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Research Paper: The Effect of Kaempferol on Autophagy and Nrf-2 Signaling in a Rat Model of Aβ1-42-induced Alzheimer's Disease

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ABSTRACT

Background: Numerous pieces of evidence support that oxidative stress is a key factor in the pathogenesis of neurodegenerative diseases, like Alzheimer's Disease (AD). Suppression of oxidative stress is an attractive strategy and flavonoids as potent natural antioxidants are extremely noticeable.

Objectives: In this study, the effects of Kaempferol (KMP) were evaluated on passive avoidance memory, hippocampal Nrf-2, and beclin-1 expression in a rat model of Aβ1-42 –induced AD.

Materials & Methods: Forty male Wistar rats weighing 200-250 g were divided into five groups (n=8); sham-operated, AD model, and KMP treatment (5, 7.5, 10 mg/kg, i.p. for three weeks). Animals received an intracerebroventricular injection of amyloid-beta (1-42) to establish an AD model. Passive avoidance memory of rats was evaluated using a shuttle box on day 21; Step-Through Latency (STL) and time spent in The Dark Compartment (TDC) were recorded. Then, hippocampus homogenates were used for biochemical and molecular analysis by real-time PCR, western blot, and ELISA.

Results: It was found that KMP improved memory evidenced by increased STL ($P \leq 0.05$) and decreased TDC ($p \leq 0.01$). KMP also increased the levels of Total Antioxidant Capacity (TAC) in the hippocampus of rats ($P \leq 0.05$). In addition, KMP enhanced the expression of Nrf-2 mRNA ($P \leq 0.001$) and beclin-1 protein in the hippocampus tissues ($P \leq 0.001$).

Conclusion: Overall, it is suggested that the memory-improving effect of KMP is mediated, at least in part, by enhancing Nrf-2 and TAC. KMP is also able to induce autophagy through the expression of beclin-1.

Keywords: Alzheimer's disease, autophagy, Nrf-2 and, Kaempferol

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Highlights

- Kaempferol isolated from Mespilus germanica leaves attenuates memory deficit in Aβ-induced Alzheimer's Disease
- Kaempferol increased the expression of Nrf-2 mRNA and total antioxidant capacity in the hippocampus of rats with Aβ-induced Alzheimer's Disease
- Kaempferol increased the expression of beclin-1 in the hippocampus of rats with Aβ-induced Alzheimer's Disease

Introduction

Alzheimer's Disease (AD) is the most common form of dementia in the elderly. It is an irreversible neurodegenerative disorder that is identified by memory loss and cognitive impairment. The pathogenesis of AD has been the subject of several studies and hypotheses, such as the amyloid cascade, the cholinergic, and tau hypotheses have been proposed; however, many molecular aspects of the disease remain unclear [1]. The Amyloid-Beta (Aβ) cascade, the most accepted hypothesis for AD, assumes that an early-stage factor in the initiation of the disease is the imbalance between the generation and clearance of Aβ peptides [2]. Although extracellular Aβ plaques and intraneuronal Neurofibrillary Tangles (NFT) are the basic features of AD neuropathology, it is proposed by several studies in the literature that deficits in autophagy–lysosomal pathway are likely to precede the formation of these pathological hallmarks [3, 4].

Autophagy is a vital lysosomal mechanism, which degrades misfolded/unfolded proteins and damaged organelles in the cell [5]. It is extremely important in maintaining cellular homeostasis. Autophagy starts with the development of phagophores for the purpose of isolating the long-lived proteins and organelles, which elongates and fuses to form autophagosomes. The autophagosomes are transported towards lysosomes for lysosomal fusion. Beclin-1 plays a vital function in the development of phagophore so that it can be considered as a marker of autophagy [6]. The pivotal role of dysregulated autophagy in neurodegenerative diseases, where toxic protein aggregates and damaged organelles accumulate within particular types of neurons and contribute to neuronal dysfunction, has recently been shown in a system biology review [7].

Besides, deletion of nuclear factor E2-related factor 2 (Nrf-2) deteriorates the autophagy dysfunction in AD model mice [8]. Nrf-2 is recognized as an antioxidant transcription master regulator. In the promoter of target antioxidant genes, Nrf-2 binds to the antioxidant response element and closely controls its transcription, thereby playing a crucial role in cellular protection [9, 10]. The activation of Nrf-2 can prevent or decrease cellular damage associated with injuries to various tissues and also, genetically deleting Nrf-2 exacerbates neuronal death in different diseases [11]. Therefore, Nrf-2 is likely to be a potential therapeutic target in neurodegenerative diseases, like AD.

There is no treatment for AD to date, but the use of natural antioxidants may slow the disease's pathogenesis [12]. Kaempferol (KMP) is commonly present in many vegetables and fruits as a natural flavonoid (e.g., tea leaves, broccoli, berries, and medlar). Several studies have shown that KMP has a broad range of biological and pharmacological properties, including antioxidant, anti-inflammatory, and anticancer effects [13, 14]. The neuroprotective effect of KMP has been shown in many studies. Yang et al. demonstrated that KMP protected neurons by controlling the expression levels of apoptosis-associated proteins, including Bcl-2, Bid, apoptosis-inducing factor, and MAPK in glutamate-traded HT22 hippocampal neurons [15]. Zarei et al. suggested that KMP can improve memory via the cholinergic system in scopolamine-induced memory impairment [16]. Also, Beg et al. investigated the effect of KMP against the transgenic Drosophila. They found that exposing the AD flies to KMP slowed the impairment of climbing ability, memory, reduced oxidative stress, and acetylcholinesterase activity [17]. These findings suggest that KMP could likely attenuate AD pathology. In the current study, we investigated the effect of KMP on passive avoidance memory and Total Antioxidant Capacity (TAC) in a rat model of AD. We also examined the Nrf-2 gene expression and beclin-1 protein to define the possible protective mechanism of KMP.

Materials and Methods

Materials

Aβ1-42, Tris-HCl, NaCl, sodium deoxycholate, SDS, EDTA, Triton x-100, and cocktail protease inhibitor were purchased from Sigma (Sigma-Aldrich, USA). Antibodies against beclin-1 and beta-actin were obtained from Santa Cruz (Santa Cruz Biotechnology, Inc. USA).

Extraction and isolation of kaempferol

Mespillus Germanica leaves collected from the forests of northern Iran were confirmed by a specialist from the herbarium center of the Guilan University (Herbarium code of 6157). Medlar flavonoids were extracted and purified as described in our previous study [18]. Briefly, this was achieved by dissolving 5 g of the dried leaves in 104 mL of 70% ethanol, which was stored in a rotary evaporator at a temperature of 40°C with a speed of 40 m/s. Then, pure flavonoids were obtained using a mixture of 2 molars of hydrochloric acid and ethyl acetate in the rotary evaporator. Next, all the extract components were detected by two-dimensional paper chromatography (at 366 and 254 nm). Finally, KMP was isolated using thin-layer chromatography and high-performance thin-layer chromatography.

Animals

Forty male Wistar rats weighing 200-250 g and aged 3 months were used in this study. During 12 h light and dark periods (lights on 7:00h) with unrestricted access to food, the animals were kept in cages (standard rat laboratory diet and tap water). The room temperature was kept at 22±2°C.

The animals were divided randomly into five groups (n=8): (1) Saline/saline as a sham-operated group, (2) Aβ1-42 /saline as an AD model group; (3) Aβ1-42 / KMP (5 mg/kg), (4) Aβ1-42 /KMP (7.5 mg/kg), and (5) Aβ1-42 /KMP (10 mg/kg).

The guidelines of "institutional Guide for Care and Use of Laboratory Animals" were followed in all the experiments on animals. Furthermore, our experiments were approved by the Ethics Committee of Guilan University of Medical Sciences (Code: IR.GUMS.REC.1396.203).

Stereotaxic surgery

Using a mixture of ketamine (100 mg/kg)/xylazine (5 mg/kg), the rats were anesthetized and placed in a stereotaxic instrument (Stoelting, Chicago, IL, USA). Animals were bilaterally cannulated (22 gauge guide cannulae) according to ventricular coordinates: DV=3 mm from skull surface; anteroposterior=−0.8 mm from the bregma; lateral: 1.6mm from midline), based on the atlas of Paxinos and Watson of rat brain [19]. Microinjections were performed by a 27-gauge injector cannula that was connected to a short piece of polyethylene tube and a 10 µl Hamilton syringe.

Drugs and treatment

Aβ1-42 at 2 µg/µl concentration was dissolved in 0.9% saline and incubated for three days to aggregate at room temperature [20]. The therapeutic period was 21 days for each group. In the AD model group and Aβ+KMP groups, the rats were injected on the first day with Aβ1-42 (2 µg/µl; ICV; bilateral; 4 µl volume on each side), while in the control group, they were injected with saline (ICV; bilateral with 4 µl volume on each side) on the first day. All groups were injected over three weeks by different doses of KMP (5, 7.5, 10 mg/kg; intraperitoneal (i.p.)) or saline (1 ml/kg, i.p.) [18].

Behavioral test

The rats were tested for memory retention deficits using passive avoidance apparatus on days 20 and 21 following ICV injection of Aβ. The step-through passive avoidance apparatus consisted of two chambers (30cm×20cm×20 cm, each) one of them is made up of transparent plastic and the other one is a dark chamber with walls and ceiling made up of dark opaque plastic. A rectangular opening (8cm×8cm) was located between the two chambers, which could be closed by an opaque guillotine door. The floor was made of stainless steel rods (2 mm in diameter) spaced 1 cm apart from both chambers. The floor of the dark compartment could be electrified. The apparatus was installed in a room, which was acoustically insulated.

In order to investigate the acquisition and recovery of memory based on negative reinforcement, the step-through form of passive avoidance task was used, as described elsewhere [21]. One day before initiation of the tests, animals were placed in the light chamber and were allowed to move freely inside both dark and light encasements for 5 minutes, in order to become adapted to the apparatus. After 30 min, the habituation process was repeated again.

Then, the day after the habit of the shuttle box, rats were placed in the lighted chamber, the door was lifted after 10s, and crossover latency was recorded. After the

entrance of the rat to the dark compartment, the door was closed behind it and a shock (50Hz, 1mA, 3s duration) was delivered. Immediate memory was tested in 5min immediately after training. Also, 1 and 24 hours after training, retention tests were conducted to determine short and long-term memory. The rats were put in the lighted space, the door was opened 10 s later, and up to 300 s. The Step-Through Latency (STL) and the time spent in The Dark Compartment (TDC) were measured. The electric shocks were not applied to the grid floor during this session.

Tissue preparation

Following the behavioral test, animals were decapitated under anesthesia and their brains were quickly removed and cleaned with ice-cold saline. Then, the hippocampus was dissected and stored at -80°C. To prepare the homogenized tissue, a lysis buffer containing Tris-HCl, pH8.0, NaCl, Sodium deoxycholate, SDS, EDTA, Triton x-100, and cocktail protease inhibitor was used. Then, the lysate was subjected to centrifugation at 3000g for 10 min to collect the supernatant [20]. Protein concentrations were measured using the Bradford method. For the calibration process, bovine serum albumin was used as the reference standard.

Western blot

The total proteins were electrophoresed in 12% sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), as described before [22]. The isolated proteins bands were then moved to Polyvinylidene Fluoride (PVDF) membranes (Amersham Biosciences; USA) by western blotting and were probed with specific antibodies. Expression of beclin-1 in rat brain was determined by an antibody directed against beclin-1 (rabbit polyclonal antibody, Inc, China). As a positive control, β-actin (mouse monoclonal, sc-47778, Santa Cruz Biotechnology, Inc) was applied. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, AP7170, Razi biotech, Tehran, Iran and antirabbit IgG, DB-9572, DNA biotech, Tehran, Iran). Immunoreactive polypeptides were detected by chemiluminescence using enhanced ECL reagents (Amersham Biosciences, USA) and were used followed by an investigation of autoradiography. Finally, using a densitometric scan of films with image j software (NIH, USA), band densities were measured.

Real-Time PCR

The mRNA levels of Nrf-2 were determined by quantitative real-time PCR [23]. Using TRIzol reagents, complete RNA was removed from the rat hippocampus (Qiazol, USA). It was then quantified and the purity was measured by spectrophotometry; the 260:280 ratios were 1.8-2.0. Then, the RNA was treated with DNase I to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized using the Thermo scientific cDNA synthesis package from 1 µg of total RNA (USA). Real-time PCR was performed in a 10 µl reaction using Maxima SYBER Green /ROX qPCR Master Mix (Amplicon, Denmark), specific primer sequence [(Nrf-2-forward- GAAAACGACAGAAACCTCCATC and reverse-CTCCATCCTCCCGAACCT) and (GAPDH-forward-AAG TTC AAC GGC ACA GTC AAG G and reverse-AAG TTC AAC GGC ACA GTC AAG G)], and a LightCycler 480 real-time PCR system (ABI step one, USA). Reaction procedures were as follows: an initial step at 95°C for 15 min, 40 cycles 95°C for 15 s, 40 cycles 60°C for 30 s (two times), and 60-90 °C for 30 s. GAPDH was used as an internal control and the relative fold differences were calculated by the comparative cycle threshold method.

ELISA

TAC was measured by the TAC assay kit (Zellbio GmbH, Germany). For this reason, after homogenization, the levels of TAC were determined according to the manufacturer's instruction. Briefly, 10µl of the supernatant of each sample or standard was incubated with 190µl working chromogen reagent and OD1 was read at 490 nm. Then, 50 µl of metal ion solution was applied and incubated at room temperature for 2 min. Then, a stop solution was added and the OD2 was recorded at 490nm. ΔOD is equal to OD2-OD1. TAC concentration (mM) was calculated based on a standard curve, which was drown using standard ΔOD absorbance against the standard concentrations [23].

Statistical analysis

Data are reported as Mean±SEM (Standard Error Mean). Data were analyzed by One-Way Analysis of Variance (ANOVA) followed by a Tukey's post-hoc test for multiple comparisons, except for some behavioral data, which were evaluated by the Kruskal-Wallis test. A value of $P<0.05$ was considered to be significant. SPSS v. 23 software was used for statistical analysis (IBM Corporation, Chicago, IL, USA).

Results

Effect of Kaempferol on passive avoidance memory

Passive avoidance test results in late retrieval showed that STL significantly reduced in the Aβ group (21.57± 7.2; P=0.029) compared to the saline group (139.37± 26.5). However, treatment with KMP at 10mg/kg (156±15.78; P=0.004) significantly increased STL on the retrieval day. In addition, TDC increased significantly in the Aβ group (63.42±10.3, P= 0.031) compared to the saline group (10±5.3). A significant reduction was detected in the Aβ±KMP 10 group (12.37±13.1, P= 0.011) in comparison with the Aβ group. These data suggest that KMP treatment (10 mg) had beneficial effects on the memory deficit of rats caused by Aß1-42 (Figure 1).

Effect of Kaempferol on the expression of NRF-2 mRNA in the hippocampus of rats

Nrf-2 mRNA expression significantly down-regulated (0.006± 0.0004) in the Aβ group compared to the saline group (0.039±0.008, P= 0.02). However, the mRNA expression of the hippocampal Nrf-2 significantly increased in the Aβ±KMP 10 group (0.045±0.01, P=0.005) compared to the Aβ group (Figure 2).

Effect of Kaempferol on the expression of Beclin-1 protein in the hippocampus of rats

Western blot analysis of the hippocampus was performed to assess beclin-1 protein expression levels. A one-way ANOVA showed differences between groups. As illustrated in Figure 3, the western blot assay revealed

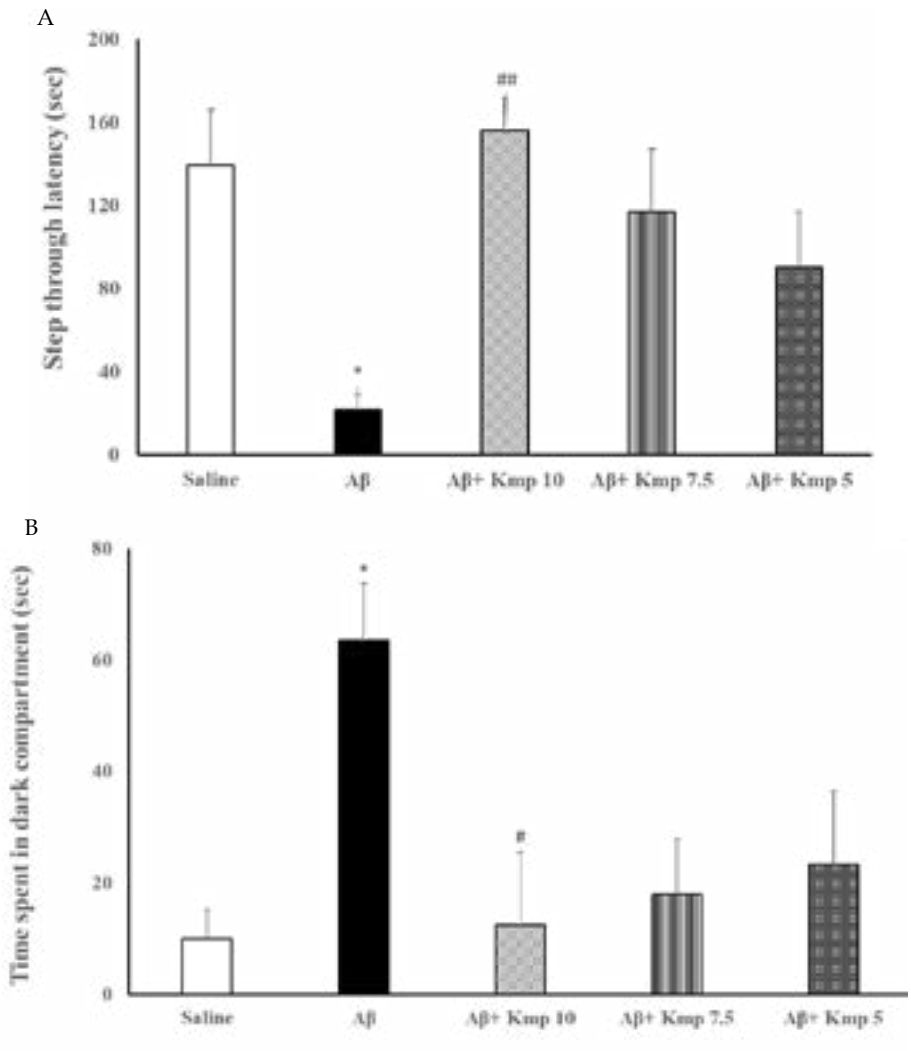

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Figure 1. Effect of Amyloid-Beta (Aβ) and Kaempferol (KMP) (5, 7.5, and 10) on passive avoidance memory in the Shuttle box

A: Changes in step-through latency; and B: Total time spent in the dark compartment 24 hours after acquisition. Data are presented as Mean±SEM. *Compared to the saline group; #Compared to the Aβ group (Kruskal-Wallis test; n=8).

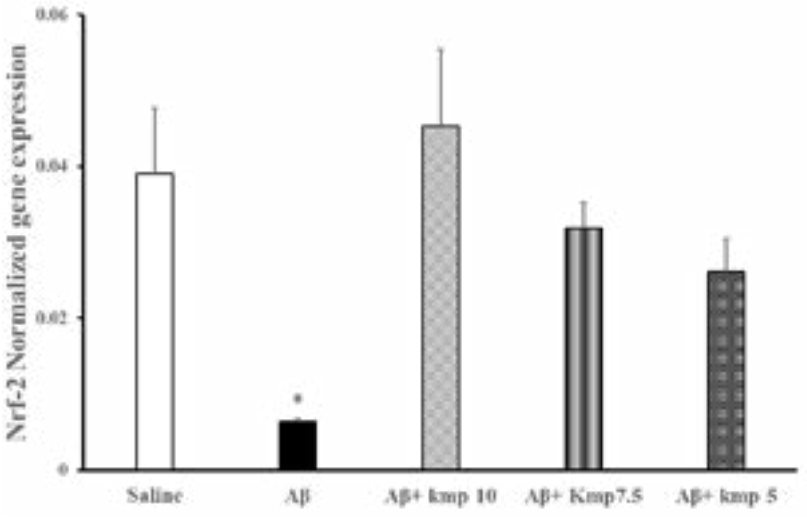

Figure 2. Effect of Amyloid-beta (Aβ) and kaempferol (KMP) (5, 7.5, and 10) on the relative expression of Nrf-2 mRNA in the hippocampus of rats

GAPDH was selected as the reference gene. Each Bar represents Mean±SEM. *Compared to the saline group; #Compared to the Aβ group (one-way ANOVA and Tukey Post-Hoc Test; n=6).

that Aβ treatment down-regulated the relative expression of beclin-1 (0.82±0.02) in the hippocampus compared to the saline group (1.09±0.04) (P=0.004). However, the Aβ±KMP 5 (1.09±0.01, P=0.004), Aβ±KMP 7.5 (1.40±0.05, P=0.00), and Aβ±KMP 10 (1.90±0.05, P=0.00) groups showed higher levels of beclin-1 protein in the hippocampus compared to the Aβ group.

Effect of Kaempferol on TAC levels in the hippocampus of rats

The levels of TAC significantly decreased in the Aβ group (1.05±0.007) compared to the saline group (1.47±0.029, P=0.00) as shown in Figure 4. However, animals treated with 10mg of KMP (1.38±0.03) showed a significant increase in the levels of TAC compared to the Aβ group. No significant change was observed between the Aβ+KMP 7.5 mg (1.15±0.1) and Aβ+KMP5 (1.06±0.03) groups compared to the Aβ group (P=0.651 and P=1, respectively).

Discussion

In this study, the effect and possible mechanism of KMP on the Aβ-model of AD in rats were investigated. The results achieved in our study showed that: 1) ICV injection of Aβ1-42 impaired memory and decreased the Nrf-2 mRNA and beclin-1 protein expression and TAC levels in the hippocampus of rats; 2) KMP (10mg/kg) reversed Aβ-induced memory impairment and increased the level of TAC and the relative expression of Nrf-2 mRNA and beclin-1 protein. Accumulation of Aβ1-42 in the brain is known to be responsible for the production of senile plaque that disrupts synapses and leads to memory impairment and cognitive dysfunction [2]. Aβ interacts with different types of receptors, like insulin, RAGE, NMDA, nicotinic receptors, and ryanodine receptors. In addition, this molecule shows a broad range of toxic mechanisms, including mitochondrial dysfunction, calcium imbalance, oxidative stress, microglial activation, and apoptosis [24]. Aβ also forms insoluble assemblies (aggregation/accumulation), which are able to induce neurotoxicity [25]. As Aβ can be internalized or produced inside the cell, provides the opportunity to facilitate neurofibrillary tangle formation [26].

Growing numbers of natural products have been researched over the last two decades in terms of their effects on neurodegenerative diseases. Flavonoids, through multiple direct and indirect effects, show suppression of inflammation, oxidative stress, and cell death. One of these flavonoid, KMP, was found to attenuate cognitive function [13]. KMP like the other flavonoids is able to cross the blood-brain barrier and may enhance many aspects of brain health. Our previous study showed that KMP improved spatial memory, via its antioxidant and anti-inflammatory properties in an Ovariectomized (OVX) rat model of sporadic dementia [18]. In addition, KMP improved memory by a reduction in oxidative stress and acetylcholinesterase activity

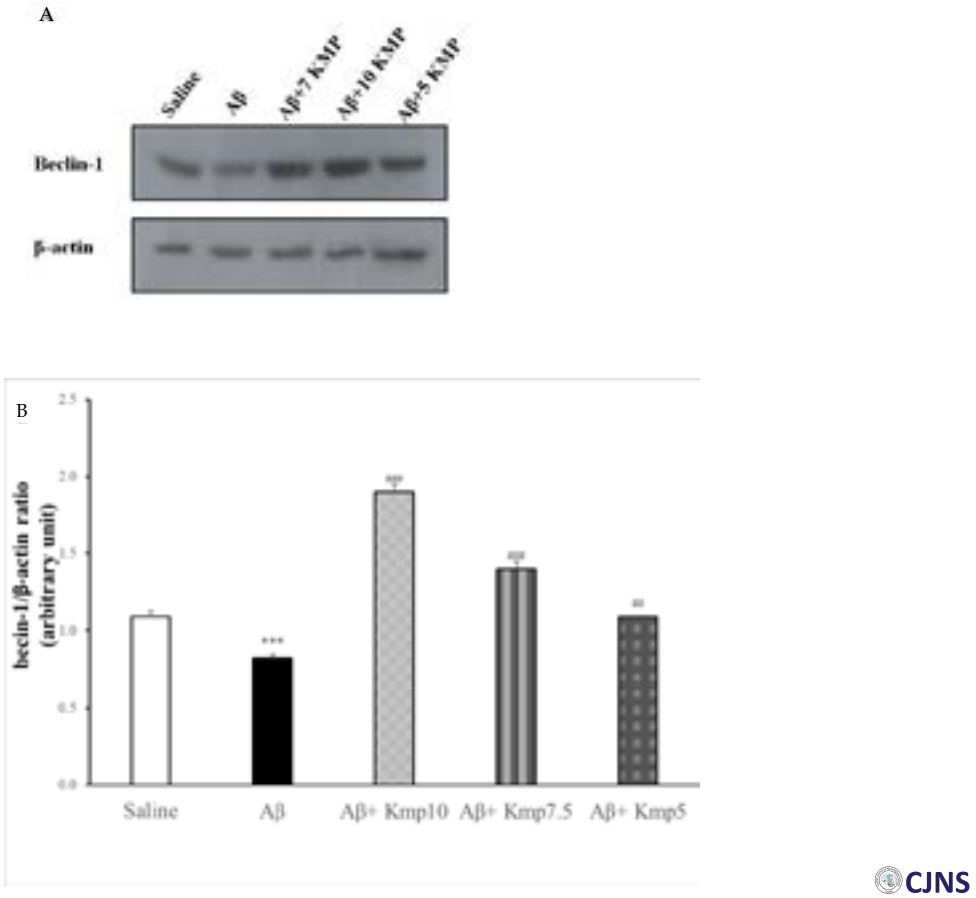

Figure 3. Effect of amyloid-beta (Aβ) and kaempferol (KMP) (5, 7.5, and 10) on the expression of beclin-1 in the hippocampus of rats

A: Western blot analysis showed beclin-1 levels in different groups; B: Quantification of protein levels of beclin-1. B-actin was used as a loading control. Each Bar represents Mean±SEM. *Compared to the saline group; #Compared to the Aβ group (one-way ANOVA and Tukey Post-Hoc Test; n=3).

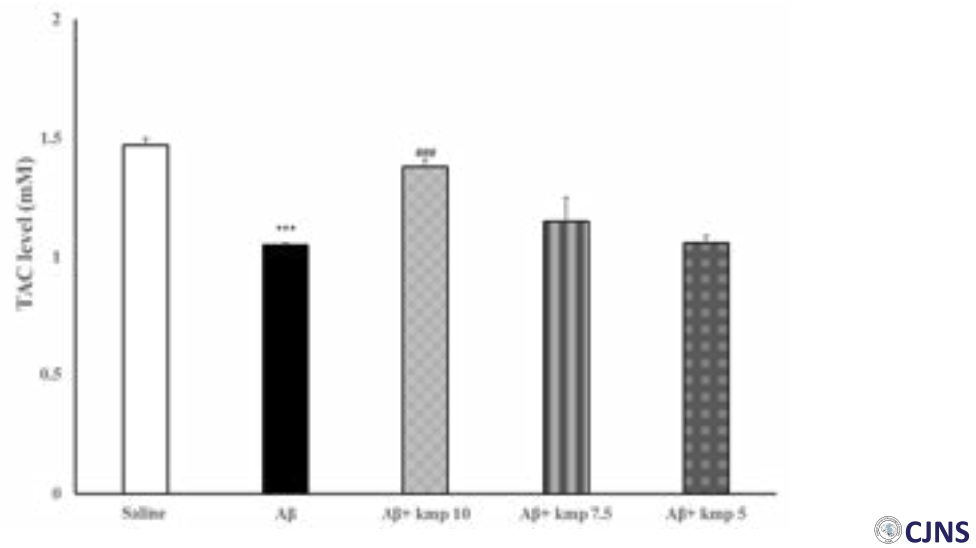

Figure 4. Effect of Amyloid-Beta (Aβ) and Kaempferol (KMP) (5, 7.5, and 10) on the total antioxidant capacity levels in the hippocampus of rats

Each Bar represents Mean±SEM. * Compared to the saline group; #Compared to the Aβ group (one-way ANOVA and Tukey Post-Hoc Test; n=6).

in transgenic Drosophila [17]. The present study demonstrated that KMP reversed Aβ1-42 -induced passive avoidance memory impairment in the shuttle box.

Of all the mechanisms involved in AD pathology, oxidative stress is the most noteworthy. Numerous studies have provided evidence that aggregated Aβ peptides are involved in ROS generation [27-29]. The imbalance between ROS generation and removal creates oxidative stress. The neurons existing in the entorhinal cortex, hippocampus, frontal cortex, and amygdala are more vulnerable to oxidative stress harm and further AD progression [30].

Our results showed that chronic KMP treatment elevated the level of total antioxidant capacity in the hippocampus of animals. In agreement with our result, Li et al. (2011) report that pretreatment with KMP markedly rehabilitated the superoxide dismutase and glutathione peroxidase operations in the MPTP model of Parkinson's Disease [31]. Furthermore, Alnumeir et al. (2015) found that KMP administration to diabetic rats elevated enzymatic (superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase) and nonenzymatic (vitamins C and E) antioxidants in the liver, kidney, and heart [32]. It has been shown that KMP could stop the self-assembly and consequently abrogate the toxicity of Aβ peptides to cells [33]. Hence, KMP could be neuroprotective not only because of its antioxidant effect but also because of its ability to interfere with Aβ pathogenesis.

We also demonstrated that this oxidative stress prevention can be induced, at least in part, through Nrf-2 restoration. Nrf-2 is a critical signaling pathway for oxidative stress because it regulates the expression of several antioxidant enzymes, such as SOD, catalase, HO-1 [34, 35]. Our results indicated that the expression of Nrf-2 mRNA decreased in AD animals. However, a 21-day KMP treatment could effectively reverse Nrf-2 mRNA expression levels. In line with our findings, Hussein et al. (2018) suggested that Nrf2 expression in the brain tissue plays an important role in the neuroprotective effects of KMP in chlorpyrifos-induced memory impairment in rats [23].

Because of its fast turnover, cells display low Nrf-2 protein levels under physiological conditions. In response to oxidative stress, it is accumulated and translocated to the nucleus. In the nucleus, Nrf-2 manages the transcription of ARE-containing genes, such as superoxide dismutase, catalase, NO-1, and HQ-1 that cause an antioxidant defense system in cells [36].

Another relevant result found in our study was that KMP treatment induced the expression of beclin-1 protein in the hippocampus of AD animals. In this regard, Filomeni et al. (2012) found that KMP induced autophagy in SH-SY5Y cells. According to their results, KMP induced the formation of autophagosomes and was an autophagic enhancer with the potential ability for therapy [37]. Aggregation of misfolded and unfolded Aβ proteins in the neurons forms the basis of one of the hypotheses in AD. These proteins are translocated into the mitochondria leading to the disruption of mitochondrial oxidative phosphorylation, organelle damage, and finally, neuronal death. Autophagy is a physiologic mechanism to eliminate these proteins and damaged organelles in the cytoplasm [6]. However, the role of autophagy in the pathogenesis of AD continues to be controversial. Some studies have demonstrated the down-regulation of autophagy morphologically and genetically in AD brains [38]. On the other hand, research has shown that autophagy is involved in AD pathogenesis. Using a transgenic model of AD, Nilson et al. (2013) reported the role of autophagy in Aβ metabolism, and more importantly, they found that it mediates the secretion of Aβ to extracellular space [39]. Thus, the role of autophagy in AD is not clear. Our findings suggest that probably part of memory improvement in animals was due to autophagy activation.

Interestingly, KMP activates both Nrf-2 and beclin-1 expression in the hippocampus of rats in our study. Jo et al. indicated that the activation of Nrf-2 induced the autophagy pathway [40]. Furthermore, Joshi et al. (2014) found that deletion of Nrf-2 causes autophagy dysfunction in the AD mice model [8]. Nrf2 has been shown to facilitate autophagy via the activation of the autophagosome cargo protein p62/sequestosome-1 (p62/SQSTM1) and other genes linked to autophagy. Nrf-2 knockdown decreases autophagic flux, while pharmacological activation of Nrf-2 enhances autophagy, and this corresponds strongly with changes in p62/SQSTM1 rates [41, 42].

Conclusion

To sum up, we reported that KMP isolated from Mespilus Germanica leaves was able to attenuate Aβ-induced memory impairment in rats. We proposed that this effect is mediated, at least in part, by enhancing Nrf-2 and TAC. KMP is also able to induce autophagy through the expression of beclin-1.

Ethical Considerations

Compliance with ethical guidelines

All study procedures complied with the ethical guidelines of the Declaration of Helsinki 2013. Also, All experiments were carried out following the policies of the "institutional Guide for Care and Use of Laboratory Animals" and permission of the local Ethics Committee of Guilan University of Medical Sciences (Code: IR.GUMS.REC.1396.203).

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Authors' contributions

Conceptualization, methodology, writing the original draft: Adeleh Jafari and Kambiz Rohampour; Methodology, supervision, review, and edition of the draft: Adeleh Jafari, Parvin Babaei, Kambiz Rohampour; Data collection: Samira Rashtiani; Funding acquisition: Adeleh Jafari.

Conflict of interest

The authors declared no conflicts of interests.

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