รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาผลของ dichloromethane fraction ที่สกัดจากผลหมากในการลดอาการ morphine withdrawal ในหนู

โดย ดร. เอกสิทธิ์ กุมารสิทธิ์

โครงการเสร็จสิ้นเมื่อวันที่ 30 มิถุนายน 2548

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สัญญาเลขที่ MRG4[illegible]2

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ก่อนอื่นกระผมในฐานะหัวหน้าโครงการวิจัยต้องขอบพระคุณ สกว และ สกอ ซึ่งเป็นแหล่งทุนที่ทำให้ผู้วิจัยมีโอกาสได้เริ่มทำงานวิจัย มิฉะนั้น คงเป็นการยากที่จะมีโอกาสได้พัฒนาทักษะทางด้านการวิจัยได้ เนื่องจากการเป็นอาจารย์รุ่นใหม่ มีภาระที่จำเป็นต้องเรียนรู้ใหม่และต้องทำหลายอย่าง อีกทั้งขาดงบประมาณสำหรับการที่จะเริ่มต้นทำการวิจัย การได้รับทุนจึงทำให้มีความสะดวกขึ้นหลายอย่าง และที่สำคัญที่สุด ผลของการได้เริ่มโครงการนี้ ทำให้กระผมสามารถที่จะทำการวิจัยต่อยอดขึ้นไปได้ ดังนั้นจึงนับว่า สกว และ สกอ เป็นผู้สร้างนักวิจัยอย่างแท้จริง

นอกจากนี้กระผมต้องขอขอบพระคุณ ศ. ดร. ปิยะรัตน์ กลางกัลยา ซึ่งเป็นอาจารย์ที่ปรึกษาโครงการวิจัย คอยให้คำแนะนำที่จำเป็นในการแก้ปัญหา รวมทั้งให้กำลังใจเสมอมาจนกระทั่งทุกอย่างสำเร็จลุล่วงไปด้วยดี

(ดร. เอกสิทธิ์ กุมารสิทธิ์)
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หมายเหตุ: ชื่อโครงการได้รับอนุมัติให้เปลี่ยนแปลงจากหัวข้อเดิมคือ การศึกษา Sites of action และ Chronic effects ของ Mitragynine ที่สกัดจากใบกระท่อม ในสมองหนู

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(คำหลัก)

1. บทคัดย่อ

มีการศึกษาที่พบว่า สารสกัด dichloromethane fraction จากผลหมากยับยั้งการทำงานของเอนไซม์ monoamine oxidase type-A ซึ่งแสดงให้เห็นว่า สารนี้มีคุณสมบัติคล้ายกับยาต้านอาการซึมเศร้า ในการศึกษานี้ได้ตั้งสมมติฐานว่า สารสกัดนี้สามารถบรรเทาความรุนแรงของอาการถอนมอร์ฟีนในหนูที่ติดมอร์ฟีนได้ โดยหนูทดลองถูกทำให้ติดมอร์ฟีนโดยการฉีดมอร์ฟีน 3 ครั้งต่อวันเป็นเวลา 3 วัน ในวันที่ 4 หนูได้รับมอร์ฟีนเพียง 1 ครั้ง หลังจากนั้น 2 ชั่วโมง หนูได้รับ naloxone เพื่อชักนำให้เกิดอาการถอนแบบเฉียบพลัน พบว่าการฉีดสารสกัดเพียง 1 ครั้ง สามารถเลื่อนระยะเวลาของการเกิดอาการถอนมอร์ฟีนโดยใช้พฤติกรรมการกระโดดครั้งแรกเป็นข้อบ่งชี้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม นอกจากนี้สารสกัดยังลดจำนวนการกระโดดของหนูรวมทั้งอาการท้องร่วงและการขับปัสสาวะที่เกิดขึ้นในช่วงถอนมอร์ฟีนอย่างมีนัยสำคัญ การค้นพบนี้ชี้ให้เห็นว่า สารสกัด dichloromethane fraction จากผลหมากสามารถบรรเทากลุ่มอาการถอนมอร์ฟีนอย่างมีนัยสำคัญในสัตว์ทดลอง

Abstract

There is evidence demonstrating that the dichloromethane fraction from *Areca catechu* nut inhibits monoamine oxidase type-A. This suggests that the dichloromethane fraction has an antidepressant-like property. In the present study, we hypothesized whether the fraction reduces the severity of naloxone-precipitated morphine withdrawal in morphine-dependent mice. Animals were rendered dependent on morphine by repeated injection (s.c.) of morphine sulphate three times a day for three days. On day 4, they were injected (i.p.) with naloxone two hours after the tenth injection of morphine sulphate to induce withdrawal signs. A single injection of dichloromethane fraction significantly delayed the onset of withdrawal jumping behavior in a concentration-dependent manner compared to that of saline controls ($p < 0.01$). The dichloromethane fractions also significantly decreased jumping numbers and faecal and urinary excretions during the withdrawal period ($p < 0.05$). These findings indicate that the dichloromethane fraction from *Areca catechu* significantly alleviates morphine withdrawal symptoms in animals.

2. Introduction

Areca nut (*Areca catechu*) is commonly used as an ingredient of betel quid, which also includes leaf of the creeping vine piper betel and lime with or without tobacco. Betel quid chewing has been popular, especially in many Southeastern Asian

countries [1;2]. In Taiwan, betel quid is chewed by a large number of both genders [3], probably because this oral habit is more socially accepted than other types of drug abuse. It is estimated that areca nut is chewed by approximately 10% of the world population [4]. Mostly, it is consumed for masticatory and psychoactive purposes [5]. It has been proven that addiction can be induced following prolonged chewing [6].

Whole nut consumption is related to an increased risk of oral submucous fibrosis in human [7-9]. Aqueous extract of areca nut was also demonstrated to induce submucous fibrosis in animal models [10;11]. Especially, when combined with smoking and alcohol drinking, betel quid chewing has about a 100-fold risk of oral cancer compared to that of subjects without this oral habit [12]. Areca nut and betel quid are both on their own also carcinogenic [13]. Thus, isolation of specific ingredients from areca nut with selective actions may contribute more benefits.

Among various alkaloid constituents from areca nut, alkaloids in dichloromethane fraction were found to be biologically active both *in vivo* and *in vitro* [14]. This study demonstrated that the dichloromethane fraction potently inhibits monoamine oxidase-A activity. It means that the fraction has the potential to restore or increase bioavailability of monoamines, 5-hydroxytryptamine or noradrenaline in the brain. Additionally, forced swimming and tail-suspension tests supported that the dichloromethane fraction obviously has antidepressant activity.

Chronic administration of opiate substance produces tolerance and dependence. Abrupt cessation of opiate administration results in withdrawal syndrome [15]. Conventionally, methadone, a μ receptor agonist, has been used to relieve withdrawal signs [16]. However, methadone also produces side effects and withdrawal by itself [17-19]. Some antidepressants and other non-opiate substances have been found and used for prevention of withdrawal syndrome. Classical antidepressants such as fluvoxamine or sertraline were found to reduce opioid withdrawal syndrome [20]. In addition, venlafaxine was also demonstrated to attenuate morphine dependence and withdrawal [21]. According to the antidepressant-like activity of the dichloromethane fraction, we hypothesized that the fraction has therapeutic effect, especially for treating drug-addicted patients.

The present study was aimed to determine whether the dichloromethane fraction from *Areca catechu* nut has suppressive effect on withdrawal signs in morphine dependent mice. Following repeated injection of morphine, naloxone, a nonspecific opiod antagonist was used to precipitate a withdrawal syndrome [22]. Effects of the dichloromethane fraction on morphine withdrawal were determined by evaluating jumping behavior and faecal and urinary excretions. These specific behavioral signs reflect the severity of withdrawal syndrome [23;24].

3. Method

3.1 Source of chemicals

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

3.2 Plant material

Areca catechu nuts were purchased from a local market in the Province of Songkhla, Thailand. Plant material was identified by Dr. Niwat Keawpradub, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The dichloromethane fraction was isolated as previously described [25]. Briefly, kernels of mature areca nuts were chopped and dried. Then, they were crushed into powder and soaked in 70% ethanol for at least 6 days. The filtrate was evaporated to obtain the aqueous ethanol extract. The extract was partitioned with *n*-hexane to remove plant lipid. The aqueous methanol layer was further partitioned with dichloromethane. The dichloromethane layer was collected and evaporated to obtain dichloromethane fraction.

3.3 Animals

Male Swiss albino mice (30 – 35 g) used in this study 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 tab water were available *ad libitum*. They were acclimatized to these conditions for at least

one week prior to the experimental use. 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.

3.4 Development of morphine dependence

Mice were rendered dependent on morphine using the method previously described [26]. Briefly, morphine sulphate was injected (s.c.) three times daily at 0800, 1200 and 1600 (50, 50 and 75 mg/kg, respectively) for three days. On day 4, only a single morning dose of morphine (50 mg/kg) was injected before naloxone injection.

3.5 Observation of morphine withdrawal

Withdrawal signs were precipitated by injection of naloxone (1.5 mg/kg, i.p.) two hours after the final injection of morphine. Immediately after naloxone injection, animals were placed individually on filter paper in an observable cylindrical plastic (15 cm in diameter and 50 cm in height). Behaviors of animals were recorded by using digital video camera. Faecal material and urine excreted during a 30-min period of withdrawal were measured.

3.6 Drugs and dichloromethane fraction treatments

One hour before the administration of naloxone, mice were given either normal saline or one of the following doses of dichloromethane fraction (i.p.): 75, 125 and 175 mg/kg. Saline containing antidepressant either imipramine (20 mg/kg), or fluvoxamine (20 mg/kg) were also injected for comparisons.

3.7 Statistical analysis

Experimental data were expressed as mean values ± SEM of the numbers of jumping and weights of faecae and urine during withdrawal period. Differences were determined using multiple comparisons versus control group (Dunn's Method). Differences with $P \leq 0.05$ were considered statistically significant.

4. Results and discussion

4.1. Effects of dichloromethane fraction on jumping behavior induced by naloxone precipitation in morphine-dependent mice

Numbers of jumping were counted and analyzed minute-by-minute. All groups exhibited jumping behavior immediately following naloxone injection. The precise data reveal that they had different onsets of their jumping behavior. Fig. 1 shows that imipramine, fluvoxamine and a 75 mg/kg dose of the dichloromethane fraction exhibited relatively equal onsets (53.7 ± 3.9, 63.6 ± 4.4 and 76.7 ± 7.5 seconds respectively) to that of saline (56.3 ± 9.5 second). The 125 and 175 mg/kg doses of the dichloromethane fraction exhibited significant longer onsets with 107.1 ± 9.4 and 149.8 ± 22.6 seconds respectively. These dichloromethane fractions significantly delayed the onset period in a dose-dependent manner compared with the control group.

Imipramine, fluvoxamine and the 75 mg/kg dose of dichloromethane fraction showed peaks of the withdrawal sign with relatively equal jumping numbers to that of saline (about 22 time/min). The 125 and 175 mg/kg doses of dichloromethane fraction exhibited jumping only 12.2 ± 3.1 and 8.9 ± 2.5 time/min respectively at peak. These two doses of the fraction significantly reduced the numbers of jumping at withdrawal peaks by 44.6% and 59.5% respectively compared to that of control group. Thereafter, all jumping responses rapidly returned to almost zero within about 6 min after naloxone injection. Total numbers of jumping over a 5-min period were also analyzed and are shown in Fig.2.

4.2. Effects of dichloromethane fraction on faecal and urinary excretions as naloxone-precipitated withdrawal signs in morphine-dependent mice

Values of faecal and urinary excretions during a 30-min period of withdrawal were expressed in grams as shown in Fig. 3. A simple analysis was performed between groups receiving imipramine, fluvoxamine and three doses of dichloromethane fraction (75, 125 and 175 mg/kg) versus saline. Compared to saline, all doses of dichloromethane fraction significantly decreased both faecal and urinary excretions, whereas imipramine and fluvoxamine did not show any significant effect.

The present results demonstrate that the dichloromethane fraction is clearly effective in alleviating the incidence of withdrawal jumping in morphine-dependent mice.

Jumping is one of the most common signs used to assess the severity of morphine withdrawal [23;27]. Defecation or diarrhea is also commonly found during opiate withdrawal [28;29]. The present data support that these signs are specific responses of a withdrawal syndrome.

These results also support the previous findings which concluded that the dichloromethane fraction inhibits MAO-A and act as an antidepressant [30]. With this activity, the fraction could increase bioavailability and enhance neurotransmission of monoaminergic, serotonergic and noradrenergic systems in brain. Activation of these systems have been found to reduce the severity of opiate withdrawal [31-33]. Thus, it is possible that the dichloromethane fraction may activate at least one of these systems to affect morphine withdrawal.

In conclusion, the alleviating effects of the dichloromethane fraction on both signs strongly confirm the promising property of the fraction for clinical purposes. Further studies are needed to investigate the central mechanism and identify specific sites of action of this fraction in the brain.

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Fig.1

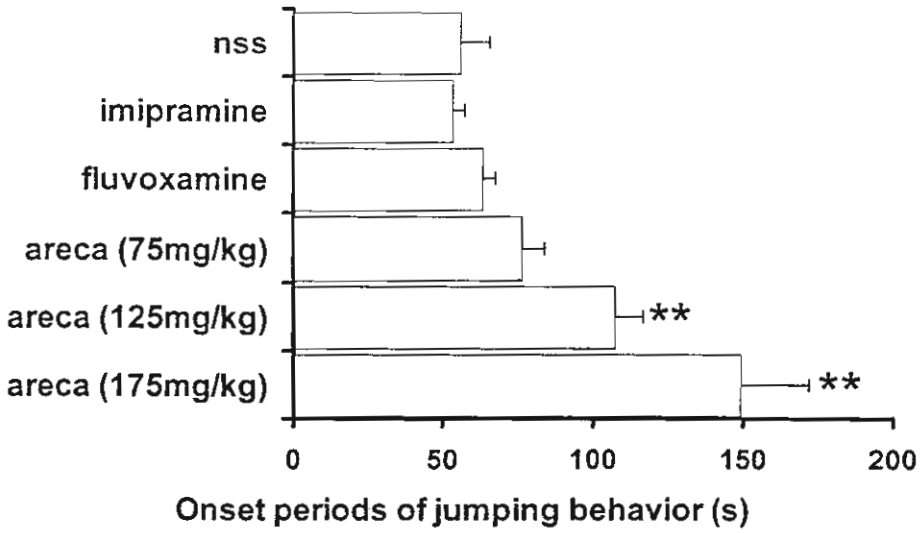

nss
imipramine
fluvoxamine
areca (75mg/kg)
areca (125mg/kg)
areca (175mg/kg)
**
**
0
50
100
150
200
Onset periods of jumping behavior (s)

Fig.2

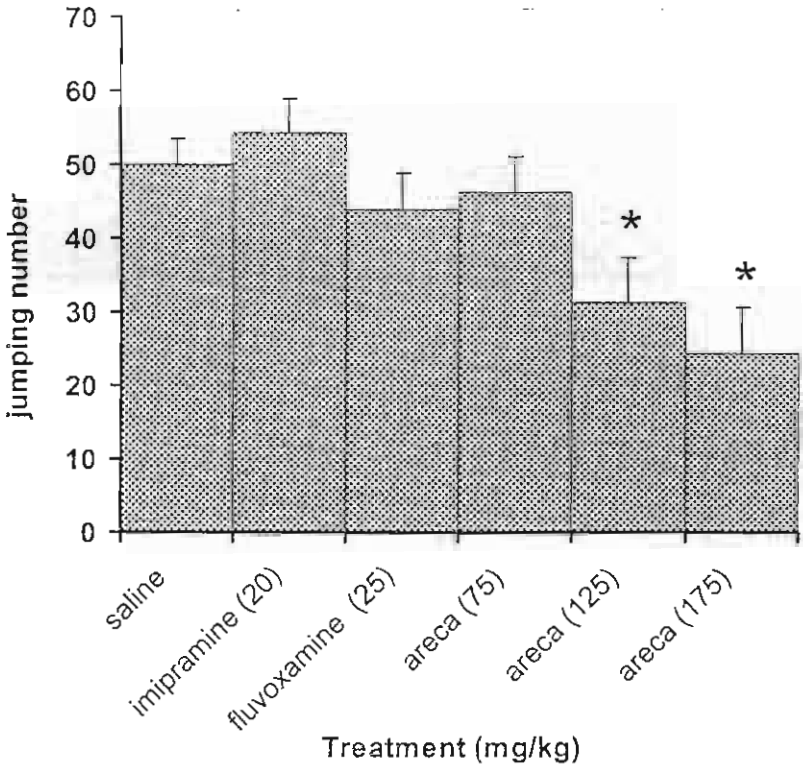
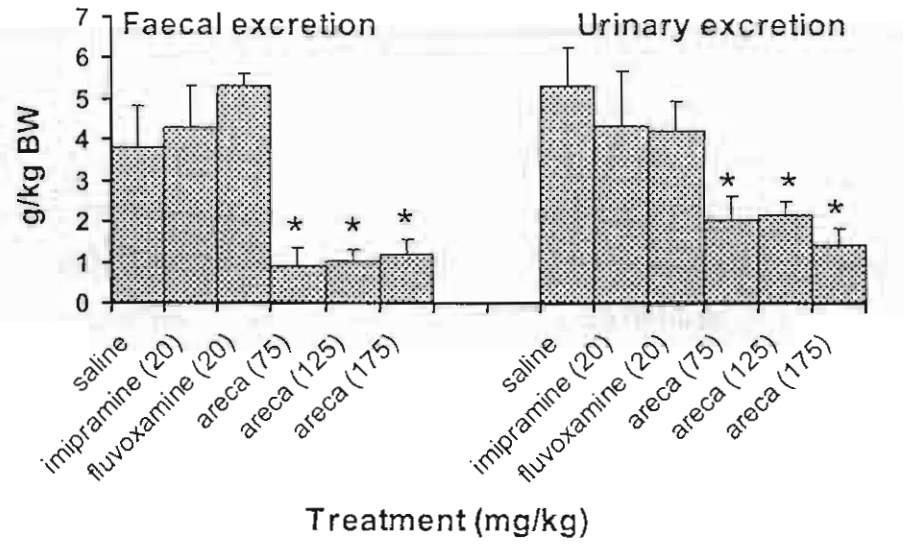

70
60
50
40
30
20
10
0
jumping number
*
*
saline
imipramine (20)
fluvoxamine (25)
areca (75)
areca (125)
areca (175)
Treatment (mg/kg)

Fig.3


Faecal excretion
Urinary excretion
g/kg BW
0
1
2
3
4
5
6
7
*
saline
imipramine (20)
fluvoxamine (20)
areca (75)
areca (125)
areca (175)
Treatment (mg/kg)

6. ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

จากผลการทดลองจะเห็นได้ว่า สารสกัด dichloromethane fraction จากผลหมากมีสรรพคุณในการบรรเทาอาการถอนมอร์ฟีนได้อย่างชัดเจน ถึงแม้จะเป็นการศึกษาในสัตว์ทดลองก็ตาม แต่หมากก็ถูกบริโภคโดยมนุษย์มาเป็นเวลานาน อาจทำให้พอคาดการณ์ถึงแนวโน้มได้ว่ามีความเป็นไปได้สูงที่สารสกัดจากหมากจะสามารถใช้ได้ในคน นอกจากนี้ ประเทศไทยก็มีทรัพยากรพืชชนิดนี้อยู่มาก ซึ่งเป็นข้อได้เปรียบเมื่อเปรียบเทียบกับนานาประเทศ ดังนั้นจึงเป็นที่น่าสนใจที่จะนำมาค้นคว้าวิจัยต่อเนื่องขึ้นไปในระดับสูงเพื่อให้เกิดประโยชน์และมูลค่าเพิ่มสูงสุดของทรัพยากรของประเทศทั้งในแง่คลินิกและเชิงพาณิชย์

7. Output

7.1 ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Authors: [a]Ekkasit Kumarnsit*, [b]Niwat Keawpradub, [c]Uraporn Vongvatcharanont, [d]Kitja Sawangjaroen, [e]Piyarat Govitrapong

Title: Suppressive effects of dichloromethane fraction from the *Areca catechu* nut on naloxone-precipitated morphine withdrawal in mice

Journal: Fitoterapia (2005 in press and currently online)

8. ภาคผนวก

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Suppressive effects of dichloromethane fraction from the *Areca catechu* nut on naloxone-precipitated morphine withdrawal in mice

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Abstract

In the present study, we investigated the effect of the dichloromethane fraction from *Areca catechu* nut on the severity of naloxone-precipitated morphine withdrawal in morphine-dependent mice. A single intraperitoneal injection of dichloromethane fraction at dose of 125 and 175 mg/kg significantly delayed the onset of withdrawal jumping behavior in a concentration-dependent manner compared to that of saline controls. The dichloromethane fractions also significantly decreased jumping numbers and faecal and urinary excretions during the withdrawal period.

Keywords: *Areca catechu*; Morphine withdrawal; Antidepressant; Naloxone; Jumping

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1. Introduction

Areca nut (*Areca catechu*) is commonly used as an ingredient of betel quid, which also includes leaf of the creeping vine piper betel and lime with or without tobacco. Betel quid chewing has been popular, especially in many Southeastern Asian countries [1,2]. In Taiwan, betel quid is chewed by a large number of both genders [3], probably because this oral habit is more socially accepted than other types of drug abuse. It is estimated that areca nut is chewed by approximately 10% of the world population [4].

Mostly, it is consumed for masticatory and psychoactive purposes [5]. It has been proven that addiction can be induced following prolonged chewing [6].

Whole nut consumption is related to an increased risk of oral submucous fibrosis in human [7–9]. Aqueous extract of areca nut was also demonstrated to induce submucous fibrosis in animal models [10,11]. Especially, when combined with smoking and alcohol drinking, betel quid chewing has about a 100-fold risk of oral cancer compared to that of subjects without this oral habit [12]. Areca nut and betel quid are both on their own also carcinogenic [13]. Thus, isolation of specific ingredients from areca nut with selective actions may contribute more benefits.

It has been previously shown that among various alkaloid constituents from areca nut, alkaloids in dichloromethane fraction were found to be biologically active both in vivo and in vitro. This fraction potently inhibits monoamine oxidase-A activity and thus restores or increases bioavailability of monoamines, 5-hydroxytryptamine or noradrenaline in the brain. Additionally, forced swimming and tail-suspension tests supported that the dichloromethane fraction has antidepressant activity [14].

Chronic administration of opiate substance produces tolerance and dependence. Abrupt cessation of opiate administration results in withdrawal syndrome [15]. Conventionally, methadone, a μ receptor agonist, has been used to relieve withdrawal signs [16]. However, methadone also produces side effects and withdrawal by itself [17–19]. Some antidepressants and other non-opiate substances have been found and used for prevention of withdrawal syndrome. Classical antidepressants such as fluvoxamine or sertraline were found to reduce opioid withdrawal syndrome [20]. In addition, venlafaxine was also demonstrated to attenuate morphine dependence and withdrawal [21]. According to the antidepressant-like activity of the dichloromethane fraction, we hypothesized that the fraction has therapeutic effect, especially for reducing the withdrawal syndrome in drug-addicted patients.

Thus, the present study was aimed to determine whether the dichloromethane fraction from *A. catechu* nut has suppressive effect on withdrawal signs in morphine dependent mice. Effects of the dichloromethane fraction on morphine withdrawal were determined by evaluating jumping behavior, faecal and urinary excretions. These specific behavioral signs reflect the severity of withdrawal syndrome [22,23].

2. Experimental

2.1. General

The following drugs were used: morphine sulphate (Zentiva, SK), naloxone (Sigma, Germany), imipramine (Sigma, Germany) and fluvoxamine (SUN, India).

The drugs were dissolved in normal saline and given to animals in a volume of 5 ml/kg.

2.2. Plant material

A. catechu nuts, purchased from a local market in the Province of Songkhla, Thailand, were identified by Dr. Niwat Keawpradub, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The dichloromethane fraction was isolated as previously described [24].

2.3. Animals

Male Swiss albino mice (30–35 g) used in this study were bred at the animal house of the Prince of Songkla University. They were housed in a group of 10 mice per cage and maintained under 12/12 dark/light cycle and controlled temperature (22 °C). Standard commercial food pellets and water were available ad libitum. They were acclimatized to these conditions for at least 1 week prior to the experimental use. 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.

2.4. Development of morphine dependence

Mice were rendered dependent on morphine using the method previously described [25]. Briefly, morphine sulphate was injected (s.c.) three times daily (8.00, 12.00 and 16.00) at 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.

2.5. Observation of morphine withdrawal

Withdrawal signs were precipitated by injection of naloxone (1.5 mg/kg, i.p.) 2 h after the injection of morphine. Immediately after naloxone injection, animals were placed individually on filter paper in an observable cylindrical plastic (15 cm in diameter and 50 cm in height). Behaviors of animals were recorded by using digital video camera. Faecal material and urine excreted during a 30-min period of withdrawal were measured.

2.6. Drugs and dichloromethane fraction treatments

One hour before the administration of naloxone, mice were treated with normal saline or injected intraperitoneally with the dichloromethane fraction at dose of 75, 125 and 175 mg/kg. Two other groups were treated with imipramine (20 mg/kg), or fluvoxamine (20 mg/kg).

2.7. Statistical analysis

Experimental data were expressed as mean values ± SEM of the numbers of jumping and weights of faecal material and urine during withdrawal period. Differences were

determined using multiple comparisons versus control group (Dunn's method). Differences with $P \leq 0.05$ were considered statistically significant.

3. Results and discussion

Numbers of jumping were counted and analyzed minute-by-minute. All groups exhibited jumping behavior immediately following naloxone injection. Fig. 1 shows that imipramine and fluvoxamine exhibited relatively equal onsets (53.7 ± 3.9 and 63.6 ± 4.4 s, respectively) to that of saline (56.3 ± 9.5 s). The dichloromethane fraction delayed the onset period in dose-dependent manner. In particular, the 125 and 175 mg/kg exhibited significant longer onsets with 107.1 ± 9.4 and 149.8 ± 22.6 s, respectively.

Also, the total number of jumping over a 5-min period was reduced by the treatment with 125 and 175 mg/kg of the dichloromethane fraction (Fig. 2).

Values of faecal material and urine excreted during a 30-min period of withdrawal are expressed in g and reported in Fig. 3. Compared to saline, all doses of dichloromethane fraction significantly decreased both faecal and urinary excretions, whereas imipramine and fluvoxamine did not show any significant effect.

The present results demonstrate that the dichloromethane fraction is effective in alleviating the incidence of withdrawal jumping in morphine-dependent mice. Jumping is one of the most common signs used to assess the severity of morphine withdrawal [22,26]. Defecation or diarrhea is also commonly found during opiate withdrawal [27,28].

These results also support the previous findings which concluded that the dichloromethane fraction inhibits MAO-A and act as an antidepressant [29]. With this activity, the fraction could increase bioavailability and enhance neurotransmission of monoaminergic, serotonergic and noradrenergic systems in brain. Activation of these systems has been found to reduce the severity of opiate withdrawal [30–32]. Thus, it is possible that the dichloromethane fraction may activate at least one of these systems to affect morphine withdrawal.

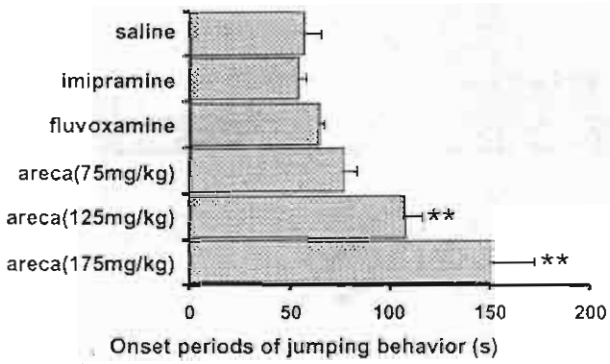

Fig. 1. Effects of dichloromethane fraction from *A. catechu* nut on the onset of naloxone-induced jumping behavior in morphine-dependent mice. The onset periods were measured immediately after naloxone injection until the first jumping. Each group had 7 to 12 mice. Data are means ± S.E.M. $^{**}P < 0.01$ different from the control group.

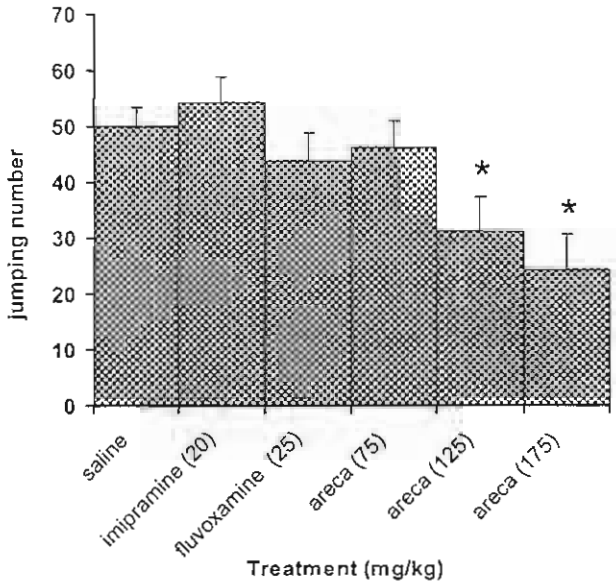

Fig. 2. Effects of dichloromethane fraction from *A. catechu* nut on total number of jumping during 5-min period of morphine withdrawal. Data are means ± S.E.M. $^*P<0.05$ different from the control group.

In conclusion, the alleviating effects of the dichloromethane fraction on both signs strongly confirm the promising property of the fraction for clinical purposes. Further studies are needed to investigate the central mechanism and identify specific sites of action of this fraction in the brain.

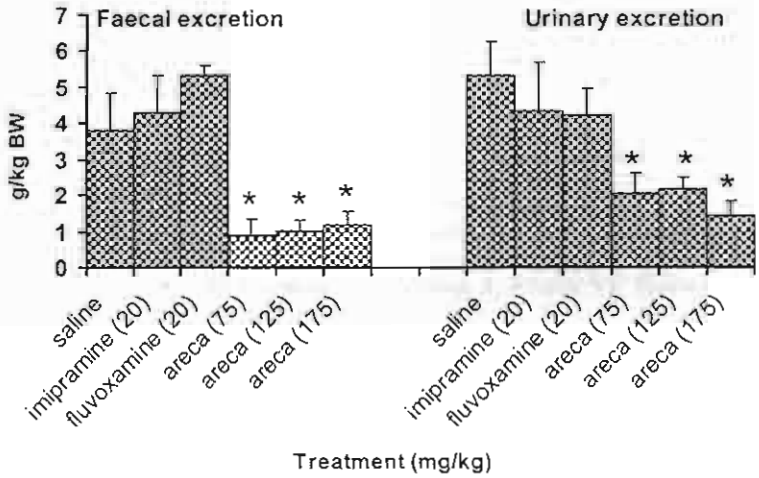

Fig. 3. Effects of dichloromethane fraction from *A. catechu* nut on faecal and urinary excretions in morphine-dependent mice induced by naloxone injection. Weights of faecal material and urine excreted during a 30-min period of morphine withdrawal were measured. Data are means ± S.E.M. $^*P<0.05$ different from the control group.

Acknowledgements

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