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LC-MS-based metabolomic profiling and anti-acetylcholinesterase study of selected *Salvia* subg. *Perovskia* species

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ABSTRACT

Objective(s): This study focused on three *Salvia* subg. *Perovskia* species, *Salvia yangii*, *Salvia abrotanoides*, and *Salvia artemisioides*, aromatic perennials native to Iran's Baluchestan region and traditionally used in European, Chinese, Iranian, and Ayurvedic medicine to manage memory impairment and dementia. The research aimed to characterize the extracts and essential oil (EO) metabolites, and to evaluate their anti-acetylcholinesterase potential to identify bioactive compounds associated with cognitive health.

Materials and Methods: In this study, a high-performance liquid chromatography-based approach coupled with linear ion trap-Orbitrap mass spectrometry (HPLC-LTQ Orbitrap) and GC-MS techniques were used for metabolomics of extracts and essential oils, respectively. Four pure compounds (carnosol, carnosic acid, cryptotanshinone, and 1α-hydroxydemethylsalvicanol), as well as extracts and EOs, were tested for their ability to inhibit acetylcholinesterase using the Ellman method.

Results: LC-MS analysis of ethyl acetate extracts revealed 55 compounds, most of which were diterpenoids. The primary components found in all three species were carnosol, deoxocarnosol, carnosic acid, rosmanol, and ethoxyrosmanol. The most effective inhibitors of acetylcholinesterase (AChE) were found to be the pure substances cryptotanshinone and 1α-hydroxydemethylsalvicanol, which demonstrated 15.78±0.1% and 12.7±0.1% inhibition at 450 µg/ml, respectively. The EtOAc extract of *Salvia abrotanoides* aerial part (AP) showed 12.96%±0.1% inhibition, while *S. atriplicifulia* AP showed 7.83%±0.3% inhibition. EOs were weak inhibitors, not exceeding 5%.

Conclusion: The findings indicate that pure compounds from selected plants hold promise for developing dietary supplements for Alzheimer's disease (AD), whereas extracts and EOs exhibited limited effects and no significant synergy.

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Introduction

Salvia, known as 'Maryam Goli' in Persian, is an important genus within the Lamiaceae family, comprising over 1000 species. The subgenus *Perovskia* within the extended genus of *Salvia* encompasses several Central Asian medicinal and aromatic species, among which *Salvia yangii* (formerly known as *Perovskia atriplicifolia*) and *S. abrotanoides* are the most widespread. Both species, along with *Salvia artemisioides*, are distributed in the Taftan region of Sistan and Baluchestan province in southeastern Iran (1, 2). Traditional medicine systems, including those of Europe, China, and Ayurveda, have long recognized the therapeutic potential of *Salvia* plants. For instance, Nao Li Kang, a Traditional Chinese Medicine formulation that incorporates *Salvia*, has demonstrated efficacy in approximately 40% of AD patients (3). Prominent herbalists such as John Gerard (1597), Nicholas Culpeper (1652), and John Hill (1756) acknowledged that sage could enhance mental acuity and sensory perception, thereby benefiting memory and cognitive function. These traditional uses, along with the anti-oxidant properties of Salvia species, are thought to help slow age-related cognitive decline (4). Salvia plants have shown promise in treating AD due to their cholinergic activity. This is significant because many current nootropic and anti-dementia drugs work by inhibiting cholinesterase to enhance cholinergic neurotransmission (5). Research indicates that various *Salvia* species and their terpenoid compounds, particularly abietane diterpenoids, may possess potent inhibitory activity against cholinesterases (6). Additionally, these plants exhibit a range of other beneficial biological activities, including effects on amyloid-beta (Aβ), neurotrophins, oxidative stress, and inflammation, as well as antimicrobial and anxiolytic/antidepressant properties (7-9). Preventative and symptomatic treatments for AD are increasingly adopting a multi-target drug strategy that focuses on several key areas, including anti-inflammatory and antioxidative mechanisms, estrogenic pathways, nicotinic receptors, nerve growth factors, and the formation of neurofibrillary tangles and Aβ plaques (10). Certain natural insecticides, including essential oils (EOs), work by disrupting the insect nervous

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system, ultimately leading to paralysis. These insecticides primarily target octopamine synapses and GABA receptors and inhibit acetylcholinesterase (11, 12). Our previous investigations demonstrated that essential oils (EOs) from *Salvia* species exhibit notable insecticidal effects, which are likely attributable to their terpenoid constituents. Our findings demonstrate that essential oils from *Salvia* species exhibit notable insecticidal effects, likely attributable to their terpenoid constituents (13). Additionally, our previous work investigated the anti-inflammatory effects of tanshinone-related diterpenes from *Perovskia artemisioides* (*S. artemisioides*) roots. These compounds were found to significantly inhibit nitric oxide release and the expression of pro-inflammatory enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (14). Furthermore, research indicates that oxidative stress plays a critical role in the onset and progression of several pathologies, including neurodegenerative disorders, inflammatory diseases, cancer, and diabetes mellitus (15). These species have also shown a significant relationship between their phenolic content and their ability to scavenge free radicals (16). Building upon our established research background and these collective findings, the present study was designed to further investigate the effects of these species on cholinesterase activity.

Continuing our studies on the discovery of bioactive constituents from Iranian *Salvia* species, we herein characterized the specialized metabolites of the ethyl acetate (EtOAc) extract from the aerial parts using a high-performance liquid chromatography (HPLC)- based approach coupled with linear ion trap-Orbitrap mass spectrometry (LTQ-Orbitrap). Furthermore, in pursuit of novel acetylcholinesterase (AChE) inhibitors, this study examined the extracts derived from Salvia species' roots and aerial parts, their EOs (rich in monoterpenoids), and isolated diterpenoid compounds, including carnosol, carnosic acid, cryptotanshinone, and 1α-hydroxydemethylsalvicanol.

Materials and Methods

Chemicals

The solvents (water, methanol, and acetonitrile) were obtained from Romil Ltd (Cambridge, United Kingdom). Sigma-Aldrich provided the following chemicals: acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel, 1000 units), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATChI). All chemicals involved were of analytical reagent standard (98.0–99.9%).

Plant material

Branches, leaves, and roots of three *Salvia* species (*S. yangii*, *S. abrotanoides*, and *S. artemisoides*) were collected from Taftan Mountain in the Sistan and Baluchestan Province (Iran) during the flowering season in June and July 2022. The region has an extremely arid, warm, windy, and dusty bioclimate. The collection was conducted by Professor Dr. Valiollah Mozafarian of the Research Institute of Forests and Rangelands in Tehran Province, who identified the plants. Voucher specimens (SCH-230, SCH-231, and SCH-232) were deposited in the herbarium of the Department of Production and Utilization of Medicinal Plants at the University of Saravan.

LC-MS analysis of EtOAc extract

The study employed an Accela HPLC system connected to an ESI source and a high-resolution mass analyzer (LTQ-Orbitrap XL), both provided by Thermo Fisher Scientific. Specialized metabolites were identified using tandem MS analyses, following initial full-mass and data-dependent scans in positive ion mode. ESI-MS/MS experiments were conducted with a collision energy of 35.0%. Chromatography was performed on a C18 reversed-phase Phenomenex Luna MS C18 column (5 µm, 150 mm × 2.0 mm; Aschaffenburg, Germany), using a binary mobile phase consisting of ultrapure water (0.1% formic acid) and acetonitrile (0.1% formic acid). The gradient increased from 5% to 60% acetonitrile over 35 min, and then to 100% over 55 min, at a flow rate of 0.200 ml/min and with an injection volume of 10.0 µl (17).

Essential oil extraction and compound identification

The essential oils obtained by hydrodistillation from the aerial parts of the species were analyzed using a DB-5 fused silica capillary column, a Thermo Quest GC-FID, and a Finnigan GC-MS instrument. Helium was used as the carrier gas. The ionization voltage was set at 70 eV, and the temperatures were set at 200 °C and 250 °C for the ion source and the interface, respectively. The mass range analyzed was 45-456 amu (18). Kovats indices, calculated for n-alkanes (C8-C21), were used to identify volatile compounds by comparison with published data and mass spectral patterns from the NIST library (19). The quantities of volatile constituents were expressed as a percentage of peak surface area relative to the total peak surface area from the flame ionization detector (FID) at 250 °C.

Isolation of pure compounds

The powdered roots and aerial parts of the plant were extracted by maceration with EtOAc at room temperature. The extract was concentrated under vacuum to afford a reddish-green, gummy residue. In a previous study, these extracts were fractionated by column chromatography on silica gel and further purified by semi-preparative HPLC-UV, enabling the isolation and identification of known diterpenoids. The structures of these compounds were determined using 1D and 2D NMR spectroscopy (7, 14). The NMR spectra are shown in the supplementary figures (S1-S8).

Bioassays

Acetylcholinesterase inhibition assay

The assay method was based on the work of Ellman *et al.* (1961) (20). A typical reaction mixture consisted of 25 µl of AChE solution at a final assay concentration of 0.06 U/ml and 50 µl of 50 mM Tris-HCl buffer solution (pH 8.25) containing 3 mM DTNB (5,5'-dithiobis (2-nitrobenzoic acid)). Subsequently, 5 µl of the sample solution (oils and individual constituents) in 53% ethanol (EtOH) was added. The reactants were combined in a 96-well, flat-bottomed polystyrene microtiter plate and preincubated at 30 °C for various durations. To initiate the reaction, 25 µl of ATChI was added to reach a final concentration of 15 mM, and each sample was tested in triplicate. Hydrolysis measurements were taken after a 6-min incubation to reduce enzyme inhibition by ethanol. Controls contained 5 µl of 53% ethanol instead of the inhibitor solution and were also tested in triplicate. Two blanks were prepared in triplicate to check for non-enzymatic hydrolysis. During the kinetic run, the Titertek Multiskan MCC/340 microplate reader measured

Table 1. Acetylcholinesterase (AChE) inhibitory activity of Salvia species extract and pure compounds and Galantamine as positive control

Extracts and pure compounds	Concentration (µg/ml)	AChE (inhibition %)
Salvia abrotanoides aerial parts	450	13.00±0.2
Salvia artemisoides aerial parts	450	0.24±0.43
Salvia yangii aerial parts	450	7.83±0.09
S. yangii roots	450	1.70±0.1
S. artemisoides roots	450	0.60±0.3
Cryptotanshinone	450	15.78±0.1
Carnosol	450	11.34±0.5
Carnosic acid	450	5.14±0.54
1α-Hydroxydemethylsalvicanol	450	12.17±0.1
Galantamine	120	50±0.6
Extracts and pure compounds	Concentration (µg/ml)	AChE (inhibition %)
S. abrotanoides aerial parts	450	13.00±0.2
S. artemisoides aerial parts	450	0.24±0.43
S. yangii aerial parts	450	7.83±0.09
S. yangii roots	450	1.70±0.1
S. artemisoides roots	450	0.60±0.3
Crypto tanshinone	450	15.78±0.1
Carnosol	450	11.34±0.5
Carnosic acid	450	5.14±0.54
1α-Hydroxydemethylsalvicanol	450	12.17±0.1
Galantamine	120	50± 0.6

absorbance at 412 nm for 6 min at 30 °C using Genesis-Lite Windows microplate software. Galantamine was used as a positive control. The results of the AChE inhibitory activity are shown in Tables 1 and 2.

The percent inhibition was obtained using the formula outlined below:

(Absorbance of control−Absorbance of sample)/ Absorbance of control×100.

Statistical analysis

All bioassays were performed in triplicate (n=3). The mean±standard deviation (SD) was calculated for each inhibition value.

Results

Identification of volatile compounds in Salvia species

The GC-MS analysis of the three studied *Salvia* species EOs is shown in Table S1. A total of thirty-seven compounds were identified in *S. yangii*, twenty-four compounds in *S. abrotanoides*, and twenty-one compounds in *S. artemisioides*, representing 99.03 %, 98.75 %, and 98.39% of the total essential oil compositions, respectively. *S. yangii* had the highest yield of essential oil among the species studied. The results showed that the essential oils of *S. abrotanoides* and *S. yangii* contain a significant proportion of oxygenated compounds. In contrast, the essential oil of *S. artemisoides* is primarily composed of hydrocarbons.

LC-HRMS analysis of specialized metabolites in Salvia species

A phytochemical study was performed using an analytical method based on LC-ESI/LTQ Orbitrap/MS/MS^n to identify metabolites in the EtOAc extract of the aerial parts of *Salvia* species. All samples showed a comparable qualitative fingerprint in their chromatograms (Figure 1), and a comprehensive compilation of all identified compounds is outlined in Table S2. The analysis revealed that the Salvia species extracts contained several terpenoid derivatives, in particular a sesquiterpene (5), a range of diterpenes (1, 3, 4, 6-52, 54, and 55), a triterpene (53), and a flavonoid (2). Overall, the samples were particularly rich in diterpenoid derivatives, including abietane and nor-abietane diterpenoids, seco-nor-abietane diterpenoids (9, 35, 36, and 55), icetexane diterpenoids (25, 42, 44, and 45), tanshinones (1, 14, 22, 25, 27, and 44), tropolone diterpenoids (3, 8, 29, 34, and 38), lactone and spirolactone diterpenoids (21 and 39). The ESI-MS/MS spectra of the tanshinone derivatives revealed a characteristic fragmentation pattern.

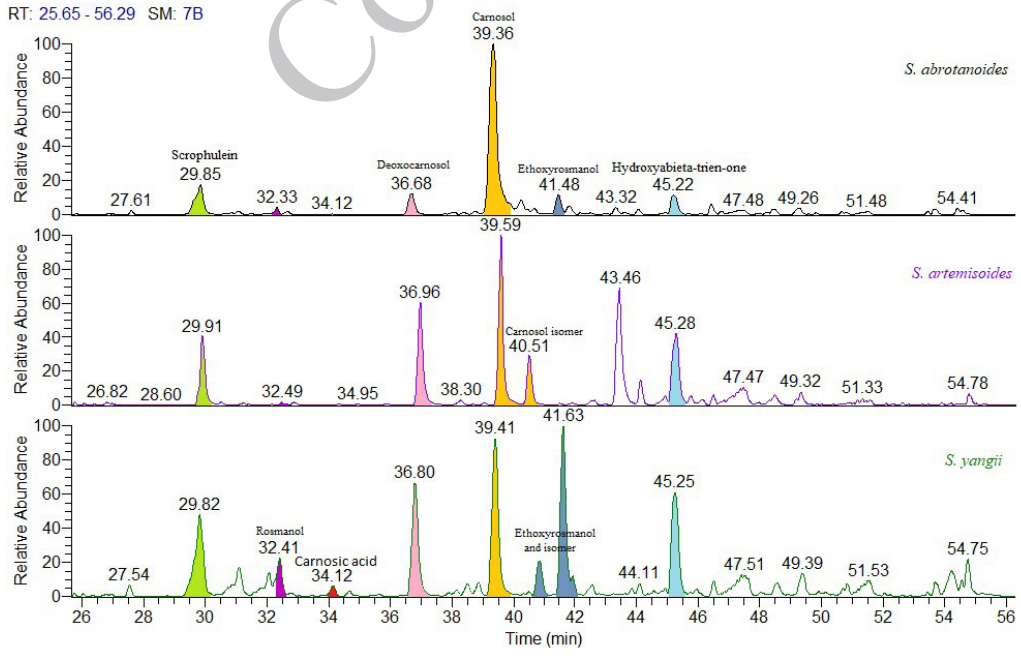

Figure 1. LC-MS chromatogram of metabolites from the ethyl acetate (EtOAc) extract of *Salvia* spp. aerial parts

Table 2. Enzyme inhibitory activity of *S. yangii, S. abrotanoides, S. artemisioides, mixture of S. yangii and S. artemisoides* EOs and galantamine as positive control

Plants EO	Major compounds	Concentration (µg/ml)	AChE (inhibition %)
S. yangii	1,8-cineole (12.25%) and linalyl acetate (11.68%)	450	2.16±0.08
Mixture of *S. yangii* + *S. artemisoides*	1,8-cineole (30.03%), linalyl acetate (11.68%), *δ*-3-carene (37.22%) and veridiflorol (20.24%)	450	3.77±0.07
S. abrotanoides	geranyl acetate (56.34%), and 1,8-cineole (10.23%)	450	4.51±0.41
S. artemisoides	*δ*-3-carene (28.59%), veridiflorol (19.44%), and 1,8-cineole (17.78%)	450	3.90±0.02
Galantamine		120	50±0.6

AChE: Acetylcholinesterase; EO: Essential oils

Specifically, the spectra of compounds 1, 14, 22, 25, 27, and 44 showed the loss of H_2O $(M+H-18)^+$, which was attributed to enolic rearrangement of a carbonyl group. This was followed by the loss of CO from the fragment ion, resulting in the ion $(M+H-46)^+$. This finding is consistent with those reported in the literature for tanshinone derivatives (21). Peaks corresponding to compounds 7, 11, 13, 32, 33, and 36 showed a significant fragment ion, $(M+H-CO_2)^+$, indicating a fragment typically formed by decarboxylation of the lactone function, which aligns with findings reported in the literature on carnosol and rosmanol derivatives (22). Several key spectral fragments support the presence of compounds 9, 35, and 55. Notably, the $(M+H-H_2O)^+$ fragment ion at m/z 313 indicates the loss of one hydroxyl group, while the $(M+H-2H_2O)^+$ ion at m/z 295 indicates the loss of two hydroxyl groups. Additionally, the fragment ions $(M+H-CO_2)^+$ at m/z 287 and $(M+H-2CO_2)^+$ at m/z 243 correspond to the loss of two carboxyl groups. Furthermore, the fragment ion $(M+H-CO-H_2O-43)^+$ at m/z 242, associated with the radical cleavage of the isopropyl group, provides additional evidence for the identification of these compounds, each of which contains two carboxyl moieties. Peaks corresponding to other diterpenoids were analyzed and identified by comparing their characteristic fragmentation patterns with those reported in the literature (23). Neutral pentacyclic triterpenoids with only one or two hydroxyl groups are not easily ionized during electrospray ionization (ESI). The presence of betulinic acid (**53**) in these species can be clearly detected by the fragment ions associated with the loss of a hydroxyl group $(M+H-H_2O)$ at m/z 439 and the loss of a carboxyl group $(M+H-CO_2)$ at m/z 413. The ESI mass spectrum showed the presence of scrophulein (**2**) by displaying a demethoxylated molecule $(M+H-CH_3)^+$ at m/z 300. Further loss of CH_3 from this fragment produced the ion $(M+H-30)^+$ at m/z 285, which is consistent with the literature data on flavonoids (24).

AChE inhibitory activity potential of the pure compounds

The inhibitory effects of various purified compounds, including cryptotanshinone, 1*α*-hydroxydemethylsalvicanol, carnosol, and carnosic acid, were investigated on AChE (Table 1). These compounds are present in high quantities in *S. artemisioides* (7) and are typical of three types of terpenoids: norditerpenoids, icetexane, and abietane diterpenoids. As shown in Table 1, the compounds studied in *Salvia* species were significantly more potent AChE inhibitors than the EOs, with inhibition of around 15%. The AChE inhibitory activity of the pure compounds showed that cryptotanshinone and 1*α*-hydroxydemethylsalvicanol are the most potent inhibitors, with 15.78±0.1% and 12.17 ±0.1% inhibition at 450 µg/ml, respectively.

AChE inhibitory activity potential of the essential oil

Yield and chemical composition of essential oils

EOs from each species were analyzed, and their yields and chemical compositions are documented in Table S1. Additionally, GC-MS analysis revealed the presence of 42 components in the isolated EOs from the three species. According to Table 2, the *in vitro* AChE inhibition assay results for EOs indicated moderate-to-low inhibitory activity. Analysis of cholinesterase inhibition revealed that the *S. abrotanoides* EO, which is rich in geranyl acetate (56.34%) and 1,8-cineole (10.23%), exhibited the strongest AChE inhibitory activity at 4.51±0.41%. Moreover, *S. artemisioides*, which contains *δ*-3-carene (28.59%), viridiflorol (19.44%), and 1,8-cineole (17.78%), showed an inhibition value of 3.90 ± 0.02%. A mixture of *S. yangii* and *S. artemisioides* showed an inhibition value of 3.77±0.07%. In contrast, *S. yangii*, containing 1,8-cineole (12.25%) and linalyl acetate (11.68%), exhibited a lower inhibitory activity of 2.16±0.08%. Notably, 1,8-cineole was detected as the main compound across all Salvia species, making it ubiquitous among the isolated EOs. Contrary to our previous research on potato aphids, the combined essential oils did not exhibit significant inhibitory effects on AChE. However, further research is needed to identify the specific bioactive compounds responsible for the desired biological effects. In addition, there is a significant need for studies on the toxicology and bioavailability of these essential oils.

Discussion

Tanshinones belong to the class of diterpenoids and consist of nor-abietane quinones, which are typically red or orange. They are associated with various pharmacological activities, including anti-aging, anticonvulsant, anticancer, and antioxidant properties, mainly through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) (11). Among the natural oxygenated norditerpenoids derived from the traditional Central Asian medicinal plant *P. atriplicifolia*, Ślusarczyk *et al.* reported that only the compounds (15R)-1-oxoaegyptinone A and isograndifoliol exhibited moderate AChE inhibition, with IC_{50} values

of 329.8 μM and 342.9 μM, respectively. Our results are consistent with those of Ślusarczyk *et al.* (2020), demonstrating that oxygenated norditerpenoids moderately inhibited AChE activity (11). Compounds derived from *P. atriplicifolia* were evaluated for their ability to inhibit cholinesterase activity. Notably, oleanane and ursane derivatives exhibited the highest efficacy, with IC_{50} values of 9.50 mM for acetylcholinesterase and 13.52 mM for butyrylcholinesterase, compared with galantamine, which showed an IC50 of 8.51 mM (25). Furthermore, Wong *et al.* (2010) demonstrated that the diterpene cryptotanshinone extracted from the roots of *S. miltiorrhiza* is an inhibitor of both human acetylcholinesterase and butyrylcholinesterase with IC_{50} values of 4.09 and 6.38 μM, respectively. The results showed that chronic oral administration of it can reverse scopolamine-induced cognitive deficits in rats. Docking analyses of cryptotanshinone in the active sites of the two cholinesterases suggested that it interacts primarily via hydrophobic interactions with the allosteric site within the active-site gorge (26). Galantamine was used as the positive control in this study. This reversible, competitive AChE inhibitor and FDA-approved drug acts through dual mechanisms: it inhibits AChE and acts as an allosteric potentiating ligand for nicotinic acetylcholine receptors (27, 28). The higher apparent IC_{50} observed for galantamine is primarily attributed to the high substrate concentration (15 mM ATChI) used in our protocol. Under the Cheng–Prusoff principle (IC_{50} = Ki * (1 + (S)/Km)), high substrate levels shift the apparent IC_{50} of competitive inhibitors to significantly higher values (29), a kinetic phenomenon supported by cholinesterase assays. Jamshidnejad-Tosaramandani *et al.* (2021) reported a significant shift in the inhibitory concentration of rivastigmine at elevated substrate levels. Moreover, using electric eel AChE (Electrophorus electricus) instead of the human enzyme resulted in reduced binding affinity and higher IC50 values due to structural differences in the active-site gorge (30).

Although the findings of this study regarding the inhibitory effects of cryptotanshinone and other diterpenoids differ from those of galantamine and the potency reported by Wong *et al.*, a key difference in how cryptotanshinone acts as a cognitive and memory-enhancing agent, as well as an anti-Alzheimer's agent, may be due to mechanisms beyond direct enzyme inhibition. Cryptotanshinone's distinct effects, compared with galantamine, could be attributed to its ability to modulate Aβ aggregation or to its anti-inflammatory and antioxidant properties (8, 14). These effects may be related to the mitigation of β-amyloid aggregation or other mechanisms. According to Perry's research, the focus of Alzheimer's disease drug development is shifting toward a multi-target drug strategy. This approach acknowledges the complex pathology of AD by targeting multiple biological pathways, including neuroinflammation and oxidative stress (10).

The anti-Alzheimer effect and memory-enhancing properties of *Salvia* species may be related to their impact on Aβ aggregation or other mechanisms. Ayoub *et al.* (2022) reported the potential therapeutic benefits of *Salvia officinalis* cultivated in Jordan and *Salvia microphylla* cultivated in Egypt on acetylcholinesterase activity, β-amyloid deposition, and oxidative stress associated with scopolamine-induced AD. Both extracts contained significant amounts of the major secondary metabolites, including methyl carnosate, carnosic acid, carnosol, rosmanol, and salvianolic acids. Their study revealed that treatment with *extracts of Salvia species* reversed the histological alterations induced by scopolamine and significantly reduced β-amyloid deposition. In addition, *Salvia* treatment decreased markers of both oxidative stress and acetylcholinesterase activity (31).

Inhibiting amyloid-β aggregation is a promising strategy for the treatment of AD. According to the literature, cryptotanshinone alleviates oxidative stress and reduces abnormally aggregated protein levels in *Caenorhabditis elegans* models of AD. The accumulation of Aβ leads to mitochondrial dysfunction and the release of reactive oxygen species (31). This excess of ROS exacerbates Aβ toxicity and stimulates neuroinflammation, leading to neuronal apoptosis (32). In another study, Ding *et al.* (2022) investigated the effects of cryptotanshinone on Aβ aggregation and inflammation in cerebrovascular endothelial cells. Their results indicated that this compound has the potential to inhibit Aβ1-42 aggregation by prolonging the nucleation phase, reducing the growth-phase slope, and modulating the final fluorescence intensity in a concentration-dependent manner (33). In addition, our investigation showed that cryptotanshinone had the highest activity in reducing nitric oxide release. It was also tested for its effects on nitrotyrosine formation and ROS release and effectively inhibited ROS release (7).

Carnosic acid and carnosol are two prominent diterpenes found in the genera Rosmarinus and *Salvia*. As shown in Table S2, these compounds were characterized by LC-MS profiling in the studied species. These ligands' selectivity for AChE is most likely due to their ability to fit and exploit the extra space within the active-site gorge. Both compounds possess a catechol group, which is an ortho-dihydroxybenzene moiety. This characteristic is shared by many phenolic substances known to inhibit cholinesterase enzymes. AChE inhibitors function by preventing the breakdown of acetylcholine, thereby increasing its levels in the brain, which may serve as a treatment option for neurodegenerative diseases like Alzheimer's disease. Numerous studies have examined the effects of these compounds, which are well known for their strong anti-inflammatory and antioxidant capabilities (34). Due to their phenolic structure, they can effectively reduce cellular ROS either by direct scavenging or by indirectly enhancing antioxidant defenses (35, 36). Numerous studies have highlighted the significance of carnosic acid as a neuroprotective agent, demonstrating its therapeutic efficacy against disorders caused by neuronal injury. The primary potential neuroprotective mechanisms of carnosic acid include inducing autophagy, alleviating oxidative stress, attenuating apoptosis, protecting against Aβ-mediated neurodegeneration, and exerting protective effects in models of neuronal injury (36).

Salvia species are associated with neuroprotective properties. The small size and lipophilic nature of essential oil constituents typically allow them to cross the blood-brain barrier easily, suggesting their potential as a treatment for neurodegenerative diseases (37). Several studies have demonstrated that *Salvia* essential oils and extracts can modulate cholinesterase enzyme activity. Consistent with the results of the current study, previous research has shown that the essential oil of *Salvia lavandulifolia* exhibited potent and selective inhibitory activity against AChE, with an IC50 of 3 μg/ml, and moderately inhibited butyrylcholinesterase (BChE) by 22% at 0.5 mg/ml. It was also found to reduce

AChE activity specifically in the striatum of rats; however, no such effect was observed in the hippocampus or cortex (38). Additionally, *S. potentillifolia* essential oil exhibited potent BChE inhibition, with 65.7% inhibition at 200 µg/ml, compared to a more modest 21.9% AChE inhibition at the same concentration (38). The anticholinesterase potential of *Salvia* species has been attributed to their rich phytochemical profiles, particularly their terpene and phenolic constituents. For instance, the essential oil of *S. mirzayanii*, containing δ-cadinene (11.72%), α-terpinyl acetate (10.56%), spathulenol (6.53%), and α-cadinol (5.76%), showed the most potent AChE inhibition (40.6% at 500 µg/ml) and BChE inhibition (72.7% at 500 µg/ml) among the evaluated *Salvia* species (38). Docking studies on the essential oil of *Salvia tomentosa* revealed that compounds such as δ-cadinene, viridiflorol, γ-muurolene, and α-caryophyllene exhibited the highest binding affinities to AChE. This makes them the most promising inhibitors of this enzyme, as demonstrated by research by Kocer *et al.* (39).

However, it is important to note that most of the monoterpenoids found in *Salvia* subg. *Perovskia* essential oils have weak AChE inhibitory activity. This information should be carefully considered when using these essential oils in organic farming applications, as they may be effective insecticides. Still, their limited AChE inhibitory activity may not be suitable for certain therapeutic applications, such as the management of neurodegenerative diseases. Further investigation is essential to fully elucidate the structure–activity relationships and optimize the AChE inhibitory potential of the bioactive constituents present in these *Salvia* essential oils.

Conclusion

This study aimed to identify natural, safe resources for treating AD by evaluating three Salvia species. Advanced chromatographic and spectrometric analyses revealed a range of diterpenoids in their specialized metabolites and essential oils. Bioassays showed that pure compounds, especially cryptotanshinone and 1α-hydroxydemethylsalvicanol, exhibited significant AChE inhibitory activity, outperforming crude extracts and essential oils. The EtOAc extracts showed moderate anti-AChE activity, whereas the essential oils had minimal effects. These results suggest that future anti-Alzheimer's drug development should prioritize the structural optimization of pure, isolated compounds derived from natural products. Future studies should outline a clear roadmap for upcoming research, including dose-response studies and *in vivo* models, to support these conclusions and provide a more direct translational route toward rational phytotherapy.

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Ethics Approval Statement

Not applicable.

Consent for Publication

All authors give consent for the data to be published.

Authors' Contributions

Z S conceived the study and drafted the manuscript. Z A and N D assisted with data collection and LC-MS analysis. Z A also contributed to table preparation and data analysis. All authors reviewed and approved the final version.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

No AI tools were used in the preparation of this manuscript.

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