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Original article

Screening seven Iranian medicinal plants for protective effects against β-Amyloid-induced cytotoxicity in cultured cerebellar granule neurons

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Abstract

Background and objectives: Alzheimer's disease (AD) as a neurodegenerative disorder is the most common form of dementia in the elderly. According to the amyloid hypothesis, accumulation of amyloid beta (Aβ) plaques, which are mostly constituted of Aβ peptide aggregates, triggers pathological cascades that lead to neuronal cell death. Thus, modulation of Aβ toxicity is the hopeful therapeutic approach for controlling the disease progression. Recently, several studies have indicated promising findings from herbal extracts against Aβ cytotoxicity. The aim of the present study was to assess the protective effect of the methanol extract of seven medicinal plants from Iran on Aβ-induced toxicity in primary neuron culture. **Method:** The methanol extracts of plants were prepared by maceration method. Primary cerebellar granule neurons (CGNs) were taken from male mice at postnatal days 6-7 and cultured in cell culture medium containing 10% FBS and 25 mM KCl. After seven days *in vitro* (DIV7), the cells were incubated with aggregated Aβ (10 μM) alone or in combination with different concentrations of extracts in the cultured medium for 24 h and cell viability was assessed by MTT assay. **Results:** Our results indicated that *Sanguisorba minor, Cerasus microcarpa, Ferulago angulata, Amygdalus scoparia and Rosa canina* extracts significantly ameliorated Aβ-induced toxicity which indicated the protective effect of these extracts. Protective effects were not observed for *Stachys pilifera and Alhagi pseudalhagi* extracts. **Conclusion:** Based on the protective effects of these plants against Aβ-induced toxicity, we recommend greater attention to their use in the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease, *Sanguisorba minor, Cerasus microcarpa, Ferulago angulata, Amygdalus scoparia*

Introduction

Alzheimer's disease (AD), is a progressive neurodegenerative disorder. It is the most prevalent form of dementia in the elderly. AD is characterized by short-term memory deficit at the first stage which is followed by spatial disorientation, planning disorders, and psychosis at advanced stages [1]. AD is a disease with complex pathophysiology. It is histopathologically characterized by considerable synaptic loss and neuronal cell death in the parts

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of the brain responsible for cognitive functions, especially in the cerebral cortex and hippocampus. The significant loss of cholinergic neurons in the basal forebrain nuclei has been observed in AD brain [2]. According to these findings, the first hypothesis for the description of the pathophysiology of AD was "cholinergic deficit hypothesis", then one of the early therapeutic strategies for the AD was restoring of cholinergic transmission by using acetylcholinesterase inhibitors [3]. Currently approved medicines for AD therapy, are acetylcholinesterase inhibitors such as tacrine, donepezil, and rivastigmine. These medicines improve only the clinical symptoms of the patient and have little effects on disease progression [4].

The two pathological hallmarks of AD are the presence of extracellular senile plaques which mainly consist of amyloid-β (Aβ) peptide aggregates and intracellular neurofibrillary tangles constituted by hyperphosphorylated aggregates of the microtubule-associated protein tau. The finding that Aβ peptide aggregates have neurotoxic properties leads to the generation of the "amyloid cascade hypothesis". According to this hypothesis, it is believed that the neuropathogenesis of AD may be triggered by the progressive accumulation of Aβ peptide in the brain which is derived from the proteolysis of Aβ precursor protein (APP) [5]. The mechanisms proposed for the neurotoxicity of Aβ includes oxidative stress, mitochondrial dysfunction, and depletion of cellular ATP, excitotoxicity, and induction of inflammatory responses. Triggering these mechanisms results in activation of cell death pathways, which induce neuronal death. Then it has been considered that prevention of Aβ toxicity by medicinal intervention would be an important therapeutic approach to control the onset and progressive of AD [6,7].

In fact, AD is a disease with complex pathology and new attempts are aiming to produce therapeutic agents that target disease progress through different pathways and cure the disease by modifying its pathology [8]. Herbal extracts have several components with different pharmacological effects then it is suggested that they can effectively treat complex diseases such as AD [9,10]. In recent years, several studies have demonstrated neuroprotective effects against Aβ toxicity by using herbal extracts such as *Ginkgo biloba* [11], *Melissa officinalis* [12] and *Satureja bachtiarica* [13]. Thus herbal medicines appear to be a promising resource to find more effective therapeutic agents for treating AD patients. In the present study, protective effect of seven medicinal plant extract including *Sanguisorba minor, Cerasus microcarpa, Ferulago angulata, Alhagi pseudalhagi, Stachys pilifera, Amygdalus scoparia* and *Rosa canina*, has been investigated. Previous studies have indicated that these medicinal plants have cholinesterase inhibitory activity [14-16] and in the present study protective effect of these extracts against Aβ-induced cytotoxicity in cultured cerebellar granular neuron was investigated.

Experimental

This study was an *in vitro* experimental study performed on cultured cerebellar granular neurons (CGNs) obtained from mice.

Chemicals

Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10000 U/mL), and trypsin (0.25%) were purchased from Gibco (USA). Poly-D-lysine (PDL) was purchased from Santa Cruz Biotechnology (USA). All other materials were purchased from Sigma (USA).

Plant material

Aerial parts of *Cerasus microcarpa* (Rosaceae), *Alhagi pseudalhagi* (*Fabaceae*), *Stachys pilifera* (Lamiaceae), *Amygdalus scoparia* (Rosaceae), *Ferulago angulata* (Apiaceae) and fruits of *Rosa canina* (Rosaceae) were collected from Kohgiluyeh va Boyerahmad province, Iran, and aerial parts of *Sanguisorba minor* (Rosaceae) collected from Hamedan province, Iran. The species were identified at the Herbarium of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. The voucher numbers were registered as 2870,

3281, 2175, 1998, 2800, 2343 and 3545 respectively.

Extraction

The plant materials were dried in shade and ground. Ten g of each plant powder was macerated with methanol: water (1:10) for 24 h. Then the extract was filtered and concentrated using a rotary evaporator. It was dried afterwards using a freeze dryer.

Primary culture of cerebellar granule neurons

Duo to the postnatal development of granule cells and formation of the most homogeneous neuronal population in culture, the cultured cerebellar granule neurons have been frequently used as a model for studying cellular and molecular mechanisms of neural cell death [17]. The cerebella was dissected from early postnatal mice, digested with trypsin, and triturated to obtain a single cell suspension. Then, the cells were cultured on PDL-coated cell culture plates in DMEM that consisted of 10% FBS, 4.5 g/L glucose, 25 mM KCl, 100 mU/L insulin and 1% v/v penicillin and streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Non-neuronal cell growth was inhibited by the addition of cytosine β-D-arabinofuranoside (Ara-C) to the cell culture medium at a final concentration of 20 μM, 48 h after cell plating. The medium was not changed during the culture period. After 7 days of *in vitro* (DIV7), more than 95% of the cells were detected in culture as neurons characterized by MAP2 protein immunostaining; therefore, the DIV7 cells were used for the experiments [18]. The Medical Ethics Committee of Tarbiat Modares University approved the procedures.

Cell culture treatments

For evaluation of the protective effects of the extracts against Aβ-induced cytotoxicity, CGNs were plated onto PDL-coated 96-well plates at 1×10^5 cells/well. On DIV7, cells were incubated with Aβ (10 μM) alone or with different concentrations of plant extracts for 24 h. The stock solution of Aβ peptide (1 mM) was prepared by dissolving 1 mg Aβ peptide in 1 mL sterile deionized water. The solution was stored at -80 °C until use. Prior to use, the Aβ peptide was aggregated by incubation at 37 °C for 3 days. The plant extract was dissolved in DMSO and further diluted with culture medium. The final concentration of DMSO in the medium was 0.5% or less.

The effect of extracts on the viability of CGNs was measured for determination of non-cytotoxic concentration of extracts. CGNs were plated onto PDL-coated 96-well plates at 1×10^5 cells/well. On DIV7, the cells were incubated with 200 μg/mL of each extract for 24 h. After the incubation period, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability assay

The percent of surviving neurons was estimated by the MTT reduction assay. The culture medium was removed and replaced with the medium that contained 0.5 mg/mL MTT reagent. After a 4-hour incubation period, the medium was replaced by 100 mL DMSO to dissolve the formazan crystals. Absorbance was measured at a wavelength of 570 against 630 nm as the reference wavelength. Results are expressed as a percentage of the control [18].

Statistical analysis

Data have been presented as mean±SEM of three separate experiments. Statistical analysis was performed by GraphPad Prism 5 software. Statistical differences were estimated by one-way ANOVA followed by the Newman-Keuls Multiple Comparison Test. *p* values of less than 0.05 were considered statistically significant.

Results and Discussion

Figure 1A has shown the effect of extracts (200 μg/mL) on the viability of CGNs. The results indicated that extracts had no cytotoxic effects on CGNs at 200 μg/mL concentration except for *Stachys pilifera* extract which indicated cytotoxic effect at this concentration. Therefore, the effect of different concentrations of *Stachys pilifera* extract on the viability of CGNs was tested. The results have been shown in figure 1B.

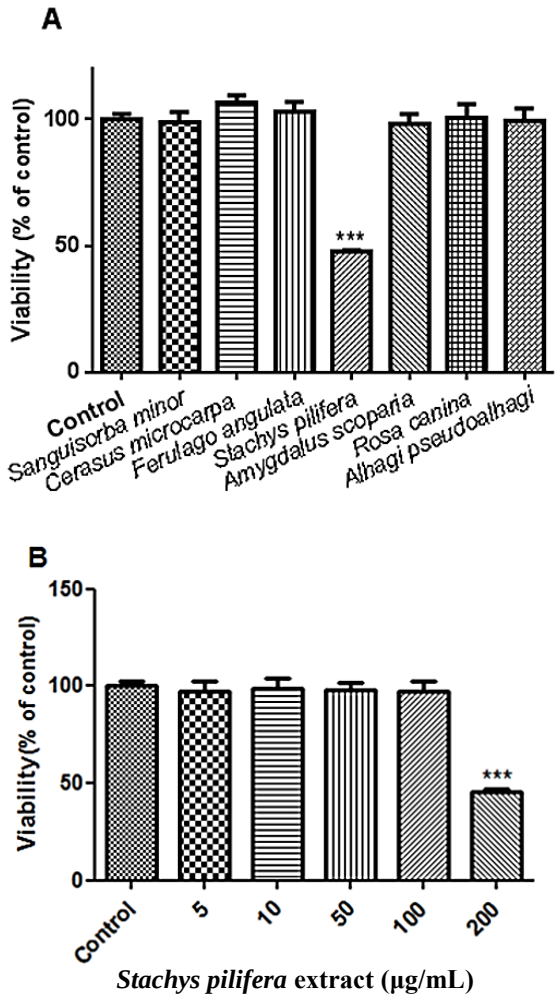

Figure 1. Effect of extracts (200 µg/mL) on the viability of the CGNs (A); effect of different concentrations of *Stachys pilifera* extract on the viability of the CGNs (B); ***$p<0.001$ *vs.* control

Stachys pilifera extract had no cytotoxic effect on CGNs at the concentrations less than 100 µg/mL. Protective effect of extracts at the concentration of 100 µg/mL against Aβ-induced cytotoxicity has been shown in figure 2. Among the extracts, *Cerasus microcarpa, Sanguisorba minor, Ferulago angulata, Rosa canina* and *Amygdalus scoparia* extracts demonstrated a significant protective effect. *Alhagi pseudalhagi* and *Stachys pilifera* extracts did not show significant protective effect against Aβ-induced cytotoxicity on CGNs. Protective effects of different concentration of *Cerasus microcarpa, Sanguisorba minor, Ferulago angulata, Rosa canina* and *Amygdalus scoparia* extracts have been shown in figure 3. A dose-dependent protective effect was observed for *Cerasus microcarpa, Amygdalus scoparia* and *Rosa canina* extracts (Figures 3B, D, E). The *Ferulago angulata* extract showed complete protective effect at concentration of 50 µg/mL and this effect decreased at high concentrations (Figure 3C) also the highest protective effect of *Sanguisorba minor* extract was observed at concentrations of 50 and 75 µg/mL (Figure 3A).

Several mechanisms have been proposed for Aβ toxicity in cell culture models and one of the important mechanisms is oxidative stress. The Aβ peptide directly or indirectly -through impairment of mitochondrial function- produces reactive oxygen species (ROS) which damage the cellular macromolecules such as lipids, proteins, and DNA and finally leads to cell death [19]. Medicinal plant extracts contain natural antioxidants can ameliorate Aβ-induced oxidative stress which has been indicated in several studies [20,21].

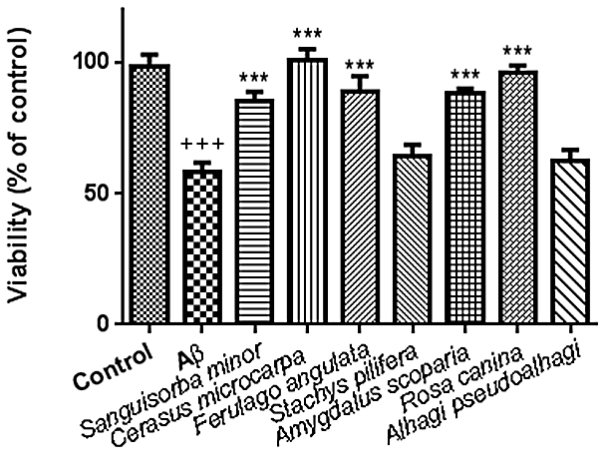

Figure 2. Protective effect of extracts (100 µg/mL) against Aβ (10µM) induced cytotoxicity on the CGNs; +++$p<0.001$ *vs.* control, ***$p<0.001$ *vs.* Aβ treated

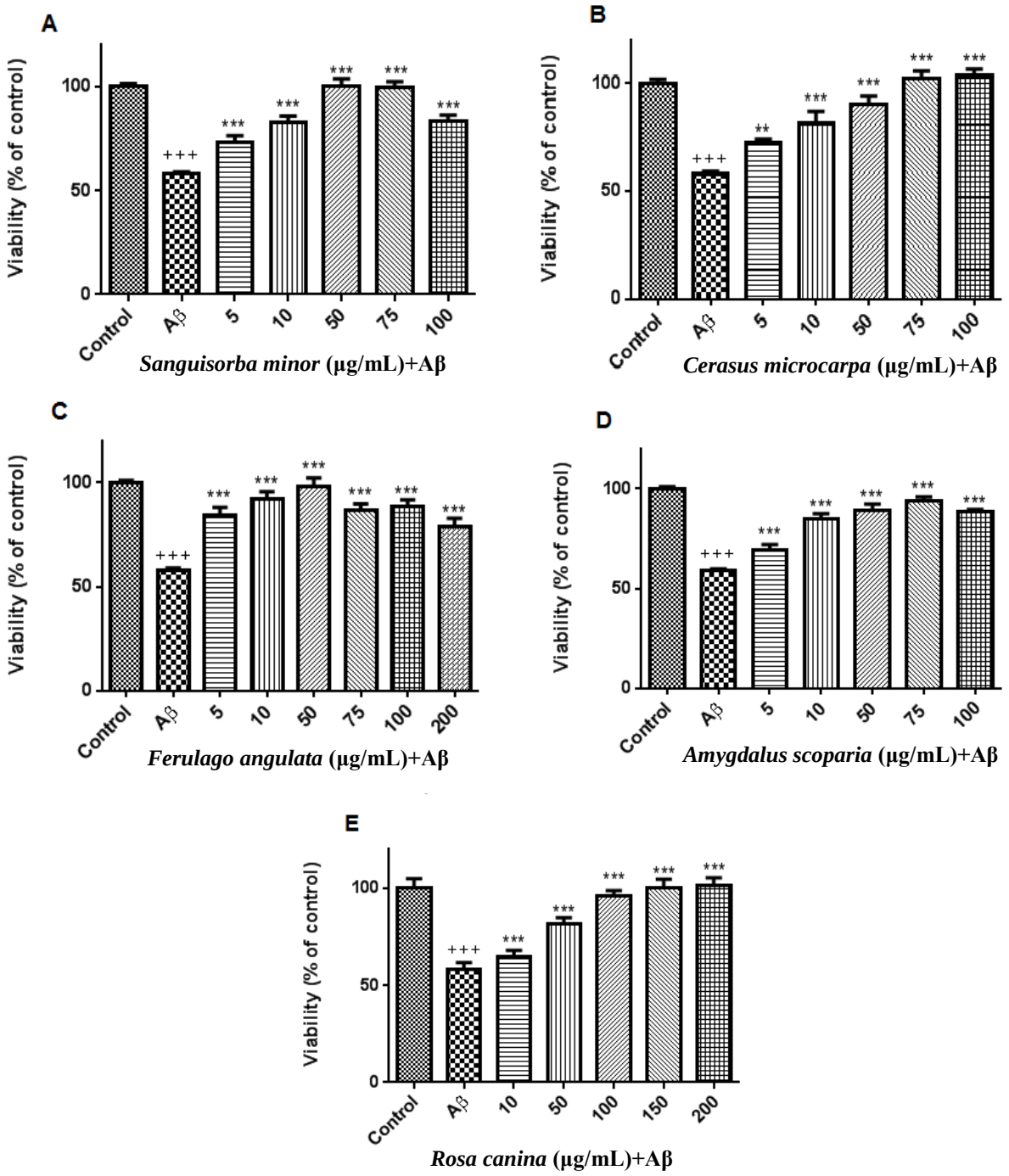

Figure 3. Protective effects of different concentration of extracts against Aβ (10 µM) toxicity on the CGNs. *Sanguisorba minor* (A), *Cerasus microcarp* (B), *Ferulago angulata* (C), *Amygdalus scoparia* (D), *Rosa canina* (E)
+++p<0.001 *vs.* control, **p<0.01, ***p<0.001 *vs.* Aβ treated

Effective plant extracts which have been studied in the present study belong to Rosaceae and Apiaceae families. Plants in these families are rich in flavonoid and polyphenolic compounds. These natural products have potent antioxidant activity and protect cells against oxidative damage directly through scavenging free radicals or indirectly through potentiating cellular antioxidant defenses such as induction of antioxidant enzymes and up-regulation of cell survival signaling pathways [22]. *Sanguisorba minor* has antioxidant and anti-inflammatory effects. High total polyphenols content has been reported for *Sanguisorba minor*. The main polyphenolic compounds isolated from this plant are gallic acid, ellagic acid, and quercetin [23]. The neuroprotective effects of these polyphenolic compounds have been reported previously [24-26]. It is suggested that the protective effect of *Sanguisorba minor* extract against Aβ-induced toxicity may be partly attributed to its polyphenolic compounds.

According to the cholinergic deficit hypothesis, acetylcholinesterase inhibitors have been investigated and currently used for the treatment of AD [3]. Also, it has been reported that acetylcholinesterase enzyme potentiates Aβ toxicity in cell culture models. Indeed, Aβ peptide has a tendency to bind peripheral anionic site of the enzyme and this attachment facilitates its aggregation and increases toxicity. Then cholinesterase inhibitors that compete with Aβ on the peripheral anionic site can attenuate Aβ toxicity in cell culture 27]. Previous studies have indicated that extracts which have been evaluated in the present study, have acetylcholinesterase inhibitory effects which can participate in their protective effect [14-16].

Several studies have suggested that another mechanism may be involved in the protective effect of herbal extracts against Aβ-induced toxicity such as inhibition of NMDA receptors, stimulation of nicotinic and dopaminergic receptors, and inhibition of mono amino oxidase enzyme-B (MAO-B) [28]. Whether these mechanisms are involved in the protective effects of the studied extracts or not are unclear and needs further investigations. *Ferulago angulata* is a plant from Apiaceae family. It is reported that *Ferulago* genius are rich in coumarin compounds [29]. They have MAO inhibitory property and some of them also have both MAO and AChE inhibitory activities [30]. On the other hand, protective effects of MAO-B inhibitors and dopamine agonist againsts Aβ toxicity have been reported [31]. Recently, researchers have been attempted to synthesize new compounds with both MAO and AChE inhibitory activities and have indicated that these compounds are more effective in AD therapy [32]. Then it is suggested that protective effects of *Ferulago angulata* extract against Aβ toxicity is due to coumarin compounds with MAO and AChE inhibitory activities.

Cerasus microcarpa, Amygdalus scoparia and *Rosa canina* are from Rosaceae family. The biological effects of the extract from the aerial parts of *Cerasus microcarpa* and *Amygdalus scoparia* have not been much studied. Only the AChE inhibitory activity was reported for these extracts [15]. The neuroprotective effect for *Rosa canina* in the combination of other herbal extracts have also been reported [33]. Because of little information about the biological effects of these extracts and their constituents, further investigation is needed to clarify the exact mechanism of neuroprotective effect of these extracts which is our plan for our future studies.

In conclusion, our study showed that *Sanguisorba minor, Cerasus microcarpa, Ferulago angulata, Amygdalus scoparia* and *Rosa canina* extracts could significantly protect against Aβ neurotoxicity and it is recommended that these extracts be further investigated for finding effective compounds for use in AD therapy.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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