



ENHANCING COGNITIVE FUNCTION OF HEALTHY WISTAR RATS WITH AQUEOUS EXTRACT OF *Centella asiatica*

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ABSTRACT

Background: *Centella asiatica* (L.) Urb is a native herb from Asian countries such as India, China, and Indonesia. This herb has been widely used as a cure for various diseases. However, studies investigating the aqueous extract of *Centella asiatica* as a nootropic in healthy individuals are still very limited.

Objective: This study aims to investigate the potential of aqueous extract of *Centella asiatica* in enhancing cognitive function of healthy male Wistar rats.

Methods: Rats were randomly allocated to four treatment groups, i.e. without treatment and aqueous *Centella asiatica* extract at doses of 200, 400 and 800 mg/kg. To determine enhancement of cognitive function, novel object recognition (NOR) test was conducted after the course of treatment. Acetylcholine content was assessed by enzyme-linked immunosorbent assay.

Results: There was a significantly high preference index towards the novel object in the NOR test in groups treated with 200 mg/kg and 800 mg/kg of the aqueous extract compared to control. This was further confirmed by a significant increase of brain acetylcholine content in rats treated with 200 mg/kg of the extract.

Conclusion: Therefore, this study confirms that the aqueous extract is effective in enhancing cognitive performance of healthy Wistar rats.

Keywords : *Centella asiatica*, Acetylcholine, Novel object recognition, Cognitive function, Cognitive performance

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INTRODUCTION

Centella asiatica L, (Urb) also known as pegagan (Indonesia) or gotu kola (India), is a plant from the family of Apiaceae present in several Asian countries.[1] This plant has long been used to cure a number of conditions in traditional Indian medicine, such as skin eczema, leprosy, diarrhea, amenorrhea and dementia.[2] In the last two decades, there have been studies reporting the effect of *Centella asiatica* (CA) extract on various diseases, particularly in the field of neuroscience. Both whole extract and isolated compounds of CA have been shown to possess antioxidant effects and are able to modulate brain neurotransmitters thereby contributing to the improvement of epilepsy [3], anxiety [4], and cognitive function deficits.[5,6]

Centella asiatica extract has been shown to facilitate increased acetylcholine levels in the brain.[7] Acetylcholine is an ester product of acetic acid and choline, secreted by both central and peripheral nerve cells. It plays a role in improving memory and learning through its activity on nicotinic receptors.[8] The activation of these nicotinic receptors has been shown to modulate the course of Alzheimer's disease, resulting in improved cognitive function.[9] Thus, CA extract is a potential drug for individuals with cognitive deficit due to the course of a disease. However, studies investigating the potential of CA extract as a supplement in healthy individuals, are very limited. This supplemental use is based on the concept of nootropics in enhancing cognition in healthy individuals. Cognitive enhancers are generally used to alleviate cognitive deterioration in individuals with neurological diseases.[10] However, research has shown that even in 'healthy' subjects (without neurological

complaints), a decrease in cognitive performance is also possible, which can be due to oxidative stress.[11,12] This provides a plausible rationale for the use of cognitive enhancers in healthy subjects. Furthermore, Furey et al.[13] confirmed that healthy individuals treated with the cholinesterase inhibitor, physostigmine, exhibited enhanced cognition. Therefore, this study aimed to investigate the properties of aqueous CA extract in enhancing cognitive function of healthy Wistar rats.

MATERIAL AND METHODS

The present study was conducted at the Biotechnology and Animal Laboratory at the Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia. This study was reviewed and ethically approved by the Commission of Ethics in Medical Research of the Faculty of Medicine, Universitas Sriwijaya (Ethical Approval Certificate No. 130/kepkrsmhfkunsri/2017). Experiments related to the use of animals has complied with the relevant regulations for the care and use of animals. General experimental procedures in this study are depicted in Figure 1.

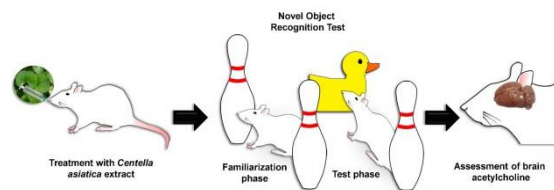


Figure 1. Experimental procedures

Animals

This study used 24 healthy white male Wistar rats, aged 2-3 months, weighing 200-300 g. Rats were obtained from a registered farm in Bandung, Indonesia. Rats were examined by a registered veterinarian and confirmed with

a health certificate issued by the local Livestock Division. Rats were housed at a temperature of $25\pm 2^{\circ}\text{C}$ and 12 hours light:dark cycle with food and water available *ad libitum*. They were acclimatized for 7 days before being randomly allocated into four experimental groups, i.e. control (untreated), and aqueous *Centella asiatica* extract at doses 200 mg/kg, 400 mg/kg, and 800 mg/kg.

Plant Extract

The plant was verified as *Centella asiatica* (L.) Urb (local name *pegagan* or *kaki kuda*) by the Plant Taxonomy Laboratory of Universitas Sriwijaya. as issued in the Letter of Plant Determination No. 174/UN9.1.7/4/PP/2017.

The extract was freshly prepared from aerial parts of CA. Plants were cultivated and air-dried for 10 days. Dry aerial parts were ground until fine powder was formed. The powder was weighed and brewed in distilled water which was already heated at 90°C with a ratio of 1:20 (1 gram of powder in 20 ml of distilled water). The mixture was allowed to stand for 30 minutes at room temperature before undergoing filtration. This was repeated twice to get a maximum yield of extract from the same batch of powder. The filtrate was then evaporated in a rotary evaporator (Heidolph, Germany) according to the manufacturer's settings for aqueous solvent (vacuum 72 mbar, rotation 50 rpm, vapor temperature 32.1°C , and bath temperature 60°C) until the extract volume decreased to 10-15% initial volume before being dried further. The dried extract was weighed accordingly and re-suspended in distilled water for experimental use. Three doses were used in this study, 200 mg/kg, 400 mg/kg, and 800 mg/kg. The extract was given per oral by using an oral-gastric tube with a final volume of 2 ml.

Novel Object Recognition Test

After acclimatization, rats were given treatment accordingly for 21 days before being evaluated by the novel object recognition (NOR) test. NOR test is a behavioral test that can be used to detect the efficacy of a drug on cognitive function.[14] This test is based on the internal drive of rats to explore unfamiliar objects (novel objects) in their surroundings. According to Ennaceur and Delacour[14], the average rat spends about 15 minutes to 1 hour to explore new objects in its surroundings.

This study used a modified protocol by Mathiasen et al.[15] The test was performed within one day, aiming for a short retention interval, which is 15 minutes. Rats were placed into a 50 x 50 x 35 cm plastic box with opaque walls lined with black cardboard on the outer surface. A camera was mounted on top of the box to record rat behavior during the test. The test was divided into 3 phases, the habituation phase (3 minutes), the familiarization phase (3 minutes), and the test phase (3 minutes).

In the habituation phase, rats were placed inside the empty box for 3 minutes. In the familiarization phase, the box was filled with 2 identical objects in the form of the same colored plastic bowling pins which were oppositely arranged in a diagonal fashion. Rats were placed in the middle of both objects, facing the wall of the box to avoid bias preference against one of the objects. They were left to explore both objects in the box for 3 minutes. After that, rats were returned to their cage for 15 minutes for memory retention.

In the 3-minute test phase, one of the bowling pins was replaced with a novel object in the form of a plastic duck. The

researcher recorded and calculated the time required for the rats to explore both the familiar (bowling pin) and the novel object (duck). The criteria of rat exploration was if the animal directed its nose towards the object within a distance of ≤ 2 cm and/or stuck its nose to the object. The distance was confirmed by comparing the experimental videos to a control video where the distance was measured real-time. The duration of exploration on the familiar object (bowling pin) and novel object (duck) was calculated as the preference index, which was expressed as the percentage of the exploration time for the familiar or novel object against the total exploration time for both objects.[16]

Enzyme-linked Immunosorbent Assay (ELISA)

After the 21-day treatment, rats were sacrificed by inhalation of chloroform, and whole brains were evacuated. Specimens were weighed before being minced with scissors. To inhibit the activity of acetylcholinesterase on the tissues, tubes containing the specimens were placed into boiling water for 10 minutes.[3] This study used an ELISA kit (Sunlong, China) for rat acetylcholine in accordance to the manufacturer's instructions. The sample was further homogenized with a tissue homogenizer, then centrifuged at 867 g for 20 minutes at 4°C. The supernatant was transferred into a new tube for ELISA. This study used 6 concentrations of the acetylcholine standard (Sunlong, China) for the determination of the standard curve. The ELISA plate was inserted into the microplate reader to determine absorbance (optical density-OD) at a wavelength of 450 nm.

Phytochemical screening

Phytochemical screening was performed to identify the class of active compounds contained in the aqueous extract. Identification of alkaloids was carried out using Wagner's test, whereas steroid/triterpenoids used Lieberman-Burchard method.[17] Tannin and phenol were assessed by addition of 1% and 2.5% FeCl_3 , respectively. Saponin was evaluated by addition of distilled water followed by vigorous shaking. Flavonoid was identified by using thin layer chromatography (TLC) using an F254 silica gel plate (Merck, Germany) developed with 60% ethyl acetate – 40% methanol. After the plate has been developed, it was exposed to NH_3 gas followed by brief heating at 100°C before being visualized under ultraviolet light at 366 nm.

Data analysis

The standard curve was created using Microsoft Excel. Data were analyzed with SPSS v.19 statistical software. The analysis included normality test with Shapiro-Wilk test, followed by bivariate analysis (Mann-Whitney or independent t-test) and multivariate (ANOVA) followed by Tamhane's *T2 post hoc* test. Values were significant if $p \leq 0.05$.

RESULTS

NOR Test

NOR test is a rapid behavioral assessment for preliminary screening of cognitive function. This assay stems from the instinctive behavior of rats to explore novel objects, without reinforcing stimuli such as hunger or fear. In this study, treatment with 200 and 800 mg/kg of CA extract significantly enhanced preference index of novel object compared to familiar object (Figure 2).

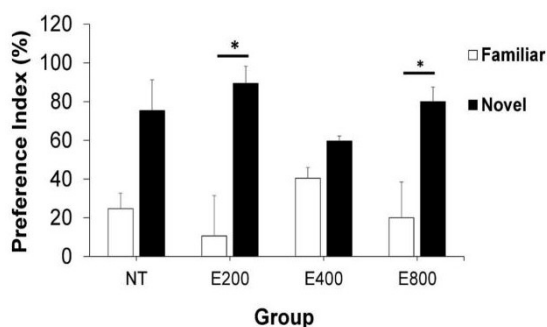


Figure 2. Preference index for familiar and novel object

(NT: no treatment; E200: 200 mg/kg extract; E400: 400 mg/kg extract; E800: 800 mg/kg extract); n = 6 independent experiments. Data are presented as mean \pm SEM. *p < 0.05

Acetylcholine content in the brain

Acetylcholine content in the brain was determined by ELISA. The standard curve was created by using 6 concentrations, 6.25 pg/ml, 12.5 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml, and 150 pg/ml. Linear regression was applied, hence the equation $y = 0.0024x + 0.0257$ with $R^2 = 0.9768$. Figure 3 shows a significant increase of acetylcholine content in brains of rats treated with 200 mg/kg of CA extract compared to control.

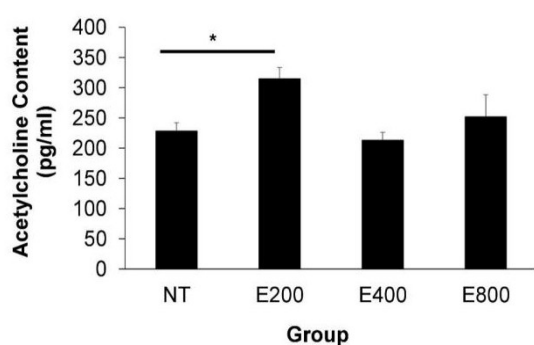


Figure 3. Acetylcholine content in the brain (NT: no treatment; E200: 200 mg/kg extract; E400: 400 mg/kg extract; E800: 800 mg/kg extract); n = 6 independent experiments. Data are presented as mean \pm SEM. *p < 0.05

Active compounds in *Centella asiatica* aqueous extract

The aqueous CA extract is approximately 10-15% of dry weight of the herb (Figure 4). Phytochemical tests and thin layer chromatography were performed to detect active compounds contained in the extract. This study confirmed the presence of tannin, phenol, flavonoid and steroid in the extract (Figure 5).



Figure 4. *Centella asiatica* Fresh (A) and air-dried *Centella asiatica* (B) used in the present study

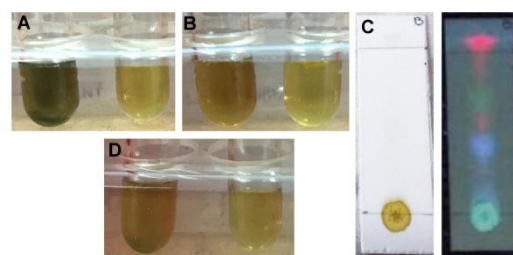


Figure 5. Phytochemical screening of active compounds
Identification of (A) tannin, (B) alkaloid, (C) flavonoid and (D) steroid.

DISCUSSION

To achieve a less-biased evaluation of cognitive performance, this study used the NOR test which does not require external stimuli pressuring the rats to succeed. This is different to the concept of reward and punishment or other external stimuli (e.g. hunger in operant conditioning [18] and T-Maze [19], or fear in elevated plus maze test [20] and passive avoidance test [21]. The test in the present

study also used a relatively short retention interval i.e. 15 minutes, to focus on retention of short term memory. This is certainly different from the Morris water maze test which involves repeated learning, thus allowing stronger memory association as it uses several days of training.[7]

Two identical objects which were introduced in the familiarization phase of the NOR test are termed as 'familiar objects'. Retention time allows rats to establish a recognition memory towards those objects. Therefore, exposure to one familiar object and one novel object during the test phase triggers retrieval of the recognition memory, causing the rats to explore the latter instead.[16,22] Although insignificant, there was a notable increase of preference index for novel objects compared to familiar objects in the non-treated rats. However, this study showed that only those treated with 200 and 800 mg/kg of aqueous CA extract significantly increased the preference index for novel object compared to familiar object. This suggests that this extract specifically enhanced memory retention, similar to that demonstrated by Jared et al.[18]

Previous studies have reported that extracts of CA was effective in improving spatial learning[20,21], and cognitive function in stress-induced.[7,23] These CA extracts contained triterpenoids, such as asiaticoside and madecassoside, which exhibited acetylcholinesterase inhibitory activity in vitro[24] and in vivo[3] and are associated with neuroprotection.[19,25]

However, the present study showed that the aqueous CA extract did not contain triterpenoids, but was positive for tannin, alkaloid, flavonoid, and steroid (Figure 5). The presence of triterpenoids in a CA extract is highly dependent on the location and diverse environmental

condition[26–29]. Orhan et al.[28] pointed out that extracts obtained from CA planted in different regions, such as China, Turkey, and India had different concentrations of triterpenoids such as asiaticoside and madecassoside, from most abundant to none. However, they demonstrated that even with little or no triterpenoid content, the extracts obtained from Turkish and India CA showed modest inhibition of butyrylcholinesterase.[28] This selective inhibition is likely to be the cause of increased acetylcholine content in the brain, as both butyryl cholinesterase and acetylcholinesterase hydrolyze acetylcholine.[30,31] In fact, previous studies using butyrylcholinesterase inhibitors have demonstrated elevated levels of acetylcholine which was associated with improved cognitive function.[30,31] This possible mechanism in the present study suggests the involvement of compound(s) other than triterpenoids which may contribute to enhanced cognition.

In addition, the presence of flavonoids in the extract of the present study may also contribute to improved cognition. The mechanisms by which flavonoids enhance memory and learning has been well documented, including modulation of synaptic plasticity via PI3-kinase pathways in the hippocampus[32] and an increase of nitric oxide levels which enables vasodilation leading to increased cerebral blood flow.[33,34] Flavonoids have been reported to be responsible for the scavenging activity of aqueous CA extract, which has an IC₅₀ of 31.2 µg/ml compared to that of ascorbic acid (2.5 µg/ml).[35] This antioxidant capacity, as well as the modulation of voltage-dependent anion channel (VDAC) in the mitochondria, was cytoprotective for neural cell lines.[36]

CONCLUSION

This study demonstrated that short term memory of healthy cohorts can be enhanced by consuming aqueous CA extract. The recommended dose was 200 mg/kg, as treatment with this particular dose was confirmed with elevated brain acetylcholine content. In absence of triterpenoids, this suggests other indirect mechanisms contributing to this increase, such as modest butyrylcholinesterase activity and the role of flavonoids. The diverse environment where the CA is cultivated is likely to play an important factor in determining the composition of chemical constituents of its extract, prompting necessary qualitative standardization. Further toxicity studies are also required to investigate safety issues in the continuous and intermittent use of this aqueous extract in healthy cohorts.

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