



Neuroprotection and anxiety like behavior reduction of *Allium hirtifolium* and *Astragalus hamosus* in the A β -injected rat

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Abstract

Background and objectives: Traditional medicine is an important approach to achieve new therapeutic strategies in basic and clinical pharmacology. *Allium hirtifolium* Boiss. and *Astragalus hamosus* L. have been mentioned in Iranian Traditional Medicine references for a kind of dementia with features and symptoms similar to those of Alzheimer's disease (AD). In the present study, the neuroprotective effect of these herbs has been evaluated as new therapies in neurotoxicity model.

Methods: Two separate groups of rats were fed with *A. hirtifolium* or *A. hamosus* extract (100 mg/kg/day) from 1 week before amyloid beta (A β) injection, for 16 consecutive days. One day after the last oral administration, behavioral test was done. The effect of these two extracts were assessed in anxiety-like behavior test using elevated plus maze. Furthermore, molecular pathways involved in apoptosis were assessed by Western blotting analysis. **Results:** The results showed that oral administration of both *A. hirtifolium* and *A. hamosus* decreased anxiety-like behavior and ameliorated the effect on apoptosis factors including Bax, Bcl-2 and caspase-3 in the rats with intra-hippocampal injection of A β . **Conclusion:** The results of this study revealed the potential neuroprotective properties of *A. hirtifolium* and *A. hamosus* as herbal remedies that could play a role in fostering healthy aging and be considered as useful candidates in decreasing AD related symptoms.

Keywords: *Allium hirtifolium*, Alzheimer's disease, anxiety, apoptosis, *Astragalus hamosus*

Introduction

Alzheimer's disease (AD) is the most common type of dementia in the elderly, and is characterized by behavioral disturbances and psychological symptoms [1,2]. The prevalence of AD is one in nine people above 65 years in the United States. In addition to memory decline which is common in patients suffering from AD, anxiety symptoms are also a source of concern [1,2]. Behavioral disturbances associated with

this disease could hurt families and caregivers [3,4]. The treatment of behavioral symptoms of AD is imperative in improving the condition of the patients and their caregivers [3,5]. Amyloid beta (A β) peptide which is the most common hallmark of AD pathogenesis [6] causes neuronal apoptosis [7] in the central nervous system. Apoptosis is the main reason for cognitive decline in AD [8]. Two of the main apoptotic

factors are changes in Bax/Bcl-2 ratio and cleavage of caspase-3 [9]. A β accumulation in the hippocampus causes apoptosis and anxiety-like behavior [10].

Medicinal herbs have the potential to be developed into optimum pharmaceuticals for complex situations such as AD because of their multi-function and multi-target characteristics [11,12]. In Iranian Traditional Medicine (ITM), numerous plants have been introduced for treatment of memory related disorders [13-15]. Among these plants based on the importance and accessibility, *Astragalus hamosus* L. and *Allium hirtifolium* Boiss. were chosen.

Persian shallot "*Mu-sir*" (traditional name) [16,17], with the scientific name of *Allium hirtifolium* Boiss., belongs to Amaryllidaceae family. *Allium* genus has more than 900 species in the world [18,19]. *Allium hirtifolium* grows as a wild plant in the Zagros mountains [18], and is one of the most frequently used spices in Iran [20].

Spices that exhibit antioxidant activity receive immense attention as food supplements to improve cognitive impairment against AD [21]. Phenolic compounds present in spicy plants possess bioactive properties which protecting cellular systems against oxidative stress [22]. *Allium hirtifolium* has been shown to have various pharmacological properties such as antioxidant, antimicrobial [23], anticancer [24], anti-inflammatory [25], antiatherosclerotic [26], antidiabetic [27], immunomodulatory [28], antinociceptive [29], and acetylcholinesterase inhibitory properties [25].

Another plant used in this study was "*Iklil-ul-Malik*" (traditional name) [16,30] with the scientific name of *Astragalus hamosus* L., belongs to Fabaceae family. *Astragalus* genus is the largest genus of flowering plants, containing up to 3000 species, and Iran is one of the most important centers of diversity of this genus [31]. Recently it was shown that *A. hamosus* has protective effect on biological systems. In this regard, anti-inflammatory (pods) [32], analgesic [33], cytoprotective and antioxidant (aerial parts) [34] activities of *A. hamosus* have been reported.

Hence, we investigated the effect of oral administration of *A. hirtifolium* and *A. hamosus* on changes in the main apoptotic factors (Bax, Bcl-2, caspase-3) in the hippocampus of A β -injected rats and anxiety-like behavior induced by A β .

Experimental

Plant material

Allium hirtifolium bulbs and *A. hamosus* fruits were purchased from a local market in Tehran. Their scientific names were authenticated and a specimen was deposited at the Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences (332HMS, 333HMS, respectively). *Allium hirtifolium* bulbs were extracted twice with ethanol (80%) at room temperature. The extract was concentrated utilizing a rotary evaporator and dried using a vacuum drying oven [16,28]. The powdered *A. hamosus* fruits were extracted employing the decoction method based on the preparation method in the ITM. The mixture was filtered and the filtrate was freeze-dried [15,35]. The extracts were kept in a closed container and protected from light at 4-8 °C until use.

Animals

All animals were kept following approval from the animal care committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran and Guide for the Care and Use of Laboratory Animals of the National Institute of Health, Bethesda, United States of America [36]. Male Wistar rats (200–250 g) were obtained from the Pasteur Institute of Iran, Tehran. Rats were habituated to the laboratory for 7 days before the experiment. Animals were kept four per cage, at a temperature of 22±2 °C with free access to standard laboratory chow and water, under a 12h light–dark cycle (lights on at 7:00 a.m.).

Oral administration of plant extract

A. hirtifolium and *A. hamosus* extracts were dissolved in water separately and fresh solutions were prepared every day to feed the rats with a volume of 1 mL using gavage tube, one week

before surgery to 24 h before behavioral testing (in general, for 16 consecutive days). Gavage (100 mg/kg) was used daily between 8:00 to 9:00 AM.

Body weight gain

To determine the effect of *A. hirtifolium* or *A. hamosus* extracts on body weight gain in the rats, the weight was assessed twice before feeding the rats with the extracts and 7 days after oral administration of the mentioned extracts.

Preparation of A β

Preparation of A β powder (GenScript, Piscataway, USA) was carried out according to the method described previously to obtain the concentration of 50 ng/ μ L [37].

Surgery

The animals were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) and were placed in a stereotaxic apparatus (Stoelting, Wood Dale, USA). After cutting along the midline, the retracting of scalp was performed and the different parts including the bregma were dried and cleaned with the help of sterile cotton. Based on the Paxinos and Watson atlas of rat brain, the stereotaxic for dorsal hippocampus was determined [38] (anterior-posterior, -3.8 mm; lateral, ± 2.2 mm from the central line, and ventral, -2.7 mm down from the surface of the skull). By means of a Hamilton syringe (1 μ L/side), A β (50 ng/ μ L) injection was introduced into both side of the CA1 region of the hippocampus for 1 to 3 minutes. The needle was left in place to facilitate the diffusion of the injected materials and thereafter, the scalp was sutured.

Experimental groups

Rats were randomly assigned into six groups (eight rats per experimental group) as follows: (1) Vehicle group that only received phosphate buffered saline (PBS) (1 μ L/ side) into the CA1 region and 1 mL of water every day using gavage tube for 16 days; (2) A β -group, which received bilateral intra-CA1 injection of A β (50 ng/ μ L

PBS per side) and 1 mL of water by gavage; (3) *Allium hirtifolium* group that received PBS (1 μ L/ side) bilaterally into the CA1 region and 100 mg/kg *A. hirtifolium* dissolved in 1 mL of water by gavage; (4) The *A. hirtifolium* and A β group, received both *A. hirtifolium* (100 mg/kg) orally and A β (50 ng/ μ L PBS) into the CA1 region; (5) *Astragalus hamosus* group that received PBS (1 μ L/ side) bilaterally into the CA1 region and 100 mg/kg *A. hamosus* dissolved in 1 mL of water by gavage; (6) The *A. hamosus* and A β group, received both *A. hamosus* (100 mg/kg) orally and A β (50 ng/ μ L PBS) into the CA1 region.

Elevated plus maze (EPM) test

The EPM test was carried out on the ninth day after stereotaxic surgery. The EPM is a rodent model of anxiety paradigm that is employed as a screening test for putative anxiolytic or anxiogenic effects [39]. The wooden and sign (+) shaped maze, possessing four arms (two closed and two open) with a platform at the center (10 cm \times 10 cm), was elevated 50 cm from the floor. The closed arms characteristics were 50 cm length, 10 cm width and 40 cm height, while the features of the open arms were 50 cm length and 10 cm width. The animals were individually placed in the center of the apparatus and were allowed to explore freely for 5 min. The percentage of time spent in the open arms [OAT%: (time in open arm/time in "open+closed" arm) \times 100] and the percentage of number of entrances to open arms [OAE%: (number of open arm entries/number of "open+closed" arm entries) \times 100] were counted as anxiety index. The total of closed and open arm entries were considered as an index for the locomotor activity [40].

Western blot test

Ten days after surgery (after the completion of maze test), the hippocampi were dissected and flash frozen in liquid nitrogen immediately. Thereafter, the tissue was stored at -80 °C. The hippocampal tissues were removed from the rats' brain, lysed in a buffer solution including Tris-HCl, SDS, NaCl, Sodium deoxycholate, EDTA,

Triton X-100 and cocktail protease inhibitor (Roche, Penzberg, Germany). Bradford test was used to determine the total concentrations of proteins using serum albumin as a standard. In this method, the proteins, which were loaded into the wells existed on the SDS-PAGE gel and were separated based on their molecular weights. The assay was continued by electroblotting the proteins on the polyvinylidene difluoride membranes (Millipore, USA) and covering the membrane with blocking solution. Addition of the primary antibodies against caspase-3, Bax, Bcl-2 and β -actin (Cell Signaling Technology, USA) paved the way for recognition of the level of these proteins by means of a secondary antibody (Cell Signaling Technology) which was conjugated with horseradish peroxidase enzyme. The substrate of electrochemiluminescent (Amersham Bioscience, USA) made the immunoreactive bands detectable on the autoradiography that was visualized on Kodak films. Subsequently, the software of Image J provided the opportunity to quantify the outcomes obtained from this experiment.

Statistical analysis

Data obtained from Western blot and other results were expressed as the Mean \pm SEM (standard error of mean) and were processed using GraphPad Prism® 5.0. Comparison among the groups was carried out by means of ANOVA (one-way) using Tukey's post hoc test. $p < 0.05$ was considered to be significant ($n = 8$).

Results and discussion

The effect of oral administration of *A. hirtifolium* and *A. hamosus* on body weight gain has been shown in table 1. During one week of oral administration of *A. hirtifolium* or *A. hamosus* (before stereotaxic surgery), body weight gain for the two groups of rats treated with the mentioned extracts did not show any significant changes, compared to the control group which received only 1 mL of water daily by gavage. The data showed feeding with *A. hirtifolium* and *A. hamosus* had no effect on body weight.

Table 1. Effects of oral administration of *Allium hirtifolium* and *Astragalus hamosus* on body weight gain

Groups	body weight gain (g) after 1 week
Control (water)	19.2 \pm 1.2
<i>A. hirtifolium</i>	16.3 \pm 2.2
<i>A. hamosus</i>	19.7 \pm 1.9

The values were measured 1 week after feeding. Data are mean \pm SEM; the results did not show any significant difference between each group compared to the control group.

In order to evaluate anxiety-like behavior, EPM test was used on the ninth day after stereotaxic surgery. The EPM has been validated to assess the anxiolytic effects of the pharmacological agents [39]. In the current study, data analysis showed that the injection of $A\beta$ into the hippocampus significantly decreased OAT% and OAE% in comparison with the vehicle group (both $p < 0.001$). In addition, data analysis showed that oral administration of each extract of *A. hirtifolium* and *A. hamosus* significantly increased OAT% (both $p < 0.05$) and OAE% ($p < 0.001$ and $p < 0.05$, respectively) in $A\beta$ -injected rats compared to the $A\beta$ -group (figures 1A and B). Moreover, locomotor activity did not show any significant changes among the different experimental groups (figure 1C). Our data exhibited that oral administration of *A. hirtifolium* and *A. hamosus* in sham animals (group 3 and 5) had no effect on anxiety like behavior and movement. According to the results of the study, the oral administration of the mentioned extracts decreased $A\beta$ -induced anxiety-like behavior. In the present study, Western blot analysis of hippocampal lysates was carried out to detect the level of Bax, Bcl-2 and cleaved caspase-3 proteins. The high Bax/Bcl-2 ratio is affiliated with greater vulnerability to apoptotic activation [41]; in addition, caspase-3 cleavage is required as typical hallmark of apoptosis [42]. Six groups were run together on single blots and bands densities were normalized to β -actin (Figure 2A). As shown in figures 2B and C, the Bax/Bcl-2 ratio and the level of cleaved caspase-3 increased about 1.6- and 2.2-fold, respectively in the hippocampi of $A\beta$ -injected group compared to the vehicle group.

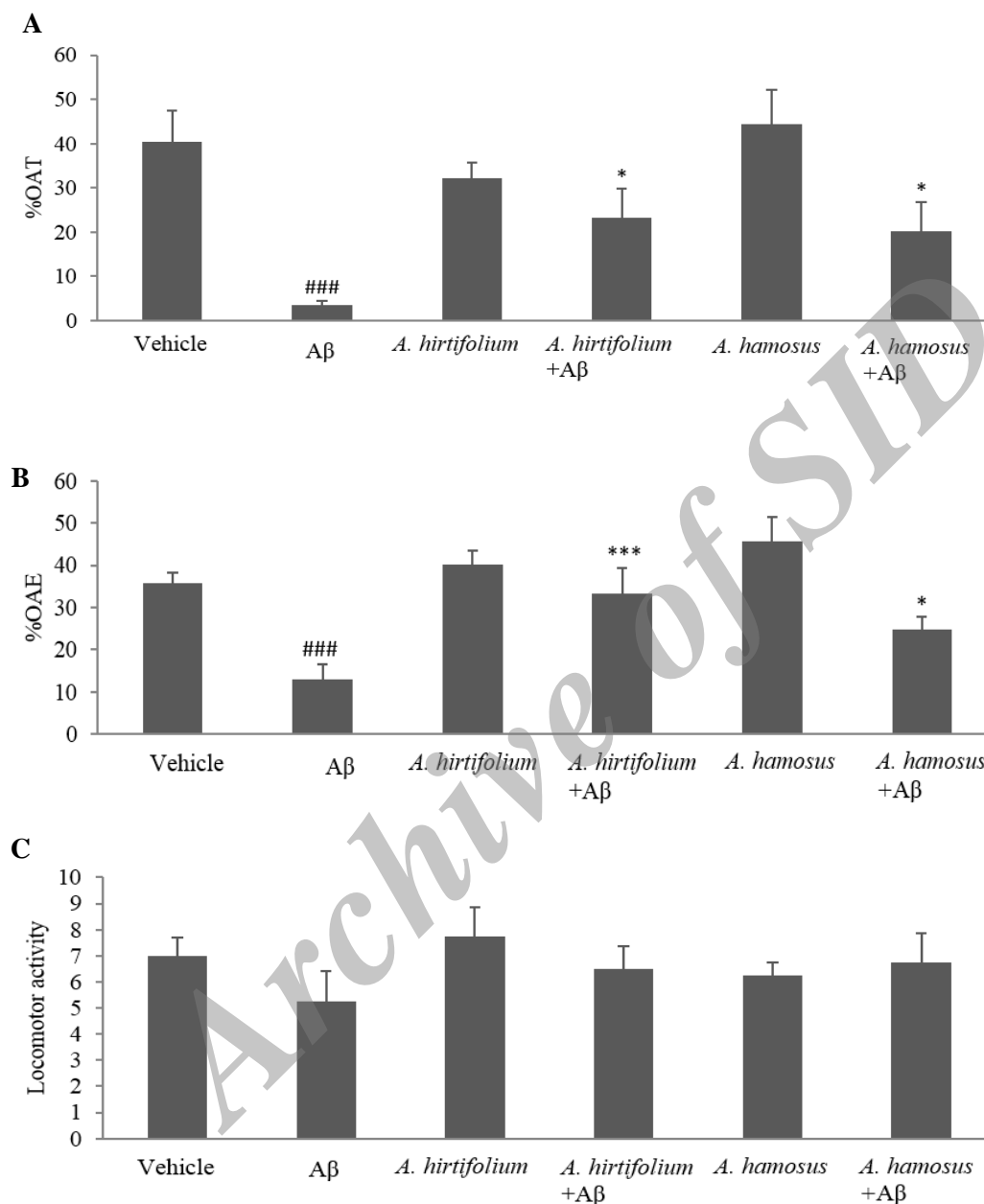


Figure 1. Effect of the oral administration with each of the extracts of *Allium hirtifolium* and *Astragalus hamosus* on anxiety-related behavior in the rats injected with Aβ (50 ng/μL). Each value represents the mean ± SEM. OAT% (A), OAE% (B) and locomotor activity (C) during 5 minutes exposure to EPM. Significant differences: ### $p < 0.001$ different from the vehicle group. * $p < 0.05$ and *** $p < 0.001$ different from the Aβ-injected group.

Besides, the outcomes obtained from Western blotting assay showed that oral administration of *A. hirtifolium* in A β -injected rats diminished the Bax/Bcl-2 ratio and caspase-3 cleavage about 42% and 30% respectively compared with the A β -injected group. Furthermore, oral administration of *A. hamosus* decreased Bax/Bcl-2 ratio and caspase-3 cleavage about 47% and 49%, respectively, in comparison with A β -injected group, 10 days after surgery.

Studies have shown that A β is neurotoxic *in vitro* and *in vivo* [43] and its injection into hippocampus tissue can lead to apoptosis and anxiety-like behavior [7,10,44]. The results obtained from the present study revealed that the oral administration of the mentioned extracts reduced anxiety-like behavior in the rats which received A β . Moreover, the outcomes of the Western blotting test of Bax, Bcl-2 and caspase-3 showed that both *A. hirtifolium* and *A. hamosus* had an ameliorating effect on A β induced apoptosis.

Studies have shown that hippocampal apoptosis has an important effect on the creation of anxiety-related behavior [45,46]. The main components of the apoptosis in neurons are proteins of the Bcl-2 and caspase families [43]. Bax is a pro-apoptotic member of the Bcl-2 family and interacts with Bcl-2 [43]. One of the major caspases involved in neuronal apoptosis is caspase-3 which plays some key roles in apoptotic cell death [42]. Studies have suggested that selective caspase inhibition might be a possible therapeutic strategy in AD [43]. Researchers have shown an effective relationship between the function of mitochondria and anxiety disorders [47,48]. One of the major modulators of mitochondrial function is Bcl-2 proteins located in the inner mitochondrial membrane and reduction in the amount of this protein can be counted as the main factor for anxiety disorders [48].

In the current study, oral administration of *A. hirtifolium* and *A. hamosus* increased Bcl-2 and decreased proapoptotic factor in the hippocampi of rats, which consequently can be effective on

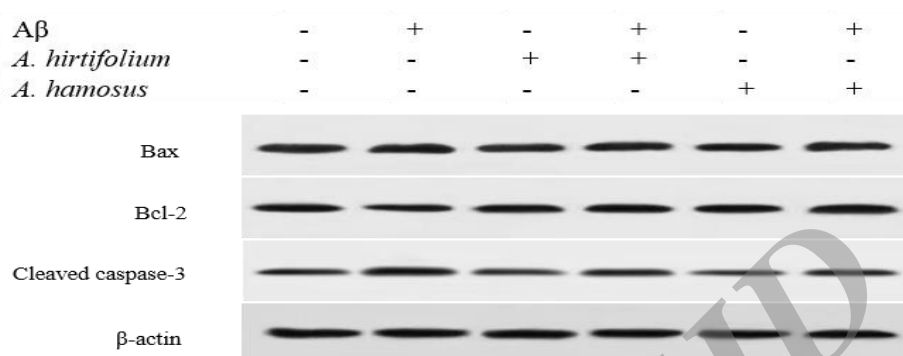
anxiety-like behaviors reduction.

These beneficial protective effects of *A. hirtifolium* in neurotoxicity induced by A β injection could probably be due to the antioxidant capacity of its phenolic and organosulfur compounds [49-52]. Polyphenols have been considered as modulators and reducers of oxidative stress [53, 54]. Dietary polyphenols are capable of producing positive effects on mental health [55] and are particularly effective against anxiety [56]. The neuroprotective benefits exhibited by *A. hirtifolium* may be due to the polyphenol components which improve a number of pathological situations including neurodegenerative disorders [57]. In addition, organosulfur compounds content of this plant such as diallyl disulfide [27,58] and diallyl thiosulfinate (allicin) [24,28,59,60] contribute to this feature.

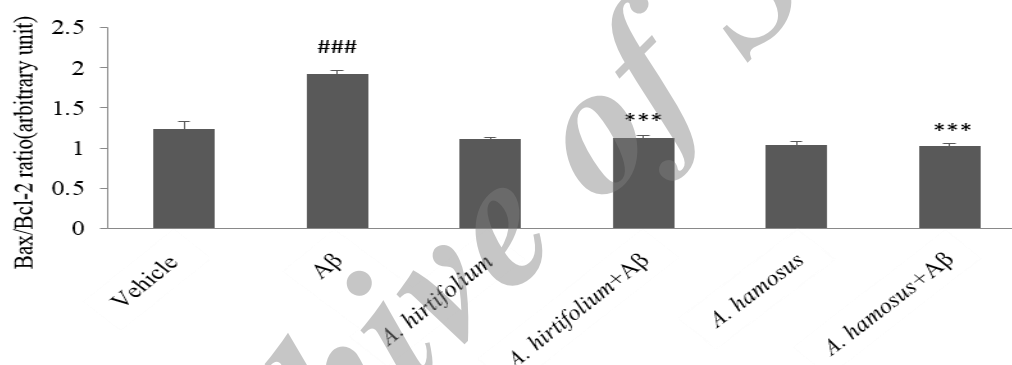
Astragalus plant species are rich sources of flavonoids [61,62]. Flavonoids such as isoquercitrin, astragalin, hyperoside and rhamnocitrin 4'-beta-D-galactopyranoside, were isolated from the aerial parts of *A. hamosus* [62]. Polyphenolic compounds like flavonoids in the plant may contribute to neuroprotective properties [55,56]. The species also contains selenium, an important micronutrient, which deficiency seems to have important relation with AD [63,64]. The neuroprotective effects may be caused by a complex mixture of phytochemicals and bioactive compounds in *A. hirtifolium* and *A. hamosus* and diverse compounds may complement or synergize to produce their beneficial actions [65].

In the present study, both *A. hirtifolium* and *A. hamosus* showed neuroprotective properties. Oral administration of *A. hamosus* resulted in more reduction of caspase-3 as a proapoptotic molecule than *A. hirtifolium*, but no significant difference in behavioral tests were observed. This could be as a result of the influence of *A. hamosus* on other molecular pathways involved in anxiety, which was not considered in this research and can be examined in future studies. As *A. hirtifolium* and *A. hamosus* proposed in

A



B



C

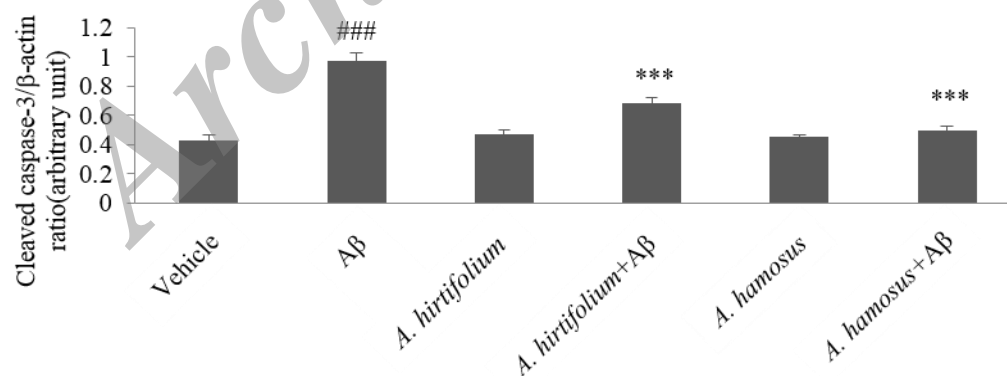


Figure 2. Western blot analysis to measure the effect of oral administration with each of the extracts of *Allium hirtifolium* and *Astragalus hamosus* on Bax, Bcl-2 and cleaved caspase-3 levels in the hippocampus of rats injected with A β (50 ng/ μ L) (A, B, C); one representative western blot is shown. The values represent the mean \pm SEM. Significant differences: ^{###} $p < 0.001$ different from the vehicle group. ^{***} $p < 0.001$ different from the A β -injected group.

traditional medicine documents to have positive effect on nervous system, our data provide their neuroprotective effect on A β -injected rat model of Alzheimer disease.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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