RESEARCH ARTICLES

Neutralization of *Naja kaouthia* Venom in Mice by *Trigonostmon reidioides* Craib. and *Areca catechu* Linn. Extracts.

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Abstract

This study was aimed to investigate the inhibitory effects of *Trigonostemon reidioides* Craib. and *Areca catechu* Linn. on lethality and myotoxicity of *Naja kaouthia* venom in mice. In neutralization study, filtrate of water extracts from each plant or mixed-plants was pre-incubated with *N. kaouthia* venom at 37°C, 1 h prior to intramuscular injection. Water extracts from *A. catechu* or mixed-plants showed neutralization activity. *A. catechu* extract at dose 0.2 mg/mouse completely protected mice receiving the LD_{100} dose (8 µg/mouse) of *N. kaouthia* venom. Mixed-plant extract (*T. reidioides*: *A. catechu*) at a dose ratio of 2.4:0.8 mg/mouse prolonged the survival time of mice receiving LD_{100} of venom. It increased % survival of mice from 0% to 66.67%. The extract of *T. reidioides* did not have the neutralization activity.

In anti-lethal activity study, only the unfiltered water extract of mixed-plants (*T. reidioides*: *A. catechu*) at a dose ratio of 1.2:0.4 mg/mouse increased survival of mice from 6.25% to 31.25%, when given 1 h prior to the venom injection. It also decreased creatine phosphokinase (CPK) activity induced by *N. kaouthia* venom (4 µg/mouse) from 2,632 ± 498 units/L to 585 ± 139 units/L. In conclusions, preparation of *T. reidioides* and *A. catechu* could inhibit lethality and myotoxicity of *N. kaouthia* venom.

Key words : *Naja kaouthai* venom, *Trigonostemon reidioides* Craib., *Areca catechu* Linn., neutralization, anti-lethal activity

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ฤทธิ์ต้านพิษงูเห่าโดยสารสกัดโลดทะนงแดงและเมล็ดหมากในหนูถีบจักร

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของสารสกัดโลดทะนงแดงและเมล็ดหมากในการยับยั้งพิษที่ก่อให้เกิดการตายและการทำลายกล้ามเนื้อของพิษงูเห่าไทย (*Naja kaouthia*) ในหนูถีบจักร ในการศึกษาฤทธิ์การทำลายพิษงูเห่า ใช้สารสกัดน้ำที่ได้จากโลดทะนงแดงหรือเมล็ดหมากหรือที่ผสมกัน และกรองก่อนใช้ นำมาผสมกับพิษงูเห่า และแช่ไว้ในอุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ก่อนที่จะให้หนูถีบจักรโดยการฉีดเข้ากล้าม พบว่าสารสกัดจากเมล็ดหมากหรือสารสกัดจากโลดทะนงแดงผสมกับเมล็ดหมาก มีฤทธิ์การทำลายพิษงูเห่า สารสกัดจากเมล็ดหมากในขนาด 0.2 มิลลิกรัม/หนู สามารถป้องกันการตายได้ 100 เปอร์เซ็นต์ เมื่อให้พิษงูเห่าในขนาดที่ทำให้ตายทั้งหมด (LD_{100}, 8 ไมโครกรัม/หนู) สารสกัดของสมุนไพรผสม (โลดทะนงแดง:เมล็ดหมาก) ในขนาด 2.4:0.8 มิลลิกรัม/หนู สามารถยืดเวลาการรอดชีวิตของหนูถีบจักรที่ได้รับพิษงูเห่าในขนาด LD_{100} สารสกัดนี้ทำให้การรอดชีวิตเพิ่มขึ้นจาก 0 เปอร์เซ็นต์ เป็น 66.67 เปอร์เซ็นต์ สารสกัดโลดทะนงแดงไม่มีฤทธิ์การทำลายพิษงูเห่า

ในการศึกษาฤทธิ์ต้านการตายจากพิษงูเห่า พบว่าสารสกัดน้ำจากสมุนไพรผสม (ไม่กรอง) เท่านั้นที่มีฤทธิ์ต้านการตายจากพิษงูเห่า เมื่อให้ในขนาด (โลดทะนงแดง:เมล็ดหมาก) 1.2:0.4 มิลลิกรัม/หนู ทำให้เปอร์เซ็นต์การรอดชีวิตเพิ่มขึ้นจาก 6.25 เปอร์เซ็นต์ ของหนูควบคุม เป็น 31.25 เปอร์เซ็นต์ เมื่อให้สารสกัดสมุนไพรผสม 1 ชั่วโมง ก่อนการให้พิษงูเห่า และยังพบว่าสารสกัดน้ำนี้ ทำให้การทำงานของเอ็นไซม์ครีเอทีนฟอสโฟไคเนส ที่ถูกกระตุ้นให้เพิ่มขึ้นด้วยพิษงูเห่า (4 ไมโครกรัม/หนู) มีค่าลดลงจาก 2,632 $\pm$ 498 Units/L ไปเป็น 585 $\pm$ 139 Units/L โดยสรุป สารสกัดสมุนไพรผสมของโลดทะนงแดงและเมล็ดหมากสามารถยับยั้งการตายและการทำลายกล้ามเนื้อจากพิษงูเห่าได้

คำสำคัญ: พิษงูเห่า, โลดทะนงแดง, เมล็ดหมาก, ฤทธิ์การทำลายพิษ, ฤทธิ์ต้านการตาย

Introduction

Snakebite is one of the most important public health problems of tropical countries including Thailand. Approximately 7,000 cases of snakebite are reported annually. The actual number of bites may be much higher with many unreported events. The cobra, *Naja kaouthia* is a common poisonous snake found throughout Thailand. It is once an important cause of death. The cobra is considered very dangerous and its venom produces systemic poisoning due to rapid action of neurotoxin causing respiratory paralysis and death. The toxin composes of neurotoxin, cardiotoxins, enzymes and proteins[1]. In addition to the respiratory crisis, local reaction of the bitten site is also a serious problem. Though not life threatening, the local reaction may prolong the duration of hospitalization and it may increase morbidity in some cases [2].

The combined preparation of root of Lot Thanong Daeng (*Trigonostemon reidioides* Craib.) and Mak seed (*Areca catechu* Linn.) has been used against snakebite by folk healers and physicians at Kabchoeng Hospital in Surin province for many years. However, their actions against snakebite are still unclear. Water extract from root of Lot Thanong Daeng has been reported to prolong survival time of snake envenomation in mice [3].

Chemical constituents in Lot Thanong Daeng root are a mixture of steroid palmitate (β-sitosteryl palmitate, stigmasteryl palmitate, campesteryl palmitate and cholesteryl palmitate), a mixture of long chain acid (C_{16}-C_{35}), a mixture of steroid (β-sitosterol, stigmasterol and campesterol), acetyl aleuritolic acid, trigonostemone (1,1,7-trimethyl-3,6,9-trimethoxy-2-phenanthrenone), 5-hydroxy-6,7-dimethoxycoumarin, 5,7-dihydroxy-6-methoxy coumarin, a mixture of long chain amide (C_{44}-C_{48}), a mixture of steroid glycoside, 5α-stigmastane-3,6-dione and water soluble constituents such as sugars, amino acids and chloride salts[4]. Sitosterol and stigmasterol have been reported to have anti-snake venom activity. They are able to neutralize the lethal dose of South American rattlesnake venom[5] and inhibit myotoxicity of crotalid venoms[6].

A. catechu Linn. (Palmaceae) is commonly known as Areca Palm, Betel Palm, Betel Nut Palm, and locally known in Thai as Mak or Makmia. Its nuts contain alkaloids namely, arecoline, arecaine, arecaidine, guvacoline, guvacine, and traces of choline. Tannins, gallic acid, gum, oily matter, and a number of amino acids are also among the constituents found[7]. Recently, Ruenraroengsak, 2002[8] reported that the seed of *A. catechu* contains high tannin contents. It contains both hydrolysable and condensed tannins. These tannins could inhibit lethal activity of snake venom in mice, inhibit acetylcholinesterase activity and protect necrosis in rats.

Therefore, we aimed to investigate the inhibitory effects of preparation from root of Lot Thanong Daeng and Mak seed on lethality and myotoxicity of *N. kaouthia* venom in mice. This research study has been ethically approved by the Ethical Committee on Animal and Human Research Studies, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Materials and Methods

Venom

Lyophilized *N. kaouthia* venom was obtained from Queen Saovabha Memorial Institute, Thai Red Cross Society, and was preserved at 2-8°C. It was dissolved in 0.9% saline and was frozen until used. Venom concentration was expressed in terms of dry weight.

Plants

The roots of Lot Thanong Daeng and dry Mak seeds were brought from Kabchoeng Hospital. The voucher specimens were identified by the Faculty of Pharmacy, Mahidol University. Both of them were ground into small pieces and made into powder. These powders were

kept in desiccators at room temperature until use.

Animals

Male Swiss albino mice weighed 18-20 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom province. They were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University under controlled environmental conditions (room temperature 25±1°C with 12-hours light/dark cycle, relative humidity of approximately 60%) with free standard mouse pellets and tap water. Mice were acclimatized for 3 days before experimentation.

Median lethal dose (LD_{50})

Median lethal dose (LD_{50}) was defined as the least amount of venom (µg dry weight) injected intramuscularly to animals and resulted in 50% death within 24 h. The venom solution with the doses of 3-12 µg/mouse were prepared in 0.9% saline. Venom solution was injected intramuscularly at the volume of 0.1 ml to each mouse. Eight mice were used for each test dose. Control animals were injected with 0.9% saline only. The percent death of animals was recorded within 24 h after the injection. The LD_{50} was calculated by probit analysis.

Neutralization of lethal venom effect

The powder of mixed-plants (*T. reidioides* 1.5 g: *A. catechu* 0.5 g) was dissolved with 50 ml distilled water. The solution was stirred for 5 min and filtered. The lethal dose (LD_{100}) of *N. kaouthia* venom (8 µg/mouse) was pre-incubated with 0.02 ml, 0.04 ml and 0.08 ml of the mixed-plants filtrate in 0.9% saline to final volume of 0.1 ml, at 37°C for 1 h. The pre-incubated solution of 0.1 ml was injected intramuscularly to left thigh of mice. The doses of mixed-plants (*T. reidioides*: *A. catechu*) were 0.6:0.2, 1.2:0.4 and 2.4:0.8 mg/mouse calculated as crude plant. The single plant were also tested using either powder of *T. reidioides* (1.5 g) or *A. catechu* (0.5 g), each powder was dissolved with 50 ml distilled water and filtered. The 0.02 ml filtrated of each plant was pre-incubated with *N. kaouthia* venom (8 µg/mouse) in 0.9% saline to final volume of 0.1 ml. at 37°C for 1 h. The percent survival was recorded within 24 h. Control group was pre-incubated only with snake venom and 0.9% saline to a final volume of 0.1 ml. Six mice were used in each group.

Inhibition of lethal venom effect

The mixed-plants powder (*T.reidioides* 0.15 g + *A. catechu* 0.05 g) was suspended in 50 ml distilled water and stirred for 5 min before use. Mice were starved 4 h before starting the experiment. Group 1 was fed with 0.2 ml mixed-plants solution. One hour after feeding, the *N. kaouthia* venom (6 µg /mouse) was injected intramuscularly to left thigh of mice. Group 2 was fed with same dose (0.2 ml) of mixed-plants solution and repeated again 30 min after the first feeding. Venom was injected intramuscularly 30 min after the second feeding. Mice were allowed to access to food after venom injection. The percent survival was recorded within 24 h. Control group was fed with distilled water and the venom was injected at 1 h after feeding. Sixteen mice were used in each group.

Inhibition of myotoxicity effect

Inhibition of myotoxic effect of venom was measured by quantitation of plasma creatine phosphokinase (CPK) activity as described previously by Mukherjee and Maity, 2002[9]. The mixed-plants powder (*T.reidioides* 0.15 g + *A. catechu* 0.05 g) was suspended in 50 ml distilled water and stirred for 5 min. Mice were starved 4 h before feeding with 0.2 ml mixed-plants solution. The sublethal dose of *N. kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 4 µg/mouse in 0.1 ml. Venom (0.1 ml) was injected intramuscularly 1 h after feeding. Control mice were injected with venom alone. Mice were sacrificed under ether anesthesia 4 h after venom injection. Blood sample was collected from inferior vena cava and

centrifuged at 3,000 rpm for 10 min. Plasma was assayed for creatine phosphokinase (CPK) activity by Professional Laboratory. Six mice were used in each group.

Statistical analysis

Data are expressed as mean ± S.E. Statistical significance was assessed by one-way ANOVA followed by Tamhane's T2. Values of $p<0.05$ were considered to indicate a significant difference.

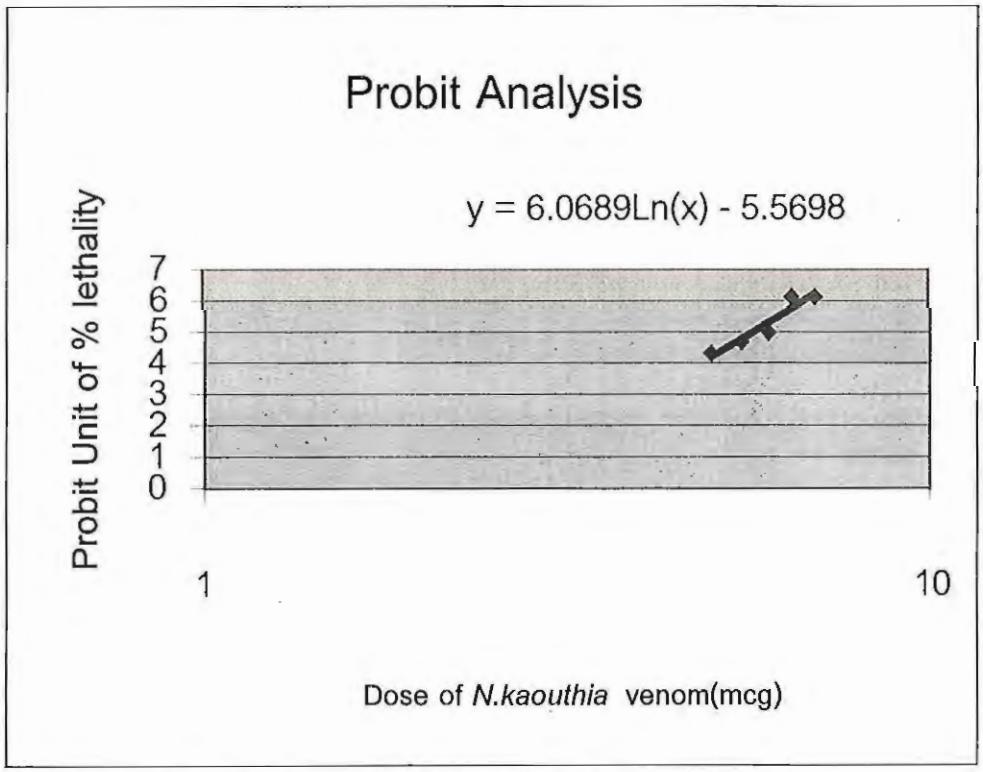
Results

Body weight and feed intake

Lethality data of *N. kaouthia* venom are shown in Table 1. Median lethal dose (LD_{50}) was 5.71 µg/mouse as calculated by probit analysis (Figure 1)

Table 1 The percent death of mice receiving various doses of *N. kaouthia* venom

Dose (µg/mouse)	Dead animal/total	% Death
5.0	2/8	25
5.5	3/8	37.5
6.0	4/8	50
6.5	7/8	87.5
7.0	7/8	87.5



LD_{50} was calculated from y = 6.0689 Ln (x) – 5.5698
LD_{50} is probit unit = 5, so at LD_{50} y = 5
5 = 6.0689 Ln (x) – 5.5698
Ln (x) = 10.5698/6.0689
X = 5.7067

Figure 1 Probit analysis for median lethal dose (LD_{50}) of *N. kaouthia* venom

Neutralization of lethality

N. kaouthia venom at dose 8 µg/mouse produced 100% death of mice. The water extracts of *A. catechu* alone and mixed-plants showed neutralization of *N. kaouthia* venom. The extract of *A. catechu* alone at dose 0.2 mg/mouse exhibited 100% protection of mice from the lethal dose of *N. kaouthia* venom. The percent survival was increased and the survival time was prolonged in mice receiving mixed-plants pre-incubated with venom (Table 2).

Table 2 The percent survival and survival time of mice injected with pre-incubated plant extracts and *N. kaouthia* venom

Treatment groups	Survival mice /total	% Survival	Survival time (min) mean ± S.E.
Control	0/6	0	106 ± 6.76
Mixed-plants (0.6:0.2 mg/mouse)	4/6	66.67	1,003 ± 276.26
Mixed-plants (1.2:0.4 mg/mouse)	4/6	66.67	1,038 ± 254.92
Mixed-plants (2.4:0.8 mg/mouse)	4/6	66.67	1,145 ± 192.34 *
T. redioides 0.6 mg/mouse	0/6	0	88 ± 6.44
A.catechu 0.2 mg/mouse	6/6	100	1,440 ± 0.00 *

* Significantly different from control, $p < 0.05$.
Data of survival time was calculated from all mice (died and survived mice).
For survived mice the survival time was calculated from 24 h (1,440 min).

Inhibition of lethality

The water extract of mixed-plants (*T. reidioides* : *A. catechu*) increased percent survival of mice fed 1 h before injection of *N. kaouthia* venom. The dose of *N. kaouthia* venom at 6 µg/mouse did not produce 100% death. The water extract of *T.reidioides*: *A.catechu* at dose 0.6:0.2 mg/mouse increased percent survival from 6.25% to 18.75% and at dose 1.2:0.4 mg/mouse increased percent survival to 31.25% (Table 3).

Table 3 The percent survival of mice feeding with plant extracts 1 h before injection of snake venom

Treatment groups	Survival mice/total	% Survival	Survival time (min) mean ± SE
Control	1/16	6.25	172 ± 84.68
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	3/16	18.75	401 ± 130.35
T.reidioides + *A.catechu* (1.2:0.4 mg/mouse)	5/16	31.25	531 ± 158.38

Data of survival time was calculated from all mice (died and survived mice).
For survived mice the survival time was calculated from 24 h (1,440 min).

Inhibition of myotoxicity effect

Injection of *N. kaouthia* venom induced myonecrosis as measured by plasma CPK activity which increased from 102 ± 2.63 units/L in normal mice (untreated control) to 2,632 ± 498.64 units/L (venom injected mice). The water extract of *T. reidioides* and *A. catechu* at the dose of 0.6:0.2 mg/mouse fed two times significantly decreased the CPK activity (Table 4).

Table 4 Inhibition of myotoxicity by plant extracts (n= 6)

Groups	CPK (units/L) Mean ± S.E.	% Inhibition
Normal untreated mice	102 ± 2.63	-
Control (venom injected mice)	2,632 ± 498.64	0
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	1,729 ± 607.36	34.31
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse) feeding two times	585 ± 139.38*	77.77

* Significant different from control, $p<0.05$.

Discussion and Conclusions

The water extract of Mak seed (*A. catechu* Linn.) significantly neutralized lethal dose of *N. kaouthia* venom when pre-incubated with snake venom before injected intramuscularly to mice. All mice in this group survived, whereas all control mice died. In the same way, extracts of mixed-plants (*T. reidioides* and *A. catechu*) also neutralized lethal dose of *N. kaouthia* venom. Four mice survived from total of six mice. When increasing dose of mixed-plants extract survival time of mice also increased. On the other hand, the extract of *T. reidioides* alone did not neutralized *N. kaouthia* venom, all mice in this group died and survival time did not differ from control. Therefore, the neutralization of *N. kaouthia* venom may be the effect of *A. catechu* only. However, effect of *A. catechu* presented in mixed-plants extract had the lesser activity than extract from *A. catechu* alone. This result may be related to pH of the extracts and/or the tannin content in the *A. catechu*. Extract of *A. catechu* had higher pH than extract mixed with *T. reidioides*. When increased dose of mixed-plants extract the capacity of neutralization of snake venom was also increased, so it seems to be dose dependent. This result was similar to previous study reported by Ruen-raroengsak, 2002 [8].

The water extract of mixed-plants increased percent survival of mice when administered orally 1 h before envenomation. Inhibition of lethal activity by mixed-plant extract seems to be dose dependent. In addition, this water extract also inhibited myotoxicity as shown by the decrease in plasma creatine phosphokinase (CPK) activity induced by envenomation.

In conclusions, the preparation of *T. reidioides* and *A. catechu* could inhibit lethality and myotoxicity of *N. kaouthia* venom in mice. The results of this investigation provides scientific support for the use of preparation from *T. reidioides* and *A. catechu* in treating snakebite patients as previously described by folk healer and doctor at Kabchoeng Hospital, Surin Province.

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วิทยานิพนธ์ปริญญามหาบัณฑิตสาขาวิชาชีววิทยา มหาวิทยาลัยมหาสารคาม 2541.

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