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**Acetylcholinesterase-inhibitory activity of Iranian plants: Combined HPLC/
bioassay-guided fractionation, molecular networking and docking
strategies for the dereplication of active compounds**

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Acetylcholinesterase-inhibitory activity of Iranian plants: Combined HPLC/bioassay-guided fractionation, molecular networking and docking strategies for the dereplication of active compounds

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Graphical abstract

Acetylcholinesterase-inhibitory activity of Iranian plants: Combined HPLC/bioassay-guided fractionation, molecular networking and docking strategies for the dereplication of active compounds

Highlights

Prangos ferulacea was the most active plant against the acetylcholinesterase enzyme.

Metabolites were analyzed by bioassay-guided fractionation and molecular networking.

Phytochemical analysis of the active extract led to the identification of 17 compounds.

Flavonoids showed a high affinity to the enzyme based on docking score values.

Abstract •

In order to search for discovery of acetylcholinesterase (AChE) inhibitors, as a therapeutic strategy for treatment of the Alzheimer's disease, twenty-five Iranian plants have been evaluated by an *in vitro* enzymatic Ellman method and molecular docking study. Each plant was successively extracted by *n*-hexane, ethyl acetate and methanol to obtain a total of 75 extracts. The inhibiting

effect of extracts was measured by a colorimetric assay in 96-well microplates. The *n*-hexane extract of *Prangos ferulacea* showed the highest AChE inhibitory activity with 75.6% inhibition at a concentration of 50 µg/mL. The chemical composition of this extract was investigated in detail based on a combination of HPLC/bioassay-guided fractionation and molecular networking techniques. The results led to the identification of seventeen compounds, one of them was a fatty acid derivative, two compounds had flavonoid structure and others were furanocoumarin type compounds. *In vitro* analysis showed that the subfraction F_{10f} was the most potent inhibitor against the activity of AChE with an IC₅₀ value of 25.2 µg/mL and good docking scores of its constituents confirming its high activity.

Keywords

Acetylcholinesterase inhibitors, Ellman assay, Molecular networking, HPLC-ESI-QToF-MS/MS, Molecular docking, *Prangos ferulacea*

1. Introduction

Dementia is one of the biggest global public health challenges facing our generation because of increasing elderly population in different countries. Dementia is a syndrome that can be caused by a number of progressive disorders that affect memory, thinking, behavior and the ability to perform everyday activities [1]. Alzheimer's disease is the most common type of dementia, which is characterized by a loss or decline of memory, difficulties with language and problem-solving and other behavioral abnormalities in the patient, mostly due to a deficit of cholinergic functions in the brain. Alzheimer's disease (AD) occurs in $\geq 40\%$ of people over the age of 85. Alzheimer's Disease International estimated there were about 47 million people living with dementia worldwide in 2016 and this number is expected to increase to more than 131 million by 2050 [2]. Alzheimer is a multifactorial disease and therefore demands multiple therapeutic approaches. One of the most important therapeutic strategies is controlling the level of acetylcholine (ACh), as a neurotransmitter in cholinergic synapses, through blocking the degradation of ACh using acetylcholinesterase inhibitors (AChEIs) [3]. Acetylcholinesterase acts as a key enzyme for hydrolyzing acetylcholine into choline and acetic acid. A few AChE inhibitors such as tacrine, galantamine, donepezil and rivastigmine have been used for treatment of Alzheimer's disease [4]. The search for new AChE inhibitors is of great interest.

Medicinal plants are rich resources of different bioactive constituents that can be used for the treatment of several diseases and inhibition of various enzymes. Therefore, the empirical approach to discover new drugs from the systematic screening of plant extracts or plant-derived substances coupled with ethnopharmacological knowledge still remains an important strategy for finding new lead compounds. One of the major challenges in natural products drug discovery is the selective isolation and identification of valuable lead compounds from a complex crude extract. HPLC-based activity profiling has been used successfully for the rapid localization and characterization

of bioactive natural products (NPs) in extracts [5, 6]. Dereplication, using computational techniques for the rapid identification of known compounds, is another important approach aspect of drug discovery and metabolomics [7, 8]. Current dereplication strategies include hyphenated techniques such as HPLC-NMR, HPLC-MS, HPLC-NMR-MS and HPLC-MS/MS [9, 10]. Molecular networking (MN), as one of the most widely used dereplication procedures, is a strategy that aids rapid identification of known metabolites in complex biological matrices using MS and MS/MS analyses. High-resolution MS data provide the precursor ion of constituents which when combined with MS/MS data can further aid dereplication of secondary metabolites in natural products by acquiring highly resolved and accurate MS/MS spectra. Since the MS/MS spectra are linked to the chemical structure of the fragmented metabolites, MN is able to group molecules according to their structural similarities. The use of MS/MS data to build molecular networks can identify compounds that are structurally related but requires MS/MS data in integrated compound databases for their identification [11]. There are different free web-based databases available including the Human Metabolome Database (HMDB) [12], LipidMaps [13], MassBank [14], National Institute of Standards and Technology (NIST) [15] and other databases that have been used for dereplication of natural products. The Global Natural Products Social (GNPS) Molecular Networking website includes a suite of libraries containing a large number of MS/MS spectra of known natural products for the purpose of dereplication [16].

To date, numerous plant extracts world-wide have been evaluated for AChEI activity [17, 18], but most Iranian species have not been studied from this aspect. Thus, to discover new herbal compounds with an AChE inhibitory effect, this study focused on the screening of 25 Iranian plants by the spectrophotometric method of Ellman on an ELISA microplate reader [19] coupled with bioassay-guided fractionation and molecular networking analysis of the most effective extracts. Some of these plants are used for food and fodder and they have also been used in Iranian

traditional herbal medicine for the treatment of different diseases. *Alhagi mannifera* (Taranjebin; family fabaceae) has been used in Iranian folk medicine as a laxative, antipyretic and expectorant and to treat hyperbilirubinemia [20, 21]. Fresh or dried leaves of *Chaerophyllum macrospermum* and *Kelussia odoratissima* (both family apiaceae) are used as a flavoring in soups and foods. *Eupatorium cannabinum* (family asteraceae), *Glaucium fimbriigerum* and *Papaver commutatum* (both in family papaveraceae) have also been used as sedatives, antitussives, analgesics, hypnotics, anticough and remedies for treatment of liver diseases. They contain many biological active constituents such as alkaloids and flavonoids which can be used for treatment of Alzheimer disease [22]. *Prangos ferulacea*, belonging to the Apiaceae or Umbelliferae family, is traditionally used as a laxative and against ruminant parasites and neurological difficulties and as a flavoring additive in yogurt [23]. It is widely used as an animal fodder because of its valuable nutritive properties [22]. Literature review revealed that a number of coumarin and furanocoumarin derivatives have been reported from this species [23]. Herein, we report the AChE inhibition activity of different plant extracts and the isolation of the active compounds from the most effective extract. Also, we generated a network of MS/MS data from three active subfractions of the *n*-hexane extract of *P. ferulaceae* to demonstrate how molecular networking can be used to dereplicate known compounds and identify close analogues. Furthermore, we have studied the key binding interactions between the most active identified compound and its AChE inhibitory activity through docking analysis.

2. Experimental

2.1 General experimental procedures

NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 500.13 MHz for ^1H NMR and 125.77 MHz for ^{13}C NMR. A 1 mm TXI microprobe with a z-gradient was used for ^1H -detected experiments. HPLC Bioassay-guided fractionation was done using a Knauer HPLC

system by SunFire C₁₈ column (250 mm × 4.6 mm) and Knauer photodiode array detector (PDA). The absorbance values were determined with a multi-well plate spectrophotometer (Elx 800 Microplate Reader, Bio-TEK, Winooski, Vermont, USA) at 570 nm. UHPLC-MS analyses were performed on a Waters Acquity UPLC system coupled to a Waters XevoTM quadrupole time-of-flight (QToF) mass spectrometer and equipped with an electrospray source with lockspray interface for accurate mass measurements. Acetylcholinesterase enzyme (AChE) from bovine erythrocyte, acetylthiocholine iodide (ATCI) and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Silica gel (70–230 mesh) was used for column chromatography, and precoated silica gel F₂₅₄ (20×20 cm) plates for TLC (both from Merck).

2.2 Plant material

Twenty-five different Iranian plants were collected from various regions of Iran and identified by Dr. Ali Sonboli. The voucher specimens have been preserved in the herbarium of the Medicinal Plants and Drugs Research Institute (MPDRI) of Shahid Beheshti University, Tehran, Iran (Table 1).

2.3 Extraction

The aerial parts of plants were dried at room temperature and ground into a fine powder in a mechanical grinder. A 20 g portion of each plant was weighed accurately in a digital balance and successively extracted with *n*-hexane, ethyl acetate and methanol using a maceration method at room temperature (3 × 200 mL). The extracts of each solvent were combined and evaporated to dryness under reduced pressure at 45°C to obtain solventless *n*-hexane, ethyl acetate and methanol extracts and further used in an Ellman assay.

2.4 Determination of AChE inhibitory activity

The AChE activity of the extracts and purified compounds and also HPLC-based activity profiling were measured according to the method developed by Ellman et al. with some modifications [19]. In this method, AChE hydrolyzes the substrate acetylthiocholine iodide (ATCI) to produce thiocholine. The reaction between 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and thiocholine causes the formation of the yellow 5-thio-2-nitrobenzoate anion, which can be detected at 405 nm. Crude extracts were prepared in two concentrations of 50 and 200 µg/mL. Concentration of the microfractions of the *n*-hexane extract of *P. ferulacea* for determination of its activity profile was 20 µg/mL. The purified compounds were also tested at concentrations ranged from 10-200 µg/mL. All samples were dissolved in DMSO and then diluted with buffer A (50 mM Tris-HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M MgCl₂.6H₂O) to give solutions with 10% of DMSO. In brief, 25 µL of 15 mM ATCI in water, 125 µL of 3 mM DTNB in buffer A, 50 µL of buffer B (50 mM Tris-HCl, pH 7.9) and 25 µL of sample were mixed together in a 96-well plate and the absorbance was measured at 405 nm five times every 15 s. The enzymatic reaction was initiated by addition of 25 µL of AChE (0.22 U/mL in buffer B) and the plate was incubated at 25 °C for 10 min. Then the absorbance was measured again eight times every 15 s. The final DMSO concentration was 1% in each well. Each assay was done in triplicate. Galantamine was used as a positive control. The percent inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample (5% DMSO in buffer A) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of the enzyme with the test sample. To avoid any increase in activity due to the extracts or spontaneous hydrolysis of substrate, the rate of reaction before addition of the enzyme was subtracted from the rate of reaction after adding the enzyme.

2.5 HPLC bioassay-guided fractionation

Selected extract was injected into the analytical HPLC system for activity profiling. An aliquot of 600 μg of the *n*-hexane extract of *Prangos ferulacea* was separated on a SunFire C18 column (3.5 μm , 10 $\times$ 150 mm; Waters) with water (solvent A) and acetonitrile (solvent B) using the following gradient: 45% B to 100% B in 20 minutes, hold for 5 min and then changed to 45% B and hold again for 4 minutes. The flow rate was 0.5 ml/min. Twenty-five one-minute fractions were collected into 96 deep well plates and dried using a nitrogen evaporator device (Ultravap, Porvair) at 40°C and submitted to bioassay. Typical evaporation time was 2 h. The dried microfractions were dissolved in 5 μL of DMSO and then diluted with 95 μL of water. This gave the stock solutions which could be used for the assays.

2.6 Extraction and isolation

The dried aerial parts of *Prangos ferulacea* (250 g) were powdered and extracted at room temperature with *n*-hexane (3 $\times$ 2L) for two days each. The extracts were concentrated under reduced pressure to afford 4.5 g of *n*-hexane extract. This extract was fractionated by open column chromatography (63–200 μm , 3.5 $\times$ 50 cm, 300 g silica gel) eluting as a gradient from 100% *n*-hexane to 100% EtOAc and then followed by increasing concentrations of MeOH (up to 50%) in EtOAc. The flow rate was approximately 15 mL/min. The effluents were combined to give 12 fractions (1–12) based on TLC patterns (bands were detected on TLC under UV or by heating after spraying with 5% phosphomolybdic acid in EtOH). Fractions were also monitored by HPLC-PDA analysis and compared with the activity profile in order to localize the bioactive compounds. They were found in F₄, F₅, F₈ and F₁₀ fractions. Fraction 4 [0.8 g, eluted with *n*-hexane-EtOAc (80 : 20)] was subjected to silica gel column chromatography (63–200 μm , 1.5 $\times$ 50 cm) and eluted with CHCl₃-EtOAc [(90 : 10), 3.0 L] to afford five subfractions (F_{4a}–F_{4e}). Subfraction F_{4c} was recrystallized from ethyl acetate to afford compound **1** (imperatorin) [24]. Subfraction F_{4b} was further separated on silica gel preparative TLC to give compound **5** [2-(4-hydroxyphenyl) ethyl

triacontanoate, **5**] [25]. From fraction **7** [0.4 g, eluted with *n*-hexane–EtOAc (70:30)] crude solid was obtained, which was triturated with acetone to afford compound **2** (heraclenin) [26]. Fraction **8** [0.35 g, eluted with *n*-hexane–EtOAc (35:65)] was separated by silica gel column chromatography (63–200 μ m, 1.5×50 cm) and dichloromethane–acetone (90:10) as the mobile phase to afford compound **3** (sprengelianin) [27]. Fraction **10** [0.4 g, eluted with EtOAc–MeOH (95:5)] was subjected to another silica gel CC (63–200 μ m, 2×40 cm) eluted with chloroform–acetone [95:5, 0.5 L; 90:10, 1 L] to give eight subfractions, of which subfraction F_{10f} was further recrystallized to afford compound **4** (oxypeucedanin) [28]. Subfraction F_{10b} was an orange solid, which after trituration with acetone and methanol was deduced to be as same as compound **2** but some minor compounds were observed on its TLC and HPLC analyses. Also, NMR spectra of compounds **3** and **4** showed some signals that were not in relation to the major identified compounds. Further, HPLC analyses confirmed the presence of compounds with low amount beside major identified compounds. Due to the high activity of mentioned fractions, identification of their constituents was the interest of this research to discover the effective chemical compounds against the AChE enzyme. The isolation of minor compounds was not possible by chromatographic methods because of the low amount of the fractions. So, a molecular networking method was performed on fraction **8** (F₈) and subfractions F_{10b} and F_{10f} to completely identify their constituents.

2.7 Mass spectrometry analysis

Chromatography was performed on a Waters Acquity UPLC system coupled to a Waters XevoTM quadrupole time-of-flight (QToF) mass spectrometer and equipped with a Z-spray electrospray ionisation (ESI) source with lockspray interface for accurate mass measurements. ESI MS data were acquired in positive ion mode over the 100–1000 *m/z* range using a survey scan method with lock correction allowing simultaneous acquisition of MS and MS/MS data for each sample. A

leucine enkephalin (0.4 $\mu\text{g/ml}$ solution in 50% acetonitrile with 0.1 % formic acid) showing ions at m/z 278.1134 and 556.2771 in positive mode was used for lock mass correction. Samples were prepared in methanol at 2 mg/ml and typically 5 μl were injected for each LC-MS analysis. A 5 μm SunFireTM C-18 column (250 $\times$ 4.6 mm) was maintained at 25°C and eluted at a flow rate of 0.4 mL/min with water + 0.1% acetic acid (solvent A) and methanol + 0.1% acetic acid (solvent B). Fraction F₈ was eluted using the following gradient: 0-5 min, 70% B; 5-10 min, to 75% B, 10-15 min, 75% B; 15-25 min, to 100% B; 25-35 min, 100% B; 35-36 min, to 70% B (return to initial conditions) and 36-40 min, 70% B. subfractions F_{10b} was eluted by the following gradient: 0-30 min, from 70% to 100% B; 30-35 min, 100% B; 35-36 min, to 70% B (return to initial conditions) and 36-40 min, 70% B. The gradient of: 0-20 min, 50% B; 20-30 min, to 100% B; 30-37 min, 100% B; 37-38 min, to 50% B (return to initial conditions) and 38-43 min, 50% B was used to elute subfraction F_{10f}. Spectra were acquired using a capillary voltage of 3.0 kV, a detector voltage of 2.2 kV and a sampling cone and extraction cone voltages of 25 V and 4.0V, respectively. The source and desolvation temperatures were set at 150°C and 250°C, respectively, with a cone and desolvation flow of 50 L/h and 600 L/h, respectively. MS/MS survey scans were set up as data dependent acquisition where the 8 most intense ions detected in the MS spectrum were selected as MS/MS precursors. Typically, the threshold for MS/MS targeting was set to 50 with targeted ions subsequently excluded from targeting for 15 seconds. MS/MS scans were obtained for selected ions with CID fragmentation, isolation width of 2.0, normalized collision energy of 36 V, activation Q of 0.250, and activation time of 30 ms.

2.8 Molecular networking

Molecular networks were created using the online work flow at the GNPS website (<http://gnps.ucsd.edu>). The data were clustered with MS-Cluster with a parent mass tolerance of 0.2 Da and a MS/MS fragment ion tolerance of 0.08 Da to create molecular networks where each

node is a consensus MS/MS spectrum and edges between the nodes indicate the degree of similarity between consensus spectra. A network was created in which edges were filtered to have a cosine score above 0.65 and more than five matched peaks. The spectra in the network were then searched against GNPS's spectral libraries. The data visualization of molecular families was performed on Cytoscape 2.8.3 using the Spring Embedded layout plug-in and displayed as a network of nodes and edges. In Cytoscape, attributes of molecular networks are tuned to help data interpretation such as edge thickness, which can be proportional to the cosine score, node size, which can be correlated to precursor ion intensity and or node color, which can be used to map various metadata associated with samples (group mapping).

2.9 Molecular docking study

The molecular docking study was carried out using the Glide application in the Schrodinger package 2016-2 (Schrodinger, LLC). The acetylcholinesterase enzyme protein structure file (PDB ID: 1EVE) was taken from PDB and was edited using protein preparation on Maestro 10.6, where all water molecules and co-crystallized ligands were removed. Partial atomic charges were assigned according to the optimized potentials for the liquid simulations (OPLS3) force field. The grid box was centered at particular residues of the protein and was generated with the Grid generation application with coordinates $x = 2.023$, $y = 63.295$, $z = 67.062$. The dimensions of the active site box were set at $40 \times 40 \times 40 \text{ \AA}$ [29]. The structures were then subjected to impact minimization with a cut off RMSD of $0.3 \text{ \AA}$. The Ligand Preparation module (LigPrep) in Maestro was used to prepare the ligands in this study. The 2D structures of the desired compounds were drawn on ChemDraw Professional 15.0 and 3D structures generated using Chem3D suite. The structures were saved in SDF format and prepared for docking. The drawn ligands were geometry optimized using the OPLS3 force field. Docking was carried out using Glide-5.5. Flexible ligand

docking was accomplished for all of the compounds (1-17). Galantamine was used as the positive control.

3. Results and discussion

3.1 Screening

Twenty-five Iranian medicinal plant materials were collected and extracted with different organic solvents (including *n*-hexane, ethyl acetate and methanol) to yield seventy-five extracts. The inhibitory activity of these extracts was evaluated *in vitro* against the AChE enzyme at two concentrations of 50 and 200 µg/mL. As shown in Table 1, the highest AChE inhibitory activity was observed for the *n*-hexane extract of *Prangos ferulacea* with 75.6% inhibition at a concentration of 50 µg/mL, followed by the EtOAc extract of *Prangos ferulacea*, the *n*-hexane extract of *Hedysarum atropatanum*, the EtOAc extract of *Glaucium fimbriigerum* and the EtOAc extract of *Plumbago europaea*, which showed inhibition values of 63.8, 60.9, 61.1 and 58.0%, respectively. The lack of reported AChE inhibitory activity of constituents of *P. ferulaceae* made this plant species particularly interesting, so it was further subjected to HPLC-based activity profiling with the *n*-hexane extract of *Prangos ferulacea* as the most active extract against AChE enzyme.

3.2 Isolation and identification of active compounds

P. feruaceae gave the highest AChE inhibitory activity of its *n*-hexane extract and was investigated in detail. A bioassay-guided fractionation method was performed to identify the bioactive components of the plant extract using a previously validated protocol [30]. In brief, 600 µg of extract was separated on an analytical C18 column. The column effluent was collected into a 96 well plate every minute to obtain twenty-five subfractions. They were dried and re-dissolved in a small amount of 10% DMSO in buffer A. Then, the activity profile was confined based on a

bioactivity test that showed the zones of activity of the extract were concentrated at three different time windows including R_{t1} 2.5-4 min, R_{t2} 6-8 min and R_{t3} 12-13 min, of which the second time window contained the most dominant peaks in the chromatogram. Fig. 1 indicates the HPLC chromatogram and the activity profiling of the time-based microfractions.

For the isolation of targeted compounds, extraction of the plant was performed on a larger scale (250 g of dried plant) and subjected to column chromatography to give twelve combined fractions with the aid of TLC analysis. To ensure the effective isolation of the most potent compounds, fractions were analyzed by HPLC-PDA and TLC to match fractions containing targeted compounds with the profile of activity. Fractions F_2 , F_7 , F_8 and F_{10} were purified by column chromatography and preparative TLC and characterized with the aid of NMR spectroscopy and molecular networking to afford fourteen known furanocoumarin compounds, two flavonoids and one fatty acid derivative.

Structures of compounds **1-5** were elucidated by 1D and 2D NMR analyses (COSY, HSQC-DEPT and HMBC) and confirmed by comparing their NMR data with those reported in the literature. The structures were determined to be imperatorin (**1**) [24], heraclenine (**2**) [26], sprengeianin (**3**) [27], oxypeucedanin (**4**) [28] and 2-(4-hydroxyphenyl) ethyl triacontanoate (**5**) [25]. A molecular networking technique was used to deduce the structures of compounds **6-17** as well as some identified compounds by NMR with the use of HRMS and MS/MS data and comparison with databases. Global Natural Products Social (GNPS) has collected available MS/MS spectral libraries of a large number of known natural products including those from MassBank [14], NIST [15] and ReSpect [31]. Altogether, these third-party libraries total 212,230 MS/MS spectra representing 12,694 unique compounds. The molecular network was generated from direct injection of the subfractions F_{10b} , F_{10f} and F_8 which each subfraction collection and also their overlapping are represented by nodes in different colors (Fig. 2). Every node is indicating a

particular ion detected by MS and the edges are representing the relationship between different nodes, with thicker lines between two particular ions representing more relationship and vice versa. Then, nodes of the network were dereplicated in an automated batch mode against the GNPS databases, which led to the identification of fifteen constituents including three isolated compounds heraclenine (**2**), sprengelianin (**3**), oxypeucedanin (**4**) as well as methoxsalen (**6**), oroselol (**7**), isopimpinellin (**8**), marmesin (**9**), phellopterin (**10**), heraclenol (**11**), oxypeucedanin hydrate (**12**), [3-hydroxy-2-methyl-4-(7-oxofuro[3,2g]chromen-9-yl) oxybutan-2-yl] (Z)-2-methylbut-2-enoate (**13**), 8-[2-(3-methylbutoxy)-3-hydroxyl-3-methylbutoxylpsoralen (**14**), rivulobirin A (**15**), quercetin (**16**) and querciturone (**17**). MS/MS fragmentation patterns of all identified compounds were annotated to verify that the fragmentation patterns were related (supporting Information, S1-S15).

Except compounds **16** and **17** which have a flavonoid structure, all other compounds are furanocoumarins that have previously been reported from several different species of the *Prangos* genus [32-34]. Individual identified compounds of each fraction were recognized with more investigation of molecular network. The constituents of subfraction F_{10b} were compounds **2**, **3**, **6**, **8**, **9**, **11**, **13** and **14** of which, compounds **2**, **3**, **8** and **9** were also found in F₈. Compounds **4**, **7**, **10**, **12**, **15**, **16** and **17** included the chemical compounds of subfraction F_{10f}. Heraclenine (**2**), sprengelianin (**3**) and oxypeucedanin (**4**) which were the major compounds of the analyzed fractions previously isolated and identified by NMR data provide confirmation between the results of the molecular networking technique and identification of compounds by purification and structural analysis. Chemical structures of the isolated compounds and also of the identified compounds from molecular networking are shown in Fig. 3. A review of the literature showed that all of the pure compounds are reported for the first time from *P. ferulacea*.

3.3 Anticholinesterase inhibitory activity

During this study, the isolated compounds (**1**, **2** and **5**) and concentrated subfractions (F_{10b}, F_{10f} and F₈) were screened for their *in vitro* AChE inhibitory activity at different concentrations using a 96-well microplate reader according to the method developed by Ellman. These compounds inhibited AChE activity in a dose dependent manner. As presented in Table 2, F_{10f} was found to be the most potent inhibitor against AChE with an IC₅₀ value of 25.2 µg/mL, followed by F_{10b} (34.9 µg/mL) and F₈ (38.2 µg/mL). IC₅₀ values of 65.1, 56.8 and 130.3 µg/mL were recorded for compounds **1**, **2** and **5**, respectively. In this study, galantamine was used as the positive control, which had an IC₅₀ value of 1.4 µg/mL.

3.4 Molecular docking

All 17 of the identified compounds were docked into the active site of acetylcholinesterase enzyme (PDB code: 1EVE) by employing the Glide module (Schrodinger suite). *In silico* studies revealed the affinity, activity and binding orientation of ligands to the target protein 1EVE. The study showed that the inhibitory activity of compounds with free hydroxyl groups was better than others. Docking results of ligands are shown in Table 3, while interactions between the most active compound (**17**) and the enzyme are shown in Fig. 4. It is clear that there are two important types of interactions with the active site of enzyme including H-bond and π - π stacking interactions with the G score value of -10.901 kJ/mol. It is shown that the hydroxyl groups of querciturone (**17**) are linked to the active site through five H-bond interactions with TYR70, ASP72, PHE288, ARG289 and TYR334 amino acids. Also, PHE290 is responsible for the π - π interaction of **17** with the enzyme. Among furanocoumarins, compound **15** was the best inhibitor with docking score of -10.40 kJ/mol due to H-bond and π - π stacking interactions of the ligand with ARG289 and TYR121 of the enzyme, respectively. Comparing the results of *in vitro* and *in silico* analyses indicated that subfraction F_{10f} had the best inhibitory activity on AChE, which could be due to the presence of some active compounds such as querciturone (**17**), heraclenol (**11**) and rivulobirin A (**15**).

The anti-AChE activity of flavonoids [35, 36] as well as furanocoumarins [37, 38] has previously been performed by Ellman method and reported in the literature supporting our results. Many MD analyses have also been performed to evaluate the interaction of different naturally occurring ligands such as flavonoids [7] and furanocoumarins [39] with the AChE enzyme.

4. Conclusion

In this study, the AChE inhibitory activity of twenty-five Iranian plants has been evaluated by the Ellman method. Four furanocoumarin type compounds and one fatty acid derivative were obtained from phytochemical analysis of the *n*-hexane extract of *P. ferulacea*, as the most active extract. A molecular networking technique was then used to clarify the unidentified compounds of the active subfractions, which led to the identification of a further fourteen furanocoumarins and two flavonoids. Molecular docking analysis of all 17 compounds was also performed on an AChE target with *in silico* results in good agreement with experimental results. Based on *in vitro* analysis, subfraction F_{10f} was the most potent inhibitor against the activity of AChE with an IC₅₀ value of 25.2 µg/mL, with strong docking scores further confirming its activity. These results propose that the *n*-hexane extract of *P. ferulacea* could be considered as AChE inhibitor due to presence of biologically active constituents including flavonoids and furanocoumarins. Further studies such as *in vitro* and *in vivo* analyses are suggested to evaluate the AChE inhibitory activity of the active compounds querciturone (**17**), heraclenol (**11**) and rivulobirin A (**15**).

Conflict of Interest

The authors declare no conflict of interest.

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Legends for Figures

Fig. 1 HPLC-based activity profile of the one-minute microfractions of the *n*-hexane extract of *P. ferulaceae*. Colored rectangles represent three retention time groups indicating more than 40% inhibition of AChE activity. The selected extract was fractionated on a SunFire C18 column (3.5 μ m, 10 $\times$ 150 mm; Waters) with water (solvent A) and acetonitrile (solvent B) using the following gradient: 45% B to 100% B in 20 minutes, hold for 5 min and then changed to 45% B and hold again for 4 minutes. The flow rate was 0.5 ml/min.

Fig. 2 The molecular network of *P. ferulaceae* subfractions with a cosine similarity score cut off of 0.65. In the network, each collection is represented by different colors. The pink, red and green circles are representing of F_{10b}, F_{10f} and F₈ nodes, respectively. Overlapping nodes are indicated by gray circles.

Fig. 3 Chemical structure of the identified compounds in the active subfractions of the *n*-hexane extract of *P. ferulacea*.

Fig. 4 Docked molecule of querciturone (**17**) in the active site of AChE (PDB code: 1EVE). The principal amino acids are evident as the vicinity of the docked molecule.

Table 1. AChE inhibitory activity of the *n*-hexane, ethyl acetate and methanol extracts of the studied Iranian plants

No.	Species	Collection area	Voucher number	Percent of inhibition (%) ^a					
				Conc. = 50 µg/mL			Conc. = 200 µg/mL		
				<i>n</i> -Hexane	EtOAc	Methanol	<i>n</i> -Hexane	EtOAc	Methanol
1	<i>Alhagi mannifera</i>	Tochal	MPH-2531	20.5 ± 2.3	30.1 ± 0.5	21.8 ± 1.5	54.3 ± 1.4	58.6 ± 0.7	35.1 ± 0.4
2	<i>Alkana trichophylla</i>	Darake	MPH-2532	40.8 ± 1.1	34.2 ± 1.3	30.0 ± 0.8	71.2 ± 0.8	65.6 ± 0.5	59.3 ± 1.2
3	<i>Cerinth minor</i>	Sis	MPH-2533	15.1 ± 1.6	22.0 ± 0.8	24.7 ± 0.4	38.9 ± 2.6	48.0 ± 0.9	59.2 ± 0.4
4	<i>Chaerophyllum macrospermum</i>	Tochal	MPH-2534	39.4 ± 0.8	31.7 ± 0.6	36.3 ± 1.5	66.3 ± 3.1	52.1 ± 1.2	60.9 ± 1.3
5	<i>Dactylorhiza iberica</i>	Sis	MPH-2535	12.1 ± 0.7	36.3 ± 1.4	28.9 ± 1.2	35.0 ± 1.4	61.3 ± 1.1	55.1 ± 2.4
6	<i>Eupatorium cannabinum</i>	Darake	MPH-2536	34.3 ± 1.6	36.7 ± 0.9	45.3 ± 2.3	68.6 ± 1.7	75.8 ± 0.8	68.8 ± 0.6
7	<i>Glaucium fimbriigerum</i>	Taleghan	MPH-2537	31.7 ± 3.0	61.1 ± 0.5	57.1 ± 1.6	64.2 ± 0.9	84.2 ± 1.3	76.3 ± 1.8
8	<i>Hedysarum atropatanum</i>	Sis	MPH-2538	60.9 ± 1.3	49.4 ± 1.7	52.0 ± 0.5	85.1 ± 2.3	81.1 ± 1.6	75.1 ± 0.8
9	<i>Heliotropium europaeum</i>	Velenjak	MPH-2539	33.2 ± 1.8	27.2 ± 0.7	30.3 ± 1.7	75.3 ± 1.4	64.2 ± 0.8	60.4 ± 0.5
10	<i>Hesperis kurdica</i>	Sis	MPH-2540	37.4 ± 0.6	38.6 ± 0.6	48.2 ± 1.3	71.7 ± 1.1	66.7 ± 0.4	81.2 ± 1.9
11	<i>Kelussia odoratissima</i>	Samsami	MPH-1835	22.9 ± 2.3	28.2 ± 1.3	36.1 ± 0.5	51.3 ± 0.6	55.9 ± 1.8	70.9 ± 1.4
12	<i>Muscari longipess</i>	Sis	MPH-2541	48.6 ± 2.1	35.0 ± 1.0	30.7 ± 1.4	81.9 ± 1.9	61.0 ± 0.6	52.4 ± 2.8
13	<i>Nepeta heliotripochya</i>	Sis	MPH-2542	17.4 ± 0.5	19.7 ± 1.2	25.0 ± 1.8	48.2 ± 1.2	45.3 ± 1.7	58.3 ± 1.2
14	<i>Nonnea anchusoides</i>	Sis	MPH-2543	19.0 ± 1.5	29.1 ± 0.4	23.1 ± 0.9	57.8 ± 2.6	60.2 ± 0.8	50.6 ± 0.4
15	<i>Onosma microcarpum</i>	Sis	MPH-2544	37.8 ± 2.2	32.3 ± 0.8	34.8 ± 0.7	70.2 ± 0.8	59.4 ± 1.3	70.3 ± 1.6
16	<i>Ornithogalum brachystachys</i>	Sis	MPH-2545	29.2 ± 1.6	36.5 ± 0.6	41.4 ± 1.2	61.8 ± 0.7	68.7 ± 2.1	79.2 ± 0.5
17	<i>Papaver commutatum</i>	Sis	MPH-2546	15.3 ± 0.6	37.2 ± 0.9	35.3 ± 1.6	40.4 ± 1.3	64.2 ± 0.6	72.4 ± 1.8
18	<i>Parietaria judaica</i>	Zirab	MPH-2547	34.1 ± 1.2	49.1 ± 0.8	41.9 ± 1.3	48.0 ± 1.5	72.1 ± 0.9	81.9 ± 1.2
19	<i>Plumbago europaea</i>	Darake	MPH-2548	27.3 ± 2.4	58.0 ± 0.7	42.2 ± 0.3	55.1 ± 1.2	85.2 ± 0.9	60.2 ± 1.4
20	<i>Prangos ferulacea</i>	Tochal	MPH-2549	75.6 ± 2.8	63.8 ± 1.3	23.2 ± 1.9	86.6 ± 1.8	81.4 ± 0.7	59.6 ± 0.6
21	<i>Pulicaria gnaphalodes</i>	Velenjak	MPH-2550	28.2 ± 3.1	41.4 ± 1.5	35.2 ± 0.7	63.1 ± 0.8	69.4 ± 1.6	75.1 ± 2.6
22	<i>Salvia urmiensis</i>	Tekab	MPH-1220	30.5 ± 0.9	32.5 ± 1.3	23.6 ± 1.4	67.7 ± 2.8	62.1 ± 1.2	57.3 ± 0.4
23	<i>Scutellaria pinnatifida</i>	Sis	MPH-2551	13.1 ± 2.1	12.8 ± 0.4	15.4 ± 0.9	42.1 ± 1.2	45.3 ± 2.4	49.4 ± 1.7
24	<i>Sorzonera latifolia</i>	Sis	MPH-2552	24.6 ± 1.7	22.3 ± 0.7	34.1 ± 1.3	49.3 ± 0.6	51.9 ± 1.5	62.2 ± 1.2
25	<i>Vincetoxicum funebre</i>	Taleghan	MPH-2553	39.3 ± 2.5	52.1 ± 1.4	48.9 ± 1.0	78.7 ± 1.8	73.4 ± 1.0	63.8 ± 0.4

^a Data are expressed as mean ± standard deviation (n = 3).

Table 2. Acetylcholinesterase inhibitory activity of the purified compounds and concentrated fractions

sample	IC ₅₀ (μg/mL) ^a
F _{10f}	25.2 ± 0.9
F _{10b}	34.9 ± 1.3
F ₈	38.2 ± 1.1
Compound (2)	56.8 ± 2.5
Compound (1)	65.1 ± 1.7
Compound (5)	130.3 ± 3.8
Galantamine	1.4 ± 0.1

^a Data are expressed as mean ± standard deviation (n = 3).

Table 3. Molecular docking analysis of identified compounds from *P. ferulaceae* against the AChE enzyme

Comp.	Docking score (KJ/mol)	Comp.	Docking score (KJ/mol)
1	-7.122	10	-7.608
2	-8.465	11	-7.944
3	-8.29	12	-9.317
4	-7.555	13	-8.779
5	-7.486	14	-9.098
6	-6.027	15	-10.398
7	-6.265	16	-8.582
8	-6.483	17	-10.901
9	-7.086	Galantamine	-9.406

Fig. 1

Fig. 2

Fig. 3

Fig. 4