

Aqueous Extract of *Lavandula Angustifolia* Alter Protein Expression in Alzheimer Rats

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ABSTRACT

Alzheimer's disease (AD) is a progressive and socio-economical form of dementia. The currently available drugs are only able to delay the symptom progression of the disease. This study tries to evaluate protein profile of the effects of aqueous extract of lavender (*Lavandula angustifolia*) on spatial performance of AD rats. Male Wistar rats were divided into control and Alzheimeric groups (CO and ALZ respectively). Rat model of AD was established by intracerebroventricular injection of 10 mg A β 1-42 twenty days prior to administration of the lavender extract. All of the groups were introduced to task learning in Morris water maze (MWM). After the first stage of spatial learning, control and Alzheimeric animals received 200 mg/kg of the lavender extract (CE200 and AE200 respectively) for 20 days. After the second stage of MWM, Hippocampus tissues of four groups were separated and protein profile was determined by 2DE. By using progenesis same spot software totally 950 spots were detected in 4 gel groups. Injection of amyloid beta suppresses expression of 111 proteins. Progressive Effects of lavender extract on spatial memory and maze learning task can be clear by finding difference gene expression in both ALZ and AE200 groups. Comparison between CO and CE200 groups show 80 new proteins that expressed and 104 protein that suppressed in CE200. These findings can disclose the efficiency of lavender on improving memory and learning. Applying more proteomics techniques to better understanding of protein changes can lead to the development of new drug treatments for dementia.

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Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by intracellular accumulation of neurofibrillary tangles (NFT) and extracellular beta amyloid (A β) plaque^[1, 2]. A β is a toxic pro-inflammatory agent that promotes inflammatory process in the brain^[3]. Also, AD patients have defect in their cholinergic system^[4]. In AD, the hippocampus is an especially susceptible brain region and early neurodegenerative symptoms include significant deficits in the performance of hippocampus-dependent cognitive abilities such as spatial learning and memory^[5]. There is no cure for AD so far, and the disease causes progressive decline in daily activities, eventually resulting in death^[6, 7]. The current available drugs are just able to delay the symptom progression of the disease and to positively influence the quality of everyday life of patients^[8].

Aromatherapy effects of lavender in alleviation of the agitated behaviors is an aspect of treatment in patients with dementia^[9]. The hydroalcoholic, polyphenolic and essential oil of lavender is an anti-inflammatory agent^[10]. It is also reported that lavender is an effective medical plant in treating inflammation, depression, stress and headache. Also lavender extract has an anti-AChE activity that causes increase the level of ACh in brain^[11, 12]. These features can be important to treat dementia disorders such as Alzheimer.

AD had been studied with two-dimensional gel electrophoresis (2-DE) before the term proteome was introduced and today by using progressive techniques like gel-free, AD proteome are approached^[13].

In every disorder proteomic study is employed in the area of disease particularly to reveal disease relevant biomarkers. Thus, many proteomic studies in AD were involved in different part of brain diagnosis and follow up of the progression of the disorder as well as new targets for medical intervention of the disease process^[14].

In our previous study we investigated that lavender extract influences the cognitive function of control and Alzheimeric model of rats in dose of 200 mg/kg of body weight (CE200 and AE200 respectively) compared to control and Alzheimer groups that receive vehicle (CO and ALZ respectively)^[15]. In this study, effect of lavender extract on proteome profile of hippocampus of all 4 groups at the end of behavioral test was evaluated.

Materials and Methods

Animals

A total number of 50 male Wistar rats, weighing 220-280 g, were employed in the present study. The animals were kept at constant temperature under a 12:12 h light/dark cycle with free access to food and water. The subjects were first divided into control and Alzheimer group. There are 4 groups: control (CO) and Alzheimeric (ALZ) rats that received distilled water and control (CE200) and Alzheimeric (AE200) groups received 200 mg/kg of the lavender extract.

Establishment of AD model

Animal model of AD was created by intracerebroventricular (i.c.v.) injection of 10 μ g of A β 1-42 peptide (Sigma Aldrich, St. Louis, MO, USA) dissolved in distilled water, 20 days before extract administration^[16, 17]. The injection site (AP=Bregma, LR=1.5 mm, D=4 mm) was determined according to the Stereotaxic Atlas^[18]. The animals in control group were treated with the same procedure except that they received distilled water.

Preparation of lavender extract

For extract preparation, 250 g dried flowers of lavender was mixed with 1000 mL boiling water. The mixture was then stirred for 4 h in a fully packed container, filtered, and concentrated by vaporizing. The plant specimen was identified by Pharmaceutics Faculty of Shaheed Beheshti University, where voucher specimens (1092) were kept.

Extract administration

The concentrated aqueous extract of lavender was suspended in distilled water. After the first probe test, all groups were intra-peritoneally injected with either distilled water or 200 mg/kg of the aqueous extract of lavender. All animals were injected at a volume of 0.4 mL/kg body weight. The treatment was conducted once per day for 20 consecutive days.

Morris water maze test

Morris water maze (MWM) is a circular tank with 180 cm in diameter and 60 cm in height. It was positioned in the middle of a dimly lit testing room enriched with some spatial cues. The tank was filled with water (22 \pm 2 $^{\circ}$ C) up to 20 cm below the rim. A stable circular platform, measuring 10 cm in diameter was submerged 1.5 cm below the surface of the water. A video camera connected to an image analysis system was placed

above the centre of the water maze. Through running software (Radiab 7, I.R.Iran) the data related to maze navigation by the animals were tracked, digitized and stored for subsequent behavioral analysis.

Probe test

In each stage of the experiments, after completion of 20 trials of spatial task learning a probe test was conducted to evaluate the retrieval of spatial memory. In this test, the platform was removed from the pool and the animals were released from a random quadrant and allowed to swim freely for 90 s. The time spent in the target quadrant of the water maze was measured in the spatial memory retrieval trials.

Sampling

To confirm the formation of A β plaque in the brain, 20 d after A β injection, 10 rats were decapitated and hippocampus area of brain was removed for histological observation. The samples were immersed in fixative formalin for 48–72 h. Then dehydration and paraffin embedding were performed via an automated processor. The brain was sectioned and stained by Congo red according to the previous report^[19]. The staining verified the formation of A β plaque in the hippocampal area of brain in A β -treated animals. Hippocampus tissue samples were taken from all groups of rats for proteomics analysis.

Protein purification

Fresh tissue samples of hippocampus tissue were snap frozen and kept in liquid nitrogen until use. Tissue samples were powdered by microdismembrator at maximum speed for 60 seconds under liquid nitrogen conditions. Each powdered tissue sample was added to an appropriate amount of lysis buffer containing 10 mM Tris-HCl pH=7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM Phenylmethylsulfonyl fluoride (PMSF), 5 mM betamercapto ethanol, 0.5% CHAPS and 10% glycerol.25 After 30 minutes incubation on ice, the lysate was centrifuged at 16000g for 30 minutes at 4°C. Protein concentration of all samples was estimated using a Bradford based microassay.

Two dimensional SDS-PAGES

The first dimension of 2D electrophoresis was performed on the PROTEAN IEF Cell system (Bio-Rad). Next, gels were equilibrated for 15 min in equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris

Hcl pH8.8, 20% glycerol, 130mM DTT). A 12% SDS-Polyacrylamide slab gel was used for the second dimension gel electrophoresis. Equilibrated IPG strips were placed on the surface of the second dimension gels and then sealed with 0.5% agarose in SDS electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1%SDS) and were run vertically.

Silver staining

After electrophoresis, the gels were fixed with 50% methanol / 12% acetic acid / 0.5ml/lit formaldehyde for 60 min. At the end of fixation, the gels were rinsed 3 times with 50% ethanol (each time 20 min). The gels were sensitized by incubating in 0.2g/lit sodium thiosulfate for 1 min. After 3 time rinsing with distilled water (each time 20 sec), the gels were incubated in 1.9 g/lit silver nitrate / 0.8ml/lit formaldehyde for 20 min. After 3 times rinsing with distilled water (each time 20 sec) the gels were developed in 60g/lit sodium thiosulfate. The development was terminated with 12% acetic acid / 50% methanol^[20].

Bioinformatics analysis

2DE gels are scanned and gels are analyzed by non linear progenesis same spot software to compare gels together and compare the spots in one statement in gels and get the density of same spot in eachgel.

Results

Figure 1 show the staining verified the formation of A β plaque in the hippocampal area of brain in A β -treated animals. Congo red staining of sections containing the medial part (including CA1) of hippocampus in control (A) and A β -treated (B) subjects. Figure 2 illustrates the performance in the second stage of maze training after 20-day treatment with lavender extract. The rats administered with 200 mg/kg of the herbal extract significantly improved learning of the maze task in the control and alzheimeric groups. Each point represents mean \pm SEM summed results of 4 daily trials. After the second stage of the experiment, the animals were introduced to probe test. Figure 3 shows histograms of time spent in the target quadrant of water maze during probe trials. The control and the Alzheimeric rats receiving 200 mg/kg of the lavender extract spent significantly increased time in the target quadrant, as compared with their counterparts with distilled water treatment.

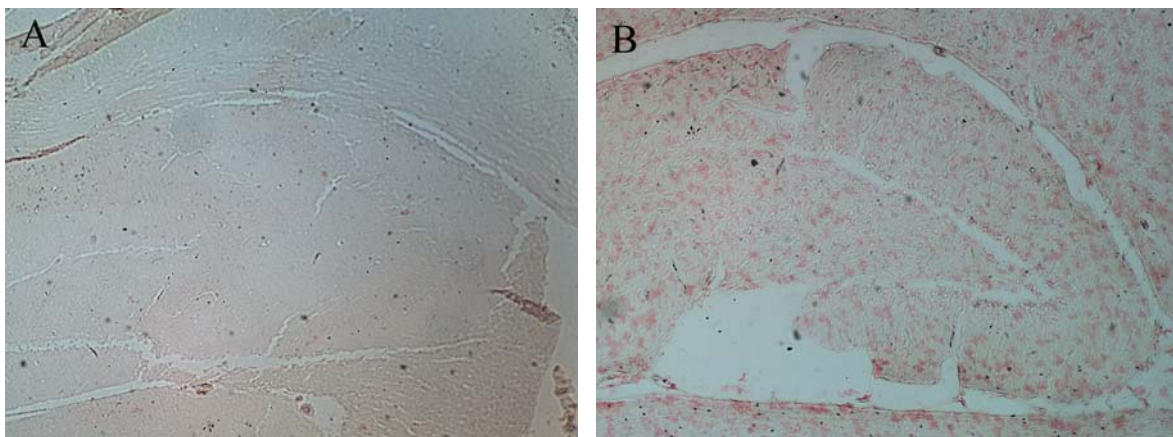


Fig. 1. Congo red staining of sections containing the medial part (including CA1) of hippocampus in control (A) and A β -treated (B) subjects. The staining verified the formation of A β plaque in the hippocampus of A β -treated rats. Red patches indicate plaques formed 20 d after A β injection. Scale bar, 100 μ m.

Protein components of hippocampus extracts from all 4 groups were separated by 2D electrophoresis and the protein spots were visualized following stain with silver staining. Figure 4, 2DE gel of the reference gel that was ALZ gel taken from result of progenesis same spot. In 4 gel groups totally produced 950 protein spots. It is necessary to remember that progenesis same spot program select one gel as

“reference gel” and situation of spots on other gels (the gels that compare with reference gel) only mark on reference gel. Figure 4 also resemble the specific statement of expression spots in the CO and ALZ groups. There are 111 protein spot just express in control and 67 spots that spatially belong to the the Alzheimer group. Comparison between ALZ group (a) and AE200 group (b) exhibited in figure 5.

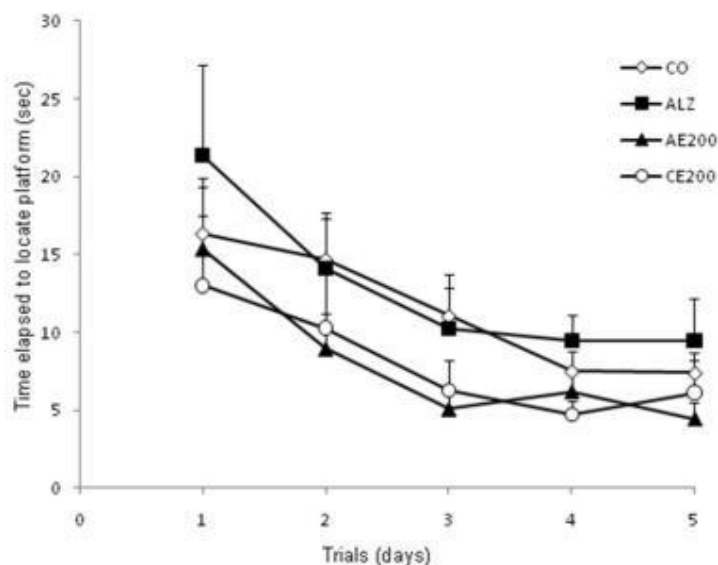


Fig. 2. performance in the second stage of maze training after 20-day treatment with lavender extract. The rats administered with 200 mg/kg of the herbal extract significantly improved learning of the maze task in the control and alzheimeric groups. Each point represents mean \pm SEM summed results of 4 daily trials.

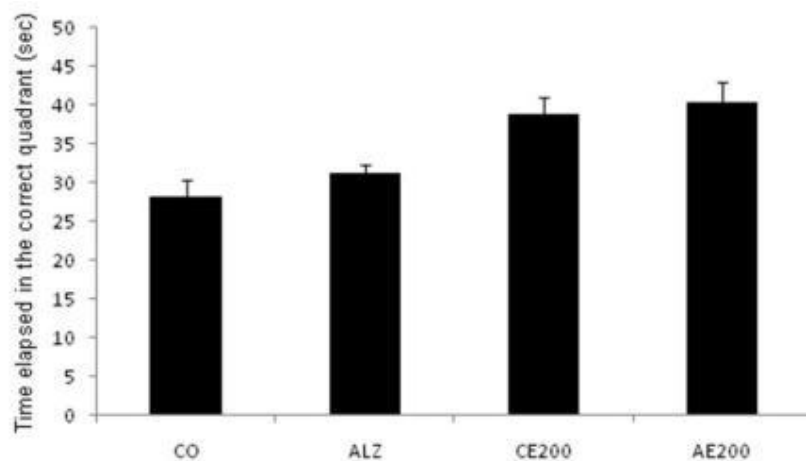


Fig. 3. After finishing the second stage of the experiment, the animals were introduced to a probe test. Histograms showing the time spent in the target quadrant of water maze during probe trials. The control and the Alzheimeric rats receiving 200 mg/kg of the lavender extract spent significantly increased time in the target quadrant, as compared with their counterparts with distilled water treatment.

Both groups just express 21 common spots that some of them were significantly ($P < 0.05$) up regulated and others were down regulated. 49 spots were observed only in ALZ gel while disappear in AE200 gel. In contrast 26 spots were expressed newly in AE200 group. The same analyses were done for CO and

CE200 groups. Results illustrate that the presence of extract can cause the appearance of 80 new protein spots and disappearance of 104 spots in CE200 group.

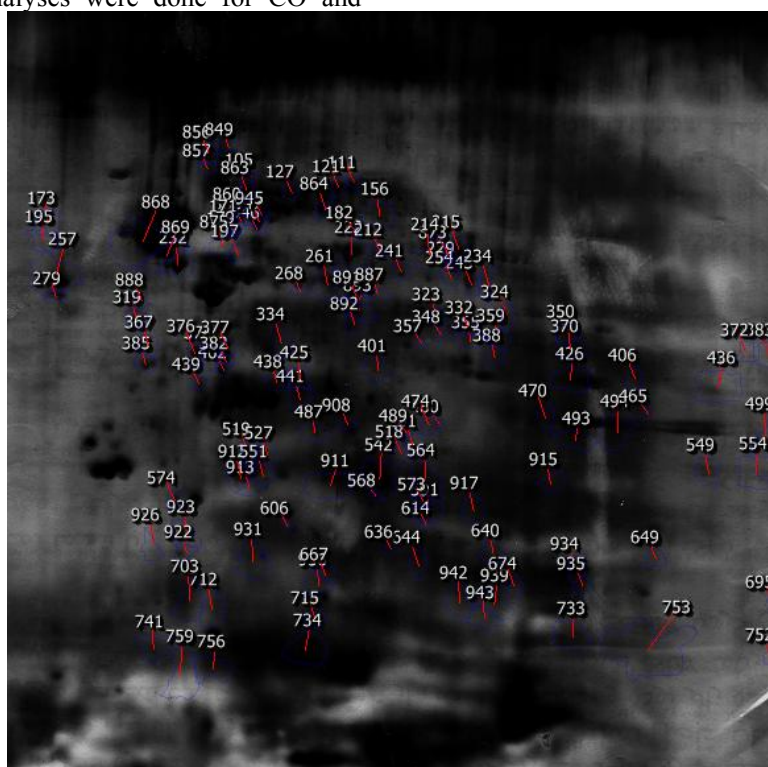


Fig. 4. 2DE gel of the reference gel that show the proteins that express in CO and ALZ.

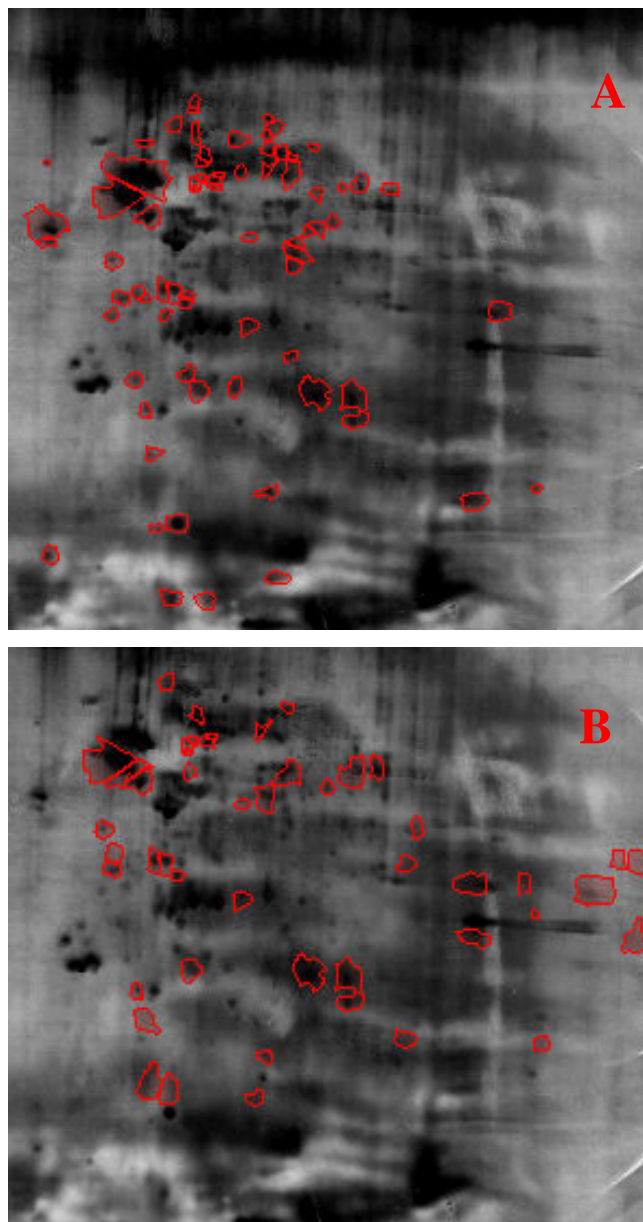


Fig. 5. 2DE gel of the ALZ and (A) AE200 (B). A and B image was taken from result of progenesis same spot so the both image depicted the reference that was the AD gel. In B the statement without any spot illustrated the same place in AE200 that newly express proteins but not express in reference image.

Table 1 shows the protein profiles that are related to the behavior alzheimeric rats in presence of extract. After comparing both alzheimeric and control group and omitting intersection proteins, recognized 16 spots newly expressed and 36 spot that suppressed in AE200. This expression pattern can introduce key proteins cause considerable improvement in the

performance of rats in the maze learning task in present of extract.

Table 1. Proteins that spatially expressed in AE200 and exactly related to the sensitivity of Alzheimer rats in presence extract.

New Expression	Suppression
712	812
493	514
111	836
324	58
383	108
703	84
465	867
372	862
388	137
406	529
436	128
667	730
426	422
649	161
640	883
923	686
	937
	178
	706
	432
	198
	738
	102
	886
	909
	852
	155
	855
	118
	98
	175
	770
	903
	186
	853
	72

Discussion

Alzheimer's disease, one of the most important neurodegenerative disorders, is characterized by deficits in learning and memory [21]. All drugs used in AD try to decrease such defects and improve quality of patient's life [8]. In this study we try to evaluate the protein profile that express under effects of aqueous extract of lavender (*Lavandula angustifolia*) on spatial performance of AD rats. Our previous study illustrated that lavender extract significantly improved the performance of control and Alzheimeric rats in the probe test, only at the dose of 200 mg/kg, as compared with their counterparts with vehicle treatment [15]. As illustrated in figure 2, performance in the second stage

of maze training after 20-d treatment with lavender extract improved learning of the maze task in the control and AD rats. The control and the AD rats according to the figure 3, receiving 200 mg/kg of the lavender extract spent significantly increased time in the target quadrant, as compared with their counterparts with distilled water treatment. Since the lavender extract could effectively reverse spatial learning deficits in Alzheimeric rats, for more information, all groups were analyzed by proteomics techniques. According to the figure 4 by using Progenesis same spot software totally 950 spots were detected in 4 gel groups (CO, CE200, ALZ and AE200). Proteomic results showed 111 proteins just express in CO group. This is illustrated that inducing of Alzheimer could suppress expression of 111 proteins while it can express 67 new proteins. One investigation of rat hippocampus proteome reported the identification of the products of 148 different genes. Six of proteins identified in hippocampus, have been linked to signal regulation and neural transmittance [22]. SNAP-25 expression levels represent early markers of synaptic loss that it is down regulate in the AD [23]. Furthermore, phosphatidyl ethanolamine binding protein (PBP) and serine/threonine protein phosphatase PP1 alpha and beta isoforms (PP1A and PP1B) closely related to memory and learning [24, 25].

The lavender treated animals in both control and Alzheimer groups demonstrated a tendency of better function in memory consolidation. In Alzheimeric animals, administration of the plant extract induced a trend of enhancement in the probe test performance. This showed that the dose of 200 mg/kg had a significant effect on memory improvement. Progressive effect of lavender extract on spatial memory and maze learning task can be clear by finding difference gene expression in both ALZ and AE200 groups. In regard to figure 5 some proteins (49 spots) were observed in ALZ but not in AE200, suggesting the absence of some proteins in AE200 animals. On the contrary, 26 proteins were only observed in AE200 but not in ALZ group.

Table 1 depicted the protein profile (16 spots newly expressed and 36 spot that suppressed) contribute to direct influence of lavender in improving the skills in AD rat. Lavender is reported to be an effective medical plant in treating inflammation, depression, stress and headache [26], so it could improve the brain activity. Besides, on the protective effect of lavender extract against AD dementia might be attributed to its anti-inflammatory property because previously the anti-inflammatory effect of lavender extract has been revealed [27]. Furthermore, in AD brains inflammatory mediators have been broadly appeared [28]. These

findings can disclose that lavender extract able to suppress expression of some principal proteins of Alzheimeric biological process or inhibit molecular function of them. Lavender may decline aging and improve learning and memory by several ways; 1) highly expression of PBP (hippocampal cholinergic neurostimulating peptides) that is involved in the function of the presynaptic cholinergic neurons of CNS [29, 30]. 2) Regulation in expression of PP1 as a potential mediator of cognitive decline during aging [31]. 3) Increase in phosphorylation of the transcription factor cyclic AMP-dependent response element binding protein (CREB). 4) Since AD patients may have defects in cholinergic system, which is associated with memory and learning [32], so increasing the level of acetylcholine (ACh) in the brain or inhibiting of AChE, an enzyme responsible for the hydrolysis of Ach and promotes A β formation may be another's effective therapy [33, 34]. It is important to attention that effective inhibitory action of lavender extract on AChE has been demonstrated [11]. Thus, enhancement of cholinergic transmission may serve as another possible mechanism underlying the effect of the extract on cognitive function of AD rats. 5) Changing cascade contains glutamate-induced excitotoxicity which consequently leads to synaptic damage and neural cell death [1, 35]. Another positive effect of aqueous extract of lavender must be reducing glutamate-induced neurotoxicity that has been reported by A. Adsersen and his coworker [12].

In conclusion, the complexity of alteration in expression proteins in treatment with herbal medicine suggests that this protein changing may have important role in the hippocampus function of Alzheimeric rats. By applying more proteomics techniques to better understanding of protein changes at the system level can gain the development of new drug treatments for dementia.

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Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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