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Original Article

Melissa officinalis Alleviates Cognitive Impairments in a Mouse Model of Accelerated Aging; Possible Involvement of the Hippocampal Sirt-1/Nrf2/NF-κB Signaling Pathway

Ghasem Khazen[1,2], Fereshteh Farajdokht[1], Saeed Sadigh-Eteghad[1], Mahtab Rajabi-Jourshari[1], Zahra Sheikhalizadeh[1], Ahad Banagozar Mohammadi[2], Mostafa Araj-khodaei[3], Sahar Khazen[4], Javad Mahmoudi[1,5]*

[1]Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Persian Medicine, Faculty of Traditional Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Research Center for Integrative Medicine in Aging, Aging Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Biotechnology, Faculty of Biological Science, Alzahra University, Tehran, Iran
[5]Medical Philosophy and History Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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Abstract

Background: Aging is associated with a wide range of cognitive impairments that affect the quality of life in the elderly. This study aimed to assess the neuroprotective properties of the hydro-alcoholic extract of *Melissa officinalis L.* (HAEMO) on cognitive impairment mediated by the D-galactose/$AlCl_3$-induced accelerated aging model.

Methods: The C57BL/6 mice were allocated into young and aged control groups, and three aged groups received different doses of HAEMO for 60 days. The performance of mice was assessed using the Lashley III maze and the novel object recognition test to assess cognitive function. Hippocampal tissue was examined not only for oxidative stress indicators (TAC, MDA, GPx and SOD) but also used for estimations of Sirt-1, Nrf2, NF-κB, IL-6, and TNF-α proteins.

Results: The results indicated improved spatial and recognition memories in the HAEMO-received aged animals. The behavioral advantages were probably linked to diminished lipid peroxidation, increased antioxidant enzyme activities, and enhancement of the hippocampal Sirt-1/Nrf2 pathway. Moreover, the HAEMO regimen reduced inflammatory markers (NF-κB, TNF-α, and IL-6) in the hippocampus.

Conclusion: HAEMO exerts a modulatory effect on the hippocampal Sirt-1/Nrf2/NF-κB pathway, offering a neuroprotective approach against progressive oxidative stress and neuroinflammation that develop with aging. Additional research is necessary to completely understand the therapeutic potential of HAEMO in the preservation of cognition during aging.

Introduction

Aging is an unavoidable biological phenomenon marked by progressive deteriorations in physical and mental capacities.[1,2] It remains a major risk factor for age-related conditions such as neoplastic, neurological, and metabolic disorders. These conditions often cause functional deficits that impair daily life, putting physical, emotional, and financial strain on family members and caregivers.[3]

Aging-induced cognitive deficits are multifactorial and can be attributed to various events, including inflammation, mitochondrial failure, oxidative stress, the buildup of misfolded or aggregated molecules like amyloid beta, synaptic dysfunction, and neuronal loss.[4-6] In aging, the brain undergoes structural and functional changes, specifically in areas responsible for cognitive processes, such as the hippocampus and prefrontal cortex, which negatively impact cognitive capacities.[7,8] Several studies have demonstrated that memory deficits resembling normal aging are the result of a deteriorated antioxidant system and increased oxidative stress and inflammatory responses, while cognitive performance can be enhanced by the overexpression of antioxidants.[9-12]

High energy demands, impaired mitochondrial function, and declined antioxidant enzyme effectiveness in aging brains increase the amount of both reactive nitrogen and oxygen species, which surpass the brain's capacity to efficiently eliminate these harmful active compounds.[13-16] Moreover, the progressive deposition of defective biological molecules and cells in senescence may promote microglial activation and subsequent neuroinflammatory reactions.[17] The nuclear factor erythroid 2-related factor 2 (Nrf2) protein, as a cellular sensor of oxidative stress, in conjunction with sirtuin 1 (Sirt-1), forms the Nrf2/Sirt-1 pathway, a regulatory mechanism that contributes to the

***Corresponding Author:** Javad Mahmoudi, Emails: mahmoudi2044@gmail.com, Mahmoudij@tbzmed.ac.ir

response to both oxidative stress and inflammation.[18,19] Upon activation, Nrf2 traffics to the nucleus to enhance the cellular antioxidant capacity, partly by regulating the transcription of heme oxygenase 1 and superoxide dismutase (SOD) genes.[20]

Sirt-1, an NAD^+-dependent deacetylase, affects the cellular redox balance by deacetylation of Nrf2, hence enhancing its transcriptional activity.[21,22] Moreover, Sirt-1 interacts with the nuclear factor kappa B (NF-κB) and modifies its acetylation level, thereby inhibiting the transcription of genes responsible for the regulation of inflammatory cytokines.[23,24] Evidence indicates that the Sirt-1/Nrf2 pathway is negatively affected by aging, leading to elevated oxidative stress and neuroinflammation, as well as age-associated mitochondrial dysfunction, ultimately contributing to diminished cognitive function.[25-27] Hence, promoting the activity of this pathway holds great potential as a highly promising approach in the management of cognitive deficits.

The toxicity mediated by the concomitant administration of the aldohexose sugar D-galactose and aluminum chloride (D-gal/$AlCl_3$) speeds up the aging process, profoundly hinders learning and memory, and serves as a valuable model for the investigation of potential treatments for aging-associated memory deficits.[28-30]

Recently, natural products with potential neuroprotective properties have attracted increasing attention for the management of age-related conditions, particularly those possessing both antioxidant and anti-inflammatory capacities.[31] *Melissa officinalis* L. has been recognized as a routine remedy for more than 2,000 years, primarily in herbal medicine literature for its therapeutic benefits. Ancient figures like Theophrastus, Pliny the Elder, and Hippocrates have endorsed its therapeutic properties, valuing it as a beneficial herbal remedy.[32] In traditional Persian medicine, it is known as Badranjboya and has been recommended by some famous traditional Iranian physicians, including Avicenna (Ibn Sina), Razes, and Aghili Khorasani, as a remedy for treating insults affecting the central nervous system and peripheral organs (i.e., depression, psychosis, obsession, and palpitation).[33-35] Consistent with historical evidence, recent studies have also established that *M. officinalis* may have tranquilizing, anti-anxiety, and cognitive-enhancing effects.[36,37]

Basically, the capacity of *M. officinalis* extract to enhance learning and memory is most likely due to the synergistic effects of rosmarinic acid, phenolic acids, flavonoids, and terpenoids. These chemicals collectively improve neuronal survival and plasticity, modify cholinergic neurotransmission, and decrease oxidative stress and neuroinflammation.[38-40] Preclinical research has also shown that *M. officinalis* L. targets the hippocampal Nrf2/HO-1 pathway to enhance neurotrophic factor and reduce the burden of oxidative stress and inflammatory reactions.[36,41] In light of this, *M. officinalis* L. may have great potential for developing therapeutic approaches aimed at preserving cognitive function during the aging process.

This study aimed to investigate whether the hydro-alcoholic extract of *M. officinalis* L. (HAEMO) may improve learning and memory function, as well as the associated molecular pathways, in a D-gal/AlCl3-mediated aging mouse model.

Methods

Animals and study design

A total of fifty C57BL/6 mice (Male, 26-28 g) were used in this study. They were kept under standard conditions (24 ± 2 °C and a photoperiod of 12 hours of light per day) with unrestricted access to tap water and conventional rodent pellets. All of the experimental procedures were conducted in accordance with the guidelines published by the National Institutes of Health (No. 85-23, amended 1985) and received approval from the regional Committee (Approval No. IR.TBZMED.AEC.1402.008).

After acclimating to the laboratory setting for seven days, the mice were allocated into five groups (n = 10 in each): I) Young control, II) Aged control, III) Aged + M50, IV) Aged + M75, and V) Aged + M150. A combination of subcutaneous (s.c.) D-gal (60 mg/kg, Daejung Chemicals & Metals Co., Ltd., Gyeonggi-do, Korea) and intragastric gavage (p.o.) of $AlCl_3$ (200 mg/kg, Sigma Chemical Co., St. Louis, USA) was administered once daily for 60 consecutive days in order to model an aging process. The mice in group I (young control) received normal saline instead of D-gal/$AlCl_3$ for 60 successive days via the same routes described for D-gal (s.c.) and $AlCl_3$ (p.o.).[42]

For treatment, the animals in groups I and II received distilled water for 60 days and groups III-V received the HAEMO regimen at doses of 50, 75, and 150 mg/kg (Figure 1). All solutions were administered at the constant volume of 0.2 ml/mice via gastric gavage. Moreover, the extract was given 60 min before D-gal/$AlCl_3$ administration. The standardized HAEMO extract (containing 5% rosmarinic acid), prepared from plant material collected in Iran's East Azerbaijan province (herbarium number: Fph-Tbz 4031) and previously standardized in our laboratory, was used as the treatment in this study.[37]

Behavioral analysis

Behavioral assessments were conducted using a ceiling-mounted camera for video recording, and subsequent analysis was performed using a computer-assisted tracking system (Noldus EthoVision® 11.5, Netherlands). All experimental procedures were conducted by an investigator who was unaware of the group allocations and treatment conditions in order to reduce the potential for bias.

Novel object recognition (NORT)

The three-phase NORT protocol, with each phase lasting 5 minutes, was conducted to assess the mice's instinctive proclivity for novelty. On day 1, during the habituation

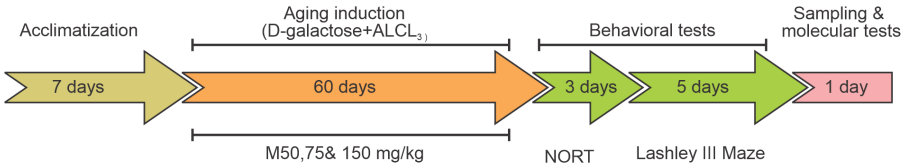

Figure 1. Timeline of study. M50, M75, and M150: 50, 75, and 150 mg/kg of hydro-alcoholic extract of *Melissa officinalis*. NORT: novel object recognition test

phase, the mice were gently located at the center of the test apparatus without any objects and allowed to explore the entire arena of the apparatus. The protocol continued on the second day for familiarization, during which two identical objects (A1 and A2) were positioned 10 cm apart, and the animals were allowed to freely explore the objects. On the final day, the testing phase began by replacing one of the familiar objects with a new one, and the mice were allowed to explore the objects. Finally, the discrimination index (DI) was obtained as below: $DI = (T_{novel} - T_{familiar}) / (T_{novel} + T_{familiar})$. T_{novel} and $T_{familiar}$ represent the time spent exploring the novel and familiar objects, respectively.[43]

Lashley III Maze

A five-day protocol was used with a white Plexiglas® Lashley III Maze. The maze comprises three main elements, including four interconnected runways, a starting box, and a goal box. The maze is covered with a transparent top lid to prevent the rodent from escaping during the test. The rodents were motivated by placing a food reward in the goal box prior to each session. Subsequently, the animals were placed inside the start box for nearly 10 seconds, after which the partition door separating the start box from the initial runway was raised, permitting the mice to traverse and investigate the runways for 5 minutes. The latency to access the goal box and the number of committed errors were both recorded during this period. The mice were manually guided toward the goal box if they were unable to complete the assignment within 5 minutes, and their latency was recorded as 5 minutes.[43]

Tissue sampling

Immediately after the cognitive tasks, the mice received an intraperitoneal injection of a xylazine HCl and ketamine HCl mixture (10 and 80 mg/kg, respectively). Sampling was then performed following the recently introduced technique for the rapid extraction of brain tissue, with modifications made to adapt the method for use in mice.[44] Then, the hippocampal samples were carefully isolated and were cryopreserved at -80 ˚C for molecular analysis.

Oxidative stress markers

Once the supernatants of the specimens were obtained following homogenization in a 1.15% KCl solution and subsequent centrifugation (1,000 rpm for 10 minutes at 4 ˚C), then the quantities of proteins were measured by the Bradford assay kit (DB0017, DNAbioTech, Tehran, Iran) and the samples were used for the following assays:

Malondialdehyde (MDA) levels, which serve as a biomarker of lipid peroxidation, were analyzed using the thiobarbituric acid reactive substances (TBARS) test. The reaction between 2-thiobarbituric acid and MDA produced a red-pink complex, which was quantified by spectrophotometry at 532 nm.

The total antioxidant capacity (TAC) was assessed by quantifying the sample's capacity to convert ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}). The reduction procedure yields a blue complex formed by Fe^{2+} and 2, 4, 6-Tri (2-pyridyl)-s-triazine. The complex was then spectrophotometrically measured at 593 nm.

The commercial spectrophotometric kits, RANSEL and RANSOD (both from Randox Laboratories Ltd., Crumlin, United Kingdom), were employed to quantify the enzymatic activity of glutathione Peroxidase (GPx) and SOD at wavelengths of 340 nm and 505 nm, respectively.

Western blotting

RIPA buffer enhanced with a protease inhibitor cocktail was employed to lyse frozen tissues. The prepared lysates were centrifuged (12,000 g for 15 minutes at 4 ˚C) and the quantities of proteins were estimated using the Bradford assay kit (DB0017, DNAbioTech, Tehran, Iran) following the directions supplied by the manufacturer. A 12.5% polyacrylamide gel was used to run 20 µg of each sample using SDS-PAGE, and then the protein was transferred to a 0.2 µm PVDF membrane (Bio-Rad Immune-BlotTM, Bio-Rad Laboratories, California, USA). Once the blocking procedure was finished, the membranes were then incubated (12 hours, at 4 ˚C) with primary antibodies (including anti-Nrf2 [1:1000; Cat No. 12721S, Cell Signaling, Leiden, The Netherlands], anti-SIRT-1 [1:1000; Cat No. ab189494, Abcam, Massachusetts, USA], anti-NF-κB-p65 [0.5 µg/ml; Cat No. ab16502, Abcam], anti-pNF-κB-p65 [1:2000; Cat No. ab264271, Abcam], anti-IL-6 [1:1000; Cat No. ab259341, Abcam], anti-TNF-α [1:1000; Cat No. ab183218, Abcam], and anti-β-actin [1:5000, Cat No. ab8227, Abcam] as an internal reference control). Following three rounds of TBST (Tris buffered saline + 0.1% Tween 20) washing, the membranes were incubated for one hour at room temperature with a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG H&L, 1:10000; Cat No. ab6721, Abcam). Signal detection was accomplished by employing ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), which were then observed on radiography film (Kodak, New York, USA) with an exposure time of 30 s. The publicly available ImageJ software (version 1.62, NIH, Bethesda, MD, USA)

was used for analysis of the protein bands.

Statistical analysis

The latency to complete the task and the number of errors committed during the test days in the Lashley III maze were analyzed using a two-way analysis of variance (ANOVA). Data obtained from the testing phase of the NORT and from the biochemical assays were analyzed by one-way ANOVA. Post hoc Tukey's tests were conducted using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, California, USA). In addition, a paired Student's t-test was used to analyze the time spent exploring each object during the familiarization phase of the NORT. Results are expressed as mean ± SEM, with differences considered statistically significant at $p < 0.05$. All data satisfied the normality criterion as verified by the Kolmogorov-Smirnov or Shapiro-Wilk tests.

Results

The Lashley III task

The two-way ANOVA repeated measures showed not only significant effects for group ($F_{(4, 125)} = 174.9$, $P < 0.001$), but also for day ($F_{(4, 125)} = 21.32$, $P < 0.001$) regarding the latency to access the target box. However, their interaction (group × day) was not significant ($F_{(16, 125)} = 1.095$, $p = 0.3667$). Additionally, significant effects of group ($F_{(4, 125)} = 169.7$, $P < 0.0001$) and day ($F_{(4, 125)} = 19.08$, $P < 0.0001$) were observed for number of committed errors. However, their interaction remained nonsignificant ($F_{(16, 125)} = 1.079$, $P = 0.3821$). Subsequent intergroup analysis revealed that the time taken to access the target box (Figure 2A) and average number of committed errors (Figure 2B) were significantly greater in the aged animals during 2nd-5th days of training (at least $P < 0.05$) when compared to the mice in young group, which indicated the impact of D-galactose/$AlCl_3$ to render spatial memory dysfunction. Administration of HAEMO (75 mg/kg) decreased latency on the day 5 of training ($P < 0.05$) and lowered the number of errors on the days 4 and 5 (at least $P < 0.05$) as compared to the mice in aged group. Additionally, the Aged + M150 group reached the target box earlier and made fewer errors than the aged control mice during the 3rd to 5th days of the training phase (at least $P < 0.05$ for both parameters).

The NORT

The study groups exhibited no notable differences in motor activity during the habituation session ($P > 0.05$, data not presented). The paired student's t-test revealed

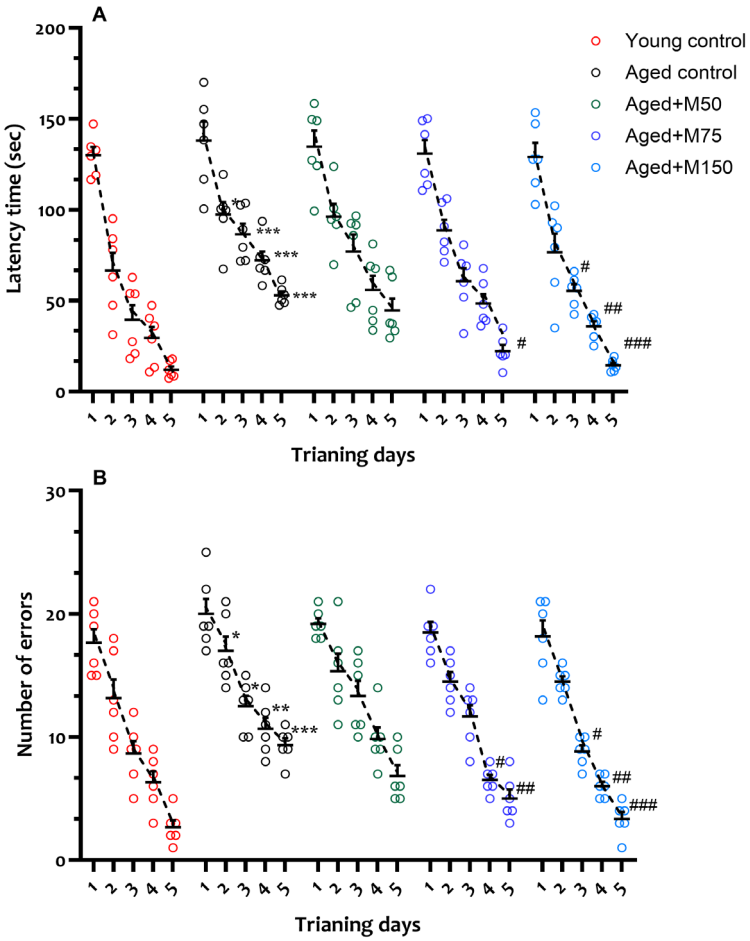

Figure 2. The effect of different doses of hydro-alcoholic extract of *Melissa officinalis* on (A) latency time and (B) number of errors in Lashley III Maze. Data are presented as mean ± SEM (n = 6). Two-way ANOVA, followed by Tukey post-hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. young control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. aged control group. [M50, M75, and M150: 50, 75, and 150 mg/kg of hydro-alcoholic extract of *Melissa officinalis*]

that the mean exploration times for objects A1 and A2 remained consistent throughout the familiarization session, suggesting a lack of object preference (Figure 3A). Also, in the test session, only the mice in the young and Aged + M150 groups exhibited longer time for exploring the unfamiliar object compared to the familiar one (Figure 3B, $P<0.01$). Moreover, one-way ANOVA analysis of DI revealed a significant difference among study groups ($F_{(4, 25)}=4.511$, $P=0.007$). Also, mice in the aged control group had a significant lower DI value (Figure 3C, $P<0.05$) than the young mice. Moreover, HAEMO at dose of 150 mg/kg significantly improved DI in the aged mice ($P<0.05$).

Hippocampal oxidative stress

There were substantial differences in oxidative stress biomarkers across the study groups. The amount of MDA ($F_{(4, 25)}=7.878$, $P=0.0003$) as well as SOD ($F_{(4, 25)}=12.48$, $P<0.0001$) and GPx ($F_{(4, 25)}=6.592$, $P=0.0009$) activities, and TAC ($F_{(4, 25)}=44.83$, $P<0.0001$) showed significant variations in the study groups.

As illustrated in Figure 4, the administration of D-gal/$ALCL_3$ could significantly increase MDA levels ($P<0.001$, Figure 4A) and lowered SOD ($P<0.001$, Figure 4B) and GPx activities ($P<0.001$, Figure 4C) as well as TAC ($P<0.001$, Figure 4D) relative to the mice in young control group. Conversely, 150 mg/kg of HAEMO significantly reduced MDA levels while enhancing the antioxidant activities of both SOD ($P<0.01$) and GPx ($P<0.01$) in aged mice. Furthermore, HAEMO at doses of 75 and 150 mg/kg resulted in significant increases in TAC levels ($P<0.01$ for both doses) compared to the aged control group.

hippocampal Sirt-1/Nrf2/NF-κB pathway

A one-way ANOVA of the immunoblotting results revealed significant differences in the hippocampal levels of Sirt-1 ($F_{(4, 10)}=12.16$, $P=0.0007$), Nrf2 ($F_{(4, 10)}=7.523$, $P=0.0046$), the ratio of p-NF-κB/ NF-κB ($F_{(4, 10)}=24.02$, $P<0.0001$), TNF-α ($F_{(4, 10)}=44.52$, $P<0.0001$), and IL-6 ($F_{(4, 10)}=25.12$, $P<0.0001$) proteins across the groups. Induction of aging by D-galactose/$AlCl_3$ significantly down-regulated both Sirt-1 and Nrf2 levels relative to the young control group. In contrast, the aging process resulted in an increased ratio of p-NF-κB/NF-κB and elevated levels of TNF-α and IL-6, reflecting the effect of induced model for development of neuroinflammtotion. However, treatment with HAEMO at a dosage of 150 mg/kg markedly increased the levels of Sirt-1 ($P<0.05$,

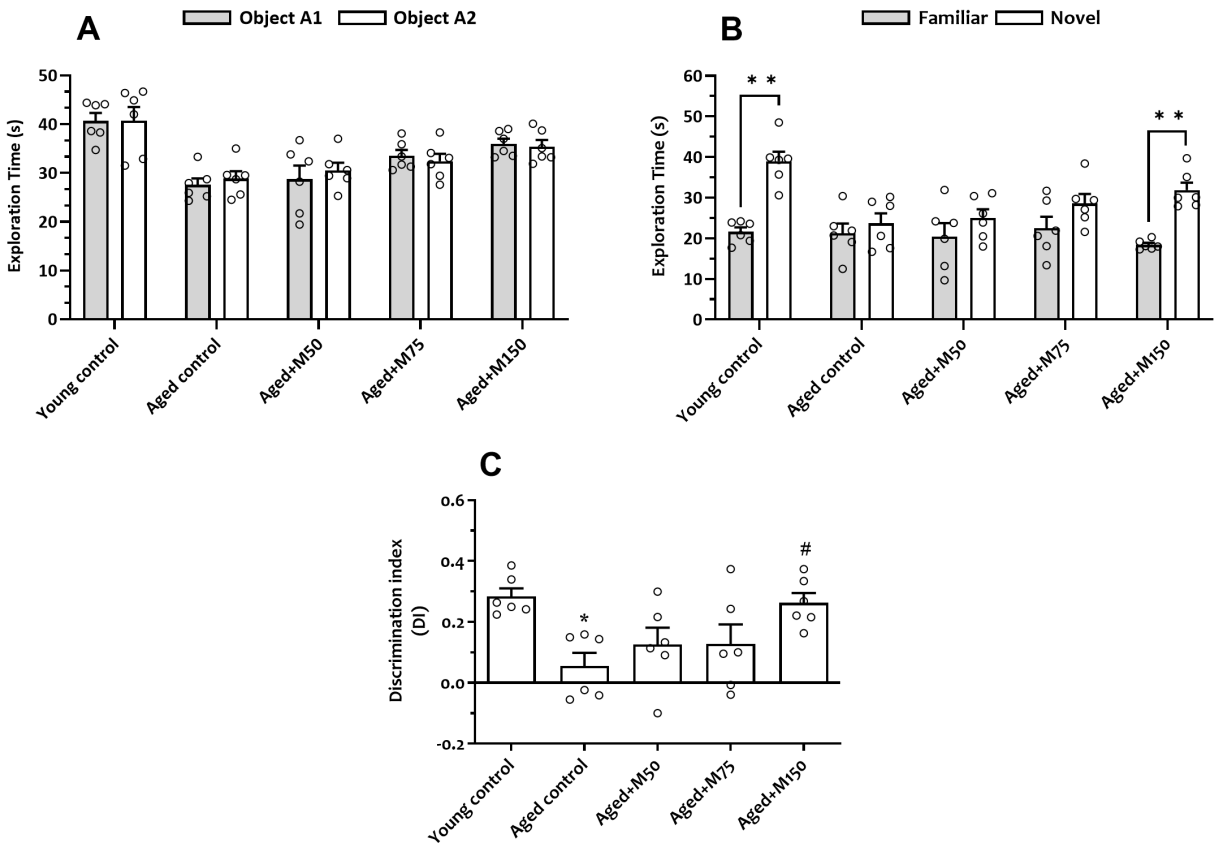

Figure 3. Effects of different doses of hydro-alcoholic extract of *Melissa officinalis* on recognition memory. (A) Exploration time of two similar objects in the habituation phase, (B) exploration time of familiar or novel objects in the testing phase of the NOR test. $^{**}P<0.01$ shows comparisons between familiar and novel objects. Paired Student's t-test. (C) Discrimination index among study groups. One-way ANOVA, followed by Tukey post-hoc test. $^{*}P<0.05$ vs. young control group, $^{\#}P<0.05$ vs. aged control group. Values are represented as mean ± SEM (n = 6). [M50, M75, and M150: 50, 75 and 150 mg/kg of hydro-alcoholic extract of *Melissa officinalis*]

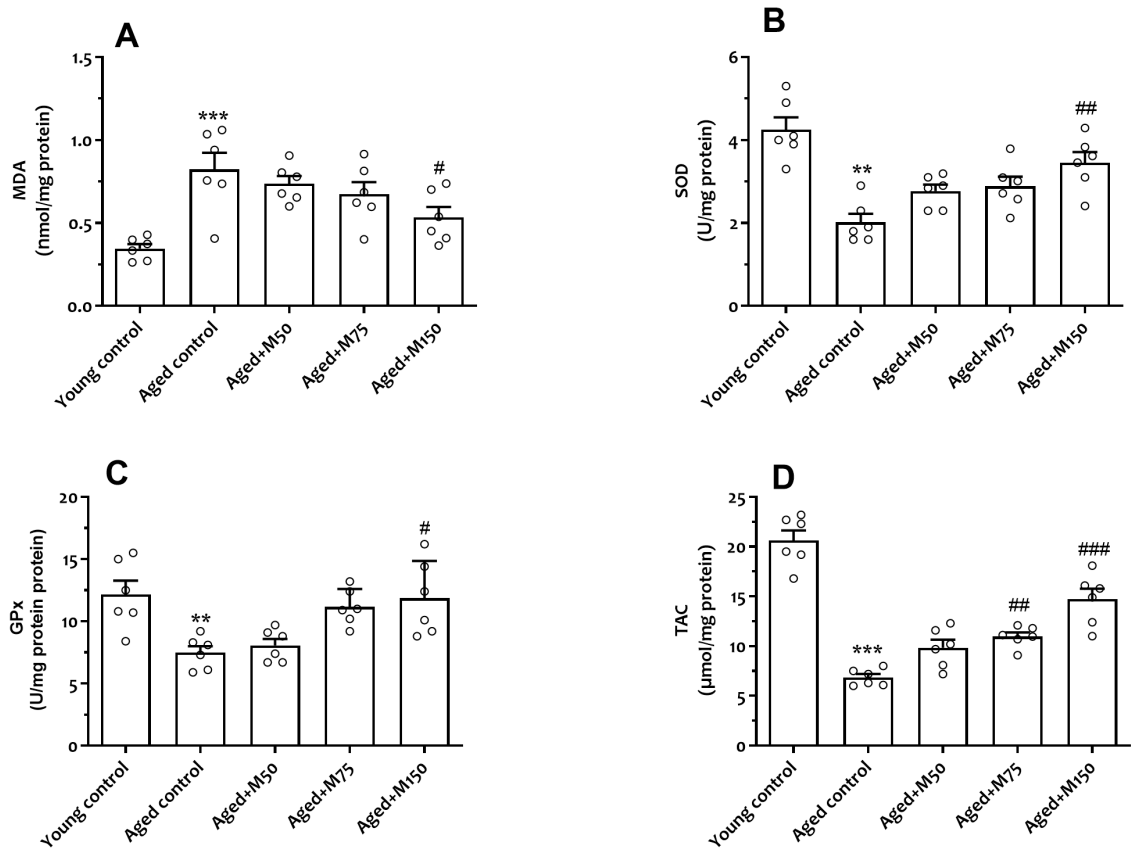

Figure 4. Effects of hydro-alcoholic extract of Melissa *officinalis* on (A) malondialdehyde (MDA) levels, (B) superoxide dismutase (SOD) activity, (c) glutathione peroxidase (GPx) activity, and (D) total antioxidant capacity (TAC) in the hippocampus of study groups. Data are presented as mean ± SEM. (n = 6). One-way ANOVA, followed by Tukey post-hoc test. $^{**}P<0.01$ and $^{***}P<0.001$ vs. young control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. aged control group. [M50, M75, and M150: 50, 75, and 150 mg/kg of hydro-alcoholic extract of *Melissa officinalis*]

Figure 5A and F) and Nrf2 ($P<0.05$, Figure 5B and F), compared to the aged control group. Moreover, all doses of HAEMO significantly reduced the ratio of p-NF-κB/NF-κB (at least $P<0.05$ for all doses; Figure 5C and F). Notably, only the HAEMO treatment at 150 mg/kg effectively decreased TNF-α ($P<0.05$; Figure 5D and F) and IL-6 ($P<0.001$; Figure 5E and F) levels in the hippocampus when compared to the aged control group.

Discussion

Our findings provide convincing evidence that HAEMO can improve cognitive deterioration in a mouse model of artificial aging, probably via tuning of oxidative stress and inflammatory reactions in the hippocampus.

The mechanism of cognitive decline in aging is multifaceted, comprising a complex combination of multiple cellular pathways, resulting in neurotoxicity and diminished cognitive function.[17,45] The aging process is often associated with excessive buildup of free radicals and diminished antioxidant defenses.[46] Furthermore, it is linked to inflammaging, a condition characterized by persistent low-level inflammation marked by elevated pro-inflammatory cytokines, establishing an unfavorable environment for neurons, leading to a deterioration in cognitive function.[47] Additionally, neuroinflammation and oxidative stress are closely linked mechanisms that have a substantial effect on cognitive malfunction in the elderly.[48] These two elements often work in tandem and establish a vicious cycle, causing neuronal damage through mechanisms like excitotoxicity, synaptic loss, and mitochondrial malfunction. These events impede synaptic transmission and plasticity, which are crucial for cognitive abilities.[10,48,49]

Long-term D-gal and $AlCl_3$ administration have been proven to speed up the emergence of age-related symptoms such as cognitive decline compared to natural aging, enabling researchers to investigate age-related consequences in a shorter time frame.[30,50] D-gal/$AlCl_3$ can induce a range of degenerative alterations in cells that mimic aging and neurodegenerative disorders, including aberrant cholinergic system function, neurological inflammation, oxidative damage, and amyloid-beta buildup, all of which contribute to the progressive loss of neurological function.[50,51] Our findings proved that D-gal/$AlCl_3$ regimen led to significant impairments in learning and memory. Our results are consistent with prior studies[52,53] that reported similar cognitive impairments following D-gal treatment. In contrast, the administration of HAEMO enhanced learning and memory capabilities in the Lashley III maze and NOR tests. These outcomes are aligned with previous preclinical and clinical studies highlighting the cognitive-enhancing effects of HAEMO

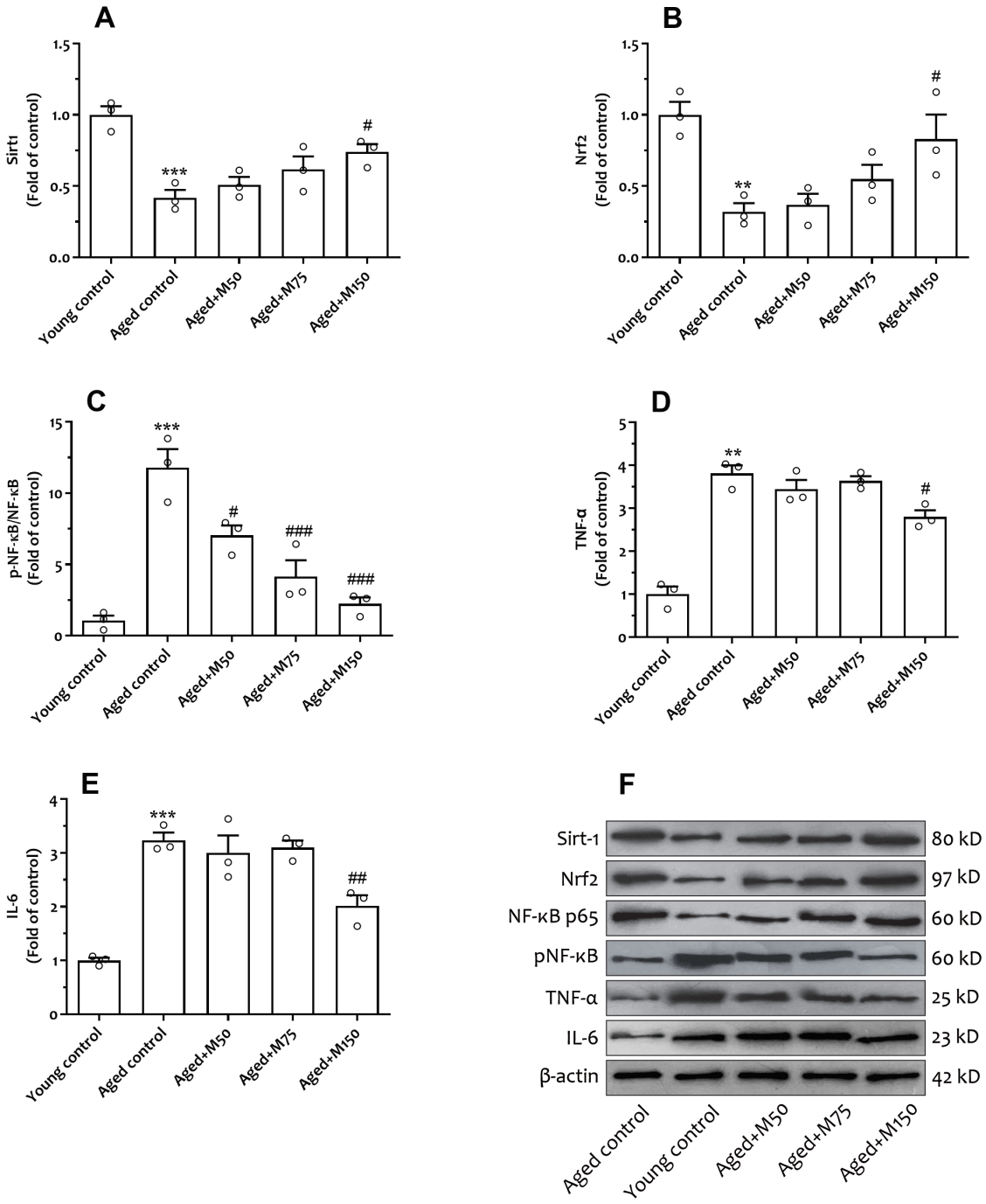

Figure 5. The Effects of hydro-alcoholic extract of Melissa *officinalis* on hippocampal protein levels of (A) Sirt-1, (B) Nrf2, (C) p-NF-κB/NF-κB, (D) TNF-α, and (E) IL-6 (F) Representative images of protein bands detected by Western blotting. Values are shown as mean ± SEM. (n = 3). One-way ANOVA, followed by Tukey post-hoc test. $^{**}P<0.01$ and $^{***}P<0.001$ vs. young control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. aged control group. [M50, M75, and M150: 50, 75, and 150 mg/kg of hydro-alcoholic extract of *Melissa officinalis*]

in Alzheimer's disease[54-56] and diabetes[36] animal models.

Combining D-gal with $AlCl_3$ causes oxidative stress and neuroinflammation through a diversity of interconnected processes. An excessive level of D-gal causes galactose oxidase to oxidize into aldose and hydrogen peroxide, leading to an abnormal increase in the production of free radicals and a reduction in the capacity to neutralize them. Concurrently, $AlCl_3$ disrupts the electron transport chain of mitochondria, enhances lipid peroxidation, and inhibits critical antioxidant enzymes, including SOD, catalase, and GPx.[57,58] Moreover, $AlCl_3$ undermines the blood-brain barrier integrity, which increases the cerebral penetration of D-gal and multiplies its neurotoxic effects.[59] Similar to previous reports,[60-62] we identified increased levels of lipid peroxidation and reduced activities of critical antioxidant enzymes in the hippocampus of the aged animals. These alterations suggest that the antioxidant defense mechanism is weakened, which causes an imbalance between free radical production and neutralization. In contrast, HAEMO-treated animals showed a significant

improvement in oxidative stress markers. These effects highlight the potential of HAEMO to bolster the brain's antioxidant defenses, thereby mitigating oxidative stress in the aging model. Rosmarinic acid, the predominant and bioactive compound in HAEMO, displays strong antioxidant capabilities by neutralizing free radicals and chelating metal ions, while also exhibiting notable anti-inflammatory effects mediated partly via inhibition of cytokines. The extract also includes several flavonoids and phenolic acids that synergistically enhance its antioxidant capacity and exhibit neuroprotective characteristics.[38-40,63]

Moreover, oxidative stress can directly promote inflammatory reactions by activating redox-sensitive transcription machineries such as NF-κB, resulting in pro-inflammatory gene expression and inflammatory mediator release from activated microglia and astrocytes.[64] This inflammatory response worsens oxidative stress, leading to the initiation of a vicious cycle that finally contributes to cognitive dysfunction in the aging brain.[65,66] In this study, we also found upregulated levels of p-NF-κB/NF-κB, as well as TNF-α and IL-6, in the hippocampus of aged mice. However, HAEMO decreased the mentioned items. Based on these findings, HAEMO may prevent cognitive deterioration in aged mice by disrupting the vicious cycle formed by the interplay of oxidative stress and inflammation in the hippocampus. In line with our results, previous studies supported the antioxidant and anti-inflammatory effects of HAEMO.[37,40,41]

Besides, this study showed down-regulation of both Sirt-1 and Nrf2 proteins in the hippocampus of the aged animals. These results were in agreement with prior research that suggests the Sirt-1/Nrf2 pathway is impaired by aging.[15,26,27] However, treatment with HAEMO markedly increased the levels of both Nrf2 and Sirt-1, suggesting that HAEMO can activate the Sirt-1/Nrf2 pathway. These findings were associated with improved antioxidant defenses, reduced inflammation, and better cognitive performance in the aged animals. Additionally, a study revealed that two weeks of HAEMO treatment decreased inflammatory and oxidative stress markers by up-regulating Nrf2 mRNA in the hippocampal regions of pilocarpine-received rats.[41] Another study revealed that HAEMO extract mitigated redox imbalance and inflammation linked to hyperthyroidism-induced hepatic injury via augmenting Nrf2 activity.[67] Contrary to the fact that there is no direct evidence of HAEMO's effect on Sirt-1, rosmarinic acid, its main constituent, has been shown to have anti-inflammatory and antioxidant benefits through modulation of the Sirt-1/Nrf2 pathway.[68,69]

There is an increasing body of research that indicates that both natural and synthetic compounds can affect the Sirt-1/Nrf2/NF-κB pathway and offer neuroprotective benefits in a variety of models of cognitive impairment and neurodegeneration.[70-73] For instance, resveratrol, a polyphenol present in grapes, has been demonstrated to activate Sirt-1 and Nrf2, thereby enhancing antioxidant defenses and reducing neuroinflammation, thereby enhancing cognitive function in aging models.[74-76] Curcumin, the primary active component of turmeric, also augments Nrf2 activity and suppresses NF-κB signaling, thereby enhancing its antioxidant and anti-inflammatory properties that promote neuroprotection.[77-79] Similarly, metformin, a prevalent antidiabetic medication, has been reported to activate Sirt-1 and Nrf2 while suppressing NF-κB, resulting in enhanced mitochondrial function, reduced oxidative stress, and superior cognitive outcomes in animals.[80,81] Additionally, bioactive substances like quercetin[82,83] and sulforaphane [84,85] affect Sirt-1/Nrf2/NF-κB signaling, promoting neuroprotection and reducing cognitive impairments. These findings emphasize the importance of the Sirt-1/Nrf2/NF-κB axis in neuronal health and preventing age-related cognitive decline. Our study confirms that HAEMO protects the brain through this pathway, adding to the growing body of evidence that addressing these molecular pathways can prevent and treat cognitive decline.

There are some limitations to our study that warrant consideration. First, our experiments were conducted exclusively in male mice. Our findings are restricted in their broader applicability due to the well-established disparities in neurobiological processes and treatment responses between males and females. Future research including both sexes will be essential to determine whether the neuroprotective effects of HAEMO observed here extend to female subjects as well. Second, the duration of HAEMO administration was limited to 60 days. While this timeframe was adequate for modeling accelerated aging and detecting significant cognitive and molecular changes, it does not fully capture the prolonged and gradual nature of aging in humans. Extended studies will be necessary to assess the long-term efficacy and safety of HAEMO as a preventive intervention. Lastly, our study was designed to evaluate the preventive, rather than therapeutic, potential of HAEMO. By initiating treatment before the onset of aging-related impairments, we cannot draw conclusions about the extract's effectiveness in reversing established cognitive deficits. Future investigations should address both preventive and therapeutic paradigms, as well as explore different dosing strategies, to more fully elucidate the translational potential of HAEMO in mitigating age-related cognitive decline.

Conclusion

This study underscored the potential of HAEMO for age-related cognitive deterioration. The extract could improve cognitive performance by diminishing oxidative stress and inflammation, primarily by activating the Sirt-1/Nrf2 pathway. Further detailed research is required to identify the exact mechanisms that that may be involved in the long-term effects of HAEMO on cognitive function in aging.

Authors' Contribution

Conceptualization: Fereshteh Farajdokht, Saeed Sadigh-Eteghad,

Javad Mahmoudi.
Data curation: Zahra Sheikhalizadeh, Mostafa Araj-khodaei, Sahar Khazen.
Formal analysis: Saeed Sadigh-Eteghad, Mostafa Araj-khodaei.
Funding acquisition: Javad Mahmoudi.
Investigation: Ghasem Khazen, Mahtab Rajabi-Jourshari, Zahra Sheikhalizadeh, Saeed Sadigh-Eteghad, Mostafa Araj-khodaei.
Methodology: Ghasem Khazen, Ahad Banagozar Mohammadi.
Project administration: Saeed Sadigh-Eteghad, Fereshteh Farajdokht
Resources: Ghasem Khazen, Javad Mahmoudi.
Software: Mostafa Araj-khodaei, Mahtab Rajabi-Jourshari, Zahra Sheikhalizadeh.
Supervision: Ahad Banagozar Mohammadi, Javad Mahmoudi.
Validation: Fereshteh Farajdokht, Mostafa Araj-khodaei
Visualization: Fereshteh Farajdokht, Javad Mahmoudi, Sahar Khazen.
Writing–original draft: Fereshteh Farajdokht, Ghasem Khazen, Mahtab Rajabi-Jourshari, Zahra Sheikhalizadeh.
Writing–review & editing: Fereshteh Farajdokht, Saeed Sadigh-Eteghad, Javad Mahmoudi.

Competing Interests

The authors have no relevant financial or nonfinancial interests to disclose.

Data Availability Statement

The data used for preparation the current manuscript are available from the corresponding author upon reasonable request.

Ethical Approval

All procedures were conducted in accordance with the National Institute of Health Guidelines (NIH; Publication No. 85-23, amended 1985) and received approval from the regional Ethics Committee of Tabriz University of Medical Sciences (Approval No. IR.TBZMED.AEC.1402.008).

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