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The Methanolic Extract Of Rhinanthus Songaricus Alleviates Scopolamine-Induced Memory Dysfunction In Rats

Hsiu-Mei Hsieh[1], Aldarmaa Jalsrai[2*], Anu Altangerel[3], Myadagbadam Urtnasan[4], Yue Cong[5], Kristina Battsogt[6]

[1] National Natural University of Taiwan, Department of Life sciences, Taipei, Taiwan
[2,3,4] Institute of Traditional Medicine and Technology, Mongolia
[5] Pharmaceutical College, Henan University, Kaifeng City, China
[6] Department of Molecular Biology and Biochemistry Rutgers University–New Brunswick

[*]Corresponding author: jaldarmaa@gmail.com

Abstract: *Rhinanthus songaricus (RS), a hemiparasitic plant belonging to the family Orobanchaceae, is native to Mongolia. This study aimed to evaluate the neuropharmacological effects of a methanol extract from the aerial parts of R. songaricus in a scopolamine-induced rat model of Alzheimer's disease.*

Phytochemical screening for flavonoids was performed via thin-layer chromatography (TLC) and ultraviolet spectrophotometry. To assess neuropharmacological effects, rats were orally treated with RS methanolic extract (100 or 200 mg/kg, orally) for 14 days. During this treatment period, cognitive impairment was induced by intraperitoneal injection scopolamine (2 mg/kg, i.p.) for 10 consecutive days.

Phytochemical analysis revealed the presence of apigenin and luteolin flavones, with total flavone content equivalent to 16 mg luteolin per gram of extract. Scopolamine significantly induced locomotor deficits, anxiety-like behavior, and memory impairments. Pretreatment with RS extract at 100 and 200 mg/kg significantly reversed these effects in behavioral tests. Scopolamine also significantly increased acetylcholinesterase (AChE) activity in the hippocampus; this increase was significantly attenuated by RS extract at 200 mg/kg, but not at 100 mg/kg. No significant changes were observed in angiotensin-converting enzyme (ACE) activity across all groups.

The methanol extract of R. songaricus exhibited neuroprotective effects, likely mediated by reducing AChE activity in the hippocampus. These findings suggest that RS extract may have potential benefits in alleviating Alzheimer's-like symptoms.

Keywords: *Luteolin, Memory Deficits, Rhinanthus songaricus*

1. INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder, primarily characterized by the excessive production and accumulation of amyloid-beta peptides and the formation of extracellular neurofibrillary tangles, leading to memory loss and cognitive decline. [Jack Jr C.R et al 2019]. Moreover, the progressive neuropathological and neurobehavioral changes associated with Alzheimer's disease are frequently accompanied by additional symptoms like depression, aggression, confusion, and social withdrawal, which all have a significant impact on the quality of life of patients [Livingston et al., 2020]. Currently, more than 50 million people worldwide are affected by dementia, and the incidence rate is increasing by 20% annually (Monfared Tahami AA et al 2022].

Reactive oxygen species (ROS) and free radicals play a significant role in the pathogenesis of Alzheimer's disease (AD), contributing to oxidative stress, neuronal damage, and neurodegeneration [Bhatt S et al 2021]. In this context, medicinal plants have emerged as a promising source of bioactive compounds with antioxidant, anti-inflammatory, and neuroprotective properties, making them potential candidates for the development of future therapeutic agents against AD [Muhammad A 2017].

Species of the genus **Rhinanthus** (family Orobanchaceae) are known to be rich in bioactive compounds, including phenolic acids, flavonoids, anthocyanins, and saponins, which exhibit a wide range of pharmacological properties [Zhang 2021]. Various Rhinanthus species have been traditionally used to treat ailments such as bacterial infections affecting the eyes and ears, gastrointestinal disorders, and symptoms of the common cold [Gonchig E 2008]. Several studies have reported that R. angustifollia exhibits antibacterial and anti-tumor properties [Giordani C 2020]. Despite their rich phytochemical profile, the potential effects of Rhinanthus species on neurodegenerative diseases remain unexplored. One such species is Rhinanthus songaricus (RS), which has not yet been studied for its chemical composition or pharmacological properties. Notably, this is the only species of the Rhinanthus genus that grows in Mongolia. Identifying the bioactive phytochemicals in its extract is essential for the development of new therapeutic agents.

Scopolamine (SCO) is a muscarinic receptor antagonist known to induce memory impairment in both humans and animals by blocking cholinergic neurotransmission and increasing oxidative stress. It is widely used to induce Alzheimer's disease (AD)-like neurodegeneration in experimental animal models.

This study was therefore designed to evaluate the neuroprotective potential of the methanol extract of R. songaricus in an in vivo model of Alzheimer's disease (AD) induced by Scopolamine.

2. MATERIALS AND METHODS

2.1 Reagents

Scopolamine hydrobromide (SCO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetylcholinesterase enzyme (AChE), and angiotensin-converting enzyme (ACE) assay kits were purchased from BioTech. All other chemicals, including the reference compounds apigenin (98%) and luteolin (98%), were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.2 Plant material and Preparation extract

The aerial parts of RS were collected from Tulu Mountain, Tsaagan soum, Bulgan Province, Mongolia, in early August 2024. The plant specimen was identified and authenticated by Prof. E. Munkh-Erdene from the Institute of Botany, Mongolian Academy of Sciences. A total of 500 g of the dried aerial parts were powdered and extracted with methanol at room temperature for 48 hours. The extract was filtered, and the solvent was removed under reduced pressure at temperatures below 50 °C to yield a solid extract. The final extract was stored at 4 °C until further use.

The dried and powdered aerial parts of the plant (250 g) were macerated in 99.9% methanol at a 1:10 (w/v) ratio and kept at room temperature for 7 days. The mixture was then filtered through Whatman No. 1 filter paper. The resulting filtrate was concentrated using a rotary evaporator and subsequently lyophilized. The lyophilized extract was stored and used for subsequent studies.

2.3 Determination of Total Flavones by Colorimetric Method

The total flavones content in the methanol extract of RS was determined using a colorimetric method as previously described [Favaro G 2007]. Briefly, 1 g of the aerial part of RS was mixed with 50 mL of 100% methanol and heated under reflux at 70–80°C for 1 hour. The extract was allowed to cool, filtered, and transferred to a 50 mL volumetric flask. The volume was adjusted with methanol to obtain Solution A.

From Solution A, 1 mL was taken and transferred to another 50 mL volumetric flask. Then, 2 mL of 2% aluminum chloride solution and a drop of 30% acetic acid were added. The volume was brought up to 50

mL with 100% methanol to obtain Solution B. The absorbance of Solution B was measured at 400 nm using a UV-Vis spectrophotometer.

A blank solution was prepared by mixing 1 mL of 100% methanol with 2 mL of 2% aluminum chloride, omitting the plant extract. Concentration of stock solution was 0.02 mg/ml

The total flavones content was calculated as Luteolin equivalents (LE) using the following equation:

$$X = \frac{C \times V_1 \times V_3 \times 100}{a \times V_2 \times (100 - W)} \times 100$$

X – Total luteolin content, %
C– Concentration of standard Luteolin, mg/ml
a – mass of the sample, mg
V1, V2, V3 – Dilution of test solution, ml (50, 0.4, 10)
W- Moisture, %

2.4 Animals and Treatments
Adult male Wistar rats (200–250 g) used in this study were obtained from the Experimental Animal Center in Ulaanbaatar, Mongolia. Upon arrival, the animals were housed under controlled laboratory conditions: a 12:12 h light-dark cycle (lights on at 6:00 a.m.), a temperature of 20 ± 2°C, and relative humidity of 55–60%, with free access to food and water. The rats were allowed a two-week habituation period prior to the start of the experiments. All procedures were carried out in accordance with the ethical guidelines of the Mongolian and European Animal Ethics Committees.

The rats were randomly divided into four groups (n = 6 in each group). The RS extract was given orally once a day for 14 days. From Day 3 to Day 14, all groups except normal control (Group 1) was pretreated by scopolamine (SCO, 2 mg/kg, i.p.) once daily to induce memory dysfunction [Tancheva L et al. 2022]. On the 11th day, Open field test was performed to assess the locomotor activity. Memory performance was assessed on Day 12 using the NOR (Novel Object Recognition). On days 13 and 14, memory impairment was additionally examined using PA (passive avoidance) test. Group 1 (Normal Control): Received distilled water. Group 2 (Negative Control): Received distilled water. Group 3: Received RS methanol extract at 100 mg/kg, and SCO. Group 4: Received RS methanol extract at 200 mg/kg and SCO. **All drugs were prepared in distilled water and administered at dosages determined from previously published research.**

2.5 Novel object recognition (NOR) test
The NOR test was conducted to assess recognition memory, according to the method of Kozela et al. (2020). The open field (OF) apparatus (60 × 60 × 60 cm) was used, with uniform light intensity across the entire area. **Training 1 (T1):** On Day 1, each rat was placed in the arena for 2 minutes with two identical objects (wooden cubes, 10 × 10 × 10 cm) positioned in two opposite corners. The rat was then allowed to explore the objects for an additional 5 minutes. **Training 2 (T2):** A plastic bottle was used as a novel object (N) in place of one familiar objects (F) following a 60-minute retention period in the home cage. The rat was returned to the middle of the arena and allowed to explore both objects for another 5 minutes. All sessions were recorded using a video camera. The time spent exploring the novel object (TN) and the familiar object (TF) was measured using stopwatch. Exploration of an object such as when rats touched or sniffed the object with their forepaws and nose.

The Recognition index (RI) was calculated as follows:

$$RI = \frac{TN}{(TN+TF)} * 100$$ (Jang and Lee, 2023).

2.6 Passive Avoidance Test

The passive avoidance test was conducted according to the method described by Kim CY et al [2014], using a commercial apparatus (Ugo Basile, USA). The apparatus consisted of two equal-sized compartments one illuminated and one dark each measuring 22 × 22 × 30 cm. The floor of both compartments was made of stainless steel rods that could deliver an electric shock. The compartments were separated by a guillotine-type sliding door. During the acquisition trial, each rat was placed in the light compartment and allowed to explore for 30 seconds. After this habituation period, the door between the compartments was opened. When the rat entered the dark compartment, the door was closed instantly, and a foot shock (1 mA, 3 seconds) was given through the grid floor. Twenty-four hours later, a retention trial was conducted to assess memory retention. The rat was again placed in the light compartment, and after 30 seconds, the door to the dark compartment was opened. The latency to enter the dark compartment (Step-Through Latency, STL) was recorded. No shock was given during this trial. An STL of 300 seconds was given if the rat was failed to enter the dark compartment within 300 seconds.

2.7 Tissue preparation

After 14 days of treatment, and following the completion of behavioral tests, the animals were sacrificed under steam chloroform anesthesia. The entire brain of each animal was immediately removed, rinsed in ice-cold normal saline to eliminate blood, and stored at -80 °C. Each brain was then bisected sagittally, and the hippocampus from both hemispheres was rapidly isolated and homogenized in 10% (w/v) of 0.1 M phosphate-buffered saline (PBS, pH 7.4) using a YG-IIn homogenizer. The homogenates were centrifuged at 12,000 rpm for 15 minutes. The supernatants were collected and stored at -80 °C until further use. Protein content was estimated using the bovine serum albumin (BSA) as the standard.

2.8 Measurement of AChE, and ACE

Acetylcholinesterase (AChE) enzyme, ACE angiotensin converting enzyme levels in the hippocampus were measured using ELISA kits, following the manufacturer's instructions (Shanghai MLBIO Biotechnology Co. Ltd., China).

2.9 Statistical analysis.

The software GraphPad Prism, version 5.0 was used to analyze the results. Data were expressed as mean ± standard error mean (SEM) and analyzed by one-way ANOVA using Tukey's multiple comparison test. P values of 0.05 or less were considered statistically significant.

3. RESULT

3.1 The phytochemical compositions of RS extracts

TLC Analysis of Methanol Extract Using Solvent System (Hexane–Ethyl Acetate–Acetic Acid, 20:10:2).

The methanol extract of R. S. was subjected to thin-layer chromatography (TLC) to identify the presence of apigenin and luteolin, using various solvent systems. The solvent system comprising hexane–ethyl acetate–acetic acid (20:10:2) yielded distinct bands corresponding to the standard compounds. The presence of luteolin and apigenin in the extract was confirmed based on their Rf values and color characteristics, which matched those of the standards: luteolin (Rf = 0.25) and apigenin (Rf = 0.35) (Figure 1). Spectral comparison further supported the identification, as two bands observed in the methanol extract aligned precisely with those of the apigenin and luteolin standards.

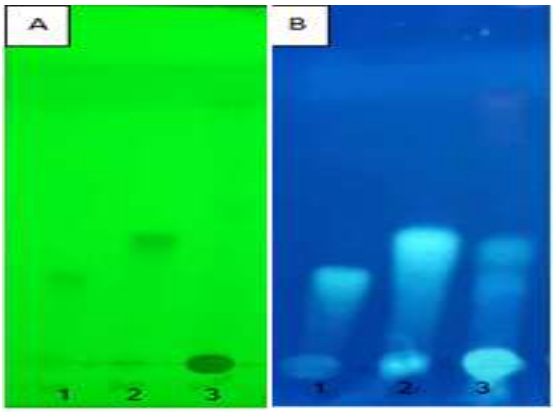

Figure 1. Thin-layer chromatography (TLC) fingerprint of methanol extract of RS.
Lane 1: Luteolin standard; **Lane 2**: Apigenin standard; **Lane 3**: Methanol extract of RS.
A. Visualization under UV light at 254 nm; **B.** Visualization under UV light at 365 nm.

The total flavone content was quantified using luteolin as the standard, and the result was expressed as milligrams of luteolin equivalents per gram of extract (mg LuE/g). A standard calibration curve for luteolin was established with the equation y = 0.0125x – 0.0085 and a high coefficient of determination (R^2 = 0.9984) within the concentration range of 2.0–10.0 mg/ml. Based on this calibration, the methanol extract was found to contain a total flavone content of 16 ± 0.14 mg LuE/g.

3.2 Behavioral tests

Table 1 summarizes the findings of the open field test including measurement of locomotor activity and anxiety-related behavior. The scopolamine treated group showed a significant decrease in the number of crossings and rearing compared to the control group indicating a marked reduction in locomotor activity in rats. Pretreatment with R songaricus methanolic extract (RS) at dose of 100 mg/kg significantly increased the number of crossings and rearings in animals compared to the scopolamine group ($p<0.01$). Although, RS at dose 200 mg/kg also increased the number of crossings and rearings, there was no significant difference between the 200 mg/kg RS group and the scopolamine group.

The time spent in the center of the arena was significantly reduced in the rat scopolamine-treated group compared to the control group ($p<0.01$) indicating anxiety like behavior. However, the time spent in the center of arena was significantly increased compared to the scopolamine treated group when pretreated with 100 mg/kg of R. songaricus methanolic extract which suggests anxiolytic activity.

TABLE 1. Summary result of the Open field test.

Parameters	Number of crossing	Number of rearing	Time spent in the center
Control	46.14 ± 2.01	13.12 ± 1.2	3.1 ± 1.3
SCO	23.11 ± 1.2 ##	5 ± 1.8 ##	0.98 ± 0.5##
RS (100 mg/kg)	54.66 ± 3.2 **	14.67 ± 1.9 **	3.42 ± 1.1**
RS (200 mg/kg)	36.01 ± 1.7	9.43 ± 1.6	0.84 ± 0.9

Data are expressed as mean SEM (n=6), ## $p<0.01$ vs control; ** $p<0.01$ vs SCO group, SCO- Scopolamine.

The results of NOR test, summarized in Fig.2A. Short-term recognition memory was assessed using the recognition index percentage. In the scopolamine treated group, the recognition index was significantly reduced to 34.04% compared to 71.046% in normal control group ($p<0.05$), indicating impaired

recognition memory. Pretreatment with RS methanol extract at dose of 200 mg/kg significantly increased recognition index to 70.08% compared to the SCO-treated group, demonstrating a clear preference for the novel object. However, pretreatment with 100 mg/kg RS extract improved the recognition index to 59.34% countering scopolamine-induced impairment, but this improvement was not statistically significant. The NOR test results show that RS methanolic extract effectively counteracts SCO-induced cognitive deficits and restores memory function (Fig. 2A).

The effect of RS on scopolamine induced memory dysfunction was evaluated using the PA test. In Fig.1B, show the data of the conducted test. The scopolamine (SCO)-treated group exhibited a significantly reduced latency time (22.23 ± 1.9 seconds) compared to the control group (235.4 ± 14.35 seconds, $P < 0.001$), indicating marked impairment in passive avoidance memory (Fig. 1B). In contrast, latency periods of the groups pretreated with RSME 100 and 200 mg/kg were significantly longer than those of the SCO-treated group, suggesting a protective effect against memory impairment. These findings demonstrate that administration of the RS methanolic extract improved memory retention. (Fig. 2B).

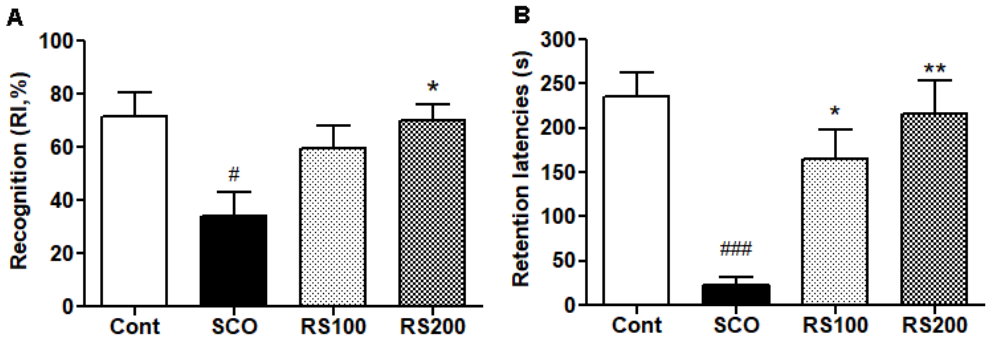

Figure 2. Effects of RS methanolic extract on SCO-induced memory impairment in rats. A - NOR test: The RI in the test NOR is measure of the ability to discriminate between new and familiar objects. B - Passive avoidance test: Latency time to enter dark chamber in retention trials. Data are expressed as mean SEM (n=6), # p<0.05, ### p< 0.001 vs control; *p<0.05; **p<0.01 vs SCO group.

3.3 Effect on Acetylcholinesterase (AChE) and angiotensin converting enzyme activities

As shown in Figure 3A, in the rat hippocampus acetylcholinesterase (AChE) activity was significantly higher in the scopolamine treated group compared to the control group ($p<0.01$). Pretreatment with 200 mg/kg of RS methanolic extract significantly reduced AChE activity compared to the scopolamine-treated group ($p<0.01$). However, pretreatment with 100 mg/kg of RS methanolic extract did not show a significant effect on scopolamine-induced AChE activity.

Likewise, Figure 3B, shows that angiotensin-converting enzyme activity in the hippocampus did not show significant changes in any of the treatment groups compared to the control group.

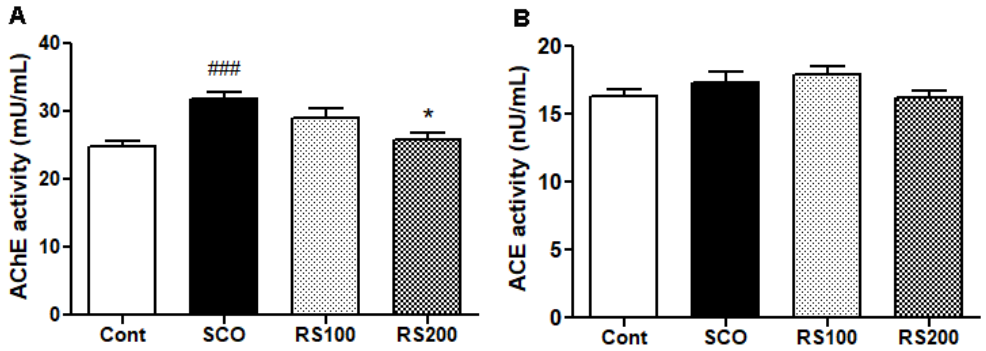

Figure 3. Effect of RS methanolic extract on biochemical parameters in hippocampus rats. A- AChE activity in hippocampus rats. B – ACE- angiotensin converted enzyme activity in hippocampus rats. Data are expressed as mean SEM (n=6), ### p< 0.001 vs control; *p<0.05; vs SCO group.

3.4 Discussion

In the present study, we evaluated the effects of Rhinanthus songaricus (RS) methanolic extract in a scopolamine-induced Alzheimer's disease (AD) model in rats by conducting behavioral tests and analyzing biochemical parameters in the hippocampus. Rhinanthus songaricus has sparked interest due to is rich phytochemicals and largely unexplored bioactive potential. Increasing evidence demonstrated that flavonoids such as luteolin and apigenin showed anti-amnestic properties on scopolamine-induced memory dysfunction, indicating a possible role for flavonoids in alleviating cognitive deficits associated with AD

[Mosal OS 2021; Ahmad S 2021; Kim Y 2021].

In our phytochemical analysis, the RS methanolic extract was found to contain flavones, including apigenin and luteolin, with luteolin quantified at 16 mg/g of extract. This composition suggests that RS extract may possess anti-amnestic and neuroprotective properties.

Scopolamine (SCO) blocks cholinergic signaling and impairs learning and memory in humans and rodents, is widely used in rodents making as a model of Alzheimer's disease.

The open field test is commonly used to assess locomotor activity and anxiety-like behavior in rodents [Seibenhener Ml et al. 2010] Our data showed that rats treated with scopolamine (2 mg/kg for 10 days) impaired locomotor activity reducing number of crossings and rearings in rats compared to the control group. Additionally, the scopolamine-treated rats spent less time in the center of the arena, indicating anxiety-like behavior relative to controls. These data are consistent with the results of other studies that higher doses of scopolamine may cause suppression of locomotor activity, impair cognitive function, and induce anxiety [Klinkenberg I 2010]. We found that pretreatment with 100 mg/kg of RS methanolic extract significantly increased of locomotor activity and showed anxiolytic activity by increasing all parameters in the open field test compared to scopolamine treated group. RS methanolic extract at a dose of 200 mg/kg did not show significant effect in OF test. These results showed that RS methanolic extract ameliorated behavioral dysfunction caused by Scopolamine, suggesting that it may have memory- improving properties [Botton H 2010].

To evaluate the effects of RS methanolic extract on scopolamine-induced memory impairment, we conducted the Novel Object Recognition (NOR) and Passive Avoidance (PA) tests

In the NOR test, scopolamine significantly reduced the recognition index compared to the control group, indicating a clear impairment in recognition memory. Pretreatment with RS methanolic extract at dose of 200 mg/kg significantly increased the recognition index, effectively reversing the scopolamine-induced memory deficits.

Consistent with pervious reports that scopolamine impairs performance in passive avoidance tasks, our results demonstrated that scopolamine administration significantly decreased the latency to enter the dark compartment, suggesting notable memory impairment [Botton H 2010]. Pretreatment with RS methanolic extract in both tested doses (100 and 200 mg/kg) significantly increased latency to enter dark compartment compared to the scopolamine treated group, indicating its protective effect against scopolamine-induced memory impairment in the passive avoidance test.

To partially clarify the mechanisms underlying the effects of RS methanolic extract, we analyzed the levels of acetylcholinesterase (AChE) activity and angiotensin-converted enzyme (ACE) activity in hippocampus of rats.

The hippocampus plays important role in cognition, particularly learning and memory formation [Eichenbaum H 2004]. Other hand, there ample evidence demonstrates that administration of scopolamine can lead to reduced neuronal density in the hippocampus [Seifhosseini S 2011, Jananshan M 2013].

The results of biochemical analysis show that the scopolamine group significantly increased the activity of AChE compared to the control group. This finding is consistent with evidence from other studies which have demonstrated that scopolamine administration in rodents leads to increased AChE activity in the hippocampus [Rahimzadegan M 2012]. Pretreatment with 200 mg/kg of RS methanolic extract effectively reversed the scopolamine-induced increase in acetylcholinesterase (AChE) activity, thereby protecting the animals from learning and memory impairment. Notably, pretreatment with a lower dose of 100 mg/kg did not significantly affect the AChE activity compared to the scopolamine group, suggesting a dose-dependent protective effect of RS methanolic extract against memory deficits.

Likewise, we examine involvement angiotensin-converting enzyme (ACE) in protective effect of RS methanolic extract in hippocampus rat.

Angiotensin-converting enzyme (ACE) is best known for its role in the renin-angiotensin system, where it catalyzes the conversion of the non-vasoactive precursor angiotensin I (ang I) into the potent vasoconstrictor octapeptide angiotensin II (Ang II) [McKinley M 2003]. Moreover, increasing evidence demonstrated that ACE mediated the cleavage of amyloid-beta peptides, which accumulation is hallmark of Alzheimer's disease. Differences in ACE activity may lead to change Amyloid accumulation [Miners S et al 2009].

Despite numerous data suggesting the possible involvement of angiotensin-converting enzyme (ACE) in the pathological process of Alzheimer's disease [Hou D 2021], our results did not show significant changes in any of the treatment groups compared to the control group.

4. CONCLUSION

These findings provide the first insight into the improving memory and neuroprotective potential of Rhinanthus songaricus (RS) methanolic extract. Our results suggest that further analysis of RS is necessary to determine its the chemical composition and pharmacological properties, in order to assess its therapeutic potential of this plant.

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ABBREVIATIONS
AChE acetylcholinesterase enzyme
ACE angiotensin-converting enzyme
RS Rhinanthus songaricus

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CONFLICT OF INTEREST The authors declare no conflict of interest.

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AUTHORS CONTRIBUTIONS
AJ, KB performed experiments, data acquisition, analyzed results, and wrote the article, AA collected experimental data and participated in completing the relevant experiments. UM performed a study on phytochemistry, data acquisition, and data analysis. HMH contributed to writing article and final form.

AJ, AA, UM, KB and HMH confirmed the authenticity of all raw data and agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

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