Altered Micro-RNA Regulation and Neuroprotection Activity of *Eremostachys labiosiformis* in Alzheimer's Disease Model

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Keywords

Eremostachys labiosiformis · Alzheimer's disease · Amyloid beta · Apoptosis

Abstract

Background: Amyloid- β peptide (A β) is involved in the formation of senile plaques in Alzheimer's disease (AD), and causes neuronal cell death by inducing oxidative stress. **Objective:** We investigated the protective effect of *Eremostachys labiosiformis* extract against the A β -induced toxicity in SH-SY5Y cells. **Methods:** Methanolic extract from the aerial parts of *E. labiosiformis* was prepared by percolation method at room temperature. SH-SY5Y cells were treated and incubated with different concentrations of the extract for 1 h, before addition of A β . Cytotoxicity was measured 24 h after the addition of A β to the medium using MTT and reactive oxygen species (ROS) assays. Effective doses were evaluated using real-time polymerase chain reaction to evaluate expression of miR-212 and miR-132. The results were analyzed using SPSS software (16). **Results:** Exposure of SH-SY5Y cells to A β significantly affected the viability of cells and increased ROS levels. The results revealed that 1.2 and 2.5 μ g/mL of the *E. labiosiformis* extract reduced A β -induced deterioration. Only 2.5 μ g/mL of the extract could reduce

ROS levels. In addition, 5 μ g/mL of the extract increased the expression of the miRNAs, which was reduced after exposure to A β . **Conclusion:** Based on the antioxidant and protective effects of the *E. labiosiformis* extract on expression of miR-132 and miR-212 and ROS level, this herb could be used as a suitable candidate for future studies on neurodegenerative diseases including AD.

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Introduction

Alzheimer's disease (AD) is known as the most prevalent form of senile dementia among the elderly, and the number of patients with AD is rising rapidly. AD is characterized by brain shrinkage, formation of senile plaques, and nerve cell loss. The disease disturbs the patient's normal life and has serious economic implications for families and societies. Hence, prevention, diagnosis, and treatment of AD have attracted considerable attention [1, 2].

The 2 main causes of AD are formation of β -amyloid senile plaques and neurofibrillary tangles containing tau proteins. Amyloid beta (A β) is produced by amyloid precursor proteins through proteolytic processes. Accumulation of A β causes cytotoxicity via oxidative stress, mito-

chondrial dysfunction, loss of synapses, deterioration of nicotine receptors, apoptosis, and inflammatory responses. Accumulation of A β also results in formation of reactive oxygen species (ROS), which target and deteriorate DNA, RNA, and lipids [2–4]. Antioxidant herbal compounds prevent ROS formation, detoxify the cells, and inhibit the progression of diseases. Herbal compounds have recently attracted considerable attention since they can also improve symptoms of complicated chronic diseases via nonspecific multifactorial mechanisms [5–7].

Neurodegenerative diseases including AD and Parkinson's disease are non-homogenous diseases that affect the central nervous system and lead to apoptosis in different parts of the brain. Attempts to elucidate pathogenic mechanisms have not been completely successful, and researchers now believe that another level of adjusting neuronal homeostasis may exist [8, 9].

Plant cells produce 2 types of compounds: primary metabolites and secondary metabolites. Primary metabolites are directly involved in growth and metabolism. These metabolites include carbohydrates, lipids, proteins, and nucleic acids. Secondary metabolites result from biosynthesis of primary metabolites. The most important secondary metabolites are alkaloids, phenols, steroids, lignin, tannins, and flavonoids [10].

Eremostachys labiosiformis (Popov) Knorring from the *Lamiaceae* family, is a flowering plant native to Iran, Afghanistan, and Turkmenistan [11]. Sixteen species of this plant grow in Iran, and are traditionally used for treatment of various diseases [12]. A mature *E. labiosiformis* is 40–60 cm tall and flowers in spring. Studies on the phytochemical composition of this plant revealed that it contains alkaloids, saponins, flavonoids, and tannins. It also exhibits antibacterial properties [11]. The extract of this plant exhibited anticancer effects on several types of cancer cell lines [13]. However, no study has been yet carried out on the therapeutic effects of *E. labiosiformis* on AD.

MicroRNAs (miRNAs) are a group of small noncoding RNAs (containing approximately 19–25 nucleotides) that play an important role in biological processes such as cell proliferation, cell differentiation, apoptosis, strength, and fat metabolism [9, 14]. A recent study indicated that expression of miR-212 and miR-132 decreases in the brain cells of AD patients, which results in apoptosis. However, this effect can be blocked by re-expression of these miRNAs. They concluded that these miRNAs are able to exert this effect by directly regulating expression of P300, FOX3a, and PTEN as the main components of the APK pathway. Silencing these pathways by these miRNAs prevents apoptosis. Thus, the miR-132/miR-212/

Fig. 1. *Eremostachys labiosiformis* plant.

PTEN/FOX3a pathway is involved in deterioration of nerve cells in AD [15].

The present study aims to study the protective effects of *E. labiosiformis* on the cellular model of AD for the first time.

Materials and Methods

Extraction

This study was approved by the Ethics Committee of Golestan University of Medical Sciences (code: IR.goums.Rec.1394.253). To obtain the extract, the plant was first soaked in methanol. Aerial parts of the plant (flower, stem, and leaf) were washed and air-dried in the dark at room temperature (22–25 °C) for 1 week. Briefly, 150 g of the air-dried parts (leaves 50 g, stem 50 g, and flower 50 g) was ground into fine powder. Next, 100 g of aerial parts of the *E. labiosiformis* powder was mixed with 80% methanol; furthermore, to prevent chemical changes under the influence of the chemical interactions induced by sunlight radiation on the plant constituents and evaporation of the solvent, the Erlenmeyer flask was covered with foil and its lid with parafilm. The flask was placed on a shaker at 120 rpm at room temperature for 48 h. The resulting extract was filtered several times with Whatman filter papers to obtain a transparent solution. To remove the organic solvent and increase the concentration of the extract, a distillatory was used in vacuum at 40 °C. The required amount of the extract was kept at 4 °C, while the remaining extract was dried in an oven at 40 °C for 48 h for long-term storage. The latter portion was then stored at 4 °C. Figure 1 shows the *E. labiosiformis* (Popov) Knorring plant.

Cell Culture

SH-SY5Y cells were supplied by Pasteur Institute of Iran in frozen vials. The cells were placed in Eagle's Minimal Essential Medium consisting of 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 lg/mL). The cells were incubated in 95% humidity and 5% CO₂ at 37 °C. All experiments were conducted 24–48 h after culturing the cells. All experiments were carried out on similar passages. Cells were grown in a 6-well plate and permitted to attach and grow for 24 h. Then the cells were incubated with Aβ peptide and different concentrations of *E. labiosiformis* for 1 h. *E. labiosiformis* was added 30 min before Aβ peptide.

Cell Viability Analysis

After counting the cells with Neobar slides, 200 μL of Eagle's Minimal Essential Medium containing 5,000 cells were loaded onto wells of a 96-well plate. After incubation for 24 h, the medium in each well was replaced with different concentrations (0.6–5 μg/mL) of the *E. labiosiformis* extract. Next, the cells were incubated for a day with Aβ. The medium was replaced with fresh medium. Then, 10 μL of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) stock solution (5 μg/mL) were added to each well of the plate. After 2 h of incubation, the content of the wells was removed, and 100 μL of dimethyl sulfoxide was added to each well. The plate was shaken gently several times until a purple color was observed. Light absorbance for each well was read with an ELISA reader at 490 nm. The study groups are summarized in Table 1.

ROS Assay with Dichlorofluorescein Diacetate

ROS assay was performed for all study groups using dichlorofluorescein diacetate (DCF-DA), a non-fluorescent compound that produces 2,7-DCF after combining with oxygen free radicals. For this purpose, after treatment of cells with the extract, we added 1 mM of DCF-DA to each well. The plate was incubated for 10 min at 37 °C. Each well was rinsed with phosphate buffered saline 3 times, and then absorbance was read with a fluorescent reader (FLX800, Bio Tek, USA; excitation 485 nm, emission 538 nm). In addition, the radiation emitted by ROS was recorded by an ELISA reader.

Extraction of miRNAs and Determination of Expression Profiles of Pooled miRNAs

Total mRNA content of cells was extracted using a miRNeasy kit (Qiagen, West Sussex, UK; Valencia, CA, USA) according to the manufacturer's guidelines. A NanoDrop spectrophotometer (Thermo Scientific, Loughborough, UK; Wilmington, DE, USA) was used to determine mRNA quality and quantity. RNA dilutions were prepared using nuclease-free water.

In each condition, reverse transcription of miRNA (250 ng) from cells was conducted using stem-loop multiplex primer pools (Applied Biosystems, Paisley, UK; Foster City, CA, USA), facilitating reverse transcription of 48 different miRNAs in 8 real-time (RT) pools. Quantitative polymerase chain reaction (qPCR) was performed for miRNAs in a 7900HT fast RT-PCR system with TaqMan arrays (TaqMan TLDA miRNA assay version 1.0; Applied Biosystems). Overall, 197 human miRNAs were completely homologous (100%) to human miRNA. In addition, miRNAs were expressed differentially at a 1.5-fold change threshold.

Stem-Loop Reverse Transcription and RT-qPCR of miRNAs

Reverse transcription was performed for each qPCR using 250 ng of total RNA and a high-capacity reverse transcription kit

Table 1. Study groups

Groups	
Control	SH-SY5Y cell + cell culture media
Vehicle	SH-SY5Y cell + dimethyl sulfoxide solution (DMSO)
Group 1	β-Amyloid (50 μM)
Group 2	β-Amyloid (50 μM) + <i>E. labiosiformis</i> extract (0.6 μg/mL)
Group 3	β-Amyloid (50 μM) + <i>E. labiosiformis</i> extract (1.2 μg/mL)
Group 4	β-Amyloid (50 μM) + <i>E. labiosiformis</i> extract (2.5 μg/mL)
Group 5	β-Amyloid (50 μM) + <i>E. labiosiformis</i> extract (5 μg/mL)

(Applied Biosystems). MiRNA reverse transcription was performed using RT-specific primers (miRNAs including miR-212 and miR-132; Applied Biosystems). In addition, qPCR was performed individually using miR-132 and miR-212 TaqMan miRNA assays (Applied Biosystems) and 7900HT fast RT-PCR system. For normalization of miRNA expression, *RNU19* was used as the house-keeping gene. The comparative CT method (2–ΔΔCT) was applied to identify the relative fold changes in the targeted gene expression.

Data Analysis

Data are presented as mean ± SEM. Analysis of variance test was carried out for inter-group comparisons, followed by post-hoc Tukey's test for pairwise comparisons. *p* values <0.05 were regarded as statistically significant.

Results

Study of the Effect of *E. labiosiformis* Extract on SH-SY5Y Cells via MTT Assay

First, various concentrations of β-amyloid and *E. labiosiformis* extract were used to treat SH-SY5Y cells. As shown in Figure 2, after 24 h of treatment with β-amyloid, SH-SY5Y cells showed cell death with a dose-dependent trend. When treated with concentrations >25 μM, the number of the living cells decreased significantly. We used 50 μM concentration for the rest of the experiments, which had a 50% cell mortality rate. β-Amyloid decreased the viability of SH-SY5Y cells and this toxicity was dose dependent. A significant toxic effect was observed in groups that received 10, 25, 50, 75, and 100 μM β-amyloid. However, 50 μM of β-amyloid, which resulted in 55.5 ± 1.38%. As shown in Figure 3, different doses of the *E. labiosiformis* extract were applied to treat SH-SY5Y cells, and no significant effect on survival of the cells was observed after 24 h of treatment. As shown in Figure 4, to examine the protective effect of *E. labiosiformis* extract on toxicity induced by β-amyloid in SH-SY5Y cells, different doses of *E. labiosiformis* extract (0.6, 1.2, 2.5, and 5 μg/mL) were tested

Fig. 2. Effect of different β -amyloid ($A\beta$) doses on the survival of SH-SY5Y cells in MTT assays (data are presented as mean $\pm$ standard error). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significantly different versus control cells.

Fig. 4. Effect of different *E. labiosiformis* doses on the survival of SH-SY5Y cells treated with β -amyloid ($A\beta$ -50 μ M) in MTT assays (data are presented as mean $\pm$ standard error). ** p value < 0.01 , *** p value < 0.001 , significant difference from the control group + $p < 0.05$, +++ p value < 0.001 , significant difference from the 50 μ M $A\beta$ group.

Fig. 3. Effect of different *E. labiosiformis* doses on the survival of SH-SY5Y cells in MTT assays.

Fig. 5. Effect of different *E. labiosiformis* doses on SH-SY5Y cells exposed to β -amyloid ($A\beta$ -50 μ M) in ROS assays (data are presented as mean $\pm$ standard error). ** $p < 0.01$, *** p value < 0.001 , significant difference from the control group ++ p value < 0.005 , significant difference from the 50 μ M $A\beta$ group.

on cells treated with 50 μ M of β -amyloid. Based on the findings, 1.2 and 2.5 μ g/mL of *E. labiosiformis* extract showed a significant protective effect.

Analysis of the Effect of *E. labiosiformis* Extract on SH-SY5Y Cells via ROS Assay

As shown in Figure 5, the intracellular ROS level was measured in the presence of 50 μ M of $A\beta$ and 0.6, 1.2, 2.5, and 5 μ g/mL of *E. labiosiformis* extract (after 24 h of

treatment). When treated with 50 μ M β -amyloid, intracellular ROS level increased significantly, and 2.5 μ g/mL *E. labiosiformis* extract significantly reduced the ROS effect.

Analysis of the miR-212 and miR-132 Expression in SH-SY5Y Cells Treated with *E. labiosiformis* Extract

MiR-132 and miR-212 expression in SH-SY5Y cells treated with β -amyloid (50 μ M) and 1 dose of *E. labiosi-*

Fig. 6. Group 4 Effect of *E. labiosiformis* extract (2.5 μg/mL) on SH-SY5Y cells treated with β-amyloid (Aβ-50 μM) in RT-PCR of miR-132 and miR-212 (data are presented as mean ± standard error). ** $p < 0.01$, *** p value < 0.001 , significant difference from the control group ++ $p < 0.01$, +++ $p < 0.001$, significant difference from the 50 μM β-amyloid group.

formis extract (2.5 μg/mL) were studied using the RT-PCR technique. As shown in Figure 6, miR-132 and miR-212 expression decreased significantly in the β-amyloid (50 μM) group. Moreover, the *E. labiosiformis* extract treatment (2.5 μg/mL) expression drastically increased the miRNA (miRNA-132 and miRNA-212) expression.

Discussion

Numerous studies have shown the preventive and therapeutic effects of herbal compounds on AD. Long-term use of such medicinal plants can inhibit onset or progression of AD due to their antioxidant and anti-inflammatory properties, and by regulating the expression of genes involved in these processes. By modulating intracellular pathways, these plants can help prevent development and progression of AD [5, 16–18]. *E. labiosiformis* contains phenolic compounds with medicinal effects. Different species of this plant have been traditionally used in Iran for treatment of rheumatism, joint pain, scars, and snakebites. Studies have shown that different species of this plant have anti-inflammatory effects [13]. Our study is the first to investigate the protective properties of the *E. labiosiformis* extract using a cellular model of AD. The tested concentration of the *E. labiosiformis* extract had no lethal effect on SH-SY5Y cells, and inhibited Aβ-induced cell death. Concentrations of 1.2 and 2.5 μg/mL of the extract showed protective effects on SH-SY5Y cells treated with 50 μM of Aβ.

A previous study has shown the anti-cancer effects of the *E. labiosiformis* extract on several cancer cell lines [13]. The lethal effect on cancer cells and the protective effect on cell culture models of AD might be due to the modulatory effect of different compounds present in this plant on the intracellular pathways. Compounds with antioxidant properties can prevent ROS production or reduce the levels of existing ROS. Herbal chemopreventive compounds affect different intracellular pathways and exert their therapeutic effects with minimum side effects [5].

Treatment with the *E. labiosiformis* extract had no lethal or harmful effect on the cells. Moreover, treatment with 2.5 μg/mL of the extract reduced ROS levels in cells exposed to Aβ. Therefore, it can be concluded that the *E. labiosiformis* extract has antioxidant and protective properties. These results are in line with the results of previous studies on other *E. labiosiformis* species [13].

We evaluated the expression of miR-132 and miR-212 to study the effect of the extract on intracellular pathways. These miRNAs are involved in several processes related to the development and progression of AD. Expression of these miRNAs decreases in AD, schizophrenia, and Huntington's disease [19, 20]. It was also shown that aberrant expression of these miRNAs increases Aβ production and senile plaque deposition, which are involved in the development and progression of AD [21]. In our study, the expression of these miRNAs decreased drastically compared to the control group after treating the cells with Aβ (50 μM). However, treatment with 2.5 μg/mL of *E. labiosiformis* extract drastically increased the expression of these miRNAs, which indicates the protective role of the extract in prevention of Aβ-induced apoptosis.

Conclusion

Our study was the first to assess the effects of *E. labiosiformis* on a cellular model of AD. The results showed that the extract has protective effects and increases the expression of miR-132 and miR-212. Based on the protective effect observed in the MTT assay and the antioxidant properties observed in the ROS assay, it can be concluded that the extract of *E. labiosiformis* could be a suitable candidate for future studies on AD and other neurodegenerative diseases.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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Authors' Contributions

M.R.S.-B.: collected, analyzed and interpreted the data, and drafted the manuscript. L.E.: conceived the idea, designed the study, and revised the manuscript. M.J.: assisted in data analysis and preparing the final version of the manuscript. S.A.B.: collected the data and drafted the manuscript. All authors read and approved the final manuscript.

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