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Anti-Amnesic Effect of *Curculigo orchioides* Plant Extract and Its Function on Acetylcholinesterase Enzyme

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and one of the most predominant causes of dementia in older people. Preventing acetylcholine from breakdown by an excess amount of acetylcholinesterase is a proven therapy for AD. Traditionally *Curculigo orchioides* is known for its antioxidant activity, enzyme inhibition activity, and other medicinal uses. In this study, in vitro acetylcholinesterase, inhibitory activity was investigated, and methanolic extract of the plant showed significant activity. This study aims to investigate cholinesterase inhibitory activity, learning, and memory-enhancing activity of *Curculigo orchioides.*
Materials and Methods: The crude methanol extract of the dried powder was prepared by the cold extraction method. *In-vitro* acetylcholinesterase (AChE) inhibitory activities were determined by the modified Ellman's method. To confirm learning and memory-enhancing effects, the scopolamine-induced memory impairment Swiss-albino mice were used, and to find the anti-amnesic effect of the extract, a passive shock avoidance task was applied.
Results: Results proved that scopolamine-induced cognitive dysfunction decreased significantly by administration of the plant extract solution in the passive avoidance task, and inhibited brain

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acetylcholinesterase activity. While running an in-vitro AChE inhibitory test the IC50 was 0.782±0.067µg/ml.
Conclusion: The results suggested that methanolic extract of *Curculigo orchioides* can inhibit AChE in-vitro. Besides this, it provides data that proves its ability to improve whole brain AChE activity and enhance memory.

Keywords: Curculigo orchioides; cholinesterase inhibitor; passive avoidance; alzheimer's disease.

1. INTRODUCTION

Preventing acetylcholine (ACh) from degradation in synapses of neurons by inhibiting cholinesterase enzyme (especially acetylcholinesterase) is one of the most preferred therapies for treating the symptoms of Alzheimer's disease (AD) [1]. In the brain, the pivotal role of the acetylcholinesterase (AChE) enzyme is to breakdown ACh into acetate and choline and terminate the nerve impulse transmission at the cholinergic synapses. Patients with AD suffers from cognitive decline and due to neurodegeneration, the number of neurotransmission also decreases. For this reason, inhibition of AChE increases the availability of ACh in the synapses by preventing early breakdown. This process indicates as an accepted strategy for not only the treatment of AD, but also myasthenia gravis, and a few form of dementia [2-4]. After the introduction of the first cholinesterase inhibitor, it became the first line of choice for the therapy of mild to moderate AD. A few synthetic drugs (e.g. donepezil, tacrine), and natural product-based drug (e.g. rivastigmine) are used nowadays for the treatment of cognitive dysfunction, and memory loss associated with AD [5,6]. Most of the available drugs are based on the modification of the manifestations of AD. However, a large number of these drugs lack selectivity, and brings several potential side effects with them, while natural products have successfully proven to be a potential source of useful AChE inhibitors. A number of these cholinesterase inhibitors have been reported to have some unwanted effects including gastrointestinal disturbances and poor bioavailability, which demands to find out a better AChE inhibitors from the natural resources [7].

Medicinal plants are used as a potential, promising, and affordable source of AChE inhibitors [8-11]. Many plant origin AChE inhibitors have already proven their efficacy. Among them, galanthamine and rivastigmine (plant-derived bioactive molecule) are currently approved drugs for AD. However, these drugs only offer symptomatic relief from AD without preventing disease progression [12,13]. Besides this, some other plants and their extracts are also proven to improve the AChE condition both in-vivo and in-vitro [14,15]. *Curculigo orchioides* (Family: Amaryllidaceae) is a perennial herbaceous plant with tuberous rootstock. The herb is commonly found in sandy regions of hotter parts of Southeast Asian countries. This herb is well known for its medicinal uses [16,17]. Several studies reveal that *C. orchioides* is a rich source of bioactive phytochemical compounds. Bioactive molecules generally occur in small amounts in different parts of the plant and exert a more subtle effect compared to the nutritious elements [18]. The bioactive molecules can influence several cellular activities that modify the risk of various diseases. *C. orchioides* is a potent source of polyphenols, tannins, alkaloids, triterpenoids, glycosides, and flavonoids [19]. Besides, these various triterpenoid glycosides were isolated from the rhizomes [20]. Due to the presence of bioactive molecules, *C. orchioides* exert antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, anti-microbial, anticancer, hepato-protective, and cardio-protective properties [21-25]. Though, *C. orchioides* is a rich source of antioxidants and anti-inflammatory agents, there is no sufficient study that provide information regarding memory enhancing capability, and cholinesterase enzyme inhibitory activities of this plant. Thus, the evaluation of the in-vitro cholinesterase inhibitory activity, and in-vivo memory enhancing property of *Curculigo orchioides* were carried out to find on what extent it enhances memory, and inhibits cholinesterase enzymes.

2. MATERIALS

A. Drugs and Chemicals

Active ingredients like acetylcholinesterase enzyme (Electric-eel), acetylthiocholine iodide (ATCI), and 5,5´-dithiobis(2-nitro) benzoic acid (DTNB), physostigmine, and scopolamine were collected from Sigma (Japan). Other solvents,

salts, and buffers were collected from the Pharmacology and Advanced Analytical lab of the Pharmacy Department, East West University.

B. Plant material

The plant was collected from the Rajshahi and Bogura district of Bangladesh in June, 2020. After collection, the plant was identified by an expert taxonomist. A voucher specimen (Voucher No. 017-20-008) has been deposited at the Department of Pharmacy, East West University, Bangladesh.

2.1 Extraction of Plant Material

Collected whole plants were cut into small pieces, and dried into the hot air oven at 50^0C to remove extra moisture from the plant. The dried plant material was then grinded and formed a fine powder of 550g. After that, the powder was macerated in methanol to extract the bioactive components (like polyphenols, alkaloids, tannins, glycosides etc.) from the powder. The solvent was then filtered through filter paper to remove undissolved suspended material, and then evaporated to dryness under reduced pressure in a rotary evaporator at 55^0C to obtain the crude methanol extract (26.12 g) for the final experiment.

A. Animals

As test animal, swiss-albino mice (*Mus musculus*) of either sex, weight around 20-25g, were used in two in-vivo experiments including passive shock avoidance test, and whole brain acetylcholinesterase activity. These mice were kept in a group of five in plastic cages under a controlled environment of a 12/12 hour light-dark cycle. The room temperature was adjusted to 20 ± 2^0C with controlled humidity of 50±5% and these mice were allowed free access to food and water. Before starting the experiment, mice were brought group-wise to the experimental room, and waited for 1hr for the behavioral settlement. The reason behind this was to habituate the mice to the laboratory environmental conditions, and not to keep those in a panic condition. Handling and experimentation of those mice were conducted in the pharmacology laboratory by following the international ethical guidelines concerning the care and use of laboratory animals.

B. Animal Treatments

Experimental mice were divided into 6 groups, each group filled with 5 members. The first group (Group 1) was termed as the control animals group that received DMSO (1 ml/100 g, i.p.) for seven consecutive days. Each mouse of this group was injected with saline intra-peritoneally every one hour after the last dose and 30 min before training session.

The second experimental group (Group 2) was treated with Scopolamine. These mice received DMSO (1 ml/100 g, i.p.) for seven days. One hour after the last dose and 30 min before the training session, the mice were treated with scopolamine dissolved in saline (1 mg/kg, i.p.).

Groups 3 to 5 received different doses of *C. orchioides* (100, 200, 400 mg/kg i.p.) for seven days with Scopolamine. Here, scopolamine was used with different concentration of plant extracts to observe the overall improvement of the brain activity. One hour after the last dose and 30 min before the training session, the mice received scopolamine dissolved in saline (1 mg/kg, i.p.). Retention of the mice (memory) was recorded after 24 h.

Group 6 were treated with Physostigmine, as it is used as an established anticholinesterase agent. It was dissolved in normal saline, and administered (0.2 mg/kg, i.p) to the positive control group followed by scopolamine. Animals that exhibited anti-amnestic effects were then sacrificed. Brains were dissected, and stored at -80°C till further investigation.

2.2 Methods

A. *In vitro* Cholinesterase Inhibition Assay [26]

Modified Ellman's colorimetric method was applied to investigate *in-vitro* AChE inhibitory assay, and ATCI was used as a substrate. The hydrolysis rate of AChE was monitored spectrophotometrically. Extract of *C. orchioides* (various concentrations) and Physostigmine was mixed with an AChE enzyme solution (200µL), and incubated at room temperature for 30 min. Then Ellman's reaction mixture (400 µl of 0.35 mM ATCI, 200 µl of 0.7 mM DTNB) was mixed with an extraction buffer (50 mM Tris.HCl buffer, 50 mM $MgCl_2$, 50mM NaCl, 1% Triton X-100, pH 8.0) to adjust its final volume (3ml). Absorbance was taken at 412nm after 30 min of incubation for 37°C. Using nonlinear regression analysis, the IC50 (the concentration of extraction at which 50% of the enzyme is inhibited) of the plant extract was calculated. To calculate this a software program (GraphPad Prism, version

5.01, Inc., 2007, San Diego California USA) was used.

B. Passive Shock Avoidance (Step-Through) Paradigm [27]

For testing passive shock avoidance of the mice, a step-through passive avoidance apparatus for mice was used (Ugo Basile, Italy). Each of the test animals went through two individual sessions. The first one was the training session, and the final one was the test session. The passive avoidance apparatus consisted of two different compartments, named light and dark.

Training session: During the training session of the mice, each mouse was trained by gently placing it in the light compartment. If the mouse stepped to the dark compartment putting all its paws on the grid floor, the door closed automatically, and an electric shock of 1mA was delivered to it for 1sec. If a mouse failed to step through within the cut-off time (90 sec), then it was not allowed for further analysis.

Test session: After twenty-four hours of training of the mice, every single mouse was introduced to the light compartment. The latency of the mouse to step through the dark compartment was recorded as behavior of passive avoidance of shock which indicates memory acquisition, with an upper cut-off time of 300 sec. During the test session, no electric shock was delivered to the mouse.

C. Estimation of Whole Brain Acetylcholinesterase Activity [28]

To estimate the whole brain acetylcholinesterase activity, modified Ellman's colorimetric method was applied. After the behavioral testing, the mice were decapitated. The brains of the mice were dissected out immediately, and placed in ice-cold buffer saline. The whole brain was weighed, and homogenized in 0.1M Phosphate buffer pH 8.0 (10% w/v). Homogenized mice brain tissue was centrifuged for 10 minutes at 15000 rpm. During centrifugation, the temperature was adjusted at 4^0C to prevent enzyme degradation. An amount of 0.4ml supernatant was added to the other reagents, including 400µl of 0.35mM ATCI, 200µl of 0.7 mM DTNB and extraction buffer (50 mM Tris.HCl buffer, 50 mM $MgCl_2$, 50mM NaCl, 1% Triton X-100, pH 8.0) to adjust a final volume of 3ml. Absorbance of this mixture was taken at 412nm after 30 min of incubation for 37°C. Any change in absorbance due to hydrolysis of the substrate was corrected by subtracting the rate of the reaction before adding the enzyme to the mixture. All the tests were carried out twice and in triplicate.

D. Statistical Analysis

In vitro assay of AChE inhibitory activity was triplicated. The enzyme inhibitory activity of the plant extract was determined from dose response curves, and defined as the IC_{50} value, and expressed as mean ± standard deviation. The calculation was done by using GraphPad Prism, version 5.01, Inc., 2007, San Diego California USA. Other data were expressed as mean ± standard error, and analyzed by one-way ANOVA followed by Tukey test to assess significant differences among the treatment groups. Probability values of less than 0.05 were considered statistically significant.

3. RESULTS

A. *In vitro* Cholinesterase Inhibition Assay and Determination of IC50

Methanolic extract of the whole plant provides promising in vitro AChE inhibitory activity. IC50 of the extract found 0.782±0.067µg/ml, whereas IC50 of the standard physostigmine was 0.042 ± 0.017µg/ml.

B. Step-Through Latencies of Mice in Passive Avoidance Paradigm

When compared with the controlled group of the mice ($p<0.001$), a significant decline in the latency time was observed in the scopolamine-induced mice. The test groups of the mice those treated with *C. orchioides* provides a significantly delayed latency time in retention trials when compared with scopolamine-treated mice ($p< 0.05$, respectively) (Fig. 1). A concentration of dependent latency time was observed, i.e. the latency time of mice increases with increased extract concentration. In the control group, the latency time was 113.17±3.23 sec. In group 2, latency time decreased to 57.81±2.56 sec. In group 3, group 4, and group 5 the latency time was 61.72±2.76, 68.96±3.11, and 87.19±2.76 sec respectively. Finally in group 6, (physostigmine, 0.2mg/kg) it was 107.32±2.19 sec.

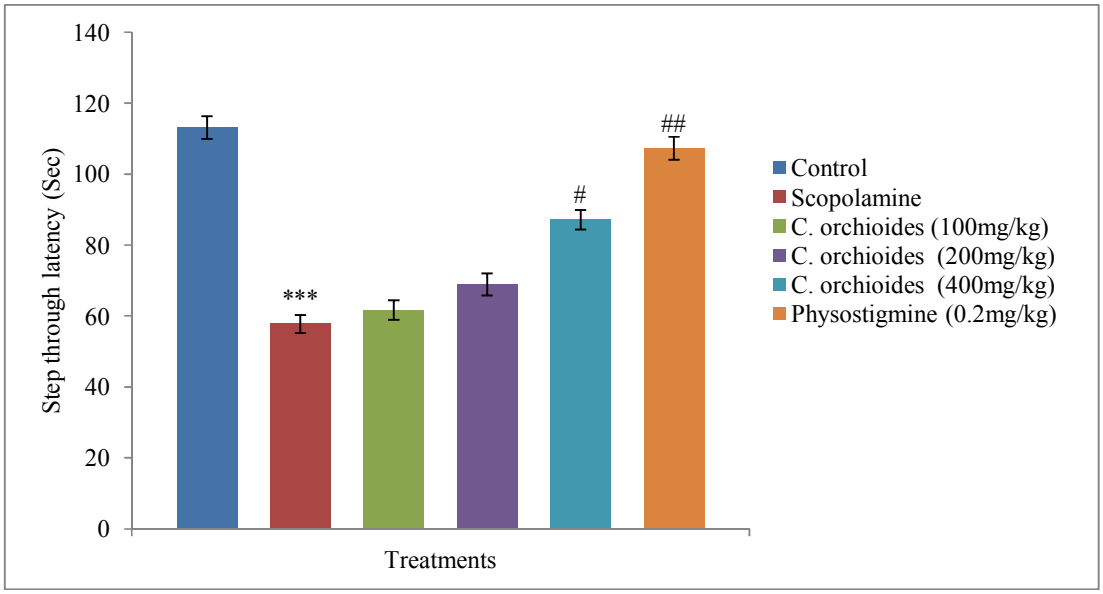

Fig. 1. Effects of *Curculigo orchioides* extract on scopolamine-induced amnesia of a step-through passive avoidance task in mice. The extract was intraperitoneally administered for 7 days. Scopolamine (1 mg/kg, i.p.) was injected 1 h following treatments of day 7, followed 30 min later by the training session. Step through latency values are presented as means ± S.E.M. (n = 5). #p<0.05, ##p< 0.01, compared to scopolamine-treated group, ***p<0.001 compared to control group (one-way ANOVA followed by Tukey test)

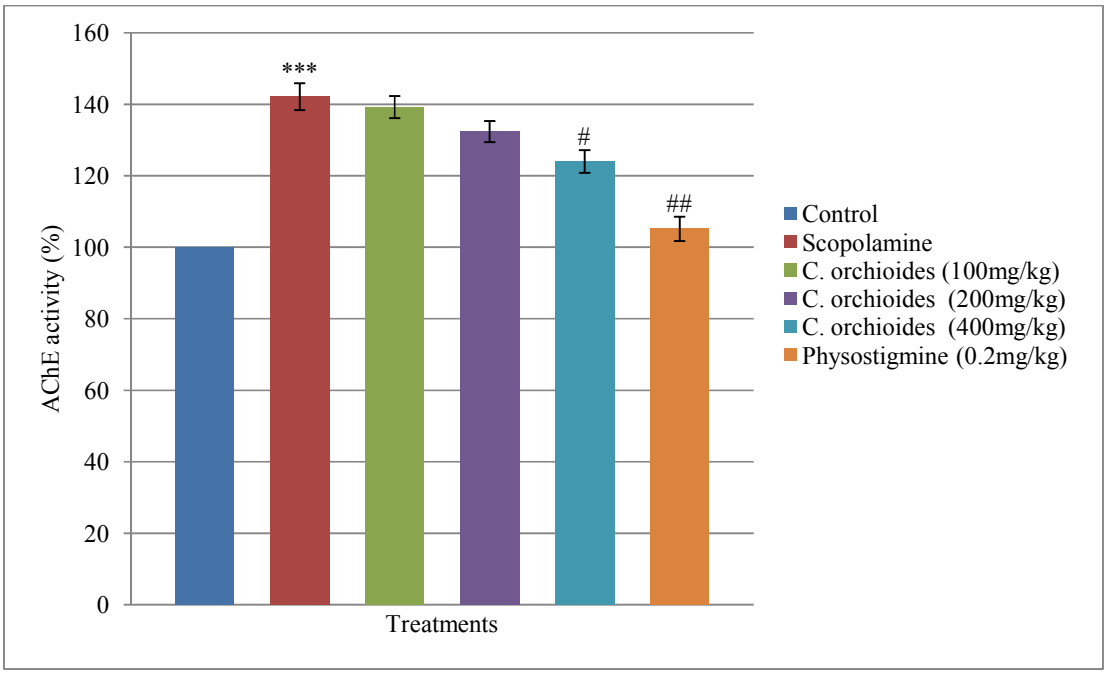

Fig. 2. Inhibitory effects of *Curculigo orchioides* extracts against brain AChE activity in scopolamine treated mice. Inhibition value of AChE (%) was calculated and compared to that of control group. Data represent the mean ±S.E.M (n=5) and were analyzed by one-way ANOVA followed by Tukey test. #p<0.05, ##p< 0.01 compared to scopolamine-treated group, ***p< 0.001 compared to control group

C. Total Brain AChE Activity

In total brain AChE activity, scopolamine treated mice had shown significantly high AChE activity in comparison to the control group ($p<0.001$) (Fig. 2). With the increase in the extract concentration in mice, the total AChE activity decreases. Group of mice treated with 400 mg/kg of C*. orchioides* significantly inhibited AChE activity in comparison to scopolamine-treated mice ($p<0.01$). In this test, physostigmine was used as a standard.

4. DISCUSSION AND CONCLUSION

AChE is recognized as one of the key enzymes in the cholinergic nervous system. To alter the cholinergic deficiency in AD, and treat the symptoms of AD patients several therapies were designed mostly based on AChE inhibition. These inhibitors enhance cholinergic transmission by inhibiting AChE into the brain with modest and transient therapeutic effects. Several studies suggested that cholinesterase inhibitors could act on multiple therapeutic targets of AD progression, such as prevention of the formation of beta-amyloid plaques, antioxidant activity, and modulation of amyloid precursor protein processing [29]. However, a scarcity of lead compound that lowers the level of AChE, simultaneously having a higher central nervous system penetration, is still in demand. Plants from different geographical origin are examined nowadays to find the appropriate lead compound that can inhibit AChE without any major side effects. This study showed that the methanolic extract of the *C. orchioides* exhibited cholinesterase inhibitory activity in both in vitro and in vivo conditions. A number of bioactive molecules have already been discovered from this plant having antioxidant and anti-inflammatory activities [19,20]. There is a link between the presence of antioxidant and anti-inflammatory agent with cholinesterase inhibitory activities [30].

Passive avoidance step through model mice were used in this study to evaluate memory retention of mice. These test result help to study learning and memory mechanisms in the test mice. The results give a clear idea that the extract of the plant has the ability to modify cognitive processes. The results also showed that the extract of the plant have an impact in the improvement of learning and memory processes in a scopolamine-impaired memory model. There is some connection in the AChE inhibition and memory improvement. Because the performance of a working memory task in a mice is significantly more sensitive to disruption of cholinergic mechanisms by AChE, in the hippocampus, than performance of reference memory task [31]. As the plant extract inhibit the AChE, so it possesses some ability to improve memory function of a mice. While testing with mice, a concentration of dependent latency time was observed, and the latency time of mice increases with the concentration of plant extracts. This increased latency time indicates the improvement of the learning and memory process.

In the scopolamine-induced mice model, AChE activity of the total brain was observed. In scopolamine-induced amnesia, the activity of AChE was found in increased level because of the increased calcium influx followed by an oxidative stress in the cell. A concentration-based activity was found in this test also. With the increased amount of extract, a decreased amount of AChE was found. In the concentration 400mg/kg body weight of the mice, it provides excellent enzyme inhibition activity. Methanol extract of this plant is composed of polar bioactive molecules. From this study, if a single molecule is responsible for the overall activity, then it is difficult to identify.

However, this research can ensure the presence of both in-vitro and in-vivo AChE inhibitory activity as well as memory retention capability. These activities can be the result of an individual molecule or a synergistic effect of the combination of several bioactive components. Further study is needed to identify the exact molecule with these effects.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Handling and experimentation of those mice were conducted in the pharmacology laboratory by following the international ethical guidelines concerning the care and use of laboratory animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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