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Cognitive Enhancing Effects of Androst-16-ene-3,6-diol Isolated from *Lysimachia christinae* on Scopolamine-Induced Impaired Memory Function

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Abstract – The ability of androst-16-ene-3,6-diol, a compound derived from *Lysimachia christinae*, to enhance cognitive function was assessed in mice with scopolamine-induced amnesia. through behavioral and biochemical studies. The Morris water maze test demonstrated significant improvements in spatial learning and memory, with treated mice showing reduced escape latency, shorter path lengths, and enhanced swimming trajectories compared to scopolamine-treated controls. In the passive avoidance test, androst-16-ene-3,6-diol effectively restored retention memory, getting about 65% of the performance detected in control mice group. Biochemical analysis revealed that androst-16-ene-3,6-diol notably diminished acetylcholinesterase activity in the cortex and hippocampus, critical regions for learning and memory. These effects were comparable to those of donepezil, a standard acetylcholinesterase inhibitor used as a positive control (1 mg/kg orally). The findings indicate that androst-16-ene-3,6-diol exerts robust cognitive-enhancing effects by mitigating scopolamine-induced cholinergic deficits through acetylcholinesterase inhibition. This highlights its potential as a promising therapeutic candidate for memory impairment and neurodegenerative disorders.
Keywords – Androst-16-ene-3,6-diol, *Lysimachia christinae*, Alzheimer's disease, Cognitive enhancing activity, Acetylcholinesterase

Introduction

Alzheimer's disease is a progressive neurological disorder affecting individuals over 65, characterized by amyloid plaques, tau tangles, neuroinflammation, and neuronal degeneration. It begins with memory impairment and advances to cognitive decline. A key feature is the dysfunction of the cholinergic system, which relies on acetylcholine to support memory and learning.[1] In Alzheimer's disease, cholinergic neuron loss and reduced acetylcholine levels drive memory and cognitive decline, highlighting the system's role and guiding therapeutic strategies.[2] Neuronal degeneration in the basal forebrain's acetylcholinergic systems is a hallmark of Alzheimer's. Treatments like acetylcholinesterase inhibitors such as, physostigmine, tacrine, donepezil, improve spatial memory deficits in behavioral studies.[3–5]

Scopolamine, a muscarinic receptor antagonist that disrupts central cholinergic function, induces temporary cognitive impairments. These impairments, which include difficulties in sustaining attention, processing information, and acquiring new knowledge, are observed in both rodents and humans and closely mirror the memory deficits seen in Alzheimer's disease.[6–9] Consequently, the scopolamine-induced amnesic mouse model has become a widely utilized research tool. This model provides critical insights into the mechanisms of Alzheimer's disease and facilitates the development of potential therapeutic interventions.[10,11]

Previously, we explored the neuroprotective properties of androst-16-ene-3,6-diol, a significant bioactive compound derived from *Lysimachia christinae*. Our research focused on its ability to counteract glutamate-induced neurotoxicity in HT-22 cells, a commonly used neuronal cell line. Androst-16-ene-3,6-diol was shown to exert powerful antioxidative effects, significantly lowering intracellular calcium ion (Ca^{2+}) levels and cellular peroxide accumulation in neurons exposed to glutamate. It also played a critical role in preserving glutathione (GSH) levels, an essential antioxidant, while mitigating oxidative damage in cultured

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cortical neurons.[12]

Building on these results, we extended our investigation to assess whether androst-16-ene-3,6-diol could improve scopolamine-induced memory deficits in mice. Using the passive avoidance test and the Morris water maze test, two well-established tools for evaluating cognitive function, we evaluated the effects of androst-16-ene-3,6-diol on learning and memory. These behavioral studies provided valuable insights into its potential as a therapeutic agent for cognitive impairments.

To further investigate the mechanisms of cognitive activity of androst-16-ene-3,6-diol, we analyzed its effect on acetylcholinesterase activity in the cortex and hippocampus of scopolamine-treated mice. By inhibiting acetylcholinesterase, cholinergic transmission in the brain can be enhanced, leading to improved cognitive function—particularly in conditions marked by cholinergic deficits, such as Alzheimer's disease. This study aimed to shed light on the cognitive-enhancing potential of androst-16-ene-3,6-diol, examining its possible therapeutic applications for memory impairment. The findings could contribute to the development of new strategies for treating neurodegenerative diseases and other disorders characterized by cognitive decline.

Experimental

Experimental animals – For this experiment, male ICR mice, aged 4 weeks and weighing around 25 grams, were chosen to evaluate cognitive enhancement. They were obtained from Kangwon Life Science Co. in Gangwon Province, Korea, and housed at the Kangwon National University Animal Care Center. After a one-week acclimatization period, the mice were kept under controlled environmental conditions: a temperature of 23 ± 1°C, 60% humidity, and a 12-hour light-dark cycle, with ad libitum access to food and water. All procedures were conducted in compliance with the Kangwon National University Animal Care and Use Guidelines (IACUC, KIACUC, 06/2021), and the study followed the ARRIVE guidelines to ensure ethical conduct in animal research.

Compounds or drugs – Androst-16-ene-3,6-diol was extracted from the entire plant of *L. christinae*.[13] High-purity scopolamine (> 98%) was procured from Sigma (St. Louis, MO, USA), along with phosphate-buffered saline (PBS) and carboxymethyl cellulose (CMC). Scopolamine was employed as a cognitive deficit inducer, mimicking Alzheimer's disease pathology by elevating acetylcholinesterase activity. Meanwhile, donepezil, obtained from Samjin Pharmaceutical Co., Ltd. (Seoul, Korea) with a purity exceeding 95%, served as a standard therapeutic reference. Scopolamine is a well-recognized agent for inducing Alzheimer's-like dementia by reducing acetylcholine levels in the brain through enhanced acetylcholinesterase activity, leading to impaired memory and cognition. Conversely, donepezil, a widely used acetylcholinesterase inhibitor, prevents the degradation of acetylcholine. This action sustains elevated acetylcholine levels in neuronal cells, which is crucial for memory retention and cognitive function. Due to its efficacy in improving cognitive performance, donepezil is commonly utilized in Alzheimer's disease treatment. By maintaining higher concentrations of acetylcholine in the brain, it supports cognitive processes and serves as a benchmark drug in studies investigating potential therapeutic agents for dementia.[14]

Drug administration – The study comprised six groups of mice, each consisting of six subjects: a control group, a scopolamine group, a positive control group treated with donepezil (1 mg/kg), and three groups treated with varying doses of androst-16-ene-3,6-diol (1, 5, and 10 mg/kg). These dosages were based on prior in vitro research and potential clinical trial considerations. No deaths or signs of toxicity were reported, and organ inspections post-assay showed no abnormalities compared to the control group. Both androst-16-ene-3,6-diol and donepezil were administered orally 90 minutes before scopolamine injection. The control group received a 0.5% carboxymethyl cellulose (CMC) solution. Scopolamine (1 mg/kg) was given subcutaneously 30 minutes before behavioral testing. The treatment lasted four days, with an additional dose on the training day for the passive avoidance test. Cognitive function was assessed using the Morris water maze and passive avoidance tests.

Morris water maze test – The water maze test was carried out using a modified version of the Morris method to assess spatial memory.[15] For the water maze test, a circular pool (90 cm diameter, 40 cm height) was filled with water maintained at 20 ± 1°C, with 500 mL of white milk added to obscure the escape platform. The pool was divided into four quadrants, and the platform was submerged 1 cm below the surface in one of the quadrants. The platform's dimensions were 10 cm in diameter and 26 cm in height. Swimming activity was recorded using a smart video-tracking system (Smart ver. 2.5.21) connected to a camera, measuring time, distance, and speed. The primary metric, escape latency, was the time it took for the mouse to find and remain on the platform. On the first day, a 60-second trial without the platform allowed the mice to familiarize themselves with the setup. The mice were humanely euthanized via cervical dislocation after the experiment. The escape latency, or time to find the platform,

was recorded. If the platform wasn't found within 120 seconds, the trial was stopped. At the end of the experiment, the mice were humanely euthanized. The subsequent four days included test trials, with each mouse allowed up to 120 seconds to locate the platform; failure to do so resulted in an escape latency of 120 seconds.

Passive avoidance test – The passive avoidance test was performed with adjustments based on our previous work. The apparatus consisted of two compartments (17 cm × 12 cm × 10 cm) separated by a guillotine door and featuring an electrical grid floor. The test procedure included three stages: acquisition, training, and testing. During the acquisition stage, mice were placed in the light compartment and allowed to explore for 40 seconds. The guillotine door then opened, and once the mouse entered the dark compartment, the door closed. The training phase took place 24 hours later, with mice again placed in the light compartment. After 30 seconds, the door opened, and when the mouse entered the dark compartment, it received a mild foot shock (0.1 mA/10 g body weight for 2 seconds). The test phase occurred 24 hours after training, where each mouse was placed in the light compartment, and the time taken to enter the dark compartment was recorded, with a maximum of 180 seconds. At the end of the experiment, the mice were humanely euthanized by cervical dislocation.

Acetylcholinesterase (AChE) inhibition assay – An acetylcholinesterase (AChE) inhibition assay was performed to assess the effects of androst-16-ene-3,6-diol on AChE activity using a modified Ellman method. Brain tissues were collected from mice within 30 minutes after completing the Morris water maze or passive avoidance test. Hippocampi were isolated and homogenized in ice-cold sodium phosphate buffer. The homogenates were centrifuged to obtain clear supernatants. The assay mixture included hippocampal supernatant, sodium phosphate buffer, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and acetylcholine iodide (AChI). The reaction was initiated by adding AChI, and the production of thiocholine was monitored at 412 nm using a microplate spectrophotometer. All measurements were performed in triplicate, with blanks included to account for non-enzymatic reactions.

Statistics – The results of the Morris water maze, passive avoidance test, and AChE activity assay were presented as mean values with standard error of the mean (S.E.M.). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test for pairwise comparisons. Data were processed using IBM SPSS Statistics (version 26). Statistical significance was assessed at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results and Discussion

The Morris water maze test was employed to investigate the cognitive-enhancing effects of androst-16-ene-3,6-diol, derived from *L. christinae*, in alleviating scopolamine-induced memory deficits (Fig. 1). In the control group, the escape latency decreased significantly over the four days of testing, reaching 36.1 seconds on the final day. Conversely, the scopolamine-treated group exhibited a prolonged and unchanging escape latency, peaking at 112.6 seconds on day four, confirming that scopolamine effectively induced memory impairment. The memory-enhancing potential of androst-16-ene-3,6-diol was assessed over the course of four days, with notable improvements in each treatment group observed after the third day. The donepezil-treated group had an escape latency of 59.3 seconds on day three, which further reduced to about 48.9 seconds by day four. The groups treated with androst-16-ene-3,6-diol showed a dose-dependent reduction in escape latency compared to the scopolamine group. At a dosage of 10 mg/kg, the escape latency was 54.7 seconds, while the 5 mg/kg and 1 mg/kg doses resulted in latency times of 78.3 seconds and 92.8 seconds, respectively (Fig. 2).

The swimming distance covered by the control group was significantly shorter compared to that of the scopolamine-treated group. The control group swam a total distance of 376 cm, reflecting normal cognitive function. In contrast, the scopolamine-treated group exhibited a swimming distance of 732 cm, nearly double that of the control group, indicating a pronounced impairment in memory and spatial learning induced by scopolamine. Over the four-day testing period, administration of androst-16-ene-3,6-diol effectively reduced the excessive swimming distance caused by scopolamine in a dose-dependent manner. Mice treated with 1 mg/kg of androst-16-ene-3,6-diol swam 645 cm, showing a moderate improvement compared to the scopolamine-treated group. Further reductions were observed with

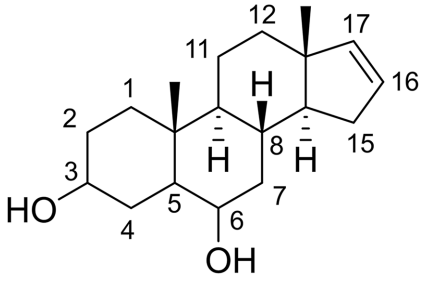

Fig. 1. The chemical structure of androst-16-ene-3,6-diol isolated from *L. christinae* extract.

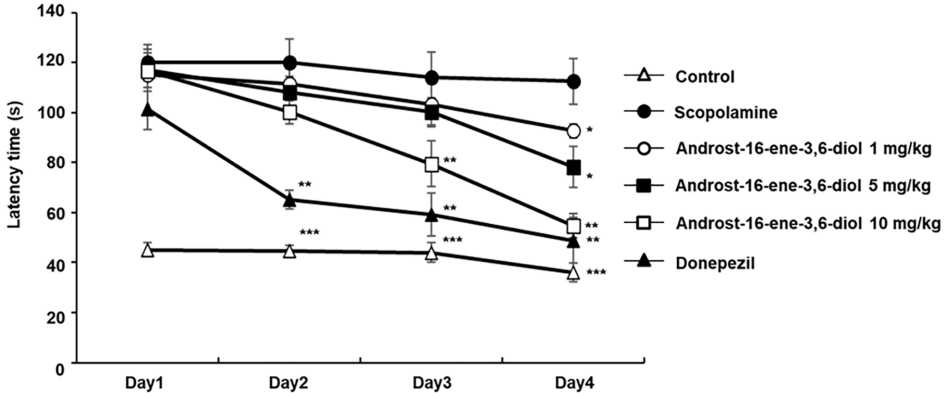

Fig. 2. The spatial memory enhancing activity of androst-16-ene-3,6-diol by Morris water maze test. Data are mean escape latencies ± SD (n = 6). (*p < 0.05, **p < 0.01 and ***p < 0.001 versus scopolamine treated group.)

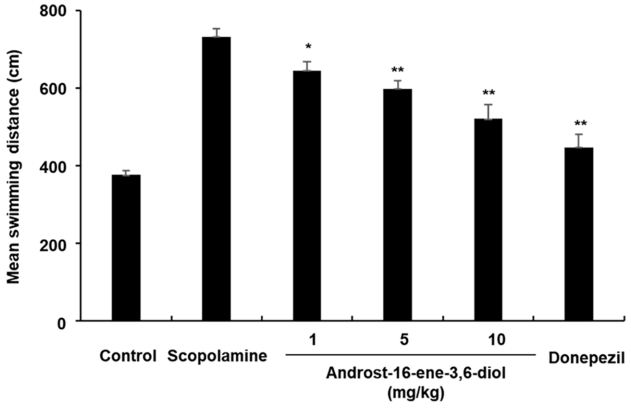

Fig. 3. The effect of androst-16-ene-3,6-diol on mean swimming distance. Data are mean escape latencies ± SD (n = 6). (*p < 0.05, **p < 0.01 and ***p < 0.001 versus scopolamine treated group.)

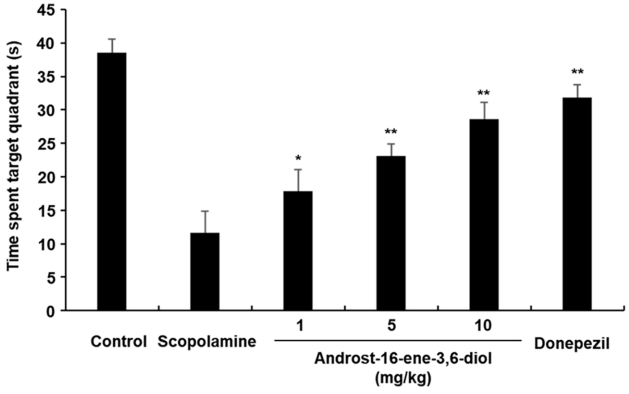

Fig. 4. The effect of androst-16-ene-3,6-diol in the probe trial. Data are mean escape latencies ± SD (n = 6). (*p < 0.05, **p < 0.01 and ***p < 0.001 versus scopolamine treated group.)

higher doses, as mice treated with 5 mg/kg and 10 mg/kg swam distances of 599 cm and 521 cm, respectively. These findings demonstrate a clear trend of enhanced cognitive recovery and memory improvement with increasing doses of androst-16-ene-3,6-diol (Fig. 3).

The probe test was designed to evaluate memory retention by recording the amount of time the mice spent in the target quadrant, which previously housed the escape platform. Results showed that the control group spent significantly more time in the target quadrant (38.5 seconds) compared to the scopolamine-treated group (11.6 seconds). This difference highlights the effective memory retention in the control group and the memory impairing effect of scopolamine. The reduced time spent in the target quadrant by the scopolamine-treated mice further confirms the cognitive deficits induced by the drug, emphasizing its impact on spatial memory performance. Administration of androst-16-ene-3,6-diol improved memory retention in a dose-dependent manner, as indicated by increased time spent in the target quadrant. Mice treated with 1 mg/kg of androst-16-ene-3,6-diol spent 17.9 seconds in the target quadrant, while those receiving 5 mg/kg and 10 mg/kg spent 23.1 seconds and 28.6 seconds, respectively. These findings suggest that higher doses of androst-16-ene-3,6-diol effectively counteract scopolamine-induced memory impairment (Fig. 4).

To confirm that the reduced time to find the target platform in the androst-16-ene-3,6-diol-treated group was not influenced by changes in motor abilities, the swimming speed of the mice was examined. This evaluation was necessary to distinguish between cognitive improvements and physical enhancements. The results revealed no significant variation in the average swimming speeds across all groups, including the control group, the scopolamine-treated group, the androst-16-ene-3,6-diol-treated group, and the donepezil-treated group. These findings indicate that the improved performance in the androst-16-ene-3,6-

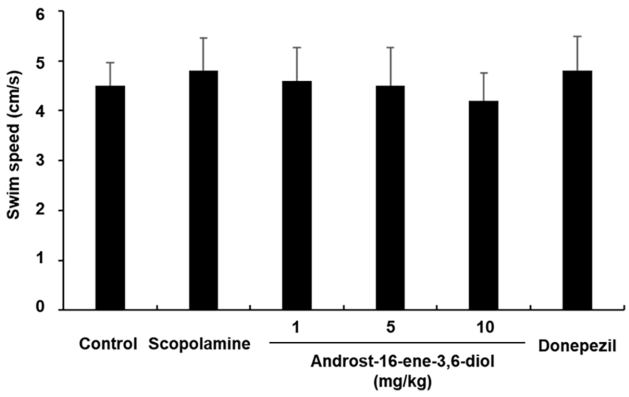

Fig. 5. The effect of androst-16-ene-3,6-diol on swim speed. Data are mean escape latencies ± SD (n = 6). (*p < 0.05, **p < 0.01 and ***p < 0.001 versus scopolamine-treated mice.)

diol-treated group is attributed to cognitive enhancement rather than alterations in physical capabilities (Fig. 5). The mean swimming speeds of the mice across all groups were similar, indicating that motor function was unaffected by the treatments. This suggests that the observed improvements in the groups treated with androst-16-ene-3,6-diol and donepezil were not attributable to changes in locomotor activity. Rather, these effects were specifically linked to enhanced spatial memory. This interpretation is reinforced by the consistent swimming speeds, which isolate the memory-enhancing properties of scopolamine, donepezil, and androst-16-ene-3,6-diol from any influence on physical performance.

To assess the protective effects of androst-16-ene-3,6-diol on scopolamine-induced cognitive deficits, the Morris water maze test was used. Scopolamine, known to impair cholinergic signaling, causes learning and memory deficits and increases hippocampal AChE activity.[16] The test measured escape latency, reflecting the time it took for mice to find a submerged platform. The findings revealed that androst-16-ene-3,6-diol significantly shortened escape latency in a dose-dependent fashion, with higher doses enabling faster platform discovery. Compared to scopolamine-treated mice, those receiving androst-16-ene-3,6-diol showed notably improved performance in locating the platform. However, no significant differences in average swimming speed were observed among the control, scopolamine-treated, and androst-16-ene-3,6-diol-treated groups for the four-day testing period. This indicates that the reduced escape latency in the androst-16-ene-3,6-diol-treated mice was driven by improved memory performance rather than enhanced motor abilities. Additionally, mice treated with androst-16-ene-3,6-diol spent more time in the target quadrant, where the platform had been located, compared to those treated with scopolamine. This suggests that androst-16-ene-3,6-diol promoted memory retention, enabling the mice to effectively recall and identify the platform's location. Taken together, the results demonstrate that androst-16-ene-3,6-diol improves spatial learning and memory without influencing swimming speed. Among the compounds derived from *L. christinae*, androst-16-ene-3,6-diol exhibited the strongest cognitive-enhancing effects in the Morris water maze test.

The passive avoidance test, a widely recognized method for assessing long-term memory and evaluating the potential for memory enhancement, was employed to explore the enduring cognitive benefits of androst-16-ene-3,6-diol treatment. This test involves placing the mice in an environment where they learn to avoid an area associated with an unpleasant stimulus. By measuring the latency time to enter the dark compartment, the test provides valuable insight into the ability of animals to retain information over an extended period. In this study, we aimed to determine whether treatment with androst-16-ene-3,6-diol could not only improve immediate memory function but also enhance long-term memory retention. The results helped evaluate the compound's potential in promoting sustained cognitive improvement, suggesting its possible therapeutic application in memory-related disorders.[17] During the acquisition trial, the time it took for the mice to avoid the unpleasant stimulus was recorded as latency. This measurement provided an indication of their ability to learn and recall the association between the environment and the aversive stimulus. The findings showed no substantial variation in latency times between the groups, implying that the initial learning capabilities were comparable across all experimental conditions. Specifically, the average latency times were 2.66 s for the control group, 2.12 s for the scopolamine-treated group, and 2.08 s for the donepezil-treated group. For the androst-16-ene-3,6-diol-treated groups, the average latency times were 2.17 s at 1 mg/kg, 2.54 s at 5 mg/kg, and 2.18 s at 10 mg/kg (Fig. 6).

In the test trial, the latency time for the scopolamine-treated group was significantly shorter than that of the control group, confirming that scopolamine successfully induced memory impairment. The control group had a mean latency of 43.87 s, while the scopolamine-treated group showed a significant decrease, with a latency of 12.11 s. In contrast, the androst-16-ene-3,6-diol-treated groups exhibited a dose-dependent increase in latency times, with averages of 25.33 s, 26.42 s, and 28.99 s for the 1 mg/kg, 5 mg/kg, and 10 mg/kg doses, respectively (Fig. 6). The passive avoidance test was used to assess the

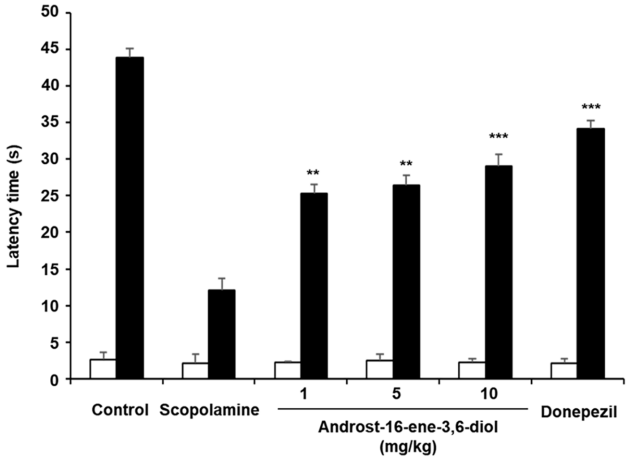

Fig. 6. The cognitive enhancing activity of androst-16-ene-3,6-diol by the passive avoidance test. Data are mean latency times (s) ± SD (n = 6). (*p < 0.05, **p < 0.01 and ***p < 0.001 compared with the scopolamine treated group).

effects of androst-16-ene-3,6-diol on scopolamine-induced memory deficits. While there were no significant differences in learning ability during the acquisition phase, the androst-16-ene-3,6-diol-treated groups showed a clear, dose-dependent increase in latency times during the test phase, compared to the scopolamine-treated group. This suggests that androst-16-ene-3,6-diol effectively enhances memory and helps reverse scopolamine-induced cognitive impairments.

The impact of androst-16-ene-3,6-diol on AChE activity in the hippocampus was evaluated. Scopolamine treatment resulted in a 148.6% increase in AChE activity compared to the control group. However, androst-16-ene-3,6-diol significantly reduced AChE activity in a dose-dependent manner (p < 0.05). At doses of 1 mg/kg, 5 mg/kg, and 10 mg/kg, AChE activity was reduced by 122.5%, 121.8%, and 116.7%, respectively (Fig. 7).

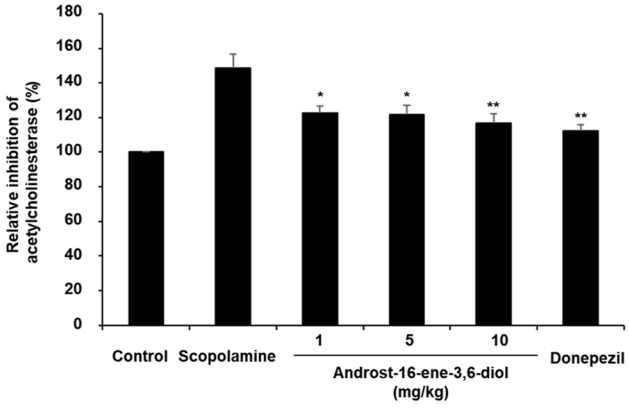

Fig. 7. The acetylcholinesterase inhibitory activity of androst-16-ene-3,6-diol. Data were means ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the scopolamine treated group (n = 3).

To better understand the memory-boosting effects of androst-16-ene-3,6-diol, we delved into its impact on enzyme activity. Acetylcholine is crucial for cognitive functions like learning and memory. In Alzheimer's disease, lower acetylcholine levels contribute to cognitive decline, often linked to higher acetylcholinesterase (AChE) activity, which breaks down acetylcholine. This makes AChE a key target for Alzheimer's treatments. Inhibiting AChE helps preserve acetylcholine, potentially improving memory and cognitive function. Androst-16-ene-3,6-diol's ability to reduce AChE activity may therefore help improve cognitive performance in Alzheimer's disease.[18,19] These AChE inhibitors, including donepezil, galantamine, and tacrine, have been developed as therapeutic options for memory loss, particularly in conditions like Alzheimer's disease. These drugs function by inhibiting acetylcholinesterase (AChE). By preventing the degradation of acetylcholine, these inhibitors assist in preserving elevated levels of this neurotransmitter within the brain, thereby improving cholinergic function and potentially enhancing cognitive abilities such as memory and learning. This mechanism aims to mitigate the cognitive decline associated with acetylcholine deficiency.[19,20] Androst-16-ene-3,6-diol showed the potent AChE activity, with higher concentrations leading to a more significant reduction in enzyme activity. By decreasing AChE activity, androst-16-ene-3,6-diol helps maintain higher acetylcholine levels in the brain, which are essential for cognitive processes such as memory and learning. As a result, the compound may help alleviate scopolamine-induced memory deficits by preserving acetylcholine levels, thus enhancing cognitive function. This mechanism suggests that androst-16-ene-3,6-diol could be a potential candidate for improving memory in conditions where acetylcholine activity is impaired. Androst-16-ene-3,4-diol does show acetylcholinesterase (AChE) inhibitory activity, but its effect is not very strong. According to previous studies from our laboratory, it also demonstrates neuroprotective activity. Given that it may act through various mechanisms to show cognitive enhancing activity, it is reasonable to attribute its effects to repeated administration. Also, we think that conducting a behavioral test or an AChE activity assay after a single administration could provide further insights into the effects and mechanism of action of Androst-16-ene-3,6-diol.

In conclusion, androst-16-ene-3,6-diol, a compound from *L. christinae* extract, effectively reduces scopolamine-insulted memory disorder in a dose-dependent manner. This effect might be due to its inhibition of acetylcholinesterase (AChE), an enzyme that breaks down acetylcholine, a neurotransmitter vital for memory and

cognition. By inhibiting AChE, androst-16-ene-3,6-diol helps maintain higher acetylcholine levels, increasing the brain function related to memory and cognition. These results indicated that androst-16-ene-3,6-diol could be a potential candidate for developing the new medicine to treat for neurodegenerative diseases, including Alzheimer's disease.

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Conflict of Interest Statement

The authors have declared that there are no conflicts of interest.

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