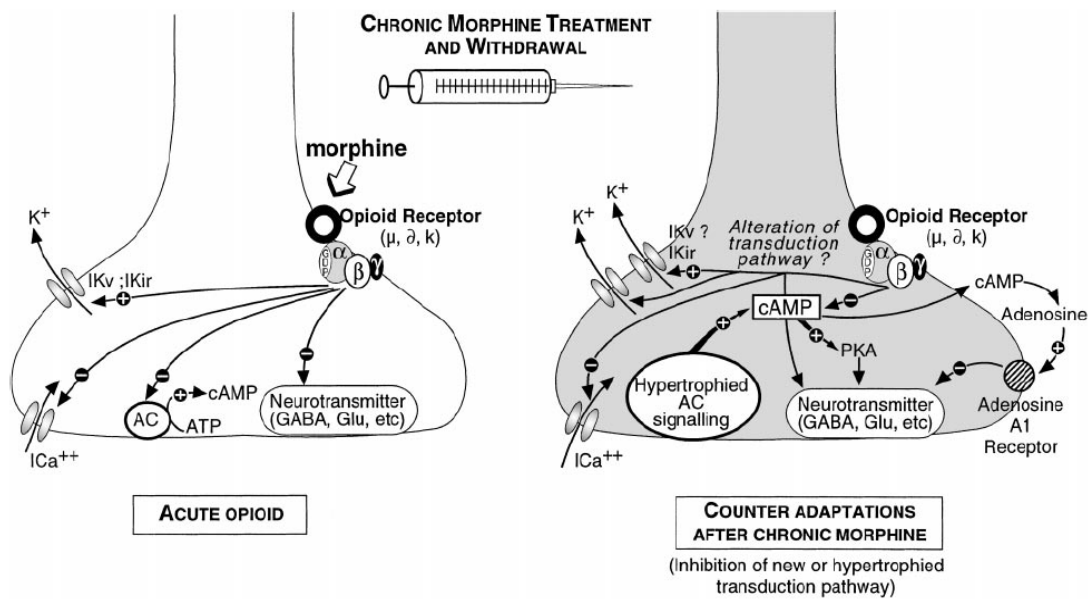
Figure 2.5 Regulation of transmitter release from synaptic terminals after acute and chronic opioid treatment. Acutely administered opiates inhibit transmitter release by: the activation of K^+ conductance, inhibition of Ca^{2+} conductance, or other mechanisms that has not been well characterized. Opiates inhibit also adenylyl cyclase (AC); however, it does not seem to play a role in the acute inhibition of transmitter release. After withdrawal from chronic administered opiates, the inhibition of transmitter release by opioids is changed 1) Opioids no longer activate voltage-dependent K^+ currents to inhibit release. 2) There is an upregulation of AC that increases transmitter release by activation of PKA. 3) The upregulated AC is sensitive to inhibition by opioids and represents a new, morphine-induced effector. 4) The increased AC activity increases the production of cAMP that is metabolized to adenosine such that adenosine tone and thus presynaptic inhibition mediated by A1 adenosine receptors is enhanced at some synapses (Williams et al., 2001).

Physical dependence of opioids

Chronic administration of morphine results in the development of physical dependence, as evidenced by the appearance of distressing physical symptoms. The symptoms of withdrawal can be induced either by abrupt termination of morphine treatment or by injecting an opioid receptor antagonist, such as naloxone, nalorphine

and naltrexone. In morphine-dependent subjects, the nature of the symptoms depends on the species studied. In rodents, such as rats and mice, morphine physical dependence has been characterized by antagonist-precipitated withdrawal signs that include the appearance of jumping, teeth chattering, wet dog shakes, ptosis, and diarrhea. Among such signs, jumping is widely considered the most sensitive and reliable index of withdrawal intensity in rodents (especially mice), and is the most commonly used. Although the withdrawal syndrome resulting from chronic morphine treatment tends to be more intense and/or of longer duration than that observed following its acute injection, the symptoms themselves are qualitatively similar. Because part of the withdrawal syndrome is induced by noradrenergic hyperactivation (Narita et al., 2001). Neurotransmitters involved in the withdrawal symptoms are shown in Table 2.4.

Table 2.4. Neurotransmitters implicated in the motivational effects of withdrawal from drugs of abuse (Koob and Moal, 2008)

Neurotransmitter	Functional effect
↓ Dopamine	Dysphoria
↓ Serotonin	Dysphoria
↓ γ-Aminobutyric acid	Anxiety, panic attacks
↓ Neuropeptide Y	Antistress
↑ Dynorphin	Dysphoria
↑ Corticotropin-releasing factor	Stress
↑ Norepinephrine	Stress

The locus coeruleus (LC) is the main brain region playing an important role in the opioid withdrawal syndrome, including jumping, rearing, and hyperactivity, in morphine-dependent rats (Table 2.5). The LC where the μ-opioid receptors are dense is the largest cluster of noradrenergic (NAergic) neurons in the brain, and represents the

primary source of NAergic innervation of the limbic system and the cerebral and cerebellar cortices (Narita et al., 2001).

Table 2.5. Some withdrawal signs observed in rats and/or mice in respect to their origin (central) and the involved neurotransmitter/receptor sites (Maldonado et al., 1992; Maldonado and Koob, 1993)

Withdrawal sign	Brain area/brain nucleus	Neurotransmitter receptor site
Wet dog shakes	Forebrain, lower diencephalon /brain stem, anterior hypothalamus, hypothalamus, medial thalamus, nucleus raphe magnus, amygdala, substantia nigra, locus coeruleus, hippocampus, nucleus accumbens	5-HT, DA, Ach, μ-receptor site
Jumping	medial thalamus, raphe nuclei, central/dorsal amygdala, locus coeruleus, periaqueductal gray	DA, μ-receptor site
Rearing	locus coeruleus, periaqueductal gray, nucleus accumbens	μ-receptor site, DA
Teeth-chattering	basal ganglia, substantia nigra, nucleus accumbens	DA
Grooming	medial/lateral hypothalamus, nucleus accumbens, cerebellum	DA
Chewing	amygdala, substantia nigra, locus coeruleus, nucleus accumbens	DA, Ach

Classes of drug treatments of opioid addiction

Goals for the treatment of addiction include preventing withdrawal symptoms, reducing drug craving, normalizing any physiological functions that are disrupted by drug use and targeting the treatment agent to the specific site of action or physiological system that is affected by the drug of abuse. Common medications for opioid dependence are shown in Table 2.6.

Opioid agonist

Agonist drugs bind to opioid receptors and exert similar effects to natural endogenous opiate. There are three agonist drugs having shown effective treatment.

Methadone is a μ-opioid receptor agonist and is a potent opiate analgesic with properties to produces morphine like effects, half-life 24-36 hours. It is the most commonly used medication for the treatment of opioid withdrawal. However, there are many adverse effects including nausea, vomiting, euphoria, drowsiness, constipation and respiratory depression. With continuous treatment, tolerance develops to most of these effects and dependence.

Levomethadyl acetate (LAAM) is derivative of methadone. With an action that is qualitatively similar to morphine, a prototype μ-opioid agonist and long duration of action 48-72 hours. Its use has been associated with prolongation of the QT interval, ventricular tachycardia, angina pectoris, myocardial infarction and cardiac arrest.

Buprenorphine is a partial μ-opioid agonist and κ-opioid antagonist can be given by sublingual or parenteral routes, has a long half-life (20-73 hours), and has a relatively low abuse potential. Its use has been found side effects after administration such as nausea, vomiting, constipation and respiratory depression.

Similar structure of all drugs causes drug addiction. They are considered highly addictive, therefore, their administrations should be under controlled condition (Ling and Compton, 2005).

Opioid antagonist

Naloxone and naltrexone are opioid antagonists blocking μ-opioid receptors. They have many adverse effects such as nausea, vomiting, headache, stupor and sedative. Because they are different from opioid agonists, they do not have positive reinforcement effects. Furthermore, they may be associated with precipitated opioid withdrawal symptoms if they are used too soon after stopping opioid use (Comer et al., 2006).

Non opioid drugs

Clonidine and lofexidine, the anti-hypertensive drugs, acted by stimulating α_2-adrenergic receptors and have been used effectively in the reduction of opioid withdrawal symptoms by reducing the noradrenergic hyperactivity and suppressing autonomic signs and symptoms of opioid withdrawal such as sweating, piloerection, tingling, nausea and vomiting. Nevertheless, their uses have side effects such as sedation and hypotension (Gowing et al., 2008).

Psychosocial and behavioral treatments

There are numerous psychosocial and behavioral approaches currently being used in the management of an opioid dependent individual. These include cognitive-behavioral therapy, relapse prevention, psychotherapy, and psychopharmacological. Pharmacological treatment is usually restricted to the management of intoxication, withdrawal syndromes, drug-induced aggression or behavioral changes, medical complications and, in some cases, there is a need to use agonist compounds that bind competitively to the same receptors that mediate the effects. Combined substance use disorders require special attention, because treatment directed at opioid dependence alone is unlikely to lead to cessation of other substance use. Treatment is generally similar to that for individual substances. Increased frequencies of behavioral monitoring, intensified councilling, contingency contracting, referral to specific self-help groups and specialized pharmacological treatments have all been used with varying degree of success (Veilleux et al., 2010).

Table 2.6. Common medications for opioid dependence (Nicholls et al., 2010)

Medication	Action	Indication	Dosage	Frequency	Adverse Effects
Buprenorphine	Partial opioid agonist	Withdrawal and maintenance	2-32 mg sublingual	Daily or 3 times per week	Respiratory depression, headache, constipation
Clonidine	α_2-adrenergic antagonist	Withdrawal	0.1-0.3 mg orally	Every 6 hours	Bradycardia, hypotension, dry mouth, drowsiness
Levomethadyl acetate (LAAM)	Opioid agonist	Maintenance	25-100 mg orally	3 times per week	QT prolongation, Constipation
Methadone	Opioid agonist	Withdrawal and maintenance	20-100 mg orally	Daily	Constipation, respiratory depression, dizziness, nausea, sedation
Naltrexone	Opioid antagonist	Withdrawal and maintenance	50-100 mg orally	Daily or 3 times per week	Anxiety, nausea, myalgia

A novel approach for the treatment of adverse effects of drugs abuse is a use of natural products, because they implies more therapeutic efficacies, safety, low toxicities and lesser potential for physical dependence than other opioids. Studies have the efficacies of medicinal herbs for the opioid dependence.

Animal models in drug addiction research

Animal models are very important tool in the work toward understanding the addiction process and in the search for medical intervention. Science faces many ethical and technical difficulties in the research of drug dependence in human subjects. Nevertheless, more refined methods are available for the evaluation of reinforcing properties of a drug and for modeling different features of human addictive behavior (Gerrits et al., 2003).

Locomotor activity test

Development of behavioral measurements of locomotor activity and exploration was in part relevant in various rodent models as an initial screen for pharmacological effects predictive of therapeutic efficacy of a drug in humans. Locomotor activity measurement is commonly used in mice to study sensitivity to the locomotor activating or depressing effects of a drug. Locomotor activity and exploration are involved in many behavioral and physiological functions. Indeed, they are mediated by neurotransmitters affected by many drugs, such as benzodiazepines, opiates, and psychostimulants, and consequently are changed in response to these drugs administration. Moreover, alterations of locomotor activity and exploration can have important consequences for paradigms that aim to study more specific processes including learning, memory reward and anxiety (Oades et al., 1986).

Drug self-administration (SA)

The self-administration paradigm is an important tool for screening drugs for abuse potential (drug-taking behavior) and to elucidate the rewarding effects of drugs. There are various possible routes of administration including intravenous, subcutaneously, orally, intracranial and inhalation. This paradigm is based on positive reinforcement, which means that a specific behavior (e.g. lever pressing) has a positively perceived effect (i.e. drug administration). This positive effect will recording the number of times an animal produces a response which result in an increase of the lever pressing since the animal wants more of the positive perceived feeling. During this

procedure drugs can be injected via a catheter implanted in the jugular vein, but also via drinking-bottles (e.g. ethanol) or food pellets (e.g. sucose). To increase the motivational value of the reward, a stimulus (e.g. light) can be used (Gerrits et al., 2003).

Conditioned place preference (CPP)

The CPP paradigm is a useful tool for studying the affective properties of drugs, and is routinely used in concert with standard research techniques in neuroscience. After prolonged abuse of a drug, the environment where the effects of the drug have been experienced can contribute to drug dependence. By repeated association of a specific environment with the effects of the drug, the environment can, in time, in absence of the drug induce craving or a feeling of the drug. The environments differ with respect to their distinctive perceptual features (visual, tactile, olfactory and auditory cues). CPP is a way to evaluate preferences for environmental stimuli that have been associated with positive or negative reward. An animal is exposed to a new environment containing two (or more) compartments. One of these compartments is paired repeatedly with the administration of a specific drug, while in the other compartment vehicle is administered. During testing, when no drugs are administered, the animal is able to choose between one of the two compartments. CPP occurs when the animal specifically prefers the compartment where the drug was previously administered above the vehicle-treated location. Comparison of the time spent in the drug- or non-drug associated environment. An increase in time spent in the environment previously paired with the drug provides information about the rewarding properties of a drug (Gerrits et al., 2003).

Withdrawal model

The withdrawal model holds that individuals seek to consume drugs primarily in order to escape aversive states. As opposed to being motivated by a reward, these individuals seek to relieve withdrawal related discomfort. Physical dependence to drug has been described as expression of withdrawal symptoms in long term used as a result of abrupt withdrawal of drug, reduction in drug doses and precipitated withdrawal

symptoms with administration of drug antagonists. However, motivational measures of abstinence have proven to be more sensitive measures of drug withdrawal and powerful tools for exploring the neurobiological bases for the motivational aspects of drug dependence (Hajhashemi et al., 2007).

Mitragyna speciosa Korth. (Kratom)

Mitragyna speciosa Korth., (*M. speciosa*) is categorized in the family Rubiaceae and it is called kratom in Thai and "Biak-Biak" in Malaysia. It is a tropical tree indigenous to Thailand, Malaysia, Indonesia, Myanmar and other areas of South East Asia and Africa. The leaves of kratom have been traditionally used in Thailand. Different routes of administration include chewing fresh or dried leaves, smoking, cooking, and making it into a tea. The medicinal properties of this plant had previously reported due to its opium-like effect and cocaine-like stimulant in combating fatigue and as hard work tolerance and increase labor work efficiency and tolerance under the hot sunshine atmosphere (Suwanlert, 1975). Additionally, it has been used to relief pain, wound poultice, cure for fever, cough suppressant, and diarrhea. Furthermore, it is often used as a substitute for opium when opium is unavailable and treatment for opiate addiction and weaning of morphine addiction (Suwanlert, 1975; Jansen and Prast, 1988). In many countries, kratom use remains uncontrolled and illegal. It can be purchased at a wide variety of shops online and website for more self-treatment of opioid withdrawal using kratom (Boyer EW et al., 2007; Boyer EW et al., 2008).

After kratom consumption, the user will become happy, strong and active. However, the following side effects were found in long term use of kratom such as dry month, frequent urination, anorexia, weight loss, constipation, and moreover, the feces are black and small in shape similar to goat feces. Typical withdrawal symptoms of kratom addicts include for example, aggression, tearfulness, wet nose, rhinorrhea, aching in the muscles and bones, inability to work, and jerky movement of the limbs (Suwanlert, 1975).

In 1943, the Thai government passed the Kratom Act 2486 that made planting of the tree illegal. In 1979, the Thai government enacted the Narcotics Act B.E. 2522, placing kratom along with marijuana in Category V of a five category classification of narcotics. Kratom remains a popular drug of abuse in Thailand. In December 2006, kratom is the third most popular drug within southern Thailand, after methamphetamine and marijuana. It has been reported that young Thai militants drink a "4x100" kratom formula to make them "more bold and fearless and easy to control." The two "4x100" kratom formulas are described as a mixture of a boiled kratom leaves and mosquito coils and cola or a mixture of boiled cough syrup, kratom leaves and cola served with ice. Kratom is a controlled substance in Thailand, Bhutan, Australia, Finland, Lithuania, Malaysia and Myanmar (Assanangkornchai et al., 2006).

Morphology of kratom

Kratom is a large tree, 10-30 m tall; bole 60-100 cm in diameter, bark greyish, shallowly sculptured and with pustular lenticels; each node with 2 serial buds; terminal vegetative bud ellipsoid, slightly flattened. Leaves opposite, simple, entire, oblong-ovate, 8-15 cm x 4-10 cm, base broadly rounded, apex abruptly acuminate, glabrous or veins beneath puberulous, veins 12-15 pairs; petiole (1-)2-5 cm long; stipules lanceolate, 2 cm long, pubescent, with 9 veins, inside with colleters at base (Figure 2.6). Inflorescence terminally on lateral branches, composed of 3(-7) globose heads, 1 head subsessile between 2 others on long peduncles, 5 cm long; head 2.5 cm in diameter when flowering, 1.5 cm when fruiting, receptacle hairy, leafy bracts up to 4 cm long, petiolate, interfloral bracteoles up to 3.5 mm long. Flowers bisexual, 5-merous, sessile; calyx cup-shaped, up to 2 mm long, 5-lobed; corolla yellowish-white turning deep yellow, funnel-shaped, 5-8 mm long, lobes 5, 3 mm long, thickened at apex, margin revolute, a conspicuous ring of hairs inside at base of lobes; stamens 5, on intersection with lobes, anthers lanceolate, cordate, conspicuously protruding from the corolla; ovary inferior, 2-celled, style exserted, 13 mm long, stigma rounded, 2 mm long. Fruit composed of 2 cocci, exocarp thin, splitting loculicidally along its length, 10-ridged; seeds numerous. Seed shortly winged on 2 sides, lower wing shortly bifid or notched.

In Thailand, Krotom was found in central part of the country such as Nakhonpathom, Pathumthani, and Nonthaburi. Moreover, it can be found in the South of Thailand such as Suratthani, Nakhonsrithammarat, Trung, Satun, Pattalung, Songkhla, Yala, Pattani, and Narathiwat. In Thailand, there are two varieties of kratom; green and red veins. In the present study, we choose the red veins, because of its pharmacologic properties and widespread use (Chua and Schmelzer, 2001).

Figure 2.6 *Mitragyna speciosa* Korth. (Assanangkornchai et al., 2006)

Chemical compounds of kratom

Kratom leaves contain several alkaloids and other chemical substance. Chemicals constant were identified structure including indole alkaloid, oxindole alkaloid, flavanoids, phytosteroi and tannis. The major chemicals are indole alkaloids such as mitragynine, paynantheine, isospeciofoline, speciogynine, mitraciliatine, rhynchophylline, isorhynchophylline, speciophylline, speciociliatine, corynoxeine, ciliaphylline, mitragynaline, 3-dehydromitragynine, isomitraphylline, rhynchociline, isospecionoxeine, mitragynalimic acid, ciliaphylline, corynantheidaline, mitralactonine, specionoxeine and 7α-hydroxy-7H-mitragynine (Shellard et al., 1978; Houghton and Said, 1986; Jansen and Prast, 1988; Houghton et al., 1991; Ponglux et al., 1994; Takayama et al., 1999; Yamamoto et al., 1999). The oxindoles are mitraphylline and speciofoline. Several flavonoids such as apigenin, astragalin, cosmosin, hyperoside,

and also the polyphenols kaempferol, quercetin, and rutin. More than 32 alkaloids have been isolated from kratom leaves. Mitragynine was the major constituent (about 66% of crude extract or about 0.25% of dry leaf; Figure 2.7). The alkaloid content varies from location to location and from time to time (Shellard, 1974; Takayama, 2004). Mitragynine can be separated from kratom leaves only and was used to identify of kratom leaves (Shellard, 1974; Ponglux et al., 1994).The alkaloid content of the leaves of *Mitragyna speciosa* is about 0.5%, about half of which is mitragynine. An average leaf weighs about 1.7 grams fresh or 0.43 grams dried. Twenty leaves contain approximately 17mg of mitragynine (Amattayakul, 1960).
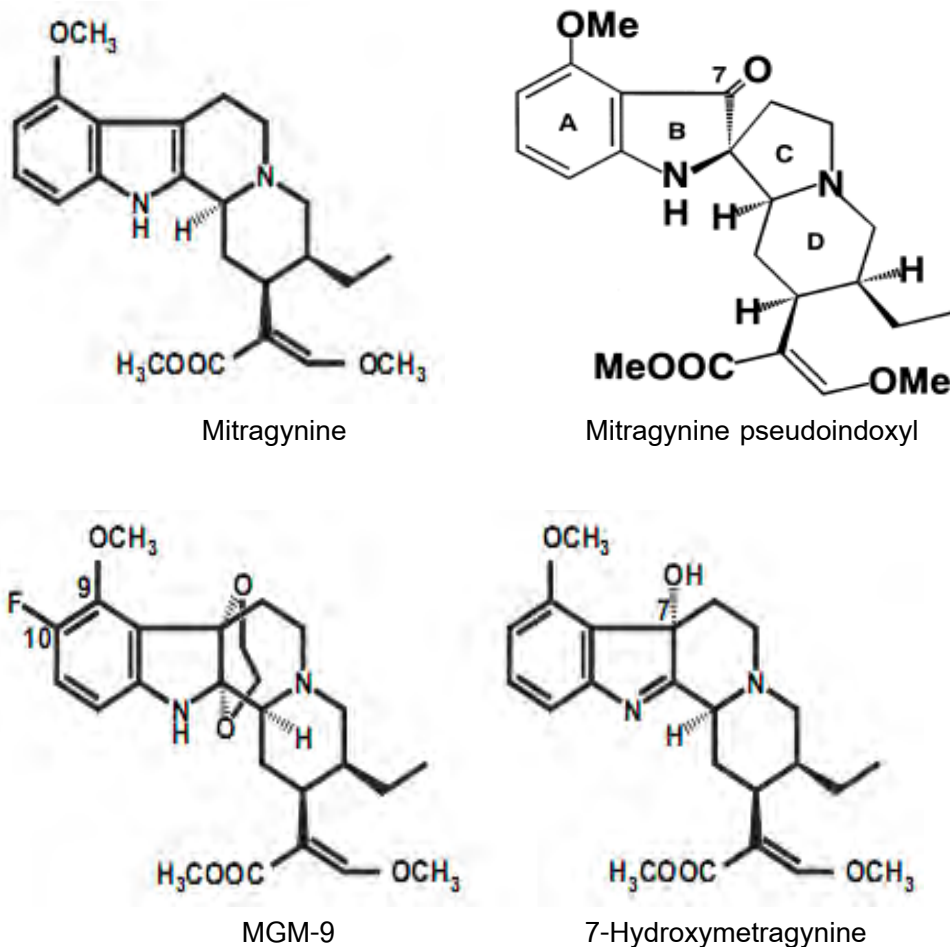

Mitragynine   Mitragynine pseudoindoxyl

MGM-9   7-Hydroxymetragynine

Figure 2.7 Chemical structures of mitragynine, mitragynine pseudoindoxyl, MGM-9, and 7-Hydroxymetragynine (Matsumoto et al., 2008)

Pharmacological effects of *Mitragyna speciosa* extracts and mitragynine

1) Antitussive effect

Mitragynine was showed antitussive activity in dog similar with codeine which inhibited vomiting and had addictive affects as same as opioids (Macko et al., 1972).

2) Smooth muscle contraction effect

Effect of mitragynine on smooth muscle contraction using electrically stimulated contraction, showed that mitragynine inhibited electrically stimulated contraction of rabbit ileum, cat ileum and guinea pig ileum through the opioid receptor (Grewal et al., 1932b).

Another study found that mitragynine inhibited electrically stimulated contraction of guinea-pig ileum through the opioid receptor (Watanabe et al., 1997).

Recent study found that mitragynine inhibited electrically stimulated contraction of guinea-pig vas deference through blocking neuronal Ca^{2+} ion channel (Matsumoto et al., 2005).

3) Antinociceptive effect

The antinociceptive of mitragynine was studied by tail-pinch and hot plate test in mice. The result demonstrates mitragynine has an antinociceptive activity through the supraspinal opioid receptors and its action dominantly mediated by μ- and δ-opioid receptor in vitro and in vivo studies (Matsumoto et al., 1966a, b; Thongpradichote et al., 1998). Competitive binding study found that mitragynine bound to 3 types of receptors with the highest affinity in μ- followed by κ- and δ-opioid receptors (Yamamoto et al., 1999).

Oral administration of mitragynine at dose of 200 mg/kg significantly reduced the number of writhing and produced significant analgesia when tested by the hot tail-flick (Idid et al., 1988).

The study of analgesic activities of methanolic and alkaloid extract of *Mitragyna speciosa* (*M. speciosa*) leaves were using hot plate test in mice and tail flick test in rats. Oral administration of methanolic and alkaloid (100 and 20 mg/kg, respectively) exhibited significantly antinociceptive in hot plate test. The antinociceptive action of both methanol and alkaloid extracts were blocked by naloxone (2 mg/kg, i.p.) in mice. These results suggest that the methanol and alkaloid extracts of *M. speciosa* leaves possess the analgesic activity which partly acted at opioid receptors in the supraspinal opioid system. (Reanmongkol et al., 2007).

Intraperitoneal administration of the methanolic extract of *M. speciosa* leaves (100 and 200 mg/kg) produced significant antinociceptive in all of the nociceptive models evaluated (writhing test, hot plate test in mice and the formalin test in rats) (Mossadeq et al., 2009).

4) Inhibited gastric acid secretion

Injection of mitragynine (3-30 μg) into the fourth cerebroventricle, like morphine, inhibited 2-deoxy-D-glucose-stimulated gastric acid secretion. The inhibitory effect of mitragynine (30 μg) was reversed by naloxone (100 μg). These results suggest that mitragynine has a morphine-like action on gastric acid secretion in the CNS (Tsuchiya et al., 2002).

5) Gastrointestinal tract effect

Acute intraperitoneal administration of *M. speciosa* leaves (MS) extracts (45 and 50 mg/kg) significantly decreased in food and water intakes in rats. The long-term administration of the MS extract (40 mg/kg) for 60 consecutive days also significantly suppressed weight gaining. (Kumarnsit et al., 2006).

Oral administration of methanolic extract of *M. speciosa* leaves at 50, 100, 200, and 400 mg/kg exhibited significantly against castor oil-induced diarrhea and also inhibited intestinal transit in a dose-dependent manner (Chittrakorn et al., 2008).

6) Antidiabetic effect

In vitro study the antidiabetic activities of aqueous, methanolic and alkaloid extracts and mitragynine of *M. speciosa* leaves by evaluating glucose transport in muscle cells. Protein levels of glucose transporters (GLUTs) were measured by Western blot. The result showed that it significantly increased the rate of glucose uptake which was associated with increase in GLUT1 protein content. This recent study demonstrated the effect of *M. speciosa* leaves in stimulating glucose transport in muscle cells, implicating the folkloric use of *M. speciosa* leaves for diabetic treatment (Purintrapiban et al., 2008).

7) Anti-inflammatory effect

The anti-inflammatory activity of the methanolic extract of *M. speciosa* leaves was studied in rats using carrageenan-induced paw edema and cotton pellet-induced granuloma. Oral administration of methanolic extract of *M. speciosa* leaves at 100 and 200 mg/kg produce significant inhibition of the development of paw edema and at dose 200 mg/kg it caused significant inhibition of the growth of granuloma tissue. (Mossadeq et al., 2009).

8) Antibacterial effect

In vitro study of the antibacterial properties of aqueous, alkaloid and methanolic extracts of *M. speciosa* leaves were evaluated by broth dilution method. The extracts showed antimicrobial activity against *Samonella typhi* and *Bacillus subtilis*. The minimum inhibitory concentrations (MICs) of extracts determined by the broth dilution method range from 3.12 - 6.25 mg/ml. The alkaloid extract was found to be most effective against all of the tested organisms (Parthasarathy et al., 2009).

9) Antioxidant effect

In vitro study of the antioxidant properties of aqueous, alkaloid and methanolic extracts of *M. speciosa* leaves were evaluated using (2, 2-diphenyl-1-picrylhydrazyl, DPPH) radical scavenging method. The DPPH IC_{50} values of the aqueous, alkaloid and

methanolic extracts were 213.4, 104.81 and 37.08 μg/ml, respectively and total phenolic content were 66.0, 88.4 and 105.6 mg CAE/g, respectively while the total flavanoid were 28.2, 20.0 and 91.1 mg CAE/g, respectively. The antioxidant activities were correlated with the total phenolic content. This result presented that high antioxidant activity of methanolic extract could be possibly due to its high phenolic content (Parthasarathy et al., 2009).

10) CNS effect

Oral administration methanolic extract at 50, 100 and 200 mg/kg and alkaloid extract at 5, 10 and 20 mg/kg of *M. speciosa* leaves did not show effect on locomotor activity (Reanmongkol et al., 2007).

Oral administration of aqueous extract at 100, 200 and 500 mg/kg of *M. speciosa* leaves had no significant effect on spontaneous motor activity (Kumarnsit et al., 2007).

Intraperitoneal administration of mitragynine (1, 5, 10 and 30 mg/kg) showed significantly changed the locomotor activity in rats which the compound reduced the activity (Moklas et al., 2008).

11) Cognitive effect

To study cognitive effects of mitrgynine using object location task test. Chronic intraperitoneal administration for 28 consecutive days of mitragynine at all doses 5, 10 and 15 mg/kg did not showed any significant discrimination between the object that had changed position and the object that had remained in a constant position. These results suggest that chronic administration of mitragynine can altered the cognitive behavioral function in mice (Apryani et al., 2010).

12) Antidepressant effect

Intraperitoneal administration of mitragynine (1, 10, 20 and 30 mg/kg) were attenuated the head-twitch response when induced by 5-methoxy-N-dimethyltryptamine (5-MeO-DMT) in mice and a dose dependent manner. It is by stimulation of postsynaptic

α_2-adrenoceptor or blockade of 5-HT$_{2A}$ receptors. The 5-HT$_{2A}$ receptor is known to participate in various psychiatric disorders suggested that mitragynine might have an effect to reduce psychotic disorders (Matsumoto et al., 1997).

The antidepressant-like activity of intravenous administration of alkaloid extract of *M. speciosa* leaves at 60 and 90 mg/kg decreased immobility time in the forced swimming test (FST) in mice (Kumarnsit et al., 2007).

Intraperitoneal administration of mitragynine at 10 and 30 mg/kg could significant reduce the immobility time of mice in both FST and tail suspension test (TST) without any significant effect on locomotor activity (Farah Idayu et al., 2010).

13) Decrease ethanol withdrawal symptoms

Oral administration of the aqueous extract of *M. speciosa* leaves at 300 mg/kg exhibited a reducing effect on the ethanol withdrawal behaviors such as rearing, displacement and head weaving probably due to antidepressant activity (Kumarnsit et al., 2007).

Toxicology of kratom

Intraperitoneal administration mitragynine at 920 mg/kg in mice had no tremor and seizure (Macko et al., 1972).

In vitro study kratom extract cytotoxicity was assessed by flow cytometry analysis (FACS) by using HEK-293 and SH-SY5Y treatment with kratom extract were 50, 100, 250, 500 and 1,000 μg/ml. This result found that kratom extract leaded to dose-dependent cytotoxicity (Saidin and Gooderham, 2007).

In acute toxicity study, the oral medial lethal dose (LD$_{50}$) of the methanol and alkaloid extracts of *M. speciosa* leaves in mice were 4.90 g/kg and 173.20 mg/kg, respectively (Reanmongkol et al., 2007).

The toxicity to brine shrimp of mitragynine, alkaloid and aqueous extract of *M. speciosa* leaves were 44, 62 and 98 μl/ml respectively (Moklas et al., 2008).

Acute toxicity of methanolic extract *of M. speciosa* leaves in rats, demonstrated that oral administration of methanolic extract at 100, 500 and 1,000 mg/kg, all animals were scarified after 14 days. These results found that methanolic extract caused increase blood pressure an hour of drug administration. The highest dose also induced acute hepatotoxicity and mild nephrotoxicity but it had no effect on body weight, food and water consumption, absolute and relative organ weight and hematology parameters (Harizal et al., 2010).

CHAPTER III
MATERIALS AND METHODS

Materials
Animals

Male Wistar rat weighing 200-250 g and male ICR mice weighing 18-25 g from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhonprathom, Thailand served as experimental subjects in the study. The animals were housed in the animal facility of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions of temperature ($25\pm2\ ^{\circ}C$), 50-60% of humidity and 12 hr/12 hr light/dark cycles. The animals were kept under laboratory conditions for one week prior to the start of the experiments and allowed food and water *ad libitum*. At the end of each experiment, the animals were sacrificed with carbon dioxide asphyxiation. This study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Appendix).

Preparation of the ethanolic extract of *Mitragyna speciosa* leaves

The ethanolic extract of *Mitragyna speciosa* leaves was prepared using maceration technique. In brief, the fresh leaves of *Mitragyna speciosa* were dried in a hot air oven (50 °C), crushed to powder and macerated in 95% ethanol. Finally, the solvent was evaporated under vacuum and dried using rotary evaporator (Buchi, Japan) to leave the ethanol extract.

Chemicals

The following drugs were used

- 0.9% Normal saline solution (NSS; Otsaka, Thailand)

- 0.5% Carboxymethyl cellulose solution (CMC; Sigma, USA)

- Morphine sulphate (5-100 mg/kg, Temad, Tehran, Iran)

- Methadone hydrochloride (1 mg/kg, Alchymars, Italy)

- Naloxone hydrochloride (3 mg/kg, Sigma, USA)

- Naltrindole hydrochloride (5 mg/kg, Sigma, USA)

- Norbinaltorphimine hydrochloride (10 mg/kg, Sigma, USA)

- Ethanolic extract of *Mitragyna speciosa* leaves (MS; 50-400 mg/kg)

Morphine, methadone, naloxone, naltrindole and norbinaltorphimine were dissolved in 0.9% normal saline solution. MS were suspended in 0.5% carboxymethyl cellulose solution. Morphine was used as a standard drug for locomotor activity, conditioned place preference and withdrawal tests. Methadone was used as a standard drug for conditioned place preference and withdrawal tests. Volumes of drugs orally administered were 10 ml/kg and 5 ml/kg for rat and mice, respectively. While, volumes of drugs administered by intraperitoneal injection were 0.5 ml/300 g and 0.3 ml/30 g body weight for rat and mice, respectively.

Instruments and apparatus

- Syringes (1 ml and 3 ml)

- Needles (No.27 length 1")

- Feeding tube No. 16 length 3" for rat

- Feeding tube No. 18 length 2" for mice

- Automatic pipettes

- Pipettes tips

- Mortar and pestle

- Beakers

- Glass cylinders (diameter 30 cm, height 70 cm)

- Weighing machines (Sartorius TE 612, Germany; Mettler, Toledo AG245,
   Switzerland)

- Vortex (Bohemia G-506E, NY USA)

- Locomotor activity cage (UGO Basile, Comerico, Italy)

- Conditioned place preference apparatus

Methods

1. Locomotor activity test

The effect of MS on locomotor activity in mice was examined using an activity cage (length 35 cm, width 23 cm, height 20 cm, UGO Basile, Comerico, Italy) (Figure 3.1). Their movements are automatically recorded by an array of infrared photobems. Temperature, sound and light conditions were maintained uniform during the course of the experiments. The drug administration took place with either intraperitoneal (i.p.) 0.9% normal saline solution (NSS; 0.3 ml/30 g), morphine (MO; 5 mg/kg) or oral administration of 0.5% carboxymethyl cellulose solution (CMC; 5 ml/kg) and various doses of MS (50, 100, 200, and 400 mg/kg). Each mouse was placed in an activity cage immediately after drug administration and horizontal movement of the mouse was recorded every 5 minutes for 75 minutes. (Capasso et al., 1996).

Figure 3.1 Locomotor activity cage

2. Conditioned place preference test

The conditioned placed preference (CPP) method of Spyraki et al. in 1982 with some modification was used for evaluating the motivation properties of various doses of MS (50, 100, 200, and 400 mg/kg) in rats. CPP apparatus (length 25 cm, width 80 cm, height 36 cm) was made of acrylic resin and consisted of three compartments. The middle compartment consisted of an (length 25 cm, width 11 cm) area painted grey the

guillotine door. The lateral compartment (length 25 cm, width 34 cm) offered different stimuli in odor, color, and texture. One compartment was white wall with a smooth white floor, the other was black wall with a mesh floor and white vertical stripes. A drop of 2% acetic acid was placed at the right center of the compartment with a mesh floor. Install a 60-W red light directly overhead for illuminating all boxes. The animals were observed through VDO camera (Figure 3.2).
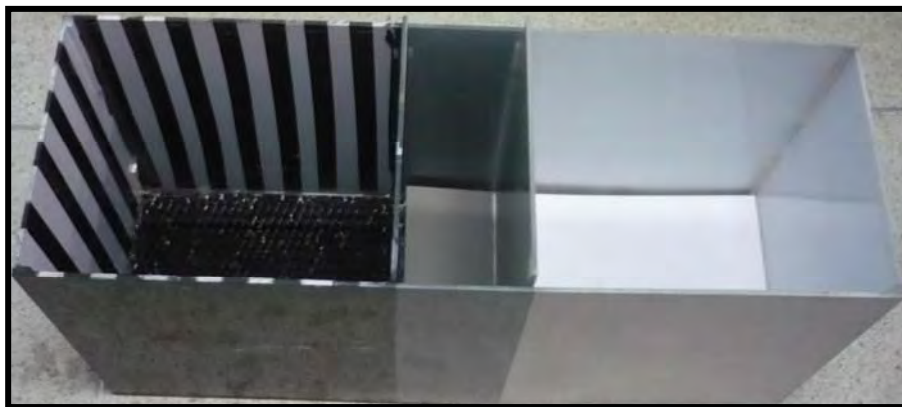

Figure 3.2 Conditioned place preference apparatus

The CPP consisted of a 12 days schedule, with three phases: preconditioning, conditioning, and test phase.

Preconditioning phase (Phase I)

During the preconditioning phase, animals were adapted to the experimental conditions. Each rat was placed into the middle chamber of the apparatus for 5 min then the guillotine doors which separate two compartments were opened and the animal was allowed to move freely to explore all three compartments for 15 min each day for three consecutive days. On day 3, the time spent by the rats in each compartment was recorded. The compartment occupied for the shorter time was designated as the drug-paired side.

Conditioning phase (Phase II)

The conditioning phase consisted of eight injections of drug or vehicle on alternate days. Each rat was received 0.9% NSS or test drugs in eight consecutive days (four days of 0.9% NSS and four days of test drugs). On conditioning days 1, 3, 5 and 7, rats were received various doses of test drugs before they were confined to the drug-paired compartment for 30 min. On the in-between days, each rat was received either 0.9% NSS injection or 0.9% NSS orally and confined to the opposite side for 30 min.

Test phase (Phase III)

On day 12, as in the preconditioning phase, the guillotine door separating the two compartments was raised and the drug-free rats were allowed free access to all compartments for 15 min. The time spent in drug-paired compartment was recorded for each animal and the change of preference was calculated as the difference (in seconds) between the time spent in the drug-paired compartment on the testing day, and the time spent in this compartment in the preconditioning day. Conditioned place preference was defined by an increase in the time spent in the drug-paired compartment during a preference test. Increasing in the time spent in the drug-paired compartment suggests the presence of the positive reinforcing effects.

2.1. Effect of MS on conditioned place preference

On conditioning day 1, 3, 5, and 7, rats were received either intraperitoneal morphine 5 mg/kg or oral administration of 0.5% CMC and various doses of MS (50, 100, 200, and 400 mg/kg) before they were confined to the drug-paired compartment for 30 min. Rats which received morphine were placed into the drug-paired compartment immediately after injection, while animals which received 0.5% CMC and various doses of MS (50, 100, 200, and 400 mg/kg) were placed to the drug-paired compartment at 30 min after oral administration. On the in-between days, each rat was received either 0.9% NSS injection or 0.9% NSS orally and confined to the opposite side for 30 min. The time spent by the animal in each compartments were observed through VDO camera.
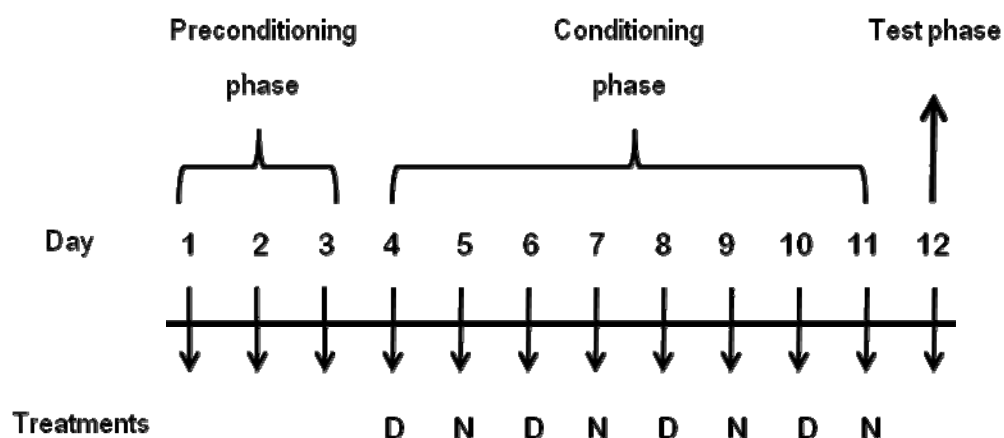
Remark: N = 0.9% NSS

D = MO, CMC or various doses of MS

Figure 3.3 Diagram of the ethanolic extract of *Mitragyna speciosa* leaves (MS)

treatment on CPP

2.2. Effect of MS on morphine-induced conditioned place preference

On conditioning day 1, 3, 5, and 7, rats were pretreated with 0.5% CMC or various doses of MS (50, 100, 200, and 400 mg/kg) orally 30 min before receiving morphine 5 mg/kg intraperitoneally and were confined to the drug-paired compartment for 30 min. On the in-between days, each rat was received either 0.9% NSS injection or 0.9% NSS orally and confined to the opposite side for 30 min. The time spent by the animal in each compartments were observed through VDO camera.
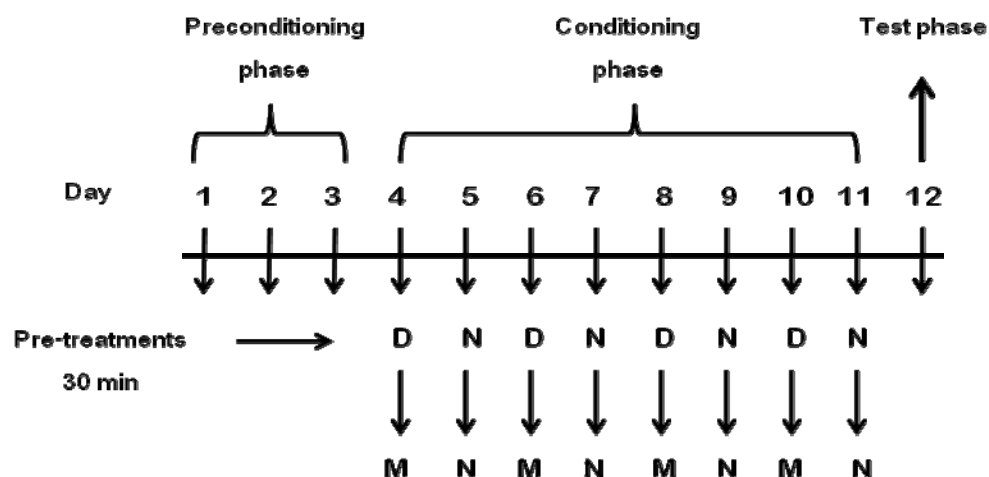
Remark: N = 0.9% NSS

D = 0.5% CMC or various doses of MS

M = MO

Figure 3.4 Diagram of the ethanolic extract of *Mitragyna speciosa* leaves (MS) treatment on morphine-induced CPP

3. Withdrawal effects

The withdrawal model holds that individuals seek to consume drugs primarily in order to escape aversive states. As opposed to being motivated by a reward, these individuals seek to relieve withdrawal related discomfort. Physical dependence to opioids has been described as expression of withdrawal symptoms as a result of abrupt withdrawal of opioids, reduction in opioid doses or administration of opioid antagonists (Hajhashemi et al., 2007). Previous studies found that antinociceptive effect of MS acted through μ- and δ-opioid receptors (Matsumoto et al., 1996a, b; Thongpradichote et al., 1998). This study employed naloxone (μ-opioid antagonist), naltrindole (δ-opioid antagonist), and norbinaltorphimine (κ-opioid antagonist) to evaluate the withdrawal effects of MS.

3.1. Precipitated withdrawal symptoms of acute MS treatment with naloxone

Mice in control group were administered orally with 0.5% CMC (5 ml/kg; p.o.). Treatment groups were administered with morphine (100 mg/kg; i.p.) or various doses of MS (50, 100, 200, and 400 mg/kg; p.o). At two hours after administration, withdrawal symptoms of all groups were precipitated with naloxone (3 mg/kg i.p.), then they were immediately placed in a glass cylinder. Withdrawal symptoms consisting of jumping (raising all limbs off the ground rapidly); wet dog shakes (a paroxystic shudder of the head, neck and trunk, reminiscent of the purposeful movement seen in dogs); rearing (lifting the forepaws off the ground); grooming (perform facial strokes, lick and scratch the body, and gnaw at the extremities); straub tail (erection of the tail) and C-shaped tail (C-shape roll tail). The frequency of these behaviors was recorded for each animal through VDO camera for 30 min (El-Kadi and Sharif, 1998; Cao et al., 2002).

Figure 3.5 Glass cylinder

3.2. Precipitated withdrawal symptoms of acute MS treatment with naltrindole

Mice in control group were administered orally with 0.5% CMC (5 ml/kg; p.o.). Treatment groups were administered with morphine (100 mg/kg; i.p.) or various doses of MS (50, 100, 200, and 400 mg/kg; p.o). At two hours after administration, withdrawal symptoms of all groups were precipitated with naltrindole (5 mg/kg; i.p.), then they were immediately placed in a glass cylinder. Withdrawal symptoms consisting of jumping

(raising all limbs off the ground rapidly); wet dog shakes (a paroxystic shudder of the head, neck and trunk, reminiscent of the purposeful movement seen in dogs); rearing (lifting the forepaws off the ground); grooming (perform facial strokes, lick and scratch the body, and gnaw at the extremities); straub tail (erection of the tail) and C-shaped tail (C-shape roll tail). The frequency of these behaviors was recorded for each animal through VDO camera for 30 min (El-Kadi and Sharif, 1998; Cao et al., 2002).

3.3. Precipitated withdrawal symptoms of acute MS treatment with norbinaltorphimine

Mice in control group were administered orally with 0.5% CMC (5 ml/kg; p.o.). Treatment groups were administered with morphine (100 mg/kg; i.p.) or various doses of MS (50, 100, 200, and 400 mg/kg; p.o). At two hours after administration, withdrawal symptoms of all groups were precipitated with norbinaltorphimine (10 mg/kg; i.p.), then they were immediately placed in a glass cylinder. Withdrawal symptoms consisting of jumping (raising all limbs off the ground rapidly); wet dog shakes (a paroxystic shudder of the head, neck and trunk, reminiscent of the purposeful movement seen in dogs); rearing (lifting the forepaws off the ground); grooming (perform facial strokes, lick and scratch the body, and gnaw at the extremities); straub tail (erection of the tail) and C-shaped tail (C-shape roll tail). The frequency of these behaviors was recorded for each animal through VDO camera for 30 min (El-Kadi and Sharif, 1998; Cao et al., 2002).

3.4. Precipitated withdrawal symptoms of chronic MS treatment with naloxone

Chronic morphine dependence in mice was induced by intraperitoneal administration. Dose of morphine was increased each day from the first day to the seventh day by 10, 20, 30, 40, 50, 60, and 70 mg/kg, respectively. Morphine was injected twice daily (at 08.00 am and 04.00 pm). Vehicle group was treated with 0.5% CMC (5 ml/kg) twice daily. Treatment groups were treated orally with various doses of MS (50, 100, 200, and 400 mg/kg) twice daily. Withdrawal symptoms of all groups were precipitated with naloxone (3 mg/kg; i.p.) two hours after the first dose of the seventh day. Immediately after naloxone injection, each mouse was placed in a glass cylinder and withdrawal symptoms are observed and recorded through VDO camera for 30 min (Matsumoto et al., 2004).

3.5. Effect of MS pretreatment on morphine withdrawal

Morphine dependence was induced by intraperitoneal administration. Dose of morphine was increased each day from the first day to the seventh day to 10, 20, 30, 40, 50, 60, and 70 mg/kg, respectively. Morphine was injected twice daily (at 08.00 am and 04.00 pm). Vehicle group was pretreated orally with 0.5% CMC (5 ml/kg) twice daily. Standard group was pretreated with methadone (1 mg/kg) intraperitoneally. Treatment groups were pretreated orally with various dose of MS (50, 100, 200, and 400 mg/kg) twice daily. All groups were pretreated 30 min before morphine injection every day and withdrawal symptoms were precipitated with naloxone (3 mg/kg; i.p.) two hours after the first dose of the seventh day. Immediately after naloxone injection, each mouse was placed in a glass cylinder and withdrawal symptoms were observed and recorded through VDO camera for 30 min (Matsumoto et al., 2004).
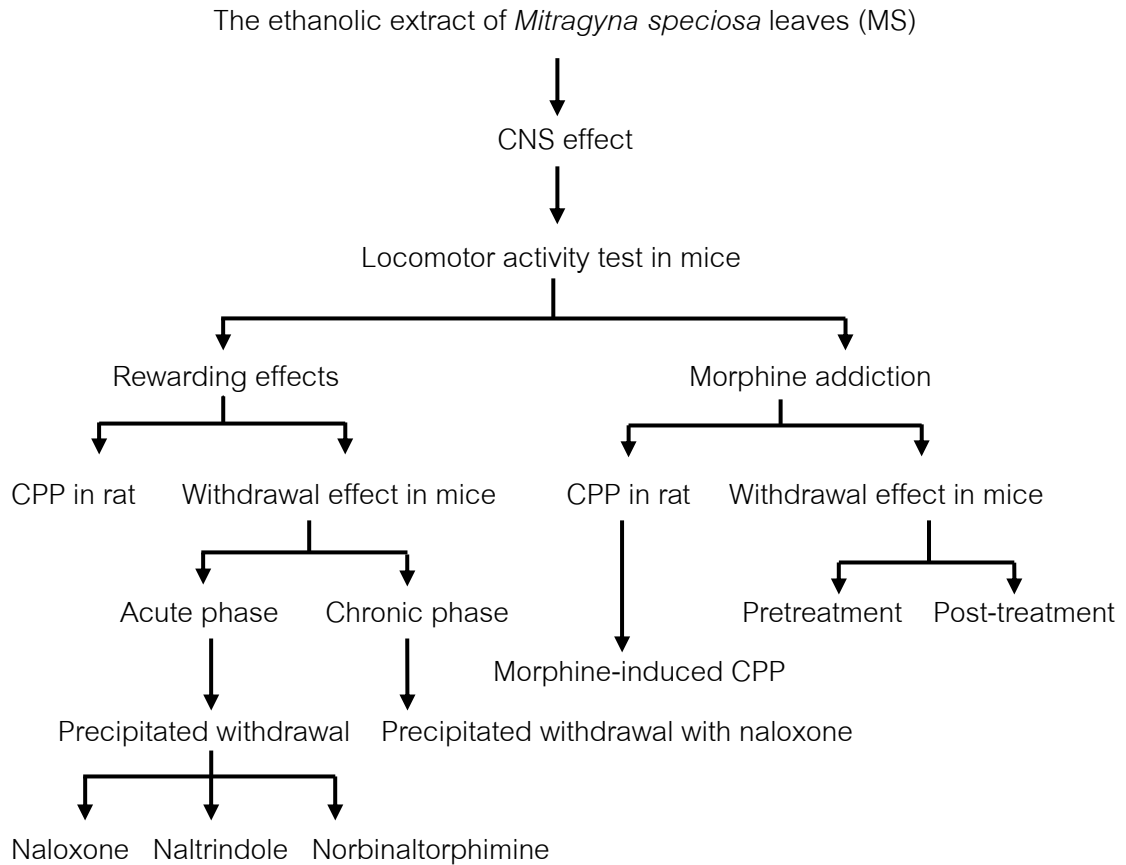
3.6. Effect of MS post-treatment on morphine withdrawal

Morphine dependence was induced by intraperitoneal administration. Dose of morphine was increased each day from the first day to the seventh day to 10, 20, 30, 40, 50, 60, and 70 mg/kg, respectively. Morphine was injected twice daily (at 08.00 am and 04.00 pm). On day 7, 2 hours after the first dose of morphine, all groups were treated with tested substances. Vehicle group was treated orally with 0.5% CMC (5 ml/kg). Standard group was treated with methadone (1 mg/kg) intraperitoneally. Treatment groups were treated orally with various doses of MS (50, 100, 200, and 400 mg/kg). Thirty minutes after treatment, withdrawal symptoms were precipitated with naloxone (3 mg/kg; i.p.). Immediately after naloxone injection, each mouse was placed in a glass cylinder and withdrawal symptoms were observed and recorded through VDO camera for 30 min (Matsumoto et al., 2004).

Statistical analyses

The data were presented as the mean ± S.E.M.. Statistical analyses were performed with Student's paired t-test or one way analysis of variance (ANOVA) and followed by Dunnett's test and Fisher's LSD test where applicable. Values of $p < 0.05$ was considered statistically significant.

Experimental design

The ethanolic extract of *Mitragyna speciosa* leaves (MS)

↓

CNS effect

↓

Locomotor activity test in mice

Rewarding effects   Morphine addiction

CPP in rat Withdrawal effect in mice CPP in rat Withdrawal effect in mice

Acute phase Chronic phase   Pretreatment Post-treatment

Morphine-induced CPP

Precipitated withdrawal Precipitated withdrawal with naloxone

Naloxone Naltrindole Norbinaltorphimine

Remarks: CPP   = Conditioned place preference

    Naloxone   = μ-opioid antagonist

    Naltrindole   = δ-opioid antagonist

    Norbinaltorphimine = κ-opioid antagonist

CHAPTER IV

RESULTS

Effect of MS on locomotor activity

To determine the effect of various doses of MS on the central nervous system, locomotor activity was investigated in mice after administration of the test substances. Total locomotor activity counts of 0.9% NSS, morphine (5 mg/kg; i.p.), 0.5% CMC and various doses of MS (50, 100, 200 and 400 mg/kg; p.o.) were 2134.25 ± 249.85, 2123.13 ± 203.66, 2202.50 ± 180.55, 2216.25 ± 184.99, 2358.1 ± 208.35, 2182.75 ± 147.34, and 2331.50 ± 176.56 counts, respectively. No significant difference was found among all groups ($p<0.05$; Figure 4.1).
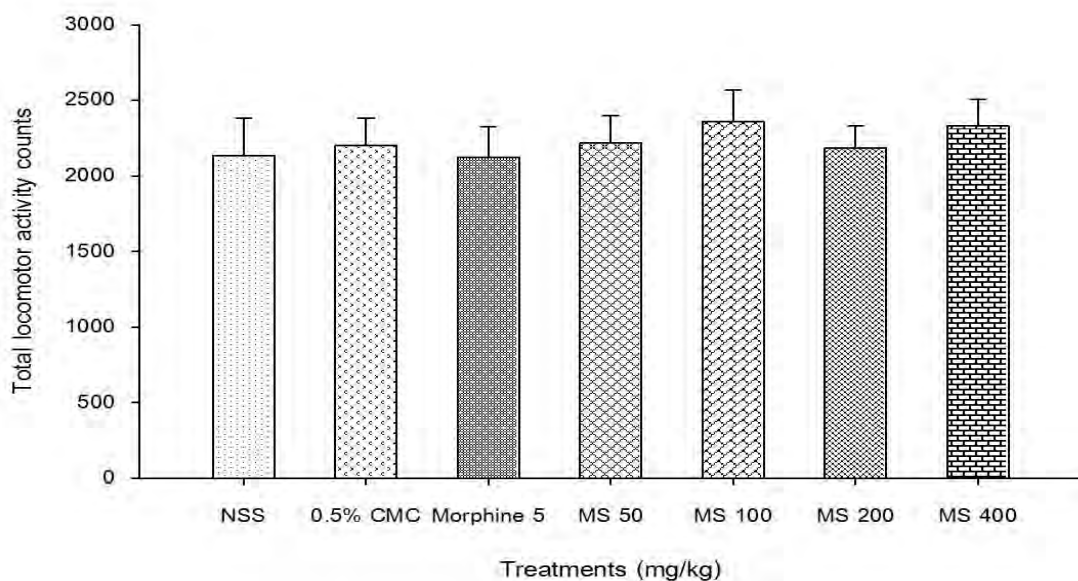
Figure 4.1 Locomotor activity in mice produced by NSS, morphine (5 mg/kg; i.p.), 0.5% CMC, and various doses of MS (50-400 mg/kg; p.o.). Each value represents mean ± S.E.M. N=8 for all groups.

Effect of MS on conditioned place preference

In order to investigate the rewarding effects of various doses of MS, the conditioned place preference test (CPP) in rats was used. All treatments were pretreated 30 min before starting the experiments. The time spent in the drug-paired compartment of morphine (5 mg/kg; i.p.) in the test phase (240.50 ± 22.21 sec) significantly ($p<0.05$) increased when compared with the preconditioning phase (127.75 ± 13.36 sec; Figure 4.2). Moreover, the time spent in the drug-paired compartment of morphine (5 mg/kg; i.p.) in the test phase was also significantly ($p<0.01$) different when compared to 0.5% CMC (142.38 ± 16.01 sec; Figure 4.2). No significant difference was observed between the time spent in the drug-paired compartment in the test phase and the conditioning phase of all doses of MS tested (Figure 4.2).
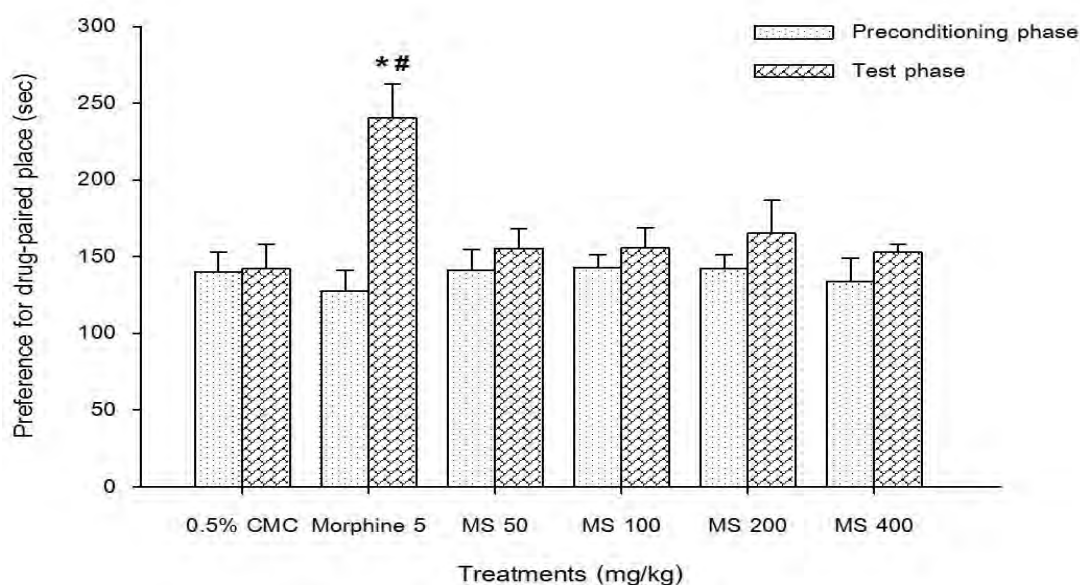

Figure 4.2 Conditioned place preference in rats produced by morphine (5 mg/kg; i.p.) Each values represents mean ± S.E.M. N=8 for all groups. *$p<0.05$ significantly different from preconditioning phase. #$p<0.01$ significantly different from 0.5% CMC.

Effect of MS on morphine-induced conditioned place preference

   In order to investigate the effect of various doses of MS pretreatment on rewarding effects of morphine, the conditioned place preference test in rats was used. Pretreatment with methadone (1 mg/kg; i.p.), the positive control, significantly ($p<0.05$) decreased morphine-induced place preference. Pretreatment with all doses of MS significantly ($p<0.05$) decreased % change of place preference induced by morphine when compared to 0.5% CMC (Figure 4.3).
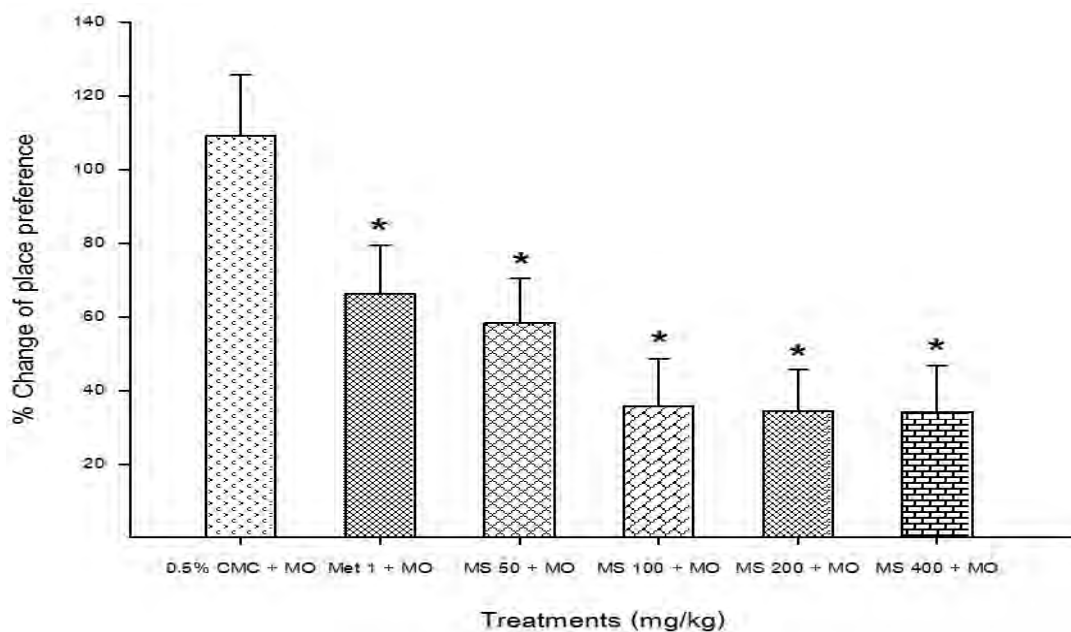

Figure 4.3 Effects of 0.5% CMC, methadone (Met 1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on morphine (MO)-induced conditioned place preference in rats. Percent change of place preference equals the difference in time (s) spent in the white compartment between preconditioning and test phases over the time spent in the preconditioning phase. Each value represents mean± S.E.M. N=8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.

Precipitated withdrawal symptoms of acute MS treatment with naloxone

To determine withdrawal symptoms caused by μ-opioid receptor activation of various doses of MS acutely administered, precipitated withdrawal symptoms with naloxone, a μ-opioid receptor antagonist, was performed in mice. Morphine (100 mg/kg; i.p.) significantly ($p<0.05$) showed withdrawal symptoms precipitated by naloxone, especially jumping and straub tail when compared to 0.5% CMC (Figure 4.4 and 4.7). In contrast, all doses of MS (50, 100, 200 and 400 mg/kg) did not show any significant withdrawal symptoms after naloxone injection when compared to 0.5% CMC (Figure 4.4-4.9).
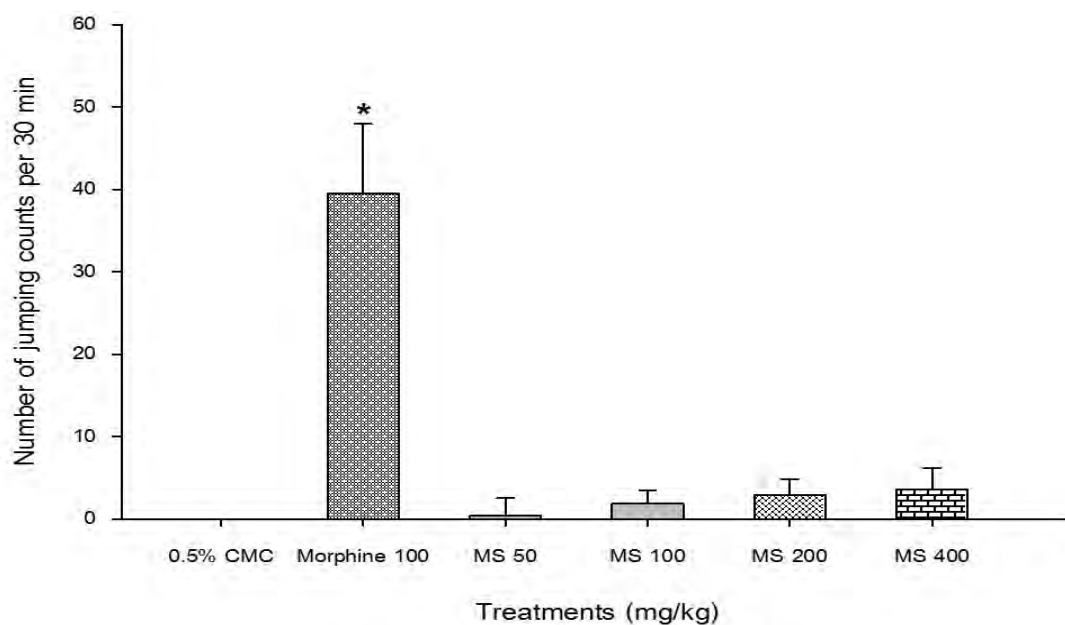

Figure 4.4 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
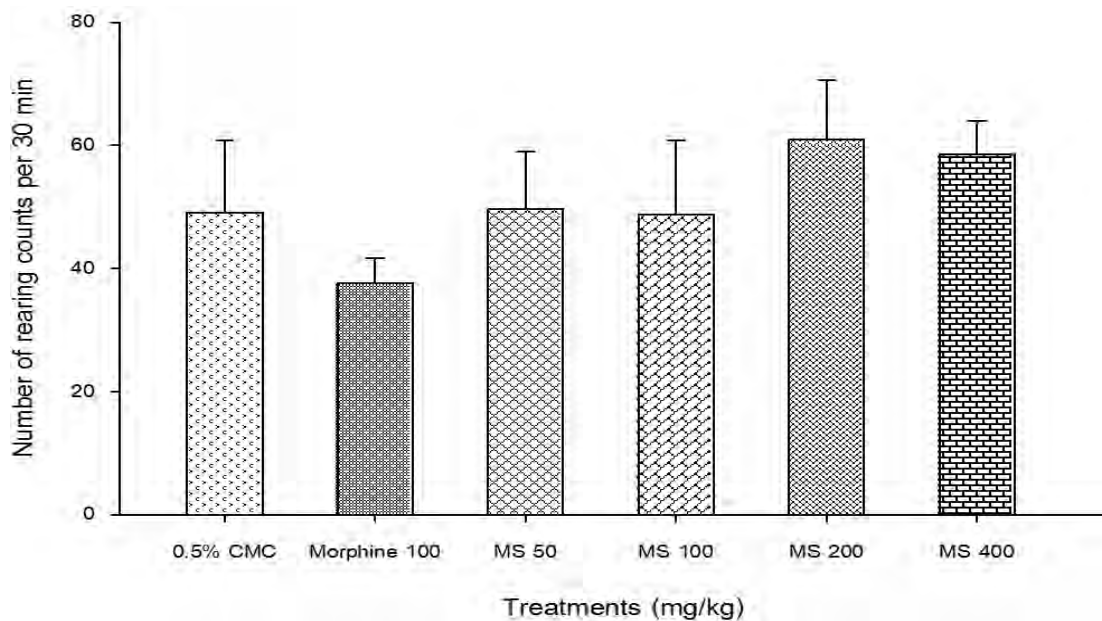
Figure 4.5 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
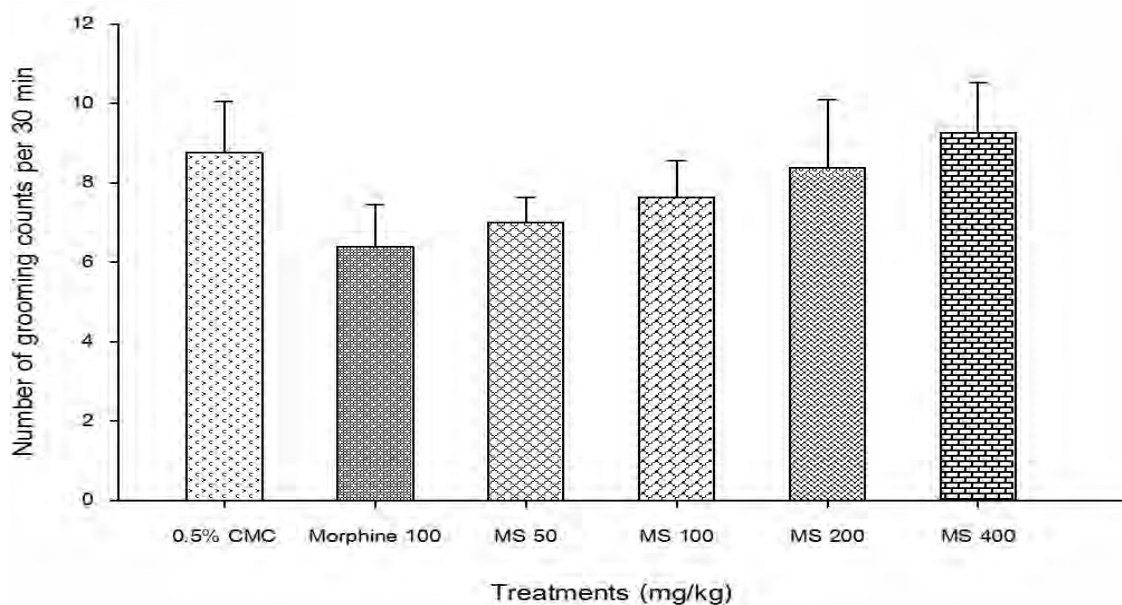

Figure 4.6 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
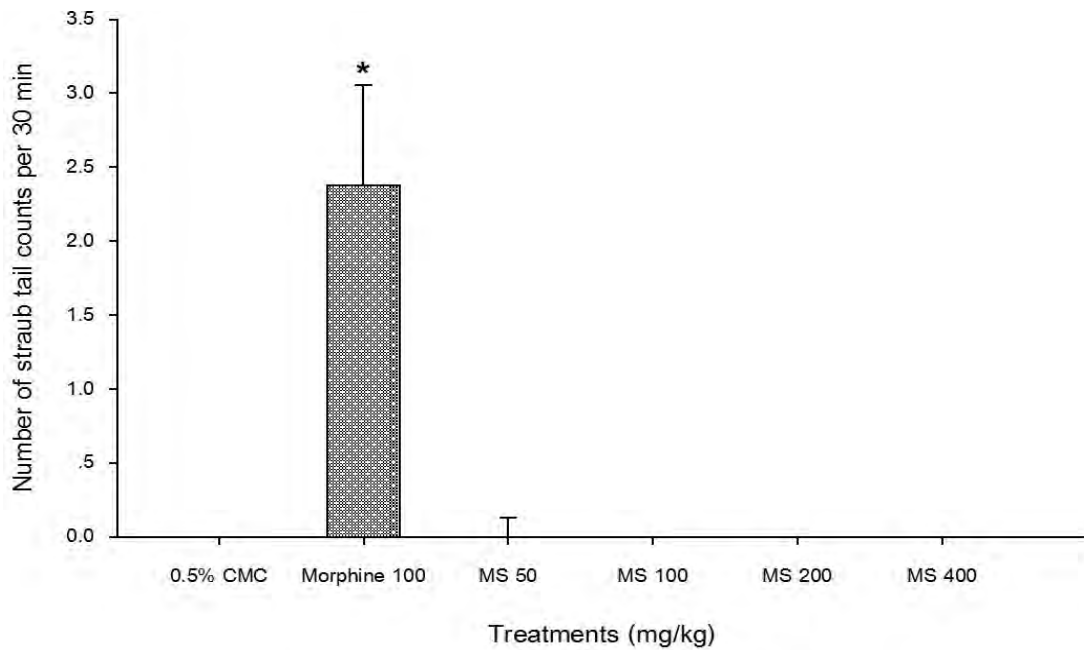
Figure 4.7 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
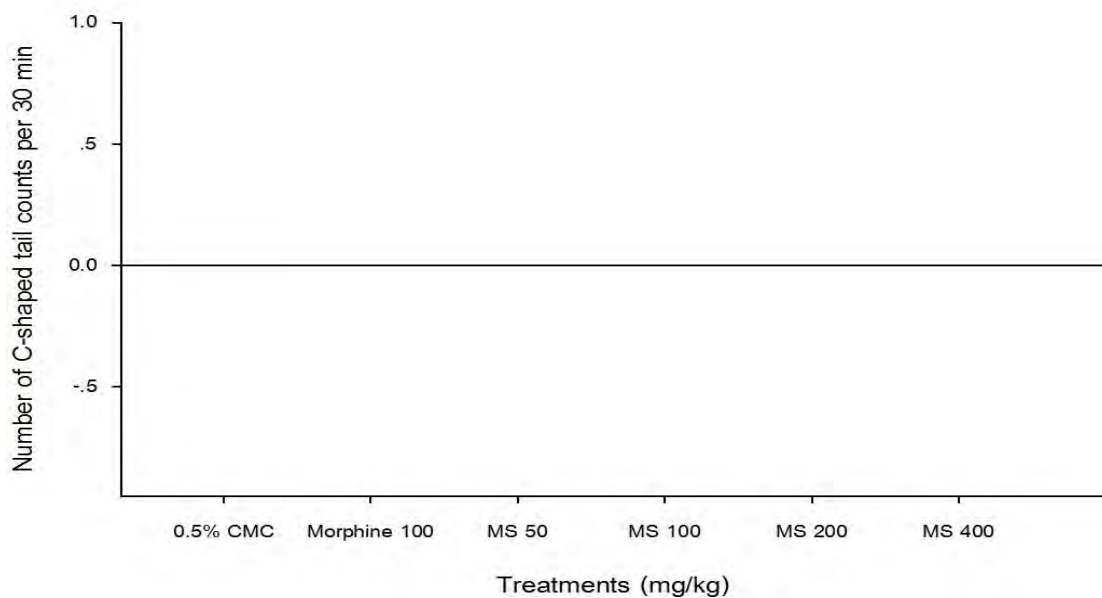

Figure 4.8 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
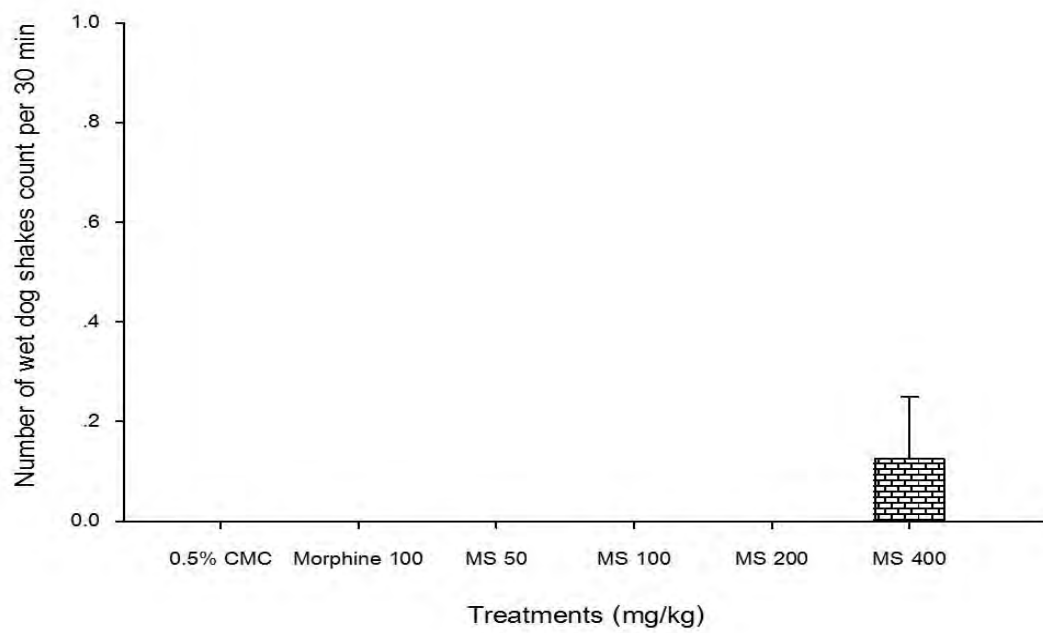
Figure 4.9 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Precipitated withdrawal symptoms of acute MS treatment with naltrindole

To determine withdrawal effects caused by δ-opioid receptor activation of various doses of MS acutely administered, precipitated withdrawal symptoms with naltrindole, a δ-opioid receptor antagonist, was performed in mice. Morphine (100 mg/kg; i.p.) did not show any significant withdrawal symptoms after naltrindole injection (Figure 4.10-4.15). Similarly, all doses of MS (50, 100, 200 and 400 mg/kg) also did not showed any significant withdrawal symptoms after naltrindole injection (Figure 4.10-4.15).
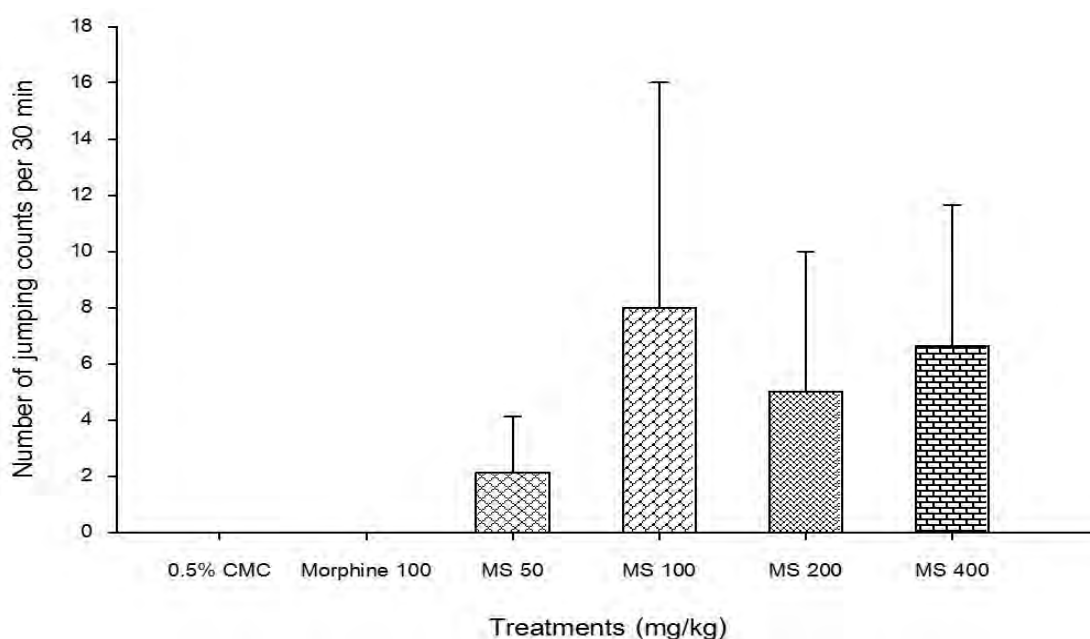

Figure 4.10 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.11 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.
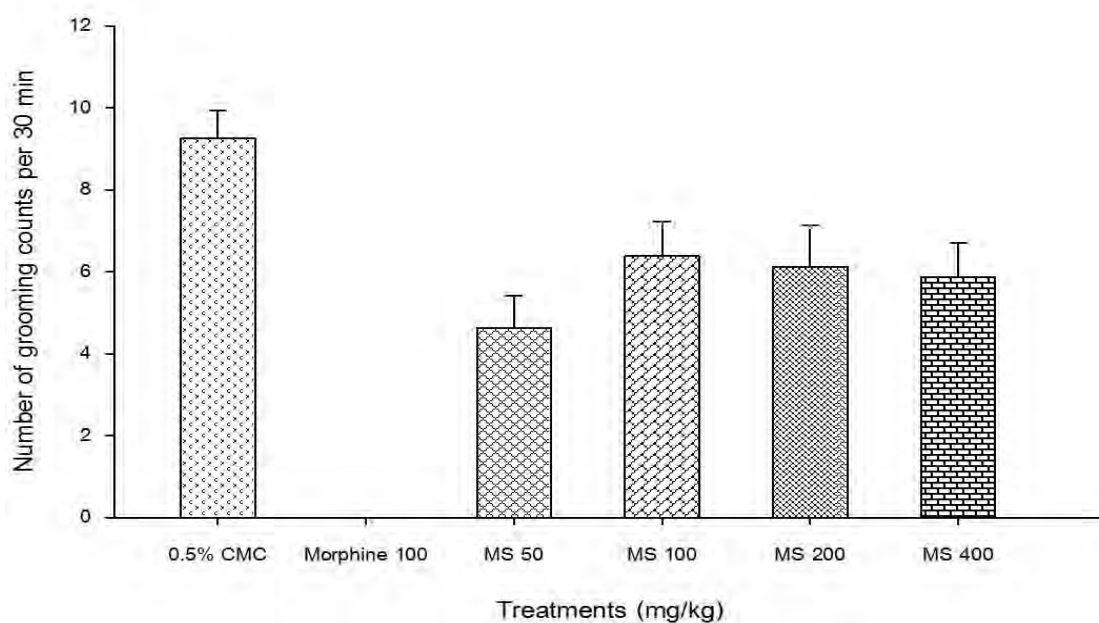
Figure 4.12 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.
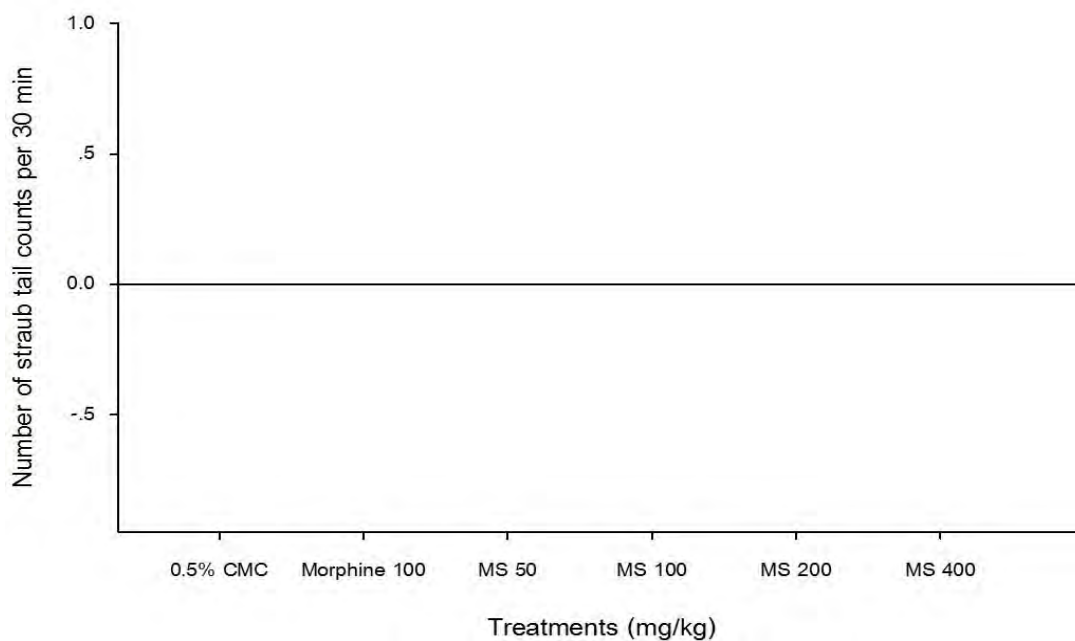
Figure 4.13 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.
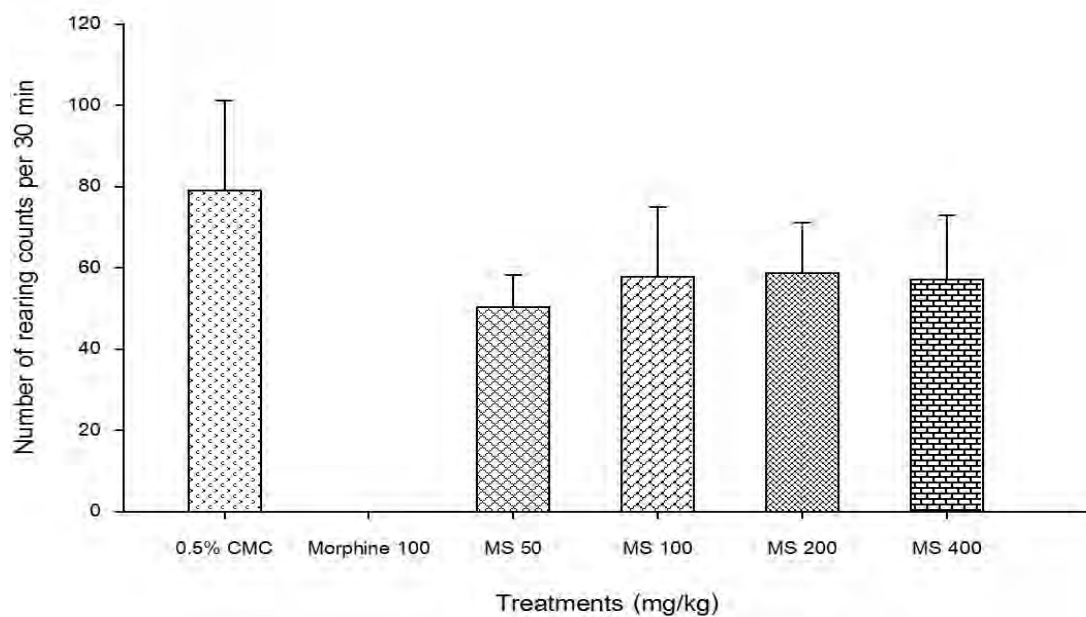

Figure 4.14 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.15 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Precipitated withdrawal symptoms of acute MS treatment with norbinaltorphimine

To determine withdrawal effects caused by K-opioid receptor activation of various doses of MS acutely administered, precipitated withdrawal symptoms with norbinaltorphimine, a K-opioid receptor antagonist, was performed in mice. Morphine (100 mg/kg; i.p.) did not show any significant withdrawal symptoms after norbinaltorphimine injection (Figure 4.16-4.21). Similarly, all doses of MS (50, 100, 200 and 400 mg/kg) also did not showed any significant withdrawal symptoms after norbinaltorphimine injection (Figure 4.16-4.21).

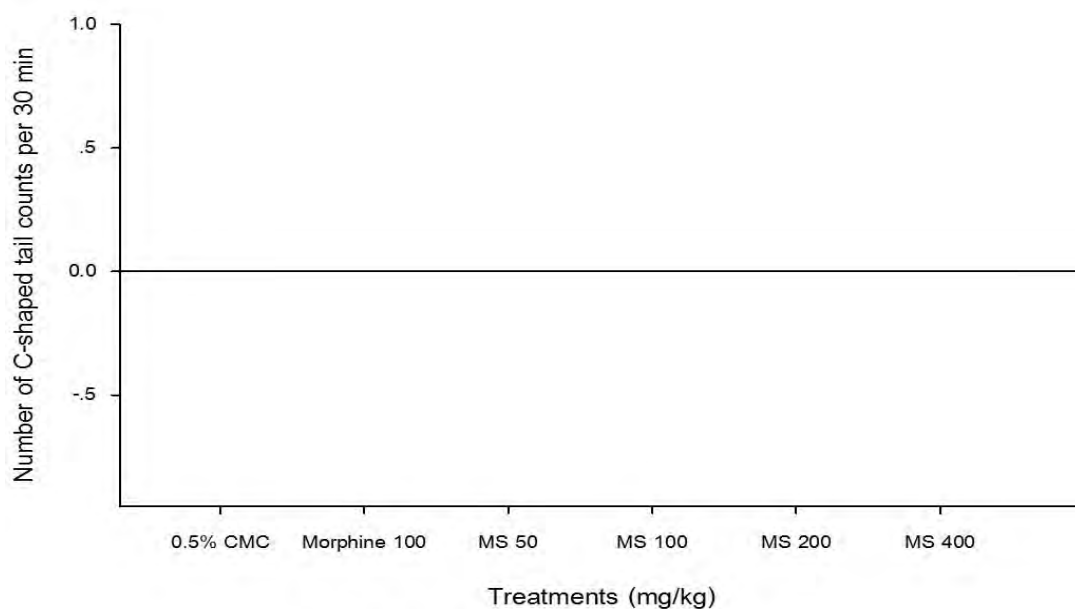**Figure 4.16** Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.
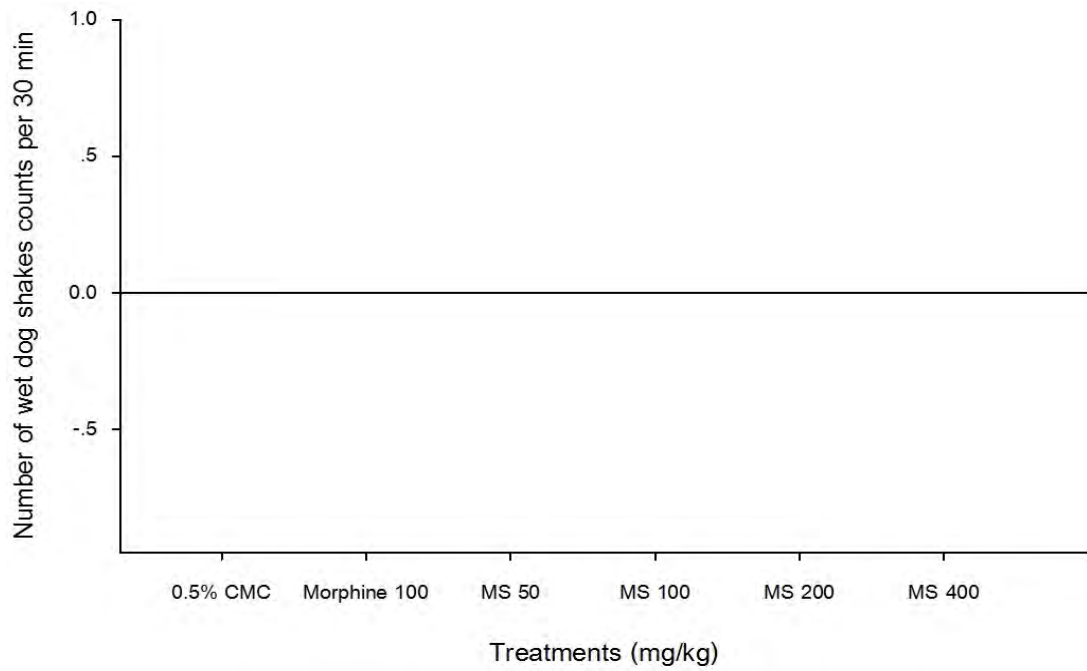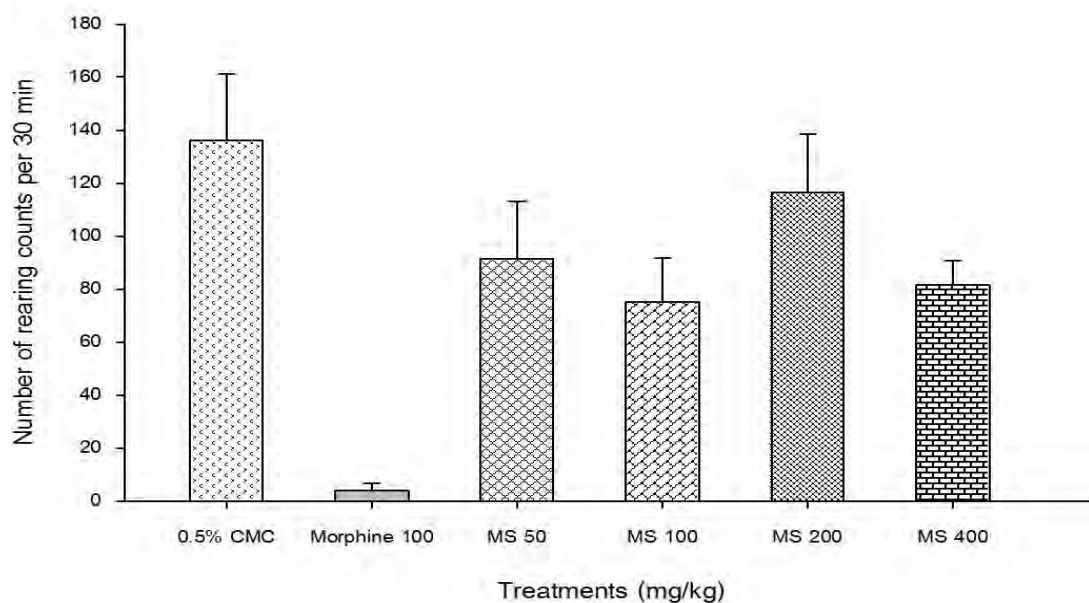
Figure 4.17 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.18 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.19 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.20 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.
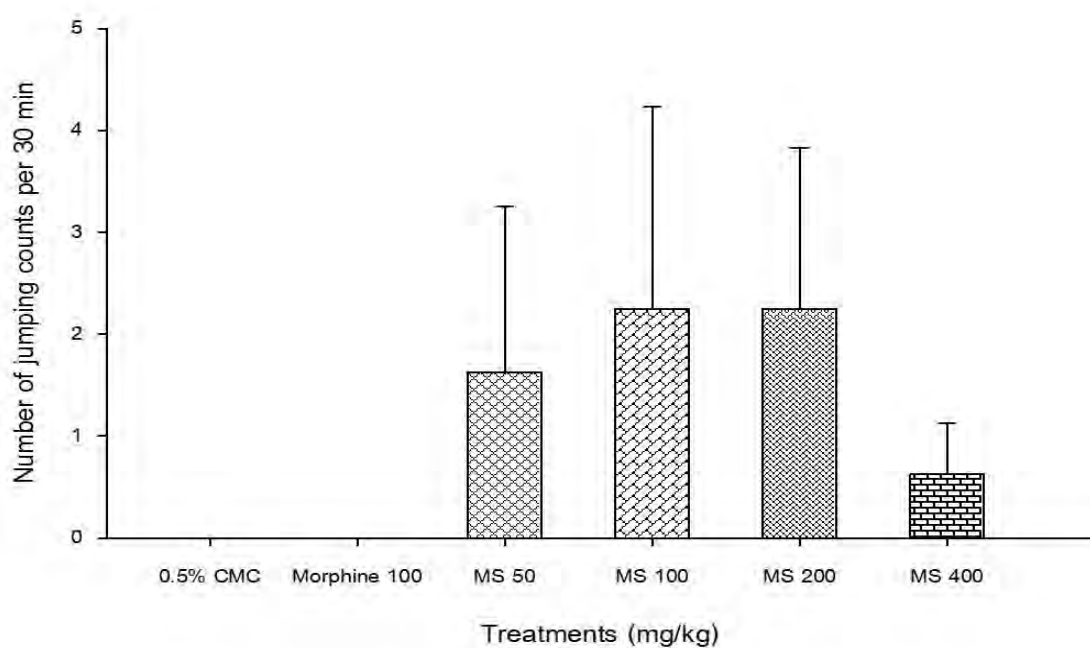
Figure 4.21 Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Precipitated withdrawal symptoms of chronic MS treatment by naloxone

        To determine withdrawal effects caused by μ-opioid receptor activation of various doses of MS chronically administered for 7 days, precipitated withdrawal symptoms with naloxone, a μ-opioid receptor antagonist, was performed in mice. Chronic administration of morphine (increasing dose from 10-70 mg/kg; i.p.) showed significant ($p<0.05$) withdrawal symptoms including jumping, straub tail, C-shaped tail, and wet dog shakes behaviors when compared to 0.5% CMC (Figure 4.22, 4.25-4.27). On the other hand, chronic treatment of all doses of MS (50, 100, 200 and 400 mg/kg) did not showed any significant withdrawal symptoms after naloxone injection when compared to 0.5% CMC (Figure 4.22-4.27).

Figure 4.22 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
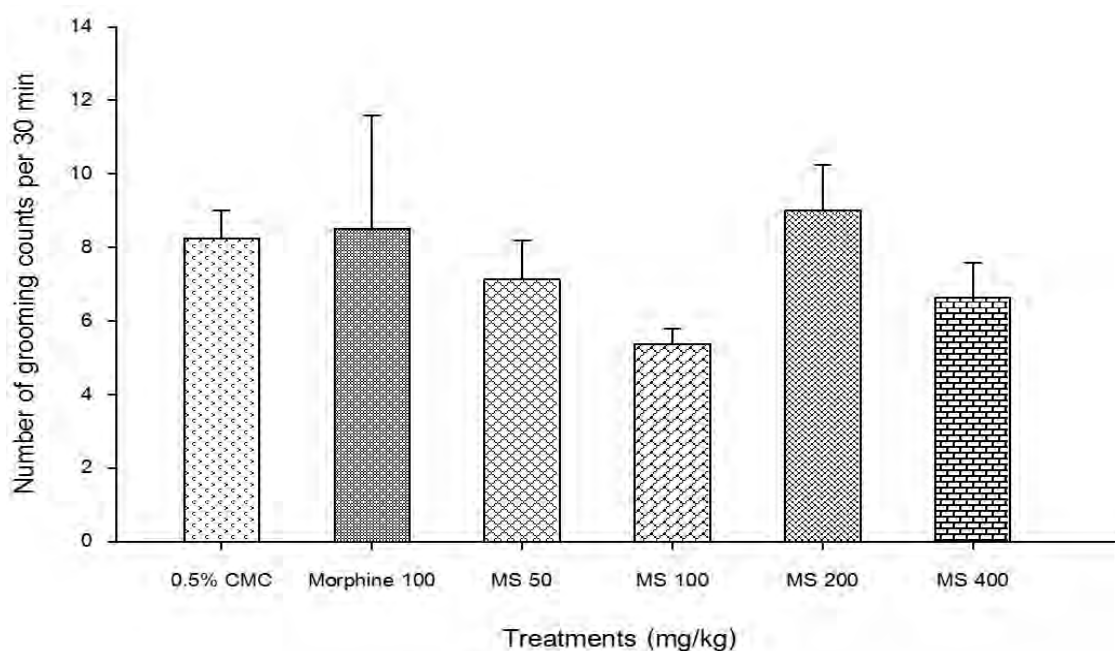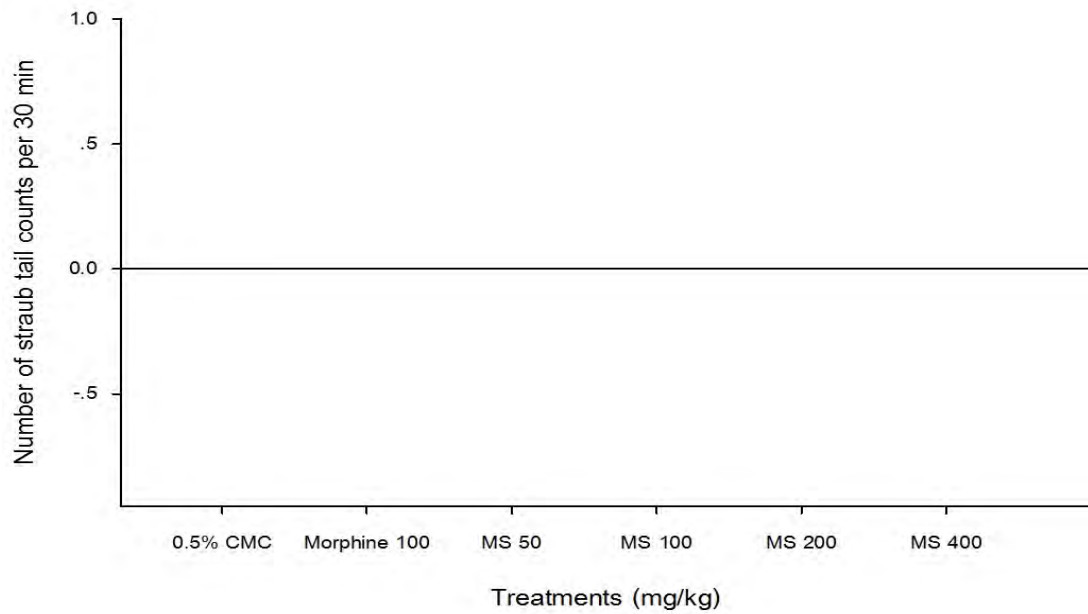
Figure 4.23 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.24 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M. N = 8 for all groups.

Figure 4.25 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
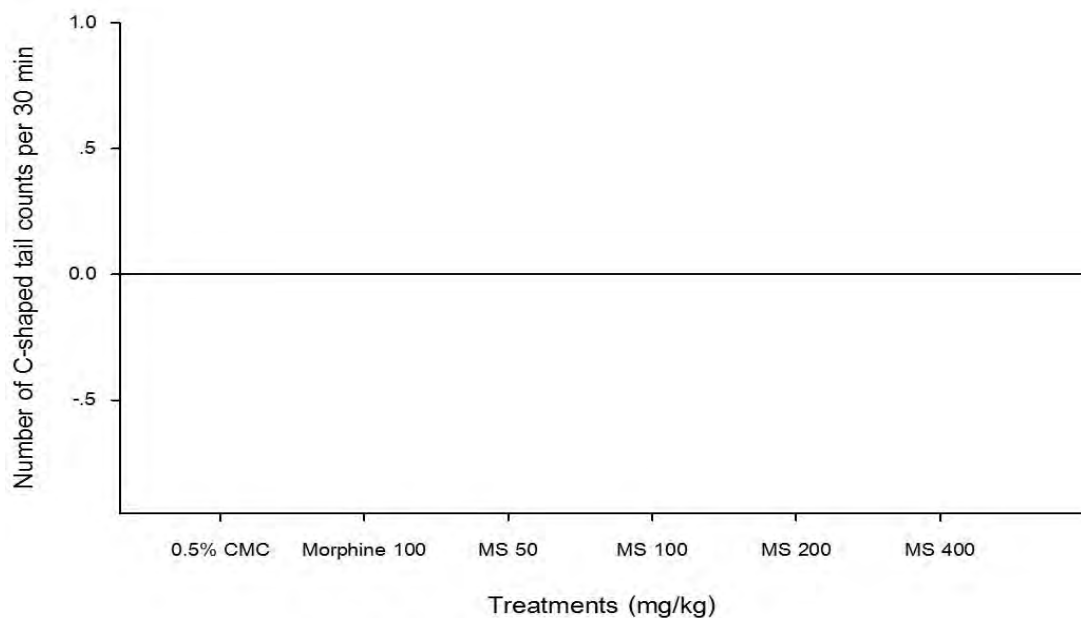

Figure 4.26 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
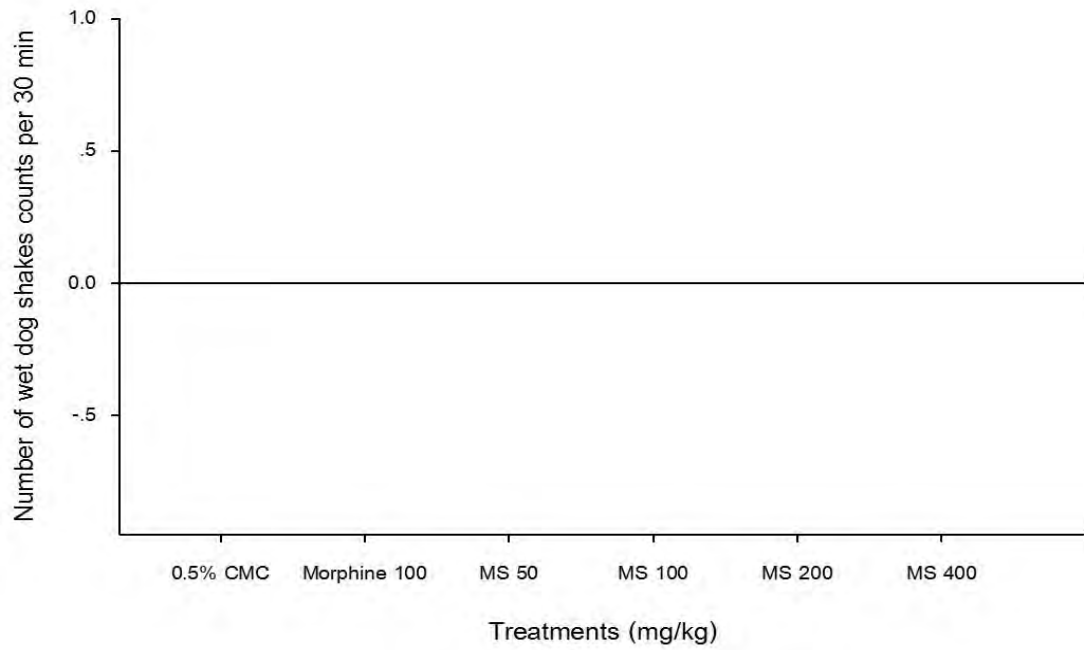
Figure 4.27 Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.

Effect of MS pretreatment on morphine withdrawal

To investigate the effect of pretreatment with various doses of MS on morphine dependence mice, withdrawal symptoms of morphine precipitated with naloxone were observed. Pretreatment with methadone (1 mg/kg; i.p.) significantly ($p<0.05$) decreased naloxone-precipitated withdrawal symptoms including jumping and straub tail behaviors when compare to 0.5% CMC (Figure 4.28 and 4.31). Pretreatment with all doses of MS (50, 100, 200 and 400 mg/kg) significantly ($p<0.05$) reduced only jumping behavior of morphine dependence mice in a dose-dependent manner (Figure 4.28).
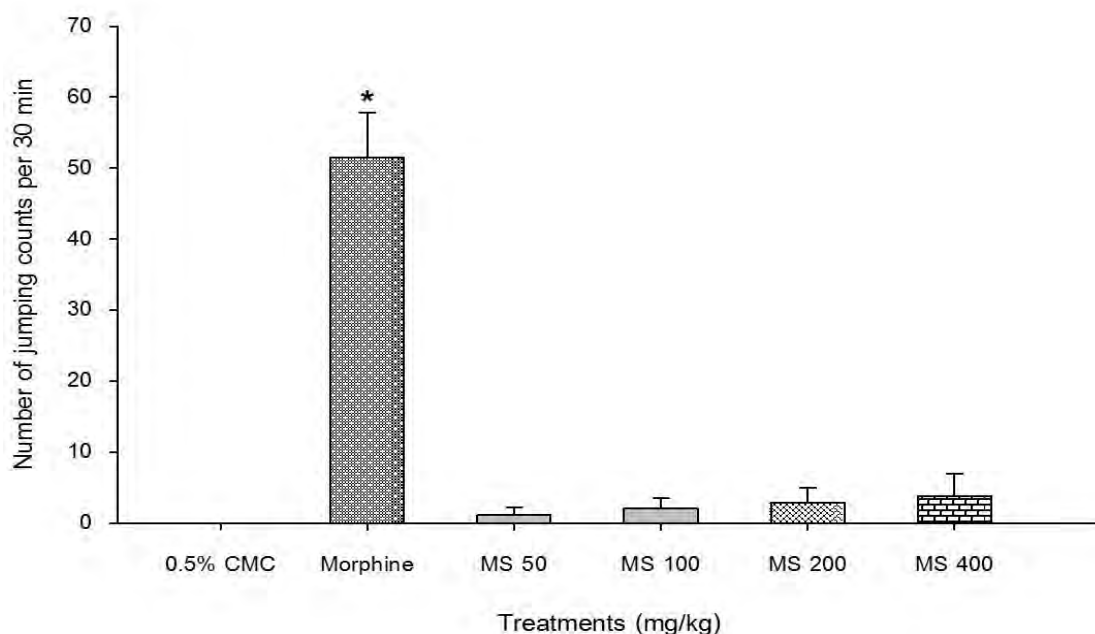

Figure 4.28 Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.

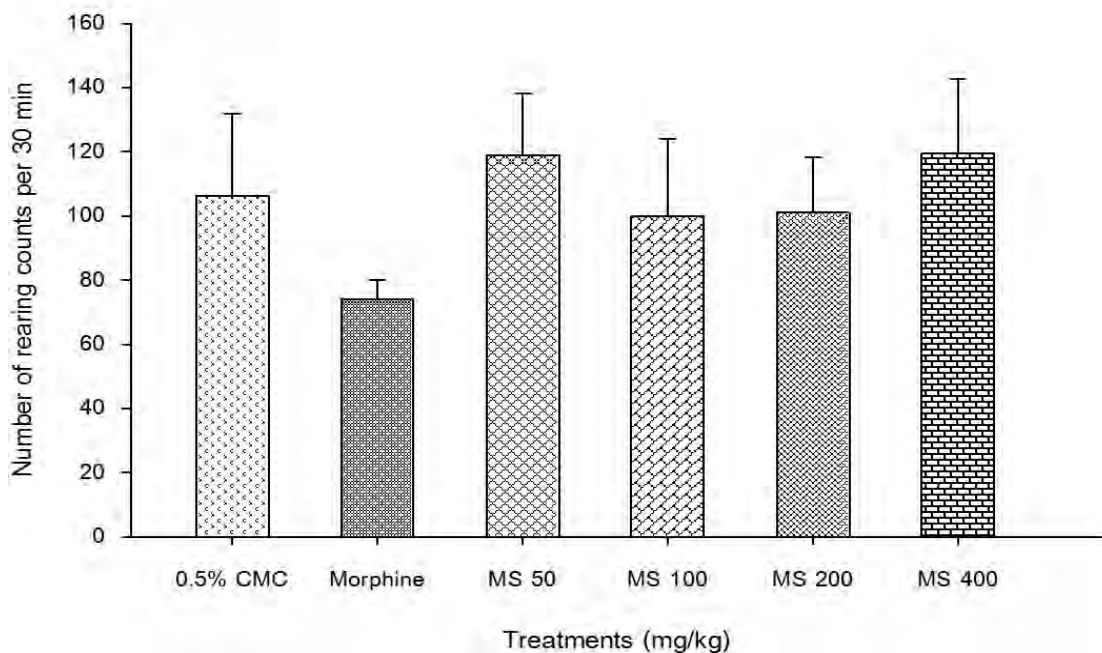**Figure 4.29** Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
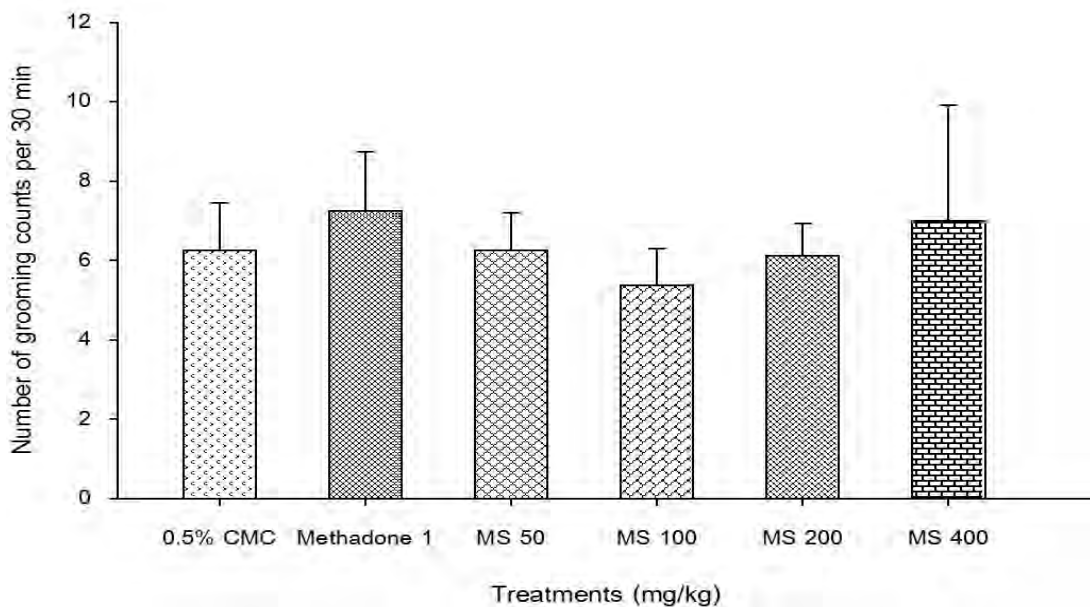
Figure 4.30 Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
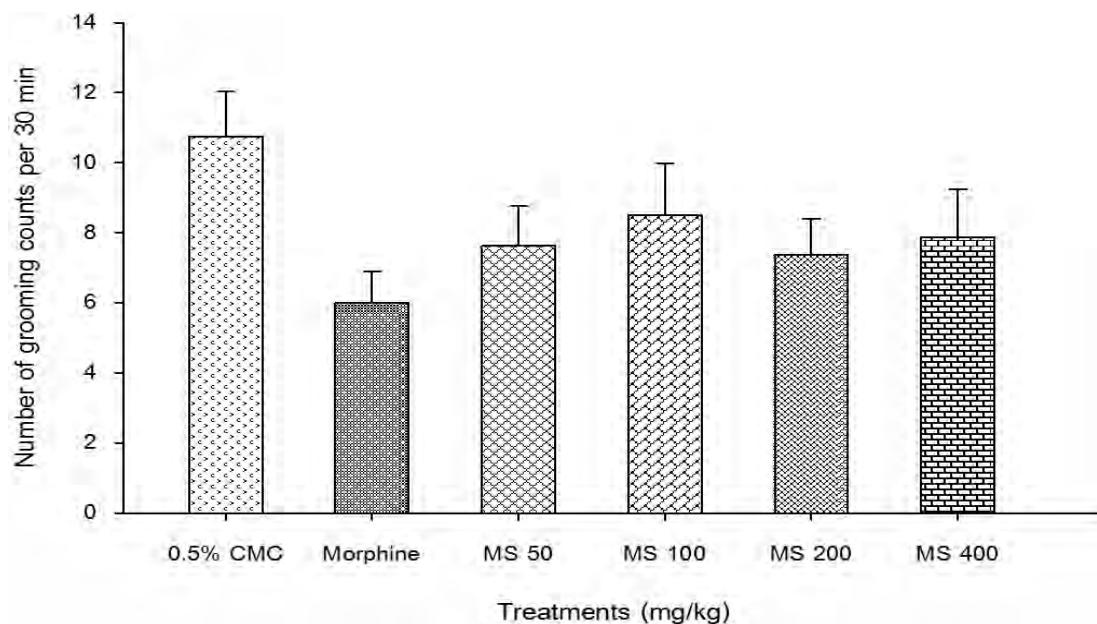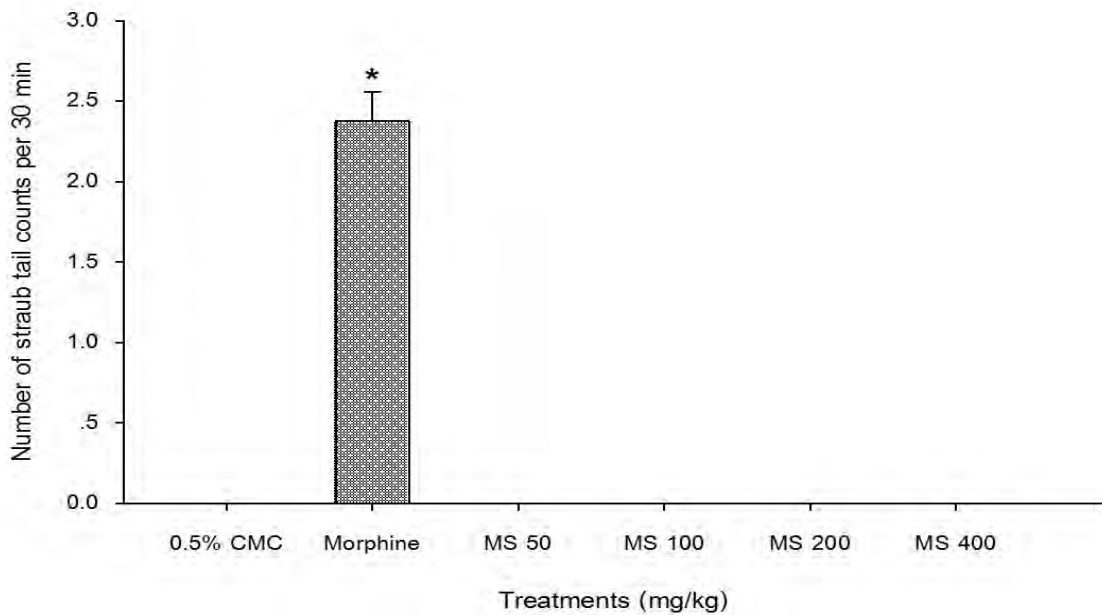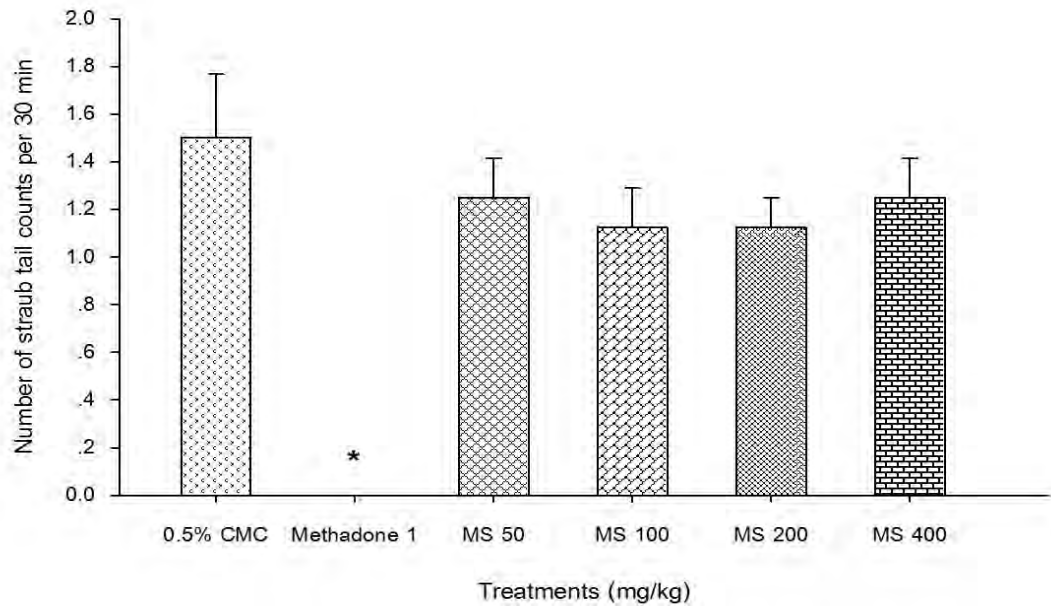
Figure 4.31 Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *p<0.05 significantly different from 0.5% CMC.
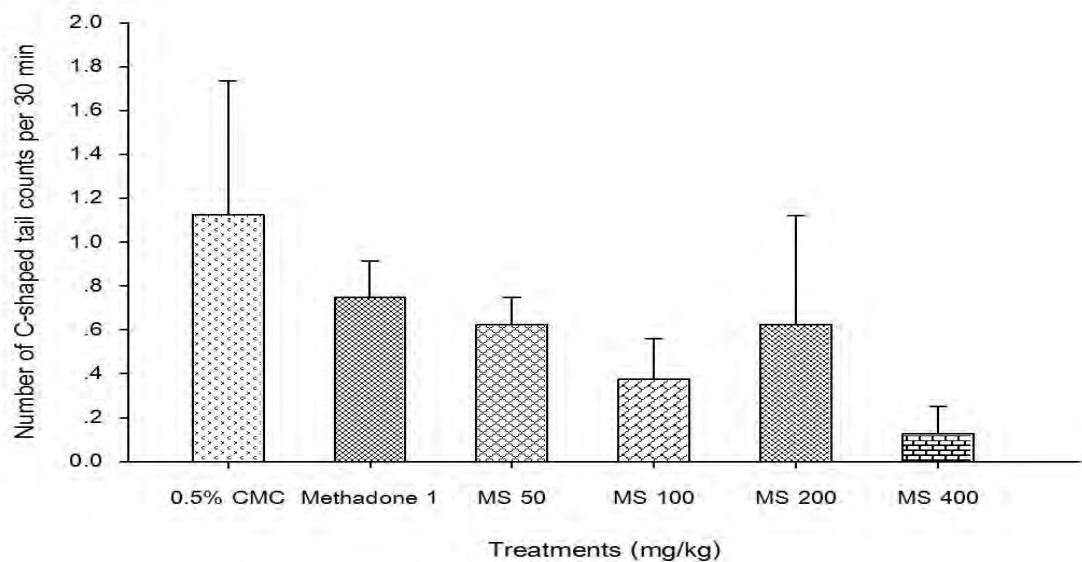
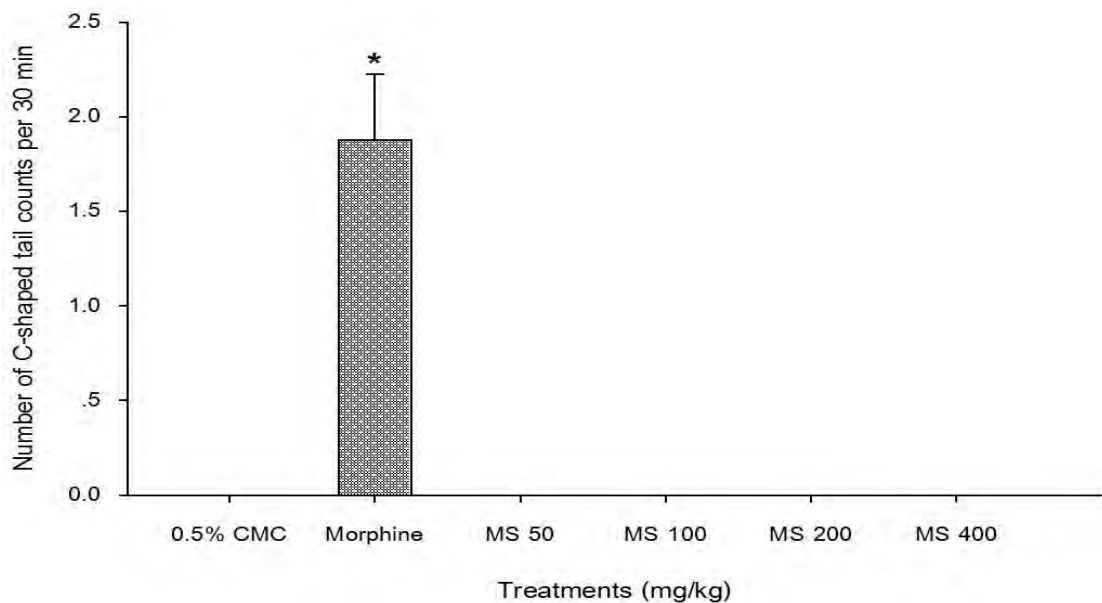**Figure 4.32** Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
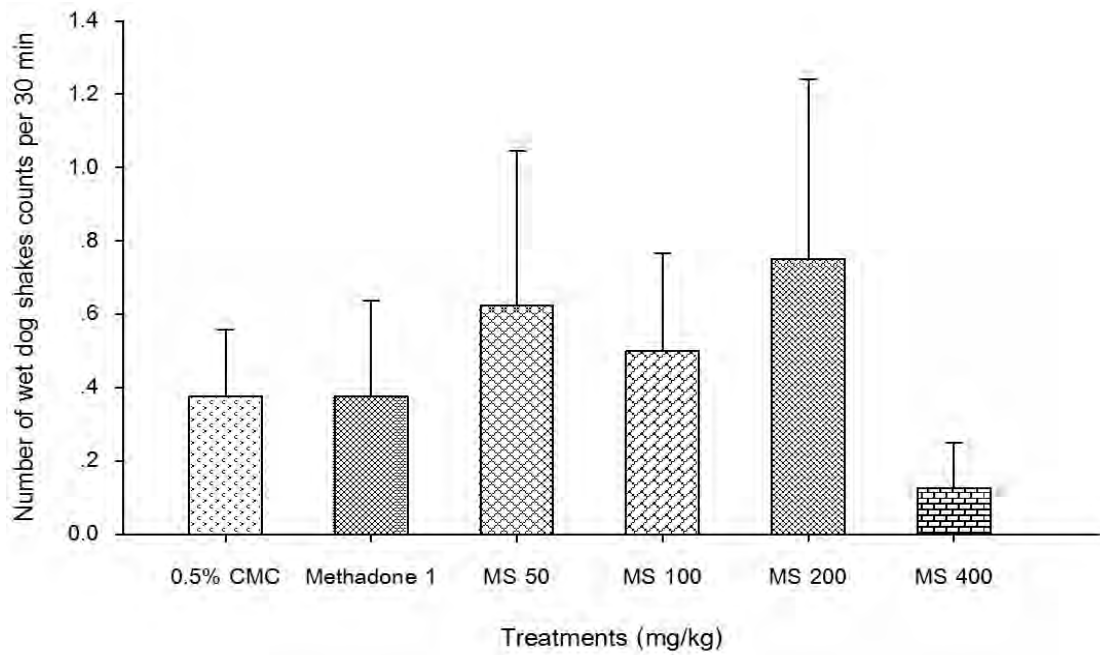
Figure 4.33 Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.

Effect of MS post-treatment on morphine withdrawal

To investigate the effect of various doses of MS on morphine dependence mice, withdrawal symptoms of morphine precipitated with naloxone were observed. Methadone (1 mg/kg, i.p.) significantly ($p<0.05$) decreased only jumping behavior of morphine dependence mice when compared to 0.5% CMC (Figure 4.34). All doses of MS (50, 100, 200 and 400 mg/kg) also significantly ($p<0.05$) decreased jumping behavior of morphine dependence mice when compared to 0.5% CMC (Figure 4.34).
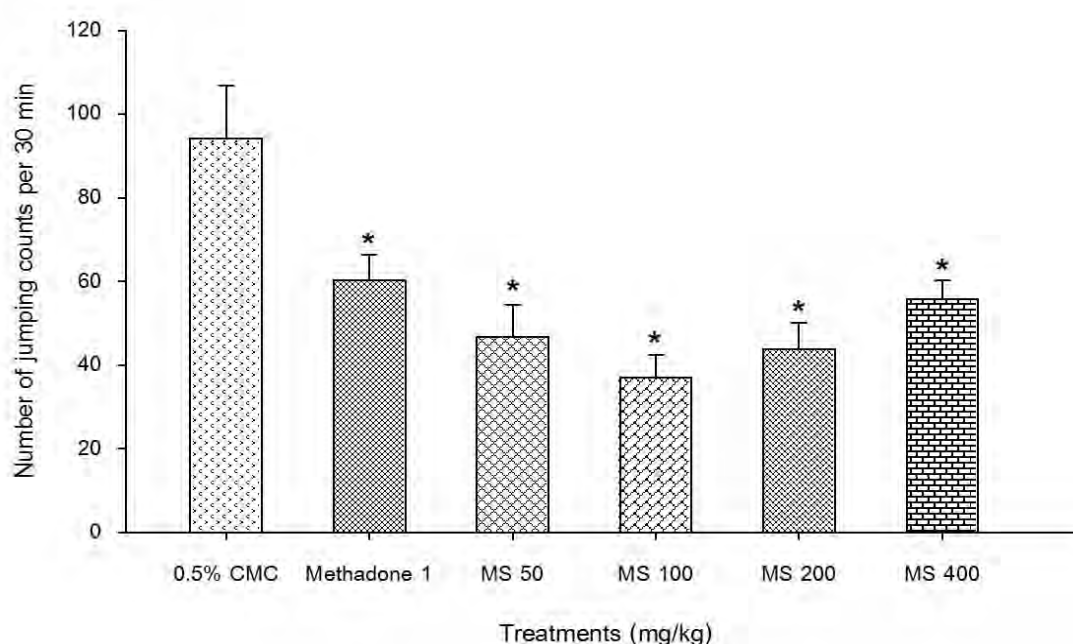
Figure 4.34 Effects of post-treatment of .5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC.
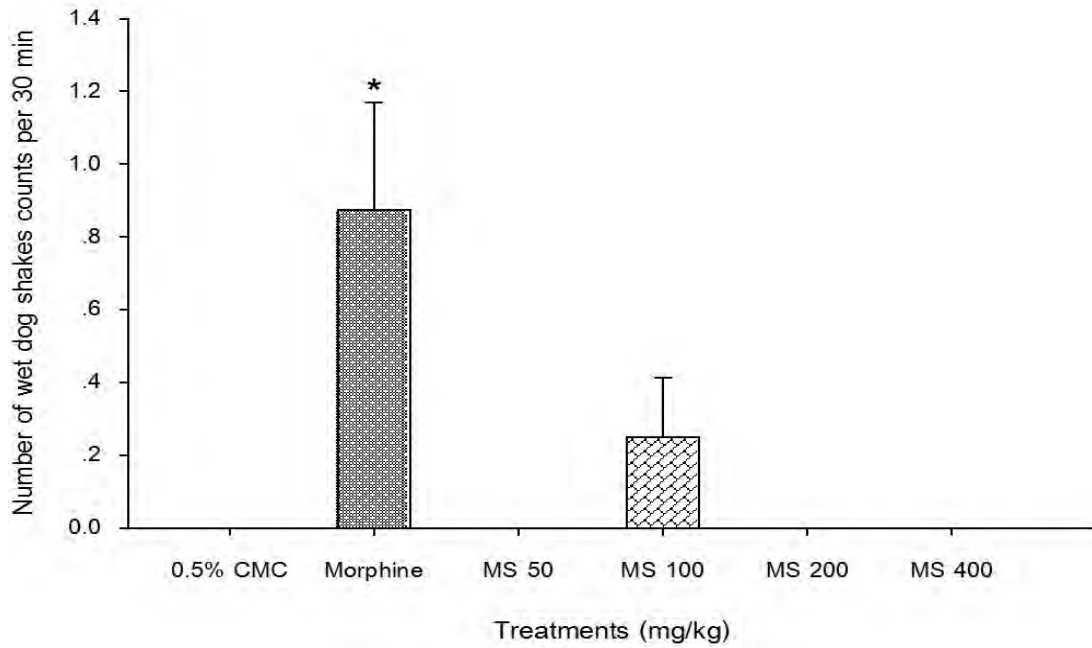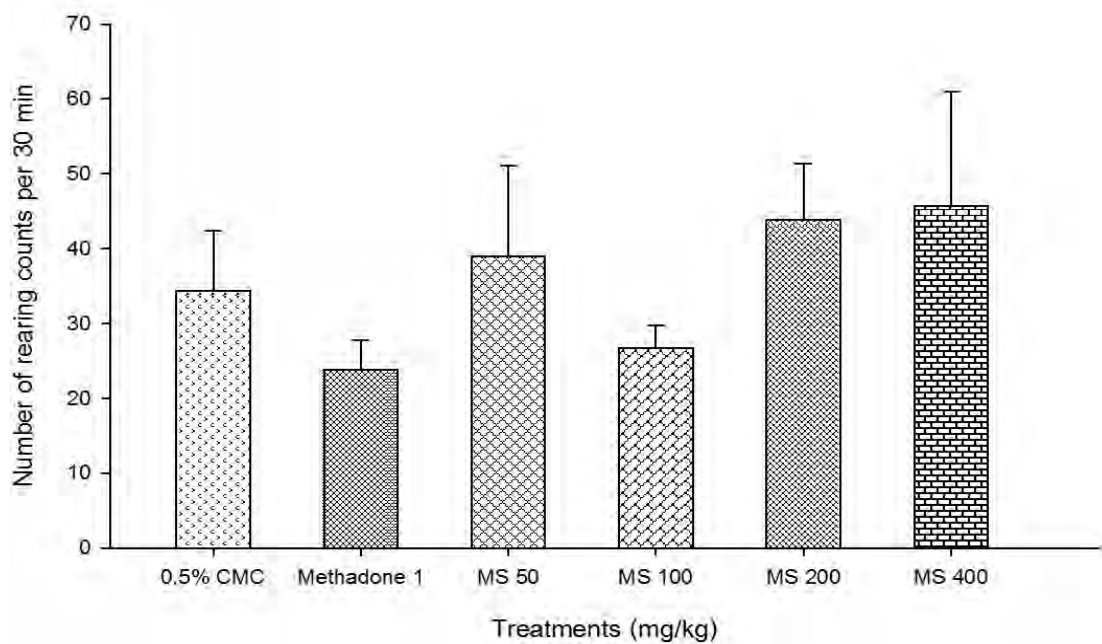
Figure 4.35 Effects of post-treatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on rearing behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
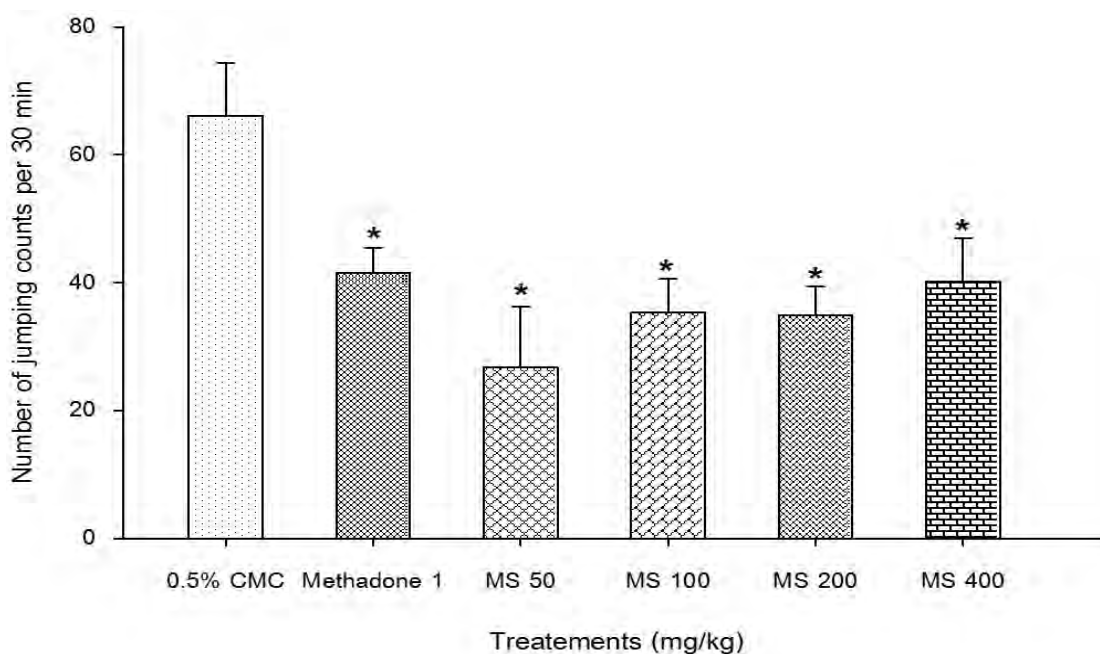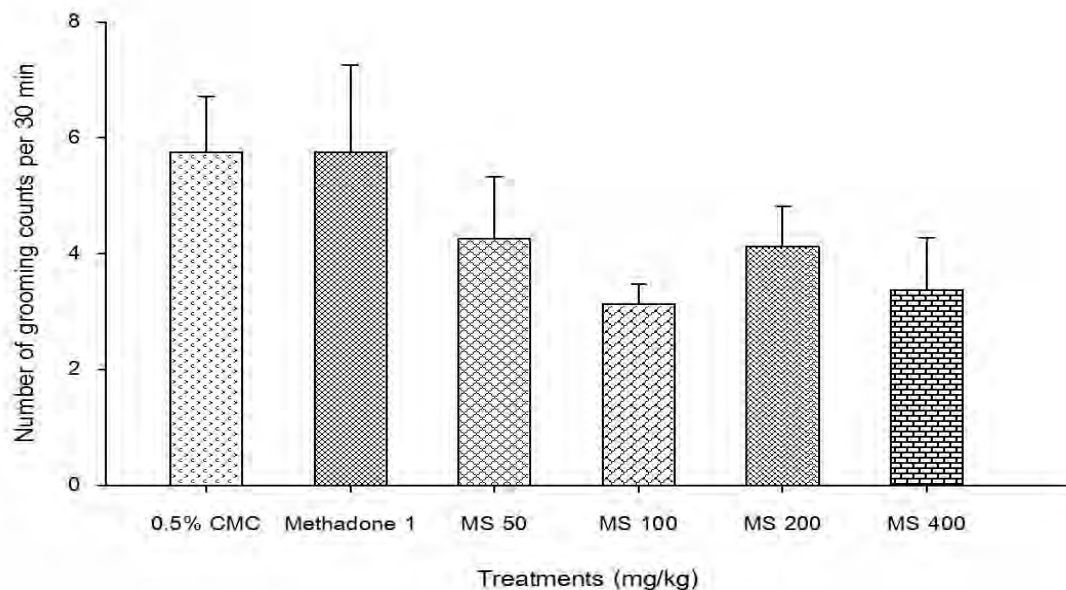

Figure 4.36 Effects of post-treatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on grooming behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
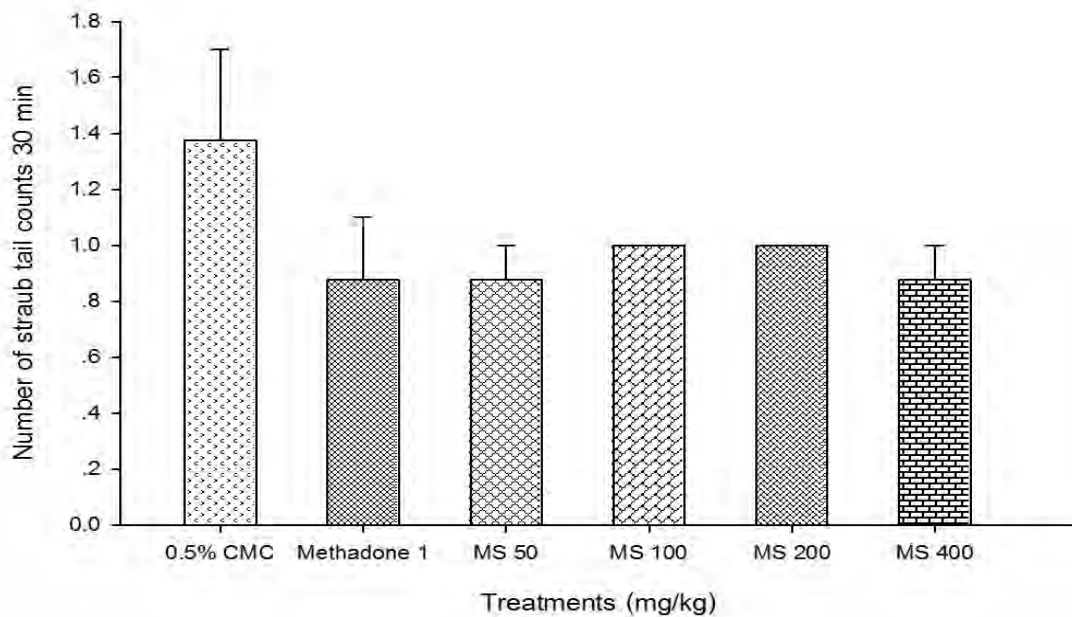
Figure 4.37 Effects of post-treatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on straub tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
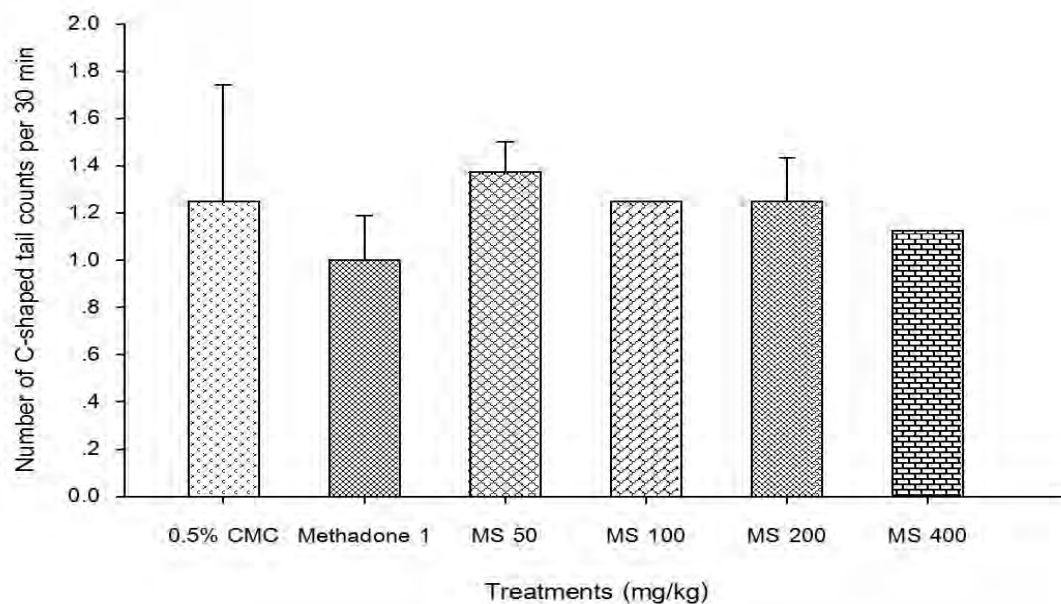
Figure 4.38 Effects of post-treatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on C-shaped tail behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.
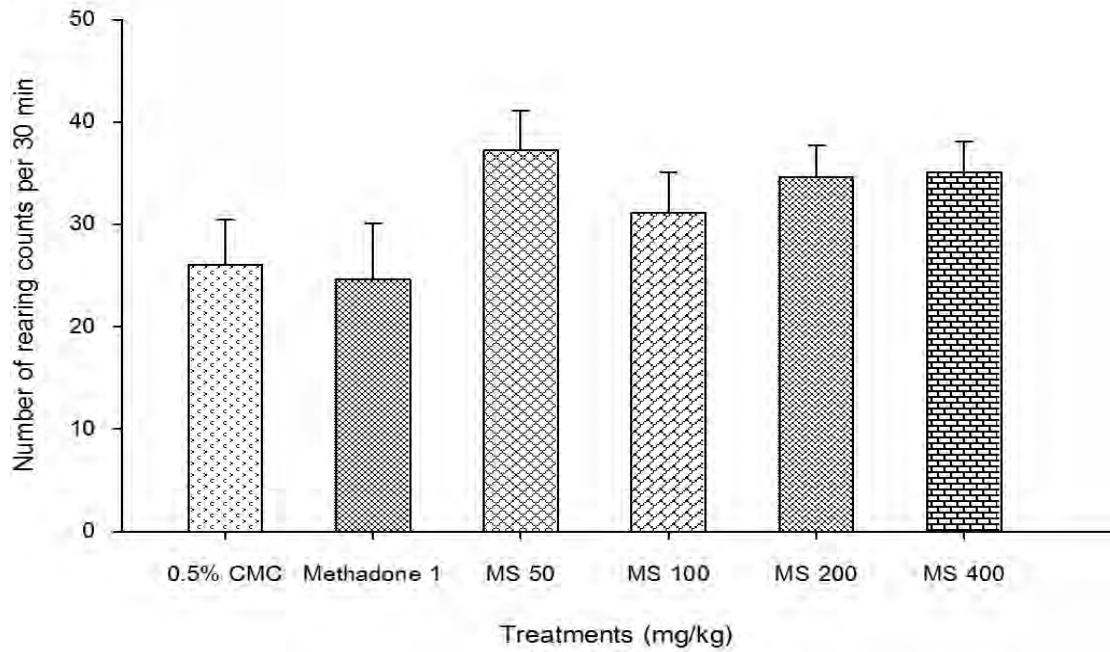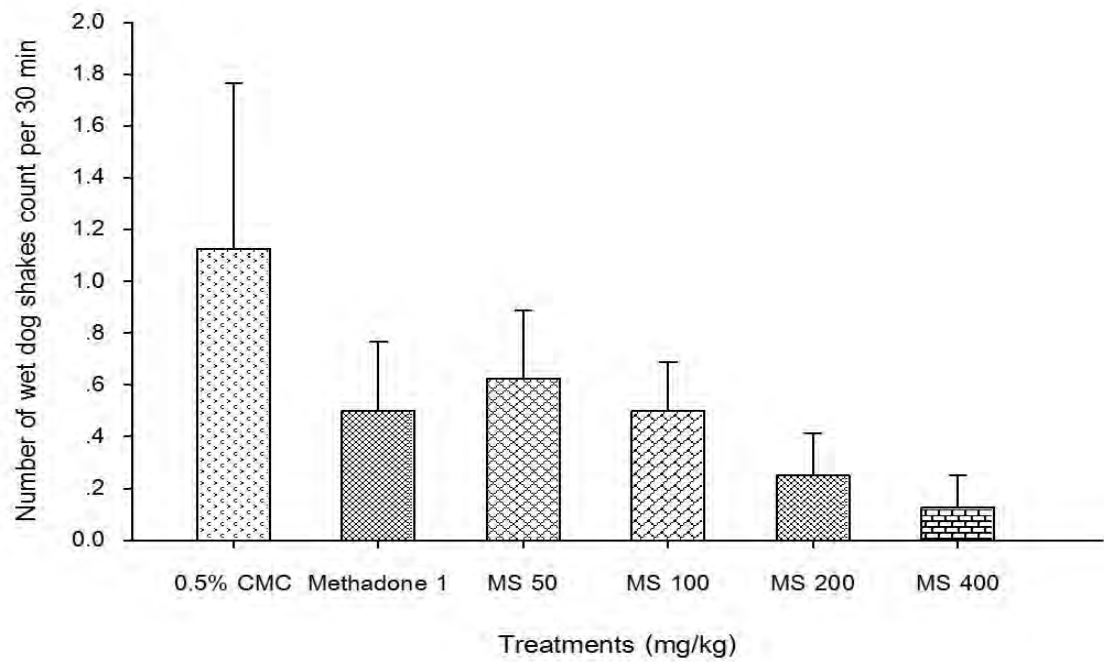
Figure 4.39 Effects of post-treatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The problem of drug abuse exists in most of the societies over the world. Nevertheless, the problem has become more complex and alarming in the recent years. Of the various substance use disorders, opioid dependence has a significantly major impact on mortality and morbidity (Gautam et al., 2000). Kratom (*Mitragyna speciosa*) is a medicinal herb indigenous to Southeast Asia whose components mitragynine and 7-hydroxymitragynine agonize the μ-opioid receptor with high affinity (Matsumoto et al., 2004). Kratom is recognized increasingly as a remedy for opioid withdrawal by self-treat individuals for chronic pain. The natural history of kratom use, including its clinical pharmacology and toxicology are poorly understood.

The present study was aimed to determine the effects of the ethanolic extract of *Mitragyna speciosa* (kratom) leaves (MS) on the locomotor activity, rewarding system, and morphine addiction utilizing locomotor activity, conditioned place preference (CPP) and precipitated withdrawal tests.

Our results showed that all doses of MS (50, 100, 200, and 400 mg/kg, p.o.) did not affect the locomotor activity of mice similar to the previous reports of Kumarnsit et al. (2007) and Reanmongkol et al. (2007) as shown in Figure 4.1. These results demonstrated that orally administered MS within this dose range was neither stimulating nor sedative.

The conditioned place preference (CPP) procedure is widely used in rodents for assessing drug effects on motivational processes (Schechter and Calcagnetti, 1993). This procedure is used to measure the positive reinforcing properties of test compounds. This procedure is less time consuming than more complex techniques used to detect drug abuse, such as self-administration or drug-discrimination paradigms, and is sensitive to the reinforcing effects of many substances abused by

humans. Previous study demonstrated that the rewarding effects of opioid receptor agonists including morphine and heroin can be conditioned to environment stimuli which have previously signaled their administration (Tzschentke, 1998). Another study also demonstrated that morphine can produce CPP and the maximum effect was achieved with intraperitoneal 5 mg/kg of morphine (Sahreaei et al., 2005). We then utilized CPP test to determine the rewarding effect of MS and the effect of MS on morphine-induced CPP.

In the present study, the reference drug, morphine (5 mg/kg i.p.), showed a strong rewarding effect while all doses of MS (50-400 mg/kg p.o.) did not show positive reinforcing effect in the CPP model (Figure 4.2). MS at this dose range by itself was not capable of producing a conditioned place preference. It is known that morphine acted through μ-opioid receptor which produces rewarding effects via activation of the mesocorticolimbic dopaminergic system (Koob, 1992; Xi and Stein, 2002). Several studies have suggested that the mesocorticolimbic dopaminergic system, originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc) and other forebrain regions, play an important role in mediating the rewarding activities of opioids. For example, administration of μ-opioid receptor agonist into VTA induced CPP (Phillips and Lepiane, 1980) and self-administration in rats (Devine and Wise, 1994). Moreover, μ-opioid receptor knock-out mice do not show place preference for an environment paired with morphine (Mattes et al., 1996). It is hypothesized that morphine acts on μ-opioid receptor within VTA which are located on GABAergic neurons and brings about its inhibiting influence on dopaminergic neurons (Hyman and Malenka, 2001). Therefore, we concluded that this extract may not have μ-opioid receptor agonist property strong enough to produce CPP or the constituents of this extract may antagonize effect of each other to reduce the rewarding effects.

We then examined the effect of MS on morphine-induced CPP. Our results showed that morphine (5 mg/kg i.p.) produced significant CPP. Methadone (1 mg/kg i.p.) and all doses of MS (50, 100, 200, and 400 mg/kg, p.o.) significantly suppressed

morphine-induced CPP. These findings provide the first demonstration that MS could attenuate morphine-induced CPP in rats.

In order to investigate the withdrawal effects and the mechanisms involved after acute administration of MS, precipitated withdrawal symptoms with naloxone, naltrindole and norbinaltorphimine (μ-, δ- and κ-opioid antagonists, respectively) were used. Morphine exhibited significant withdrawal symptoms such as jumping and straub tail with only naloxone treatment and confirmed its action through μ-opioid receptor. Acute treatments of all doses of MS did not show significant withdrawal symptoms with all antagonist administrations.

Chronic administration of opiate substance produces tolerance and dependence. Abrupt cessation of opiate administration results in withdrawal syndrome (Gold et al., 1978). Chronic administration of morphine exhibited more significant withdrawal symptoms including jumping, straub tail, C-shaped tail, and wet dog shakes than acute treatment. Chronic treatments of MS at all doses tested did not show any significant withdrawal symptoms when precipitated with naloxone. Together with the results from CPP model, it can be concluded that all doses of MS did not have rewarding effects.

The effect of pretreatment and post-treatment of MS on morphine withdrawal symptoms were also evaluated. Pretreatment with methadone significantly lowered the number of naloxone-precipitated morphine withdrawal jumping and straub tail, while all doses of MS significantly attenuated only jumping behavior in chronic morphine-dependent mice in a dose-dependent manner. Jumping is one of the most common signs used to assess the severity of morphine withdrawal (Broseta et al., 2002). The lowest dose of MS pretreatment (50 mg/kg) seemed to have the highest efficacy. Treatment with methadone and all doses of MS especially at the dose of 100 mg/kg lowered the number of naloxone-precipitated morphine withdrawal jumping in chronic morphine-dependent mice. Therefore, MS at the dose of 100 mg/kg p.o. has a potential

to be used for the treatment of morphine withdrawal. This result is in agreement with a case report that the patient used kratom leaves in the self-treatment of chronic pain and opioid withdrawal (Boyer et al., 2008). Moreover, there were some additional studies which supported the use of MS. The alkaloid extract of *Mitragyna speciosa* leaves exhibited antidepressant-like activity in the forced swimming test in mice. These findings suggested that MS might produce antidepressive action partly through activation of the dorsal raphe nucleus (Kumarnsit et al., 2007). Kumarnsit et al. (2007) found that MS could inhibit ethanol withdrawal symptoms which might be due to its antidepressant effect. Additionally, Farah Idayu et al. (2010) evaluated the antidepressant effect of mitragynine, the major constituent of kratom in the mouse forced swim test (FST) and tail suspension test (TST). They found that mitragynine reduced the immobility time of mice in both FST and TST. Some antidepressants and other non-opiate substances have been found and used for prevention of opiate withdrawal syndrome. Classical antidepressants such as fluvoxamine and setraline were found to reduce opioid syndrome (Gray, 2002). In addition, venlafaxine was also demonstrated to attenuate morphine dependence and withdrawal (Lu et al., 2001). According to the antidepressant-like activity of MS, we hypothesized that MS has therapeutic effect especially for reducing the withdrawal syndrome in drug-addicted patients. Conventionally, methadone, a μ-receptor agonist, has been used to relieve withdrawal signs (McMillan et al., 1976). However, methadone also produces side effects and withdrawal by itself (Beswick et al., 2003).

Previous study from Matsumoto et al. (1996a) indicated that mitragynine, the predominant alkaloid of kratom, itself can induce antinociception by acting on the brain, and the supraspinal opioid systems are at least partly involved in the antinociceptive action of mitragynine in mice. Further study reported that the antinociception of mitragynine dominantly mediated by μ- and δ-opioid receptor subtypes and the selectivity of mitragynine for the supraspinal opioid receptor subtypes differ from that of morphine in mice (Matsumoto et al., 1996b). Additional study suggested that both descending noradrenergic and serotonergic systems are involved in the antinociceptive

activity of supraspinally administered mitragynine on the mechanical noxious stimulation, while the descending noradrenergic system predominantly contributes to the effect of supraspinal mitragynine on the thermal noxious stimulation. The mechanisms underlying the suppressive action of mitragynine on the nociceptive response may differ from those of morphine in mice (Thongpradichote et al., 1998).

The results from high throughput molecular screening of mitragynine activity at central nervous system receptors indicated that mitragynine binds μ- and κ-opioid receptors, but has additional receptor affinities that might augment its effectiveness at mitigating opioid withdrawal (Boyer et al., 2008). The clinical implication of these results is that μ-opioid receptor agonism may avert withdrawal symptoms, while κ-opioid agonism attenuates reinforcement and produce aversion (Narita et al., 2001). It may be concluded that pretreatment and post-treatment with MS in morphine dependence mice could reduce withdrawal jumping behavior by acting through κ-opioid receptor more than μ-opioid receptor. In addition, mitragynine through putative α_2-adrenergic agonist activity (Boyer et al., 2008) may mimic adjunctive therapies for opioid withdrawal such as clonidine. Mitragynine, therefore, may exert several convergent pharmacological effects that could attenuate opioid withdrawal systems and blunt cravings.

Adverse effects of kratom are poorly described. Although mitragynine agonize μ-opioid receptor, respiratory depression, coma, pulmonary edema and death have not been associated with human kratom ingestion (Boyer et al., 2008). Furthermore, the protracted use of MS as a single therapy did not appear to produce any significant adverse effects. The risk of kratom and outcomes from its long term use are still unknown (Boyer et al., 2008).

In conclusions, the ethanolic extract of *Mitragyna speciosa* leaves did not have psychostimulating, sedative or rewarding effects and also did not produce any significant precipitated withdrawal symptoms after acute or chronic uses. Furthermore, it could inhibit morphine withdrawal symptoms. We concluded that MS which is a natural

product might be helpful in the treatment of morphine and other opioid addiction, especially in reducing the opioid withdrawal symptoms. Since MS has been shown to possess several pharmacological properties without rewarding effects, it may have a potential to be developed for clinical purposes. Nevertheless, the chemical structural characterization, potency evaluation, and detailed mechanism of this extract should be further investigated.

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APPENDIX

Effects of MS on locomotor activity in mice

Group	Total locomotor activity (counts/75 min)
NSS	2134.25 ± 249.85
0.5% CMC	2202.50 ± 224.14
Morphine 5 mg/kg	2123.13 ± 203.66
MS 50 mg/kg	2216.25 ± 184.99
MS 100 mg/kg	2358.13 ± 222.85
MS 200 mg/kg	2182.75 ± 147.34
MS 400 mg/kg	2331.50 ± 176.56

Table 1: Locomotor activity in mice produced by NSS, morphine (5 mg/kg; i.p.), 0.5% CMC, and various doses of MS (50-400 mg/kg; p.o.). Each value represents mean ± S.E.M. N=8 for all groups.

Effect of MS on conditioned place preference in rats

Group	Time spent in preference for drug-paired place (sec)	
	Preconditioning phase	Test phase
0.5% CMC	140.25 ± 12.77	142.38 ± 16.01
Morphine 5 mg/kg	127.75 ± 13.36	240.50 ± 22.21*[#]
MS 50 mg/kg	141.13 ± 13.91	155.63 ± 12.69
MS 100 mg/kg	142.63 ± 8.53	155.88 ± 13.28
MS 200 mg/kg	142.25 ± 9.21	165.25 ± 21.40
MS 400 mg/kg	133.75 ± 15.34	152.88 ± 5.26

Table 2: Conditioned place preference in rats produced by morphine (5 mg/kg; i.p.) Each values represents mean ± S.E.M. N=8 for all groups. *$p<0.05$ significantly different from preconditioning phase (Student's paired t-test). [#]$p<0.01$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.

Effect of MS on morphine-induced conditioned place preference in rats

Group	Time spent in preference for drug-paired place (sec)	
	Preconditioning phase	Test phase
0.5% CMC	128.88 ± 16.41	252.63 ± 26.52
Morphine 5 mg/kg	141.75 ± 15.20	227.13 ± 16.43
MS 50 mg/kg	127.60 ± 8.45	198.50 ± 14.43
MS 100 mg/kg	141.80 ± 14.15	181.60 ± 10.70*
MS 200 mg/kg	152.00 ± 18.87	198.40 ± 28.10
MS 400 mg/kg	133.30 ± 8.94	176.00 ± 15.05*

Table 3: Effects of 0.5% CMC, methadone (Met 1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on morphine (MO)-induced conditioned place preference in rats. Each value represents mean± S.E.M. N=8 for all groups. *$p<0.05$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.

Effect of MS on morphine-induced conditioned place preference in rats

Group	% Place preference
0.5% CMC + Morphine 5 mg/kg	109.02 ± 16.60
Methadone 1 mg/kg + Morphine 5 mg/kg	66.21 ± 13.26*
MS 50 mg/kg + Morphine 5 mg/kg	58.36 ± 11.94*
MS 100 mg/kg + Morphine 5 mg/kg	35.71 ± 13.03*
MS 200 mg/kg + Morphine 5 mg/kg	34.40 ± 11.35*
MS 400 mg/kg + Morphine 5 mg/kg	34.23 ± 12.56*

Table 4: Effects of 0.5% CMC, methadone (Met 1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on morphine (MO)-induced conditioned place preference in rats. Percent change of place preference equals the difference in time (s) spent in the white compartment between preconditioning and test phases over the time spent in the preconditioning phase. Each value represents mean± S.E.M. N=8 for all groups. *p<0.05 significantly different from 0.5% CMC were performed by one-way ANOVA and Fisher's LSD test for comparison.

Effect of MS on precipitated withdrawal symptoms with naloxone

	Number of times symptom occurred (counts/30 min)					
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	0.00 ± 0.00	49.13 ± 11.64	8.75 ± 1.29	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Morphine 100 mg/kg	39.50 ± 8.42*	37.63 ± 3.97	6.38 ± 1.07	2.38 ± 0.68*	0.00 ± 0.00	0.00 ± 0.00
MS 50 mg/kg	0.38 ± 2.16	49.63 ± 9.29	7.00 ± 0.63	0.00 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
MS 100 mg/kg	1.88 ± 1.61	48.75 ± 11.97	7.63 ± 0.92	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 200 mg/kg	2.88 ± 1.94	60.88 ± 9.76	8.38 ± 1.72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 400 mg/kg	3.63 ± 2.56	58.50 ± 5.45	9.25 ± 1.26	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.13

Table 5: Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.

Effect of MS on precipitated withdrawal symptoms with natrindole

Number of times symptom occurred (counts/30 min)						
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	0.00 ± 0.00	79.00 ± 22.13	9.25 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Morphine 100 mg/kg	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 50 mg/kg	2.13 ± 1.99	50.38 ± 7.90	4.63 ± 0.78	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 100 mg/kg	8.00 ± 8.00	57.88 ± 17.04	6.38 ± 0.84	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 200 mg/kg	5.00 ± 5.00	58.75 ± 12.40	6.13 ± 1.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 400 mg/kg	6.63 ± 5.03	57.13 ± 15.85	5.88 ± 0.83	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 6: Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by naltrindole in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.

Effect of MS on precipitated withdrawal with norbinaltorphimine

Number of times symptom occurred (counts/30 min)						
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	0.00 ± 0.00	136.13 ± 25.07	8.25 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Morphine 100 mg/kg	0.00 ± 0.00	4.00 ± 2.81	8.50 ± 3.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 50 mg/kg	1.63 ± 1.63	91.50 ± 21.72	7.13 ± 1.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 100 mg/kg	2.25 ± 1.98	75.13 ± 16.47	5.38 ± 0.42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 200 mg/kg	2.25 ± 1.58	116.50 ± 22.09	9.00 ± 1.24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 400 mg/kg	0.63 ± 0.50	81.50 ± 9.34	6.63 ± 0.96	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 7: Effects of 0.5% CMC, morphine (100 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by norbinaltorphimine in mice. Each value represents mean ± S.E.M.. N = 8 for all groups.

Precipitated withdrawal symptoms of chronic MS treatment by naloxone

Number of times symptom occurred (counts/30 min)						
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	0.00 ± 0.00	106.25 ± 25.73	10.75 ± 1.28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Morphine	51.50 ± 6.36*	73.88 ± 6.13	6.00 ± 0.91	2.38 ± 0.18*	1.88 ± 0.35*	0.88 ± 0.30*
MS 50 mg/kg	1.13 ± 1.13	119.00 ± 19.12	7.63 ± 1.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 100 mg/kg	2.00 ± 1.51	100.00 ± 23.94	8.50 ± 1.49	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.16
MS 200 mg/kg	2.88 ± 2.07	101.13 ± 17.11	7.38± 1.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MS 400 mg/kg	3.75 ± 3.22	119.38 ± 23.35	7.88 ± 1.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 8: Effects of chronic treatment of 0.5% CMC, morphine (10-70 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p < 0.05$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.

Effect of MS pretreatment on morphine withdrawal

Number of times symptom occurred (counts/30 min)						
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	66.13 ± 8.15	26.00 ± 4.43	6.25 ± 1.21	1.50 ± 0.27	1.13 ± 0.61	0.38 ± 0.18
Methadone 1 mg/kg	41.50 ± 4.89*	24.63 ± 5.50	7.25 ± 1.49	0.00 ± 0.00*	0.75 ± 0.16	0.38 ± 0.26
MS 50 mg/kg	26.75 ± 9.43*	37.25 ± 4.03	6.25 ± 0.94	1.25 ± 0.16	0.63 ± 0.13	0.63 ± 0.42
MS 100 mg/kg	35.38 ± 5.21*	31.13 ± 3.98	5.38 ± 0.92	1.13 ± 0.16	0.38 ± 0.18	0.50 ± 0.27
MS 200 mg/kg	34.88 ± 4.51*	34.63 ± 3.05	6.13 ± 0.81	1.13 ± 0.13	0.63 ± 0.50	0.75 ± 0.49
MS 400 mg/kg	40.13 ± 6.80*	35.13 ± 2.94	7.00 ± 2.90	1.25 ± 0.19	0.13 ± 0.13	0.13 ± 0.13

Table 9: Effects of pretreatment of 0.5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p<0.05$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.

Effect of MS post-treatment on morphine withdrawal

Number of times symptom occurred (counts/30 min)						
Group	Jumping	Rearing	Grooming	Straub tail	C-shaped tail	Wet dog shakes
0.5% CMC	94.25 ± 12.58	34.38 ± 7.97	5.75 ± 0.96	1.38 ± 0.32	1.25 ± 0.49	1.13 ± 0.64
Methadone 1 mg/kg	60.25 ± 6.17*	23.88 ± 3.96	5.75 ± 1.51	0.88 ± 0.23	1.00 ± 0.19	0.50 ± 0.27
MS 50 mg/kg	46.63 ± 7.86*	39.00 ± 12.02	4.25 ± 1.08	0.88 ± 0.13	1.38 ± 0.13	0.63 ± 0.26
MS 100 mg/kg	37.00 ± 5.50*	26.75 ± 3.05	3.13 ± 0.35	1.00 ± 0.00	1.25 ± 0.00	0.50 ± 0.19
MS 200 mg/kg	43.75 ± 6.32*	43.88 ± 7.49	4.13 ± 0.69	1.00 ± 0.00	1.25 ± 0.18	0.25 ± 0.16
MS 400 mg/kg	55.63 ± 4.55*	45.75 ± 15.26	3.38 ± 0.91	0.88 ± 0.13	1.13 ± 0.00	0.13 ± 0.13

Table 10: Effects of post-treatment of .5% CMC, methadone (1 mg/kg; i.p.) and various doses of MS (50-400 mg/kg; p.o.) on jumping, rearing, grooming, straub tail, C-shaped tail and wet dog shakes behavior precipitated by naloxone in mice. Each value represents mean ± S.E.M.. N = 8 for all groups. *$p < 0.05$ significantly different from 0.5% CMC were performed by one-way ANOVA and Dunnett's test for comparison.
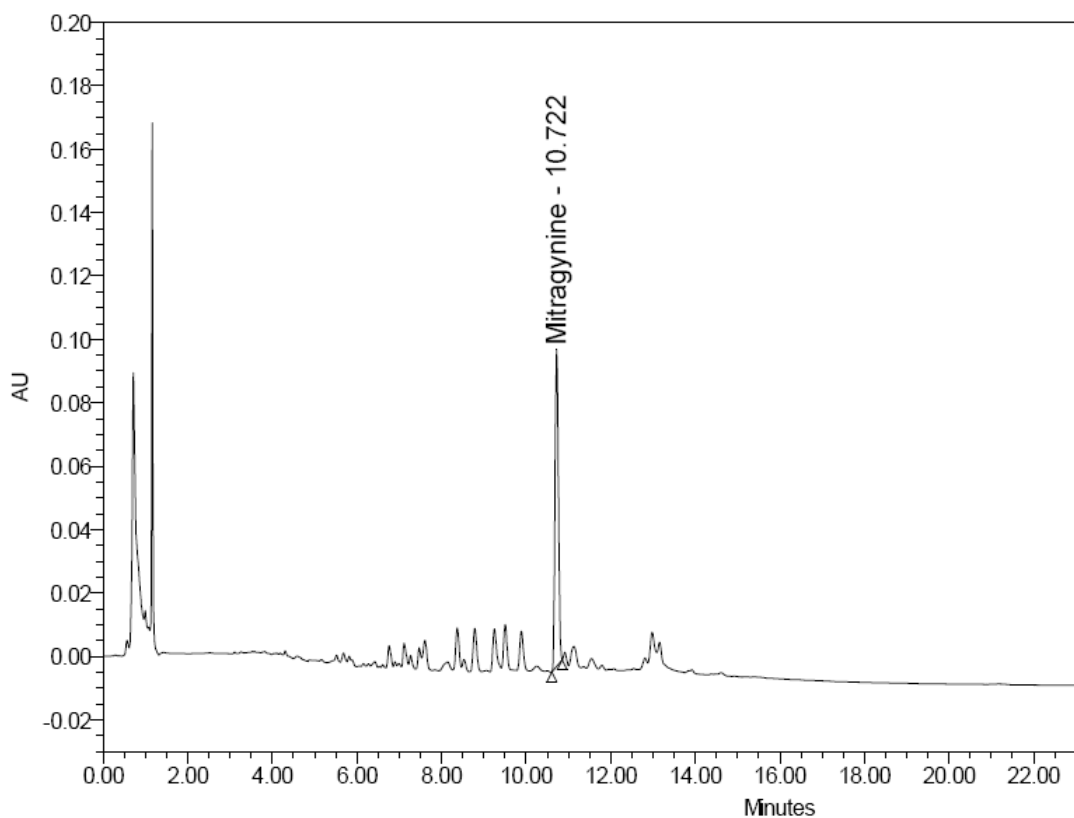
Figure: HPLC Chromatogram of the ethanolic extract of *Mitragyna speciosa* leaves

Wave length: 225 nm

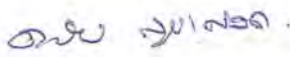Column: XBridge®C18 column

Organic modifiers: acetonitrile (ACN)

Acid modifier: ammonium hydroxide

Rate of change ratio of carrier solvent

Time (min)	Ratio (v/v)		
	10% NH$_4$OH	Water	Acetonitrile
0.1	10	80	10
12	0	0	100
22	0	0	100

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 10-33-014	**Approval No. 10-33-014**

Protocol Title
Effects of the ethanolic extract of *Mitragyna speciosa* leaves on morphine addiction in mice and rats

Principal Investigator
Pasarapa Towiwat, Ph.D.

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

Date of Approval May 17, 2010	**Date of Expiration** May 17, 2011

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

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

   The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.
   This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

BIOGRAPHY

Miss Supaporn Aunlamai was born on December 2, 1977 in Phrae, Thailand. She received Bachelor degree of Nursing Science in 2000 from Thai Red Cross college of Nursing Bangkok, Thailand. She worked as a nurse at Chulalongkorn hospital Bangkok, Thailand since 2000-2007. In 2008, she becomes a graduate student in the master's degree at science program in Pharmacology, Interdisciplinary Program in Pharmacology, Graduated School, Chulalongkorn University.

Poster presentation entitled "Effect of the Ethanolic Extract of *Mitragyna speciosa* Leaves on Conditioned Place Preference" of Miss Supaporn Aunlamai was presented in Thai Journal of Pharmacology Vol. 33, Suppl. 2, 2011, Proceedings of 33rd Pharmacological and Therapeutic Society of Thailand Meeting 17-19 March 2011 at Diamond Plaza Hotel, Had Yai, Songkhla, Thailand.