

Satureja bachtiarica ameliorate beta-amyloid induced memory impairment, oxidative stress and cholinergic deficit in animal model of Alzheimer's disease

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Abstract Extracellular deposition of Beta-amyloid peptide (A β) is the main finding in the pathophysiology of Alzheimer's disease (AD), which damages cholinergic neurons through oxidative stress and reduces the cholinergic neurotransmission. *Satureja bachtiarica* is a medicinal plant from the Lamiaceae family which was widely used in Iranian traditional medicine. The aim of the present study was to investigate possible protective effects of *S. bachtiarica* methanolic extract on A β induced spatial memory impairment in Morris Water Maze (MWM), oxidative stress and cholinergic neuron degeneration. Pre-aggregated A β was injected into the hippocampus of each rat bilaterally (10 μ g/rat) and MWM task was performed 14 days later to evaluate learning and memory function. Methanolic extract of *S. bachtiarica* (10, 50 and 100 mg/Kg) was injected intraperitoneally for 19 consecutive days, after A β injection. After the probe test the brain tissue were collected and lipid peroxidation, Acetylcholinesterase (AChE) activity and Cholin Acetyl Transferees (ChAT) immunoreactivity were measured in the hippocampus. Intrahippocampal injection of A β impaired learning and memory in MWM in training days and probe trail. Methanolic

extract of *S. bachtiarica* (50 and 100 mg/Kg) could attenuate A β -induced memory deficit. ChAT immunostaining revealed that cholinergic neurons were loss in A β - injected group and *S. bachtiarica* (100 mg/Kg) could ameliorate A β - induced ChAT reduction in the hippocampus. Also *S. bachtiarica* could ameliorate A β -induced lipid peroxidation and AChE activity increase in the hippocampus. In conclusion our study represent that *S. bachtiarica* methanolic extract can improve A β -induced memory impairment and cholinergic loss then we recommended this extract as a candidate for further investigation in treatment of AD.

Keywords Alzheimer's disease · Beta amyloid peptide · *Satureja bachtiarica* · ChAT · Oxidative stress · Memory

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease predominantly characterized by memory deficits. The impairment of memory in AD is associated with hypofunction of cholinergic system in forebrain resulted from the loss of basal forebrain cholinergic neurons which play an important role in memory formation and cognition. (Schliebs and Arendt 2006, Kar et al. 2004) Extracellular deposition of beta-amyloid peptide (A β) is the main finding in the pathophysiology of AD and is considered a critical event in initiating the synaptic loss and finally neuronal cell death (Carter and Lippa 2001, Hardy and Selkoe 2002). Several mechanisms were proposed for A β - induced neurotoxicity (Cavallucci et al. 2012, Luquin et al. 1997). but much evidence suggested that oxidative damage plays a pivotal role in A β -induced toxicity (Chen and Zhong 2014). Increased production of reactive oxygen species (ROS) was reported in the brain of animal model of AD. Oxidative damage induced by ROS, includes protein, DNA,

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RNA oxidation and lipid peroxidation, have been described in the AD brain also treatment with antioxidant could ameliorate A β –induced memory deficit and antioxidants such as vitamin C and natural polyphenols were considered in AD therapy (Heo et al. 2013, Butterfield 1997). Current curative for treatment of AD are the acetylcholinesterase inhibitors such as donepezil and *N*-methyl-D-aspartate (NMDA) receptor antagonist such as memantine. These medicines have mild to moderate effectiveness and cannot prevent of disease progression (Birks 2006).

The therapeutic limitations and pharmacological side effects of these medicines along with this fact that prevalence of AD is steadily increasing, have led to further studies for finding new agents for the management of AD. Because of complex pathology of AD, new research are focused on therapeutic agent that can prevent disease progress through different pathways (Tayeb et al. 2012). Herbal medicines which contain several components with different pharmacological effects are considered for treatment of AD by the several studies and also traditional remedies have been used for treatment of dementia for several years (Perry and Howes 2011, Howes and Houghton 2012).

Lamiaceae family is the one of the largest family between plants family. There is 81 genus in this family used as herbal medicine. These plants were widely used in Iranian traditional medicine for neurological, cardiovascular and gastrointestinal disorders. Several plants from this family have been studied for their effectiveness in AD. Neuroprotection and memory-enhancing activity have been reported for some plants in this family (Orhan et al. 2012, Singhal et al. 2012, Topcu and Kusman 2014, Soodi et al. 2014, Sepand et al. 2013). *Satureja* species are medicinal plants from the Lamiaceae family which were widely used in Iranian traditional medicine as a tonic, carminative and digestive, also in several studies anti-inflammatory, anti-oxidant, anti-diabetic, anti-bacterial, antiviral, anti-leishmanial and anti-hyperlipidemic activity have been reported for plants of *Satureja* species (Naghibi et al. 2005; Tepe 2015). *S. bachtiarica* is one of the endemic species from Iran. There are few studies about the pharmacological effect of this plant. Anti-bacterial and anti-leishmania activity was reported for essential oil of *S. bachtiarica* (Mohammadpour et al. 2012, Ahanjan et al. 2011). In our previous study we screened the methanolic extract of some plants from Lamiaceae family against A β –induced toxicity in cell culture. Our results indicated that *S. bachtiarica* methanolic extract significantly ameliorated A β –induced toxicity in PC12 cells (Balali et al. 2012). This finding implies to neuroprotective activity of this extract and possible effectiveness in AD. In the present study, the protective effect of *S. bachtiarica* methanolic extract on A β -induced spatial memory impairment in Morris water maze (MWM) and against A β –induced toxicity in primary neuron culture were investigated,

in addition the effect of this extract on oxidative stress and cholinergic system were studied.

Materials and methods

Materials

Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (10,000 U/ml) and trypsin (%0.25) were taken from GIBCO, VectaStain Elite ABC Kit obtained from Vector Laboratories, Rabbit anti-Goat IgG biotin conjugate and Goat Anti-Choline Acetyltransferase (ChAT) antibody were purchased from Millipore. A β (25–35) was obtained from Alexis Biochemicals. All other chemicals were obtained from Sigma-Aldrich.

The plant materials (flowered aerial parts) were collected from wild growing areas of Iran (Chaharmahal-va-Bakhtiari Province) in June (2008) during full flowering stage. The plants were identified by Dr. Yousef Ajani. The voucher herbarium specimens were deposited at the Herbarium of Institute of Medicinal Plants, ACECR.

Preparation of plant extract

Extraction was performed by percolation method. Dried and milled aerial part of plant was extract with ethyl acetate and methanol respectively. At each step, after 24 h, the mixture was filtered and new solvent was added to the plant powder. The combined methanolic extracts were concentrated in rotary evaporator and dried in freeze drier. Methanolic extract was used in this study.

Preparation of stock solution for extract and A β

The A β (25–35) was dissolve in sterilized and deionized distilled water, and was stored in sterile microtubes at -20°C until use. Before injection aliquots were incubated for 4 days at 37°C for producing aggregated form of peptide.

Stock solution of methanolic extract was prepared by dissolving of 1 g in DMSO and further diluted by PBS before each experiment. The final concentrations of DMSO in behavioral test and cell culture were 1 % and 0.1 % respectively.

Antioxidant activity evaluation (FRAP assay)

The key solutions for performing FRAP assay were prepared as followed: a) Acetate buffer 300 mM pH 3.6; b) TPTZ (2, 4, 6-tripyridyl-striazine):10 mM in 40 mM HCl; c) FeCl $_3$. 6H $_2$ O:20 mM. The FRAP solution were prepared by mixing a, b and c in the ratio of 10:1:1 just before testing. Standard was FeSO $_4$.7H $_2$ O: 0.1–1.5 mM in methanol. FRAP solution

(3.6 mL) was incubated at 37 °C for 5 min. Then the solution was mixed with 0.4 mL distilled water and certain concentration of the plant extract (80 µL) and incubated at 37 °C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. For plotting of the calibration curve, five concentrations of FeSO₄·7H₂O (0.1, 0.4, 0.8, 1, 1.5 mM) were used and the absorbance values were measured as for sample solutions. Vitamin C was used as positive control (Hajimehdipoor et al. 2014a).

Determination of total phenolics content

The total phenolics contents of the extracts were determined spectrophotometrically according to the Folin-Ciocalteu method using chlorogenic acid (concentrations, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) as the standard. The reaction mixture was prepared by mixing the methanolic solution (1 mL) of the extract, distilled water (9 mL), Folin-Ciocalteu's reagent (1 mL) and sodium carbonate (10 mL, 7 %). Incubation at room temperature was down for 90 min and the absorbance was determined at 765 nm. The total phenolics content was expressed as chlorogenic acid equivalent in milligram per 100 g dried extract (Manayi et al. 2012).

In vitro AChE activity assay

The experiment was conducted according to modified microplate method. The sample was dissolved in methanol in concentration of 3 mg/mL. In the 96-well plates, 125 µL of 3 mM DTNB, 25 µL of 15 mM ATCH and 50 µL of phosphate buffer (pH 8), 25 µL of sample dissolved in methanol were added to the wells. The absorbance was read at 405 nm every 13 s for 65 s. 25 µL of 0.22 U/mL of AChE enzyme was then added and the absorbance was again measured every 13 s for 104 s using a microplate reader (BIOTEK) at 405 nm. Absorbance was plotted against time and enzyme activity was calculated from the slope of the line and expressed as a percentage compared to an assay using a methanol without any inhibitor. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the rate of the reaction before adding the enzyme, from the rate after adding the enzyme. Inhibition percentage was obtained by comparing the rates for the sample to the blank (methanol). Donepezil was used as the positive control (Hajimehdipoor et al. 2014b).

Assessment of protective effect of extract in cell culture

Primary cultures of cerebellar granule neurons (CGNs) were prepared from 7-day-old mouse pups, as previously described (Kramer and Minichiello 2010). Briefly, cerebella freshly dissected and briefly minced then were incubated with 0.025 % trypsin solution for 15 min at 37 °C. After inhibition of trypsin

by serum, single cell suspension prepared by mechanically disruption of digested tissue. Then, they were cultured on PDL coated cell culture plates in DMEM containing 10 % FBS, 4.5 g/l glucose, 25 mM KCl, insulin (100 mU/L), penicillin and streptomycin 1 % (v/v). Cells were maintained at 37 °C in a humidified atmosphere with 5%CO₂. The growth of non-neuronal cells was inhibited by addition of 20 µM cytosine arabinofuranoside 48 h after seeding and the medium was not changed during the culture period. After 7 days of growth in vitro (DIV7), more than 95 % of cells in culture were neurons which characterized by MAP2 protein immunostaining. Then experiments done on DIV 7. The CGNs plated onto PDL coated 96 well plate (1 × 10⁵ cell/well) were pre-incubated with different concentration of extract (0.1–100 µg/ml for 24 h, then Aβ peptide was added to the medium at final concentration of 20 µM. cell viability was assessed after 48 h by MTT assay. Briefly, after incubation the medium was replaced with fresh medium containing MTT solution (final concentration 0.5 mg/ml) and incubated for 4 h in 37 °C. Then the medium was removed and 100 µl DMSO was added to each well and mixed properly until blue formazan product completely dissolved. Absorbance was measured at 570 nm in an automated plate reader (BIOTEK) against 630 nm as reference wavelength. The results were reported as percentage of control.

Behavioral tests

Animals Male Albino Wistar rats (200–220 g) were obtained from faculty of pharmacy, Tehran University of Medical Sciences. Animals were housed in cages (five/cage), having access to food and water ad libitum. They were maintained on a 12 h-light-dark cycle at 20–22 °C temperature. All efforts were made to avoid animal suffering and reduce the number of animal used and all animal experiments were approved by ethical committee of the Tarbiat Modares University.

Treatments The animals were randomly divided into the following groups, with 8 rats in each group: the control group, the Aβ injected group, Aβ + extract groups and extract group. The Aβ was bilaterally injected into hippocampus through Stereotaxic surgery and the animals were trained in Morris Water Maze 14 days after injection. Three doses of extract (10, 50 and 100 mg/Kg) were daily injected intraperitoneally. Extract injection were began on the day of surgery and continued until the end of training (19 days) The control group received DMSO (1 %) similarly. In order to investigate just extract effects, highest dose of extract (100 mg/Kg) were administrated to non-surgical animals in a similar manner.

Stereotaxic surgery Before surgery rats were anesthetized by interaperitoneal injection of Ketamin (100 mg/Kg) and Xylazine (10 mg/Kg). They were placed into stereotaxic

device (Steolting, USA). Lidocaine and epinephrine solution (0.2 ml) was injected around the midline and a section was made along the midline. Bilateral injection was made by the 10- μ l Hamilton syringe at the CA1 area of hippocampus, 3.8 mm posterior, 2.2 mm lateral to bregma and 2.7 mm ventral to the surface of the skull, consistent with the atlas of Paxinos and Watson (1997). During 10 min, rats received volume of 2 μ l/side of A β (10 μ g/rat). After injection, the incisions were sutured.

Behavioral training and testing Fourteen day after surgery all experimental groups were trained in Morris Water Maze, for four consecutive days, as described in our previous studies (Soodi et al. 2007). Black-painted water maze was a circular pool by 136 cm diameter and 60 cm height, filled by 22 ± 2 °C water to a height of 40 cm. It divided to four equal quadrants, hypothetically. A hidden platform by 10 cm diameter was located in center of North-west quadrant (target quadrant) in order to be 1 cm under the water surface. Four trials in one block were done in each training day, at about the same time of morning. In each trial the rat was randomly placed in one of quadrants facing the nearest wall. The animal was allowed to swim in maze during a period of 90 s to find the platform, using three visual cues. mounted on the walls of the room. If an animal could not find the platform within this period, it was manually guided to platform by researcher and was allowed to rest 30 s between two consecutive trials. Directions of the animals were recorded by a video camera located just above the center of maze. The camera was linked to a computer. Spatial acquisition was evaluated by Ethovision video tracking system (Noldus Information Technology, Wageningen, Netherlands) through measuring escape latency (time to find the platform) and swimming speed (Azami et al. 2010). The probe test was performed on the fifth day. In the probe test, the hidden platform was removed, and the animal was released from the opposite of target quadrant and allowed to swim freely for 90 s. Time spent in target quadrant was measured. The visible test was performed in order to assess motivation and vasomotor coordination. For visible test evaluation, the platform was elevated above water in the center of opposite quadrant and the same measures for acquisition evaluation were assessed in this test.

Immunohistochemical staining procedure Brain tissue was obtained and processed according to standard protocols (Hosseini-Sharifabad et al. 2011). Briefly, animals were deeply anesthetized and then transcardially perfused with 100 mL of phosphate-buffered saline (PBS), followed by 300 mL of 4 % paraformaldehyde in 0.1 M phosphate buffer. The brains were then postfixed in the same fixative overnight followed by incubation in a PBS solution containing 30 % sucrose. After being embedded in optimal cutting temperature (OCT), the

brains were sectioned at 40 μ m intervals. These tissue sections were immunostained to determine the expression of ChAT protein. Free-floating tissue sections were made permeable in PBS containing 0.4 % TX-100 and 1 % normal rabbit serum for 45–60 min followed by incubation for 30 min in PBS containing 0.3 % hydrogen peroxide solution. After washing the tissue in PBS the sections were blocked for 60 min in PBS containing 0.3 % TX-100 and 3 % normal rabbit serum. The sections were then incubated for 48 h on a shaking platform at 4 °C with Anti-ChAT polyclonal antibody (diluted 1:100 in PBS containing 1 % normal rabbit serum and 0.3 % TX-100). The sections were then washed extensively 8 times for 5 min each with 1 % normal rabbit serum in PBS, followed by incubation with biotinylated anti-goat IgG secondary antibody diluted 1:200 in PBS and 1 % normal rabbit serum for 60 min at room temperature. The sections were incubated for 45 min at room temperature in avidin-biotin complex detection solution (ABC Elite Kit, Vector Labs) diluted 1:150 in PBS then the sections were incubated for 5–15 min with a PBS solution containing 0.02 % diaminobenzidine-4HCl (Sigma), 0.3 % nickel sulfate, and 0.03 % hydrogen peroxide until the desired staining intensity was achieved. Staining was stopped by washing the sections 3 times with PBS. The sections were then mounted on gelatin-coated glass slides and coverslipped. These tissue sections were analyzed with a BX51 Olympus microscope.

Preparation of brain tissue samples for biochemical analysis

After training, the animals were sacrificed by decapitation under ether anesthesia, and the hippocampuses were dissected out and homogenized in ice cold 0.1 M phosphate buffer saline (pH 7.4) with 1 % TritonX. The homogenates were then centrifuged at $3000 \times g$ for 10 min at 4 °C, and the supernatant was used.

Measurement of brain AChE activity

The AChE activity was measured as described earlier by Ellman (Ellman et al. 1961). Briefly, 0.1 M phosphate buffer saline (pH 8.0), acetyl thiocholine iodide (ATCh, 75 mM) as a substrate, and 5, 5-dithiobis (2-nitrobenzic) acid (DTNB, 10 mM) in a ratio of 150:2:5 were mixed. Absorbance was then measured immediately after the enzyme source (10 μ l) was added to the reaction mixture during 6 min at 412 nm using a microplate reader. The supernatant of brain homogenate was used as the enzyme source. Protein concentration in the supernatant was measured using the Bradford method (Bradford 1976).

Measurement of lipid peroxidation

The levels of malonyldialdehyde (MDA), the most abundant lipid peroxidation product, were determined by thiobarbituric acid reaction (TBAR) colorimetric assay. One volume of brain homogenate was mixed with two volume of TBA reagent (containing 3.75 % TCA and 0.0925 % TBA) and the mixture

incubate at 90 °C for 60 min. After cooling, centrifuged at 1000 g for 10 min and optical density of supernatant was measured in 540 nm in plate reader. MDA standard curve was established with using the stable MDA precursor, Malondialdehyde bis (dimethyl acetal).

Statistical analysis Differences in water maze performance (escape latency and speed) were analyzed by Two-way repeated measures analysis of variance (ANOVA) followed by Benferroni post test. Other data were compared by one-way ANOVA, followed by Newman-Keulz multiple comparison post hoc test. The *p* values of 0.05 and less were considered as statistically significant.

Results

Antioxidant, total phenol and in vitro AChE activity

The results of FRAP assay, total phenol content and AChE activity of the methanolic extract of *S. bachtiarica* have been summarized in Table 1. The data were obtained according to calibration curves of FeSO₄.7H₂O ($y = 1.0436x - 0.0183$, $r^2 = 0.9972$) and chlorogenic acid ($y = 2.0912x - 0.0227$, $r^2 = 0.9989$). The results of *in vitro* AChE inhibitory assay showed that the methanol extract of this plant displayed no inhibitory activity at 300 µg/ml concentration. It is revealed that polarity and chemical structure of the constituents of the plant extract could make a lot of difference in binding to AChE.

Protective effect of *S. bachtiarica* extract on Aβ-induced cell toxicity

The effect of *S. bachtiarica* extract against Aβ-induced toxicity in cultured CGNs was evaluated by MTT assay.

Table 1 The results of FRAP assay, total phenol content and anti-cholinesterase activity of the methanolic extract of *S. bachtiarica*

Plant material	Antioxidant activity (FRAP assay) ^a	Total phenol content ^b	Acetyl choline esterase inhibitory ^c
<i>Satureja bachtiarica</i> (Methanolic extract)	153.0 ± 5.5	16.0 ± 0.2	Not detected
Vitamin C	2003.1 ± 6.7	–	–
BHT	–	265.2 ± 0.4	–

^a FRAP values are indicated as mmol of FeSO₄.7H₂O equivalent in 100 g of the dried extract

^b Total phenolics content was calculated on the basis of the milligram of chlorogenic acid per 100 g of the dried extract (ChAE/100 g EXT)

^c Acetylcholine esterase inhibitory activity was measured on 300 µg/ml of the plant extract

Table 2 Acetylcholinesterase activity and MDA level in the hippocampus

Groups	AChE activity (µ mol/min/mg protein)	MDA (n mol/mg protein)
Control	0.273 ± 0.02	0.67 ± 0.12
Extract(100 mg/kg)	0.204 ± 0.03*	0.75 ± 0.18
Aβ	0.635 ± 0.06***	4.6 ± 0.23***
Extract(100 mg/kg) + Aβ	0.278 ± 0.01 ⁺⁺⁺	1.2 ± 0.14 ⁺⁺⁺

*Represent significant differences versus control group (**p* < 0.05, ****p* < 0.001)

+Represent significant differences versus Aβ-treated group (⁺⁺⁺*p* < 0.001)

Treatment of cultured CGNs in DIV 8 with Aβ for 48 h significantly decreases cell viability as compared with control group. Pretreatment of cultured CGNs with different concentration of *S. bachtiarica* extract before Aβ incubation protect them dose dependently from Aβ induced toxicity (Fig. 1). The 0.1 µg/ml of extract could slightly increase cell viability however 100 µg/ml of extract completely reverse the Aβ induced toxicity and increase cell viability up to control level. Treatment with extract (0.1–100 µg/ml) alone did not affect the cell viability as compared to control (data not shown).

Effects of *S. bachtiarica* extract on learning and memory deficits induced by Aβ25–35

According to the results, intra hippocampal injection of 10 µg of aggregated Aβ (25–35) after 14 days could impair learning ability of animals that was shown by increased escape latency during training days. (Fig. 2a) also Aβ-injected group spent less time in target quadrant in the probe test which demonstrate memory impairment in this group in compared to control group (Fig. 2b). Intraperitoneally injection of *S. bachtiarica* extract after Aβ injection for 19 days, at the dosages of 50 mg/kg

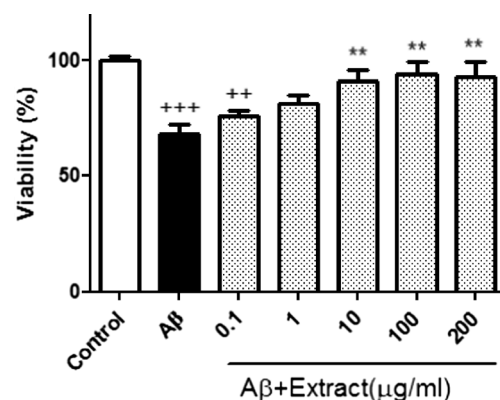


Fig. 1 Protective effect of *S. bachtiarica* methanolic extract against Aβ-induced toxicity in primary cerebellar granular neuron culture. * Represent significant differences versus Aβ-treated group (***p* < 0.01). + Represent significant differences versus control group (⁺⁺⁺*p* < 0.001)

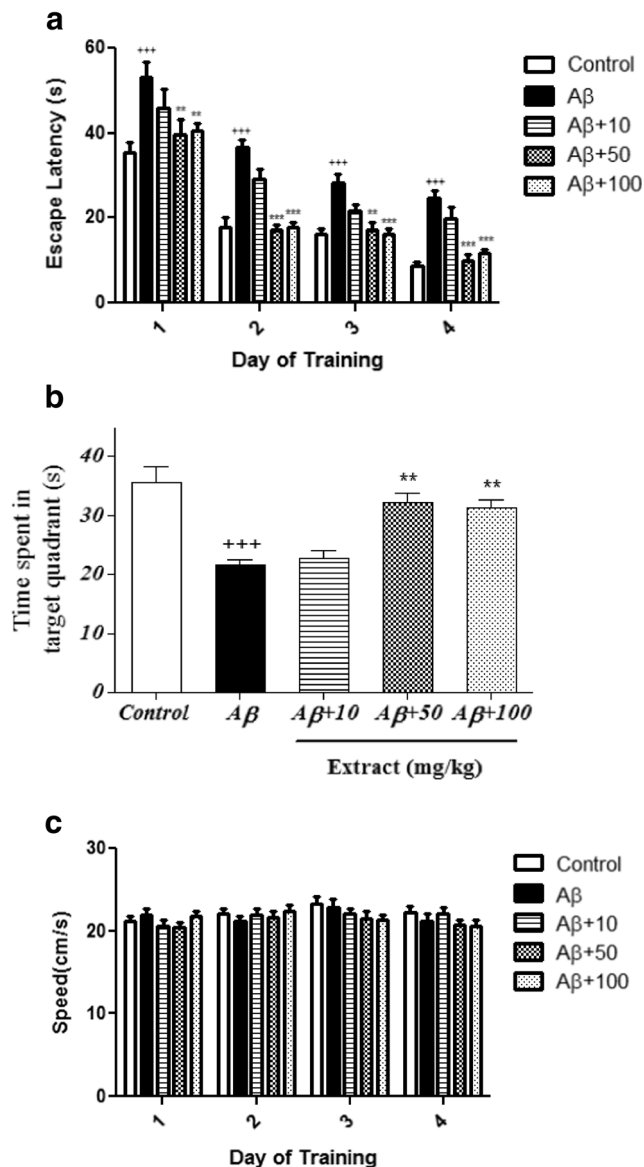


Fig. 2 The effect of *S. bachtiarica* methanolic extract on A β -induced memory impairment in Morris water maze task: Escape latency during training days (a), Time spent in target quadrant in probe test (b), speed during training days (c). * Represent significant differences versus A β -treated group (** $p < 0.01$, *** $p < 0.001$). + Represent significant differences versus control group (++ $p < 0.001$)

and 100 mg/kg significantly protected against A β _{25–35}-induced learning and memory deficits, as indicated by the decreased escape latency during training days. Also, these groups spent more time in target quadrant in the probe test, compared with that in the A β -injected group (Fig. 2a, b). The learning measures during training day and probe test were not affected by administration of extract (100 mg/kg) in naïve rats alone (data not shown). The swimming speed was not affected in all treated groups of animals (Fig. 2c), as well as, Assessment of visio-motor coordination toward the visible platform showed no significant difference in escape latency between treated groups and the control group (Fig. 3).

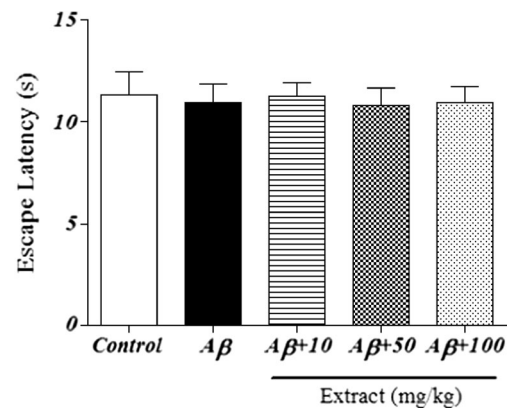


Fig. 3 The performance of animals in visible platform test

Immunohistochemical evaluation of ChAT expression in hippocampus

The rat brains were collected immediately after behavioral experiments and immunostained with anti-ChAT antibody to evaluate and compare expression of ChAT protein between control, A β -injected and A β + extract (100 mg/kg)-injected groups. As observed in Fig. 4 expression of ChAT protein has been reduced in A β -injected group in compared with the control group, while treatment of animals with extract (100 mg/Kg) after A β injection could ameliorate A β -induced reduction in ChAT expression.

Effect of *S. bachtiarica* extract on brain AChE activity and lipid peroxidation

The AChE activity and lipid peroxidation were measured in the hippocampus of animals in control, A β -injected, A β + extract (100 mg/kg)-injected and extract alone groups. Results indicated in Table 2. The injection of A β in the hippocampus significantly increased the AChE activity and MDA production in compared to control group. Treatment with *S. bachtiarica* extract after A β injection significantly attenuated the A β -induced AChE activity increase and lipid peroxidation. Also treatment with *S. bachtiarica* extract alone significantly inhibited the AChE activity in the hippocampus.

Discussion

In the present study the protective effect of *S. bachtiarica* on A β -induced toxicity in primary cell culture and in animal model of AD were investigated. In our previous study we had shown that *S. bachtiarica* methanolic extract protect PC12 cells against A β -induced cytotoxicity and then to confirm in vitro protective activity of this extract on A β -induced cytotoxicity, CGNs culture was used in the present study. CGNs culture has widely been used as a model for studying cellular and molecular mechanisms of neural cell apoptosis,

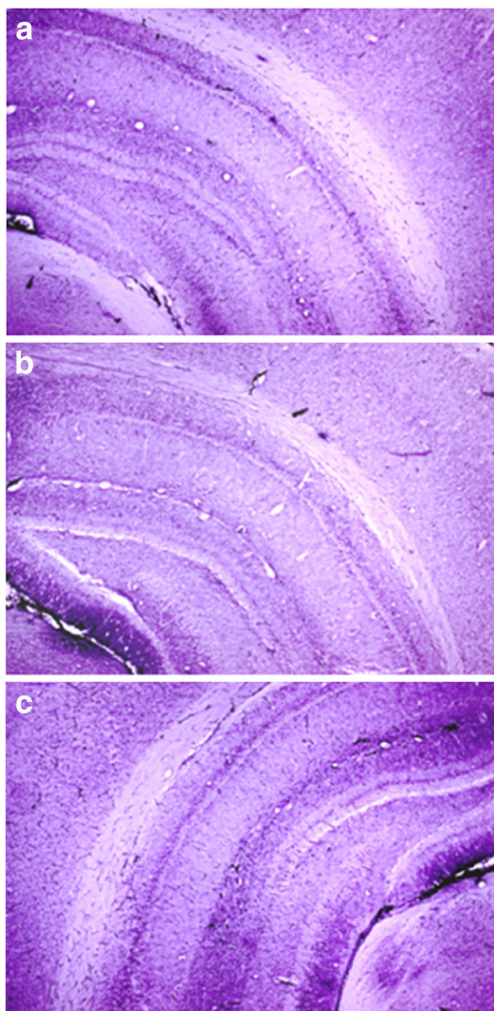


Fig. 4 Photographs of ChAT immunostaining in the dorsal hippocampus: control (a), A β -treated group (b), A β + Extract (100 mg/kg) treated group

survival, neurodegeneration and neuroprotection (Contestabile 2002). The A β -induced cytotoxicity in CGNs culture was used for studying of effectiveness of agents which are proposed for use in AD (Wei et al. 2000). Our result indicated that *S. bachtiarica* methanolic extract significantly protect cultured cerebellar granule neurons against A β -induced cytotoxicity and confirm the in vitro neuroprotective activity of this extract.

Then for further investigation of neuroprotective activity and usefulness of this extract in treatment of AD, in vivo animal model of AD was used. It has been shown that single injection of A β (25–35) in cerebroventricular or hippocampus induces learning and memory impairment in Morris Water Maze, radial arm maze and passive avoidance in rat (Sun and Alkon 2002, Yamaguchi and Kawashima 2001, Ghahremanitamadon et al. 2014) Also cholinergic deficit, oxidative stress and neuronal cell loss in the hippocampus have been reported as a result of A β (25–35) administration (Stepanichev et al. 2004). Then several studies have been used

A β - injected animals as an in vivo model of AD. In our study administration of *S. bachtiarica* extract for 19 days could ameliorate A β -induced memory deficit in MWM. On the other hand increased level of lipid peroxidation and AChE activity were observed in A β -injected animals which were attenuated following *S. bachtiarica* extract administration. These results indicate that *S. bachtiarica* extract ameliorate A β -induced oxidative stress in the hippocampus. The oxidative stress plays important role in the pathogenesis of AD and A β -induced toxicity. It has been shown that cerebroventricular A β administration increases ROS production and decrease antioxidant enzyme level resulted in oxidative stress and leads to oxidative damage in neurons such as lipidperoxidation and protein oxidation (Parks et al. 2001). It is reported that natural antioxidant ameliorate A β -induced oxidative stress in both cell culture and brain tissue may be through free radical scavenging or metal ion chelating activity.

Our study indicated that *S. bachtiarica* methanolic extract shows antioxidant activity in FRAP assay and also contain polyphenolic compounds. It is reported that plant polyphenols have potent antioxidant activity and as a result, have a beneficial effect in various diseases such as cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey and Rizvi 2009). The neuroprotective activity of natural polyphenols was reported against various neuronal injury and neurological disorders. Several polyphenols such as resveratrol, quersetine and rosmarinic acid and also plants which contain polyphenols ameliorate A β -induced oxidative stress and neural cell death through scavenging of free radicals or up regulation of cellular antioxidant defenses. Beyond the antioxidant activity, polyphenols protect neural cells through activation of cell survival signaling pathways such as MAP kinase and PI3K/Act (Vauzour 2012). Then it is suggested that polyphenols in *S. bachtiarica* methanolic extract is responsible for its antioxidant and neuroprotective activity also the role of the other compounds is not ignored and needs further investigation. The phytochemical investigation has indicated that the main polyphenols in the *S. bachtiarica* methanolic extract are luteolin, naringenin and rosmarinic acid (Khodabakhsh et al. 2010). The neuroprotective activity was reported for these compounds in several studies. Luteolin has the antioxidant, anti-inflammatory and radical scavenging activity and protect neural cells against H₂O₂-induces DNA damage (Cheng et al. 2010). Also luteolin reduce A β production an A β -induced apoptosis in SHSY5Y cell culture (Liu et al. 2011). In addition luteolin ameliorate A β -induced memory deficit in MWM performance and passive avoidance through activation of antioxidant defense and restoring the A β -induced oxidative stress (Tsai et al. 2010). Naringenin prevent neural cell death in cerebral ischemia with activation of anti inflammatory mechanism (Bai et al. 2014). Naringenin ameliorate iron-over load induced oxidative stress and neurotoxicity (Chtourou et al. 2014) also, it improve cognition function

and reduce A β production in streptozocine-induced dementia model of rat (Yang et al. 2014). Rosmarinic acid is a natural polyphenol with neuroprotective activity which was mostly found in lamiaceae family. Protective effect of Rosmarinic acid against chromium VI induced neurotoxicity has been reported (Dashti et al. 2014). It protects PC12 cells against A β -induced toxicity and oxidative stress (Iuvone et al. 2006). Rosmarinic acid ameliorates A β -induced memory impairment by proxynitrit free radical scavenging activity (Alkam et al. 2007). It is a potent mito-protectant and improves mitochondrial dysfunction in neurodegenerative diseases (Camilleri et al. 2013). Also Rosmarinic acid and luteolin inhibit beta-secretase activity an enzyme involves in A β production (Choi et al. 2008). Then it is suggested that these polyphenols may have a role in the neuroprotective activity of *S. bachtiarica* methanolic extract.

Degeneration of the cholinergic innervations in the septo-hippocampal pathway is the common pathological feature in AD (Schliebs and Arendt 2006, Kar et al. 2004). It is reported that impaired memory function are associated with reduction of ChAT activity and immunoreactivity in the animal model of AD (Yamaguchi et al. 2002). In our study, A β -injected directly into the hippocampus resulted in learning and memory impairment which associated with reduced ChAT immunostaining in the hippocampus, indicate cholinergic neuron loss. Treatment with extract in concentration which improves memory impairment could increase ChAT immunostaining revealed increase cholinergic neuron survival. In addition AChE activity, which was increased in A β -injected group, decreased in extract treated group. Also AChE activity was decreased in extract alone treated animals. Then it is deduced that *S. bachtiarica* methanolic extract has inhibitory effect on brain AChE activity, but interestingly extract did not show any direct inhibitory effect on pure enzyme activity. Then it is suggested that extract may reduce the AChE protein expression in the hippocampus. The AChE is a key enzyme in the cholinergic nervous system which hydrolysis acetylcholine and terminates its action. Current pharmacotherapy for AD is using AChE inhibitors which increase Achetylcholine level through inhibition of the cholinesterases and improve AD symptoms by facilitating cholinergic neurotransmission (Lleo et al. 2006). but alternative functions of AChE unrelated with the hydrolysis of acetylcholine are supposed in AD. It is reported that AChE activity was increase around the amyloid plaques and the expression of AChE protein is increased in AD brain. AChE protein interacts with A β peptide and increases its toxicity and deposition to form plaque. Then AChE is the target of several study for finding new therapeutic agent for treatment of AD (Campanari et al. 2014, Garcia-Ayllon et al. 2011).

In conclusion our results indicate that *S. bachtiarica* methanolic extract improve A β -induced memory impairment, oxidative stress and cholinergic dysfunction. These effect of

extract at least as part could be attributed to the polyphenolic compounds. According to this finding it is suggested that *S. bachtiarica* methanolic extract can be a candidate for further investigation as therapeutic agent in AD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ahanjan M, Ghaffari J, Mohammadpour G, Nasrolahie M, Haghshenas MR, Mirabi AM (2011) Antibacterial activity of *Satureja bachtiarica* bung essential oil against some human pathogenic bacteria. *Afr J Microbiol Res* 5:4764–4768
- Alkam T, Nitta A, Mizoguchi H, Itoh A, Nabeshima T (2007) A natural scavenger of peroxynitrites, rosmarinic acid, protects against impairment of memory induced by abeta(25–35). *Behav Brain Res* 180: 139–145
- Azami K, Etmnani M, Tabrizian K, Salar F, Belaran M, Hosseini A, Hosseini-Sharifabad A, Sharifzadeh M (2010) The quantitative evaluation of cholinergic markers in spatial memory improvement induced by nicotine–buccladesine combination in rats. *Eur J Pharmacol* 636:102–107
- Bai X, Zhang X, Chen L, Zhang J, Zhang L, Zhao X, Zhao T, Zhao Y (2014) Protective effect of naringenin in experimental ischemic stroke: down-regulated NOD2, RIP2, NF-kappaB, MMP-9 and up-regulated claudin-5 expression. *Neurochem Res* 39:1405–1415
- Balali P, Soodi M, Saeidnia S (2012) Protective effects of some medicinal plants from Lamiaceae family against beta-amyloid induced toxicity in PC12 cell. *Tehran Univ Med J (TUMJ)* 70:402–409
- Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst Rev* 25
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Butterfield DA (1997) Beta-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. *Chem Res Toxicol* 10:495–506
- Camilleri A, Zarb C, Caruana M, Ostermeier U, Ghio S, Hogen T, Schmidt F, Giese A, Vassallo N (2013) Mitochondrial membrane permeabilisation by amyloid aggregates and protection by polyphenols. *Biochim Biophys Acta* 11:28
- Campanari ML, Garcia-Ayllon MS, Blazquez-Llorca L, Luk WK, Tsim K, Saez-Valero J (2014) Acetylcholinesterase protein level is preserved in the Alzheimer's brain. *J Mol Neurosci* 53:446–453
- Carter J, Lippa CF (2001) Beta-amyloid, neuronal death and Alzheimer's disease. *Curr Mol Med* 1:733–737
- Cavallucci V, D'amelio M, Cecconi F (2012) Abeta toxicity in Alzheimer's disease. *Mol Neurobiol* 45:366–378
- Chen Z, Zhong C (2014) Oxidative stress in Alzheimer's disease. *Neurosci Bull* 30:271–281
- Cheng HY, Hsieh MT, Tsai FS, Wu CR, Chiu CS, Lee MM, Xu HX, Zhao ZZ, Peng WH (2010) Neuroprotective effect of luteolin on amyloid beta protein (25–35)-induced toxicity in cultured rat cortical neurons. *Phytother Res* 24:S102–S108

- Choi SH, Hur JM, Yang EJ, Jun M, Park HJ, Lee KB, Moon E, Song KS (2008) Beta-secretase (BACE1) inhibitors from *Perilla frutescens* var. *acuta*. *Arch Pharm Res* 31:183–187
- Chtourou Y, Fetoui H, Gdoura R (2014) Protective effects of naringenin on iron-overload-induced cerebral cortex neurotoxicity correlated with oxidative stress. *Biol Trace Elem Res* 158:376–383
- Contestabile A (2002) Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. *Cerebellum* 1:41–55
- Dashti A, Soodi M, Amani N (2014) Cr (VI) induced oxidative stress and toxicity in cultured cerebellar granule neurons at different stages of development and protective effect of Rosmarinic acid. *Environ Toxicol* 12:22041
- Ellman GL, Courtney KD, Andres Jr V, Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95
- Garcia-Ayllon MS, Small DH, Avila J, Saez-Valero J (2011) Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and beta-amyloid. *Front Mol Neurosci* 4:1–9
- Ghahremanitamadon F, Shahidi S, Zargooshnia S, Nikkhal A, Ranjbar A, Soleimani Asl S (2014) Protective effects of *Borago officinalis* extract on amyloid beta-peptide(25–35)-induced memory impairment in male rats: a behavioral study. *Biomed Res Int* 798535:11
- Hajimehdipoor H, Ajani AGY, Saeidnia S (2014a) Comparative study of the total phenol content and antioxidant activity of some medicinal herbal extracts. *Res J Pharmacognosy* 1:21–25
- Hajimehdipoor H, Mosaddegh M, Naghibi F, Haeri A, Hamzeloo-Moghadam M (2014b) Natural sesquiterpen lactones as acetylcholinesterase inhibitors. *An Acad Bras Cienc* 86(2):801–805
- Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353–356
- Heo JH, Hyon L, Lee KM (2013) The possible role of antioxidant vitamin C in Alzheimer's disease treatment and prevention. *Am J Alzheimers Dis Other Demen* 28:120–125
- Hosseini-Sharifabad A, Mohammadi-Eraghi S, Tabrizian K, Soodi M, Khorshidahmad T, Naghdi N, Abdollahi M, Beyer C, Roghani A, Sharifzadeh M (2011) Effects of training in the Morris water maze on the spatial learning acquisition and VAcHT expression in male rats. *DARU, J Pharm Sci* 19:166–172
- Howes MJ, Houghton PJ (2012) Ethnobotanical treatment strategies against Alzheimer's disease. *Curr Alzheimer Res* 9:67–85
- Iuvone T, De Filippis D, Esposito G, D'amico A, Izzo AA (2006) The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-beta peptide-induced neurotoxicity. *J Pharmacol Exp Ther* 317:1143–1149
- Kar S, Slowikowski SP, Westaway D, Mount HT (2004) Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer's disease. *J Psychiatry Neurosci* 29:427–441
- Khodabakhsh A, Gohari A, Davood A, Saeidnia S, Thesis, 1388–1389 (2010). *Phytochemical Investigation of Satureja bachtiarica*. Pharm.D, Islamic Azad university of Tehran
- Kramer D, Minichiello L (2010) Cell culture of primary cerebellar granule cells. *Methods Mol Biol* 633:233–239
- Liu R, Meng F, Zhang L, Liu A, Qin H, Lan X, Li L, Du G (2011) Luteolin isolated from the medicinal plant *Elsholtzia rugulosa* (Labiatae) prevents copper-mediated toxicity in beta-amyloid precursor protein Swedish mutation overexpressing SH-SY5Y cells. *Molecules* 16:2084–2096
- Lleo A, Greenberg SM, Growdon JH (2006) Current pharmacotherapy for Alzheimer's disease. *Annu Rev Med* 57:513–533
- Luquin MR, Martinez-Vila E, Saldice L (1997) Mechanisms of cell death in Alzheimer's disease. *Rev Med Univ Navarra* 41:34–45
- Manayi A, Mirnezami T, Saeidnia S, Ajani Y (2012) Pharmacognostical evaluation, phytochemical analysis and antioxidant activity of the roots of *Achillea tenuifolia* LAM. *Pharmacogn J* 4:14–19
- Mohammadpour G, Marzony ET, Farahmand M (2012) Evaluation of the anti-Leishmania major activity of *Satureja bachtiarica* essential oil in vitro. *Nat Prod Commun* 7:133–136
- Naghibi F, Mosaddegh M, Motamed SM, Ghorbani A (2005) Labiatae family in folk medicine in Iran: from ethnobotany to pharmacology. *IJPR* 2:63–79
- Orhan IE, Senol FS, Sener B (2012) Recent approaches towards selected Lamiaceae plants for their prospective use in neuroprotection. *Stud Nat Prod Chem* 38:397–415
- Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Med Cell Longev* 2:270–278
- Parks JK, Smith TS, Trimmer PA, Bennett Jr JP, Parker Jr WD (2001) Neurotoxic Abeta peptides increase oxidative stress in vivo through NMDA-receptor and nitric-oxide-synthase mechanisms, and inhibit complex IV activity and induce a mitochondrial permeability transition in vitro. *J Neurochem* 76:1050–1056
- Paxinos G, Watson C (eds) (1997) *The rat brain in stereotaxic coordinates*. Academic Press, San Diego
- Perry E, Howes MJ (2011) Medicinal plants and dementia therapy: herbal hopes for brain aging? *CNS Neurosci Ther* 17:683–698
- Schliebs R, Arendt T (2006) The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. *J Neural Transm* 113:1625–1644
- Sepeand MR, Soodi M, Hajimehdipoor H, Sahraei E (2013) Comparison of neuroprotective effects of melissa officinalis total extract and its acidic and non-acidic fractions against A β -induced toxicity. *Iran J Pharm Res* 12:415–423
- Singhal AK, Naithani V, Bangar OP (2012) Medicinal plants with a potential to treat Alzheimer and associated symptoms. *Int J Nutr Pharmacol Neurol Dis* 2:84–91
- Soodi M, Sharifzadeh M, Naghdi N, Ostad N, Abdollahi M, Roghani A (2007) Systemic and developmental exposure to lead causes spatial memory deficits and a reduction in COX-2 immunoreactivity in the hippocampus of male rats. *J Neurosci Res* 85:3183–3192
- Soodi M, Naghdi N, Hajimehdipoor H, Choopani S, Sahraei E (2014) Memory-improving activity of *Melissa officinalis* extract in naïve and scopolamine-treated rats. *Res Pharm Sci* 9:107–114
- Stepanichev MY, Zdobnova IM, Zarubenko II, Moiseeva YV, Lazareva NA, Onufriev MV, Gulyaeva NV (2004) Amyloid-beta(25–35)-induced memory impairments correlate with cell loss in rat hippocampus. *Physiol Behav* 80:647–655
- Sun MK, Alkon DL (2002) Impairment of hippocampal CA1 heterosynaptic transformation and spatial memory by beta-amyloid(25–35). *J Neurophysiol* 87:2441–2449
- Tayeb HO, Yang HD, Price BH, Tarazi FI (2012) Pharmacotherapies for Alzheimer's disease: beyond cholinesterase inhibitors. *Pharmacol Ther* 134:8–25
- Tepe B (2015) Inhibitory effect of *Satureja* on certain types of organisms. *Rec Nat Prod* 9:1–18
- Topcu G, Kusman T (2014) Lamiaceae family plants as a potential anti-cholinesterase source in the treatment of Alzheimer's disease. *Bezmialem Science* 1:1–25
- Tsai FS, Cheng HY, Hsieh MT, Wu CR, Lin YC, Peng WH (2010) The ameliorating effects of luteolin on beta-amyloid-induced impairment of water maze performance and passive avoidance in rats. *Am J Chin Med* 38:279–291
- Vauzour D (2012) Dietary polyphenols as modulators of brain functions: biological actions and molecular mechanisms underpinning their beneficial effects. *Oxidative Med Cell Longev* 914273:3
- Wei H, Leeds PR, Qian Y, Wei W, Chen R, Chuang D (2000) Beta-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment. *Eur J Pharmacol* 392:117–123
- Yamaguchi Y, Kawashima S (2001) Effects of amyloid-beta-(25–35) on passive avoidance, radial-arm maze learning and choline acetyltransferase activity in the rat. *Eur J Pharmacol* 412:265–272

- Yamaguchi Y, Matsuno T, Kawashima S (2002) Antiamnesic effects of azaindolizone derivative ZSET845 on impaired learning and decreased ChAT activity induced by amyloid-beta 25–35 in the rat. *Brain Res* 945:259–265
- Yang W, Ma J, Liu Z, Lu Y, Hu B, Yu H (2014) Effect of naringenin on brain insulin signaling and cognitive functions in ICV-STZ induced dementia model of rats. *Neurol Sci* 35:741–751