รายงานวิจัยฉบับสมบูรณ์

โครงการ
การศึกษาการประมวลสัญญาณประสาทของสมองส่วน reward circuit ที่ถูกชักนำโดยสารสกัดจากพืชกระท่อม
(Study of neural signaling of brain reward circuit induced by the extract of psychoactive plant kratom)

โดย

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สัญญาเลขที่ MRG 6080163

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด
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บทคัดย่อ

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ชื่อโครงการ: การศึกษาการประมวลสัญญาณประสาทของสมองส่วน reward circuit ที่ถูกชักนำโดยสารสกัดจากพืชกระท่อม

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ระยะเวลาโครงการ 2 ปี

Mitragyna speciosa Korth หรือกระท่อม เป็นพืชท้องถิ่นที่พบได้ทั่วไปในภาคใต้ของประเทศไทย เป็นที่ทราบกันดีว่าสารสกัดกลุ่มอัลคาลอยจากใบกระท่อมมีสรรพคุณลดปวดได้ดี โดยมีกลไกผ่าตัวรับกลุ่มโอปิออยด์ นอกจากนี้ยังพบว่ามีการนำกระท่อมมาใช้ทดแทนฝิ่นเพื่อลดอาการลงแดงหรือรักษาอาการเสพติดฝิ่น อย่างไรก็ตาม ยังไม่มีการศึกษาฤทธิ์ของพืชกระท่อมต่อการทำงานของสมองที่แน่ชัด ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อทดสอบผลของสารสกัดอัลคาลอยด์จากกระท่อมในหนูที่ชักนำให้มีอาการถอนมอร์ฟีน นอกจากนี้ยังทำการศึกษาความเป็นไปได้ของฤทธิ์เสพติดด้วยการประเมินผลจากสัญญาณไฟฟ้าในสมองส่วนนิวเคลียสแอคคัมเบนส์ซึ่งเป็นบริเวณสมองที่เกี่ยวข้องกับกลไกการเสพติด ทำการทดลองโดยชักนำให้หนูที่ติดมอร์ฟีนมีอาการถอนด้วยการฉีดยานาล็อกโซน ประเมินอาการถอนจากพฤติกรรมการกระโดด ปริมาณปัสสาวะ และอุจจาระ และทำการฝังขั้วบันทึกสัญญาณไฟฟ้าจากนิวเคลียสแอคคัมเบนส์ ผล

การศึกษาพบว่าสารสกัดอัลคาลอยด์จากกระท่อมขนาด 80 และ 100 mg/kg มีผลลดอาการกระโดด ปริมาณปัสสาวะ และจำนวนอุจจาระในหนูที่ชักนำให้มีอาการถอนมอร์ฟีนอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ในขณะที่การวิเคราะห์สัญญาณไฟฟ้าจากนิวเคลียสแอคคัมเบนส์พบว่า หนูที่ได้รับการฉีดมอร์ฟีนขนาด 15 mg/kg มีผลเปลี่ยนรูปแบบสัญญาณไฟฟ้าเมื่อวิเคราะห์ด้วยวิธี frequency analysis โดยพบว่ามีผลลดพลังงานในช่วงความถี่แอลฟฟ่า (9.7 – 12 Hz) และเพิ่มพลังงานในช่วงความถี่แกมม่า (30.3 – 95.7 Hz) นอกจากนี้มอร์ฟีนยังมีผลเพิ่มระดับการเคลื่อนไหวอย่างชัดเจน ในขณะที่หนูที่ได้รับสารสารสกัดอัลคาลอยด์จากกระท่อมนั้นไม่มีผลแตกต่างจากกลุ่มควบคุม ทั้งผลรูปแบบสัญญาณไฟฟ้าจากนิวเคลียสแอคคัมเบนส์และระดับการเคลื่อนไหว นอกจากนี้ยังพบว่าสารสกัดบริสุทธิ์ mitragynine ซึ่งเป็นสารอัลคาลอยด์หลักไม่มีผลลดอาการถอนมอร์ฟีนแต่อย่างใด ดังนั้นผลลดอาการถอนมอร์ฟีนจำเป็นผลจากสารกลุ่มอัลคาลอยด์หลายตัว ผลกาศึกษานี้จึงสรุปได้ว่ามีความเป็นไปได้ที่จะนำพืชกระท่อมมาใช้เพื่อบรรเทาอาการถอนมอร์ฟีน หรือรักษาอาการเสพติดยาเสพติดกลุ่มฝิ่น

คำสำคัญ: กระท่อม มอร์ฟีน ลงแดง คลื่นไฟฟ้าสมอง ยาเสพติด

Abstract

Project Code: MRG 6080163

Project Title: Study of neural signaling of brain reward circuit induced by the extract of psychoactive plant kratom

Investigator: Dr. Dania Cheaha, Department of Biology, Faculty of Science, Prince of Songkla University

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Project period: 2 years

Mitragyna speciosa Korth (MS) is an indigenous plant widely growing naturally in southern Thailand. Its alkaloid extracts have been well known to produce antinociception acting through opioid receptors. It has gained increasing attention as a plant with potential to substitute morphine in addiction treatment program. However, its action on the central nervous system is controversial. This study was aimed to test the effect of an MS alkaloid extract from this plant on naloxone-precipitated morphine withdrawal in mice. Moreover, the possible addictive effect was also evaluated by investigated the neural signaling in the nucleus accumbens (NAc) in mice. Naloxone-precipitated morphine withdrawal was induced in morphine dependent mice. The intensity of naloxone-precipitated morphine withdrawal was assessed from the mice jumping behavior and

fecal and urine excretions induced during a period of withdrawal. The local field potential (LFP) signal from the NAc were recorded by implanted intracranial electrode. The results showed that the MS alkaloid extract (80 and 100 mg/kg) significantly decreased the number of jumps compared to the control saline whereas mitragynine, a pure major constituent, did not. In addition, treatment with the MS alkaloid extract were also significantly reduce the fecal and urine excretion. In addition, the LFP power spectra and spontaneous motor activity following MS alkaloid extract (80 mg/kg) and morphine (15 mg/kg) treatments were analyzed in comparison to control levels. LFP analysis revealed that morphine significantly decreased alpha (9.7-12 Hz) and increased low gamma (30.3-44.9 Hz) and high gamma (60.5-95.7 Hz) powers in the NAc whereas MS alkaloid extract did not. Spontaneous motor activity was significantly increased by morphine but not MS alkaloid extract. In summary, the present study indicates that the MS alkaloid extract, but not mitragynine, attenuates the severity of naloxone-precipitated morphine withdrawal. On the other hand, neural signaling in the NAc and spontaneous motor activity were sensitive to morphine but not MS alkaloid extract. This indicates that the significant effects may belong to minor constituents in the whole alkaloid extract from *Mitragyna speciosa*, rather than the pure mitragynine. In addition, it could also be the result of a combined action of many ingredients as the whole extract showed a higher potency than did the individual ingredients. Altogether, treatment with the crude MS alkaloid extract may be useful for any opiate addiction treatment program.

Keywords: *Mitragyna speciosa*, Morphine withdrawal, EEG, addiction, Nucleus accumbens

CHAPTER 1

General Introduction

Psychoactive plants and plant-derived products have been used for spiritual, therapeutic and recreational purposes. Furthermore, the investigation of psychoactive plants such as Cannabis sativa (marijuana), Nicotiana tabacum (tobacco) and analogues of psychoactive plant derivatives including opium, lysergic acid diethylamide (LSD), have provided insights into essential understanding of neurochemical processes and diseases of the central nervous system (CNS).

Mitragyna speciosa Korth (MS), also known as Kratom, is a native tropical plant which has been found to exhibit some potential properties as a medicinal plant. The MS is mainly found in Thailand and Malaysia. MS leaves have been used by natives as an herbal drug for decades. They are usually chewed by individuals to improve tolerance for hard work and to relieve muscle strains. MS was also used as a substitute for opium when the opium was unavailable. It has also been used to manage opioid withdrawal symptoms by chronic opioid users. According to these effects, MS was hypothesized to mimic morphine mechanisms. Until recently, the focus had shifted to its antidepressant-like actions. Crude MS alkaloid extract was found to stimulate rat's dorsal raphe nuclei, the main site for serotonin biosynthesis and produce antidepreassant-like effects in mice (Kumarnsit et al., 2007). On the other hand, the MS extract was also found to attenuate ethanol

withdrawal symptom as effectively as a standard drug, fluoxetine (Cheaha et al., 2014). These indicate therapeutic effects of MS extract that might be partly via its antidepressant properties.

In the past few decades, the most frequent question was whether the MS has addictive effect properties. This unclear situation prevents MS application from further therapeutic uses. MS use in Thailand is illegal. They were categorized in Category V of a five category classification of narcotics as same as marijuana. Potential abuses of the MS have been reported in both human and animal studies. Previous studies on MS users in Thailand revealed that long-term MS use produced addiction-like effects (Suwanlert, 1975). However, they also reported that MS use caused much less adverse effects and lower admission rate to the hospital in comparison to other addictive drugs. This indicates milder withdrawal symptoms and less harm than that of opiates and other drugs. Several lines of evidence have indicated addiction-related effects of the MS in animal models. Chronic mitragynine administration produced withdrawal-like symptom after cessation which indicated dependence and tolerance effectd of long-term mitrgynine exposure (Yusoff et al., 2016). In addition, mitragynine also produced reward response in condition place preference (CPP) test, a standard test to examine reward effect of addictive substances. These studies strongly support addictive and reward properties of MS. However, alkaloid-riche extract from MS did not showed reward response in CPP test (Sufka et al., 2014). This suggested a higher risk of possible substance dependence for mitragynine but not the MS alkaloid-rich extract.

Growing evidence suggest that all reinforcers including the natural rewards (for example, food, water, and pheromone), psychoactive drugs and cues which predict naturally rewarding outcomes have powerful reinforcing effects partly mediated by dopamine release in the mesolimbic

dopamine (DA) system. The brain reward circuit is composed of dopaminergic neurons in the ventral tegmental area (VTA) and their projections to the striatum which includes the dorsal striatum and the nucleus accumbens (NAc), the amygdala, the prefrontal cortex (PFC), and other forebrain regions. Therefore, the mesolimbic dopamine DA system is a major target for investigating reward processing mechanism induced by addictive substances including the MS extracts.

It is clear that reward stimuli are encoded through the neural network processing within brain reward circuit. The neural mechanisms involved in the processing of reward function have been studied by using various techniques including behavioral tests, genetics and molecular techniques. Signals from neural network processing is generally complicate with plenty of 'hidden' features. Most of techniques are usually unable extract the useful information. Due to the advent of powerful computational tools, the signal processing techniques are applied to solve the complexity of electrophysiological signals. Moreover, the signal processing is also enable to process the real-time decoding of neural information. The neural network signaling shared by animal models and human subject can provide a strong link between experimental and clinical research. The conservation of brain structures and neurobiological features across mammalian species allows the translatability of results. Translational potential of neural network signaling or electrophysiological biomarkers in both animal and human studies may be used for early diagnosis, severity prediction, therapeutic target identification, and monitoring response to therapy in clinical level.

However, direct neural processing of reward circuit induced by the MS extract have not been established. Therefore, this study aimed to investigate the brain reward network signaling as

EEG fingerprint of the MS alkaloid extract. The pattern of neural network oscillations in the brain reword circuit might reveal the definite of kratom in terms of reward and addiction-related processing mechanisms.

This will examine therapeutic effect of MS alkaloid extract on morphine withdrawal. Therefore, the neural signaling of mesolimbic reward circuit in mice-exposed to acute MS alkaloid extract were characterized and identified. Finally, the reinforcing effect of MS alkaloid extract were evaluated using conditioned place preference (CPP) paradigm.

CHAPTER 2

General Methodology

Animals

Three months old male Swiss Albino (ICR) mice (figure 1A) were provided by the Southern Laboratory Animal Facility, Prince of Songkla University, Hatyai, Songkhla, Thailand. They were housed in standard environmental conditions (23-25 °C, 50-55% humidity and 12/12 hr light/dark cycle) with free access to standard commercial food pellets and water. All the tests were performed between 8.00 A.M. and 4.00 P.M. This study was carried out in accordance with guidelines of the European Science Foundation (Use of Animals in Research, 2001) and International Committee on Laboratory Animal Science, ICLAS (2004). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Plant materials and extraction

Young leaves of MS were collected from natural sources in Songkhla and Satun provinces, Thailand during 2004-2005. Authentication of the plant material was carried out at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of

Songkla University, Thailand where the herbarium voucher specimens (no. PCOG/MS001-002) have been deposited.

Extraction and isolation of the MS extract was described in a previous study (Cheaha et al., 2015b). The major alkaloid, isolated by silica-gel CC eluting with MeOH:CHCL3 (5:95), was identified as mitragynine with the standard spectroscopic methods (MS, 1H NMR and 13C NMR). According to TLC analysis, mitragynine was found to constitute about 60% of the content of the alkaloid extract. The solution of the MS alkaloid extract was prepared by dissolving the extract in co-solvent (tween 80:propylene glycol:H2O at a 1:4:4 ratio) and adjusted to desired concentrations with distilled water. The MS alkaloid extract was previously identified using high performance liquid chromatography (HPLC) (Cheaha et al., 2015b). The alkaloid extract was run and found to contain mitragynine as a major component.

Animal surgery for local field potential (LFP) electrode implantation

Method of electrode implantation was also previously described (Cheaha et al. 2015a) (fig. 1). Briefly, animals were anesthetized pre-injection intramuscularly of 16 mg/kg xylazine and follow by 50 mg/kg Zoletil® 100 (Virbac, Thailand Co. Ltd.). Then the animal's head was mounted in a stereotaxic frame (Fig. 2.1B). Local analgesic, lidocaine (Locana, L.B.S. Laboratory Ltd., Part., Thailand) was applied to the exposed tissue of the head. An incision was made at the midline to expose the skull. The silver wire electrodes (A-M system, Sequim, WA, USA) with bare diameter of 0.008" (Coated-0.011") were stereotaxically positioned on the left side of the brain (figure 1D)

including frontal cortex (AP: +4.5 mm, ML: ±1 mm), nucleus accumben (AP: +1.5 mm, ML: ±1 mm, DV: 4.3 mm), dorsal hippocampal CA1 (AP: -2.5 mm, ML: ±1.5 mm, DV: 1.5) according to mouse brain atlas (Franklin and Paxinos, 1998). The reference and ground electrodes were placed at midline overlying the cerebellum. Bipolar electrode wires were inserted in the dorsal neck muscles for electromyography (EMG). Additional holes were drilled for stainless steel anchor screws. Dental acrylic (Unifasttrad, Japan) was used to secure all electrodes on the skull. The antibiotic ampicillin (General Drug House Co., Ltd., Thailand) was applied intramuscularly (100 mg/kg) once a day for 3 days to prevent infection and allowed them to recover for at least 7 – 10 days (Fig. 2.1C).

The experiments of this study were divided into 2 main parts including

Part 1: Effects of alkaloid extracts from Mitragyna speciosa on morphine withdrawal in mice (CHAPTER 3)

Part 2: The effects of acute MS alkaloid extract treatment on neural signaling of brain reward circuit (CHAPTER 4)

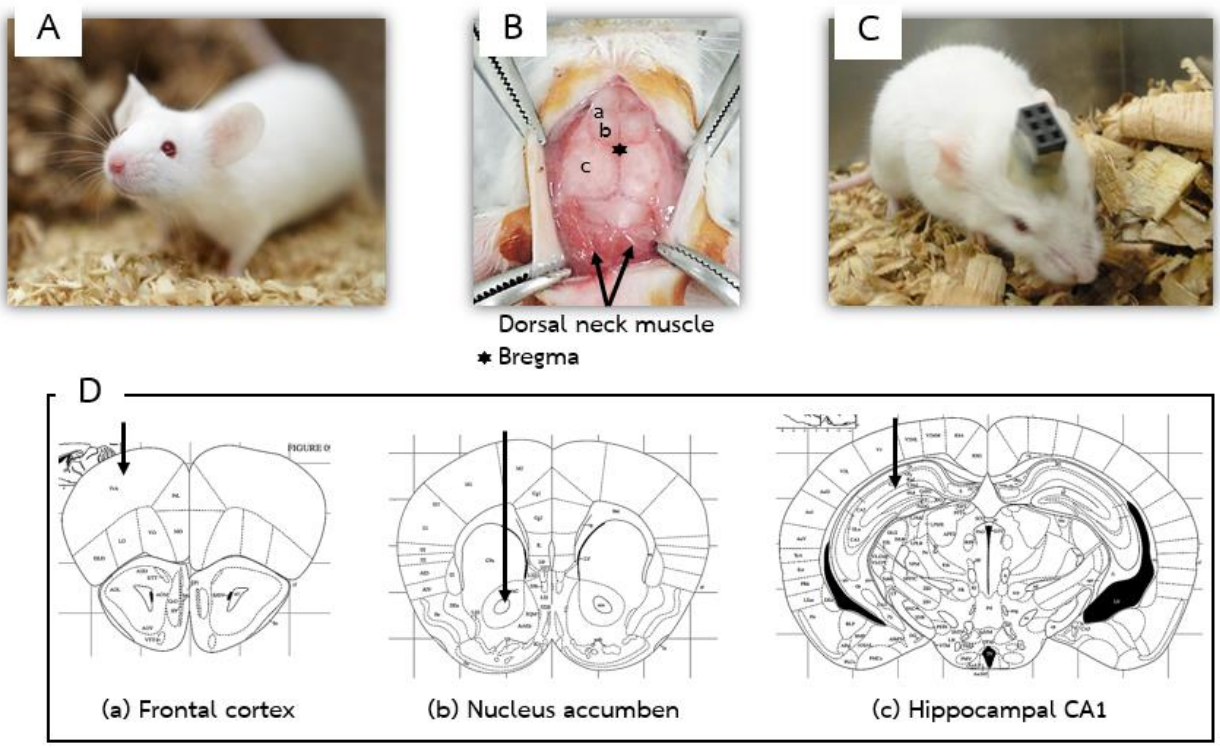

Fig. 2.1. Overview intracranial electrode implantation surgery for LFP recording. (A) Swiss albino (ICR) mice. (B) Coordination of electrode placement location on exposed skull reference with Bregma. (C) Animal after full recovery from surgery. (D) Stereotaxic positions of electrode placement according to Franklin and Paxinos, 1998.

CHAPTER 3

Effects of alkaloid extracts from *Mitragyna speciosa* on morphine withdrawal in mice

Abstract

Mitragyna speciosa Korth (MS) is an indigenous plant widely growing naturally in southern Thailand. Its alkaloid extracts have been well known to produce antinociception acting through opioid receptors. This study was aimed to test the effect of an MS alkaloid extract from this plant on naloxone-precipitated morphine withdrawal in mice. The intensity of naloxone-precipitated morphine withdrawal was assessed from the mice jumping behavior and fecal and urine excretions induced during a period of withdrawal. One-way ANOVA and multiple comparisons revealed that the MS alkaloid extract (80 and 100 mg/kg) significantly decreased the number of jumps compared to the control saline ($p = 0.003$) whereas mitragynine, a pure major constituent, did not. In addition, treatment with the MS alkaloid extract (80 and 100 mg/kg) significantly decreased dry fecal excretion ($p = 0.003$) and all doses completely abolished wet fecal excretion. The analysis also revealed the reduction of urine excretion by the MS alkaloid extract (100 mg/kg, $p = 0.025$). The level of spontaneous motor activity was not affected by treatment with the MS alkaloid extract. In summary, the present study indicates that the MS alkaloid extract, but not mitragynine, attenuates the severity of naloxone-precipitated morphine withdrawal. In particular, the MS alkaloid extracts completely prevented diarrhea induced during a withdrawal period. This indicates that the

significant effects may belong to minor constituents in the whole alkaloid extract from Mitragyna speciosa, rather than the pure mitragynine. In addition, it could also be the result of a combined action of many ingredients as the whole extract showed a higher potency than did the individual ingredients. Altogether, treatment with the crude MS alkaloid extract may be useful for any opiate addiction treatment program.

Keywords: *Mitragyna speciosa*, Morphine withdrawal, Opioid, Naloxone, Jumping

Introduction

Mitragyna speciosa (MS), locally called Kratom in Thailand, is a member of the Rubiacaea plant family. It has been used as a medicinal plant for centuries (Jansen and Prast, 1988b; Suwanlert, 1975). The antinociceptive effects of this plant are well known and have been confirmed by many studies. The main component of the MS extract, mitragynine, has consistently exhibited antinociception centrally via the descending noradrenergic and serotonergic systems (Matsumoto et al., 1996a) by acting on opioid receptors (Thongpradichote et al., 1998). However, the selectivity of mitragynine for opioid receptor subtypes is different from that of morphine (Matsumoto et al., 1996; Thongpradichote et al., 1998). In addition, the action of mitragynine on other system such as gastric acid secretion is also through opioid receptors (Tsuchiya et al., 2002). These consistent data confirm the morphine-like action of the plant and seem to support an original idea that this plant could replace morphine in treatment programs (Jansen and Prast, 1988a). Mitragynine was

demonstrated to inhibit morphine withdrawal successfully in vitro (Watanabe et al., 1997) but no in vivo demonstration has been performed. Recently, this plant has been mentioned in some local areas as being effective in preventing relapse and withdrawal symptoms in morphine addicts but without scientific proof.

Until now, mitragynine is the major indole alkaloid of MS shown to have an antinociceptive action. However, a new compound, 7-hydroxymitragynine, a minor constituent of this plant, also exhibits an even higher potency than mitragynine both in vitro (Takayama et al., 2002) and in vivo (Matsumoto et al., 2004). Moreover, other constituent alkaloids of the plant may also exert potent therapeutic effects.

Opiate substances are known to produce dependence and the abrupt cessation causes a withdrawal syndrome (Gold et al., 1978). Acute morphine withdrawal can also be induced in animal models. It emerges in morphine-dependent animals immediately after the administration of naloxone, a nonspecific opiate antagonist. Opioid receptor agonists including methadone have been used to relieve withdrawal symptoms (McMillan et al., 1976) but methadone also has side effects and can cause withdrawal by itself (Beswick et al., 2003; Gossop et al., 1989; Gossop et al., 1987). Later, levo-alpha-acetylmethadol (LAAM), a synthetic μ opioid receptor agonist, was also found to have some potential advantages with better preference (Trueblood et al., 1978). However, use of LAAM was believed to be linked with arrhythmia (Clark et al., 2002). For more effective treatment of the opiate withdrawal syndrome, partial or non-opiate substances have also been sought for treatment of opiate addiction. Some of the main targets are extracts of medicinal plants that act at least in part on opioidergic systems.

Since most studies have revealed that the MS alkaloid extract produces the antinociceptive effects by acting on opioid receptors, it was hypothesized that the MS alkaloid extract may be able to reduce the intensity of opioid withdrawal. To examine this hypothesis, the effects of the MS alkaloid extract were evaluated by the morphine withdrawal method in mice. Jumping behavior, defecation and urine excretion during a withdrawal period were measured as indicative data reflecting the intensity of morphine withdrawal. In this study, pure mitragynine and the whole alkaloid extract, containing approximately 60% mitragynine as a major alkaloid, were used to treat animals and their effects compared with those of imipramine, an antidepressant drug.

Materials and methods

Chemicals

The following drugs were used: morphine sulphate (Zentiva, SK), naloxone (Sigma, Germany), diazepam and imipramine (Sigma, Germany). The drugs were dissolved in normal saline and given to animals in a volume of 5 ml/kg.

Plant materials

Young leaves of M. speciosa Korth (Rubiaceae) were collected from natural sources in Songkla Province, Thailand. Plant materials were identified by Dr. Niwat Keawpradub, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of

Songkla University. The use of plant materials was approved by the Ministry of Agriculture of Thailand and was restricted to research purposes only.

Preparation of the extract

Extraction and isolation of alkaloids from the plant have been described in a previous study (Ponglux D et al., 1994), with some modifications. The product was collected as a dry crude alkaloid extract. The major alkaloid was isolated by silica gel column chromatography (eluted with 5% methanol in chloroform) and was identified to be mitragynine by the standard spectroscopic method (MS, 1H-NMR and 13C-NMR). According to the TLC analysis, mitragynine was found to constitute about 60% of the crude alkaloid extract.

Animals

The male Swiss albino mice (30 – 35 g) used in each experiment were bred at the animal house of the Prince of Songkla University. They were housed in a group of 10 mice per cage (20x25x35 cm) and maintained under 12/12 dark/light cycle (lights on at 0600 am) and controlled temperature (22°C). Standard commercial food pellets and filtered water were available ad libitum. They were acclimated to these conditions for at least one week prior to use in the experiment. Each animal was used only once and killed immediately after the experiment. The experimental protocols described in the present study were approved and guided by the Animals Ethical Committee of the Prince of Songkla University for care and use of experimental animals.

Development of morphine dependence

Mice were rendered dependent on morphine using the method previously described (Marshall and Grahame-Smith, 1971). Briefly, morphine sulphate was injected (s.c.) 3 times daily at 0800, 1200 and 1600 (50, 50 and 75 mg/kg, respectively) for 3 days. On day 4, only a single morning dose of morphine (50 mg/kg) was injected before naloxone injection.

Observation of morphine withdrawal

Withdrawal signs were precipitated by injection of naloxone (1.5 mg/kg, i.p.) 2 hours after the final injection of morphine. Immediately after naloxone injection, animals were placed individually on filter paper in an observable cylindrical plastic container (15 cm in diameter and 50 cm in height). The behavior of animals was recorded by using a digital video camera. Fecal materials and urine excreted during a 30-min period of withdrawal were measured. Jumping behavior was used as an experimental index of the central morphine withdrawal syndrome whereas defecation and urine excretion reflected a peripheral withdrawal syndrome (Broseta et al., 2002).

MS alkaloid treatments

One hour before the induction of withdrawal with naloxone, mice were given a single oral administration of either normal saline, mitragynine (30, 90 and 120 mg/kg), the MS alkaloid extract

(20, 40, 60, 80 and 100 mg/kg) or imipramine (20 mg/kg). Diazepam (5 mg/kg) was used as the positive control.

Spontaneous motor activity test

The spontaneous motor activity of mice was measured in a plastic cylinder (45 cm in diameter, 50 cm high). Its floor was divided into 9 roughly equal areas. One hour after treatment with the MS extract, animals were individually placed into the middle of the cylinder. The animal behavior was recorded by using the digital video camera from a top view. Spontaneous motor activity was evaluated by counting the number of grid lines crossed by both hind feet during a period of 5 min.

Statistical analysis

Experimental data were expressed as mean values ± SEM of the numbers of jumps and weights of fecal materials and urine during a withdrawal period. Differences were determined using one-way analysis of variance (ANOVA), followed by multiple comparisons versus control groups (Dunn's Method). Differences with $P < 0.05$ were considered statistically significant.

Results

Effects of MS alkaloid extract on jumping behavior induced by naloxone-precipitated morphine withdrawal

Naloxone-induced jumping behavior in morphine-dependent mice was rapidly observed following naloxone injection. In the saline control and all groups, the numbers of jumps increased from the first minute and peaked in the second minute (data not shown). Thereafter, they gradually decreased to almost zero in the fifth minute and remained unchanged for a period of 30 min. Therefore, jumping behavior during a 5-min period was counted and analyzed to represent the intensity of withdrawal for all groups. Jumping of the saline control was 46.2 ± 3.0 compared to 20.2 ± 5.4 for the diazepam control (**Fig. 3.1**). Treatment with imipramine did not have any significant effect in this experiment compared to the saline control. In the treatment groups receiving pure mitragynine, one-way ANOVA revealed a non-significant overall effect. In contrast, a more obvious suppressive effect was seen in the treatment groups that received the MS alkaloid extract. One-way ANOVA confirmed that jumping behavior of these groups was significantly decreased ($p = 0.003$). The decreases were clearly seen in groups that received the alkaloid extract at 80 and 100 mg/kg.

Effects of MS alkaloid extract on defecation during naloxone-precipitated morphine withdrawal

Defecation was evaluated from dry and wet fecal materials. Data were expressed as weight per 100 g body weight **(Fig. 3.2 A)**. One-way ANOVA showed that treatment with different doses of

the MS alkaloid extract significantly reduced dry fecal weight compared to the saline control ($p = 0.001$). The effect of the alkaloid extract was more obvious in the analysis of the wet feces. The saline control showed 0.29 ± 0.09 g per 100g BW of wet fecal matter compared to zero in all treatment groups. The analysis of total fecal matter also confirmed the significant reducing effect of the MS alkaloid extract on defecation ($p = 0.001$). No similar effect was seen in the group treated with imipramine.

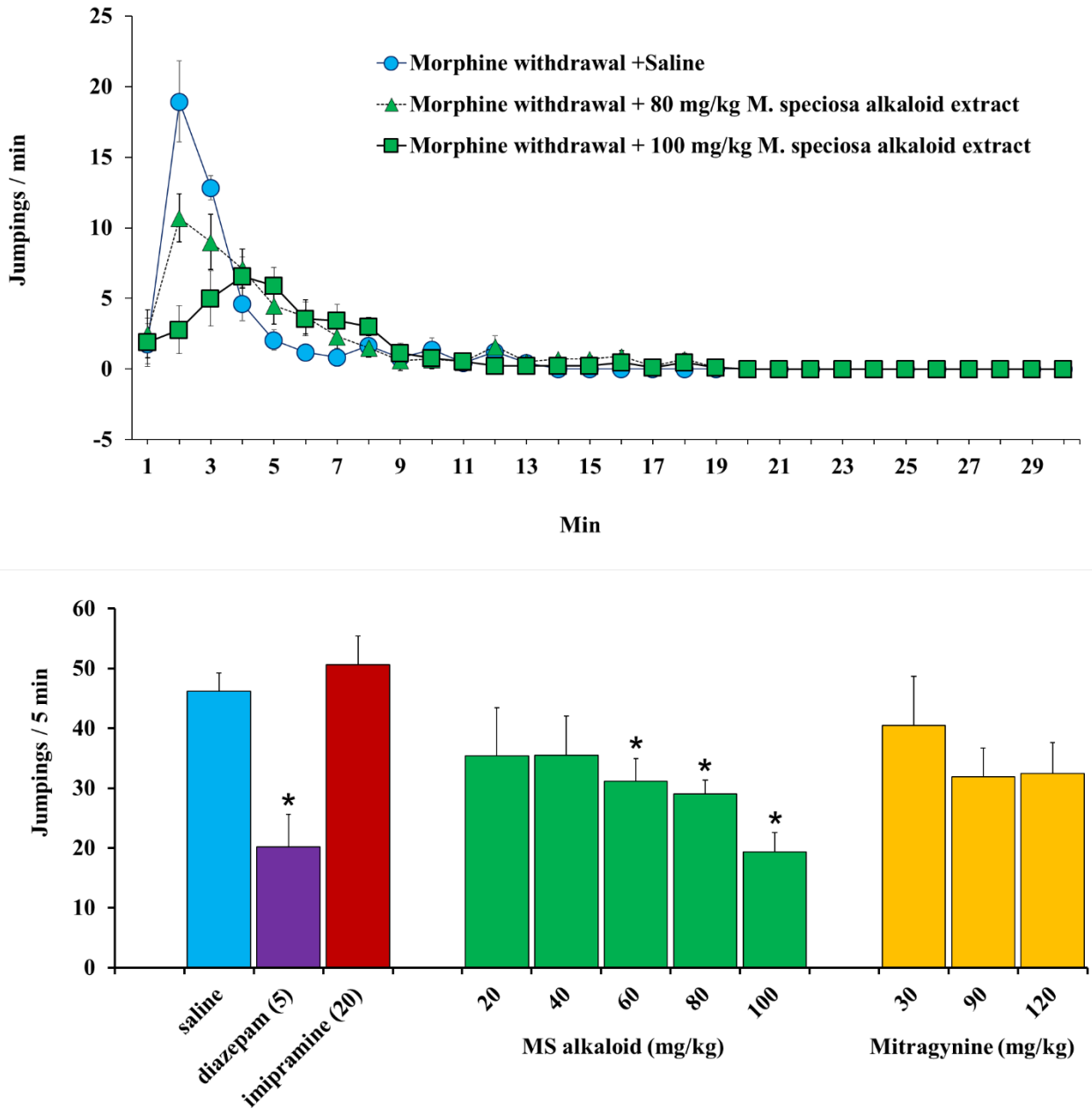

Fig. 3.1. Effects of mitragynine and crude alkaloid extract from Mitragyna speciosa on jumping behavior during a 5-min period (A) and 30-min period (B) of morphine withdrawal. Data are means ± S.E.M. * P < 0.05 different from the saline control group. (n=8-12)

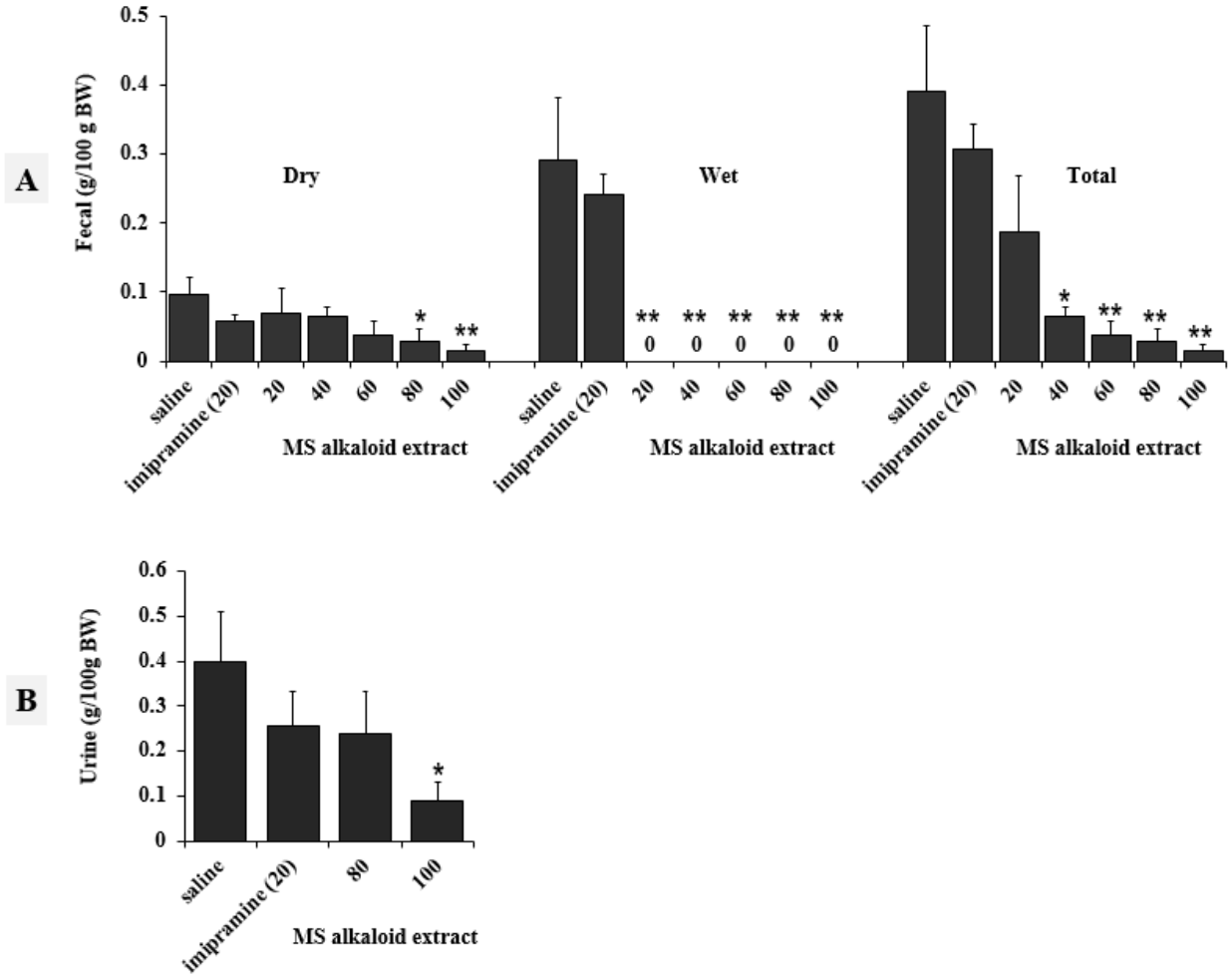

Fig. 3.2. Effects of crude alkaloid extract from *Mitragyna speciosa* on dry and wet fecal (A) and urine excretions (B) during a 30-min period of morphine withdrawal. Data are means ± S.E.M. * $P < 0.05$ and ** $P < 0.01$ different from the saline control group. (n=8-12)

Effects of MS alkaloid extract on urine excretion during naloxone-precipitated morphine withdrawal

The amount of urine was also expressed as weight per 100 mg body weight. The saline control group excreted about 0.4 g per 100 mg BW (**Fig. 3.2B**). One-way ANOVA revealed that

urine excretion was significantly decreased by treatment with the MS alkaloid extract ($p = 0.001$). All groups tested with the MS alkaloid extract (20, 60 and 100 mg/kg) showed a significant effect but the group tested with imipramine did not.

Effects of MS alkaloid extract on spontaneous motor activity

Levels of spontaneous motor activity were counted per 5 min. Overall analysis revealed that no significant effect of treatment with the MS alkaloid extract on spontaneous motor activity was seen. However, any doses higher than 100 mg/kg were likely to suppress motor activity (data not shown).

Discussion

The present study has demonstrated that the alkaloid extract from *Mitragyna speciosa* attenuated the intensity of morphine withdrawal in terms of jumping behavior. Treatment with mitragynine also tended to reduce the number of jumps but the change did not reach the significant level. The whole alkaloid extract at lower doses exhibited relatively stronger effects than did the pure mitragynine. This indicates that minor ingredients may have more promising properties for this purpose. Previously, 7-hydroxymitragynine, one of the minor alkaloids of the plant, exhibited a more potent antinociceptive effect than even morphine (Matsumoto et al., 2004a). This may explain why the crude extract was more potent than that of mitragynine. Having a high affinity to

the μ opioid receptor, 7-hydroxymitragynine could effectively retain levels of opioid receptor activation, which is low during the withdrawal period. Moreover, apart from mitragynine and 7-hydroxymitragynine, there might be some other biologically active minor constituents in this plant. On the other hand, it is also possible that there are two or more components that act on opioid receptors with different subtype selectivities. Thus, the combination of many constituents included in the same extract is likely to activate more opioid receptors in the central nervous system and therefore produce a stronger effect.

In this study, it was obvious that the MS alkaloid extract had mild effects on the jumping behavior but completely stopped diarrhea during the withdrawal period. Jumping is the sign frequently used to evaluate morphine dependence in a mice model (Ballard and McAllister, 1999; Reddy and Kulkarni, 1997). It also represents central withdrawal symptoms that may reflect craving. On the other hand, diarrhea represents peripheral symptom that reflects the level of intestinal motility during a morphine withdrawal period. The suppressive effect of the MS alkaloid extract on morphine withdrawal-induced diarrhea correlates with the previous in vitro study using smooth muscle (Watanabe et al., 1997). It has long been known that people use opium to stop diarrhea. Consistently, both synthetic agents and natural product extracts that act as opioid receptor agonists also have an antidiarrheal action (Broccardo and Improta, 1992; Shook et al., 1989). Altogether, these data suggest that the MS alkaloid extract completely suppresses diarrhea but just lessens the intensity of craving during a period of withdrawal.

Until now, methadone and LAAM are routinely used for pharmacological intervention for opiate dependence. The continuing use of these agents gives benefits in suppressing opioid

withdrawal symptoms for long durations. However, some patients also reported experiencing withdrawal symptoms during treatment (Dyer and White, 1997). Increasing the dose of methadone has benefits in suppressing withdrawal symptoms but also leads to some risks both physically and psychologically (Kreek, 1973). Alternative treatments are needed to substitute methadone or to be used in combination with a small dose of methadone to enhance its effect. According to our findings, the MS alkaloid extract might be used as an alternative treatment in addition with other effective opioid substances. On its own, it might be too weak to suppress peak cravings. However, it may be capable of substituting for methadone later when the opiate dependence is mild.

CHAPTER 4

The Effects of Acute MS Alkaloid Extract Treatment on Neural Signaling of Brain Reward Circuit

Abstract

Mitragyna speciosa (Korth.) Havil. (M. speciosa) is among the most well-known plants used in ethnic practice of Southeast Asia. It has gained increasing attention as a plant with potential to substitute morphine in addiction treatment program. However, its action on the central nervous system is controversial. This study investigated the effects of M. speciosa alkaloid extract on neural signaling in the nucleus accumbens (NAc, brain reward center) of mice. To test possible addictive effect of M. speciosa alkaloid extract, mice were implanted with intracranial electrode into the NAc for local field potential (LFP) recording. Following M. speciosa alkaloid extract (80 mg/kg) and morphine (15 mg/kg) treatment, LFP power spectra and spontaneous motor activity were analyzed in comparison to control levels. LFP analysis revealed that morphine significantly decreased alpha (9.7-12 Hz) and increased low gamma (30.3-44.9 Hz) and high gamma (60.5-95.7 Hz) powers in the NAc whereas M. speciosa alkaloid extract did not. Spontaneous motor activity was significantly increased by morphine but not M. speciosa alkaloid extract. Taken together, neural signaling in the NAc and spontaneous motor activity were sensitive to morphine but not M. speciosa alkaloid

extract. Therefore, treatment with the M. speciosa alkaloid extract may be useful for opiate addiction treatment program.

Keywords: *Mitragyna speciose*; Morphine withdrawal; Opioid; Naloxone; Reward; nucleus accumbens

Introduction

In Thailand as well as Malaysia, MS leaves or preparations are controlled substances. In the United States, United Kingdom and Germany, they are currently not controlled substances, but are under surveillance awaiting more scientific evidence (EMCDDA/Kratom-Europa 2012; Hassan et al., 2013). MS abuse has gained significant attention in Malaysia and Thailand (Chan et al., 2005). Furthermore, the wide availability of kratom to purchase through the internet reflects an increasing demand of misusing this globally (Boyer et al., 2008).

Until recently, there were some cases that may suggest potential abuses of the MS following its prolonged use applied for chronic pain treatment and opioid replacement as alternative therapy (Boyer et al., 2008). Previous studies on MS users in Thailand revealed that long-term MS use produced addiction-like effects (Suwanlert, 1975). Regular use of MS also reported the associated with drug dependency, development of withdrawal symptoms, and craving. These symptoms became more severe with prolonged use (Singh et al., 2014). However, they also reported that MS use caused much less adverse effects and lower admission rate to the hospital

than other addictive drugs such as morphine methamphetamine did. This indicates milder withdrawal symptoms and less harm than that of opiates and other drugs.

Addictive drugs are positive reinforcers, which directly increase the probability of a response to reward stimulus. Acute drug consumption that produces euphoria state (reward stimuli) can lead to repetitive and continue drug using. Brain reward center that plays a role in positive reinforcement was identified (Olds and Milner, 1954). The most important reward pathway in brain is mesolimbic dopamine system. This neural circuit plays an important role in encoding of rewarding stimuli which includes the natural rewards (Spreckelmeyer et al., 2009), drug of abuse or psychoactive drugs (Spyraki et al., 1982) and cues which predict naturally rewarding outcomes.

Due to the fact that changes in activity of major neurotransmitter systems modulate the cortical or intracranial activity that can be detected by using EEG. Moreover, it has been clear that specific action of compounds on specific neurotransmitter system produced unique EEG pattern called electropharmacogram or EEG fingerprints (Dimpfel, 2003). These would be logical to assume specific neurotransmitter system response to pharmacological treatment from EEG pattern analysis. This indicate that EEG has been consistently used to classify drug action in the CNS. The effect of MS alkaloid extract was also studied using EEG technique to characterize its EEG fingerprint pattern. The MS alkaloid extracts selectively decreased EEG power in slow frequency range (1-20 Hz) in frontal and parietal cortices. The enhancement of serotonergic activity by antidepressant drugs such as fluoxetine, produced a decrease of slow wave EEG power similar to that of the MS alkaloid extract.

However, direct neural processing of reward circuit induced by the MS extract have not been established. Therefore, this study aimed to investigate the brain reward network signaling as EEG fingerprint of the MS alkaloid extract. The pattern of neural network oscillations in the brain reword circuit might reveal the definite of kratom in terms of reward and addiction-related processing mechanisms.

Materials and methods

Plant materials and extraction

Young leaves of MS were collected from natural sources in Songkhla and Satun provinces, Thailand during 2004-2005. Authentication of the plant material was carried out at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand where the herbarium voucher specimens (no. PCOG/MS001-002) have been deposited.

Extraction and isolation of the MS extract was described in a previous study (Cheaha et al., 2015b). The major alkaloid, isolated by silica-gel CC eluting with MeOH:CHCL3 (5:95), was identified as mitragynine with the standard spectroscopic methods (MS, 1H NMR and 13C NMR). According to TLC analysis, mitragynine was found to constitute about 60% of the content of the alkaloid extract. The solution of the MS alkaloid extract was prepared by dissolving the extract in co-solvent (tween 80:propylene glycol:H2O at a 1:4:4 ratio) and adjusted to desired concentrations with distilled water. The MS alkaloid extract was previously identified using high performance liquid

chromatography (HPLC) (Cheaha et al., 2015b). The alkaloid extract was run and found to contain mitragynine as a major component.

Chemicals

The following drugs were used: morphine sulphate (Zentiva, SK), naloxone (Sigma, Germany). The drugs were dissolved in normal saline and given to animals in a volume of 5 ml/kg. The m. speciosa alkaloid extract was dissolved in co-solvent (Tween80: propyleneglycol: H2O at a 1:4:4 ratio). A ball tipped stainless steel gavage needle was used for oral feeding.

Animals

Male Swiss albino ICR mice (7-8 weeks old) used in each experiment were bred at the animal house of the Prince of Songkla University. They were housed in a group of 10 mice per cage (20x25x35 cm) and maintained under 12/12 dark/light cycle (lights on at 0600 am) and controlled temperature (22°C). Standard commercial food pellets and filtered water were available ad libitum. All the tests were performed between 8.00 A.M. and 4.00 P.M. This study was carried out in accordance with guidelines of the European Science Foundation (Use of Animals in Research 2001) and International Committee on Laboratory Animal Science, ICLAS (2004). The experimental protocols described in the present study were approved and guided by the Animals Ethical Committee of the Prince of Songkla University for care and use of experimental animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgery for LFP electrode implantation

Method of electrode implantation was also previously described (Cheaha et al., 2015a). Briefly, animals were anesthetized by injection intramuscularly of a mixture of 150 mg/kg ketamine (Calypsol, Gedeon Richter Ltd., Hungary) and 15 mg/kg xylazine (Xylavet, Thai Maji Phamaceutical co., Ltd., Thailand). Then, the animal's head was mounted in a stereotaxic frame. Local analgesic, lidocaine (Locana, L.B.S. Laboratory Ltd., Part., Thailand) was applied to the exposed tissue of the head. An incision was made at the midline to expose the skull. The silver wire electrodes (A-M system, Sequim, WA, USA) with bare diameter of 0.008" (Coated-0.011") were stereotaxically positioned on the left nucleus accumbens (NAc) (AP: + 0.7 mm, ML: 1.3 mm, DV: 4.7 mm) according to mouse brain atlas (Franklin and Paxinos, 1998). The reference and ground electrodes were placed at midline overlying the cerebellum. Additional holes were drilled for stainless steel anchor screws. Dental acrylic (Unifasttrad, Japan) was used to secure all electrodes on the skull. The antibiotic ampicillin (General Drug House Co., Ltd., Thailand) was applied intramuscularly (100 mg/kg) once a day for 3 days to prevent infection and allowed them to recover for at least 7-10 days

Experimental procedure

After fully recovery from the surgery, animals were acclimatized to the experimental condition in the recording chamber for 2 days before testing. During the testing days, animals were

given similar condition to the habituation days and the LFPs and behaviors were monitored. Baseline activity was performed for 30 minutes before treatment. Animals were divided into 3 groups to orally receive vehicle, 80 mg/kg MS alkaloid extract and intraperitoneal injection of 15 mg/kg morphine. Post-drug recording was performed 180 minutes after drug administration. All states of the protocol were collectively illustrated in Fig. 4.1.

A concentration of alkaloid extract from MS was chosen according to preliminary result (figure 11 and 12). A moderate dose (80 mg/kg) of MS alkaloid extract was found to effectively attenuate morphine withdrawal symptoms while pure mitragynine did not. In addition, hepatotoxicity and nephrotoxicity of the high dose of mitragynine (100 mg/kg) has been reported previously (Sabetghadam et al., 2013).

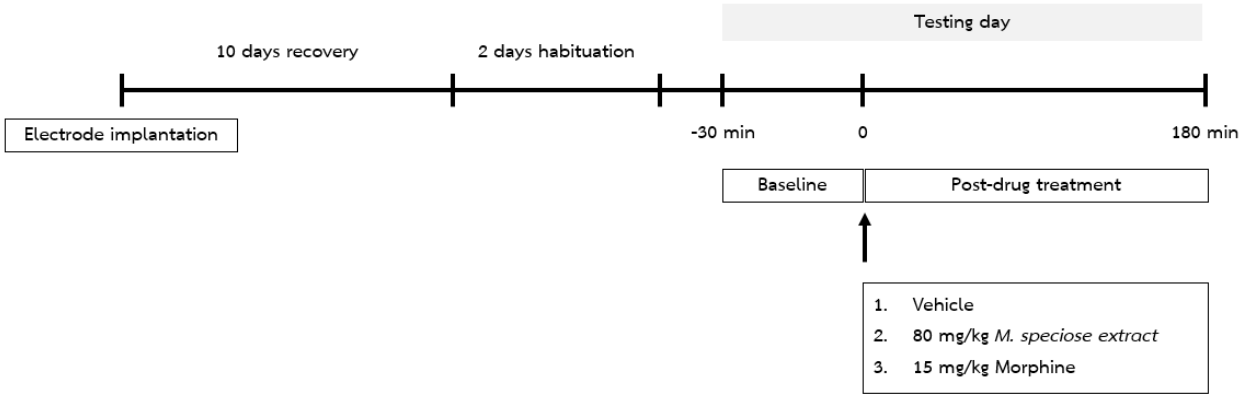

Fig 4.1. Schematic diagram of protocol for examination of acute MS alkaloid extract effects.

LFP signals acquisition and analysis

Methods in details of signal recoding and analysis were shown in Fig.4.2. and described in the previous study (Cheaha et al. 2015a). Briefly, all signals from animal electrodes were amplified

with a low-pass 1 kHz, high-pass 0.3 Hz and digitized at 2 kHz by a PowerLab 16/35 system (AD Instruments, Castle Hill. NSW, Australia) with 16-bit A/D, and stored in a PC through the LabChart 7 pro software (figure 5). For offline analysis, recorded files were overviewed by using visual inspection and only noise-free signals were used for the analysis. Fifty Hz notch filtering was applied to remove the noise from power line artifacts. To avoid 50 Hz noise, the signal from 45-55 Hz may be sometimes excluded from the further analysis. The LFP and EMG signals were processed through 1-200 Hz and 1-100 Hz band-pass digital filter, respectively. Standard LFP analysis, the frequency analysis, were performed using FFT function in LabChart 7. Synchrony analysis including coherence and phase-amplitude cross frequency coupling (PACFC) were specifically analyzed using MATLAB-based Brainstorm software (Tadel et al., 2011). Sleep-wake pattern was score manually using both EEG and EMG signals.

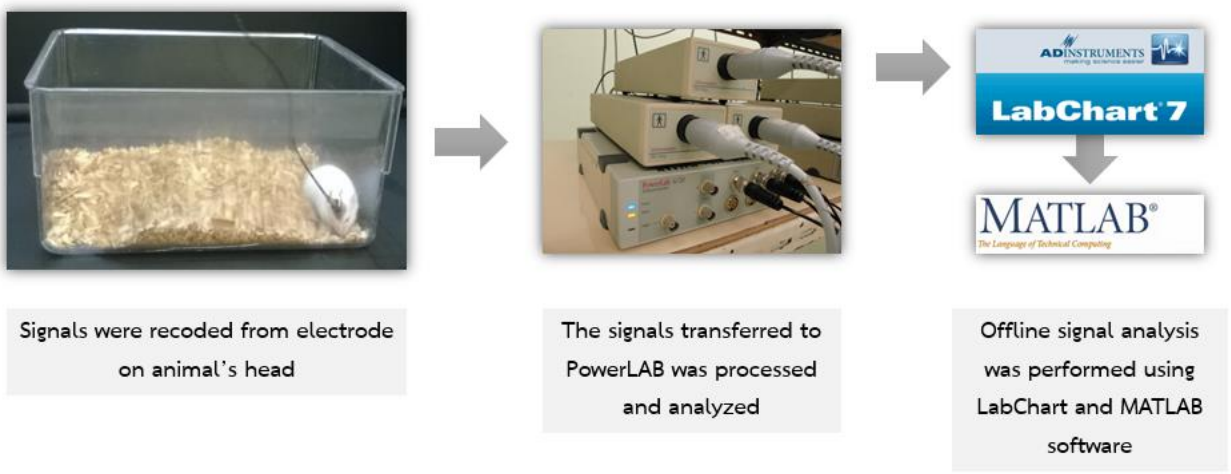

Fig. 4.2. Signals acquisition and analysis setup

Frequency analysis of LFP signals (Fig. 4.3.)

For the frequency analysis (figure 6), the digitized data were assessed by analyzing power spectral density (PSD) and spectrograms (frequency through time plots) generated by LabChart software (PowerLab Software, AD Instruments, Castle Hill, Australia) using the Fast Fourier Transform (FFT) algorithm. The setup conditions for FFT calculation are Hanning window cosine with 50% window overlap, and frequency resolution of 0.976 Hz. Then, the PSD in each frequency bin was expressed as relative power (percentage of baseline and/or percentage of total power). The average spectral powers were constructed in discrete frequency bands of each group and expressed in frequency domain based on correlations among frequency ranges and functional role of each brain areas (Cheaha et al., 2014; Cheaha et al., 2015a; Cheaha et al., 2015b; Cheaha et al., 2015c). The power was averaged across 6 discrete frequency bands: theta, 4-8 Hz; alpha, 9.7-12 Hz; beta1, 13.6-18.0 Hz; beta2, 19.5-29.3 Hz; gamma1 or low gamma, 30.3-44.9 Hz; gamma2 or high gamma 60.5-95.7 Hz. Spectral powers in discrete frequency bands of each group were averaged and expressed either in time or frequency.

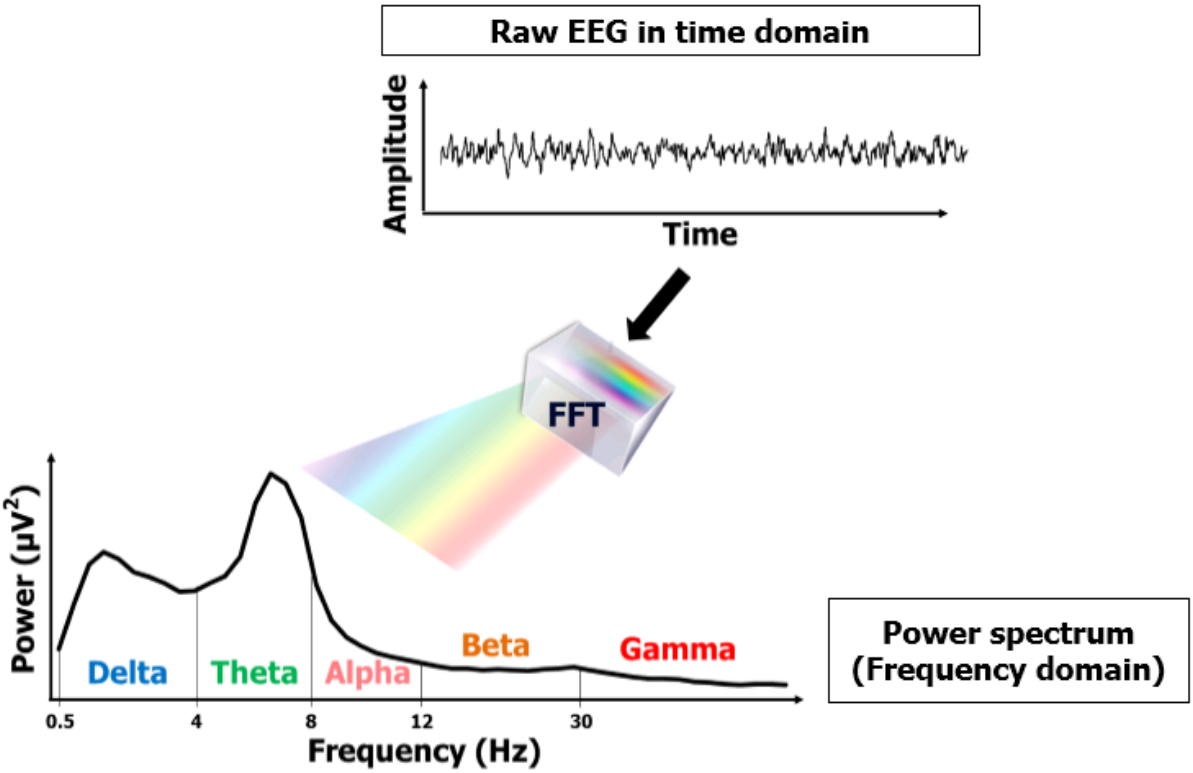

Fig. 4.3. The FFT transforms the discrete EEG signal in time domain in into the frequency domain as shown.

Recording and analysis of locomotor activity

Animal behaviors and locomotor activity were recorded using a webcam vertically mounted on the top of recording chamber. Animal image was continuously captured and transferred to the computer. Animal movement was analyzed by using visual C++ -based software specifically developed for locomotor activity research (Cheaha et al., 2015a). Basically, translocation of animal was detected with a sensitivity at 2-mm threshold. One count was considered from 1 period of

continuous translocation and 1 stop. Levels of locomotor activity were expressed as mean ± S.E.M. of locomotor count per time period.

Statistical analysis

Experimental data were expressed as mean values ± S.E.M. of the numbers of jumps and weights of fecal materials and urine during a withdrawal period. Differences were determined using one-way analysis of variance (ANOVA), followed by multiple comparisons using Turkey's Method. Differences with $P < 0.05$ were considered statistically significant.

Results

Effects of M. speciosa alkaloid extract and morphine treatment on local field potential in the nucleus accumbens

LFP signals from individual mice were continuously recorded for 3 hrs following treatment with either vehicle, m. speciosa extract (80 mg/kg BW, p.o.) or morphine (15 mg/kg BW, i.p.). Representative raw LFP tracings are shown in time domain (Fig. 4.4.). By visual inspection, relatively similar patterns were seen between LFPs of control and m. speciosa alkaloid extract but not morphine group. The latter group appeared to have additional oscillations of fast wave superimposed in slow waves of raw LFP signals. Therefore, all signals were converted into

frequency domain for LFP powers analysis (Fig. 4.4.). Data were illustrated in LFP power spectra (% total power) for 1-100 Hz frequency range. Obviously, morphine appeared to produce different pattern of LFP power spectrum from that of vehicle group. Morphine seemed to reduce slow frequency powers particularly below 30 Hz and increase powers of frequencies above 30 Hz. However, m. speciosa alkaloid extract did not change LFP powers throughout the frequency range analyzed. The LFP spectrum produced by m. speciosa extract was relatively similar to that of vehicle group. Finally, broad power spectra were divided and assigned into 6 discrete frequency ranges for statistical analysis. Multiple comparisons also indicated significant decreases in alpha and increases in low gamma and high gamma powers produced by morphine treatment. No significant difference between powers of vehicle and m. speciosa alkaloid extract groups was found.

Therefore, time-course effect of treatments on low gamma power was particularly analyzed (Fig. 4.5). Following morphine treatment, low gamma power was rapidly increased and peaked approximately within 40 minutes. Therefore, the effect of morphine gradually decreased to control level within 3 hrs. Statistical analysis also confirmed significant effect of morphine treatment on low gamma power. On the other hand, no significant change was produced by m. speciose alkaloid extract administration.

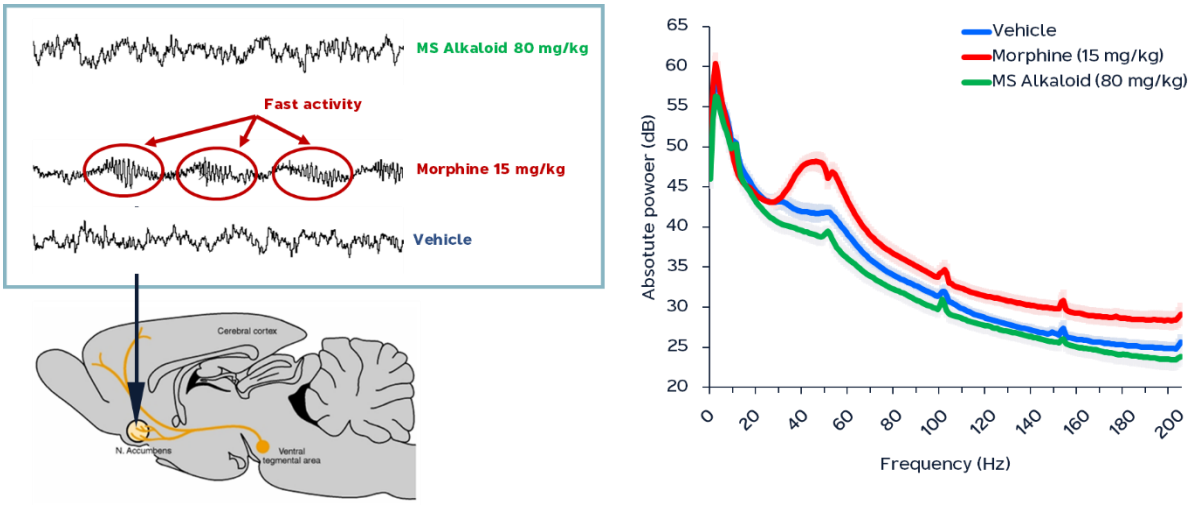

Fig. 4.4. Patterns of local field potentials following administrations with M. speciosa alkaloid extract and morphine (n = 6-8). Time domain of raw LFP signals recorded from the nucleus accumbens of representative mice that received saline (SAL), 80 mg/kg M. speciosa alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) are displayed. Power spectrums of LFP are expressed in frequency domain.

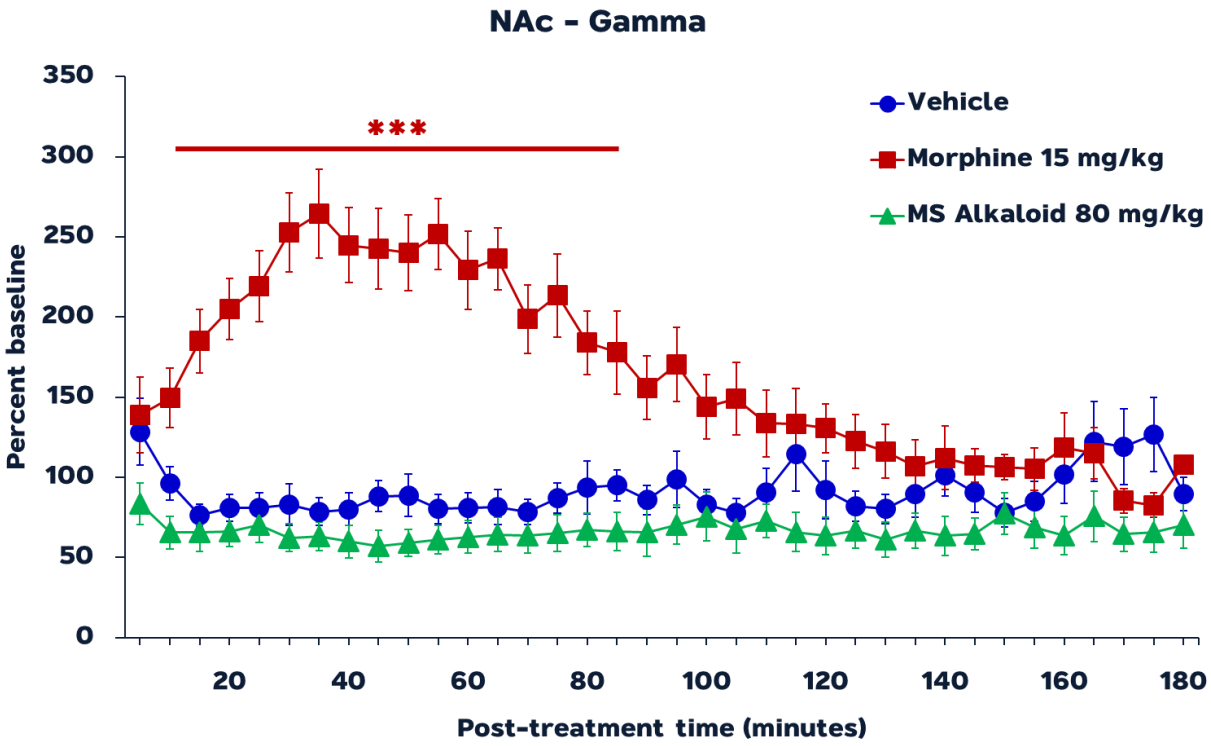

Fig. 4.5. Average percent baseline power of gamma1 range were analyzed every 5 minutes following administration with saline (SAL), 80 mg/kg M. speciosa alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) (n = 6-8). Data were compared with vehicle levels using one-way ANOVA followed by Tukey's post hoc test. ***: P < 0.001, respectively.

Effects of M. speciosa alkaloid extract on spontaneous motor activity

Levels of spontaneous motor activity were counted every 5 minutes (Fig. 4.6). Locomotor count was progressively increased following morphine treatment. The levels of locomotor activity peaked within 60 minutes and decreased to baseline level within 2.5 hrs. No significant effect of m. speciosa alkaloid extract on spontaneous motor activity was seen.

Discussion

Activation of dopaminergic brain pathways and spontaneous motor activity by drugs of addiction are well-known. Particularly, activation of the nucleus accumbens and striatum by psychostimulants and opiates is believed to play a major role in the establishment of drug dependence and withdrawal phenomena (Hyman, 1996; Koob, 1996; Nestler, 1996). Similar findings were seen following injections of cocaine and methamphetamine (Wang and McGinty, 1995; Young et al., 1991). In term of behavioral output, psychostimulants also enhance locomotor activity (Vezina, 2004). These are among the most common effects produced by addictive substances. However, *M. speciosa* alkaloid extract did not activate the nucleus accumbens and spontaneous motor activity at all. The present data may explain why kratom use for emotional purpose has not been reported. Therefore, the impact of situations and context like social, ritualistic or self-medicating factors may be discussed why kratom consumption is still widespread in some countries.

According to our findings, the M. speciosa alkaloid extract might be used as an alternative treatment in combination with other effective opioid agonists. CNS action of M. speciosa alkaloid extract is unlikely to mimic that of opiates according to LFP patterns and levels of spontaneous motor activity.

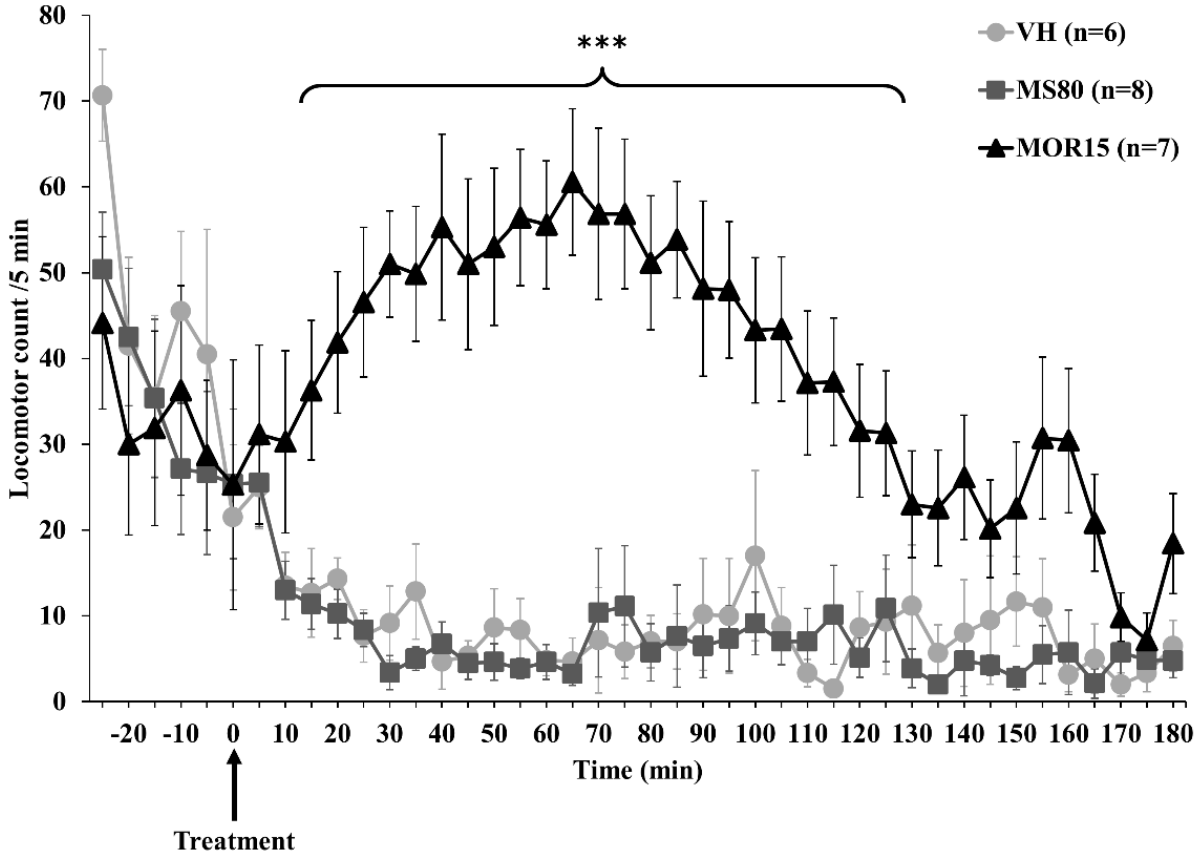

Fig. 4.6. Effects of administration with saline (SAL), 80 mg/kg M. speciosa alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) on spontaneous motor activity (n = 6-8). Locomotor counts in each group were averaged 5 minutes during baseline period and following the administration for 3 hrs. Data are expressed as means ± S.E.M. *** $P < 0.001$ for comparisons with vehicle levels (one-way ANOVA followed by Tukey's post hoc test).

References

Ballard,T.M. and McAllister,K.H., 1999. Acutely administered clozapine does not modify naloxone-induced withdrawal jumping in morphine-dependent mice. Pharmacol. Biochem. Behav. 62, 285-290.

Beswick,T., Best,D., Rees,S., Bearn,J., Gossop,M., and Strang,J., 2003. Major disruptions of sleep during treatment of the opiate withdrawal syndrome: differences between methadone and lofexidine detoxification treatments. Addict. Biol. 8, 49-57.

Boyer, E.W., Babu, K.M., Adkins, J.E., McCurdy, C.R., and Halpern, J.H., 2008. Self-treatment of opioid withdrawal using kratom (*Mitragynia speciosa* korth). Addiction. 103, 1048-1050.

Broccardo,M. and Improta,G., 1992. Antidiarrheal and colonic antipropulsive effects of spinal and supraspinal administration of the natural delta opioid receptor agonist, [D-Ala2]deltorphin II, in the rat. Eur. J. Pharmacol. 218, 69-73.

Broseta,I., Rodriguez-Arias,M., Stinus,L., and Minarro,J., 2002. Ethological analysis of morphine withdrawal with different dependence programs in male mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 26, 335-347.

Chan, K.B., Pakiam, C., and Rahim, R.A., 2005. Psychoactive plant abuse: the identification of mitragynine in ketum and in ketum preparations. Bull. Narc. 57, 249-256.

Cheaha D., Bumrungsri S., Chatpun S. and Kumarnsit E. 2015a. Characterization of in utero valproic acid mouse model of autism by local field potential in the hippocampus and the olfactory bulb. Neuroscience Research. 29:28-34.

Cheaha D., Keawpradub N., Sawangjaroen K., Phukpattaranont P. and Kumarnsit E. 2015b. Effects of an alkaloid-rich extract from *Mitragyna speciosa* leaves and fluoxetine on sleep profiles, EEG spectral frequency and ethanol withdrawal symptoms in rats. Phytomedicine. 22:1000-1008.

Cheaha D., Sawangjaroen K., Kumarnsit E. 2014. Characterization of fluoxetine effects on ethanol withdrawal-induced cortical hyperexcitability by EEG spectral power in rats. Neuropharmacology. 77:49–56.

Clark,N., Lintzeris,N., Gijsbers,A., Whelan,G., Dunlop,A., Ritter,A., and Ling,W., 2002. LAAM maintenance vs methadone maintenance for heroin dependence. Cochrane. Database. Syst. Rev. CD002210.

Dimpfel, W. 2003. Preclinical data base of pharmaco-specific rat EEG fingerprints (tele-stereo-EEG). Eur J Med Res. 8(5): 199-207.

Dyer,K.R. and White,J.M., 1997. Patterns of symptom complaints in methadone maintenance patients. Addiction 92, 1445-1455.

EMCDDA/Kratom-Europa (2012) Kratom (*Mitragyna speciosa*). Available at: http://www.emcdda.europa.eu/publications/drug-profiles/kratom/de. Accessed 10 June 2012.

Franklin, K.B.J., Paxinos, G. 1998. The mouse brain in stereotaxic coordinates. Academic: San Diego, Calif. ; London.

Gold,M.S., Redmond,D.E., Jr., and Kleber,H.D., 1978. Clonidine blocks acute opiate-withdrawal symptoms. Lancet 2, 599-602.

Gossop,M., Bradley,B., and Phillips,G.T., 1987. An investigation of withdrawal symptoms shown by opiate addicts during and subsequent to a 21-day in-patient methadone detoxification procedure. Addict. Behav. 12, 1-6.

Gossop,M., Griffiths,P., Bradley,B., and Strang,J., 1989. Opiate withdrawal symptoms in response to 10-day and 21-day methadone withdrawal programmes. Br. J. Psychiatry 154, 360-363.

Hassan, Z., Muzaimi, M., Navaratnam, V., Yusoff, N.H.M., Suhaimi, F.W., Vadivelu, R., Vicknasingam, B.K., Amato, D., von Hרזrsten, S., Ismail, N.I.W., Jayabalan, N., Hazim, A.I., Mansor, S.M., and Mרזller, C.P., 2013. From Kratom to mitragynine and its derivatives: Physiological and behavioural effects related to use, abuse, and addiction. Neuroscience & Biobehavioral Reviews. 37, 138-151.

Hyman, S.E., 1996. Addiction to Cocaine and Amphetamine. Neuron. 16, 901-904.

Jansen,K.L. and Prast,C.J., 1988. Psychoactive properties of mitragynine (kratom). J. Psychoactive Drugs 20, 455-457.

Koob, G.F., 1996. Drug Addiction: The Yin and Yang of Hedonic Homeostasis. Neuron. 16, 893-896.

Kreek,M.J., 1973. Medical safety and side effects of methadone in tolerant individuals. JAMA 223, 665-668.

Kumarnsit, E., Vongvatcharanon, U., Keawpradub, N., and Intasaro, P., 2007. Fos-like immunoreactivity in rat dorsal raphe nuclei induced by alkaloid extract of *Mitragyna speciosa* . Neuroscience Letters. 416, 128-132.

Marshall,I. and Grahame-Smith,D.G. (1971). Evidence against a role of brain 5-hydroxytryptamine in the development of physical dependence upon morphine in mice. J. Pharmacol. Exp. Ther. 179, 634-641.

Matsumoto,K., Horie,S., Ishikawa,H., Takayama,H., Aimi,N., Ponglux,D., and Watanabe,K., 2004. Antinociceptive effect of 7-hydroxymitragynine in mice: Discovery of an orally active opioid analgesic from the Thai medicinal herb Mitragyna speciosa. Life Sci. 74, 2143-2155.

Matsumoto,K., Horie,S., Ishikawa,H., Takayama,H., Aimi,N., Ponglux,D., and Watanabe,K., 2004a. Antinociceptive effect of 7-hydroxymitragynine in mice: Discovery of an orally active opioid analgesic from the Thai medicinal herb Mitragyna speciosa. Life Sci. 74, 2143-2155.

Matsumoto,K., Mizowaki,M., Suchitra,T., Murakami,Y., Takayama,H., Sakai,S., Aimi,N., and Watanabe,H., 1996. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic systems. Eur. J. Pharmacol. 317, 75-81.

Matsumoto,K., Mizowaki,M., Suchitra,T., Murakami,Y., Takayama,H., Sakai,S., Aimi,N., and Watanabe,H., 1996b. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic systems. Eur. J. Pharmacol. 317, 75-81.

McMillan,D.E., Leander,J.D., Wilson,T.W., Wallace,S.C., Fix,T., Redding,S., and Turk,R.T., 1976. Oral ingestion of narcotic analgesics by rats. J. Pharmacol. Exp. Ther. 196, 269-279.

Nestler, E.J., 1996. Under Siege: The Brain on Opiates. Neuron. 16, 897-900.

Olds, J. and Milner, P., 1954. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. Journal of comparative and physiological psychology. 47, 419-427.

Ponglux D, Wongseripipatana S, Takayama H, Kikuchi M, Aimi N, and Saiki S., 1994. A new indole alkaloid, 7 alpha-hydroxy-7H-mitragynine, from mitragyna speciosa in Thailand. Planta Med. 60, 580-581.

Reddy,D.S. and Kulkarni,S.K., 1997. Chronic neurosteroid treatment prevents the development of morphine tolerance and attenuates abstinence behavior in mice. Eur. J. Pharmacol. 337, 19-25.

Shook,J.E., Lemcke,P.K., Gehrig,C.A., Hruby,V.J., and Burks,T.F., 1989. Antidiarrheal properties of supraspinal mu and delta and peripheral mu, delta and kappa opioid receptors: inhibition of diarrhea without constipation. J. Pharmacol. Exp. Ther. 249, 83-90.

Singh D., Müller C.P., Vicknasingam B.K. 2014. Kratom (*Mitragyna speciosa*) dependence, withdrawal symptoms and craving in regular users. Drug Alcohol Depend. 139:132-137.

Spreckelmeyer K.N., Krach S., Kohls G., Rademacher L., Irmak A., Konrad K., Kircher T., Gründer G. 2009. Anticipation of monetary and social reward differently activates mesolimbic brain structures in men and women. Soc Cogn Affect Neurosci. 4(2):158-165.

Spyraki, C., Fibiger, H.C., and Phillips, A.G., 1982. Dopaminergic substrates of amphetamine-induced place preference conditioning. Brain Research. 253, 185-193.

Suwanlert, S., 1975. A study of kratom eaters in Thailand. Bull. Narc. 27, 21-27.

Tadel, F., Baillet, S., Mosher, J.C., Pantazis, D., Leahy, R.M., 2011. Brainstorm: A User-Friendly Application for MEG/EEG Analysis. Comput. Intell. Neurosci. 2011, 879716.

Takayama,H., Ishikawa,H., Kurihara,M., Kitajima,M., Aimi,N., Ponglux,D., Koyama,F., Matsumoto,K., Moriyama,T., Yamamoto,L.T., Watanabe,K., Murayama,T., and Horie,S., 2002. Studies on the synthesis and opioid agonistic activities of mitragynine-related indole alkaloids: discovery of opioid agonists structurally different from other opioid ligands. J. Med. Chem. 45, 1949-1956.

Thongpradichote,S., Matsumoto,K., Tohda,M., Takayama,H., Aimi,N., Sakai,S., and Watanabe,H., 1998. Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. Life Sci. 62, 1371-1378.

Trueblood,B., Judson,B.A., and Goldstein,A., 1978. Acceptability of methadyl acetate (LAAM) as compared with methadone in a treatment program for heroin addicts. Drug Alcohol Depend. 3, 125-132.

Tsuchiya,S., Miyashita,S., Yamamoto,M., Horie,S., Sakai,S., Aimi,N., Takayama,H., and Watanabe,K., 2002. Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. Eur. J. Pharmacol. 443, 185-188.

Vezina, P., 2004. Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. Neuroscience & Biobehavioral Reviews. 27, 827-839.

Wang, J.Q. and McGinty, J.F., 1995. Differential Effects of D1 and D2 Dopamine Receptor Antagonists on Acute Amphetamine- or Methamphetamine-Induced Up-Regulation of zif/268 mRNA Expression in Rat Forebrain. Journal of Neurochemistry. 65, 2706-2715.

Watanabe,K., Yano,S., Horie,S., and Yamamoto,L.T., 1997. Inhibitory effect of mitragynine, an alkaloid with analgesic effect from Thai medicinal plant Mitragyna speciosa, on electrically stimulated contraction of isolated guinea-pig ileum through the opioid receptor. Life Sci. 60, 933-942.

Young, S.T., Porrino, L.J., and Iadarola, M.J., 1991. Cocaine induces striatal c-fos-immunoreactive proteins via dopaminergic D1 receptors. Proc Natl Acad Sci U S A. 88, 1291-1295.

Yusoff, N.H.M., Suhaimi, F.W., Vadivelu, R.K., Hassan, Z., Rรฝmler, A., Rotter, A., Amato, D., Dringenberg, H.C., Mansor, S.M., Navaratnam, V., and Mรฝller, C.P., 2016. Abuse potential and adverse cognitive effects of mitragynine (kratom). Addiction Biology. 21, 98-110.

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Effects of alkaloid-rich extract from *Mitragyna speciosa* (Korth.) Havil. on naloxone-precipitated morphine withdrawal symptoms and local field potential in the nucleus accumbens of mice

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ABSTRACT

Ethnopharmacological relevance: *Mitragyna speciosa* (Korth.) Havil. (*M. speciosa*) is among the most well-known plants used in ethnic practice of Southeast Asia. It has gained increasing attention as a plant with potential to substitute morphine in addiction treatment program. However, its action on the central nervous system is controversial.

Aim of the study: This study investigated the effects of *M. speciosa* alkaloid extract on naloxone-precipitated morphine withdrawal and neural signaling in the nucleus accumbens (NAc, brain reward center) of mice.

Materials and methods: The effects of *M. speciosa* alkaloid extract and mitragynine, a pure major constituent, on naloxone-precipitated morphine withdrawal were examined. Male Swiss Albino (ICR) mice were rendered dependent on morphine before injection with naloxone, a nonspecific opioid antagonist, to induce morphine withdrawal symptoms. The intensity of naloxone-precipitated morphine withdrawal was assessed from jumping behavior and diarrhea induced during a period of morphine withdrawal. To test possible addictive effect of *M. speciosa* alkaloid extract, mice were implanted with intracranial electrode into the NAc for local field potential (LFP) recording. Following *M. speciosa* alkaloid extract (80 mg/kg) and morphine (15 mg/kg) treatment, LFP power spectra and spontaneous motor activity were analyzed in comparison to control levels.

Results: One-way ANOVA and multiple comparisons revealed that *M. speciosa* alkaloid extract (80 and 100 mg/kg) significantly decreased the number of jumping behavior induced by morphine withdrawal whereas mitragynine did not. Additionally, *M. speciosa* alkaloid extract significantly decreased dry and wet fecal excretions induced by morphine withdrawal. LFP analysis revealed that morphine significantly decreased alpha (9.7–12 Hz) and increased low gamma (30.3–44.9 Hz) and high gamma (60.5–95.7 Hz) powers in the NAc whereas *M. speciosa* alkaloid extract did not. Spontaneous motor activity was significantly increased by morphine but not *M. speciosa* alkaloid extract.

Abbreviations: AP, Anteroposterior; ANOVA, Analysis of variance; BW, Body weight; CNS, Central nervous system; DV, Dorso-Ventral; FFT, Fast Fourier transform; HPLC, High performance liquid chromatography; ICLAS, International Committee on Laboratory Animal Science; LAAM, levo-alpha-acetylmethadol; LC-MS, liquid chromatographic mass spectrometry; LFP, Local field potential; ML, Medio-Lateral; NAc, Nucleus accumbens; PSD, Power spectral density; TLC, Thin layer chromatography; S.E.M., Standard error of the mean

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Conclusions: Taken together, *M. speciosa* alkaloid extract, but not mitragynine, attenuated the severity of naloxone-precipitated morphine withdrawal symptoms. Neural signaling in the NAc and spontaneous motor activity were sensitive to morphine but not *M. speciosa* alkaloid extract. Therefore, treatment with the *M. speciosa* alkaloid extract may be useful for opiate addiction treatment program.

1. Introduction

Mitragyna speciosa (Korth.) Havil. (*M. speciosa*), locally called Kratom in Thailand, is a member of the Rubiacaea plant family. It has been used as a medicinal plant for centuries (Jansen and Prast, 1988; Suwanlert, 1975). In modern studies, four alkaloids namely mitragynine, 7-hydroxymitragynine, corynantheidine and speciociliatine appeared to act on opioid receptor (Takayama et al., 2002). However, kratom consumption for opium-like effect was previously reported (Suwanlert, 1975). In Malaysia, there is a record that kratom was used as an affordable opium substitute (Burkill, 1935). However, the efficacy of kratom use as an opium substitute has not been clearly elucidated. There were still many questions needed to be answered in scientific ways.

The antinociceptive effects of this plant are well known and have been confirmed by many studies. The main component of the *M. speciosa* extract, mitragynine, has consistently exhibited antinociception centrally *via* the descending noradrenergic and serotonergic systems (Matsumoto et al., 1996) by acting on opioid receptors (Thongpradichote et al., 1998). However, the selectivity of mitragynine for opioid receptor subtypes is different from that of morphine (Matsumoto et al., 1996; Thongpradichote et al., 1998). In addition, the action of mitragynine on other physiological systems such as gastric acid secretion is also mediated *via* opioid receptors (Tsuchiya et al., 2002). These consistent data confirmed morphine-like actions of the plant and seemed to support an original idea that this plant could replace morphine in treatment programs (Jansen and Prast, 1988). Mitragynine was demonstrated to inhibit morphine withdrawal successfully *in vitro* (Watanabe et al., 1997). In particular, mitragynine was found to attenuate withdrawal syndrome in morphine-withdrawn zebrafish (Khor et al., 2011). These findings appeared to suggest that among many *M. speciosa* alkaloid components, mitragynine has gained the most attention as a potential morphine substitute. However, it remained to be examined whether mitragynine or crude *M. speciosa* alkaloid extract would show therapeutic effect for opiate withdrawal treatment.

Mitragynine is a major indole alkaloid of *M. speciosa* shown to have an antinociceptive action. However, 7-hydroxymitragynine, a minor constituent of this plant, also exhibits an even higher potency than mitragynine both *in vitro* (Takayama et al., 2002) and *in vivo* (Matsumoto et al., 2004a). Moreover, other alkaloid constituents of the plant may also exert potent therapeutic effects. Previously, *M. speciosa* alkaloid extract was found to act in the central nervous system (CNS) and produce antidepressant-like action *in vivo* models (Kumarnsit et al., 2007). Therefore, mode of CNS action of *M. speciosa* alkaloid extract has been tested extensively. Ultimately, its actions in addition to opioidergic mechanisms were also evidenced. The *M. speciosa* alkaloid extract was found to produce electroencephalographic patterns in the frontal and parietal cortices similarly to that of fluoxetine, a standard antidepressant drug (Cheaha et al., 2015b). These findings suggest other mode of *M. speciosa* action and possibility to apply this extract as an antidepressant-like compound.

From some qualitative studies in human, kratom is considered addictive with its patterns of use and symptoms (Saingam et al., 2013; Ahmad and Aziz, 2012). This was found as a major concern. However, classical drugs of addiction are known to act on the mesolimbic dopamine pathway as a reward system (Seiden et al., 1993; Koob, 1992). It remained to be examined whether the *M. speciosa* extract also has actions on the brain reward areas.

Opiate substances are known to produce dependence and the abrupt cessation causes withdrawal syndrome (Gold et al., 1978). Acute morphine withdrawal can also be induced in animal models. It emerges in morphine-dependent animals immediately after the administration of naloxone, a nonspecific opiate antagonist. Opioid receptor agonists including methadone have been used to relieve withdrawal symptoms (McMillan et al., 1976) but methadone also has side effects and can cause withdrawal by itself (Beswick et al., 2003; Gossop et al., 1989, 1987). Later, levo-alpha-acetylmethadol (LAAM), a synthetic μ opioid receptor agonist, was also found to have some potential advantages with better preference (Trueblood et al., 1978). However, use of LAAM was believed to be linked with arrhythmia (Clark et al., 2002). For more effective treatment of the opiate withdrawal syndrome, partial or non-opiate substances have also been sought for treatment of opiate addiction.

The *M. speciosa* alkaloid extract was hypothesized to reduce the intensity of opioid withdrawal. The effects of *M. speciosa* alkaloid extract on morphine withdrawal symptoms were tested. Jumping behavior and diarrhea during withdrawal period were measured as indicative data to represent the intensity of morphine withdrawal symptoms in mice. In this study, pure mitragynine and *M. speciosa* alkaloid extract were used for treatment of morphine withdrawal symptoms. In addition, addictive potential of *M. speciosa* alkaloid extract was examined. Following treatment with *M. speciosa* alkaloid extract, patterns of local field potential (LFP) in the nucleus accumbens, a brain reward center, and spontaneous motor activity were evaluated.

2. Materials and methods

2.1. Plant materials

Young leaves of *M. speciosa* Korth (Rubiaceae) were collected from natural sources in Songkla Province, Thailand. Plant materials were identified by Dr. Niwat Keawpradub, the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand where the herbarium voucher specimens (no. PCOG/MS001-002) have been deposited. The use of plant materials was approved by the Ministry of Agriculture of Thailand and was restricted to research purposes only.

2.2. Extraction and analysis of alkaloid extract from M. speciosa and mitragynine

An *M. speciosa* alkaloid-rich extract and mitragynine were prepared as described in previous studies (Cheaha et al., 2015a). The same batch of *M. speciosa* alkaloid-rich extract was also used for the present study within the same time period. For *M. speciosa* alkaloid-rich extract, young leaves were dried at 45–50 °C, powdered and macerated with methanol. The filtrate was evaporated *in vacuo*. The residue was dissolved in 10% aqueous acetic acid, filtrated and washed with petroleum ether, then made into alkaline (pH 9) with 25% ammonia solution and extracted with chloroform. The combined chloroform extracts that were washed, dried over anhydrous sodium sulphate and evaporated gave 0.25% of dry crude alkaloid extract. The extract was analyzed by using high performance liquid chromatography (HPLC). Mitragynine was used as a standard. HPLC analyses revealed the dominant peak of *M. speciosa* alkaloid-rich extract with the same retention time to mitragynine (Supp. Figs. 1 and 2, inset).

For mitragynine isolation, an aliquot (2.5 g) of alkaloid extract was subjected to silica gel column chromatography, eluted with 5% methanol in chloroform to obtain a major alkaloid (1.27 g), which appeared as a single spot on thin layer chromatography (TLC) analysis (four solvent systems). According to the TLC analysis, mitragynine was found to constitute about 60% of the crude alkaloid extract.

The pure mitragynine was identified by using liquid chromatographic mass spectrometry (LC-MS). A solution was run through LC-MS (Waters 2690 liquid chromatograph (Milford, MA, USA)) coupled with a mass spectrometer LCT™ (Micromass, Manchester, UK) at the Scientific Equipment Center, Prince of Songkla University. Confirmation of *M. speciosa* alkaloid extract was performed by using mitragynine as a standard. The spectrum of the *M. speciosa* alkaloid extract revealed masses m/z of 397.2, 398.2 and 399.2 (Supp. Fig. 1). The spectrum of pure mitragynine had a single mass m/z of 399.0 (Supp. Fig. 2). They were identified as mitragynine, a major component of *M. speciosa* compared with the obtained spectral data of previous published (Janchawee et al., 2007; Houghton et al., 1991; Shellard et al., 1978).

2.3. Chemicals

The following drugs were used: morphine sulphate (Zentiva, Hlohovec, Slovakia), naloxone hydrochloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The drugs were dissolved in sodium chloride solution (0.9% w/v) and given to animals in a volume of 5 ML/kg. The *M. speciosa* alkaloid extract was dissolved in co-solvent (Tween80: propyleneglycol: H_2O at a 1:4:4 ratio). A ball tipped stainless steel gavage needle was used for oral feeding.

2.4. Animals

This study was carried out in accordance with guidelines of the European Science Foundation (Use of Animals in Research 2001) and International Committee on Laboratory Animal Science, ICLAS (2004). The experimental protocols described in the present study were approved and guided by the Animals Ethical Committee of the Prince of Songkla University for care and use of experimental animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Swiss albino ICR mice (7–8 weeks old) used in each experiment were bred at the animal house of the Prince of Songkla University. They were housed in a group of 10 mice per cage (20 × 25 × 35 cm) and maintained under 12/12 dark/light cycle (lights on at 0600 a.m.) and controlled temperature (22 °C). Standard commercial food pellets and filtered water were available *ad libitum*. All the tests were performed between 8.00 a.m. and 4.00 p.m.

There were 2 different sets of animals for 2 main experiments. The first set was rendered dependent on morphine for testing the effects of *M. speciosa* alkaloid extract on morphine withdrawal symptoms (n = 10–12). Animals in the second set were anesthetized for electrode implantation and used for electrical brain wave study (n = 6–8).

2.5. Development of morphine dependence

Mice were rendered dependent on morphine using the method previously described (Marshall and Grahame-Smith, 1971). Briefly, morphine sulphate was injected (s.c.) 3 times daily at 08:00, 12:00 and 16:00 (50, 50 and 75 mg/kg, respectively) for 3 days. On day 4, only a single morning dose of morphine (50 mg/kg) was injected before naloxone injection.

2.6. Observation of morphine withdrawal symptoms

Withdrawal signs were precipitated by injection of naloxone (1.5 mg/kg, i.p.) 2 h after the final injection of morphine. Immediately after naloxone injection, animals were placed individually on filter paper in an observable cylindrical plastic container (15 cm in diameter and 50 cm in height). The behavior of animals was recorded by using a digital video camera. Fecal materials excreted during a 30-min period of withdrawal were measured. Jumping behavior was used as an experimental index of the central morphine withdrawal syndrome whereas fecal excretion reflected a peripheral withdrawal syndrome (Broseta et al., 2002).

2.7. M. speciosa alkaloid extract treatments

One hour before the induction of withdrawal with naloxone, mice were given a single oral administration of either sodium chloride solution (0.9% w/v), mitragynine (30, 90 and 120 mg/kg) or the *M. speciosa* alkaloid extract (20, 40, 60, 80 and 100 mg/kg). One effective dose of *M. speciosa* alkaloid extract (80 mg/kg) was selected for a study of LFP and spontaneous motor activity.

2.8. Surgery for LFP electrode implantation

Method of electrode implantation was also previously described (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Briefly, animals were anesthetized by injection intramuscularly of a mixture of 150 mg/kg ketamine (Calypsol, Gedeon Richter Ltd., Budapest, Hungary) and 15 mg/kg xylazine (Xylavet, Thai Maji Phamaceutical co., Ltd., Bangkok, Thailand). Then, the animal's head was mounted in a stereotaxic frame. Local analgesic, lidocaine (Locana, L.B.S. Laboratory Ltd., Part., Bangkok, Thailand) was applied to the exposed tissue of the head. An incision was made at the midline to expose the skull. The silver wire electrodes (A-M system, Sequim, WA, USA) with bare diameter of 0.008 in. (Coated-0.01 in.) were stereotaxically positioned on the left nucleus accumbens (NAc) (AP: + 0.7 mm, ML: 1.3 mm, DV: 4.7 mm) according to mouse brain atlas (Franklin and Paxinos, 1998). The reference and ground electrodes were placed at midline overlying the cerebellum. Additional holes were drilled for stainless steel anchor screws. Dental acrylic (Unifast Trad, GC Dental Industrial Corp., Tokyo, Japan) was used to secure all electrodes on the skull. Following the surgery, animals were orally given an analgesic drug, 5 mg/kg meloxicam (Hofbic, Osoth Inter Laboratories Co., Ltd., Chonburi, Thailand). The antibiotic ampicillin (General Drug House Co., Ltd., Bangkok, Thailand) was applied intramuscularly (100 mg/kg) once a day for 3 days to prevent infection and allowed them to recover for at least 7–10 days

2.9. LFP signals acquisition and analysis

Methods in details of signal recoding and analysis were described in the previous study (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Briefly, all signals from animal electrodes were amplified with a low-pass 1 kHz, high-pass 0.3 Hz and digitized at 2 kHz by a PowerLab 16/35 system (AD Instruments, Castle Hill. NSW, Australia) with 16-bit A/D, and stored in a PC through the LabChart 7 pro software. For offline analysis, recorded files were overviewed by using visual inspection and only noise-free signals were used for the analysis. Fifty Hz notch filtering was applied to remove the noise from power line artifacts. To avoid 50 Hz noise, the signals from 45 to 60 Hz were excluded from the further analysis. The LFP signals were processed through 1–100 Hz band-pass digital filter.

For the frequency analysis, the digitized data were assessed by analyzing the power spectral density (PSD) generated by LabChart software using the Fast Fourier Transform (FFT) algorithm. The setup conditions for FFT calculation are Hanning window cosine with 50% window overlap, and frequency resolution of 0.976 Hz. Then, the PSD in each frequency bin was expressed as percentage of total power. The power was averaged across 6 discrete frequency bands: theta, 4–8 Hz; alpha, 9.7–12 Hz; beta1, 13.6–18.0 Hz; beta2, 19.5–29.3 Hz; gamma1 or low gamma, 30.3–44.9 Hz; gamma2 or high gamma 60.5–95.7 Hz.

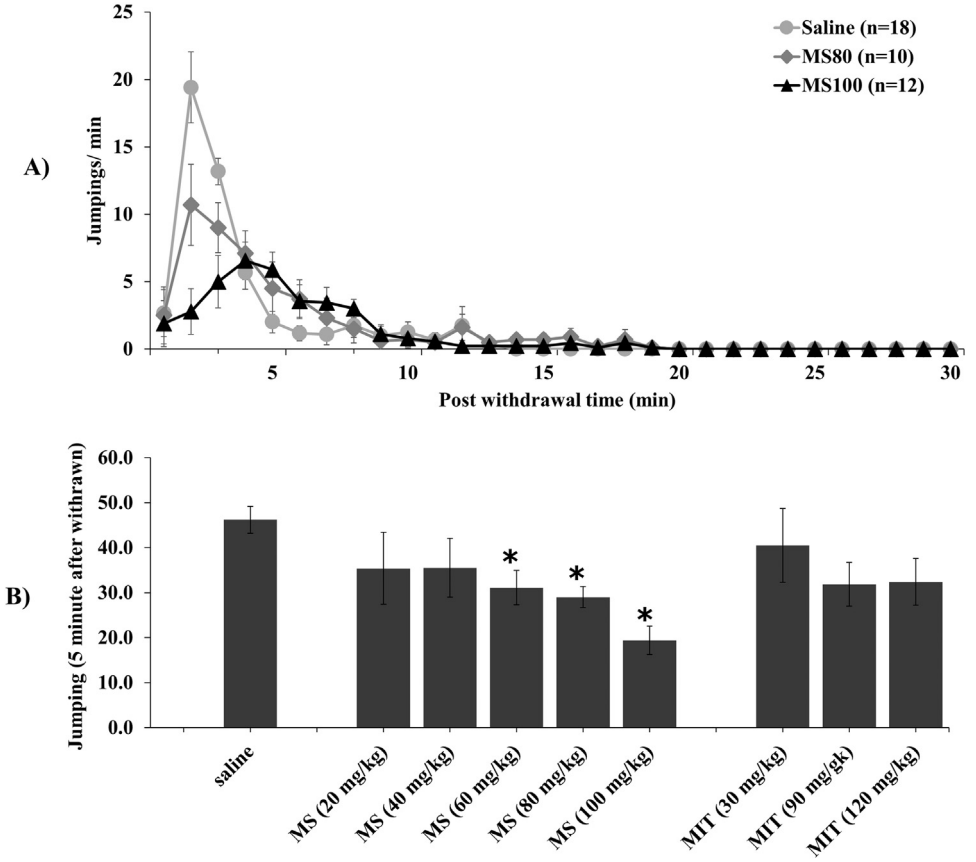

Fig. 1. Effects of *M. speciosa* alkaloid extract (MS) and mitragynine (MIT) on jumping behavior induced by naloxone-precipitated morphine withdrawal (n = 10–12). Jumping numbers of animals in control and *M. speciosa* alkaloid group (80 and 100 mg/kg) were counted minute-by-minute (A). Effects of *M. speciosa* alkaloid extracts and pure mitragynine on jumping behavior during the first 5-min period following morphine withdrawal are shown in comparison to control level (B). Representative data are expressed as means ± S.E.M. *$P < 0.05$ for comparisons with control levels.

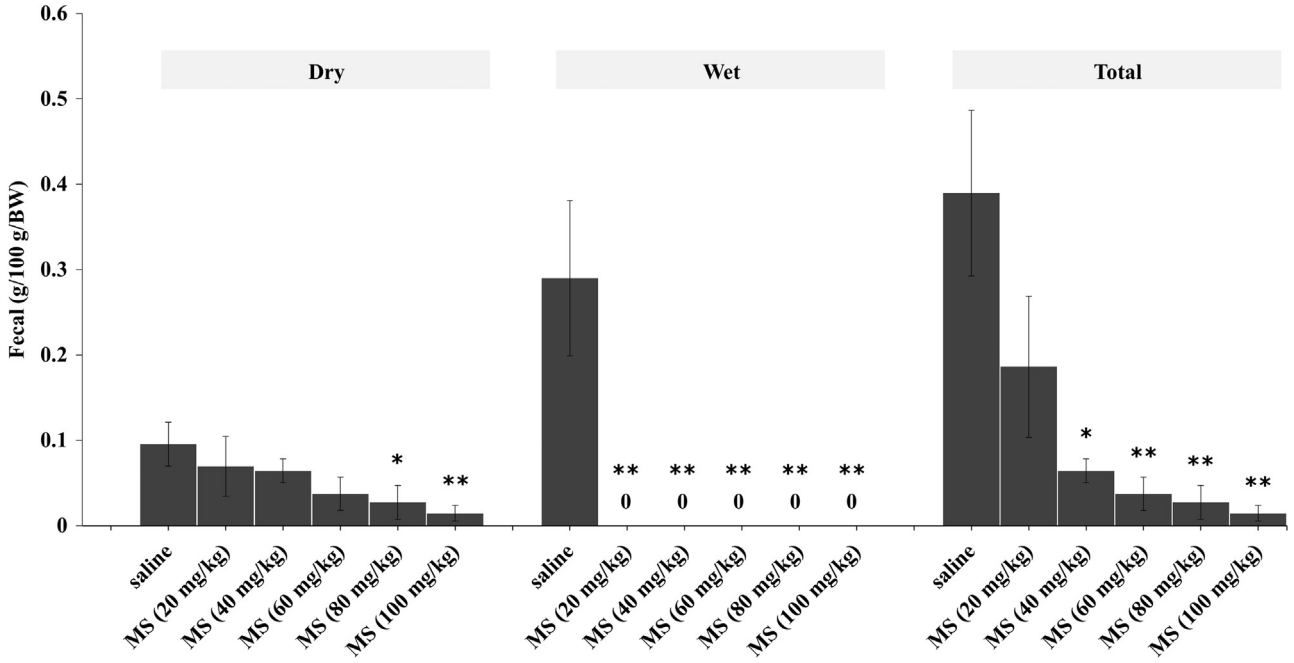

Fig. 2. Effects of *M. speciosa* alkaloid extracts (MS) on dry and wet fecal excretions induced by naloxone-precipitated morphine withdrawal (n = 10–12). Weights of fecal materials excreted during a 30-min period of morphine withdrawal were measured. Data are expressed as means ± S.E.M. *, **, ***$P < 0.05$, 0.01, 0.001, respectively, for comparisons with control levels.

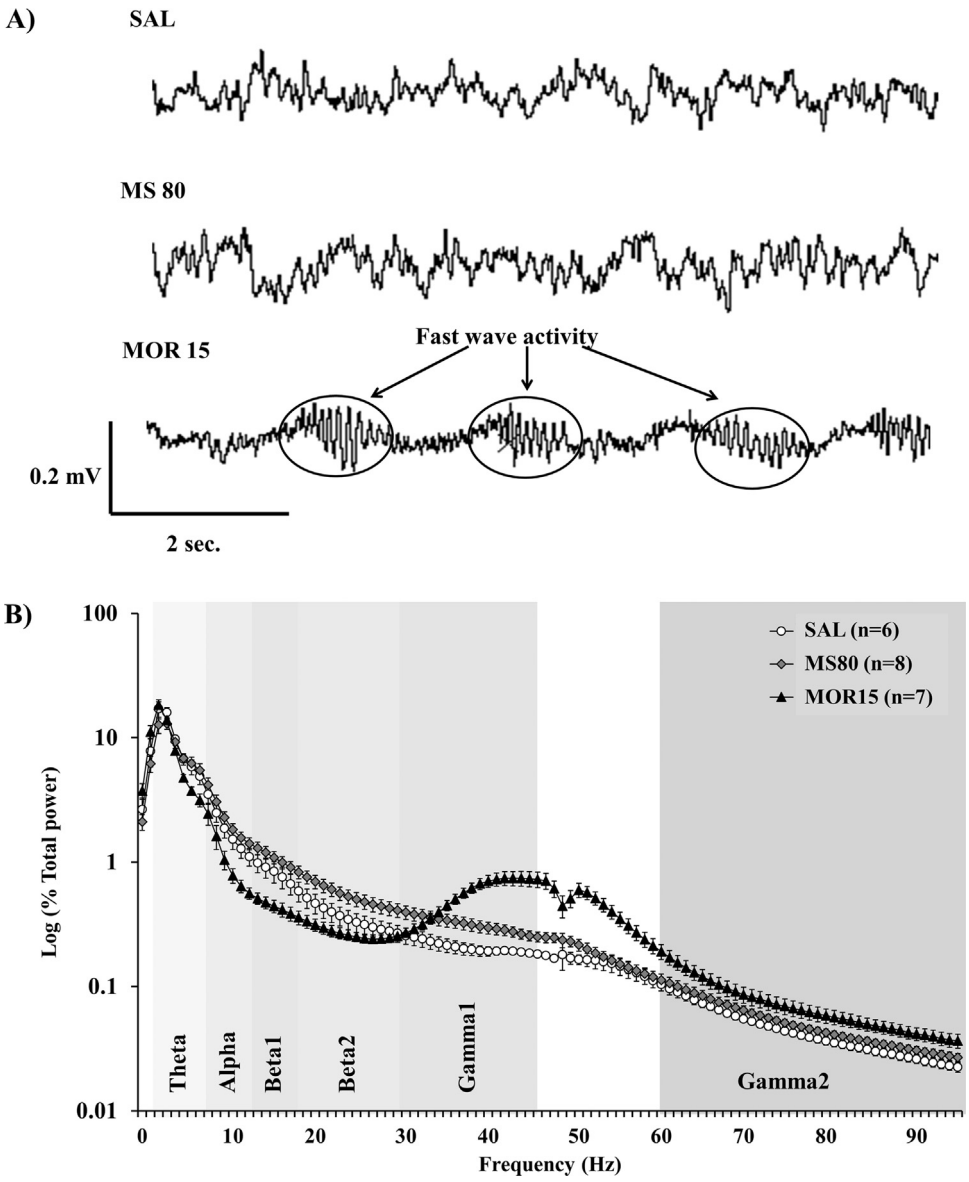

Fig. 3. Patterns of local field potentials following administrations with *M. speciosa* alkaloid extract and morphine (n = 6–8). Time domain of raw LFP signals recorded from the nucleus accumbens of representative mice that received saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) are displayed (A). Power spectrums of LFP are expressed in frequency domain (B).

Spectral powers in discrete frequency bands of each group were averaged and expressed either in time or frequency domains.

2.10. Recording and analysis of locomotor activity

Animal behaviors and locomotor activity were recorded using a webcam vertically mounted on the top of recording chamber. Animal image was continuously captured and transferred to the computer. Animal movement was analyzed by using visual C^{++} -based software specifically developed for locomotor activity research (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Basically, translocation of animal was detected with a sensitivity at 2-mm threshold. One count was considered from 1 period of continuous translocation and 1 stop. Levels of locomotor activity were expressed as mean ± S.E.M. of locomotor count per time period.

2.11. Statistical analysis

Experimental data were expressed as mean values ± S.E.M. of the numbers of jumps and weights of fecal materials and urine during a withdrawal period. Differences were determined using one-way analysis of variance (ANOVA), followed by multiple comparisons using Turkey's Method. Differences with $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Effects of M. speciosa alkaloid extract on jumping behavior induced by naloxone-precipitated morphine withdrawal

Naloxone-induced jumping behavior in morphine-dependent mice was rapidly observed following naloxone injection. Mostly, the numbers of jumping increased from the first minute and peaked in the second minute (Fig. 1A). Jumping numbers gradually decreased to almost zero within the 5th minute and disappeared thereafter. Therefore, jumping behavior during a 5-min period was counted and analyzed to represent the intensity of morphine withdrawal for all groups (Fig. 1B). Jumping number of the control animals (46.2 ± 3.0) was used for comparison with data of other groups treated with either *M. speciosa* alkaloid extract or pure mitragynine. One-way ANOVA revealed suppressive effects seen in treated groups that received *M.*

speciosa alkaloid extract. Multiple comparisons confirmed that jumping number induced by naloxone-precipitated morphine withdrawal was significantly decreased by *M. speciosa* alkaloid extract at 60, 80 and 100 mg/kg in a dose-dependent manner. However, no significant difference was seen in treated groups that received pure mitragynine (30, 90 and 120 mg/kg).

3.2. Effects of M. speciosa alkaloid extract on diarrhea induced naloxone-precipitated morphine withdrawal

Defecation was evaluated from dry and wet fecal materials. Data were expressed as weight per 100 g body weight (Fig. 2). One-way ANOVA showed that treatment with different doses of the *M. speciosa* alkaloid extract significantly reduced dry fecal weight compared to the saline control ($p = 0.001$). The effect of the *M. speciosa* alkaloid extract was more obvious in the analysis of the wet feces. The saline control showed 0.29 ± 0.09 g per 100 g body weight (BW) of wet fecal materials compared to zero in all treated groups. The analysis of total fecal materials also confirmed significant effect of *M. speciosa* alkaloid extract on defecation during morphine withdrawal period ($p = 0.001$).

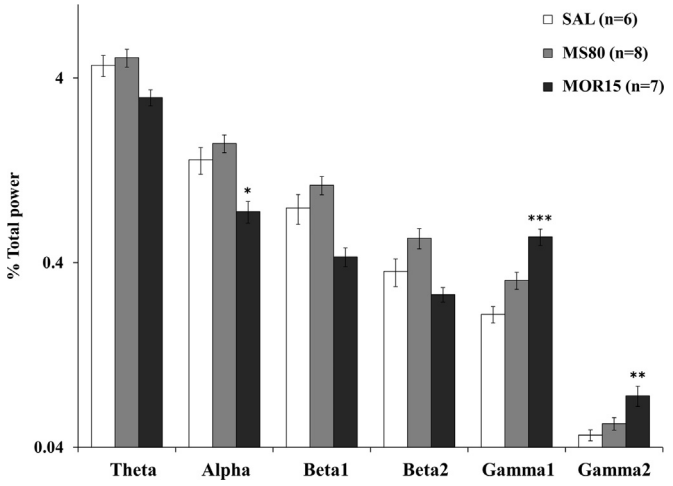

Fig. 4. Effects of administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) on powers of 6 discrete frequency ranges (n = 6–8). Averaged percent total powers of different frequency ranges are expressed as means ± S.E.M. *, **, *** $P < 0.05$, 0.01, 0.001, respectively, compared with control levels (one-way ANOVA followed by Tukey's post hoc test).

3.3. Effects of M. speciosa alkaloid extract and morphine treatment on local field potential in the nucleus accumbens

LFP signals from individual mice were continuously recorded for 3 h following treatment with either vehicle, *M. speciosa* extract (80 mg/kg BW, p.o.) or morphine (15 mg/kg BW, i.p.). Representative raw LFP tracings are shown in time domain (Fig. 3A). By visual inspection, relatively similar patterns were seen between LFPs of control and *M. speciosa* alkaloid extract but not morphine group. The latter group appeared to have additional oscillations of fast wave superimposed in slow waves of raw LFP signals. Therefore, all signals were converted into frequency domain for LFP powers analysis (Fig. 3B). Data were illustrated in LFP power spectra (% total power) for 1–100 Hz frequency range. Obviously, morphine appeared to produce different pattern of LFP power spectrum from that of vehicle group. Morphine seemed to reduce slow frequency powers particularly below 30 Hz and increase powers of frequencies above 30 Hz. However, *M. speciosa* alkaloid extract did not change LFP powers throughout the frequency range analyzed. The LFP spectrum produced by *M. speciosa* extract was relatively similar to that of vehicle group. Finally, broad power spectra were divided and assigned into 6 discrete frequency ranges for statistical analysis. One-way ANOVA revealed significant changes in theta, alpha, beta1, beta2, low gamma and high gamma (Fig. 4). Multiple comparisons also indicated significant decreases in alpha and increases in low gamma and high gamma powers produced by morphine treatment. No significant difference between powers of vehicle and *M. speciosa* alkaloid extract groups was found.

Therefore, time-course effect of treatments on low gamma power was particularly analyzed (Fig. 5). Following morphine treatment, low gamma power was rapidly increased and peaked approximately within 40 min. Therefore, the effect of morphine gradually decreased to

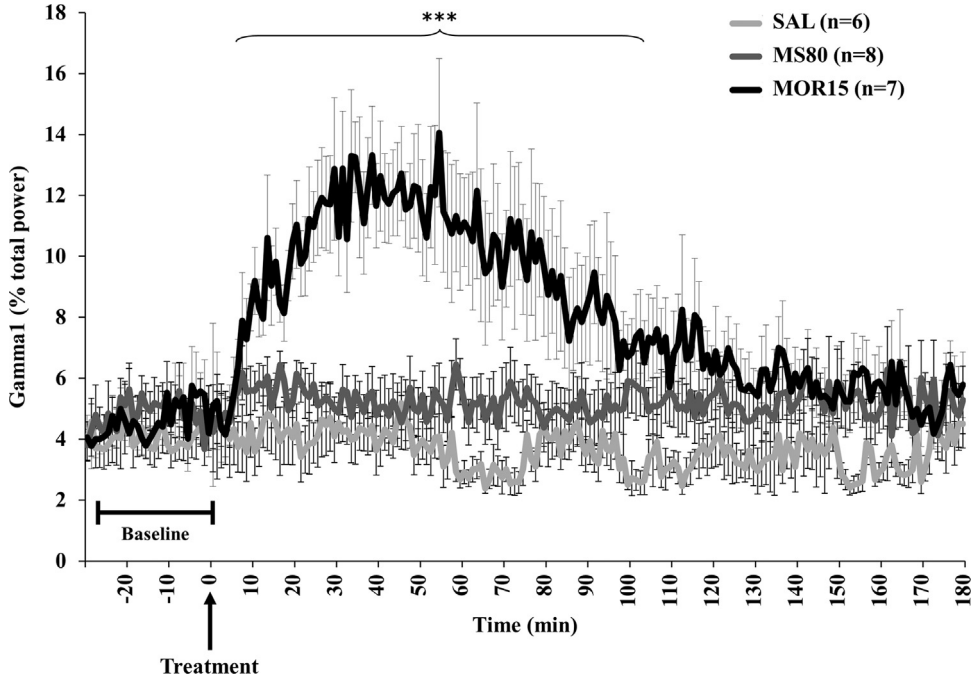

Fig. 5. Average percent total power of gamma1 range were analyzed every 5 min following administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) (n = 6–8). Data were compared with control levels using one-way ANOVA followed by Tukey's post hoc test. *, **: $P < 0.05$, 0.01, respectively.

control level within 3 h. Statistical analysis also confirmed significant effect of morphine treatment on low gamma power. On the other hand, no significant change was produced by *M. speciosa* alkaloid extract administration.

3.4. Effects of M. speciosa alkaloid extract on spontaneous motor activity

Levels of spontaneous motor activity were counted every 5 min (Fig. 6). Locomotor count was progressively increased following morphine treatment. The levels of locomotor activity peaked within 60 min and decreased to baseline level within 2.5 h. No significant effect of *M. speciosa* alkaloid extract on spontaneous motor activity was seen.

4. Discussion

The present study demonstrated that the alkaloid extract from *Mitragyna speciosa* (*M. speciosa*) significantly attenuated the intensity of morphine withdrawal symptoms. Particularly, *M. speciosa* alkaloid extract clearly reduced jumping behavior induced by morphine withdrawal. Jumping is a sign frequently used to evaluate morphine dependence in a mice model (Ballard and McAllister, 1999; Reddy and Kulkarni, 1997). It is also one of central withdrawal symptoms that may reflect craving. On the other hand, mitragynine did not reduce number of jumping. These data indicate that some minor ingredients of this plant may have more promising properties especially for this purpose.

Among various constituents of *M. speciosa*, 7-hydroxymitragynine, one of minor alkaloids of this plant, exhibited a more potent antinociceptive effect than even morphine (Matsumoto et al., 2004b). This may explain why *M. speciosa* alkaloid extract produced effective properties whereas mitragynine did not. Moreover, apart from mitragynine and 7-hydroxymitragynine, there might be some other biologically active minor constituents in this plant that act on transmitter systems and result in attenuation of morphine withdrawal symptoms. Thus, the combination of many constituents included in *M. speciosa* alkaloid extract is likely to produce stronger therapeutic effects.

In traditional use, chewing kratom leave and swallowing its crude extract are also known for total alkaloid constituents. In general, biological activities of kratom leaves rely mostly on the alkaloid constituents. The extraction in this study was performed to obtain crude alkaloid extract containing total alkaloid constituents. In terms of confirmation, the crude alkaloid *M. speciosa* extract was analyzed with HPLC and LC-MS to determine the major constituents (Supp. Figs. 1 and 2). These reports suggest that treatment with the crude *M. speciosa* extract can supply relatively more or equal alkaloids to that of chewing process.

Another highlight of the present study was the effect of *M. speciosa* alkaloid extract on diarrhea during morphine withdrawal period. Diarrhea represents peripheral symptom that reflects the level of intestinal motility during a morphine withdrawal period. The suppressive effect of the *M. speciosa* alkaloid extract on morphine withdrawal-induced diarrhea was correlated with previous report of the *in vitro* study using smooth muscle (Watanabe et al., 1997). It has long been known that people use opium to stop diarrhea. Consistently, both synthetic agents and natural product extracts that act as opioid receptor agonists also have an antidiarrheal action (Broccardo and Improta, 1992; Shook et al., 1989). Taken together, these data highlighted that *M. speciosa* alkaloid extract completely suppressed diarrhea and attenuated behavioral hyperactivity that might represent the intensity of craving during a period of withdrawal. In terms of mechanism, serotonergic neurotransmitter system has been particularly involved in attenuation of morphine withdrawal severity. Pre-injection with antidepressant drugs such as fluoxetine, clomipramine, or citalopram, significantly attenuated the naloxone precipitated syndrome (Wu et al., 2005). Additionally, fluvoxamine and sertraline, selective serotonin reuptake inhibitors, also reduced the severity of the naloxone precipitated opioid withdrawal syndrome (Gray, 2002). These findings suggest that the *M. speciosa* alkaloid extract might attenuate morphine withdrawal-induced jumping behavior partly through its antidepressant-like activity.

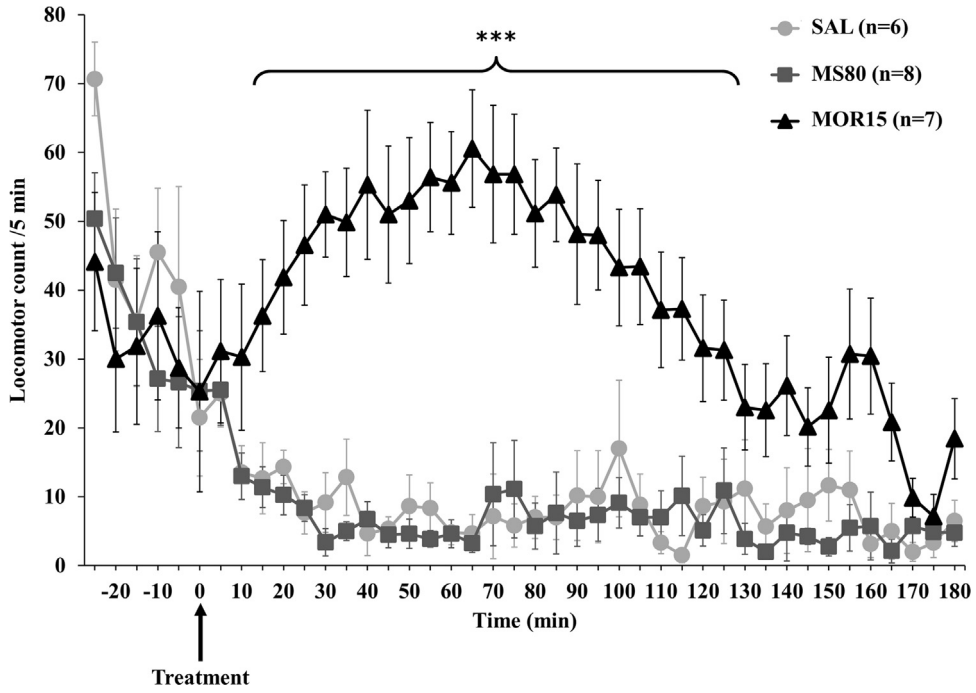

Fig. 6. Effects of administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) on spontaneous motor activity (n = 6–8). Locomotor counts in each group were averaged 5 min during baseline period and following the administration for 3 h. Data are expressed as means ± S.E.M. *** $P < 0.001$ for comparisons with control levels (one-way ANOVA followed by Tukey's post hoc test).

Activation of dopaminergic brain pathways and spontaneous motor activity by drugs of addiction are well-known. Particularly, activation of the nucleus accumbens and striatum by psychostimulants and opiates is believed to play a major role in the establishment of drug dependence and withdrawal phenomena (Hyman, 1996; Koob, 1996; Nestler, 1996). Similar findings were seen following injections of cocaine and methamphetamine (Wang and McGinty, 1995; Young et al., 1991). In term of behavioral output, psychostimulants also enhance locomotor activity (Vezina, 2004). These are among the most common effects produced by addictive substances. However, *M. speciosa* alkaloid extract did not activate the nucleus accumbens and spontaneous motor activity at all. The present data may explain why kratom use for emotional purpose has not been reported. Therefore, the impact of situations and context like social, ritualistic or self-medicating factors may be discussed why kratom consumption is still widespread in some countries.

Until now, methadone (Wang et al., 2015) and levo-α-acetylmethadol (LAAM) (Judson and Goldstein, 1979) are routinely used in pharmacological intervention for opiate dependence. Continuous use of these agents gives benefits in suppressing opioid withdrawal symptoms for long durations. However, some patients also reported experiencing withdrawal symptoms during treatment (Dyer and White, 1997). Increasing the dose of methadone has benefits in suppressing withdrawal symptoms but also leads to some risks both physically and psychologically (Kreek, 1973). Rapid transition to dependence were associated with anxiety disorders in methadone maintained patients (Karsinti et al., 2016). In particular, methadone maintained patients were found to have difficulty in learning the outcomes of positive stimulus in drug-related context (Levy-Gigi et al., 2014). This finding was believed to explain why many opiate users relapse back to using opiates over and over again throughout their lives.

Mechanisms of opioid withdrawal were found to involve various neurotransmitter systems including opioid and non-opioid systems (Freye and Latasch, 2003; Gulati et al., 2004; Puppala et al., 2006; Williams et al., 2001). These might explain why opioid alone is insufficient for morphine withdrawal treatment. Additional treatments with agonists of other neurotransmitter systems are highly recommended. The crude alkaloid extract is likely to be suitable for opioid withdrawal treatment having more constituents to produce broader spectrum of CNS actions. Therefore, alternative treatments are needed to substitute methadone or to be used in combination with a small dose of methadone to enhance treatment efficacy. According to our findings, the *M. speciosa* alkaloid extract might be used as an alternative treatment in combination with other effective opioid agonists. CNS action of *M. speciosa* alkaloid extract is unlikely to mimic that of opiates according to LFP patterns and levels of spontaneous motor activity.

Conflict of interest

All authors declared that they have no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2017.07.008.

References

Ahmad, K., Aziz, Z., 2012. *Mitragyna speciosa* use in the northern states of Malaysia: a cross-sectional study. J. Ethnopharmacol. 141, 446–450.
Ballard, T.M., McAllister, K.H., 1999. Acutely administered clozapine does not modify naloxone-induced withdrawal jumping in morphine-dependent mice. Pharmacol. Biochem. Behav. 62, 285–290.
Beswick, T., Best, D., Rees, S., Bearn, J., Gossop, M., Strang, J., 2003. Major disruptions of sleep during treatment of the opiate withdrawal syndrome: differences between methadone and lofexidine detoxification treatments. Addict. Biol. 8, 49–57.
Broccardo, M., Improta, G., 1992. Antidiarrheal and colonic antipropulsive effects of spinal and supraspinal administration of the natural delta opioid receptor agonist, [D-Ala2]deltorphin II, in the rat. Eur. J. Pharmacol. 218, 69–73.
Broseta, I., Rodriguez-Arias, M., Stinus, L., Minarro, J., 2002. Ethological analysis of morphine withdrawal with different dependence programs in male mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 26, 335–347.
Burkill, A., 1935. A dictionary of the economic products of the Malay peninsula. 2, pp. 1480–1483.
Cheaha, D., Bumrungsri, S., Chatpun, S., Kumarnsit, E., 2015a. Characterization of in utero valproic acid mouse model of autism by local field potential in the hippocampus and the olfactory bulb. Neurosci. Res. 98, 28–34.
Cheaha, D., Keawpradub, N., Sawangjaroen, K., Phukpattaranont, P., Kumarnsit, E., 2015b. Effects of an alkaloid-rich extract from *Mitragyna speciosa* leaves and fluoxetine on sleep profiles, EEG spectral frequency and ethanol withdrawal symptoms in rats. Phytomedicine 22, 1000–1008.
Clark, N., Lintzeris, N., Gijsbers, A., Whelan, G., Dunlop, A., Ritter, A., Ling, W., 2002. LAAM maintenance vs methadone maintenance for heroin dependence. Cochrane Database Syst. Rev., CD002210.
Dyer, K.R., White, J.M., 1997. Patterns of symptom complaints in methadone maintenance patients. Addiction 92, 1445–1455.
Franklin, K.B.J., Paxinos, G., 1998. The mouse brain in stereotaxic coordinates.
Freye, E., Latasch, L., 2003. Development of opioid tolerance-molecular mechanisms and clinical consequences. Anasthesiol. Intensivmed. Notfallmed. Schmerzther. 38, 14–26.
Gold, M.S., Redmond, D.E., Jr., Kleber, H.D., 1978. Clonidine blocks acute opiate-withdrawal symptoms. Lancet 2, 599–602.
Gossop, M., Bradley, B., Phillips, G.T., 1987. An investigation of withdrawal symptoms shown by opiate addicts during and subsequent to a 21-day in-patient methadone detoxification procedure. Addict. Behav. 12, 1–6.
Gossop, M., Griffiths, P., Bradley, B., Strang, J., 1989. Opiate withdrawal symptoms in response to 10-day and 21-day methadone withdrawal programmes. Br. J. Psychiatry 154, 360–363.
Gray, A.M., 2002. The effect of fluvoxamine and sertraline onthe opioid withdrawal syndrome:: A combined in vivo cerebral microdialysis and behavioural study. Eur. Neuropsychopharmacol. 12, 245–254.
Gulati, A., Bhalla, S., Matwyshyn, G., 2004. A novel combination of opiates and endothelin antagonists to manage pain without any tolerance development. J. Cardiovasc. Pharmacol. 44 (Suppl. 1), S129–S131.
Houghton, P.J., Latiff, A., Said, I.M., 1991. Alkaloids from Mitragyna speciosa. Phytochemistry 30, 347–350.
Hyman, S.E., 1996. Addiction to cocaine and amphetamine. Neuron 16, 901–904.
Janchawee, B., Keawpradub, N., Chittrakarn, S., Prasettho, S., Wararatananurak, P., Sawangjareon, K., 2007. A high-performance liquid chromatographic method for determination of mitragynine in serum and its application to a pharmacokinetic study in rats. Biomed. Chromatogr. 21, 176–183.
Jansen, K.L., Prast, C.J., 1988. Psychoactive properties of mitragynine (kratom). J. Psychoact. Drugs 20, 455–457.
Judson, B.A., Goldstein, A., 1979. Levo-alpha-acetylmethadol (LAAM) in the treatment of heroin addicts. I. Dosage schedule for induction and stabilization. Drug Alcohol Depend. 4, 461–466.
Karsinti, E., Fortias, M., Dupuy, Gl, Ksouda, K., Laqueille, X., Simonpoli, A.M., Touzeau, D., Avril, E., Orizet, C., Belforte, B., Coeuru, P., Polomeni, P., Icick, R., Jarroir, M., Bloch, V., Scott, J., Lépine, J.P., Bellivier, F., Vorspan, F., 2016. Anxiety disorders are associated with early onset of heroin use and rapid transition to dependence in methadone maintained patients. Psychiatry Res. 245, 423–426.
Khor, B.-S., Amar Jamil, M.F., Adenan, M.I., Chong Shu-Chien, A., 2011. Mitragynine attenuates withdrawal syndrome in morphine-withdrawn zebrafish. PLoS ONE 6 (12), e28340.
Koob, G.F., 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol. Sci. 13, 177–184.
Koob, G.F., 1996. Drug Addiction: the Yin and Yang of Hedonic Homeostasis. Neuron 16, 893–896.
Kreek, M.J., 1973. Medical safety and side effects of methadone in tolerant individuals. JAMA 223, 665–668.
Kumarnsit, E., Vongvatcharanon, U., Keawpradub, N., Intasaro, P., 2007. Fos-like immunoreactivity in rat dorsal raphe nuclei induced by alkaloid extract of *Mitragyna speciosa*. Neurosci. Lett. 416, 128–132.

Levy-Gigi, E., K+¬ri, S., Shapiro, A.R., Sason, A., Adelson, M., Peles, E., 2014. Methadone maintenance patients show a selective deficit to reverse positive outcomes in drug-related conditions compared to medication free prolonged opiate abstinence. Drug Alcohol Depend. 144, 111–118.
Marshall, I., Grahame-Smith, D.G., 1971. Evidence against a role of brain 5-hydroxytryptamine in the development of physical dependence upon morphine in mice. J. Pharmacol. Exp. Ther. 179, 634–641.
Matsumoto, K., Horie, S., Ishikawa, H., Takayama, H., Aimi, N., Ponglux, D., Watanabe, K., 2004a. Antinociceptive effect of 7-hydroxymitragynine in mice: discovery of an orally active opioid analgesic from the Thai medicinal herb *Mitragyna speciosa*. Life Sci. 74, 2143–2155.
Matsumoto, K., Horie, S., Ishikawa, H., Takayama, H., Aimi, N., Ponglux, D., Watanabe, K., 2004b. Antinociceptive effect of 7-hydroxymitragynine in mice: discovery of an orally active opioid analgesic from the Thai medicinal herb *Mitragyna speciosa*. Life Sci. 74, 2143–2155.
Matsumoto, K., Mizowaki, M., Suchitra, T., Murakami, Y., Takayama, H., Sakai, S., Aimi, N., Watanabe, H., 1996. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic systems. Eur. J. Pharmacol. 317, 75–81.
McMillan, D.E., Leander, J.D., Wilson, T.W., Wallace, S.C., Fix, T., Redding, S., Turk, R.T., 1976. Oral ingestion of narcotic analgesics by rats. J. Pharmacol. Exp. Ther. 196, 269–279.
Nestler, E.J., 1996. Under Siege: the brain on opiates. Neuron 16, 897–900.
Puppala, B.L., Bhalla, S., Matwyshyn, G., Gulati, A., 2006. Involvement of central endothelin receptors in neonatal morphine withdrawal. Exp. Biol. Med. 231, 1157–1160.
Reakkamnuan, C., Hiranyachattada, S., Kumarnsit, E., 2015. Low gamma wave oscillation in the striatum of mice following morphine administration. J. Physiol. Sci. 65 (Suppl), 11–16.
Reddy, D.S., Kulkarni, S.K., 1997. Chronic neurosteroid treatment prevents the development of morphine tolerance and attenuates abstinence behavior in mice. Eur. J. Pharmacol. 337, 19–25.
Saingam, D., Assanangkornchai, S., Geater, A.F., Balthip, Q., 2013. Pattern and consequences of krathom (*Mitragyna speciosa* Korth.) use among male villagers in southern Thailand: a qualitative study. Int. J. Drug Policy 24, 351–358.
Seiden, L.S., Sabol, K.E., Ricaurte, G.A., 1993. Amphetamine: effects on catecholamine Systems and Behavior. Annu. Rev. Pharmacol. Toxicol. 33, 639–676.
Shellard, E.J., Houghton, P.J., Resha, M., 1978. The Mitragyna species of Asia Part XXXI. The alkaloids of Mitragyna speciosa Korth from Thailand. Planta Med. 34, 26–36.
Shook, J.E., Lemcke, P.K., Gehrig, C.A., Hruby, V.J., Burks, T.F., 1989. Antidiarrheal properties of supraspinal mu and delta and peripheral mu, delta and kappa opioid receptors: inhibition of diarrhea without constipation. J. Pharmacol. Exp. Ther. 249, 83–90.
Suwanlert, S., 1975. A study of kratom eaters in Thailand. Bull. Narc. 27, 21–27.
Takayama, H., Ishikawa, H., Kurihara, M., Kitajima, M., Aimi, N., Ponglux, D., Koyama, F., Matsumoto, K., Moriyama, T., Yamamoto, L.T., Watanabe, K., Murayama, T., Horie, S., 2002. Studies on the synthesis and opioid agonistic activities of mitragynine-related indole alkaloids: discovery of opioid agonists structurally different from other opioid ligands. J. Med. Chem. 45, 1949–1956.
Thongpradichote, S., Matsumoto, K., Tohda, M., Takayama, H., Aimi, N., Sakai, S., Watanabe, H., 1998. Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. Life Sci. 62, 1371–1378.
Trueblood, B., Judson, B.A., Goldstein, A., 1978. Acceptability of methadyl acetate (LAAM) as compared with methadone in a treatment program for heroin addicts. Drug Alcohol Depend. 3, 125–132.
Tsuchiya, S., Miyashita, S., Yamamoto, M., Horie, S., Sakai, S., Aimi, N., Takayama, H., Watanabe, K., 2002. Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. Eur. J. Pharmacol. 443, 185–188.
Vezina, P., 2004. Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. Neurosci. Biobehav. Rev. 27, 827–839.
Wang, G.Y., Kydd, R., Wouldes, T.A., Jensen, M., Russell, B.R., 2015. Changes in resting EEG following methadone treatment in opiate addicts. Clin. Neurophysiol. 126, 943–950.
Wang, J.Q., McGinty, J.F., 1995. Differential effects of D1 and D2 dopamine receptor antagonists on acute amphetamine- or methamphetamine-induced up-regulation of zif/268 mRNA expression in rat forebrain. J. Neurochem. 65, 2706–2715.
Watanabe, K., Yano, S., Horie, S., Yamamoto, L.T., 1997. Inhibitory effect of mitragynine, an alkaloid with analgesic effect from Thai medicinal plant *Mitragyna speciosa*, on electrically stimulated contraction of isolated guinea-pig ileum through the opioid receptor. Life Sci. 60, 933–942.
Williams, J., Christie, M., Manzoni, O., 2001. Cellular and synaptic adaptations mediating opioid dependence. Physiol. Rev. 81, 299–343.
Wu, C.C., Chen, J.Y.-R., Tao, P.L., Chen, Y.A., Yeh, G.C., 2005. Serotonin reuptake inhibitors attenuate morphine withdrawal syndrome in neonatal rats passively exposed to morphine. Eur. J. Pharmacol. 512, 37–42.
Young, S.T., Porrino, L.J., Iadarola, M.J., 1991. Cocaine induces striatal c-fos-immunoreactive proteins via dopaminergic D1 receptors. Proc. Natl. Acad. Sci. USA 88, 1291–1295.