Short communication

*Mespilus germanica* leaves flavonoids improve passive avoidance memory and apoptosis in a rat model of amyloid-β neurotoxicity

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Abstract

Introduction: Alzheimer’s disease (AD) is a common progressive, neurodegenerative disorder with no preventive or curative therapy until now. Use of natural products as an important source of neuroprotective flavonoids against AD has been considered recently. In this study, the effect of *Mespilus germanica* leaves (MGL) flavonoids treatment on memory dysfunction and apoptosis in the amyloid beta (Aβ)-treated rat was investigated.

Methods: Forty-eight male Wistar rats (220-250g) were divided into 6 groups (n=8): saline, Aβ, treatment (5, 7.5 and 10 mg/kg MGL flavonoids) and positive control group. Step through the passive avoidance test was performed on the 22nd day to examine learning and memory. Immediately afterward, the animals were killed and their brains were removed to measure the levels of cytochrome c in brain homogenate.

Results: Our results showed significant improvement in passive avoidance task as flavonoid (10mg/kg) increased step-through latency (*P*=0.003) and decreased the time spent in dark compartment (*P*=0.001) significantly. In addition, the levels of cytochrome c which was significantly increased in the Aβ-injected group was reduced remarkably in the flavonoid treatment group (*P*=0.029).

Conclusion: Therefore, MGL flavonoid can improve Aβ1-42 induced memory dysfunction in rats and its effect might be partially due to their role in decreasing apoptosis.

Keywords: Alzheimer’s disease; Amyloid beta; Flavonoids; Passive avoidance; Cytochrome c; *Mespilus germanica*

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Introduction

Alzheimer’s disease (AD) is a progressive, neurodegenerative disorder and the most common cause of dementia among the elderly (Mu and Gage, 2011). Amyloid beta (Aβ) is the key molecule in AD pathogenesis. Sequential proteolysis of the amyloid precursor protein (APP) by beta and gamma-secretases (O’Brien and Wong, 2011) produces Aβ peptide with 40 to 43 amino acids. It is believed that Aβ1-42 due to great power to aggregate has the most neurotoxic effect (Knobloch et al., 2007). It is found that Aβ triggers apoptosis (Devi et al., 2006), induces inflammatory response (Macready et al., 2009), makes the neuron susceptible to free radicals, nerve
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toxins and harmful factors (Newman et al., 2007), blocks synaptic plasticity (Nakamura et al., 2001), loses functional neurons (Nakamura et al., 2001) and finally, declines memory and cognition (Lambert et al., 1998).

A line of research in AD has concentrated on apoptosis. It has been reported that apoptosis is a major cause of neuronal death in AD (Youle and Strasser, 2008). Both intrinsic and extrinsic pathways of apoptosis are involved in AD (Devi et al., 2006). In the intrinsic pathway, mitochondria release cytochrome c. It binds with APAF-1 (apoptotic protease activating factor-1) to make a complex with procaspase-9 leading to activation of caspase-9 and then caspase-3 (Asadi et al., 2015). Cytochrome C is an important component of the electron transport chain and shuttles electron between mitochondrial complexes III and IV. In addition, it plays a critical role during apoptosis. It is widely believed that the release of cytochrome c from mitochondria is one of the early stages of apoptotic cascades and is also considered as a point of no return in cell death (Jazvinšćak Jembrek et al., 2015).

Although there are five FDA approved drugs for management of AD, none of them cures or prevent disease and just offer symptomatic benefits. Over the last decade, growing evidence has been found on the potential of dietary flavonoids for helping prevention of cognitive function. Flavonoids are a large family of polyphenolic compounds synthesized by plants and provide much of the flavor and color of fruits and vegetables. They have the capacity to cross the blood-brain barrier (BBB) and have been detected in the rat brain in areas related to learning and memory shortly after oral administration (Krishnaveni, 2012). The effect of flavonoids on Aβ-induced memory impairment has been assessed in many studies (Balouchnejadmojarad, 2009; Ejaz Ahmed et al., 2013). Although the exact mechanisms are not clear, it has been suggested that flavonoids can enhance cognitive function via their neuroprotective properties and also, they can stimulate neurogenesis, therefore, enhance neuronal function (Spencer, 2007; Spencer, 2008). One of the extremely rich sources of flavonoids is Medlar. It is the fruit of Mespilus germanica in the family of Rosaceae (Gülçin et al., 2011). The medlar is an edible fruit and modern medicine has recognized its healing properties in the treatment of some diseases (Ansari et al., 2006; Ahmady et al., 2013; Ramezani et al., 2016). However, the effects of MGL flavonoids on impaired learning and memory induced by Aβ1-42 in rats has not been reported.

Based on these findings as previously reported, this study examined the possible effects of MGL flavonoids on memory dysfunction and brain cytochrome c levels in a rat model of amyloid-β neurotoxicity.

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). Cytochrome c ELISA kit was purchased from Abcam (Abcam, USA).

The preparation of Mespilus germanica leaves (MGL) flavonoids

MGL were collected from the forests of northern Iran. The extract was prepared after drying the leaves with 70% ethanol. Briefly, the extract was evaporated at 40°C and at a speed of 40 via the rotary evaporator. Then 2 molar of hydrochloric acid was added and next ethyl acetate was added and they were mixed, it was again transferred to a rotary evaporator. Finally, after complete evaporation pure flavonoids were achieved.

At first, we used two–dimensional paper chromatography (2-DPC) for detecting and diagnosis of flavonoids and were investigated in UV light (at 366 and 254nm, respectively) and was calculated Rf for all spot and were determined by Markham identification of key. We also used thin layer chromatography (TLC) for determining the type of flavonoids. After spotting and observation in UV light (at 366 and 254nm, respectively) the location and stain of spots were found and eventually, calculation Rf and the type of flavonoid were obtained.

Animals and experimental designs

Forty-eight male Wistar rats weighing 220-250g were used for experiments according to the guide for the Care and Use of Laboratory Animals (the National Academy of Sciences, 2011). Animals were caged under controlled lighting (12h light, 12h dark) and temperature (20-22°C). All rats were allowed free
access to standard rat laboratory diet and tap water. For the experiment, we divided animals randomly to six groups: (1) saline/saline as a sham-operated group, (2) Aβ1-42/saline as an AD model group; (3) Aβ1-42/flavonoid (5mg/kg); (4) Aβ1-42/flavonoid (7.5mg/kg); (5) Aβ1-42/flavonoid (10mg/kg) and (6) Aβ1-42/donepezil. Donepezil is an FDA approved drug against the AD and it is selected as a positive control.

Aβ1-42 was dissolved in distilled water at the concentration of 4μg/μl (Boyd-Kimball et al., 2005; Jafari et al., 2015). The solution was kept at room temperature for 3 days before being used to allow the peptide to aggregate.

Flavonoids (5, 7.5 and 10mg/kg; IP) or donepezil (1mg/kg) were administered once a day for 21 consecutive days, after Aβ injection. The sham and model groups received the same volume of normal saline.

Twenty-four hours after the last dose of flavonoids or donepezil or saline, rats underwent behavioral assessment for two days and then animals were sacrificed. The brain was removed and immediately frozen in liquid nitrogen and stored at -80ºC. The diagram (Fig. 1) shows a timeline of the study in all groups of animals.

**Stereotaxic surgery**
Rats were anesthetized with a mixture of ketamine (100mg/kg)/xylazine (5mg/kg) and placed into a stereotaxic instrument (Stoelting, Chicago, IL, USA). Coordinates of the tip of the guide cannula were as follows: lateral: 1.6mm from midline; dorsoventral: 3mm from skull surface; anteroposterior: −0.8mm from the bregma, according to the stereotaxic atlas of Paxinos and Watson (2005). Aβ or saline was injected into each lateral ventricle (1μl) by a Hamilton syringe (Nakamura et al., 2001). After injection, the needle was left in place for an extra 30s.

**Single trial passive avoidance test**
On days 20 and 21 after intracerebroventricular (ICV)-Aβ injection, the rats were tested for memory retention deficits using passive avoidance apparatus (Nakamura et al., 2001). The apparatus consisted of a two-compartment dark/light shuttle box (20×20×30cm high) with a guillotine door (7×9cm) separating the compartments. The floor of the dark compartment was made of stainless steel rods (2.5mm in diameter) with a distance of 1cm.

For the acquisition trial, we placed the rat in the dark compartment and opened the door 20 seconds later. Immediately after the animal entered the dark compartment, the door closed and an electric foot shock (1mA, 50Hz, 5 seconds) was delivered to the floor grids. The entrance latency to the dark compartment, step through latency (STL1), was recorded when the animal placed all four paws in the dark compartment. After 24h, the retention latency time was measured in the same way as in the acquisition trial, but foot shock was not delivered and STL2 and the time spent in the dark compartment (TDC) was recorded to a maximum of 180s. The experiment was performed between 9:00 and 15:00 in the laboratory at standard optimal conditions.

**ELISA of cytochrome c in the brain**
Levels of cytochrome c were measured by
cytochrome C ELISA kit (ab210575, USA). For this reason, the brain tissue was homogenized in lysis buffer containing Tris-HCl, pH 8.0, NaCl, sodium deoxycholate, SDS, EDTA, Triton x-100 and a cocktail protease inhibitor. Then, the lysate was subjected to centrifugation at 3000g for 10 min to collect the supernatant. Then, protein concentrations were measured using the Bradford method. Bovine serum albumin was used as the reference standard for the calibration process.

Fig.2. The effect of ICV injection of Aβ1-42 on learning and memory in rats. Step through latency (A) in acquisition day, step-through latency (B) and time spent in dark compartment (C) during the retrieval test performed 1 day after passive avoidance acquisition. Values are expressed as mean±SEM (n=8). *P<0.05 and **P<0.01 compared with saline group.
The ELISA kit special for rats was used to determine the levels of cytochrome c according to the manufacturer’s instruction. Briefly, 50µl of the supernatant (250µg/protein) of each sample associated with 50µl of the antibody cocktail was incubated with 100µl 3,3’,5,5’-tetramethylbenzidine substrates for 1 hour at room temperature. Then, stop solution was added and the OD was recorded at 450nm. The results were expressed as picogram per milligram protein.

### Statistical analysis

The data are expressed as the mean±SEM (standard error mean). All the data were analyzed by the one-way analysis of variance (ANOVA) followed by a Tukey post-hoc analysis for multiple comparisons, except for some behavioral data which were evaluated by Kruskal-Wallis test and Bonferroni correction. Also, Student’s t-test and Mann Whitney u test were used for comparison between two groups. A value of $P<0.05$ was considered to be significant.

### Results

The extract of MGL consisted of quercetin, chrysin, kaempferol, myricetin, apigenin and rutin according to TLC and 2-DPC results.

#### Effect of Aβ1-42 in memory deficit

The unpaired t-test indicated that before the acquisition trial, there was no significant difference in the time of entrance between the saline and Aβ groups indicating that the preference of the dark place between groups was the same ($P=0.94$) (Fig. 2A). However, 24 hours later, STL ($P=0.021$) was increased and TDC ($P=0.001$) was decreased in the Aβ compared to the saline group (Fig. 2B and C). These data show that the ICV injection of Aβ1-42 causes memory deficit in the Aβ group.

#### Flavonoid improves learning and memory impairment induced by Aβ1-42

We used Kruskal-Wallis and Bonferroni correction to compare groups because of abnormal distribution of data. Passive avoidance test results showed that treatment with 10mg/kg flavonoid ($P=0.003$) and donepezil (1mg/kg) significantly ($P=0.03$) increased STL compared to Aβ group (Fig. 3A).

In addition, TDC was decreased in 7.5 and 10mg/kg flavonoid ($P=0.02$ and $P=0.001$, respectively) and donepezil ($P=0.01$) compared to Aβ group (Fig. 3B). These data suggest that MGL flavonoid had beneficial effects on memory deficit of rats caused by Aβ.

#### Flavonoid reverses cytochrome c level in Aβ1-42 treated rat

To investigate the effect of flavonoid on neural apoptosis, cytochrome c levels in homogenized brain tissue (n=6) were measured. As shown in Figure 4, the contents of cytochrome c were significantly increased in the rat subjected to ICV Aβ injection ($P=0.008$). However, flavonoid and donepezil treatment decreased significantly cytochrome c in the brain homogenate relative to Aβ group (7.5mg/kg: $P=0.02$, 10mg/kg: $P=0.029$ and donepezil: $P=0.028$). The statistical comparison was finished by one-way ANOVA followed by Post hoc Tukey. These results indicated that MGL flavonoid was able to improve Aβ-induced overproduction of cytochrome c.

### Discussion

In the present study, the effect of MGL flavonoids on the cognitive function and apoptotic response in the Aβ-injected rat were investigated for the first time. Aβ1-42 is a very toxic peptide which can aggregate and accumulate on the brain. It is responsible for the production of senile plaque that disrupts synaptic function between neurons, therefore, causes memory impairment. In addition, recent evidence has shown that Aβ can enter the cell and interfere with mitochondrial function, impair electron transport chain, induce oxidative stress and trigger apoptosis (Knobloch et al., 2007; Newman et al., 2007). Most studies have used ICV injection of Aβ as a model of Alzheimer’s disease (Nitta et al., 1994; Lambert et al., 1998; Jafari et al., 2015). Although the exact mechanism is elusive, RAGE receptor increasing, changing in choline acetyltransferase activity and microglial dysfunction (Deane et al., 2003) are the proposed mechanisms. So, it is believed that ICV infusion of Aβ is a useful animal model for evaluating the Alzheimer’s type of dementia.

The majority of existing drug treatments for neurodegenerative disorders does not prevent neural death, so most of the studies on AD disease have tried to find a new approach. Growing evidence has...
proposed that flavonoids may delay the development of Alzheimer’s disease-like pathology or recent evidence found the neuroprotective effect of flavonoids (Macready et al., 2009; Rendeiro et al., 2012; Sarahroodi et al., 2012; Kamal et al., 2015). Most plants are a rich source of different flavonoids. Medlar is the native plant of Guilan and is consumed by native people, but their flavonoids have not been considered yet. Our pilot study showed that MGL contains different types of flavonoids including quercetin, myricetin, apigenin, luteolin, chrysin, kaempferol and apigenin (data not shown). In the present study, we examined the effect of chronic intraperitoneal injection of flavonoid on the AD model. Our results showed that the 21-day MGL flavonoids treatment attenuate Aβ-induced memory deficit in the rat. We showed that the time spent in the dark compartment was decreased and step through latency was increased in flavonoid (10mg/kg) treated group.

Consistent with our data, Wu et al. have reported that Scutellaria barbata flavonoids improved spatial memory dysfunction and reduced neuronal injury in the composited Aβ-treated model of Alzheimer’s disease (Wu et al., 2016). The mechanisms by which flavonoids act in the brain are not clear, but there is evidence to suggest that flavonoids can cross the BBB and have been shown to affect different aspects of synaptic plasticity, regulation of receptors activation, activation of transcription factors, regulation of gene and protein expression as well as promotion of LTP (Rendeiro et al., 2012; Williams and Spencer, 2012; Rendeiro et al., 2013). Also, by activating a number of protein kinase signaling cascades, such as the PI3 kinase (PI3K)/Akt, tyrosine kinase (Zhu et al., 2016) flavonoids can lead to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and
Differentiation. In addition, it is reported that flavonoids can inhibit the formation and aggregation of amyloid beta fibrils (Ono et al., 2003; Jiménez-Aliaga et al., 2011) and potently inhibit BACE-1 activity. Therefore, they reduce the level of secreted Aβ in primary cortical neurons (Shimmyo et al., 2008).

Apoptosis is one of the most important mechanisms of neural death. Intracellular apoptotic cascade begins with the release of cytochrome c. It is found that Aβ cause to activate apoptotic cascades by changing the pro and anti-apoptotic proteins such as increase of Bim and decrease of Bcl2 (Kudo et al., 2012). We found that the levels of cytochrome c increase in Aβ group but flavonoid treatment can reduce these levels similar to control rats. Consistent with our data, Liu et al. have shown that pinocembrin treatment reduces cytochrome c and pro-apoptotic factor such as Bax and caspase 3 in the AD model (Liu et al., 2012). We found that the levels of cytochrome c increase in Aβ group but flavonoid treatment can reduce these levels similar to control rats. Consistent with our data, Liu et al. have shown that pinocembrin treatment reduces cytochrome c and pro-apoptotic factor such as Bax and caspase 3 in the AD model (Liu et al., 2012). These results suggest that the effect of MGL flavonoids on memory impairment induced by Aβ1-42 may be derived from preventing the release of cytochrome c. However, this mechanism needs to be clarified by further studies.

**Conclusion**

In summary, our study demonstrated that MGL flavonoid chronic treatment can improve Aβ-induced memory impairment in rats. This effect might be partially due to their role in decreasing cytochrome c levels. It suggests that MGL flavonoids may be particularly useful in the treatment of neurodegenerative disease.

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**Conflict of interest**

Authors declare no conflict of interest.

**References**

