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Original Article

Effects of hydroalcoholic, methanolic, and hexane extracts of brown algae *Sargassum angustifolium* on scopolamine-induced memory impairment and learning deficit in rodents

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Abstract

Background and purpose: Properties of Alzheimer's disease, can be caused by several reasons and there is no definite treatment for it. We aimed to study the effect of the hydroalcoholic extract, methanolic and n-hexane fractions of brown algae *Sargassum angustifolium* on memory impairment in mice and rats.
Experimental approach: Hydroalcoholic extract (25, 50, 100, 200 mg/kg), methanolic (20 and 40 mg/kg) and n-hexane (40 and 60 mg/kg) fractions of *S. angustifolium* were administered for 21 days intraperitoneally before scopolamine injection (2 mg/kg) on day 21. Rivastigmine was administered for 3 weeks intraperitoneally as well. Then, cognitive function was evaluated by three behavioral tests: passive avoidance, object recognition, and the Morris Water Maze test.
Findings/Results: Scopolamine induced memory impairment and rivastigmine significantly reversed the memory dysfunction in all three tests. Hydroalcoholic extract and methanolic fraction significantly reversed scopolamine-induced memory impairment in passive avoidance by 64% and 55% and enhanced the recognition index in the object recognition test. In the Morris water maze test probe trial and training session, on days 3 and 4, the hydroalcoholic extract showed a significant decrease in time spent in the target quadrant and path length, respectively. Also, hydroalcoholic extract and methanolic fraction decreased escape latency time in training sessions on days 3 and 4, by 50% and 31% in comparison to scopolamine. N-hexane fractions had no significant effect on scopolamine-induced cognitive impairment.
Conclusion and implications: Although the n-hexane fraction wasn't effective, the administration of hydroalcoholic extract and the methanolic fraction of *S. angustifolium* enhanced scopolamine-induced memory impairment.

Keywords: Alzheimer; Morris water maze test; Object recognition; Passive avoidance; Sargassum angustifolium; Scopolamine.

INTRODUCTION

Dementia represents an intra-individual pattern of decreases in memory and thinking impairing at least two domains of cognition (1). Alzheimer's disease (AD) which is the most prevalent cause of dementia includes 50 to 75 percent of dementia cases and is a heterogeneous illness with complicated pathobiology. Extra-cellular beta-amyloid plaques and deposition of intra-cellular phosphorylated tau protein in neurofibrillary tangles seem to be the main neuropathological reasons; even though anti-cholinesterase activity, oxidative stress, and inflammatory process have a role in its pathobiology (2-5). Epidemiologic data demonstrate about 33.9 million of the world's population are suffering from AD and this number is going up in the next 40 years (6).

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Furthermore, recent studies predicted an 87% increase in AD prevalence from 2010 to 2050 in Europe. Improvement in life quality and living conditions cause population aging which leads to age-related diseases like AD (2). Although there is no definite treatment for this illness and all treatments are symptomatic, the most effective drugs include acetylcholine esterase inhibitors such as tacrine, donepezil, rivastigmine, and galantamine. These drugs' effectiveness is limited because they cannot prevent the neuro-degeneration process and also, they do not have an impressive effect on mild dementia (7).

Traditionally, plants have been consumed to reduce the symptoms associated with AD disease (8). Many herbal medicines like *Melissa officinalis, Ginkgo biloba, Panax ginseng, Boswelia serrata, Thymus vulgaris*, and *Lavandula angustifolia* were effective on memory owing to their anti-cholinesterase inhibitory, antioxidant, and anti-inflammatory effects (9-14). Seaweed is a marine macroalga comprised of many species. It is classified by photosynthetic pigments into three groups; green, brown, and red algae. Iran has about 1260 km coastal line in the southern Persian Gulf with over 250 species of marine algae that have been reported in this area such as *Sargassum angustifolium*, a type of brown algae that belongs to Sargassaceae family (15). These species of algae have anticancer (16), anti-inflammatory (15), anti-virus, and hepato-protective effects (17). Sodium oligomannate extracted from seaweeds has completed a phase III clinical trial and entered the market for the treatment of Alzheimer's in China. This oligosaccharide was found to therapeutically remodel gut microbiota, preventing the accumulation of phenylalanine and isoleucine in the brain. These two amino acids contributed to AD-associated neuro-inflammation by increasing the differentiation and proliferation of T helper cells in the brain (18).

In addition, anti-cholinesterase (17), antioxidant (19), anti-inflammatory, and neuroprotective effects have been shown in *Sargassum* algae species (20). In recent studies, the effect of *Sargassum* in inhibiting kinase, beta-secretase 1, and butyrylcholinesterase, which are AD biomarkers, has been proven (21-23). Although different species of *Sargassum* have been reported to affect de-loading amyloid beta in the hippocampus and improve memory, no reports have been issued on the anti-amnestic effects of *S. angustifolim* which is localized in the Persian Gulf (24). These findings and the possibility of the presence of sodium oligomannate in these organisms prompted us to investigate the effect of hydroalcoholic extract and methanolic and n-hexane fractions of brown algae *S. angustifolim* on scopolamine-induced memory impairment and learning deficit.

MATERIALS AND METHODS

Drugs and chemicals

Scopolamine hydrochloride was purchased from Exir, Iran and rivastigmine tartrate from Marham Daroo, Iran. Tween 80, methanol, ethanol, and n-hexane were purchased from Merck, Germany. Scopolamine and rivastigmine were dissolved in 0.9% normal saline while extracts were dissolved in saline and tween 80 (0.5%).

Plant material and extraction *procedures*

The coasts of the Persian Gulf near Bushehr province, which is a rich source of seaweed, were selected as the sampling site. Seaweeds were characterized by the Agricultural and Natural Resources Research Center of Bushehr. Voucher specimens were made and deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences (Voucher No. 2662). After air-drying at room temperature and in the shade, the algae were powdered by Moulinex's mill. After that, algae extract was prepared by ethanol 70% at room temperature. Finally, the extracts were evaporated in a vacuum and the n-hexane and methanolic fractions were separated.

Determination of total polyphenols

Total phenolic content was estimated spectrophotometrically using the Folin-Ciocalteu reagent as described by Everette (25). 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.

Animals

In this study, male Syrian mice weighing 20 to 30 g were used in passive avoidance and object recognition tests and rats weighing approximately 200 g were used in the Morris water maze (MWM) test. The temperature, humidity, and brightness of the rats' cages were standard. Animals had free access to food and water *ad libitum*. Animals were acclimated to the experimental environment 2 h before experiments. All tests were done in the range of 8:00 AM to 1:00 PM to prevent the diurnal cycle. A minimum number of animals were used due to ethical issues. All animal experiments were carried out according to the Animal Research Ethics Committee of the Isfahan University of Medical Science (Ethical No. IR.MUI.RESEARCH.REC.1399.624) and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

Mice were randomly divided into 6 groups, each group consisting of 6 mice, as described below:

Group I: 0.9% NaCl daily; group II: 0.9% NaCl daily and scopolamine 2 mg/kg on day 21 (26,27); group III: rivastigmine 1 mg/kg daily and scopolamine 2 mg/kg on day 21 (28); group IV: hydroalcoholic extract of *S. angustifolium* (25, 50, 100, 200 mg/kg) and scopolamine (2 mg/kg) on day 21; group V: the methanolic fraction of *S. angustifolium* (20 and 40 mg/kg) and scopolamine (2 mg/kg) on day 21; group VI: the n-hexane fraction of *S. angustifolium* (40 and 60 mg/kg) and scopolamine (2 mg/kg) on day 21.

All solutions were injected intraperitoneally (i.p.) for 3 weeks. Scopolamine was administered 30 min after main injections i.p. in treatment groups (2 mg/kg).

The starting dose of each treatment was based on previous research. We also administered upper and lower doses in case of effectiveness.

Passive avoidance test

A shuttle box apparatus was used for the passive avoidance test (PAT) (26). This apparatus was designed by Teknik-Azma in Iran. Long-term and short-term memory was assessed by PAT and it is considered a fear-motivated test. A guillotine gate (6 × 7 cm) was used to separate the device and divided into two chambers of the same size (25 × 25 × 20 cm). One of the chambers was dark, and the other was lit with LED light. The floor of the dark and the light chambers were made of stainless steel bars (3 mm) with space 1 cm apart. High sensitivity photoelectric transducer was used for the detection of the animal's position.

In the train session, rodents were placed in the bright room while the door was closed, and after 3 s the guillotine gate opened. Rodents faced a closed door when they entered a dark room and receive an electric foot shock (1 mA) for 3 s through stainless steel rods. After 24 h of training, the mice were returned to the light cabinet, then, the guillotine door was lifted after 3 s. The step-through latency was recorded for mice when they entered the dark chamber. One hour before the training trial, mice received *Sargassum* extract (25, 50, 100, 200 mg/kg, i.p.) or rivastigmine (1 mg/kg, i.p.) as a positive control. Thirty minutes after, 2 mg/kg, i.p. of scopolamine was used to induce memory impairment. Training and test trials were calculated according to the time required for the mouse to enter the dark compartment when the door is opened. Up to 300 s was recorded as latency to enter the dark compartment.

Object recognition test

A circular field (diameter: 32 cm; height: 20 cm) was used for the object recognition test (ORT) test. A video tracking camera was applied on the top of the box to facilitate assessments (26). In the habituation phase, mice were brought to the testing room 30 min before the start of the experiment to familiarize

themselves with the test medium. Mice were then allowed to freely explore the box in the absence of objects for 10 min. Thirty minutes after the start of the experiment, two identical objects were placed in opposite positions at the two corners of the cage. The mice were then allowed to explore similar objects inside the cage for 3 min and then return to their cage. An hour before the 5-min test, the animals were put back in the same box and one of the two objects was replaced with a new one. In this study, we tried to make all the objects used in this study the same in terms of size but different in shape and color. Also, all objects were fixed on the floor of the box to prevent movement. After each test, the entire box and its contents were cleaned with 70% alcohol to remove any olfactory cues (29). The length of time when the animal directed its nose within 2 cm distance of the object or sniffed or pawed the object was considered object exploration time. Sitting or standing on the object was not included as exploration. The recognition index (RI) in the testing phase was calculated using the following equation:

$$RI=\frac{\text{Time exploring novel object}}{\text{Time exploring novel object + time exploring familiar object)}}\times 100$$

MWM test

After performing PAT and ORT on mouse models, groups with significant differences and appropriate results were selected for the MWM test in rat models. To perform this test, a black circular pool, 120 cm in diameter and 60 cm in height, up to 30 cm was filled with water (211 °C) (30). A circular platform 10 cm in diameter and 28 cm high was placed on the pool so that 2 cm of it was submerged. Any clues, including experimenter, computer, and extra-maze clues that may be potential clues for rats were kept constant during the experiment. Rats were allowed to locate the platform in a time span of up to 60 s. Mice that found the platform were allowed to stay on it for 15 s. The animals were tested four times daily at 10-min intervals for four consecutive days. On the fifth day, by removing the platform, the mice were allowed to search for the platform for 60 s and a probe trial session was tested. Memory retention was evaluated by the platform-cross number in a probe trial. On the fifth day, the platform was removed. In this probe trial, the rats were put into the pool and allowed to swim freely in the pool for 60 s. The times for rats crossing the location of the platform were recorded.

Statistical analysis

All data were analyzed using GraphPad Prism software. Each data value is presented as the mean ± SEM. For single dependent variables in the PAT, ORT, and probe trial in the MWM test one-way ANOVA was used. Escape latency, path length, and swimming speed in the MWM test were analyzed using two-way ANOVA. Tukey post hoc test was considered for recognition of the significant difference between groups. *P*-value < 0.05 was considered statistically significant.

RESULTS

The total phenolic content

The total phenolic content of extracts was determined by Folin-Ciocalteau's method (Fig. 1). According to this test, the amount of phenolic content for hydroalcoholic extract and methanolic fraction was 37.3 and 28.3 mg GAE/g of dried extract, respectively.

PAT

There were no significant differences between groups in the training trial in the passive avoidance test (Fig. 2).

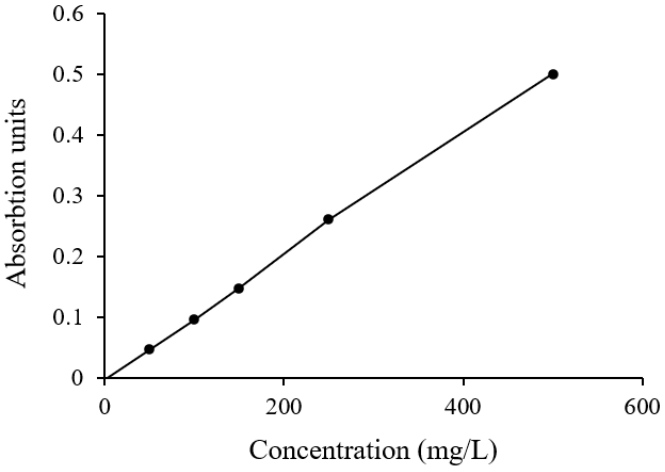

Fig. 1. Standard curve of gallic acid. Results were calculated according to a linear gallic acid regression and expressed as milligrams of gallic acid per liter.

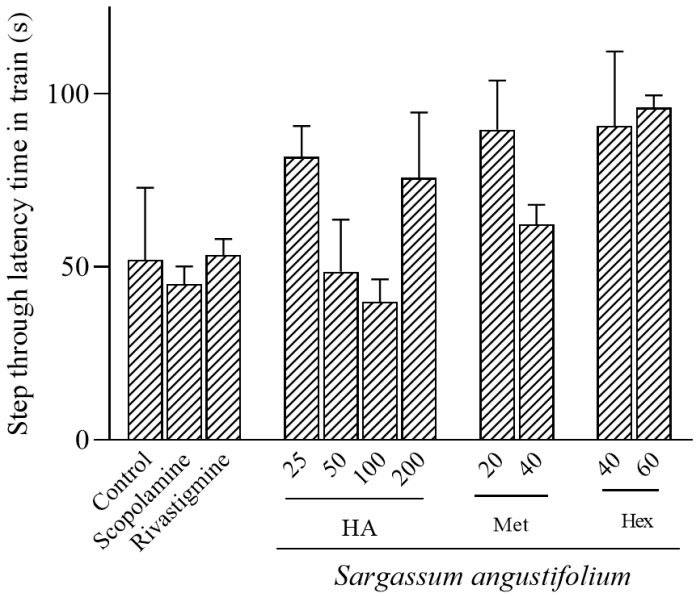

Fig. 2. Effect of *Sargassum angustifolium* extracts on step-through latency time (train) in the passive avoidance task. Step-through latency time in training and test trials was calculated according to the time required for the mouse to enter the dark compartment when the door is opened. Six animals were used per treatment group. Scopolamine was injected 30 min after the last dose of extracts on day 21. Data represent means ± SEM. HA, Hydroalcoholic extract (25, 50, 100, and 200 mg/kg); Met, methanolic extract (20 and 40 mg/kg); Hex, hexane extract (40 and 60 mg/kg).

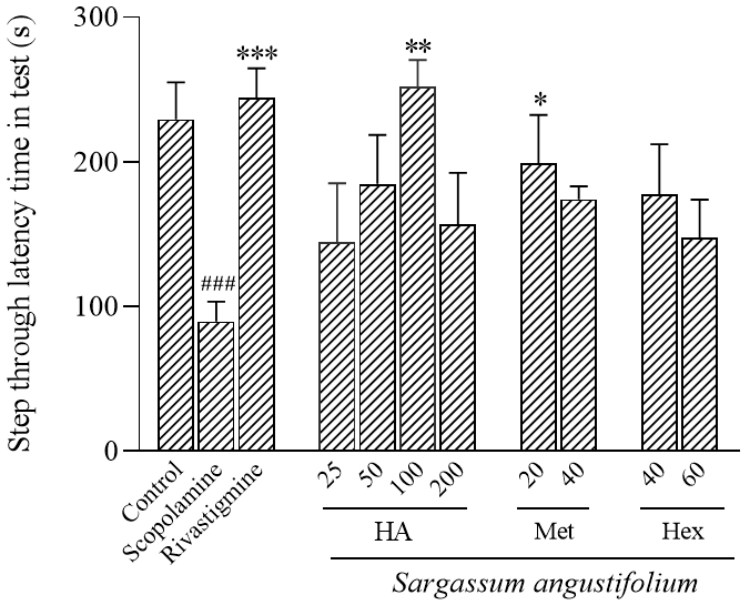

Fig. 3. Effect of *Sargassum angustifolium* extracts on step-through latency time (test) in the passive avoidance task. Step-through latency time in training and test trials was calculated according to the time required for the mouse to enter the dark compartment when the door is opened. Six animals were used per treatment group. Scopolamine was injected 30 min after the last dose of extracts on day 21. Data represent means ± SEM. HA, Hydroalcoholic extract (25, 50, 100, and 200 mg/kg); Met, methanolic extract (20 and 40 mg/kg); Hex, hexane extract (40 and 60 mg/kg). $^{###}P < 0.001$ Indicates significant differences compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus scopolamine group.

To assess whether *Sargassum* extracts administration had enhancing effects against learning and memory impairment, the PAT was carried out 24 h after the acquisition trial (Fig. 3). The step-through latency of scopolamine-treated (2 mg/kg) mice was 61% shorter than that of the vehicle-treated control mice. Rivastigmine at 1 mg/kg reversed the scopolamine-induced acquisition deficit approximately to the control level. Furthermore, among hydroalcoholic extracts (25, 50, 100, and 200 mg/kg) and methanolic fractions (20 and 40 mg/kg), only the step-through latency time of mice treated with hydroalcoholic extract at 100 mg/kg and methanolic fraction at 20 mg/kg, were significantly increased compared to scopolamine-treated mice (by 64% and 55%, respectively).

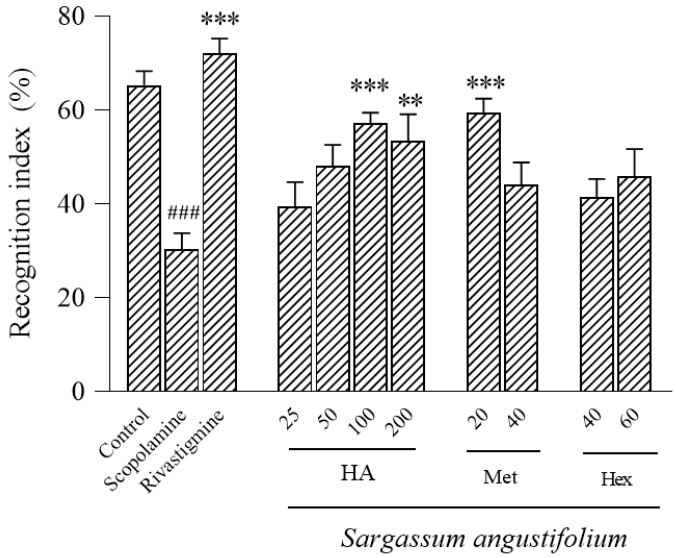

Fig. 4. Effect of *Sargassum angustifolium* extracts on recognition index in the object recognition test. HA: hydroalcoholic extract (25, 50, 100, and 200 mg/kg); Met: methanolic extract (20 and 40 mg/kg); Hex: hexane extract (40 and 60 mg/kg). Six animals were used per treatment group. Data represent means ± SEM. HA, Hydroalcoholic extract (25, 50, 100, and 200 mg/kg); Met, methanolic extract (20 and 40 mg/kg); Hex, hexane extract (40 and 60 mg/kg). $^{###}P < 0.001$ Indicates significant differences compared to control group; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus scopolamine group.

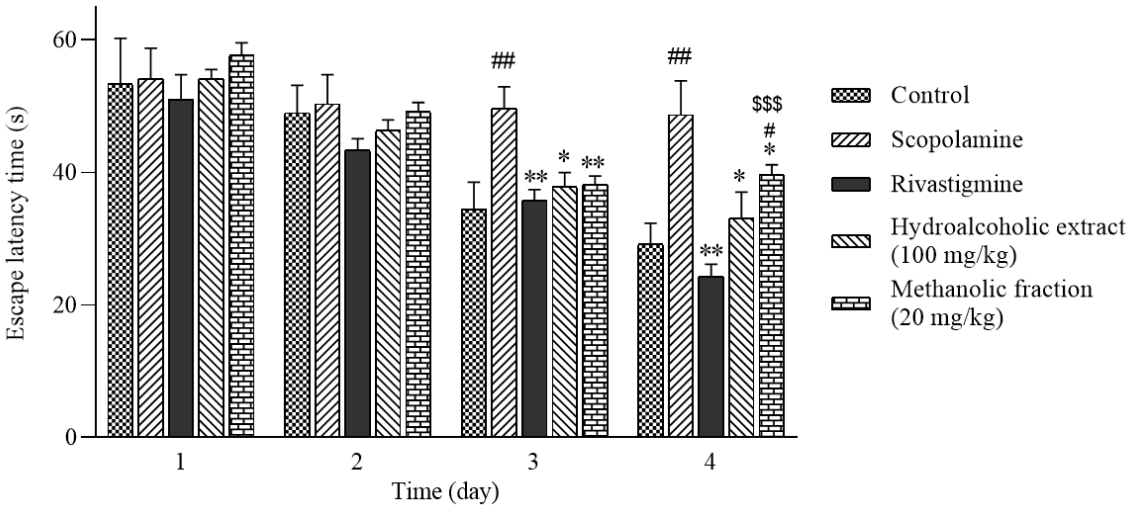

Fig. 5. Effect of *Sargassum angustifolium* extracts on escape latency time in Morris water maze Test. Six animals were used per treatment group. Scopolamine was injected 30 min after the last dose of extracts on day 21. Data represent means ± SEM. $^{#}P < 0.05$, $^{##}P < 0.01$ indicate significant differences compared to the control group in each day; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus corresponding scopolamine group in each day; $^{\$\$\$}P < 0.001$ versus corresponding rivastigmine group in each day.

ORT

Scopolamine significantly decreased RI by 50% in comparison to the control group (Fig. 4). Rivastigmine enhanced RI induced by scopolamine approximately to the control level. Of all dosages used, hydroalcoholic extracts in doses of 100 and 200 mg/kg and methanolic fraction at 20 mg/kg increased RI, while n-hexane fractions exhibited no significant effect compared to the scopolamine group.

MWM test

Figure 5 indicates escape latency time during four consecutive training days in the MWM test. On days 1 and 2, the treatment groups were not significantly different from scopolamine. However, on day 3 rivastigmine, hydroalcoholic extract (100 mg/kg) and a methanolic fraction (20 mg/kg) showed significant differences compared to the scopolamine group and the scopolamine-treated group exhibited a 30% longer escape latency time than that of the control group. Also, on day 4, scopolamine injection had increased escape latency time in comparison the control group by 39% and rivastigmine administration significantly reversed the scopolamine-induced acquisition effect compared

to scopolamine. Administration of hydroalcoholic extract (100 mg/kg) and a methanolic fraction (20 mg/kg) recovered the scape latency prolonged by scopolamine by 46.5% and 31% respectively.

Path length decreased across the 4 days of training in all five groups (Fig. 6). On days 1 and 2, treatment groups were not significantly different from scopolamine. However, on day 3, the scopolamine-treated group exhibited a 14% longer path length, than did the control group. Hydroalcoholic extract (100 mg/kg) and methanolic fraction (20 mg/kg) significantly shortened the path length prolonged by scopolamine treatment. Moreover, rivastigmine also significantly reduced path length compared with the scopolamine-treated group, approximately to the control level.

As well on day 4, scopolamine injection significantly altered escape latency distance compared to the control group by 24%. Rivastigmine injection reversed the scopolamine effect compared to scopolamine. Administration of hydroalcoholic extract (100 mg/kg) decreased escape latency distance significantly in comparison to scopolamine by 27%. Methanolic fraction (20 mg/kg) injection had a significant difference compared to the control and rivastigmine groups. There were significant decreases in path length between days one and four in all groups except for scopolamine-injected rats. There were no significant differences between groups in swimming speed in the MWM test (Fig. 7).

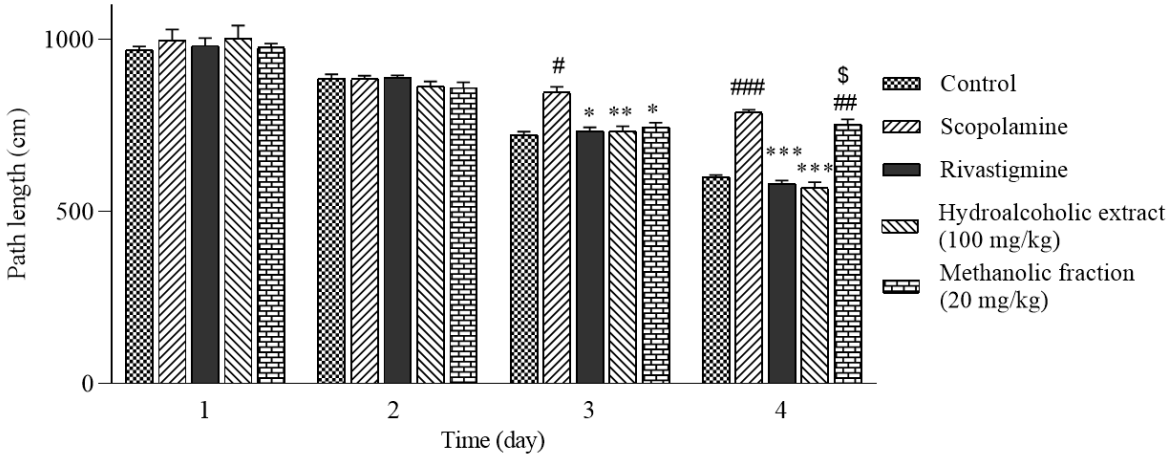

Fig. 6. Effect of *Sargassum angustifolium* extracts on path length in Morris water maze test. Six animals were used per treatment group. Scopolamine was injected 30 min after the last dose of extracts on day 21. Data represent means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ indicate significant differences compared to the control group in each day; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding scopolamine group in each day; $^{\$}P < 0.05$ versus corresponding rivastigmine group in each day.

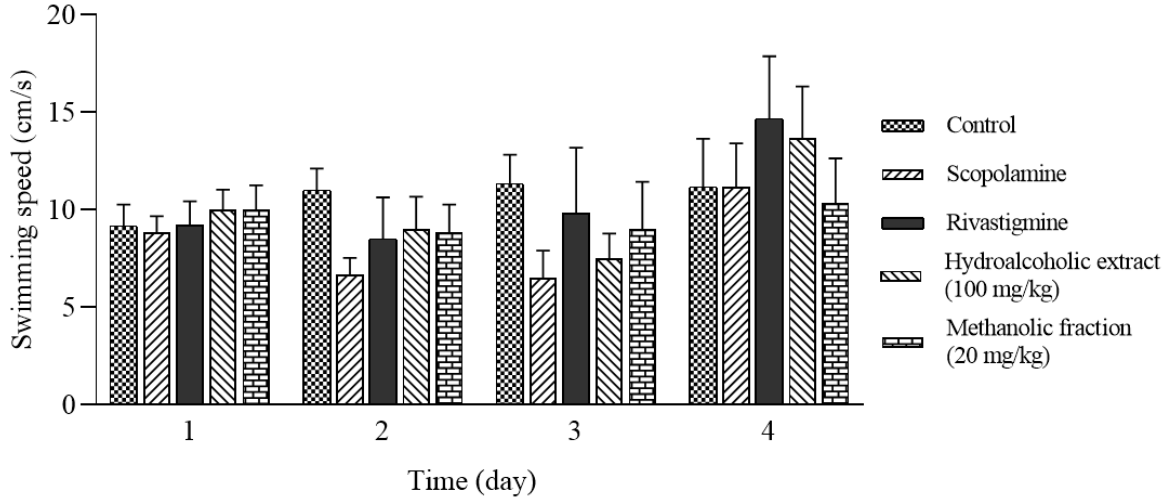

Fig. 7. Effect of *Sargassum angustifolium* extracts on swimming speed in Morris water maze test. Six animals were used per treatment group. Scopolamine was injected 30 min after the last dose of extracts on day 21. Data represent means ± SEM.

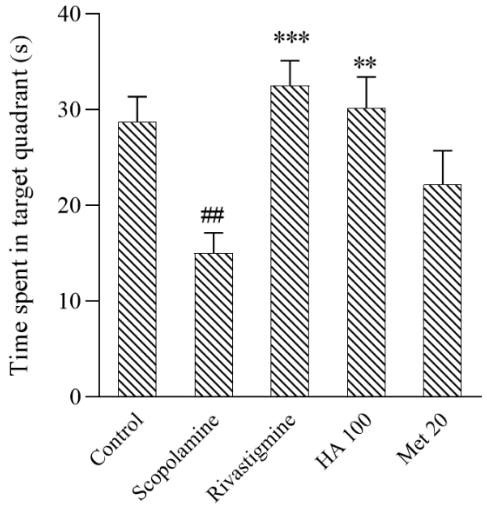

Fig. 8. The effect of administration of scopolamine, rivastigmine, and extracts on the time spent in the target zone during probe trial in Morris water maze test. Data represent means ± SEM. HA, Hydroalcoholic extract (25, 50, 100, and 200 mg/kg); Met, methanolic extract (20 and 40 mg/kg); Hex, hexane extract (40 and 60 mg/kg). $^{\#\#}P < 0.01$ Indicates significant differences compared to the control group; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus scopolamine group.

Swimming in the quadrant that previously contained the platform indicated memory for the former platform location (Fig. 8). Scopolamine injection (2 mg/kg) had a significant effect on the platform-crossed time, compared to the control group, with a 47% difference. The administration of rivastigmine completely reversed the scopolamine-induced acquisition deficit. Injection of hydroalcoholic extract (100 mg/kg) increased platform-crossed time by 54% in comparison with scopolamine.

DISCUSSION

As life expectancies increase, the number of people with learning and memory disorders increases, this consequently fuels a major increase in drug use worldwide. *S. angustifolium* is a seaweed with many species and a wide variety of pharmacological actions. The present study assessed the ameliorating effect of various extracts of Persian Gulf brown algae species of *Sargassum* on memory function.

To evaluate the effects of *S. angustifolium* extracts on cognitive function in mice, three distinct behavioral tests, namely PAT, MWM, and ORT were used. A single acute dose of scopolamine was used for impairing learning and memory in mice. Scopolamine works by blocking acetylcholine muscarinic receptors, thereby, causing disruption in the cholinergic neurons in the CNS which results in short-term and long-term memory impairments (31). In agreement with previous findings, scopolamine impaired learning, and memory in all three behavioral tests (32-34). In PAT, the hydroalcoholic extract and methanolic fraction of *S. angustifolium* ameliorated the scopolamine-induced memory deficit by increasing the latency time. The latency time is the period that animals spend in the lighted chamber during the test trial despite their natural tendency to move into the dark compartment. In this test, the methanolic fraction appeared to be more potent than the hydroalcoholic extract in reversing the memory deficit. PAT is based on negative reinforcement and is classically used to assess long-term memory (35). Similar results were obtained in ORT, whereby, hydroalcoholic extract and methanolic fraction increased RI. The ORT relies on an animal's innate predisposition toward exploring novelty (36). Data from neurophysiological studies show that the perirhinal, entorhinal, and inferior cortices play an important role in memory, and drugs capable of improving recognition memory affect these brain regions (37). In the MWM test, only the hydroalcoholic extract reversed the scopolamine-induced memory deficit by decreasing the escape latency times (spatial memory) and the path length compared to the group treated with scopolamine alone. These behavioral data clearly showed that *S. angustifolium* improved cognitive function as well as attenuated scopolamine-induced memory impairment which was evident in long-term memory.

There are several possible underlying causes for this improved learning behavior by *S. angustifolium*. Different species of *Sargassum* are known to have secondary metabolites such as terpenoid, fucoidan, alginate, phlorotannin, and laminarin (16). Each of these secondary metabolites could affect memory function. For example, terpenoids such as ginsenosides in *Panax ginseng* and bilobalide from *Ginkgo biloba* are

thought to be potential ingredients behind the memory-boosting properties of these plants. Even stronger evidence is available for the cognitive enhancement of fucoidan and alginate (35,38). Furthermore, properties like antioxidant, anti-inflammation, and anti-acetylcholine esterase activities have been linked to various species of *Sargassum* (16). Polyphenolic and flavonoid compounds of *Sargassum* extract are shown to inhibit acetylcholine esterase enzyme and reduce inflammation by inhibiting cyclooxygenase activity. The beneficial effects of *S. angustifolium* extracts could well be due to the downregulation of inflammatory signaling inside the brain (39). *S. angustifolium* extracts contain several other molecules that we are attempting to isolate and elucidate their pharmacological properties.

CONCLUSION

In summary, this study demonstrated that the hydroalcoholic extract of *S. angustifolium* was more effective in reversing the memory impairment induced by scopolamine in all three tested paradigms. The cognitive-enhancing activities of hydroalcoholic extract might result from the metabolites that could well interfere with AChE or work as antioxidants. These results suggest that *S. angustifolium* may be used as an effective agent to prevent cholinergic dysfunctions, such as AD.

Acknowledgments

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Conflict of interest statements

All authors declared no conflict of interest in this study.

Authors' contribution

M. Rabbani and A. Yegdaneh conceived and designed the research; A. Hassanzadeh and M. Rabbani conducted the experiments; A. Hassanzadeh analyzed the data and wrote the manuscript. All authors read and approved the finalized article.

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