

Original Research Article

Anti-nociceptive effect of total alkaloids isolated from the seeds of *Areca catechu* L (Arecaceae) in mice

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Abstract

Purpose: To investigate the antinociceptive effect of the total alkaloids (TA) isolated from the seeds of *Areca catechu* L. (SAC) and to elucidate the probable mechanism of action.

Methods: TA extraction conditions including concentration of ethanol, extraction temperature, liquid–solid ratio and designed pH were optimized by an orthogonal experiment $\{L_9(3)^4\}$ test. The anti-nociceptive effect of the extract in mice was evaluated by acetic acid writhing reflex test, hot plate test, capsaicin-induced nociception test, tail-flick test and formalin-induced pain test in mice. Furthermore, pretreatment of the animals with naloxone (2 mg/kg) was performed to investigate whether the anti-nociceptive effect involved the opioid route or not. The locomotor activity of TA in mice were also assessed.

Results: The optimum extraction conditions of TA were as follows: solid-liquid ratio of 1: 15 (w/v), ethanol concentration of 80 %, pH of 9.0 and extraction temperature of 70 °C. Oral administration of TA produced a marked anti-nociceptive activity in mice, and pretreatment with naloxone did not reverse the anti-nociceptive activity of TA in mice. Also, the locomotor activity of mice was not affected by TA. In addition, TA significantly down-regulated the expression levels of COX-2 in the dorsal root of mice spinal cord at 100, 200 and 400 mg/kg doses.

Conclusion: The results demonstrate that TA possesses significant anti-nociceptive effects, and the mechanisms are closely related to suppression of COX-2 expression. Overall, the results provided scientific support for the use of TA in treatment of pain.

Keywords: *Areca catechu*, Total alkaloids, Anti-nociceptive, Formalin-induced pain, Naloxone

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INTRODUCTION

Although pain can be considered as protective mechanism of the body, it disrupts the life quality of millions of people all over the world, and brings a heavy burden to both individuals and society [1,2]. Classical types of analgesic medication for treating pain include non-steroidal anti-inflammatory drugs (NSAIDs) and opioids [3]. However, long-term use of NSAIDs can induce some adverse effects, such as gastric mucosal injury and hemorrhage, and the

repeated use of opioids can induce severe drug dependence [4,5]. Thus, the clinical efficacy and application of conventional analgesics were limited by their unwanted adverse effects.

The use of natural products are increasing in treating pain as alternative therapies to the conventional medical approaches [6]. *Areca catechu* L., belonging to Arecaceae family, is widely distributed in South-Asian countries. It is a folk medicine employed to treat some diseases, such as gastrointestinal inflammation, dysentery

and diarrhea [7]. The seeds of *Areca catechu* L. (SAC), is one of Traditional Chinese Medicines used for the treatment of distended abdomen and intestinal parasitosis, tenesmus and diarrhea [8]. Alkaloids were the major active constituents of SAC which had many pharmacological activities [9]. Although, anti-nociceptive effects of this drug have been reported [10], limited evidence is available to confirm its effective constituents. The present study was designed to explore the anti-nociceptive effects of total alkaloids isolated from areca nut, which was conducive to the development of this drug.

EXPERIMENTAL

Chemicals and reagents

Acetic acid, capsaicin, naloxone hydrochloride, indomethacin, diazepam solution (1 mg/mL) and morphine solution (1 mg/mL) were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-cyclooxygenase-2 (COX-2) rabbit monoclonal IgG and goat anti-rabbit/HRP conjugate were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Other chemicals used were of analytical grade.

Preparation of total alkaloids from SAC

SAC materials were purchased from Yuzhou Chinese Herbal Medicine Professional Market (Yuzhou, China). They were authenticated by the Department of Traditional Chinese Medicine in He'nan University of Science and Technology Luoyang, China. A voucher specimen was deposited in the herbarium of the First Affiliated Hospital of He'nan University of Science and Technology (No. YPLY-2016-07-21). The extraction methods were performed as previously reported [11] with some modifications. The powder (5.0 g) of SAC was weighed and placed in a 100 mL triangular flask containing ethanol. Then an ultrasound extraction was carried out with different ethanol concentration (70, 80 and 90 %), extraction temperature (50, 60 and 70 °C), and liquid–solid ratio (1: 10, 1: 15 and 1: 20, w/v) for 30 min. The extraction solutions were collected and concentrated, and adjusted to designed pH (7.0, 8.0 and 9.0) by ammonium hydroxide. After that, the solutions were extracted by trichloromethane for three times and

finally freeze drying to obtain TA. The extraction yield of TA (mg/g) was calculated by equation (1):

$$Y \text{ (mg/g)} = W_1/W_0 \dots \dots \dots (1)$$

where Y was the yield of extracted alkaloids, W_1 was the weight of extracted alkaloids (mg), and W_0 was weight of dried powder sample (g).

Optimization of TA extraction

An orthogonal experiment $\{L_9 (3)^4\}$ test design was employed in the present study for optimizing the extraction conditions of TA. The key parameters that influenced the yield of TA including liquid–solid ratio, concentration of ethanol, extraction temperature, and the pH value were analyzed. The experimental design for the extraction of TA from SAC were shown in Table 1.

Determination of alkaloid content in TA

The content of alkaloid in TA was determined by acid dye colorimetry method [12]. Briefly, the samples were dissolved by acetic acid-sodium acetate buffer solution (pH = 5). Then the bromocresol green solution (0.8 mg/mL) was added to the samples and extracted by trichloromethane for three times. The trichloromethane solution was collected, and anhydrous ethanol solution of potassium hydroxide (0.01 mol/L) was added before detecting the absorbance at 618 nm. Arecoline hydrobromide was used as standard substance.

Animals

Female KM mice (18 - 22 g) were obtained from Laboratory Animal Center of He'nan University of Science and Technology (Luoyang, China). Animals were housed in controlled environment (temperature of 23 ± 1 °C under a 12-h light–dark cycle, 50 – 60 % humidity) for 7 days before the experiment. Feed and water were provided *ad libitum*. The experiments were approved by the Institutional Ethics Committee for Animal Care and Use of the First Affiliated Hospital of He'nan University of Science and Technology (approval no. SYDW 201602-08),

Table 1: Orthogonal experiment design (four factors and three levels)

Factor	A	B	C	D
	Solid-liquid ratio (w/v)	Ethanol concentration (%)	pH	Temperature (°C)
1	1: 10	70	7.0	50
2	1: 15	80	8.0	60
3	1: 20	90	9.0	70

and conducted in accordance with Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) [13].

Acetic acid-induced abdominal writhing test

Acetic acid-induced abdominal writhing was performed as previously reported by Wang *et al* [14]. Mice were randomly divided into five groups ($n = 10$): the control group [normal saline (NS)], three TA groups (100, 200 and 400 mg/kg), and the indomethacin group (10 mg/kg). TA, NS and indomethacin were all administered orally (i.g.) to animals (10 mL/kg) 1 h before acetic acid solution (0.7 % v/v, 10 mL/kg) injected intraperitoneally (i.p.). The number of writhing of each mouse was counted after acetic acid injection over a period of 12 min.

Hot plate test

Hot plate test was carried out using the methods as previously described by Suseem *et al* [15]. The metal surface was maintained at the temperature of 50 ± 1 °C. Latency time was defined as the duration until the mouse jumped off the plate or licked its hind paw to avoid thermal stimulation. All mice were screened before the test, and a pre-test latency (5-30 s) qualified them for the experiment. The mice were treated with TA (100, 200 and 400 mg/kg, i.g.) or NS (10 mL/kg, i.g.) one hour before the test. Morphine (10 mg/kg, i.p.) was administered 30 min before the test. Additionally, to avoid tissue damage of the mouse, a cut off time (60 s) was used.

Capsaicin-induced nociception test

The procedure used in the present study was same to that previously described by Pinheiro *et al* [16]. Capsaicin (1.6 µg/paw, 20 µL) was injected subcutaneously (s.c.) in the mice right hind paw. Animals were treated with TA (100, 200 and 400 mg/kg, i.g.) one hour before capsaicin injection. The mice in the control group received NS (10 mL/kg, i.g.), while the mice in positive control group received morphine (10 mg/kg, i.p.). Each mouse was observed individually for 5 min following capsaicin injection, and the time of the animal spent for licking the injected paw was recorded.

Tail-flick test

Antinociceptive activity of TA was further assessed using the tail-flick test [17]. Mice were treated with TA (100, 200 and 400 mg/kg, i.g.) or NS (10 mL/kg, i.g.) one hour before the test. Morphine (10 mg/kg, i.p.) was administered to

mice in the positive group 30 min before the experiment. The tail of each mouse was immersed in a hot water bath (maintained at 55 ± 1 °C). Each mouse was recorded the reaction time to flick the tail from the hot water. A cut off time of 7 s was used to prevent any injury to the tail of the mouse.

Formalin-induced pain test

The formalin-induced pain test was performed per the study of Wandji *et al* [18]. Mice were treated with TA (100, 200 and 400 mg/kg, i.g.) or NS (10 mL/kg, i.g.) one hour before the test. Morphine (10 mg/kg, i.p.) was administered to mice in the positive group 30 min before the test. Animals were injected with 5% formalin (20 µL, s.c.) into the dorsal hind paw and observed in individual cages post formalin injection. Two distinct phases were observed: phase I, 0-5 min; phase II, 15-25 min. The time of the mice spent biting or licking the injected paw or leg was recorded. Furthermore, the mechanism of action of TA was evaluated by testing their analgesic effects in animals pre-treated with naloxone (1 mg/kg, i.p.) 15 min before the formalin test.

Evaluation of effect of TA on COX-2 in formalin induced pain

After formalin test, the mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Thereafter, the spinal cord was quickly removed, an intact L4 ~ L6 dorsal root was cut and stored into -80 °C liquid nitrogen pending further experiments [19]. To evaluate the COX-2 expression in the dorsal horn of the spinal cord, total proteins of spinal cord tissue were extracted. Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with an anti-COX-2 rabbit monoclonal IgG followed by a goat anti-rabbit/HRP conjugate. The proteins were detected by using chemiluminescence reagents. Antibodies directed against β -actin were used to normalize for protein loading.

Determination of locomotor activity

The effect of the TA on spontaneous locomotor activity was assessed using the open-field test. Mice were treated with TA (100, 200 or 400 mg/kg, i.g.) or the NS (10 mL/kg), or diazepam (2 mg/kg, i.p.) one hour before the test. The number of locomotor activity of each mouse was recorded within 5 min period using a multi-

functional mice locomotor activity recorder (Yiyan technology development co., LTD, Jinan, China).

Statistical analysis

Statistical analysis was performed by SPSS version 16.0 (SPSS Inc., USA). All data are expressed as mean±SEM, and were analysed by one-way ANOVA followed by *Dunnnett's* multiple comparison. *P* < 0.05 was considered statistically significant.

RESULTS

Optimization by orthogonal experimental design

An extreme different analysis shows that the effect of the four factors on the extraction yield of TA is solid-liquid ratio > extraction temperature > pH > ethanol concentration (Table 2). According to the variance analysis in Table 3, the contributions of solid-liquid ratio (*p* < 0.01) and pH (*p* < 0.05) for the extraction yield of TA are significant, whereas ethanol concentration and extraction temperature are not significant factors. According to extreme difference analysis, the optimum extraction conditions of TA were: solid-liquid ratio of 1: 15 (w/v), ethanol concentration of 80 %, pH value of 9.0, and extraction temperature of 70 °C.

Table 2: Orthogonal design test results

Test no.	A	B	C	D	Yield (mg/g)
1	1	1	1	1	1.72
2	1	2	2	2	1.82
3	1	3	3	3	1.89
4	2	1	2	3	2.22
5	2	2	3	1	2.37
6	2	3	1	2	2.19
7	3	1	3	2	1.75
8	3	2	1	3	1.71
9	3	3	2	1	1.66
K1	1.810	1.897	1.873	1.917	
K2	2.260	1.967	1.900	1.920	
K3	1.707	1.913	2.003	1.940	
Extreme value	0.553	0.070	0.130	0.023	

Table 3: Variance analysis (orthogonal test)

Source	SS	df	F-value	Significance
A	0.519	2	519.000	<i>p</i> < 0.01
B	0.008	2	8.000	<i>p</i> > 0.05
C	0.028	2	28.000	<i>p</i> < 0.05
D	0.001	2	1.000	<i>p</i> > 0.05

To confirm the predicted optimum condition of TA, extractions under predicted optimum conditions were carried out, and the extraction

yield of TA was 2.41 ± 0.12 mg/g. Therefore, it's confirmed that the predicted conditions were the best combination of different parameters. In addition, the results of content determination showed that the average content of alkaloid in the TA was 70.75 %, indicating that alkaloids were the main constituents of TA.

Anti-nociceptive effect of TA

Acetic acid writhing reflex

The effect of TA on the writhing response in mice was shown in Figure 1. The indomethacin (10 mg/kg) significantly inhibited acetic acid induced writhing response compared with mice in the control group (*p* < 0.01). Interestingly, TA also significantly inhibited the acetic acid induced writhing response in mice compared with the control group. The inhibitory effect was in a dose dependent manner at the doses of 100, 200 and 400 mg/kg (*p* < 0.05, *p* < 0.01 and *p* < 0.01, respectively).

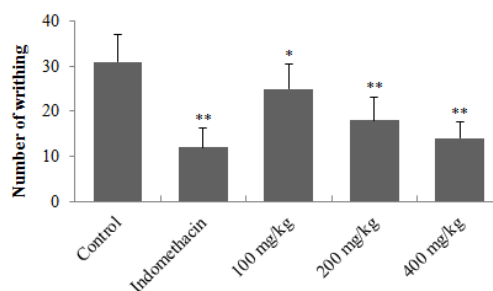


Figure 1: Effect of TA on acetic acid-induced writhing reflex in mice. **p* < 0.05, compared with control group; ***p* < 0.01, compared with control group

Latency time

In the hot plate test, morphine (10 mg/kg) displayed marked anti-nociceptive effects in mice (*p* < 0.001), compared with the control group. As shown in Figure 2, TA also significantly increased the latency time at the doses of 200 and 400 mg/kg (*p* < 0.01) as compared with the control group.

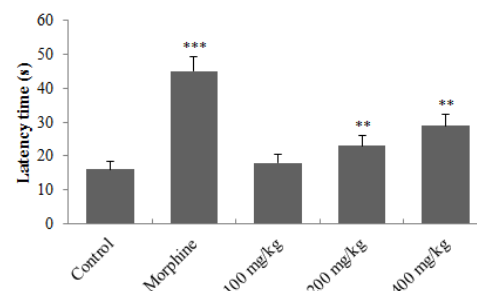


Figure 2: Effect of TA on the latency time in hot plate test. ***p* < 0.01, compared with control group; ****p* < 0.001, compared with control group

Capsaicin-induced nociception

The effects of TA on capsaicin-induced nociception in mice were shown in Figure 3. From the results, TA showed significant inhibitory effect on capsaicin-induced neurogenic nociception dose-dependently at 100, 200 and 400 mg/kg ($p < 0.05$, $p < 0.01$ and $p < 0.001$), compared with the control group. Morphine (10 mg/kg) also significantly inhibited the capsaicin-induced neurogenic nociception compared with the control group.

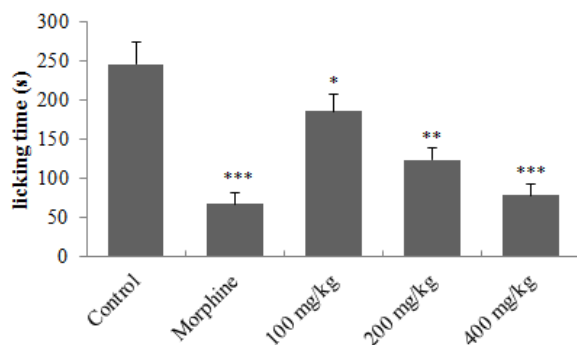


Figure 3: Effect of TA on Licking time in capsaicin-induced nociception. * $p < 0.05$, compared with control group; ** $p < 0.01$, compared with control group; *** $p < 0.001$, compared with control group

Tail-flick reaction time

TA significantly increased the reaction time on mice in tail-flick test at the doses of 200 and 400 mg/kg ($p < 0.01$ and $p < 0.001$), compared with mice in the control group (Figure 4). In addition, morphine (10 mg/kg) also produced significant analgesic activity in mice, compared with the control group.

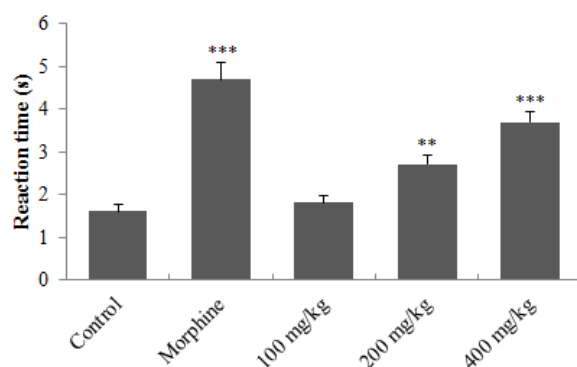


Figure 4: Effect of TA on reaction time in tail-flick test; ** $p < 0.01$, compared with control group; *** $p < 0.001$, compared with control group

Formalin-induced pain

The results of formalin-induced pain and pretreated with naloxone are shown in Figure 5.

In the phase I, TA significantly reduced the time of licking the paw or leg of mice at the doses of 100, 200, and 400 mg/kg ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), compared with the control group dose-dependently. In the phase II, TA also showed significant antinociceptive effect at the doses of 100, 200, and 400 mg/kg ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively) dose-dependently, compared with the control group.

In addition, naloxone (1 mg/kg) showed no antinociceptive effect on formalin induced pain. However, pretreated with naloxone decreased the anti-nociceptive effect of morphine ($p < 0.001$, compared with morphine group). Interestingly, the antinociceptive effect of TA (400 mg/kg) was not decreased by pretreating with naloxone ($p > 0.05$, compared with the TA group of 400 mg/kg).

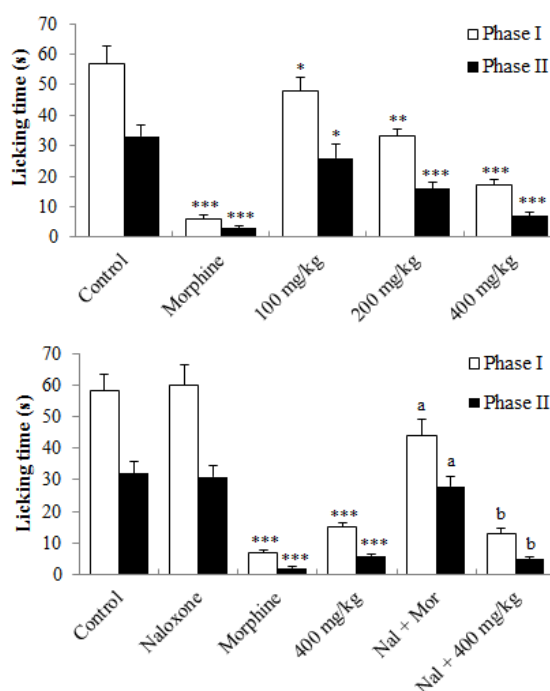


Figure 5: Effect of TA on licking time in formalin-induced pain in mice. Mor = Morphine and Nal = Naloxone; * $p < 0.05$, compared with control group; ** $p < 0.01$, compared with control group; *** $p < 0.001$, compared with control group. ^a $p < 0.001$, compared with Morphine group; ^b $p > 0.05$, compared with TA (400 mg/kg) group

Effect of TA on COX-2 in formalin induced pain

As shown in Figure 6, TA significantly down-regulated the expression of COX-2 in dorsal root of mice spinal cord at the doses of 100, 200 and 400 mg/kg, compared with control group. Furthermore, TA showed inhibitory effect on

expression of COX-2 in dorsal root of formalin induced pain mice (100, 200 and 400 mg/kg).

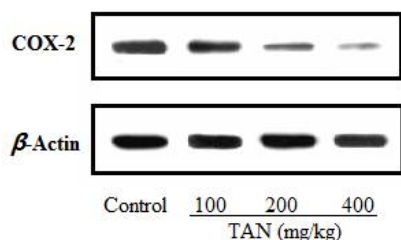


Figure 6: Effect of TA on COX-2 in formalin induced pain in dorsal root of mice spinal cord

Effect of TA on locomotor activity

As shown in Figure 7, diazepam (2 mg/kg) significantly decreased the number of locomotor activity of mice compared with the mice in the control group. However, the number of locomotor activity of mice in TA treated group had no significant difference compared with the control group. Thus, it indicated that TA had no significant effect on spontaneous activity at 100, 200 and 400 mg/kg by oral administration in mice ($p > 0.05$).

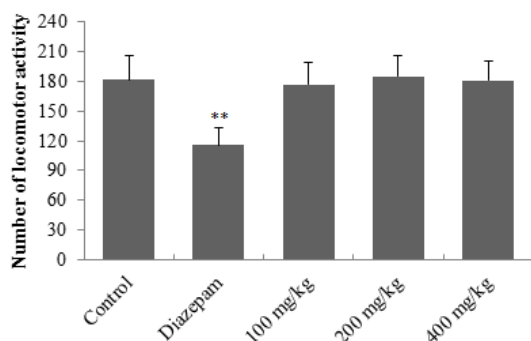


Figure 7: Effect of TA on locomotor activity in mice; ** $p < 0.01$, compared with the control group.

DISCUSSION

The present study demonstrated that oral administration of TA produces a marked anti-nociceptive activity when tested in different experimental models of nociception including acetic acid writhing reflex test, hot plate test, capsaicin-induced nociception test, tail-flick test and formalin-induced pain test.

It's reported that the acetic acid-induced writhing response is a sensitive test commonly used for screening anti-nociceptive effect, regardless of the central or peripheral causes [20]. The results indicated that TA exerted significant anti-nociceptive effects on acetic acid induced writhing reflex in mice with dose-dependent manner. Further studies on hot plate test,

capsaicin-induced nociception and tail-flick test also demonstrated that TA possesses significant anti-nociceptive effects on a variety of stimulation induced nociception. In the formalin test, the phase I (0 - 5 min) is characterized by neurogenic pain caused by a direct stimulation of nociceptors, while the phase II (15 - 25 min) is characterized by inflammatory pain [21]. In general, centrally acting drugs inhibit phase I and phase II equally, while peripherally acting drugs inhibit mainly the phase II. The results of formalin test indicated that TA had central and peripheral anti-nociceptive properties. As is known to all, naloxone is a one of non-selective opioid receptor antagonists [22]. Pretreatment with naloxone (2 mg/kg) didn't reverse the anti-nociceptive activity of TA administered orally. Thus, the results suggested that the opioid route was not involved in the observed anti-nociceptive activity of TA.

COX-2 is an inducible enzyme which is responsible for the production of the pro-inflammatory prostaglandins (PEG) [23]. TA showed obvious inhibitory effect on the expression of COX-2, indicating that it might be a potent COX-2 inhibitor. Thus, TA had the potential to develop into drugs for treating pain from COX-2 involved-inflammatory diseases.

In the evaluation of the anti-nociceptive effect drugs, it's a major concern whether the treatment of the drugs causes other behavioral alterations, such as sedation or impairment of motor coordination. These behavioral alterations might be misinterpreted as anti-nociceptive effect [24]. In the present study, the locomotor activity of mice was not affected by TA, indicating that its anti-nociceptive effect was highly selective.

CONCLUSION

The findings of the present study demonstrate that TA possesses significant anti-nociceptive effects. Its effect is not induced by the opioid route, but is closely related to suppression of COX-2 expression. Overall, the results provide some scientific support for the use of TA in the treatment of pain in traditional medicine.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by

the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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