Research Journal of Pharmacognosy (RJP) 7(3), 2020: 25-33
Received: 12 Mar 2020
Accepted: 30 May 2020
Published online: 2 June 2020
DOI: 10.22127/rjp.2020.223143.1565

Original article

Evaluation of *Asarum europaeum* L. Rhizome for the Biological Activities Related to Alzheimer's Disease

Mina Saeedi[1,2], Yasaman Vahedi-Mazdabadi[2], Arezoo Rastegari[2], Mahdieh Soleimani[3], Mahdieh Eftekhari[4], Tahmineh Akbarzadeh[2,5], Mahnaz Khanavi[2,4,6*]

[1]Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[2]Persian Medicine and Pharmacy Research Center, Tehran University of Medical Sciences, Tehran, Iran.
[3]School of Pharmacy, International Campus (TUMS-IC), Tehran University of Medical Sciences, Tehran, Iran.
[4]Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[5]Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[6]Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, Canada.

Abstract

Background and objectives: *Asarum europaeum* L. is an herbal medicine belonging to the family Aristolochiaceae. The rhizome of the plant has been used for the treatment of various diseases in complementary and alternative medicine of various countries. In Iranian traditional medicine (ITM), the aqueous extract of the rhizome has been used for the improvement and enhancement of memory. **Methods:** In the present study, the aqueous and hydroalcoholic extracts as well as different fractions of *A. europaeum* rhizome were evaluated for their cholinesterase (ChE), acetyl- and butyrylcholinesterase (AChE and BChE) inhibitory activity via modified Ellman's method. **Results:** The ethyl acetate fraction selectively showed the most suitable anti-AChE activity (IC_{50} = 99.69 µg/mL); none of the extracts or fractions demonstrated anti-BChE activity. In this regard, the ethyl acetate fraction was candidate for the investigation of further biological activities such as antioxidant activity, neuroprotectivity, and metal chelating ability related to Alzheimer's disease. It depicted favorable neuroprotectivity at concentration of 100 µg/mL against the toxicity of exposure to H_2O_2 in PC12 cells ($p \leq 0.001$, cell viability = 80/60%) and chelating ability towards zinc, iron, and copper ions. The results of antioxidant activity by DPPH assay showed that the ethyl acetate fraction was much more potent than BHA as the reference drug. **Conclusion:** The ethyl acetate fraction of *A. europaeum* L. showed potent biological activities involved in Alzheimer's disease and needs complementary investigations to develop an herbal product against Alzheimer's disease.

Keywords: Alzheimer's disease; *Asarum europaeum*; cholinesterase inhibitors; neuroprotection; traditional medicine

Citation: Saeedi M, Vahedi-Mazdabadi Y, Rastegari A, Soleimani M, Eftekhari M, Akbarzadeh T, Khanavi M. Evaluation of *Asarum europaeum* L. rhizome for the biological activities related to Alzheimer's disease. Res J Pharmacogn. 2020; 7(3): 25-33.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder which is created via multiple mechanisms in the brain including reduction of acetylcholine (ACh) [1],

*Corresponding author: khanavim@tums.ac.ir

intracellular hyper-phosphorylated tau neurofibrillary tangles [2], accumulation of extracellular beta amyloid (Aβ42) plaques [3], β-site APP-cleaving enzyme 1 or beta secretase (BACE 1) [4], redox metal dysregulation [5], and oxidative stress caused by mitochondrial dysfunction [6]. AD is usually described by the reduction or loss of cognitive functions and intelligence in patients, which finally leads to dementia. The population of patients with AD is increasing and it has been remained as a controversial health issue in all countries [7]. Failure in the treatment of AD comes back to the multifactorial nature of the disease in such a manner that there is no certain cure. There are various compounds such as bapineuzumab, crenezumab, avagacestat, etc. acting via different mechanisms in AD such as BACE1, amyloid beta and tau aggregation inhibition which have been clinically studied for the treatment of AD; however, they kept failing [8,9]. Currently available drugs such as donepezil, rivastigmine, and galantamine [10] only improve cognitive and behavioral symptoms through inhibiting cholinesterase (ChE) enzymes, lacking a large impact on the disease itself. For this purpose, a wide range of drug candidates have been designed and synthesized based on the mechanisms involved in the creation of AD [11]; however, they have not represented successful results in clinical trials [12,13]. Accordingly, looking for an effective treatment of AD is currently in high demand and subsequently natural resources have attracted lots of attention due to lower adverse effects and more diversity comparing with currently available drugs [14]. In this regard, some anti-ChE compounds such as galantamine, physostigmine, and huperzine A have been isolated from plants [15,16]. Various extracts from medicinal plants have been investigated for biological activities involving in AD and a wide range of studies have been dedicated for natural ChEIs [17-19].

Asarum europaeum L. is an herbal medicine belonging to the family Aristolochiaceae commonly known as Asarum or European wild ginger [20]. It has been traditionally used for the treatment of diseases in many countries. *Asarum europaeum* is known as "Asaroon" in Iranian traditional medicine (ITM) and the rhizome of the plant has been frequently used in herbal formulations for the improvement of memory [21,22]. Moreover, it has been used for the treatment of epilepsy, paralysis, limb numbness, obstructive jaundice, inflammation of the liver and spleen, ascites, corneal inflammation, kidney and bladder stones, joint pain, amenorrhea, difficulty urinating, general edema, sciatica, and gout [23-25]. It has also demonstrated pharmacological properties such as antimicrobial, antitumor [26], gastroprotective, antiulcer properties [27], and AChEI activity based on TLC bioautography method in recent studies [28]. In continuation of our research on the herbal ChEIs [17,18,29,30] and focusing on the medicinal properties of *A. europaeum* recommended in ITM, here in, the rhizome of the plant was investigated for anti-ChE and antioxidant activities as well as neuroprotectivity and metal chelating ability as important pathways involved in the creation of AD. The study was conducted to develop a herbal multi-target agent to overcome failure in clinical trials of single-target drugs [8,9].

Materials and Methods

Ethical considerations

The Ethics Committee of Tehran University of Medical Sciences approved this research (IR.TUMS.TIPS.REC.1397.080).

Chemicals

Acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel, 1000 unit), butylcholinesterase (BChE, E.C. 3.1.1.8, from equine serum), and all required reagents were obtained from Sigma-Aldrich.

Plant material

The dry rhizome of *A. europaeum* was purchased from the local market in Tehran, Iran in 2018. It was identified and deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran by the voucher specimen of pmp-265.

Extraction and fractionation

The rhizome of *A. europaeum* was milled using a laboratory-scale mill and then the powder was extracted.

Hydroalcoholic extract

The hydroalcoholic extract was prepared by maceration of 200 g of powdered plant in methanol-water (80:20 (v/v)) with total volume of 1500 mL for 72 h at room temperature. The

extraction was repeated three times. The collected extract was filtered off, centrifuged at 4000 rpm for 6 min (Heraeus Megafuge 1.0, England), concentrated using a rotary evaporator under vacuum (Heidolph, Germany) at low temperature, and freeze-dried (LTE science LTD, England) at -60 °C/10 μmHg for 8 h to obtain desired extract.

Aqueous extract

The powdered plant (50 g) was transferred to conical flask containing 750 mL boiling distilled water, boiled moderately for 10 min; after that, it was cooled and filtered off. The solid residue was re-extracted by 250 mL distilled water and finally the extract was filtered, centrifuged at 4000 rpm for 6 min, concentrated using a rotary evaporator under vacuum at low temperature and freeze-dried.

Liquid-liquid fractionation

The dried hydroalcoholic extract (33.13 g) was dissolved in 110 mL methanol-distilled water (80:20 (v/v)). The solution was then subsequently fractionated by a series of liquid-liquid extractions using petroleum ether (four times, totally 1300 mL), chloroform (two times, totally 300 mL), and ethyl acetate (four times, totally 1300 mL) to afford desired extracts, respectively.

AChE and BChE inhibition assay

In vitro anti-AChE activity was performed according to the modified Ellman's method [17,27]. The stock solutions of all extracts were dissolved in DMSO and each well contained 50 μL potassium phosphate buffer (KH_2PO_4/ K_2HPO_4, 0.1 M, pH 8), 25 μL MeOH-diluted solution of each sample, 25 μL enzyme with final concentration of 0.22 U/mL in buffer. They were pre-incubated for 15 min at room temperature, then 125 μL DTNB (3 mM in buffer) was added. Characterization of the hydrolysis of ATCI catalyzed by AChE was performed spectrometrically at 405 nm followed by addition of the substrate (ATCI 3 mM in water). The absorbance measurements were recorded at 405 nm. A negative control was also performed under the same conditions without inhibitor and donepezil was used as the positive control. Four different concentrations were tested for each extract in triplicate for all tests. Similarly, BChE inhibitory assay was conducted for all extracts.

Kinetic study of AChE inhibition

Estimation of the inhibition model and inhibition constant Ki were obtained from reciprocal plots of 1/V versus 1/[S] using different concentrations of the substrate acetylthiocholine [17,31] where V is the reaction velocity and S is substrate. The experiments were completely conducted according to method of ChE assay. The rate of enzymatic reaction was recorded in the presence of different concentrations of inhibitor (0, 50, 200, and 400 μg/mL) and in the absence of inhibitor. For each experiment, the reaction was initiated by adding acetylthiocholine and the absorbance was recorded at 405 nm within 2 min. Next, double reciprocal plots (1/V vs. 1/[S]) were made using the slopes of progress curves to perceive the type of inhibition. Slopes of these reciprocal plots were then plotted against the concentrations of the A-ET and Ki was determined as the intercept on the negative x-axis. All rate measurements were performed in triplicate and data analysis was performed with Microsoft Excel 2013.

Neuroprotection study assays

Rat pheochromocytoma PC12 cell line was obtained from the Pasteur Institute (Tehran, Iran) and culture media and supplements were purchased from Gibco (Paisley, UK). Cells were cultivated in DMEM supplemented with 10% fetal calf serum plus antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin). To start neuronal differentiation, PC12 cells were re-suspended using trypsin/EDTA (0.25%), seeded in 96 well culture plate (4000cells/well), and cultured for 1 week in differentiation medium (DMEM + 2% horse serum + NGF (100 ng/mL) + penicillin & streptomycin). To examine the impact of A-Et on the survival rate of neurons, the culture medium was substituted to NGF free medium and different concentrations of A-ET (1, 10, 100 μg/mL) were applied to the cells. Quercetin (3 μg/mL) was used as the positive control. A-ET was diluted in DMEM and added to each well in the volume of 10 μL. After 3 h, induction of ROS mediated apoptosis was initiated by adding H_2O_2 (400 μM) to their medium. After 12 h, MTT assay was performed (Gerlier and Thomasset, 1986). MTT solution (5 mg/mL) was added to each well in a volume of 10 μL, and 3.5 h later, 100 μL of the solubilisation solution [10% SDS in 0.01 M HCl (w/v)] was added into each well. The plates were

allowed to stand overnight in the incubator in a humidified atmosphere. Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a plate reading spectrophotometer (BioTek ELx808, USA). Each experiment was carried out in three replicates.

Chelating assay

To investigate the biometal chelating properties of A-ET, the absorbance of methanolic solution was initially recorded at a concentration of 100 µg/mL in the wavelength range of 250-600 nm. Then, to study the chelating ability of A-ET towards metal ions (Zn^{2+}, Fe^{2+}, and Cu^{2+}), an equal volume of solutions of A-ET (final concentration of 100 µg/mL) and the desired metal ion (final concentration of 20 µM) were mixed and placed at room temperature for 30 min. Then, the absorbance of the solution was read in the wavelength range of 250-600 nm and the results were compared with that obtained from A-ET [32].

DPPH radical scavenging activity

All required materials were purchased from Sigma and antioxidant activity was determined using the DPPH assay according to our previous report [33].

Statistical analysis

All experiments were performed in triplicates. The IC_{50} values were estimated graphically from log concentration of inhibitor (extract or fraction) versus percentage inhibition curves using Microsoft Excel 2013 program. One-way ANOVA was applied to assess significant differences among the treatment groups and Tukey's multiple comparisons test was accomplished to specify the level of significance by GraphPad Prism 6 software (San Diego, CA, USA). It means statistically significant when the p-value was less than 0.05.

Results and Discussion

The hydroalcoholic extract, aqueous extract, petroleum ether fraction, chloroform fraction, and ethyl acetate fraction were obtained in 21.61%, 22.3%, 10.32%, 3.13%, and 15.84% yield, respectively and they were stored at -20 °C.

Anti-ChE activity of aqueous and hydroalcoholic extracts as well as different fractions of the rhizome of *A. europaeum* L. was evaluated comparing with donepezil as the reference drug (table 1). As shown in table1, A-ET demonstrated the most considerable and selective anti-AChE activity with IC_{50} = 99.69 µg/mL whereas the other extracts and fractions demonstrated no inhibitory activity against AChE. Furthermore, all extracts and fractions depicted no activity towards BChE.

To gain an insight into the mechanism of inhibition of AChE by A-ET, a kinetic study was performed and Lineweaver-Burk reciprocal plot was provided (figure 1). It was found that A-ET acted as a competitive inhibitor resembling the substrate to bind to the active site of enzyme. Also, the inhibition constant (Ki) was calculated as 551.9 µg/mL using secondary replots of the slope versus various concentrations of A-ET.

Table 1. Anti-ChE activity of different extracts and fractions of *Asarum europaeum* rhizome

NO	Samples	AChEI [IC_{50} (µg/mL)]	BChEI [IC_{50} (µg/mL)]
1	Aqueous extract	>500	>500
2	Hydroalcoholic extract	>500	>500
3	Petroleum ether fraction (A-PE)	>500	>500
4	Chloroform fraction (A-Cl)	>500	>500
5	Ethyl acetate fraction (A-ET)	99.69±3.850	>500
6	Donepezil	0.020±0.002	1.50±0.27

[a]Data are expressed as Mean ± SD (three independent experiments

A-ET was selected as the most potent fraction of *A. europaeum* for the in vitro evaluation of neuroprotectivity caused by H_2O_2 in distinguished PC12 neuron cells (figure 2). As depicted in figure 2, the percentage of cell viabilities were calculated at the concentrations for A-ET (1, 10, 100 µg/mL) in comparison to the H_2O_2-treated group. According to our results, PC12 cells pre-treated with A-ET significantly protected neurons against H_2O_2 at 100 µg/mL (cell viability = 80.60% with p-value < 0.001). It demonstrated no significant protectivity at 1 and 10 µg/mL.

For the investigation of metal chelating ability of the fraction A-ET, the absorbance of methanolic solution of A-ET in the range of 250-600 nm was recorded and compared with those UV-visible absorption spectra obtained from treated solution of A-ET with Zn^{2+}, Fe^{2+}, and Cu^{2+} ions (figure 3). Changes in the absorption peaks and shift to longer (red shift) or shorter (blue shift) wavelengths for treated solutions confirmed the formation of different complexes between the active ingredients of A-ET and metal ions.

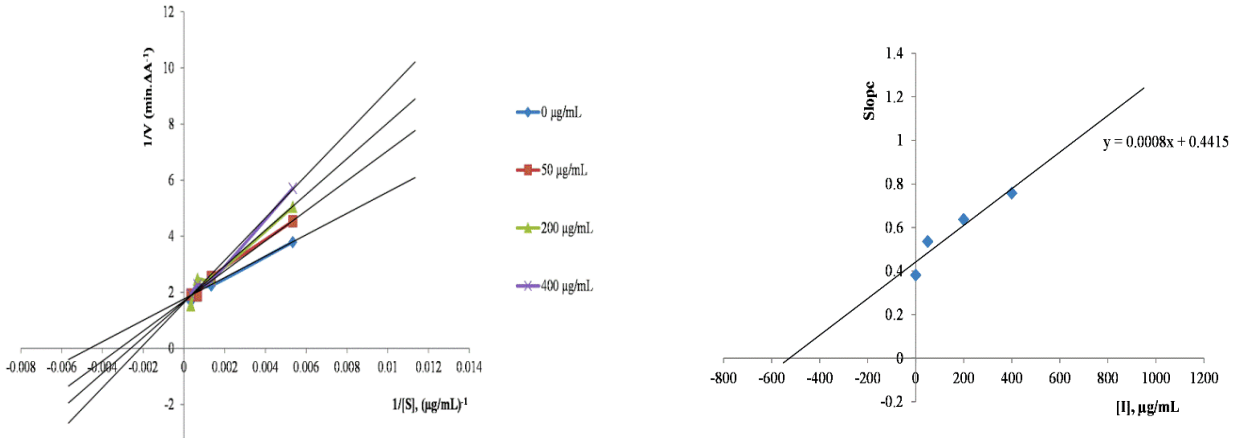

Figure 1. Left: Lineweaver-Burk plot for the inhibition of AChE by A-ET at different concentrations of acetylthiocholine (ATCh); Right: steady-state inhibition constant (Ki)

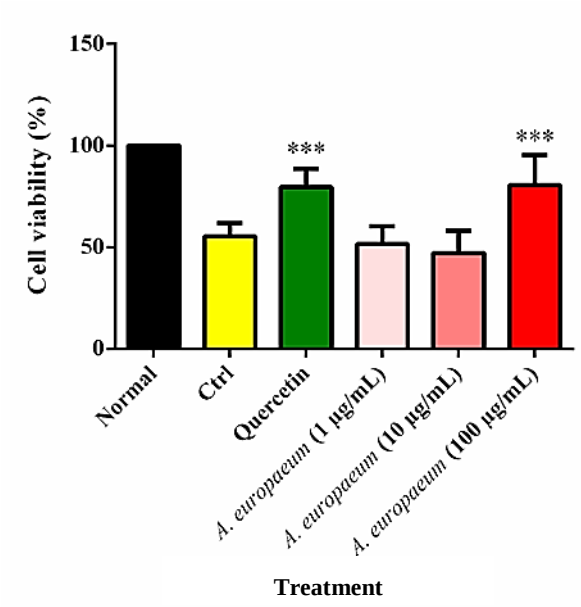

Figure 2. Neuroprotective effect of A-Et on cell viability of PC12 cells in H_2O_2-induced damage. Data were expressed as mean±SD and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed to determine the level of significance; $^{***}p < 0.001$ vs control

Hence, the compounds in this fraction are well capable of chelating the aforementioned biometals.The ethyl acetate fraction was tested for its antioxidant activity through 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity comparing with hydroxyanisole (BHA) as a standard drug. It showed suitable antioxidant activity with IC_{50} value of 45.65±0.72 comparing BHA with IC_{50} value of 91.28± 0.13 µg/mL.

The dramatic increase in the incidence and prevalence of AD among elderly people highlights the urgency of developing novel anti-AD agents. Although the "amyloid cascade" hypothesis [3] has been investigated as the most significant model of AD pathology, β-amyloid inhibitors have failed to treat the disease [8]. Multifactorial nature of AD has led researchers to consider different approaches for the pathogenesis of AD. In this regard, the relation between AD and redox metal dysregulation [5] has been emerged as a possible versatile therapeutic alternative since the presence of high concentrations of polyvalent metal cations such as Zn^{2+}, Fe^{2+}, and Cu^{2+} in senile plaques have been proved in the brains of Alzheimer's patients [34]. It has been suggested that Cu-amyloid complexes catalyze the reduction of dioxygen affording to the formation of reactive oxygen species (ROS) which plays an important role in the neuron death [35]. Also, Zn^{2+} ions are expected to be responsible for the cleavage of APP at the β-cleavage site [36]. High concentrations of Fe^{2+} ions have been considered to be involved in the pathogenesis of AD through various mechanisms such as microglia activation following with neuro-inflammation and neurodegeneration via formation of ROS. Recently, development of new iron chelators has been in the center of attention as neuroprotective agents for the treatment of neurodegenerative diseases [37]. Apart from these pathogenic factors involved in AD, the role of ChE inhibitors in the symptomatic treatment of AD can't be ignored and developing novel and efficient ChEIs are still in demand [1].

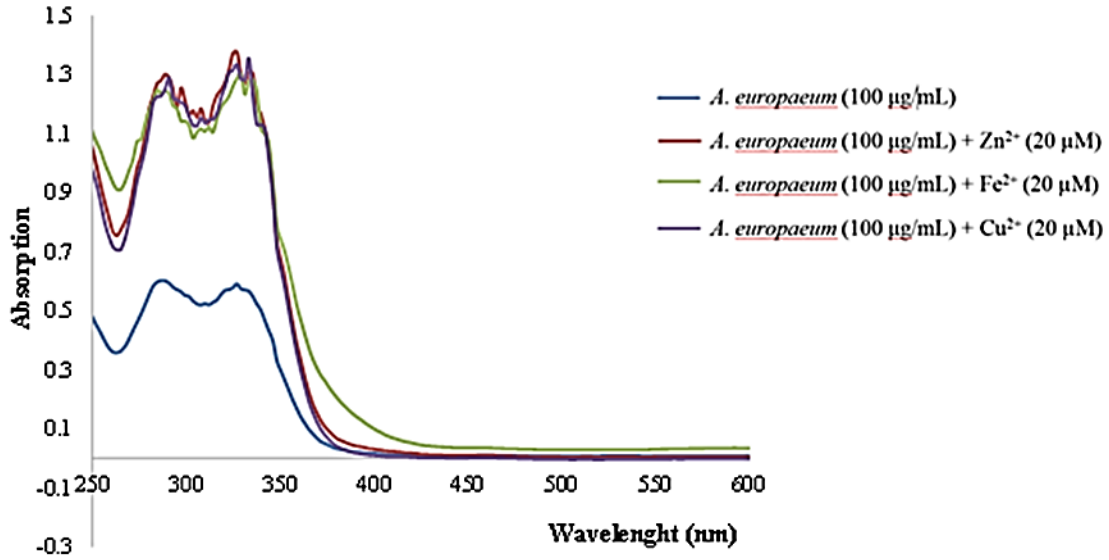

Figure 3. Metal chelating ability of **A-Et** towards Zn^{2+}, Fe^{2+}, and Cu^{2+} ions; A-ET: Fraction of ethyl acetate of *Asarum europaeum*

Natural resources as the main tools in the complementary and alternative medicines of countries, have recently attracted lots of attention in the treatment of various diseases. In this regard, a wide range of studies have endorsed the therapeutic effects of medicinal plants for the treatment of AD [15-17,38].

Asarum europaeum has been used for the treatment of different diseases in Iranian traditional medicine (ITM), and it has been frequently recommended for the improvement and enhancement of memory [21]. Considering the fact that the plants of the genus *Asarum* including *A. europaeum* L. contain secondary metabolites such as alpha-asarone and beta-asarone possessing different biological activities [20] specially neuprotectivity and anti-AD activity [39-44], the rhizome of *A. europaeum* was investigated for ChEI and antioxidant activity as well as neuroprotectivity and metal chelating ability which are important in onset and progress of AD.

In a study reported by Limón et al. [39], the effect of alpha-asarone on production of β-amyloid plaques (25-35), production of nitric oxide (NO), working spatial memory in an eight-arm radial maze, and cognitive impairment of treated male Wistar rats was investigated. The results indicated neuroprotectivity against Aβ (25-35)-caused neurotoxicity by inhibiting the effects of NO overproduction in the hippocampus and significant improvement of impairment in the spatial memory in rats. The in vitro and in vivo studies reported by Kim et al. [40] showed that alpha-asarone significantly decreased microglia-mediated neuroinflammation by inhibiting NF kappa B activation and mitigates MPTP-induced behavioral deficits in a mouse model of Parkinson's disease (PD). In addition, this metabolite diminished the MPTP-induced behavioral deficits in a mouse model of PD via suppressed microglial activation. Another study conducted by Pages et al. in various mice seizure models demonstrated that non-toxic doses of alpha-asarone (60 mg/kg) delayed onset of clonic and/or tonic seizures. Moreover, treatment of mice with the dose of 100 mg/kg induced brain antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and reductase in hippocampus and striatum to a lesser extent in cortex [41]. The study by Li et al. on beta-amyloid-induced neurotoxicity in PC12 cells showed that beta-asarone protects these cells against beta-amyloid-induced neurotoxicity via c-Jun N-terminal kinases (JNK) signaling and modulation of Bcl-2 family proteins [42]. The study by Li et al. on rats with AD confirmed that beta-asarone could improve rats' memory and learning, enhance their regional cerebral blood flow (rCBF) and cerebral metabolism, and regulate endothelin-1 (ET-1) mRNA expression in their hippocampus. The effect might be

associated with its cerebrovascular protectivity [43]. Also, the potent therapeutic activity of beta-asarone in the treatment of AD was confirmed by of Zou et al. as it could decrease beta-amyloid-induced apoptosis by the blockage of the activation of apoptosis signal-regulating kinase 1 (ASK1) in SH-SY5Y cells [44].

The literature review and our results obtained in this study revealed that the ethyl acetate fraction of *A. europaeum* could be considered as an appropriate complement useful to alleviate symptomatic treatment of AD. It could selectively inhibit AChE (IC_{50} = 99.69 µg/mL) and showed important neuroprotection against H_2O_2 at 100 µg/mL (cell viability = 80.60% with p-value <0.001). Also, considering the efficacy of the fraction via chelating Zn^{2+}, Fe^{2+}, and Cu^{2+} ions may make A-ET useful towards formation of β-amyloid plaques and production of ROS leading to neuro-inflammation and neurodegeneration. It should be noted that it depicted very good antioxidant activity by DPPH assay even more potent than BHA as the reference drug. Considering the fact that *A. europaeum* has shown low toxicity as the LD_{50} for mice in enteral administration was 417.6 mg/kg and in intra-abdominal was 310 mg/kg [45], it can be a good candidate for herbal drug discovery developments.

Acknowledgments

This study was supported by Research Council of Tehran University of Medical Sciences with project No. 97-03-33-40720.

Author contributions

Mina Saeedi designed and performed all steps and prepared the manuscript. Yasaman Vahedi-Mazdabadi contributed to the preparation of manuscript. Arezoo Rastegari performed biological activities. Mahdieh Soleimani prepared extracts and fractions. Mahdieh Eftekhari contributed to select the plant. Tahmineh Akbarzadeh supervised biological tests. Mahnaz Khanavi supervised all phases of the study.

Declaration of interest

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

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Abbreviations

A-Cl: Chloroform fraction; A-ET: Ethyl acetate fraction; A-PE: Petroleum ether fraction; NGF: Nerve Growth Factor; SDS: Sodium Dodecyl Sulfate