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

การประเมินฤทธิ์เพิ่มความจำของสารสกัดน้ำจากดอกอัญชันในหนูถีบจักรเพศผู้

Jindaporn Yimdee (จินดาพร ยิ้มดี)* Dr.Nongnut Uabundit (ดร.นงนุช เอื้อบัณฑิต)** Kowit Chaisiwamonkol (โกวิท ไชยศิวามงคล)*** Dr.Sitthichai Iamsa-ard (ดร.สิทธิชัย เอี่ยมสะอาด)**** Dr.Wiphawi Hipkaeo (ดร.วิภาวี หีบแก้ว)**** Dr.Jintanaporn Wattanathorn (ดร.จินตนาภรณ์ วัฒนธร)*****

ABSTRACT

Base on the crucial role of acetylcholinesterase enzyme (AChE) and oxidative stress is involved 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* 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 for 7 and 14 days. The object recognition test was performed on day 1, 7 and 14. Then, all animals were sacrificed and their prefrontal cortex was collected for determination of MDA levels and AChE activity. It was found that *C. ternatea* flowers 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. Further studies regarding its toxicity and, other behaviors associated with biochemical studies are still required.

บทคัดย่อ

เอนไซม์อะซีติลโคลีนและสารอนุมูลอิสระ มีผลต่อการเกิดความจำบกพร่อง นอกจากนี้ยังพบว่าสมุนไพรมีฤทธิ์เพิ่มความจำได้ จึงสนใจที่จะทำการศึกษาผลของสารสกัดน้ำจากดอกอัญชันต่อการเพิ่มความจำ ฤทธิ์ต้านสารอนุมูลอิสระ และฤทธิ์ยับยั้งการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรสในสมองส่วนพรีฟรอนทัลคอร์เท็กซ์ของหนูถีบจักร โดยแบ่งหนูเป็น 5 กลุ่ม ได้แก่ กลุ่มควบคุม กลุ่มที่ได้รับน้ำกลั่น และกลุ่มที่ได้รับสารสกัดดอกอัญชันที่ความเข้มข้น 200, 400 และ 800 มิลลิกรัมต่อน้ำหนักตัว(กิโลกรัม) สัตว์ทดลองได้รับสารเป็นเวลา 14 วัน ซึ่งความจำของหนูถูกทดสอบ ในวันที่ 1, 7 และ 14 หลังจากได้รับสาร 14 วัน หนูจะถูกทำให้สลบและเก็บสมองส่วน prefrontal มาศึกษาระดับของสารอนุมูลอิสระ และการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรส พบว่า ฤทธิ์เพิ่มความจำของสารสกัดน้ำจากดอกอัญชันสามารถคงอยู่ได้ 30 นาที หลังจากได้รับสารสกัด ซึ่งผลเพิ่มวามจำนี้อาจเกิดจาก การลดลงของปริมาณสารอนุมูลอิสระและการทำงานของเอนไซม์อะซีติลโคลีนเอสเทอเรสลดลง อย่างไรก็ตาม ยังคงต้องมีการศึกษาความเป็นพิษของสาร การทดสอบทางพฤติกรรมอื่นๆ ก่อนนำใช้ในการศึกษาทางคลินิกต่อไป

Key Words: *Clitoria ternatea* flowers, Oxidative stress, Cognitive

คำสำคัญ: ดอกอัญชัน สารอนุมูลอิสระ ความจำ

* *Student, Master of Science in Anatomy, Faculty of Medicine, Khon Kaen University*

** *Lecturer, Department of Anatomy, Faculty of Medicine, Khon Kaen University*

*** *Associate Professor, Department of Anatomy, Faculty of Medicine, Khon Kaen University*

**** *Assistant Professor, Department of Anatomy, Faculty of Medicine, Khon Kaen University*

***** *Associate Professor, Department of Physiology, Faculty of Medicine and Integrative Complementary Alternative Medicine Research group, Khon Kaen University*

Introduction

The recent evidence has been indicated that prefrontal cortex plays role in the cognitive functions including working memory, decision and attention (Gonzalez et al., 2013; Jo and Choi, 2014). Acetylcholine is the neurotransmitter which plays roles in cognitive function including attention, memory consolidation. In addition, this neurotransmitter is involved in arousal, sensory perception and in the control of motor activity, pain perception and body temperature regulation (Levin and Simon, 1998). In addition, the increasing of acetylcholinesterase (AChE) enzyme caused by the excessive lipid peroxidation (MDA) leading to the learning and memory deficit (Yang et al., 2013). The acetylcholinesterase inhibitor is commonly used to treat cognitive impairment (Stuchbury and Munch, 2005). Moreover, Naghizadeh and colleagues (2013) suggested that antioxidants may prevent and treat in cognitive impairment including Alzheimer's disease.

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 (Fukui et al., 2002). Moreover, it is known for many years that the antioxidants commonly present in medicinal plants are used for against cells damage and prevent the normal cells from oxidative stress (Pham-Huy et al., 2008).

Clitoria ternatea (Family Fabaceae) or butterfly pea is commonly used as the traditional Ayervedic medicine to treat various diseases. Jain and colleagues (2003) have been investigated the effect of *C. ternatea* on the central nervous system and they found that the aerial part of *C. ternatea* possesses cognitive enhancing, anxiolytic, antidepressant and anticonvulsant effects. However, it less evidences support of this flower on cognitive function.

Objectives of the study

In this present study, we aims to investigate the effect on cognitive enhancement, oxidative stress status and AChE inhibition of *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 Sirisa-ard (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 it was boiled at 100 $^{\circ}$C for 30 minutes and filtered through the nylon cloth. The extract was dried by lyophilizer. The percent yield of *C. ternatea* flowers water extract was 3.06. The extract contain 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 7 and 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 (Ennaceur and Delacour, 1988).

The day before test, mice were given a training session where the animals were freely to explore the box for 3 min. On the day of test, two identical objects was 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 minute 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 (T2, 30 mins after administration), one of the objects presented in T_1 (i.e., familiar object) was replaced by new object and mice was place in box for 3 min. Another trial was be performed at 6 hrs (T3) 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 level

A measurement of MDA level was described by Gupta and co-worker in 2003. 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 *n*-butanol:pyridine (15:1). Then the mixture was centrifuged at 4000 rpm for 10 minutes. The organic layer was separated. The absorbance was measured at 532 nm.

5. Determination of acetylcholinesterase inhibition

The AChE activity was measured following the method of Ellman and coworker in 1961. 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 dithiobisnitrobenzoate (DTNB) to produce yellow color. The absorbance of AChE activity was measured by spectrophotometer at 405 nm.

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 effects of *C. ternatea* flowers extract on cognitive function were showed in table 1. It was found that the water extract significantly enhanced cognitive function in animals at low dose (200 mg/kg) and medium dose (400mg/kg). 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 as compared with vehicle (0.156 ± 0.078).

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

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

* = P-value < 0.05; compared with vehicle

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

The alteration of malondialdehyde (MDA) level

The results of *C. ternatea* flowers extract on the alteration of MDA level were showed in Table 2. It was found that all doses of the extract showed 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).

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***

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

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

The alteration of acetylcholinesterase (AChE) activity

The results of *C. ternatea* flowers extract on the alteration of AChE activity were show in Table 2. It was found that all doses of *C. ternatea* flowers extract showed 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).

Discussion and Conclusions

C. ternatea (Family: Fabaceae) is commonly used in Ayervedic medicine. It has been investigated this plant in considerable details such as learning and memory enhancing, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant and sedative effects (Rai et al., 2005;Taranalli and Cheeramkuzhy, 2000). In this study has been study the flowers of this plant. It has been demonstrated that *C. ternatea* flowers extract has cognitive enhancing by increased the discrimination index. However, the enhancing effect of extract after 7 and 14 days in this study was observed in 30 minutes after extract administration whereas no significant increase discriminative index after 6 hrs of extract administration. 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 suppression effect by decreased the AChE activity and decreased MDA level in prefrontal cortex. The possible mechanism explanation for this finding may related with the AChE inhibition and the oxidative stress suppression effect of *C. ternatea* flowers extract might be promote cognitive function.

It has been reported that oxidative stress plays a crucial role in several diseases. The targets of oxidative stress are lipid, proteins and DNA (Lopez et al., 2007; Sudha et al., 2001). The principle product of oxidative stress is malondialdehyde (MDA) which is high toxic molecule (Del Rio et al., 2004). 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 to choline and acetate. The activity of AChE can inhibit by AChEI by increase acetylcholine content (Yang et al., 2013).

In addition, the recent studies showed that *C. ternatea* flowers extract significant inhibit AChE activity therefore, it might be improve cognitive function by inhibit AChE activity and suppressive the oxidative stress status.

In conclusion, *C. ternatea* flowers extract showed to be the cognitive enhancing effect by decrease the MDA level and AChE activity. Therefore, *C. ternatea* flowers extract might be the potential natural source for cognitive enhancing agent against learning and memory 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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