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Original Article

Effects of *Piper nigrum* fruit and *Cinnamum zeylanicum* bark alcoholic extracts, alone and in combination, on scopolamine-induced memory impairment in mice

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Abstract

Background and purpose: Alzheimer's disease is a progressive brain disorder that is thought to be triggered *via* disruption of cholinergic neurons and enhanced oxidative stress. Therefore, antioxidant phytochemicals with the ability to fortify cholinergic function should help in preventing the progress of the disease. This study aimed at evaluating the combinational effects of two popular herbs one with anticholinesterase activity namely *Piper nigrum* and the other with antioxidant capacity, *Cinnamomum zeylanicum*.
Experimental approach: In this study, *P. nigrum* extract (PN) (50, 100 mg/kg, ip) and *C. zeylanicum* extract (CZ) (100, 200, 400 mg/kg, ip) and their combinations were administered for 8 days before the injection of scopolamine (1 mg/kg, ip). Mice were then tested for their memory using two behavioral models, namely the object recognition test and the passive avoidance task.
Findings/Results: Administration of scopolamine significantly impaired memory performance in both memory paradigms. In the passive avoidance test (PAT) model, PN at doses up to 100 mg/kg and CZ at doses up to 400 mg/kg did not significantly alter the memory impairment induced by scopolamine. The combination of these two plant extracts did not change the PAT parameters. In the object recognition test (ORT) model, however, administration of 100 mg/kg CZ alone and a combination of PN (50 mg/kg) with CZ (400 mg/kg), significantly increased the recognition index ($P < 0.05$).
Conclusion and implications: Two plant extracts when administered alone or in combinations affected the memory performance differently in two memory paradigms. In the PAT model, the extracts did not show any memory improvement, in ORT, however, some improvements were observed after plant extracts.

Keywords: Alzheimer; *Cinnamum zeylanicum; Piper nigrum*; Scopolamine.

INTRODUCTION

Alzheimer's disease (AD) is a chronic, destructive, and progressive disorder of the central nervous system that affects neurons in the hippocampus and cortex. Pathologically, there are several theories for AD, which include; decreased levels of acetylcholine, accumulation of amyloid plaques, and neurofibrillary tangles in the brain (1) and also, oxidative stress caused by amyloid plaques (2).

Traditionally, plants have been used to enhance memory and reduce the symptoms associated with AD disease (3). The two leading drugs (galantamine and rivastigmine) that are currently used for the treatment of dementia are derived from plant sources. Curcumin, ginseng, *Ginkgo biloba* are the most popular herbs that researchers have put a seal for their effects on memory and cognition disorders (4).

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Piper nigrum (*P. nigrum*) from the Piperaceae family is one of the most widely used spices in the world. The main component of this plant is piperine, the amount of which in the plant is between 5-10% (5). Piperine and its isomer called chavicine have been significantly reduced the expression of amyloid-producing isoforms in the cerebral cortex, hippocampus, and amygdala (6). Previous studies have claimed that *P. nigrum* can inhibit acetylcholinesterase and butyrylcholinesterase enzymes by as much as 55% and 90%, respectively (7).

Cinnamomum zeylanicum (*C. zeylanicum*) belongs to the Lauraceae family, with a long history in traditional medicine. Its bark has been used as an antidiabetic, antioxidant, anticarcinogenic and anti-inflammatory agent (8). Scientists believe that *C. zeylanicum* might have therapeutic effects on AD. Cinnamon contains polyphenols that control oxidative stress by destroying reactive oxygen species (ROS) and inflammatory pathways through inhibiting lipid peroxidase enzyme (9). Also, *C. zeylanicum* has the ability to inhibit tau aggregation, the formation of amyloid β toxic oligomer species, and can correct the cognitive impairment in AD animal models (10).

There is a considerable body of evidence that supports the individual action of *P. nigrum* extract (PN) and *C. zeylanicum* extract (CZ) in improving memory in both normal brains and impaired ones (11). The effect of simultaneous administration of these plant extracts, however, has not been investigated before. Therefore, the present study was designed to evaluate the possible ameliorative effects of these plant extracts alone or in combination on cognitive impairment induced by scopolamine in mice. Scopolamine is a nonselective antimuscarinic agent that leads to progressive impairment of learning and memory principally by blocking central cholinergic signaling (12). ROS generated by scopolamine result in oxidative stress, a critical factor that results in AD-like dementia (13,14).

MATERIALS AND METHODS

Preparation of the extracts

P. nigrum fruits and *C. zeylanicum* barks were purchased from the local market of Isfahan and authenticated by a pharmacognosy specialist. In this study, plants were crushed to a fine powder (3 kg) and macerated in 20 L of ethanol 96% three times (each time 48 h) at room temperature. The extract was filtered and concentrated in a rotary evaporator. The alcoholic extract was then lyophilized to obtain dried powders of *P. nigrum* (PN) and *C. zeylanicum* (CZ) ready for use in subsequent experiments (15).

Determination of total polyphenols

Total phenolic content was estimated spectrophotometrically using Folin-Ciocalteu reagent as described by Everette (16). Briefly, the diluted reagent was mixed with plant samples. After 5 min, sodium carbonate solution (20%) was added to the mixture followed by incubation at room temperature for 120 min, subsequently, UV absorbance was measured at 765 nm using a UV-visible spectrophotometer (Jenway, UK). Total phenolic content was quantified by a standard curve obtained from various concentrations of gallic acid (50-500 μg/mL in ethanol).

The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dried extract.

Chemicals

Scopolamine in the form of 20 mg ampoules, was purchased from Exir Co (I.R. Iran). Rivastigmine 1.5 mg was purchased from Razak Co. (I.R. Iran).

Animals

Male mice (25-30 g) were supplied by the animal house of Isfahan University of Medical Sciences. The animals were housed under standard laboratory conditions and were provided with free access to standard pellet feed and water. All procedures were approved by the ethical committee of the Isfahan University of Medical Sciences (Ethics ID IR.MUI.RESEARCH.REC.1398.603) and conducted by the internationally accepted principles for laboratory animal use and care.

Treatments

Five groups of mice, 6 each, were used and received daily for 8 days: saline for vehicle group, rivastigmine (2 mg/kg, ip) as the positive control group, and either PN (50 and 100 mg/kg, ip), CZ (100, 200, and 400 mg/kg,

ip), or both PN (50 mg/kg) and CZ (100 and 400 mg/kg). All groups received scopolamine (1 mg/kg, ip) on days 8 and 9 of the experiment (Table 1). The solutions were freshly prepared daily by dissolving in water. All animals were trained prior to the assessment of cognition. Amnesia was induced with scopolamine and the training trials were started 60 min after its injection (2,9).

Behavioral assessments

Passive avoidance test

Passive avoidance test (PAT) was carried out according to the previously described method by Safavi *et al*. (17). Step-through passive avoidance is an effective behavioral task commonly used to determine the function of the brain involved in memory. The device consists of two chambers (bright and dark) that are separated by a guillotine door. On the training trial, each mouse was placed into the bright side of the compartment while the central door was closed. After 10 s the door to the dark compartment was opened and simultaneously, timing by a computer was initiated. As soon as, the mice entered the dark compartment, the door was closed and after 3 s received the foot shock (1 mA for 3 s) through the floor stainless steel bars. The test session was performed 24 h after the training session. The first time that mice entered the darkroom was recorded as step-through latency. In this study, the number of passages between rooms as well as, the total time spent by mice in the dark chamber was also recorded. The maximum delay in the bright chamber is 180 s.

Object recognition test

The object recognition test (ORT) was performed, as previously described (17). ORT was performed in a circular field with a diameter of 35 cm and a height of 30 cm. One h before the training session, mice were adjusted to the empty field for 10 min and brought back to their original cages. In the trial session, two identical objects were placed in the field within 10 cm distance from each other and 5 cm distance from the wall. Mice were then put into the place for exploration. Exploration was defined as sniffing or touching the objects with the nose (the distance to the object is less than or equal to 2 cm).

Sitting on objects was not taken as an exploratory behavior. In the training session exploration time was 3 min. After 24 h of training, one of the familiar objects was replaced by a new object that is similar in size but different in shape. After 5 min of exploration by mice, the exploration time of each object was calculated. Mice that did not meet the standard were excluded from the analysis. Mice with 0 or 100% relative exploration over a period of time, i.e. without any exploration or exploration of just one object, were also excluded from the analysis. Finally, the discrimination index (DI) and recognition index (RI) were calculated using the following equations:

$$DI = \frac{\mathrm{N} - \mathrm{F}}{\mathrm{X}} \times 100$$

$$RI = \frac{\mathrm{N}}{\mathrm{X}} \times 100$$

where, N is the exploration time of a new object, F is the exploration time of a familiar object, and X is the total exploration time of objects.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a post hoc Tukey test. $P < 0.05$ was considered significant. All data are expressed as mean ± SEM. Also, if necessary, Student's t-test was used to compare between two groups.

Table 1. Experimental procedure. Extracts of *Cinnamomum zeylanicum* (CZ) at 100, 200, and 400 mg/kg, *Piper nigrum* (PN) at 50 and 100 mg/kg, and their combination were administered as explained in the method section.

Treatment and test	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9
Normal saline	+	+	+	+	+	+	+	+	−
Scopolamine	−	−	−	−	−	−	−	+	+
Rivastigmine	+	+	+	+	+	+	+	+	−
PN	+	+	+	+	+	+	+	+	−
CZ	+	+	+	+	+	+	+	+	−
CZ + PN	+	+	+	+	+	+	+	+	−
Passive avoidance	−	−	−	−	−	−	−	Train	Test
Object recognition	−	−	−	−	−	−	−	Train	Test

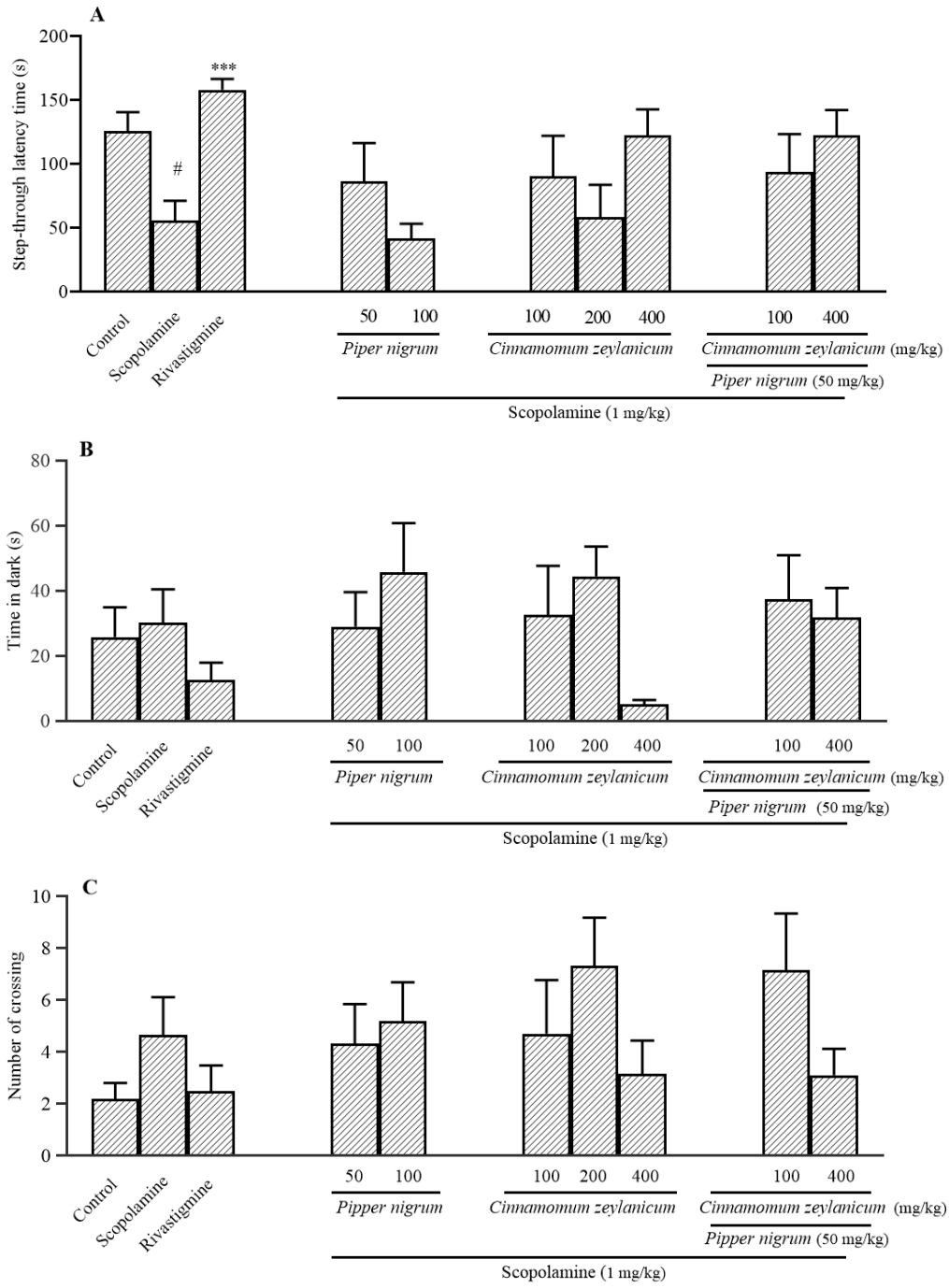

Fig. 1. Effects of *Piper nigrum*, *Cinnamomum zeylanicum* alcoholic extracts, and their combinations on memory performance in passive avoidance test. On days 8 and 9, memory deficit was induced by scopolamine (1 mg/kg, i.p.). Animals were tested after 24 h the training, for retention of memory. Cognitive performance was measured by (A) step-through latency time of entering the dark chamber for the first time, (B) total time spent in the dark chamber, and (C) the number of crossings between chambers and 24 h after train session. Results are expressed as mean ± SEM, n = 6. $^{\#}P < 0.05$ indicates significant differences compared with the control group, $^{***}P < 0.001$ versus the scopolamine group.

RESULTS

The total phenolic

The total phenolic content of extracts was determined by Folin-Ciocalteau's method. According to this test, the amount of phenolic content for CZ and PN alcoholic extracts was 164 and 27.4 mg GAE/g of dried extract, respectively.

Passive avoidance test

The latency of entering the dark chamber was recorded as a step-through latency time. Administration of scopolamine at 1 mg/kg, significantly decreased the latency time by 55% in comparison to the control group ($P < 0.05$, Fig. 1A).

Rivastigmine at 2 mg/kg reversed the scopolamine-induced acquisition deficit ($P < 0.001$). Administration of PN (50 and 100 mg/kg) and CZ (100, 200, and 400 mg/kg) did not show a significant change in the step-through latency time. A combination of PN 50 mg/kg and CZ (100 and 400 mg/kg), did not have significant effects on the step-through latency time (Fig. 1A).

Scopolamine did not significantly change the time spent in the dark chamber (Fig. 1B) and the number of crossing in comparison to the control group (Fig. 1C). In this paradigm, rivastigmine did not reverse the acquisition deficit induced by scopolamine. Administration of PN (50 and 100 mg/kg) and CZ in all doses (100, 200, and 400 mg/kg) did not alter the time spent in the dark chamber and the number of crossing between chambers (one-way ANOVA test). Neither the co-administration of PN (50 mg/kg) nor CZ (100 and 400 mg/kg), have any prominent effect on time spent in the dark chamber (Fig. 1B) or the number of crossing between chambers (Fig. 1C).

Object recognition test

Administration of scopolamine significantly decreased DI by 80% ($P < 0.01$, Fig. 2A). Rivastigmine enhanced DI induced by scopolamine approximately to the control level. PN in doses of 50 and 100 mg/kg and CZ in doses of 200 and 400 mg/kg had no significant effect on DI, while CZ at 100 mg/kg increased the DI in comparison to the control group ($P < 0.05$, Student's t-test). When we combined the PN at 50 mg/kg and CZ at either dose of 100 and 400 mg/kg, the result was not much different from separate administrations of these extracts on memory improvement (Fig. 2A).

Administration of scopolamine significantly decreased RI compared to the control group ($P < 0.05$, Fig. 2B). Rivastigmine increased RI induced by scopolamine approximately to the control level. PN in doses of 50 and 100 mg/kg and CZ in doses of 200 and 400 mg/kg did not cause a significant change in RI, but in CZ at 100 mg/kg, as in co-administration PN (50 mg/kg) with CZ (400 mg/kg), RI increased significantly ($P < 0.05$, Fig. 2B).

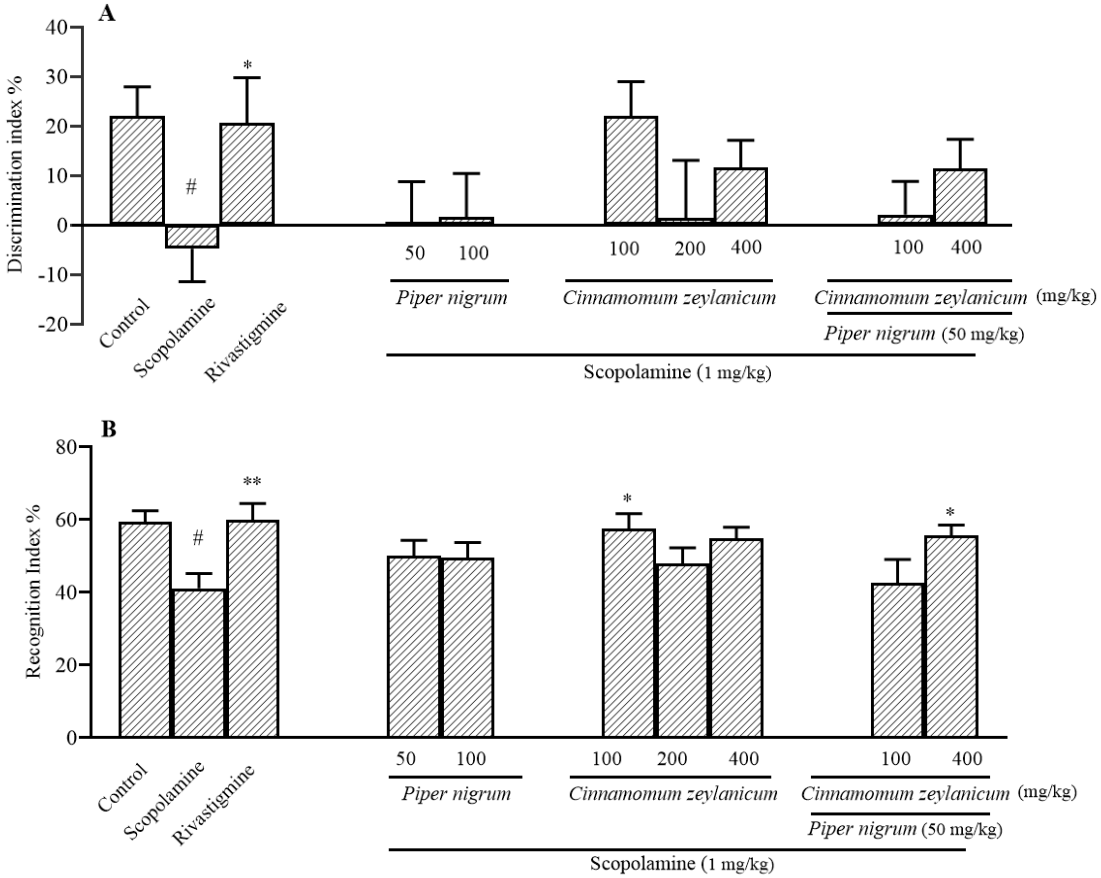

Fig. 2. Effects *Piper nigrum*, *Cinnamomum zeylanicum* alcoholic extracts, and their combinations on memory performance in the object recognition test. On days 8 and 9, memory deficit was induced by scopolamine (1.0 mg/kg, i.p.). Animals were tested after 24 h training, for retention of memory. Cognitive performance was expressed as (A) the percentage discrimination index and (B) the recognition index. Results are expressed as mean ± SEM, n = 6. $^{\#}P < 0.01$ indicates significant differences compared with the control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ against scopolamine group.

DISCUSSION

The most important pathological features of AD are due to the accumulation of amyloid beta and tau proteins in the nervous system and disorders of the cholinergic system and oxidative stress. Plants with large amounts of biologically active compounds could be used for the prevention and treatment of neurological disorders such as AD (18,19). Several studies have shown the beneficial effects of separate administration of PN and CZ extracts in different models of memory impairment (6,7,11,12). However, no one before has looked at the effect of co-administration of these extracts on memory performance. In the current study, we investigated the effects of PN and CZ extracts, alone and in combination, in scopolamine-induced memory impairment in mice.

In the present study, two models, namely, PAT and ORT were used for the assessment of memory in mice. Scopolamine significantly reduced discrimination and recognition indices in these models. Though memory impairment was seen in both models, the rate of degeneration was not the same in both paradigms. When we compared the two models, the intensity of memory impairment was greater in ORT. This could be due to different brain regions involved in memory assessments. The ORT test is mostly related to the para-hippocampus, but the PAT test usually plays an important role in the hippocampus and amygdala of the brain (20,21). In fact, non-spatial memory is measured in ORT and spatial memory in PAT (22,23). The results obtained by other researchers investigating the performance of scopolamine in these two models are in agreement with our findings (24,25). Rivastigmine at 2 mg/kg reversed the scopolamine-induced acquisition deficit. Studies have shown that rivastigmine inhibits acetylcholinesterase enzymes in the brain and this way antagonizes the deficits in working and reference memory. In a similar manner to other studies, rivastigmine in our work improved the DI and the RI in ORT and latency time in PAT (26). Together with the scopolamine data, these findings validated the methods used as indeed they were proved by other investigators.

In the present work, only CZ at 100 mg/kg, as well as, the co-administration of PN at 50 mg/kg with cinnamon at 400 mg/kg, in ORT model showed a positive effect on memory, while in other results, the administration of extracts did not demonstrate a significant effect on the learning process and memory improvement.

The total phenolic content of the alcoholic extract of PN fruit was found to be 27.4 mg GAE/g of dry extract. This amount varies between different plant species ranging from 12.03 to 22.88 mg GAE/g (27). The total phenolic content of alcoholic extract of CZ bark in our study was approximately equal to 164 mg GAE/g of dry cinnamon extract which is lower than the values (200 mg GAE/g of dry extract) found by other researchers (28,29). The extraction process that we used was carried out with 96% alcohol.

The aqueous and alcoholic extract of CZ can inhibit up to 40% of the acetylcholinesterase enzyme in a dose-dependent fashion (30,31). In some studies, the inhibitory activity for the PN has been shown to be close to 70%. Furthermore, the PN alcoholic extract has been shown to have excellent performance in inhibiting amyloid accumulation and antioxidant activity (32). Based on the above-mentioned evidences, it can be argued that ethanol can be a good solvent for extracting PN and CZ in this study.

The lack of clear effects on memory by the plant extracts in this study could be due to several factors. The length of the treatment with plant extracts administration is an important factor. We used an eight-day treatment here, while other studies have extended this period to as many as four weeks. (6,33). One reason for not extending the period of treatment with pepper is the irritating action of this plant. Animal species could be another factor in determining the response by drugs or plant extracts. This is especially true when we are dealing with memory assessment. In order to cut the cost of materials, we used mice in this study. However, it is well established that rats are generally more clever than mice. Snyder *et al*. showed that more neurons are involved in the learning processes of rats (34). In addition, Ellenbroek *et al*. showed that rats are a better

species than mice for cognitive tests because they have more stable performance over time and are less affected by non-cognitive distractions (35). Thus, these reasons suggest that rats can be a better model than mice in memory and cognitive studies. Laboratory conditions can also be important in determining any behavioral outcome. Animal husbandry, environment, social housing, etc. can simultaneously decrease the quality of life for rodents (36).

CONCLUSION

In conclusion, the results of this study showed the nature of the memory paradigm that is used to test drugs or plant extracts could be quite determining. In contrast to PAT, the ORT model seemed to be more sensitive in detecting the memory-enhancing effects of the current plant extracts. Further research is needed to isolate the major ingredient of the plants and study their underlying mechanism of action.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Author's contribution

M. Rabbani supervised the pharmacolgical experiments and prepared the manuscript. A. Yekdaneh supervised the pharmacognosy studies and M. Teymuori carried out all the experimental work. All authors read and approved the final manuscript.

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