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Therapeutic effect of *Melissa officinalis* in an amyloid-β rat model of Alzheimer's disease

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ABSTRACT

Introduction: Medicinal herbs have several components with different pharmacological effects. It has been described that *Melissa officinalis* is able to improve memory in different models of learning. Nevertheless, its influence has not been studied in animal models of AD. Here, we studied the potential therapeutic effect of *M. officinalis* in intracerebroventricular (i.c.v) amyloid-β (Aβ) model of Alzheimer's disease (AD).

Methods: Male Wistar rats weighing 260-330 g received the hydro-alcoholic extract of *M. officinalis* (50, 100, 200, 400 mg/kg; P.O), chronically for 30 consecutive days. The control group received solvent of the drug. Memory retrieval was assessed, using the passive avoidance task. Three groups of the rats received Aβ (1-42; 10 μg/rat bilaterally; i.c.v). One group received DMSO 1% (2 μL/rat; i.c.v). Twenty days later memory retrieval was assessed. The Aβ-treated rats, received *M. officinalis* (50, 100 mg/kg; P.O) or saline (1 mL/kg; P.O), chronically for 30 consecutive days. The DMSO 1%-treated rats received saline (1 mL/kg; P.O).

Results: The hydro-alcoholic extract of *M. officinalis* (50, 100, 200, 400 mg/kg; P.O) did not have a significant effect on step-through latency (STL). Aβ impaired memory retrieval by decreasing STL and increasing the time spent in the dark compartment (TDC). *M. officinalis* (50, 100 mg/kg; P.O) improved memory retrieval in AD rats by increasing STL and decreasing TDC, significantly.

Conclusion: The outcomes of the study show that *M. officinalis* has a therapeutic effect in the Aβ model of AD. It seems that the extracts of *M. officinalis* can be suggested as a powerful therapeutic herb for AD patients.

Implication for health policy/practice/research/medical education:
Melissa officinalis has the potential to improve memory deficiency and might be advantageous in patients with Alzheimer's disease.

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Introduction

Alzheimer's disease (AD) is an enduring, progressive and irreversible neurodegenerative syndrome and is the central cause of dementia in later life. Two neuropathological features of AD are neuritic plaques and neurofibrillary tangles which are respectively related to the buildup of the amyloid-beta peptide (Aβ) in the brain, and to cytoskeletal alterations that appear due to the hyper-phosphorylation of tau protein in neurons. Aβ-peptide has a crucial impact to the pathogenesis of AD (1). Based on the amyloid theory, accretion of Aβ in the brain is the major impact driving AD pathogenesis. Other aspect of the disease progression, including creation of neurofibrillary tangles comprising tau protein, is suggested to result from an inequality between Aβ production and Aβ clearance. Aβ deposit leads to oxidative stress, mitochondrial abnormalities and depletion of cellular ATP, elevated intracellular calcium and excitotoxicity and induction of inflammatory responses. All these mechanisms result in synaptic dysfunction and cause neuronal loss through activation of apoptotic and necrotic cell death pathways (2). Intracerebroventricular (i.c.v) injection of Aβ molecules leads to the buildup of Aβ plaques in the brain tissue, oxidative stress and neurotoxicity, which results in impairment of memory characteristic of the AD (3). It has been commonly used as an animal model of AD (4-6). The step-through passive

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avoidance paradigm is a learning task that has been widely utilized to assess the involvement of various treatments in learning and memory (7).

Treatment of AD is a great clinical challenge. Despite all the advances in this field, there is not a complete curative strategy for patients with AD. Due to the multifaceted pathology of AD, novel therapeutic strategies aim to stop disease progress through different pathways (8). Medicinal herbs have numerous constituents with diverse pharmacological properties and may be effective in complex diseases like AD. Hence, several studies have been considered to evaluated therapeutic effects of herbal medicines in AD therapy (9). *Melissa officinalis* is a medicinal herb from Lamiaceae family. It has been consumed as a medicinal plant for more than 2000 years (10). It has putative anti-inflammatory, antioxidant, neuro-protective, binding capacity to cholinergic receptors and anti-cholinesterase activity. It is suggested to be effective in the hindrance of various neurological diseases accompanied with oxidative stress (11) and has the potential to provide a natural treatment for AD (12). Acute injection of *M. officinalis* modulated mood and memory performance in healthy young individuals. Meanwhile, no side effects or toxicities have been described with its use (13).

Melissa officinalis is traditionally used to improve memory, however, there are not any reports assessing its effect in animal models of AD. Due to the central role of Aβ in AD pathogenesis, here we investigated the potential therapeutic activity of chronic injections of the hydro-alcoholic extract of *M. officinalis* in the Aβ rat model of AD.

Materials and Methods

Chemicals

Ketamine and xylazine were purchased from Alfasan (the Netherland). Aβ (1-42) was purchased from Sigma (USA) and dissolved in DMSO 1%. Dried leaves of *M. officinalis* were a gift from Iraninoosh Company which gathered it from Kamfiruz, Fars province, Iran in 2014.

Animals

Adult male Wistar rats (260–330 g) were obtained from the breeding colony of Department of Biology, University of Isfahan. Rats were kept four per cage in a temperature (24±1°C) well-ordered room that was upheld on a 12:12 light cycle (light on at 07:00 AM). The rats had unrestricted access to food and water. After the surgery for cannula embedding, the rats were housed alone in standard cages. All experiments were performed in agreement with the guide for the care and use of laboratory animals (USA National Institute of Health publication No. 80-23, revised 1996) and were approved by the graduate studies committee of the Department of Biology, University of Isfahan.

Preparation of the hydro-alcoholic extract of *Melissa officinalis*

One hundred grams of dried leaves of *M. officinalis* were pulverized, using a mixing instrument (Molineux, France), then were soaked in 400 mL of 96% ethanol, while mixing in a stirrer. The solution was filtered and put in a dark glass after 24 hours. The remaining crude was again soaked in 400 mL of 70% ethanol for 24 hours. The solution was again filtered and mixed with the previous solution. The extract was then concentrated in a rotary evaporator and dried in an oven in 40°C for 72 hours. The resulting extract was dissolved in saline to a final concentration of 100 mg/mL. The animals received the extract through gavage.

Surgical procedures

Rats were anesthetized with a combination of ketamine (100 mg/kg, i.p) and xylazine (10 mg/kg, i.p) and were bilaterally embedded with guide cannula (22-gauge) targeted at site 1 mm dorsal to the lateral ventricles (anterior-posterior: −0.9 mm from bregma, midline: ±1.4 mm from midline, and dorsal-ventral: −2.5 mm from dura) based on the atlas of Paxinos and Watson (14). A screw was implanted into the skull and cannulas were fixed to it with dental cement. The cannulas were locked with stainless steel stylets smeared with mineral oil to avoid clogging with blood.

Microinjection procedure

Intracerebroventricular injections were done via guide cannula with needles (27-gauge) that were joined by polyethylene tubing (PE20, Stoelting) to a 2 µL Hamilton micro syringe. The injections (2 µL total volume) were conveyed over 4 minutes bilaterally (1 µL each side), and the injection needles (extending 1mm from the end of the guide cannula) were left in place an extra minute, then they were slowly withdrawn.

Passive avoidance task

The step-through passive avoidance task was executed to evaluate memory performance, as formerly described (15). Briefly, each rat was positioned in the white compartment of the PAT apparatus facing the sliding door. After 5 seconds the door was elevated. When the animal walked into the dark compartment with all four paws, the door was closed and the rat stayed there for 20 seconds. Then the animal was removed to be sited in a temporary cage. 30 minutes later, the rat was again positioned in the white compartment for 5 seconds, then the door was elevated to let the animal enter the dark compartment and after entrance, the door was shut, but this time a controlled electrical shock of 0.3 mA lasting for 1 second was delivered. After 20 seconds, the rat was sited into the temporary cage. Two minutes later, the same testing process was reiterated. When the rat stayed in the white

compartment for a 2-minute times period, the training was ended. On the second day, a retrieval test was done to assess long-term memory. Each animal was positioned in the white start compartment for 20 seconds, then the door was elevated and the step-through latency (STL) and the time spent in the dark compartment (TDC), were recorded, up to 600 seconds.

Experiment 1

Eight rats were used in each experimental group. In this experiment, the effect of chronic injection of different doses of the hydro-alcoholic extract of *M. officinalis* was assessed on memory performance. Five groups of animals received the hydro-alcoholic extract of *M. officinalis* (50, 100, 200 and 400 mg/kg; P.O) or Saline (1 mL/kg; P.O) for 30 consecutive days. Memory retrieval was assessed using a passive avoidance paradigm.

Experiment 2

In this experiment, the effect of i.c.v administration of Aβ was assessed on memory performance in rats. One group of animals received Aβ (10 μg/rat), bilaterally. The dose of Aβ was selected according to previous reports (16). The control group received DMSO 1%. Twenty days following the injection, memory retrieval was evaluated.

Experiment 3

In this experiment, the effect of the hydro-alcoholic extract of *M. officinalis* was assessed on memory performance in Aβ-treated rats. The animals were allocated into four groups. Group 1 received DMSO 1% (2 μL/rat; i.c.v). Groups 2, 3 and 4 received Aβ (10 μg/rat; i.c.v), bilaterally. Twenty days later, memory performance was assessed. Afterwards, groups 1 and 2 received saline (1 mL/kg; P.O) for 30 consecutive days. Groups 3 and 4 received the hydro-alcoholic extract of *M. officinalis* (50 or 100 mg/kg; P.O), respectively for 30 consecutive days. Memory performance was assessed again after completion of the treatment regimen (Figure 1).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison post-hoc test, unpaired or paired *t* tests were performed using GraphPad Prism version 5.04 for Windows. In all experiments, differences were considered statistically significant at the level of $P < 0.05$. The data are presented as mean ± standard error of the mean (SEM).

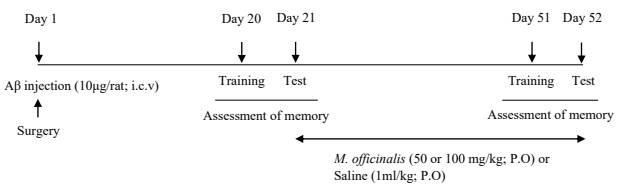

Figure 1. Diagram illustrating experimental design.

Results

Verification of cannulas placements

At the end of the experiments, each animal was euthanized with an overdose of chloroform. The brain was removed and fixed in a 10% formalin solution and sectioned 5 days later. Sections were inspected to determine the site of the cannulas targeted for the lateral ventricles. The cannula placements were confirmed using the atlas of Paxinos and Watson (14). Data from the animals with the injection sites placed outside the lateral ventricles were not used for the analysis.

The effect of the hydro-alcoholic extract of *Melissa officinalis* on memory retrieval

In the passive avoidance task, the decrease in STL and the increase in TDC indicate loss of fear memory. One-way ANOVA showed that the hydro-alcoholic extract of *M. officinalis* (50, 100, 200 and 400 mg/kg) did not have a significant effect on STL ($P > 0.05$; Figure 2).

The effect of Aβ on memory retrieval

Intracerebroventricular injection of Aβ impaired memory retrieval by decreasing STL and increasing TDC, significantly compared with the control group (Figure 3; $P < 0.05$).

The effect of the hydro-alcoholic extract of *Melissa officinalis* on memory retrieval in rats treated with Aβ

One-way ANOVA indicated a significant main effect of STL following chronic i.c.v injections of the hydro-alcoholic extract of *M. officinalis* in the Aβ-treated rats ($F [3, 31] = 5.21$; $P = 0.005$). Post hoc comparison showed that *M. officinalis* (50 and 100 mg/kg; P.O) significantly increased STL as compared to the group received Aβ-saline (Table 1; $P < 0.05$). Data analysis of this experiment

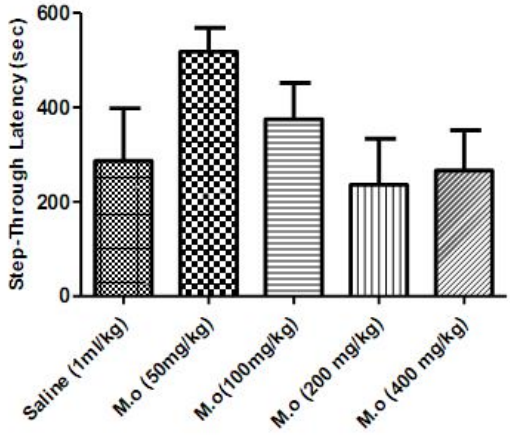

Figure 2. The effect of the hydro-alcoholic extract of *Melissa officinalis* on step-through latency (STL). Data are shown as means ±SEM and were analyzed by ANOVA.

also revealed main effect of TDC (F [3, 31] = 4.30; $P = 0.01$). Post hoc comparisons showed that *M. officinalis* (50 mg/kg; P.O), significantly decreased TDC in the Aβ-treated rats as compared to the group received Aβ-saline (Table 2; $P < 0.05$). Post-hoc comparisons also revealed that *M. officinalis* (100 mg/kg; P.O), significantly reduced TDC in the Aβ-treated rats as compared to the group received Aβ-saline (Table 2; $P < 0.01$).

Discussion

The main finding of the current study was that chronic consumption of the hydro-alcoholic extract of *M. officinalis* has a therapeutic effect in rats with AD induced by i.c.v injections of Aβ. In AD there is a gradual loss of cholinergic neurons in basal forebrain which has been suggested to cause the cognitive deficits observed in patients with AD (17). Drugs used for AD treatment are acetylcholinesterase inhibitors, which can improve cognitive impairment but cannot prevent disease progression (18). *M. officinalis* has a variety of components, of which the most important are phenolic compounds, rosmarinic acid, caffeic acid, cholinergic acid, metrilic acid; flavonoids such as luteolin, apigenin and monoterpene derivatives; the sesquiterpenes including beta-caryophyllene and germacrene; triterpenes such as oleanolic and ursolic acid; volatile oil, and tannins (19). Some of these compounds have binding capacity to nicotinic and muscarinic acetylcholine receptors and also inhibit the effects of acetylcholinesterase enzyme (20-22). Thus, by regulating the cholinergic system it might be helpful in Alzheimer's treatment and adjusting mood and cognitive processes. In *M. officinalis* extract the affinity for acetylcholine nicotinic receptor is more than muscarinic receptor (23). It was reported that nicotinic receptor stimulation, may be able to protect neurons from degeneration induced by Aβ and may have effects that stand the progress of AD.

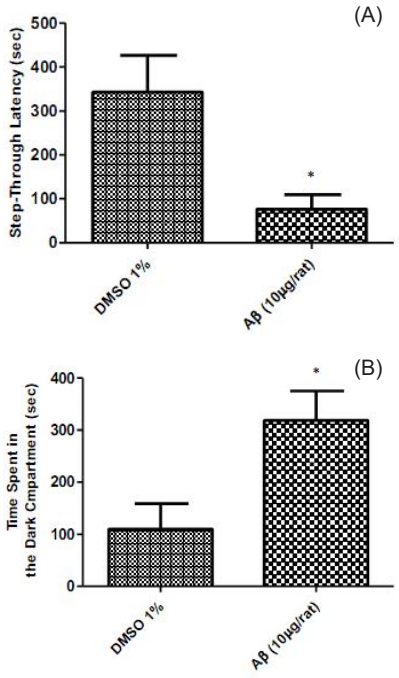

Figure 3. The effect of bilateral injection of Aβ (10 µg/rat; i.c.v) on (A) step-through latency (STL) and (B) TDC (the time spent in the dark compartment). Data are shown as means ± SEM and were analyzed by un-paired *t* test. *$P < 0.05$.

Oxidative stress is the mechanism that was assumed for Aβ toxicity and Alzheimer's etiology (24). Aβ is accumulated and creates more free radicals in the presence of free radicals. In this regard, *M. officinalis* total extract could attenuate the Aβ-induced toxicity and oxidative stress (22). Direct free radical scavenging activity has been reported for *M. officinalis*. The total extract could defend PC12 cells against hydrogen peroxide-induced cell death and oxidative stress (25). Antioxidants can ameliorate disease progression (26), so it is supposed that antioxidant activity of *M. officinalis* may have contributed to neuroprotective effect against Aβ toxicity.

Different biological activities have been designated for the major polyphenol component of *M. officinalis,* rosmarinic acid, including anti-oxidative and anti-inflammatory activities (27). Rosmarinic acid could protect against Aβ-induced memory impairment in mice, due to the direct

Table 1. The effect of *Melissa officinalis* on step-through latency (STL) in Aβ-treated rats

	21 days after surgery	52 days after surgery
	Aβ treatment of rats (single injection)	***M. officinalis* treatment of rats (30 days) treated with Aβ**
Group 1	DMSO 1% (2 µL/rat; i.c.v)	Saline (1 mL/kg; P.O)
	274±79.00	400±97.00
Group 2	Aβ (10 µg/rat; i.c.v)	Saline (1 mL/kg; P.O)
	51.28±15.00	66.50±20
Group 3	Aβ (10 µg/rat; i.c.v)	*M. officinalis* (50 mg/kg; P.O)
	76.33±29.41	365.70±93.23*
Group 4	Aβ (10 µg/rat; i.c.v)	*M. officinalis* (100 mg/kg; P.O)
	60.63±20.29	309.00±63.97*

Data are shown as means ± SEM and were analyzed by paired *t* test. * $P<0.05$.

Table 2. The effect of *Melissa officinalis* on TDC (the time spent in the dark compartment) in Aβ-treated rats

	21 days after surgery	52 days after surgery
	Aβ treatment of rats (single injection)	***M. officinalis* treatment of rats (30 days) treated with Aβ**
Group 1	DMSO 1% (2 µL/rat; i.c.v)	Saline (1 mL/kg; P.O)
	86.43±25.10	95±15.00
Group 2	Aβ (10 µg/rat; i.c.v)	Saline (1 mL/kg; P.O)
	231.10±40.00	250±49.00
Group 3	Aβ (10 µg/rat; i.c.v)	*M. officinalis* (50 mg/kg; P.O)
	228.40±38.16	79.56±40.90*
Group 4	Aβ (10 µg/rat; i.c.v)	*M. officinalis* (100 mg/kg; P.O)
	291.70±59.91	8.71± 2.63**

Data are shown as means ± SEM and were analyzed by paired *t* test. * $P<0.05$.

peroxynitrite scavenging activity (28). Rosmarinic acid protected mice against impairment of memory induced by i.c.v injection of Aβ (28). It could also lower Aβ depositions in ovariectomized rats and improve short-term spatial memory (29).

The caffeic acid and the ursolic acid found in *M. officinalis* were reported to have neuro-protective effects. Ursolic acid is a triterpenoid with hydroxyl radical scavenging activity and it augmented the activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (30). Caffeic acid improved Aβ-induced cognitive damage through inhibition of lipid peroxidation and NO production (31). Caffeic acid also exerted neuro-protective and anti-dementia effects by preventing the loss of neural cells and synapses in mice ischemic brain injury (32).

Another active component of *M. officinalis*, luteolin could improve Aβ-induced memory deficiency and increased the activity of choline acetyl transferase, superoxide dismutase and glutathione peroxidase in hippocampal tissue. Luteolin also inverted the augmented activity of acetylcholine esterase. In hippocampi homogenate, the amount of acetylcholine augmented, but malondialdehyde reduced. Moreover, luteolin could raise Bcl-2/Bax ratio. Luteolin could protect AD rats against Aβ-induced memory deficiency through regulating the cholinergic system and inhibiting oxidative damages (33). Luteolin also improved learning and memory in Aβ-induced cognition damage in rats by activating the anti-oxidation system (34). Luteolin reduced the impairment of passive avoidance memory induced by scopolamine by increasing the activities of the brain muscarinic and nicotinic receptors (35).

Apigenin, another component of *M. officinalis*, attenuated cognitive deterioration in diabetic rats via suppressing oxidative stress, nitric oxide and apoptotic cascades synthase pathway (36). Oral administration of apigenin for three month rescued learning deficits and improved memory retention in APP/PS1 double transgenic AD mice. Apigenin also affected APP processing and prevented Aβ load, the relief of Aβ deposition, and the decrease of insoluble Aβ levels. Moreover, apigenin showed superoxide anion scavenging effects and enhanced antioxidative enzyme activity of superoxide dismutase and glutathione peroxidase (37).

Oral administration of β-Caryophyllene, another compound found in *M. officinalis* prohibited cognitive deficiency in APP/PS1 mice, and this positive cognitive effect was concomitant with reduced β-amyloid load in the hippocampus and the cerebral cortex. Moreover, β-caryophyllene reduced astrogliosis and microglial activation and the levels of cyclooxygenase-2 protein and the mRNA levels of the tumor necrosis factor-α and interleukin-1β in the cerebral cortex (38).

Due to the above mentioned beneficial effects of the components of *M. officinalis* on improvement of memory, oxidative stress, inflammatory conditions, neuro-protection, anti-cholinesterase activities and Aβ clearance, it seems that *M. officinalis* can be suggested as a powerful therapeutic herb for AD patients.

In accordance with our results, *M. officinalis* enhanced spatial memory in the Morris water maze in streptozotocin-induced memory impairment in rats and reduced amyloid plaques (39). Also, in a clinical trial it could improve disease signs in patients with mild to moderate AD (40). Based on the outcomes of the current study and these studies, it appears that *M. officinalis* have components with a great potential to treat AD.

Conclusion

As a conclusion, the outcomes of the current study indicated potential therapeutic effects of the hydro-alcoholic extract of *M. officinalis* in the i.c.v Aβ model of AD. The anti-inflammatory, anti-oxidant, neuro-protective and anti-cholinesterase activities of the extract are proposed to be involved in the observed results. Further studies are requisite to clarify the mechanisms of this activity and identify the responsible components of the extract.

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Authors' contributions

SB contributed to the conception and design of the work, drafting and revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. BS contributed to conducting the study.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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