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Molecular docking and ex vivo and in vitro anticholinesterase activity studies of *Salvia* sp. and highlighted rosmarinic acid

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Background/aim: To evaluate acetylcholinesterase (AChE) inhibitory activity and antioxidant capacity of the major molecule from *Salvia* sp., rosmarinic acid, as a drug candidate molecule for treatment of Alzheimer disease (AD).

Materials and methods: The AChE inhibitory activity of different extracts from *Salvia trichoclada*, *Salvia verticillata*, and *Salvia fruticosa* was determined by the Ellman and isolated guinea pig ileum methods, and the antioxidant capacity was determined with DPPH. The AChE inhibitory activity of the major molecule rosmarinic acid was determined by in silico docking and isolated guinea pig ileum methods.

Results: The methanol extract of *Salvia trichoclada* showed the highest inhibition on AChE. The same extract and rosmarinic acid showed significant contraction responses on isolated guinea pig ileum. All the extracts and rosmarinic acid showed high radical scavenging capacities. Docking results of rosmarinic acid showed high affinity to the selected target, AChE.

Conclusion: In this study in vitro and ex vivo studies and in silico docking research of rosmarinic acid were used simultaneously for the first time. Rosmarinic acid showed promising results in all the methods tested.

Key words: *Salvia*, rosmarinic acid, acetylcholinesterase, isolated guinea pig ileum, computational screening

1. Introduction

Alzheimer disease (AD) is characterized by loss of cognitive function leading to dementia. The main symptoms associated with AD involve a decline in cognitive dysfunction, primary memory loss, and, in the later stages of the disease, language deficits, depression, agitation, mood disturbances, and psychosis (1). The pathological features of AD include neurotic plaques composed of amyloid- β -peptide (A β) cores, neurofibrillary tangles of hyperphosphorylated tau protein, and neurotransmitter deficits (2). Acetylcholine has a functional key role in cognitive functions including learning and memory, arousal, and attentional processes in the brain (3). Acetylcholinesterase (AChE) is the key enzyme in the breakdown of acetylcholine; therefore, the inhibition of AChE is considered one of the treatment strategies against several neurological disorders such as AD, senile dementia, ataxia, and myasthenia gravis (4). AChE

inhibitors are the only agents approved by the US Food and Drug Administration for the treatment of AD. Within the past few years, some synthetic compounds (tacrine, rivastigmine, donepezil, and galanthamine) have become available for clinical use; however, none of them have the ability to stop the disease. Thus, there is still great urgency for finding new drug candidates for AD treatment.

Several findings suggest that oxidative stress may play an important role in the pathogenesis of AD. First, the brains of patients with AD contain lesions that are typically associated with exposure to free radicals. In addition, oxidative stress in the brains of AD patients is indicated by elevated cerebral levels of endogenous antioxidants that scavenge free radicals (5).

Moreover, in vitro studies suggest that exogenous antioxidants reduce the toxicity of A β in the brains of AD patients. Antioxidants may decrease the level of oxidative stress in the brain and thereby reduce the amount of DNA

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damage, neuronal cell death, and aggregation of A β within the brain (5). Notably, oxidative stress leads to activation of stress-activated protein kinases. One stress-activated protein kinase in particular, p38 MAP kinase, appears to be crucial in AD because it promotes tau protein hyperphosphorylation (6).

In the 16th century *Salvia officinalis* was described as being good for the memory. In clinical trials the ethanolic extracts and volatile oils of *S. officinalis* and *S. lavandulifolia* have been shown to be effective in mild scaled AD patients, even in low doses (7–10). A previous study showed the protective effect of *Salvia officinalis* extract and its active ingredient rosmarinic acid on PC12 neuroblastoma cells (11). The dried root of *Salvia miltiorrhiza* has been used for the treatment of cerebrovascular disease and central nervous system deterioration in old age for over 1000 years (12).

The genus *Salvia* L. (Lamiaceae) is represented by over 900 species worldwide and by 99 species in the flora of Turkey (13). A large number of secondary metabolites, including essential oils, terpenoid compounds, and phenolic derivatives, have been isolated from the genus, and these feature prominently in the pharmacopoeias of many countries throughout the world (14–16). Traditional medicinal uses prompted us to investigate this herb further. In our previous study the antiinflammatory effects of 3 *Salvia* species were compared (17).

The aim of this study was to evaluate the anticholinesterase and antioxidant effect of extracts of *S. trichoclada* Benth., *S. verticillata* L., and *S. fruticosa* Mill. (Lamiaceae) and their main active ingredient rosmarinic acid by in vitro, ex vivo, and in silico methods.

2. Materials and methods

2.1. Plant material

The aerial parts of *Salvia trichoclada* Benth. were collected from Hakkari - Yüsekova (A. Donmez, HUB 11036) in June 2002. Those of *S. fruticosa* Mill. (syn. *S. triloba*) were collected from Antalya - Kemer in April 2004 (P. Gurbuz, HUEF 08014), and those of *S. verticillata* L. were harvested from Ankara - Karagöl (L.O. Demirezer, HUEF 08011) in June 2005. The voucher specimens of the plants were deposited at the herbarium of the Hacettepe University Faculty of Pharmacy (HUEF) and the herbarium of the Hacettepe University Faculty of Science (HUB).

2.2. Chemicals and standards

2,2-Diphenyl-1-picrylhydrazyl (DPPH), (S)-acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), physostigmine, and AChE (0.5 U/mg) derived from electric eels (AChE True Cholinesterase, EC 3.1.1.7 Type VI-S) were purchased from Sigma-Aldrich (Steinheim, Germany). Buffer components and other chemicals were of the highest purity available commercially and supplied by Carlo Erba (Milan, Italy).

2.3. Extraction and isolation

Thirty grams of aerial parts of the plants were extracted separately under reflux in a water bath with 150 mL of acetone at 37 °C for 1 h. After filtration, acetone extracts were evaporated under vacuum at 37 °C to dryness, and the plant residue was extracted with 150 mL of methanol and quantitatively filtered. After the evaporation process, methanol extracts were suspended in 150 mL of water and then extracted with 150 mL of chloroform and 150 mL of n-butanol respectively. Each extract was evaporated separately under vacuum at 37 °C to dryness.

The isolation and structural elucidation of compounds from *Salvia* species were reported previously (18).

2.4. Preparation of the *Salvia trichoclada* volatile oil

The whole plant sample (100 g) was cut into small pieces and subjected to hydrodistillation for 3 h using a Clevenger type apparatus. The essential oil was dried over anhydrous sodium sulfate, stored in a refrigerator, and protected from light until further use.

2.5. Ellman method

The methanol, n-butanol, and water extracts of 3 genera of *Salvia* were tested for their AChE inhibitory activities at 0.1, 0.25, 0.5, 1.0, and 2.0 mg/mL concentrations. The anticholinesterase potential of the extracts was compared with physostigmine. Less than 50% inhibition of AChE was not considered significant. Each assay was conducted using the modified Ellman method (19). One hundred and twenty microliters of DTNB (0.125 mM in 50 mM phosphate buffer, pH 7.4), 120 μ L of 0.5 mM acetylthiocholine iodide (ATChI in bidistilled water), 600 μ L of phosphate buffer (100 mM, pH 7.4), and different concentrations of samples were dissolved in bidistilled water, and 10% methanol was added to each cuvette. Each reaction was started by adding 10 μ L of 0.28 U/mL AChE with a final concentration of 0.023 U/mL prepared in phosphate buffer. Measurements were taken every second for 2 min at 25 °C (ϵ_{TNB} , 412 nm = 14.2 mM⁻¹ cm⁻¹). The assay was conducted on 3 separate samples of each extract. Enzyme activity was calculated as a percentage of the velocities as compared to assay using buffer without any inhibitor. Inhibitory activity was calculated by subtracting the percentage of enzyme activity from 100. Every experiment was done in triplicate. Physostigmine (0.1 mM) was used as a standard AChE inhibitor in the study. The inhibition rate (%) was calculated by the following equation:

$$\text{Inhibition \%} = 100 - (100 \times V_T / V_K),$$

where V_K is the velocity of the control without inhibitor and V_T is the velocity of the test solution.

2.6. Isolated guinea pig ileum method

Each animal was sacrificed by cervical dislocation. The ileum was removed, prepared in 2-cm lengths, mounted in 10-mL tissue baths containing Tyrode's solution maintained

at 37 °C, and aerated with a mixture of carbon dioxide (5%) and oxygen (95%). The cumulative concentration–response of acetylcholine (10^{-8} to 10^{-4} M) was constructed with the presence of a vehicle as a control. Each tissue sample was washed every 15 min for a 1-h equilibrium period. The cumulative concentration–response curves of acetylcholine (10^{-8} to 10^{-4} M) were constructed for comparison of the presence of crude extract (100 µg/mL) and/or of the physostigmine standard (0.01–0.1 µM). Each plant extract was examined for spasmogenic activity.

All experimental data are presented as mean \pm standard deviation. Analysis was performed using the GraphPad Prism statistical software package (version 3.03) and $P < 0.05$ was considered statistically significant.

2.7. Ethical aspects

All rats used in the present study were cared for in accordance with the directory of the Hacettepe University Animal Care Unit, which applies the guidelines of the National Institutes of Health on laboratory animal welfare.

2.8. Free radical DPPH scavenging assay

Two hundred and thirty microliters of methanol extracts (400, 200, 100, 50, 25 µg/mL) was added to 50 µL of a 0.022% methanolic solution of DPPH. After a 30-min incubation period at room temperature, the absorbances were read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I%) was calculated as follows: $I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$.

Tests were carried out in duplicate. Ascorbic acid was used as a standard substance (20,21).

2.9. Molecular docking studies

2.9.1. Target identification

In this study, AChE was selected as a target and then computationally determined, and relative binding energies to rosmarinic acid, gallic acid, ascorbic acid, physostigmine, galanthamine, and huperzine A and their docking results were compared. Physostigmine, galanthamine, and huperzine A are currently being evaluated for their clinical efficacy in the management of AD and vascular dementia. Gallic and ascorbic acids are strong antioxidant molecules. The 3-dimensional structures of ACE (3LII) were obtained from the Protein Data Bank (PDB). These structures were determined using X-ray diffraction. The 3-dimensional structures of the targets were downloaded from the RCSB PDB. All solvent molecules and cocrystallized ligands were removed from the structures.

2.9.2. Ligand identification

The PDB files of rosmarinic acid and physostigmine were obtained by using the Discovery Studio 3.5 Client program. The 3-dimensional structures of these compounds were drawn and then converted into PDB format by using this program. The 3-dimensional structures of huperzine A (4EY5) and galanthamine (4EY6) were obtained from

the PDB. The 3-dimensional structures of ligands were downloaded from the RCSB PDB.

2.9.3. Docking receptors with rosmarinic acid using AutoDock Vina

AutoDock Vina 1.0 beta 0.2 software was used to search for the potential targets of rosmarinic acid. The graphical user interface program AutoDock Tools was used to prepare, run, and analyze the docking simulations. Atom charges, solvation parameters, and polar hydrogens were added to the receptor PDB file for the preparation of protein in docking simulations. AutoDock Vina (22–24) requires precalculated grid maps. This grid must contain the potential binding region of the target we are interested in. The grid box size was set at 20, 20, and 20 Å (x, y, and z). The spacing between grid points was 1.0 Å. After the simulations were completed, the docked structures were studied and the interactions were analyzed. Hydrogen bond interactions and the binding distance between the donors and acceptors were measured for the best conformers. AutoDock was run with the Windows XP operating system and all the AutoDock docking runs were performed 3 times. Finally, AutoDock Vina stored the potential energy arising from the interaction of flexible targets with rigid macromolecules.

3. Results

3.1. Ellman method

Methanol, n-butanol, and water extracts were prepared (0.1, 0.25, 0.5, 1.0, and 2.0 mg/mL) and tested for their AChE inhibitory activities. The anticholinesterase potential of the extracts was compared with the positive control of physostigmine.

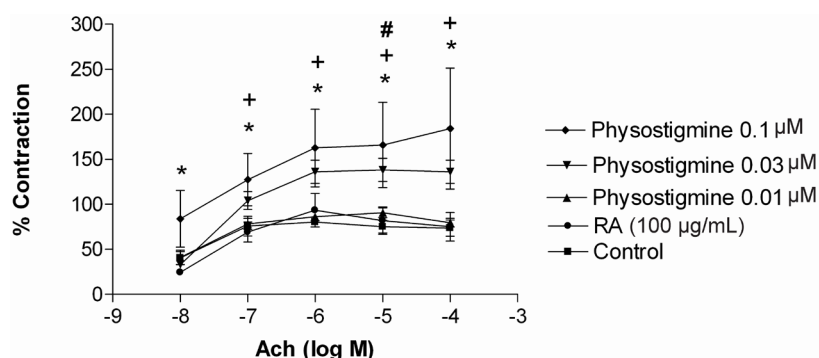
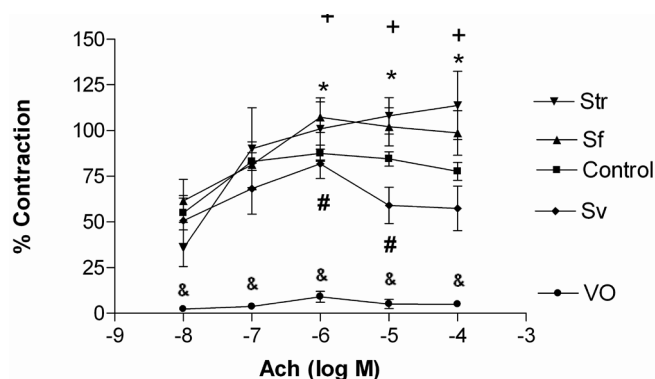
Less than 50% inhibition of AChE was not considered significant. The methanol extract of *Salvia trichoclada* inhibited AChE at a rate of $\pm 81.10\%$ at a concentration of 2 mg/mL, while other concentrations and methanol extracts of *S. verticillata* and *S. fruticosa* showed lower inhibition (Table 1).

3.2. Isolated guinea pig ileum method

Acetylcholine (10^{-9} to 10^{-4} M) elicited concentration-dependent contraction in isolated guinea pig ileum. Physostigmine (0.01–0.1 µM) ($P = 0.072$; one-way ANOVA) (Figure 1) and the methanol extract of *S. trichoclada* (100 µg/mL) (10^{-6} to 10^{-4} M; Student t-test, $P = 0.0454$; $n = 6$) (Figure 2) potentiated the effect of a cumulative dose response (contraction) of acetylcholine (10^{-8} to 10^{-4} M) in a dose-dependent fashion, suggesting an AChE inhibitory effect. The methanol extract of *Salvia trichoclada* showed better contraction response than other studied *Salvia* species, while their acetone extracts showed no inhibitory effects (Figures 2 and 3).

Table 1. Acetylcholinesterase inhibitions (%) and standard deviations of *Salvia* extracts (C in mg/mL) by Ellman method. *nd: Not detectable.

C	<i>Salvia trichoclada</i>			<i>Salvia fruticosa</i>			<i>Salvia verticillata</i>		
	n-BuOH	Water	MeOH	n-BuOH	Water	MeOH	n-BuOH	Water	MeOH
2	83.85 ± 2.7449	81.52 ± 0.4010	81.10 ± 2.6375	72.11 ± 2.4448	70.5 ± 4.9797	44.52 ± 4.1577	nd*	12.23 ± 1.1773	28.29 ± 1.5505
1	62.93 ± 1.1579	74.31 ± 1.4931	71.62 ± 0.8909	47.84 ± 0.4813	52.00 ± 2.00	25.42 ± 1.5344	43.55 ± 0.8297	12.95 ± 0.7848	15.74 ± 0.3394
0.5	45.68 ± 1.9374	62.69 ± 1.9294	63.81 ± 0.5727	33.79 ± 1.7182	36.50 ± 2.1213	15.57 ± 0.9036	27.04 ± 4.3345	9.86 ± 0.7848	13.52 ± 0.5036
0.25	30.51 ± 0.2969	23.69 ± 2.1819	57.39 ± 1.8031	21.19 ± 2.2312	21.00 ± 2.0000	10.75 ± 1.5909	16.52 ± 2.6311	5.42 ± 1.5344	9.01 ± 0.7141
0.1	nd	27.75 ± 1.8066	29.85 ± 0.7926	nd	11.5 ± 2.1213	4.80 ± 0.1524	6.35 ± 0.9192	nd	7.25 ± 0.1767

**Figure 1.** Concentration-dependent contraction responses elicited by acetylcholine (Ach) before (control) and after incubation of physostigmine standard (0.01–0.1 μ M) and rosmarinic acid (RA) (100 μ g/mL). *, +, #: significantly different from control.**Figure 2.** Concentration-dependent contraction responses elicited by acetylcholine (Ach) before (control) and after incubation of methanol extract of *S. fruticosa* (Sf), *S. verticillata* (Sv), *S. trichoclada* (Str), and volatile oil (VO) (100 μ g/mL). * Significantly different from control for Str, + for Sf, # for Sv, & for VO.

3.3. Free radical DPPH scavenging assay

Rosmarinic acid showed 90.58% and 90.40% DPPH activity at 200 μ M and 100 μ M concentrations ($IC_{50} = 24.38$), while ascorbic acid showed 91% and 90% and α -tocopherol 88% and 90% DPPH activity.

3.4. Molecular docking studies

According to the docking results of rosmarinic acid (Figure 5), the binding energies of rosmarinic acid, galanthamine, huperzine A, and physostigmine to AChE were -8.0 , -7.7 , -7.5 , and -7.0 kcal/mol, respectively (Table 2).

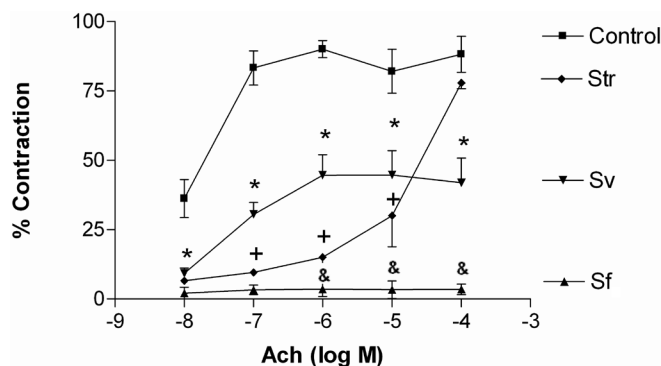


Figure 3. Concentration-dependent contraction responses elicited by acetylcholine (Ach) before (control) and after incubation of acetone extracts of Str, Sv, and Sf (100 µg/mL). + Significantly different from control for Str, * for Sv, & for Sf.

4. Discussion

Natural products have been used empirically since ancient times and a tendency is appearing today for their increased usage. The pharmaceutical industry requires potential novel drug candidate molecules. To date, no molecules could be synthesized without patterning a natural model. Therefore, the isolation of model molecules from natural sources has been very important. *Salvia* sp. is a common aromatic and medicinal plant native to Mediterranean countries and is in widespread use. As mentioned in the introduction, many studies have been carried out on *Salvia officinalis*, *S. lavandulifolia*, and *S. miltiorrhiza*. In this study, 3 different *Salvia* species, *S. trichoclada*, *S. verticillata*, and *S. fruticosa*, which grow in the Mediterranean area, were selected for guided investigation of biological activity.

In the first step, in vitro AChE inhibitory activity of the extracts from 3 *Salvia* species were studied using the colorimetric method of Ellman et al. (19).

Secondly, an ex vivo study was carried out on isolated guinea pig ileum. The enteric nervous system, which runs along the length of the gastrointestinal tract, plays an important role in coordinating the motor activity of the muscular layers. The dominant excitatory enteric neurotransmitter is thought to be acetylcholine. Two different enzymes that hydrolyze acetylcholine have been

described in the mammalian small intestine: AChE and butyrylcholinesterase (BuChE). AChE is the main enzyme responsible for the inactivation of acetylcholine, whereas the physiological function of BuChE remains unknown.

In the present study, we determined the possible inhibitory effects of *Salvia* extracts on AChE in isolated guinea pig ileum preparation, and we compared them with that of physostigmine, which is reported to inhibit AChE. In the present experimental conditions, peristalsis of the isolated guinea pig ileum persisted constantly during the experimental period. During the 10-min incubation period, none of the extracts significantly altered peristalsis of the isolated guinea pig ileum. The crude extracts of *Salvia* species did not cause a dose-dependent (100 µg/mL) spasmogenic effect in the isolated guinea pig ileum.

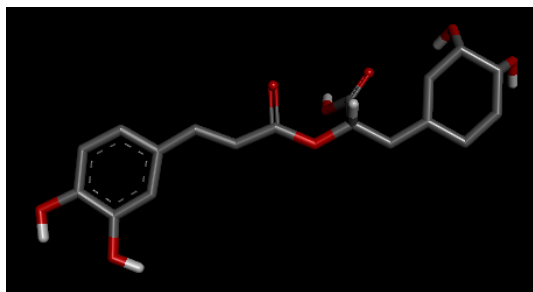


Figure 4. Rosmarinic acid.

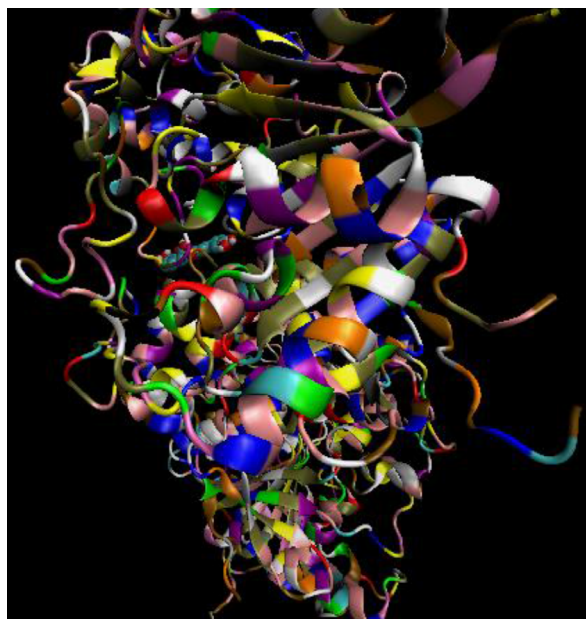


Figure 5. Molecular docking of rosmarinic acid in the active site of AChE.

Table 2. In silico docking results of rosmarinic acid (AChE).

Ligand	AutoDock Vina
	3LII (AChE)
Rosmarinic acid	-8
Galanthamine	-7.7
Physostigmine	-7.0
Huperzine A	-7.5

The volatile oil of *Salvia lavandulifolia* (Spanish sage) has been found to be an uncompetitive reversible inhibitor (10). Therefore, the volatile oil of *Salvia trichoclada* (100 µg/mL) was also investigated. As shown in Figure 2, volatile oil completely blocked the acetylcholine-induced contraction in isolated guinea pig ileum.

Furthermore, because the 10-min incubation period was not sufficient for enzyme induction, we suggest that inhibition of the contractile response might not be dependent on AChE induction. However, we speculated that the above-mentioned extracts are likely to have antagonistic activity against muscarinic receptors and these compounds are likely to compete with acetylcholine against muscarinic receptors. Further studies are necessary on this point.

As seen from the data, the Ellman and the isolated guinea pig ileum methods verified each other.

Free radicals cause various diseases including AD (5), and therefore the antioxidant effects of *Salvia* extracts were tested by the DPPH method. In our previous study, n-butanol, water, methanol, acetone, and chloroform extracts of 3 *Salvia* species were tested at 1, 5, 10, 25, 50, and 100 µg/mL. The results of the methanol extract of *S. trichoclada*, *S. fruticosa*, and *S. verticillata* at 100 µg/mL concentrations were 89.65%, 88.21%, and 86.05%, respectively (18). In a recent study, DPPH radical scavenging activities of CH₂Cl₂ and EtOH extracts of aerial parts from *S. verticillata* subsp. *amasiaca* were investigated. The EtOH extracts at 1000 µg/mL showed 81% inhibition (25). The results were similar to our findings but we represented this inhibition rate at a 100 µg/mL concentration. This may be due to the different extract preparation procedures. In another study, the DPPH radical scavenging effects of *S. fruticosa* and *S. trichoclada* were tested. According to that study the DPPH radical scavenging effects were 93% and 89% at 100 µg/mL, respectively (26). These results are also consistent with ours.

When the results of the DPPH, Ellman, and isolated guinea pig ileum methods were evaluated, it was seen that the methanol extract of *S. trichoclada* showed the best results.

The results showed us that the methanol extract of *S. trichoclada* has both AChE inhibitory effects and

antioxidant capacity. Based on these findings we want to determine active molecules that can be evaluated as drug candidates.

In recent times, computational (in silico) methods including database search algorithms, determination of quantitative structure–activity relationships, similarity search methods, computational modeling, and docking have been developed and widely applied to pharmacological hypothesis development and testing. These methods have been widely used to investigate potential drug targets and understand absorption, distribution, metabolism, excretion, and toxicity properties as well as for the physicochemical characterization of drugs for the related receptors and enzymes. To reduce the cost of drug development and save time, in silico methods can be used to make preliminary assessments and screening before in vitro and in vivo research.

In our previous study the major compound α-O-caffeoyl-3',4'-dihydroxyphenyl lactic acid (rosmarinic acid) (Figure 4) was isolated from the methanol extract of *Salvia trichoclada* and its structure was elucidated by ¹H and ¹³C NMR (18).

According to some reports (27), rosmarinic acid inhibited AChE moderately (47.3%) at doses of 1.0 mg/mL using the Ellman method. In this study, an in silico docking investigation of the interaction of rosmarinic acid with the enzyme was performed. Molecular docking is one of the most important parts of the in silico method. We used AutoDock Vina along with database search algorithms such as PDB sum and RCSB PDB and it requires precalculated grid maps, one for each atom type present in the flexible molecules being docked. It stores the potential energy arising from the interaction with rigid macromolecules. This grid must surround the region of interest in the grid macromolecule. The spacing between grid points was 1.0 Å. After the dockings were complete, the docked structures were analyzed and the interactions were seen. Hydrogen bond interactions between the ligand and enzymes were measured as binding energies and the results gave us some ideas for the experimental part of study. AChE is inhibited by a number of structurally very diverse ligands including, for example, physostigmine, huperzine A, and galanthamine, which are strong AChE inhibitors. In this part of the study AChE was selected as the target and then its relative binding energies to rosmarinic acid, physostigmine, galanthamine, and huperzine A were computationally determined and the docking results were compared. As seen from the data, rosmarinic acid showed the lowest binding energy. This means that rosmarinic acid is more effective than galanthamine, huperzine A, and physostigmine. To verify in silico results, the anticholinesterase activity of rosmarinic acid was investigated on isolated guinea pig ileum. As shown in Figure 1, rosmarinic acid did not affect the contraction

response elicited by acetylcholine in isolated guinea pig ileum.

Different plant genera are an important source of rosmarinic acid (28). Inhibition of glutathione reductase and glucose 6-phosphate dehydrogenase antioxidant enzymes by rosmarinic acid was shown in our previous study (29). Thus, the antioxidant and AChE inhibitory effects of rosmarinic acid were found to be significant with in vitro and ex vivo methods.

In this study in vitro, ex vivo, and in silico methods were used simultaneously for rosmarinic acid for the

first time and rosmarinic acid showed significant results with these methods. Our results suggest that rosmarinic acid may become a novel therapeutic candidate for the treatment of AD.

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