Evaluation of Cognitive Enhancing Effects of *Clitoria ternatea* Flowers Water Extract in Normal Male Mice
การประเมินฤทธิ์ของสารสกัดน้ำจากดอกอัญชันต่อการเพิ่มความจำในหนูถีบจักรเพศผู้

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ABSTRACT

Acetylcholinesterase (AChE) enzyme and oxidative stress play the crucial role in cognitive function. In addition, the effects of the plant extract can improve cognitive impairment. This study aimed to investigate the effect of *C. ternatea* flowers on cognitive enhancing effect, oxidative stress status and AChE inhibition in prefrontal cortex of normal male mice. The animals were grouped into five groups including control, vehicle and the extract at doses of 200, 400 and 800 mg/kg body weight. The animals were orally given substances once daily for 14 days. The object recognition test was performed on day 1, 7 and 14. Then, all animals were sacrificed and their prefrontal cortexes were collected for determination of malondialdehyde (MDA) levels and AChE activity. It was found that *C. ternatea* flowers water extract significantly improved cognitive function after administration of crude water extract at low and medium doses (200 and 400 mg/kgBW) for 14 days. The cognitive enhancement was immediately evident after 30 minutes of treatment. This improvement could be resulted from decreased MDA levels and AChE activity. However, toxicity and other behaviors associated with biochemical studies are still required for further study.

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บทคัดย่อ

เอนไซม์อะซีติลโคลีนและสารอนุมูลอิสระมีบทบาทสำคัญของต่อความจำ นอกจากนี้สารสกัดสมุนไพรยังมีฤทธิ์ปรับปรุงภาวะความจำบกพร่องได้ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของสารสกัดดอกอัญชันต่อการเพิ่มความจำ ระดับสารอนุมูลอิสระ และฤทธิ์ยับยั้งการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรสในสมองส่วน prefrontal ของหนูถีบจักรเพศผู้ สัตว์ทดลองถูกแบ่งเป็น 5 กลุ่ม ได้แก่ กลุ่มควบคุม กลุ่มที่ได้รับน้ำกลั่น และกลุ่มที่ได้รับสารสกัดดอกอัญชันขนาด 200, 400 และ 800 มิลลิกรัมต่อกิโลกรัม สัตว์ทดลองได้รับสารทุกวันเป็นเวลา 14 วัน ซึ่งการทดสอบความจำวัตถุของหนูถูกทดสอบในวันที่ 1, 7 และ 14 จากนั้นหนูจะถูกทำให้สลบและเก็บสมองส่วน prefrontal มาศึกษาระดับของสารอนุมูลอิสระ และการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรส พบว่าการให้สารสกัดดอกอัญชันขนาด 200 และ 400 มิลลิกรัมต่อกิโลกรัม เป็นเวลา 14 วัน สามารถเพิ่มความจำได้ ซึ่งฤทธิ์เพิ่มความจำนี้คงอยู่ได้ 30 นาทีหลังจากได้รับสารสกัด ซึ่งผลเพิ่มความจำนี้อาจเกิดจากการลดลงของปริมาณสารอนุมูลอิสระ และการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรสที่ลดลง อย่างไรก็ตามยังต้องมีศึกษาความเป็นพิษของสารสกัด และการทดสอบทางพฤติกรรมควบคู่การตรวจทางชีวเคมีอื่น ๆ ที่เกี่ยวข้องในการศึกษาครั้งต่อไป

Key Words : *Clitoria ternatea* flowers, Oxidative stress, Cognitive
คำสำคัญ : ดอกอัญชัน สารอนุมูลอิสระ ความจำ

Introduction

The recent evidence has been indicated that prefrontal cortex plays role in the cognitive functions including working memory, decision and attention [1, 2]. Acetylcholine is the neurotransmitter which plays roles in cognitive function including attention, memory consolidation. This neurotransmitter is also involved in arousal, sensory perception and in the control of motor activity, pain perception and body temperature regulation [3]. In addition, acetylcholine was catalyzed by acetylcholinesterase (AChE) enzyme to choline and acetate [4]. The impairment of cholinergic neurotransmission is caused of cognitive impairment especially Alzheimer's disease [5]. The acetylcholinesterase inhibitor (AChEI) is commonly used to treat this condition [6].

In biological system, the excessive oxidative stress is producing the reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (^-OH). These ROS may attack several organs to induce numerous disorders including cognitive impairment [7]. In addition, it has known for many years that the antioxidants which commonly present in medicinal plants are used to against cells damage and prevent the normal cells from oxidative stress [8]. Various studies have been demonstrated that administration of plant extract for 14 days can improve cognitive function [9, 10].

Clitoria ternatea (Family: Fabaceae) or butterfly pea is commonly used as the traditional Ayurvedic medicine to treat various diseases. The methanolic extract of aerial part

of *C. ternatea* possesses cognitive enhancing, anxiolytic, antidepressant and anticonvulsant effects [11]. In addition, the ethanol extract of *C. ternatea* leaves at doses of 200 and 400 mg/kgBW significant decreased MDA levels and AChE activity while increased antioxidant enzymes activities [12]. However, the water extract of *C. ternatea* flowers on cognitive function, oxidative stress and AChE activity in normal condition have not been reported.

Objectives of the study

In this present study, we aim to investigate the effect on cognitive enhancement, oxidative stress status and AChE inhibition effects of the C. ternatea flowers water extract in normal male mice.

Methodology

1. Plant materials and preparations

The *C. ternatea* flowers were collected from Khon Kaen province, Thailand. This plant was identified by Associated Professor Dr. Panee Sirisaard (Department of Pharmaceutical science, Faculty of Pharmacy, Chiang Mai University). The flowers were extracting by decoction method. Briefly, the C. ternatea flowers were homogenized then they were boiled at 100°C for 30 minutes and filtered through the nylon cloth. The extract was dried by lyophilizer and percent yield was 3.06. The extract contained total phenolic compound at concentration of 71.916 ± 1.816 mg/L gallic acid equivalence/mg extract.

2. Experimental animal protocols

Imprinting Control Region (ICR) male mice 8 week olds (35-40 grams) were obtained from the National Laboratory Animal Center, Mahidol Unversity, Nakornpathom, Thailand. All mice were housed under constant temperature and exposed to 12:12 light-dark cycle at the Animal Care Unit, Faculty of Medicine, Khon Kaen University, Thailand. The experimental protocols were approved by Institutional Animal Ethics Committee of Khon Kaen University (record no. AEKKU 89/2555). The mice were divided into five groups (n = 6 per group);

Group I: Control

Group II: vehicle (distilled water)

Group III: *C. ternatea* flowers extract 200 mg/kg body weight (BW)

Group IV: *C. ternatea* flowers extract 400 mg/kgBW

Group V: *C. ternatea* flowers extract 800 mg/kgBW

The animals in group II-V were orally given substances once a day for period of 14 days.

3. Determination of cognitive enhancing effect by object recognition test (ORT)

The ORT is a behavioral test that used to examine animal's memory performance based on the natural tendency of animals to explore novel objects [13].

The day before test, mice were given a training session where the animals were freely to explore the box for 3 minutes. On the day of test, two identical objects were presented in two opposite corner of the box during the first trial (T_1), and the amount of

time was taken by each mouse to complete 3 minutes of object exploration and the number of approach were recorded. Exploration was considered as directing the nose at a distance less than 2 cm to the object and/or touching it with nose or forepaw. During the second trial (T_2, 30 minutes after administration), one of the objects presented in T1was replaced by new object and mice was place in box for 3 minutes. Another trial was performed at 6 hours (T_3) after substance administration. The time spent for exploration of the familiar (F) and new (N) object were recorded and the discrimination index (DI) was calculated as follows:

$$DI = \frac{N-F}{N+F}$$

; where DI = discrimination index, N = exploration of the new object, F = exploration of the familiar object.

4. Determination of malondialdehyde (MDA) levels

A measurement of MDA levels was used to determine lipid peroxidation product. Briefly, the mixture composed of 50 µl of homogenate tissue, 50 µl of 8.1% sodium dodecyl sulphate (SDS), 375 µl of 20% acetic acid, 0.8% of thiobarbituric acid (TBA) and 150 µl of distilled water. Then the mixture was boiled at 100° C for 60 minutes. The mixture was cooled with tap water and added with 250 µl of distilled water and 1,250 µl of n-butanol:pyridine (15:1). Then the mixture was centrifuged at 4000 rpm for 10 minutes. The organic layer was separated to measure the absorbance at 532 nm [14].

5. Determination of acetylcholinesterase (AChE) inhibition

This method was working by used acetylthiocholine iodide (ATChI) as a substrate. ATChI was broken down to thiocholine and acetate by AChE. Thiocholine was reacted with dithiobis nitrobenzoate (DTNB) to produce yellow color. The absorbance of AChE activity was measured by spectrophotometer at 412 nm [15].

6. Statistical analysis

All data were presented as mean ± SEM. The statistical analysis was operated using SPSS® (version 17.0 for window®). The significant of data was performed using one way analysis of variance (ANOVA) followed by LSD post hoc test for multiple comparison. The statistical level was set up at P-value < 0.05.

Results

Cognitive enhancing effect of *C. ternatea* flowers water extract

The results of *C. ternatea* flowers extract on cognitive function were shown in table 1. It was found that the water extract significantly enhanced cognitive function in animals at low dose (200 mg/kgBW) and medium dose (400 mg/kgBW). Interestingly, the discriminative index at these concentrations significantly increased only in trial 2 at fourteen days after administration of extracts for 30 minutes before performing object recognition test. The discriminative

indexes were 0.498 ± 0.039 and 0.521 ± 0.136, respectively at P-value < 0.05; compared with vehicle (0.156 ± 0.078).

Table 1 The effects of *C. ternatea* flowers water extract on cognitive function

Group	Object recognition test (Discriminative index) T2			Object recognition test (Discriminative index) T3		
	Single dose	7-days	14-days	Single dose	7-days	14-days
Control	0.125 ± 0.074	0.192 ± 0.100	0.165 ± 0.063	0.274 ± 0.123	0.200 ± 0.090	0.199 ± 0.041
Vehicle	0.150 ± 0.098	0.210 ± 0.115	0.156 ± 0.078	0.261 ± 0.108	0.186 ± 0.118	0.289 ± 0.098
C. ternatea						
200 mg/kgBW	0.424 ± 0.246	0.492 ± 0.145	0.498 ± 0.039*	0.503 ± 0.145	0.404 ± 0.032	0.508 ± 0.174
C. ternatea						
400 mg/kgBW	0.492 ± 0.116	0.423 ± 0.168	0.521 ± 0.136*	0.403 ± 0.151	0.354 ± 0.181	0.491 ± 0.183
C. ternatea						
800 mg/kgBW	0.468 ± 0.175	0.422 ± 0.163	0.443 ± 0.144	0.201 ± 0.139	0.422 ± 0.193	0.500 ± 0.113

* = P-value < 0.05; compared with vehicle

The alteration of malondialdehyde (MDA) levels

The results of *C. ternatea* flowers water extract on the alteration of MDA levels were shown in Table 2. It was found that all doses of the extract shown the significant decreased the MDA level (0.021 ± 0.001, 0.020 ± 0.003 and 0.023 ± 0.004 respectively at P-value < 0.001, all; compared with vehicle (0.081 ± 0.015).

The alteration of acetylcholinesterase (AChE) activity

The results of *C. ternatea* flowers extract on the alteration of AChE activity were shown in Table 2. It was found that all doses of *C. ternatea* flowers extract shown the significantly decreased the activity of acetylcholinesterase enzyme (3.511 ± 0.516, 2.589 ± 0.575 and 2.035 ± 0.432 respectively, P-value < 0.001, all; compared with vehicle (9.907 ± 2.153).

Table 2 The effects of *C. ternatea* flowers water extract on MDA levels and AChE activity

Group	MDA levels (nmols of MDA/mg protein)	AChE activity (μmol/min g. protein)
Control	0.072 ± 0.012	7.325 ± 0.845
Vehicle	0.081 ± 0.015	9.907 ± 2.153
C. ternatea 200 mg/kgBW	0.021 ± 0.001***	3.511 ± 0.516***
C. ternatea 400 mg/kgBW	0.020 ± 0.003***	2.589 ± 0.575***
C. ternatea 800 mg/kgBW	0.023 ± 0.004***	2.035 ± 0.432***

*** = P-value < 0.001; compared with vehicle

Discussion and Conclusions

C. ternatea (Family: Fabaceae) is commonly used in Ayurvedic medicine. It has been investigated this plant in considerable details such as learning and memory enhancing, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant and sedative effects [16, 17]. In this study was found that *C. ternatea* flowers extract has cognitive enhancing effect by increased the discrimination index. However, the enhancing effect of extract after 14 days in this study was observed in 30 minutes (T2) after extract administration whereas after 6 hours (T3) of extract administration, the cognitive enhancing effect was not observed. This finding might be the result from the decreasing of the extract level in circular blood flow. In addition, the extract possesses AChE inhibition effects and oxidative stress suppression effect by decreased the AChE activity and decreased MDA levels in prefrontal cortex.

It has been reported that oxidative stress plays a crucial role in several diseases. The targets of oxidative stress are lipid, proteins and DNA [18, 19]. The principle product of oxidative stress is malondialdehyde (MDA) which is high toxic molecule [20]. In general, there are several mechanisms to counter act with oxidative stress by endogenous and exogenous antioxidants. Moreover, the previous studies have been reported that cognitive impairment is related with the degeneration of cholinergic neurons leading to cholinergic neurotransmission deficit. AChE is the enzyme that catalyzed acetylcholine tocholine and acetate. The activity of AChE can inhibit by AChE inhibitor by increase acetylcholine content [4].

In addition, the recent study showed that *C. ternatea* flowers extract significant inhibit AChE activity therefore, it might be improved cognitive function by inhibit AChE activity and suppress oxidative stress status. In conclusion, *C. ternatea* flowers extract shown to be the cognitive enhancing effect by decreased the MDA level and AChE activity.

Therefore, *C. ternatea* flowers extract might be the potential natural source for cognitive enhancing agent against cognitive impairment. However, the toxicity, other behavior, biochemical studies and possible active ingredients in this plant are still required before moving to clinical trial.

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