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Anti-amnesic and anti-cholinesterase activities of α-asarone against scopolamine-induced memory impairments in rats

K. VENKATESAN

Department of Pharmaceutical Chemistry, College of Pharmacy, King Khalid University, Abha, Saudi Arabia

Abstract. – OBJECTIVE: Alzheimer's disease (AD) is a neurological ailment that causes memory loss and impairments and is linked to a drop-in acetylcholine level. Acetylcholinesterase (AChE) inhibitors are used for the management of AD. In our ongoing research to search for natural AChE inhibitors from medicinal plants, we found that the *Acorus calamus* possesses memory-enhancing properties. α-Asarone is the major compound isolated from the *Acorus calamus* and it has neuroprotective action in animal models, nonetheless, its anticholinesterase activity in different brain regions was not fully understood. The purpose of this research was to determine the anti-amnesic and anti-cholinesterase activities of α-asarone against scopolamine-induced memory impairments in rats.

MATERIALS AND METHODS: The anti-cholinesterase activity of α-asarone was determined using Ellman's method in different brain areas, such as the cortex, hippocampus, and striatum. In addition, the anti-amnesic effect of α-asarone was also investigated using elevated plus-maze, passive avoidance, and active avoidance tests.

RESULTS: The effect of α-asarone on memory impairment against scopolamine-induced (1 mg/kg body weight) amnesia was evaluated. Administration of α-asarone (15 and 30 mg/kg body weight) for 14 days to rats significantly ameliorated the scopolamine-induced memory impairment as measured in the elevated plus-maze, passive avoidance, and avoidance active tests compared to the scopolamine-treated group. In this study, we also show that α-asarone treatment significantly ($p<0.05$) reduced brain acetylcholinesterase activity in the cortex, hippocampus, and striatum brain regions of amnesic rats.

CONCLUSIONS: These results confirmed that α-asarone has anti-amnesic and anti-cholinesterase potential which may be useful for the management of AD.

Key Words:
Alzheimer's disease, Acetylcholinesterase, Active avoidance test, Memory, Scopolamine, α-asarone.

Introduction

Acorus calamus (AC) Linn. rhizome extract is an ingredient in two Ayurvedic herbal formulations (Aindrarasayana and Brahmarasayana) which is used to alleviate dementia[1,2]. The rhizomes of AC have been used in Ayurvedic medicine for the management of several neurological and mental disorders[2-5]. α-Asarone and β-asarone are the main bioactive constituents isolated from the essential oil and several biological activities of these compounds have been reported[2,6]. Diverse pharmacological properties of α-asarone have been reported including sedative, neuroprotective, anti-oxidative, and anticonvulsive actions. Several recent studies[2,7-12] have reported a beneficial effect of α-asarone in the brain. Recently, α-asarone also improved cognitive impairment, protected the hippocampal neurons from damage in β-amyloid-treated rats[13], stimulated glutamate uptake, and reduced excitatory synaptic activity[14].

Alzheimer's disease (AD) is a neurological ailment characterized by impaired cognitive function and dysfunction of central cholinergic neurotransmission. Acetylcholine levels in the brain are lower in AD patients[15,16]. One of the major hallmarks that characterize AD is neurotransmitter acetylcholine and enzyme choline transferase deficiency in the different brain areas[17]. Acetylcholinesterase (AChE) has been used as a key therapeutic target for activating the central cholinergic pathway and inhibitors of this enzyme have been in the management

Corresponding Author: Kumar Venkatesan, Ph.D; e-mail: kumarve@kku.edu.sa

of AD[18-20]. The interaction of these drugs with the amyloid cascade influences the expression and/or the metabolic processing of the amyloid precursor protein and slows down one of the major pathological steps of the AD process[21]. The scopolamine-induced memory impairment animal model is frequently used in anti-amnesic drug screening. In a variety of behavioral tests, AChE inhibitors mitigate the scopolamine-induced memory impairment[22].

AChE inhibitors, both synthetic and natural, are being screened for the use in the treatment of AD. However, the FDA has approved only a few drugs including tacrine, donepezil, rivastigmine, and galanthamine. The majority of these AChE inhibitors are restricted in their use due to side effects. Medicinal plants show promising results in the management of AD in terms of their cognitive benefits. Its mechanisms of action mainly deal with the fundamental pathophysiology of the disease[23]. As a result, natural AChE inhibitors with improved efficacy, brain penetration, and safety are in high demand in the clinical setting. In spite of several studies on various behavioral aspects of α-asarone, its effect on AChE activity in different brain areas is inadequate. As a result, the purpose of this research was to determine the anti-cholinesterase activity of α-asarone in brain areas, such as the cortex, hippocampus, and striatum. In addition, this study also investigated the anti-amnesic effect of α-asarone using elevated plus-maze, passive avoidance, and active avoidance tests.

Materials and Methods

General Experimental Procedures and Chemicals

α-Asarone (Figure 1), acetylthiocholine iodide (ATCI), bovine AChE enzyme, 5, 5'-dithiobis [2-nitrobenzoic acid] (DTNB), physostigmine hemisulphate, and scopolamine hydrobromide were obtained from Sigma-Aldrich (St. Louis, MI, USA). Methanol and all other organic solvents were purchased from Merck (Darmstadt, Germany).

Animals

The study was conducted on Wistar rats of either sex weighing around 200-250 g. Before animal studies, all rats were exposed to laboratory conditions. Ethical Committee of King Khalid University approved (REC#2018-03-66) the animal studies and animal care were taken as per the recommendations.

Drug Treatment

Physostigmine hemisulphate, α-asarone, and scopolamine hydrobromide were suspended in a solution containing 0.5% methylcellulose and 1% Tween 80. Physostigmine hemisulphate, scopolamine hydrobromide, and α-asarone were administered intraperitoneally to rats. The rats were separated into five groups (n = 8). Group I, animals received saline solution (10 mL/kg body weight) for 14 days. Group II, scopolamine (1 mg/kg body weight) was administered on the 13th day. Group III, physostigmine (0.25 mg/kg, body weight) was administered to animals for 14 days. Group IV & V, α-asarone (15 mg/kg and 30 mg/kg) was administered to animals for 14 days. Amnesia was induced in animals with scopolamine hydrobromide (1 mg/kg body weight) given intraperitoneally 60 min before the first trial. Scopolamine was administered 90 minutes after the last dose was administered on the 13th day. Scopolamine-treated animals were given a saline solution (10 mL/kg body weight) for 14 days instead of a drug and scopolamine was administered on the 13th day[24]. Vehicle-treated animals received saline solution instead of scopolamine. The rats were subjected to the acquisition trial 1 h after the last administration of the drugs on day 13.

Elevated Plus-Maze Test

This test was used to evaluate memory impairment in rodents. The experimental protocol used for this test has been described previously[24]. Amnesia was induced in rats with scopolamine (1 mg/kg, i.p). The initial transfer latency was

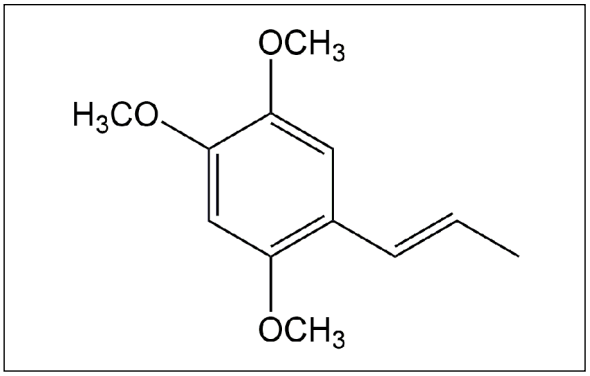

Figure 1. Structure of α-asarone.

taken 60 minutes after injection on day 13 and the retention trial was assessed one day after the first trial on the 14th day.

Passive Avoidance Task

The passive avoidance test was used to examine long-term memory. The rats were subjected to a single trial passive avoidance test. The details of the experimental procedure employed during this test have already been described in previous studies[24,25]. The rats were placed in the lighted compartment during the acquisition trial and were allowed to explore for 60 s. After 60 s of the acquisition, pressing a pedal opened the guillotine, and the training latency to cross into the dark compartment was recorded. The memory retention was measured on day 14. If the rats did not enter the dark area within 300 s, it was concluded that the rats had memorized the passive avoidance training after the acquisition trial.

Shuttle-Box Active Avoidance Paradigm

The details of this test have been described in a previous study[22]. The animals were trained on day 13, as in the passive avoidance test, and memory retention was assessed on the second day (14th day). The following parameters were counted: (1) total time, (2) number of the conditioned stimulus (avoidances, total trials), and (3) number of the unconditioned stimulus (escape, shocked trials).

Estimation of Brain Acetylcholinesterase

The rats were sacrificed by decapitation after behavioural testing. The cortex, hippocampus, and striatum of brain regions were dissected out and the brain tissues were homogenized. The homogenates were centrifuged and supernatants were collected. Each homogenate was preincubated with 0.1 mM tetraisopropyl pyrophosphoramide for 5 minutes at 37°C to suppress butyrylcholinesterase activity. AChE activity was assessed in 50 µl aliquots of the homogenates, in 96-well flat-bottom microtiter plates. Enzymatic activity was determined using Ellman's method[26]. The reaction was started by adding 0.5 mM ATCI and 0.25 mM DTNB, and both dissolved in 0.1 M phosphate buffer pH 7.4[27,28]. The microtiter plates were then placed into the microplate-reader and the yellow colour product was quantified at 405 nm.

Statistical Analysis

The data from elevated plus-maze, passive avoidance, and active avoidance tests, and brain AChE estimation were expressed as mean ± SD and analyzed by one-way ANOVA and followed by post hoc analysis using Turkey's multiple comparisons of means. The statistically significant was $p<0.05$, $p<0.001$, and $p<0.01$.

Results

Elevated Plus-Maze Test

The first day's transfer latency showed animal learning, while the second day's transfer latency reflected memory retention. Scopolamine injected before training impaired learning as indicated by increased transfer latency. Scopolamine significantly ($p<0.001$) increased transfer latency as compared to the saline-treated group. α-Asarone (15 and 30 mg/kg) showed a dose-dependent reduction in transfer latency on the fourteenth day in rats. The effect of α-asarone on transfer latency using elevated plus-maze is shown in Table I. α-asarone administered for 14 days showed a significant ($p<0.001$) effect on transfer latency on the second day as compared to the scopolamine-treated group. Physostigmine administered intraperitoneally at 0.25 mg/kg for 14 days significantly ($p<0.001$) protected the animals from scopolamine-induced impairment in memory. Both the doses of α-asarone treatment significantly ($p<0.05$, $p<0.001$) decreased transfer

Table I. Effect of α-asarone on transfer latency (TL) using the elevated plus-maze paradigm in scopolamine-induced amnesic rats.

Treatment	Transfer latency 1st day (seconds)	Transfer latency 2nd day (seconds)
Control (vehicle)	33.23 ± 3.35	25.54 ± 3.28[a]
Scopolamine (1 mg/kg)	43.23 ± 3.43	48.51 ± 6.13***
Physostigmine (0.25 mg/kg) + Scopolamine	36.12 ± 3.22	26.12 ± 3.28[a]
α-asarone 15 mg/kg + Scopolamine	28.99 ± 4.14***	22.44 ± 2.33***[a]
α-asarone 30 mg/kg + Scopolamine	25. 21 ± 2.63***	18.78 ± 1.88***[a]

[a]$p < 0.05$ *vs*. day 1, *$p < 0.05$ *vs*. control; ***$p < 0.001$ *vs*. control. Each group represents n = 8 animals.

Table II. Anti-amnesic effects of α-asarone using a passive avoidance task in scopolamine-induced amnesic rats.

Treatment	Step-through latency seconds ± SD
Control (10 ml/kg)	171 .32 ± 8.1
Scopolamine (1 mg/kg)	27.52 ± 2.9
Physostigmine (0.25 mg/kg) + Scopolamine	95.32 ± 5.82 **
α-asarone 15 mg/kg + Scopolamine	83. 44 ± 7.72**
α-asarone 30 mg/kg + Scopolamine	91.97 ± 7.82**

**$p < 0.01$, *$p < 0.05$, *vs.* the scopolamine-treated group (n = 8 animals).

latency in the acquisition trial as well as in the retention trial, indicating considerable improvement in learning and memory.

Passive Avoidance Task

The memory-enhancing efficacy of α-asarone was tested in scopolamine-induced memory impairment rats. Scopolamine (1 mg/kg, i.p.) significantly ($p<0.001$) decreased step-through latency in rats compared to the saline-treated group, indicating impairment of memory (amnesia). Treatment with scopolamine significantly shortened the latency time (16.6 % decrease in step-through latency) in the retention trial (Table II) compared to the saline-treated group. The shorter step-through latency induced by scopolamine was significantly ($p<0.01$, $p<0.05$) reversed by both the doses of α-asarone. Administrations of α-asarone for 14 days showed an increase in the step-through latency time for scopolamine-induced memory impairments compared to the scopolamine-treated group. α-Asarone at a dose of 30 mg/kg was more effective ($p<0.01$). Physostigmine (0.25 mg/kg) significantly ($p<0.05$) restored the step-through latency as compared to the scopolamine-treated group.

Shuttle-Box Active Avoidance Paradigm

In the shuttle-box test, rats treated with α-asarone (15 and 30 mg/kg) were required significantly fewer trials and time to learn the conditioned avoidance response task than rats treated with vehicle (Table III). α-Asarone showed a dose-dependent facilitative effect on the 24 hours retention of previously learned active avoidance. When compared to vehicle-treated rats, the rats pretreated with α-asarone and physostigmine required significantly fewer trials, shocked trials, and total time to re-learn the task. Furthermore, both the doses of α-asarone and physostigmine significantly attenuated the scopolamine conditioned avoidance response retention deficits, as indicated by significantly less trials, shocked trials, and the total time of the conditioned avoidance response task by rats in comparison to rats treated with scopolamine.

Reduction of Brain Acetylcholinesterase Activity

The effect of α-asarone on AChE activities in the cortex, hippocampus, and striatum is shown in Figure 2. The scopolamine treatment

Table III. Effect of α-asarone on active avoidance test in scopolamine-induced amnesic rats.

Treatment	Shocked trials	Total trials	Total time (s)
	Acquisition 1st day		
Control (vehicle)	2.72 ± 0.69	5.29 ± 0.78	135.28 ± 4.88
Scopolamine (1 mg/kg)	3.19 ± 0.72	5.41 ± 0.55	142.44 ± 5.08[b]
Physostigmine (0.25 mg/kg) + Scopolamine	2.00 ± 0.63	4.23 ± 0.53[a]	119.40 ± 5.32[c]
α-asarone 15 mg/kg + Scopolamine	2.48 ± 0.41	4.53 ± 0.61[c]	134.26 ± 3.58[c]
α-asarone 30 mg/kg + Scopolamine	2.23 ± 0.59	4.31 ± 0.49[c]	122.99 ± 3.98[c]
	Retention after 24 h		
Control (vehicle)	1.75 ± 0.32[e]	3.95 ± 0.63[e]	111.03 ± 5.25[e]
Scopolamine (1 mg/kg)	2.78 ± 0.42[b]	4.96 ± 0.65[a]	130.84 ± 6.37[b,e]
Physostigmine (0.25 mg/kg) + Scopolamine	0.59 ± 0.21[c,e]	2.72 ± 0.32[c,e]	78.25 ± 3.58[c,e]
α-asarone 15 mg/kg + Scopolamine	0.75 ± 0.32[c,e]	3.78 ± 0.42[c,e]	92.34 ± 6.11[c,e]
α-asarone 30 mg/kg + Scopolamine	0.63 ± 0.11[c,e]	3.09 ± 0.29[c,e]	82.15 ± 4.17[c,e]

[a]$p < 0.05$ *vs.* vehicle, [b]$p < 0.01$ *vs.* vehicle, [c]$p < 0.01$ *vs.* scopolamine, [d]$p < 0.05$ *vs.* acquisition, [e]$p < 0.01$ *vs.* acquisition. Each group represents n = 8 animals.

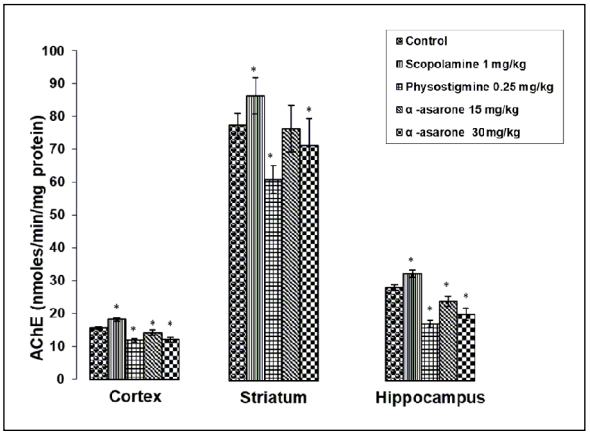

Figure 2. Effect of α-asarone on AChE (nmoles/min/mg of protein) levels in various brain regions of scopolamine-induced amnesic rat. Amnesia was induced in rats (except saline-treated group) with scopolamine (1 mg/kg body weight, i.p). *$p < 0.05$ *vs.* scopolamine-treated group. Each group represents n = 8 animals.

increased AChE levels in brain regions. Interestingly, dose-dependent inhibition of AChE was observed in various brain regions of the scopolamine-induced amnesic rats when treated with α-asarone (15 and 30 mg/kg, i.p, 14 days). α-Asarone (15 and 30 mg/kg, i.p.) significantly (p<0.05) reduced cholinesterase activity in all three brain regions of amnesic rats compared to the scopolamine-treated group. The lowest dose of α-asarone (15 mg/kg, i.p.) showed less reduction of cholinesterase activity in the striatum compared to the other regions of the rat brain. Physostigmine also showed a significant (p<0.05) reduction of cholinesterase activity in all brain regions of amnesic rats compared to the scopolamine-treated rats. The anti-cholinesterase and memory-enhancing activities of α-asarone, and the main target of α-asarone are depicted in Figure 3.

Discussion

AD is a common type of dementia in the elderly population. AD is a disorder with enormous social and economic impact, which is responsible for 50-60% of total cases among people over age 65. Prevalence studies[29-32] have indicated that in 2000 there were 25 million persons with AD worldwide, and it will rise to 114 million by 2050 if new preventive therapies do not emerge. Cholinesterase inhibitors and NMDA-receptor antagonists are the preferred treatments for Alzheimer's disease, though their therapeutic efficacy is still being debated. Therefore, we were prompted to discover natural AChE inhibitors from the Indian traditional system to manage AD.

In both humans and animals, the cholinergic pathway plays a key role in learning and memory functions. Furthermore, cholinergic deficits in various brain regions, including the cortex and hippocampus are considerable neuropathologic observations in Alzheimer's disease patients[30]. Evidence from previous studies[33,34] suggests that drugs that affect central cholinergic function can alter learning and memory. Memory impairments caused by cholinergic system disruption are inverted by AChE inhibitors.

Scopolamine hydrobromide induces memory deficits with a cholinergic dysfunction and it has been extensively used to induce amnesia in rats[24]. Therefore, this method is useful in *in vivo* model for AD. Intraperitoneal injection of scopolamine significantly increased transfer latency in elevated plus-maze test and decreased step-through latency in passive avoidance task. In this study, the treatment of α-asarone for 14 days showed a significant increase in the step-through laten-

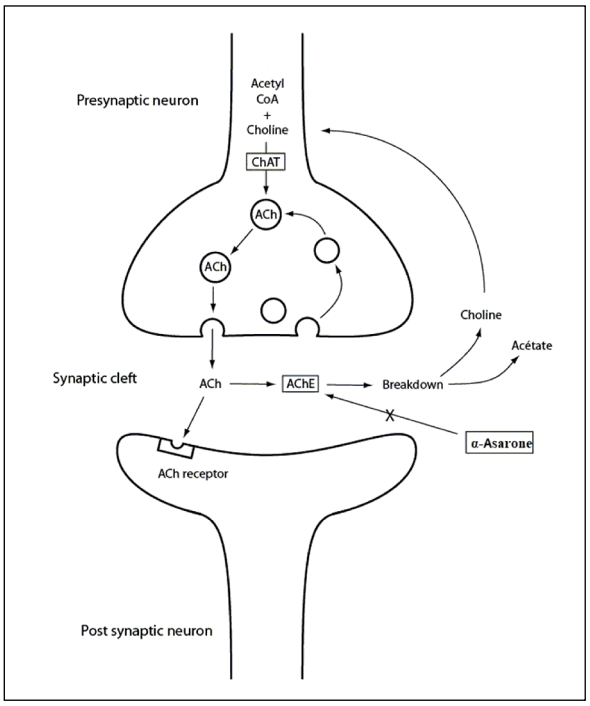

Figure 3. The mechanism of action of acetylcholinesterase and site of action of α-asarone. Acetylcholine (Ach); Acetylcholinesterase (AChE); Choline acetyltransferase (ChAT).

cy time in scopolamine-induced amnesic rats. α-Asarone also significantly decreased transfer latency in the training trial and also in the retention trial, indicating a significant improvement in learning and memory. The active avoidance test demonstrated that α-asarone (15 and 30 mg/kg, i.p.) and physostigmine required fewer trials and less time to attain the conditioned avoidance learning criterion in the training trial. When compared to vehicle-treated rats, both dosages of α-asarone and physostigmine required significantly fewer trials, shocked trials, and total time to re-learn the task after 24 hours in the retention trial. This study revealed that α-asarone was able to reverse the scopolamine-induced amnesia and proved the possible memory-enhancing potential of α-asarone.

Scopolamine causes amnesia in animals by blocking cholinergic neurotransmission in the brain. Scopolamine increased cholinesterase activity in different regions of the rat brain. This study proved that α-asarone treatment significantly reduced AChE activity in the cortex, hippocampus, and striatum regions of scopolamine-induced amnesic rats. The reduced AChE effect in different brain regions suggests an increase in acetylcholine levels, which may aid in memory retention. The memory-enhancing effect of α-asarone in this study may be due to the increase in acetylcholine level in the synaptic site through the inhibition of AChE (Figure 3). The present study suggested that the anticholinesterase effect of α-asarone may be beneficial in amnesia induced by disruption of the cholinergic system through AChE inhibition. There were no definite visible side effects of α-asarone even at a higher dose (30 mg/kg) in the present investigation.

Interestingly, previous research[35] has shown that α-asarone can penetrate the blood-brain barrier and reach effective concentrations in the brain. Furthermore, a recent study[7] revealed that α-asarone treatment increased the expression of m1 mAChR and ACh levels while decreasing AChE activity in the striatum. α-Asarone improved memory and reduced brain cholinesterase activity in the cortex, hippocampus, and striatum brain regions. Thus, the anti-amnesic effect of α-asarone in this study could be attributed to its anti-cholinesterase activity in various brain regions. Together with the preceding findings, this study strongly suggests that α-asarone may be able to slow the decline of cognitive function in AD patients.

Conclusions

Based on the results of the elevated plus-maze test, passive avoidance test, and active avoidance test in scopolamine-induced amnesic rats suggest that α-asarone had remarkable memory-enhancing activity. This study also observed a reduction of AChE activity in different brain regions by α-asarone treatment in scopolamine-induced amnesic rats. This study strongly suggests that α-asarone, which has anti-amnesic activity by inhibiting AChE activity, could be a useful therapeutic option in the management of AD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Data Availability

Data sharing is not applicable to this article.

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