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Cholinergic cognitive enhancer effect of *Salvia triloba* L. essential oil inhalation in rats

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ABSTRACT

Objective: Current treatment of Alzheimer's disease is provided by cholinesterase inhibitors. *Salvia triloba* L. (syn. *Salvia fruticosa* Mill.), a species mostly consumed as refreshing herbal tea in traditional medicine, is rich in 1,8-cineole that is known to have cholinesterase inhibiting effects. In this study, we investigated cognitive enhancer effects of *S. triloba* essential oil inhalation on healthy control rats and rats with scopolamine induced memory impairment.

Materials and Methods: *S. triloba* samples from different geographical locations of Turkey were hydro-distilled and analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The optimum sample with the highest 1,8-cineole and lowest camphor, α-thujone and β-thujone content was selected. In vitro cholinergic and antioxidant potentials of the selected essential oil were calculated. Cognitive enhancer and anti-amnestic effects of the inhaled essential oil on rats were assessed by means of Morris water maze. The bioavailability of 1,8-cineole in blood of rats was measured by GC-MS.

Results: The group that inhaled *S. triloba* significantly outperformed control group, namely faster achieving peak escape latency performance in Morris water maze. However, *S. triloba* inhalation failed to restore scopolamine induced learning impairment.

Conclusion: In this study, we report positive effects of inhaling *S. triloba* essential oil as a complementary treatment for supporting cognitive functions.

Keywords: Cognitive enhancers, Essential oil, Gas chromatography, 1,8-Cineole, Antioxidants, Morris water maze

1. INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disorder, which is characterized by impaired memory and cognitive functions, and behavioral problems leading to significant disturbances in daily activities and the most common cause of dementia [1, 2]. Acetylcholine is one of the neurotransmitters that is associated with the etiopathogenesis of AD. Drastic decrease in acetylcholine levels in the brain has been revealed as the most remarkable biochemical change of AD and major cortical cholinergic innervation by the basal forebrain in the medial septum and nucleus basalis of Meynert is known to be impaired in AD patients [3].

The animal models of AD also proved cholinergic lesions caused learning impairment, and those converging results led to the cholinergic hypothesis of AD related dementia. Accordingly, Morris water maze, is widely adopted to assess behavioral impairments in rodents due to age-related cognitive deficits and in commonly used transgenic mouse models of AD [4]. Scopolamine, a tropane alkaloid with muscarinic antagonist effects, has been the most commonly used pharmacological model to induce amnesia [5].

The cholinergic hypothesis was further supported by improvement in AD related cognitive decline by acetylcholinesterase (AChE) inhibitor therapies. Inhibition of AChE and butyrylcholinesterase (BChE) at the cholinergic synapse, increases acetylcholine levels and boosts cholinergic transmission. Although, cholinesterase inhibitors (ChE-Is) such as donepezil, rivastigmine, galantamine and the N-methyl-D-aspartate (NMDA) antagonist memantine are approved as main pharmacological options for cognitive enhancing treatment in AD, they are unfortunately not capable of reversing the neurodegenerative process [3]. Morris water maze has been commonly recruited to investigate the anti-dementia effects of

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ChE-Is on rodent models of hippocampus-dependent memory losses [6].

Several cognitive enhancer effects of cholinesterase inhibitors have also been reported in healthy population, such as improving retention of aviation skills [7], encoding items in their spatial context [8], episodic memory in young adults [9] and improved verbal memory in healthy elderlies [10]. A systematic review on the cognitive enhancing effects of ChE-Is concluded yield greater effects on low performing participants [11]. Coherent with this fact, donepezil has been reported to ameliorate attention and memory deficits due to sleep deprivation [12], and galantamine treatment has improved sustained attention in abstinent cigarette smokers [13].

Several natural metabolites are capable of selectively inhibiting both AChE and BChE enzymes. *Salvia* (sage) species are frequently used in traditional folk medicine for their memory enhancing effects [14]. The genus *Salvia* (Lamiaceae) has been distributed widely in several regions of the world, especially in the Mediterranean region and Asia, with more than 1000 species [15]. There are 100 species of *Salvia* in Turkey, 57 of which are endemic [16]. Various members of *Salvia* have significant pharmacological activities such as analgesic, anticancer, anti-inflammatory, antimicrobial, antimutagenic, antioxidant, cardioprotective and cytotoxic [17-23]. Several cholinergic enhancing activity of *Salvia* members has also been revealed via numerous *in vitro, in vivo* and *in silico* techniques [17, 18]. Especially, essential oil and extracts of numerous *Salvia* spp. have shown to have AChE and BChE inhibiting activity and antioxidant profile [24-26]. The essential oil of some *Salvia* species and the essential oils of various herbs (*Juniperus communis, Pimpinella peregrina, Lavandula angustifolia,* and *Rosmarinus officinalis*) have been reported to have positive effects on the mood and cognitive functions in the clinic when administered by inhalation [27]. 1,8-cineole in the essential oils is assumed to be the active metabolite that is associated with effects on brain functions [28]. Amongst the mentioned medicinal plants, *S. triloba* L. (syn. *S. fruticose* Mill.) that is rich in 1,8-cineole and camphor, is widely grown in Turkey and mostly consumed as refreshing herbal tea [29].

Excess reactive oxygen species (ROS) can give harm to cellular lipids, proteins or DNA by inhibiting their normal functions and this biological damage is known as "oxidative stress". Oxidative stress has been involved in many diseases including diabetes, cancer and cardiovascular disorders, as well as the aging process [30]. Furthermore, there are literature demonstrating the role of oxidative stress in either pathogenesis or prognosis of several neurodegenerative diseases including AD [31-33]. Although, limited randomized clinic intervention trials of AD have revealed mixed results on benefits from antioxidants, well established pathophysiology of oxidative stress, several *in vitro* studies, animal studies and observational studies depict antioxidants as favorable supplements [31, 34]. Besides, several ChE-Is, including derivates of donepezil and galantamine have been shown to have antioxidant activity in terms of determining their efficacy with multi-targeting [35].

Given the abundant 1,8-cineol content of *S. triloba*, its essential oil was extracted from plant material, analyzed for composition, and confirmed for its cholinesterase inhibitory activity *in vitro*. Based on this exploratory findings, we hypothesized to observe cholinergic enhancer effects of inhaling *S. triloba* essential oil on learning and memory performance of control rats and scopolamine treated amnesic rats. *In vitro* antioxidant profile of the *S. triloba* essential oil was also determined in order to examine its supplementary health promoting effects.

2. MATERIAL and METHODS

Chemicals and Drugs

All chemicals, enzymes and references used in the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The quality of all chemicals was of analytical grade. Standards used in optimization of GC–MS parameters were also purchased from Sigma.

Plant materials

Aerial parts of *S. triloba* and a commercial essential oil (STC) sample were purchased from different organic agriculture institutes from different regions of Turkey [*S. triloba* – Antalya (STA), Izmir (STI), Yalova (STY) , Balıkesir (STB)] in June 2019. Botanical identification of plant samples were verified by HB. The shade-dried upper ground parts of plant samples were kept at room temperature in air-tight containers until further use. According to the 1,8-cineole, camphor α-thujone and β-thujone amounts calculated by GC-MS analysis, the optimum *S. triloba* sample was selected.

Distillation of essential oils

Aerial parts (100 g) of the plant samples were subjected to hydro-distillation with 1L H_2O for 3h, using a clevenger-type apparatus to produce the essential oils. Condenser of the clevenger was attached to a chiller which was set to 4°C. Aerial parts of *S. triloba* samples from Antalya (STA), Izmir (STI), Yalova (STY) and Balıkesir (STB) afforded an oil with 0.015%; 0.016%; 0.018%; and 0.02% (v/w) yields, respectively. All essential oil samples were stored in amber vials under – 20°C until the day they were analyzed.

GC–MS conditions for essential oil analysis

Qualitative and quantitative analyses were performed by GC-MS. Agilent technologies 7890 A GC system was used with HP-5MS column (30 m x 0.25 mm x 0.25 µm). Oven temperature was started with 60 °C and then steadily increased to 246°C with 3 °C raise per a minute. Helium was used as mobile phase with 0.9 ml/min flow rate. Split mode was used with 50:1 ratio with 1µl sample volume. Relative Retention Indices (RRI) was calculated via comparison with (C4-C40) standards. Identification of the essential oil components were completed by comparison of their relative retention index (RRI) calculated against *n*-alkanes and relative retention times (RT) with those of authentic samples and mass spectra obtained from Wiley 7N library as well as MS literature data was used for the identification [36,

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37]. STA sample was selected for further studies due to its high 1,8-cineole content (40.48%) and significantly low α, β-thujone and camphor content (0.55, 0.80 and 5.15%, respectively).

In vitro tests

Determination of AChE and BChE inhibitory activities

AChE and BChE inhibitory activities of the fractions were studied by a previously elucidated method [38]. AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) and Horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) were used as enzymes. Acetylthiocholine iodide (AChI) and Butyrylthiocholine chloride (BChC) were used as substrate of enzymes. Adequate amounts of phosphate buffer, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution, enzyme and test substance were mixed in 96 well-plate and kept in 25 °C for 15 minutes for incubation. Then, substrates were added to mixture and results were immediately measured at 412 nm via well-plate reader. 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL concentrations of galanthamine was used as positive control to obtain a standard curve for both assays.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity assay was practiced according to method given earlier by Bardakci et al. [39]. Essential oil was diluted with dimethyl sulfoxide (DMSO):H_2O mixture (10% v/v) to adjust the concentration to 1 mg/ml and mixed with 100 µM methanolic DPPH solution. The mixture was preserved at room temperature, away from light, and the loss in absorption was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as reference compound. Moreover, vehicle control test was performed for DMSO:H_2O mixture.

Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC activity of the DMSO:H_2O (10% v/v) diluted essential oil was measured by using the modified method [39]. Equal volumes of neocuproine, $CuSO_4$ and ammonium acetate buffer were mixed. Then, the essential oil was added to mixture and then kept at room temperature for 1 h, and the absorbance was taken at 450 nm. The results were expressed as mg ascorbic acid equivalent per g DE.

Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power activity was assessed spectrophotometrically according to the method described by Bardakci et al. [39]. Correspondingly, essential oil was diluted to 1 mg/ml concentration and 10 µl of it was mixed with 250 µl FRAP solution. After an incubation period of 30 min., the absorbance was retrieved at 593 nm. BHT was used as reference. The results were expressed as mM $FeSO_4$ per g DE.

Determination of total antioxidant capacity by phosphomolybdenum method

Total antioxidant capacity was calculated by phosphomolybdenum method previously published by Bardakci et al. [39]. Diluted essential oil was mixed with the reaction mixture composed of sulfuric acid, ammonium molybdate and sodium phosphate monobasic. After incubation at 95 °C for 90 min., the absorbance was read at 695 nm. Total antioxidant capacity was expressed as mg ascorbic acid equivalent per g DE.

In vivo tests

Animals

Adult male Wistar albino rats, weighing 250-300 g were obtained from Acibadem Mehmet Ali Aydinlar University Experimental Animal Application and Research Center (ACU-DEHAM). All protocols involving animals were approved by Animal Experiments Local Ethics Committee (decision number 2020/13). Food and water were available *ad libitum*. The colony room was kept at 20–24 °C and 60-70% humidity in a 12-hour light-dark cycle. All animals were handled daily and habituated to the experiment room during 3 days preceding the experiments. *In vivo* study consisted of two sections as: behavioral study (n=24) and bioavailability study (n=6). Behavioral study animals were divided into 4 groups depending on drug injection and/or inhalation they received. The behavioral study groups were as follows: Scopolamine injection + *S. triloba* inhalation (Scopolamine + *S. triloba* group), Saline injection + *S. triloba* inhalation (*S. triloba* group), Scopolamine injection + distilled water vapor inhalation (Scopolamine group), Saline injection + distilled water vapor inhalation (control group) (n=6 per group).

Scopolamine hydrobromide was dissolved in an isotonic saline solution (0.9% NaCl). Injections were administered intraperitoneally (i.p.) daily, for 7 days 30 min before water maze test sessions. Scopolamine injections (1 mg/kg) were administered to Scopolamine group and Scopolamine + *S. triloba* group. *S. triloba* group and the control group received isotonic saline injections as sham treatment. Scopolamine dose for inducing amnesia was determined according to an extensive review that investigated effective dose in Morris water maze [5].

All animal groups were individually placed in regular laboratory rodent housing cages of 36x20x14 cm. During the inhalation session, the cage was placed in a 50x40x28 cm transparent plexiglass box (inhalation box) with the vaporizer set up. The vaporizer was designed as follows: 200 µL of the liquid substance to be delivered by inhalation (*S. triloba* essential oil or distilled water) was dropped on a beaker, and the beaker was then placed over a battery powered heater plate (55 °C). The inhalation box was hermetically sealed and the animal was left in the box for 30 minutes. During this period, bioavailability study group, *S.triloba* group and Scopolamine + *S. triloba* groups inhaled *S. triloba* essential oil, while Scopolamine group and the control group inhaled water vapor as sham treatment. Essential oil volume and duration of the implementation were determined based on the study by Bagci et al. with slight modifications [17].

Bioavailability test with GC-MS headspace

Immediately afterwards the inhalation of *S. triloba* essential oil (as described above), 1 mL blood samples were collected under isoflurane anesthesia from each rat and put into 20 mL GC-MS headspace vial and instantly sealed with an aluminum cap. Agilent 7694 E headspace sampler was used with 70 °C extraction temperature, 1 mL sample volume, 110 °C transfer

line temperature and 16 psi vial pressure for sample injection. Analysis was performed with exactly the same chromatographic method which was used for essential oil analysis. 1,8-cineole standard was used for precise determination.

Morris water maze

All groups received i.p. injection and 30 minutes of inhalation preceding the water maze sessions. The water maze consisted of a black circular tank filled with water to a depth of 30 cm at 25 ± 1 ° C. The pool was divided to four equal quadrants and according to these quadrants 4 start locations (east, north, west, and south) were determined on the perimeter of the tank. A platform (10 × 10 cm) was placed 1 cm below the water and placed at 1/3 length of tank diameter away from tank wall. Geometric shapes and banners were placed on the walls to be used as visual cues by the animals for orienting in water tank. Learning phase was of daily learning blocks for 6 days, and each learning block included 4 consecutive learning trials. In the learning trials, the animal was gently released to water pool facing towards the pool wall from one of the 4 starting locations. It was allowed to swim and climb on the platform for 90 seconds. If an animal failed to find the platform in 90 secs, it was gently picked and left over the platform. By the end of the trials, animals were allowed to rest on the platform for 30 secs, whether they could find the platform by themselves or left there by the experimenter. Starting locations were shifted on every learning trial but the location of the platform and the visual cues on the walls remained fixed through the learning phase. The time to find and climb on the platform by the animal was termed as escape latency. The escape latency for the trials with failure to find the platform is registered as 90 secs (maximum allowed search time). The mean of escape latency for 4 learning trials on a day is the daily escape latency. After completion of the 4th trial, the rat was gently dried and left under a heat lamp before returning to its home cage. The learning performance was mainly observed as the gradual decrease in escape latency on 6 consecutive days (learning phase). On the 7th day, the platform was removed and the animals were left from the second starting point (north) to swim freely for 90 seconds, termed as the probe trial. In the probe trial, the time spent to arrive at the zone where the platform was formerly located (platform latency) and the number of crossings over the platform zone (former place of the platform) are registered. In trials with failure to reach platform zone, platform latency is registered as 90 secs (maximum search time). Shorter platform latency and higher number of platform crossing were interpreted as better memory retention performance. All sessions were recorded and analyzed with Ethovision XT video tracking system (Noldus, The Netherlands).

Statistical Analysis

Each of the *in vitro* tests and analyses were done in triplicate. Escape latencies from 6 days were tested by a repeated measures ANOVA design with experimental animal study group factor with 4 levels (4 animal groups) and day factor with 6 levels (1st day, 2nd day, 3rd day, 4th day, 5th day, 6th day). Huynh-Feldt correction was applied for repeated-measures factors with more than 2 levels as correction for sphericity violation. Tukey test was used for pair-wise group comparisons (post-hoc test). In addition to the repeated measures ANOVA including whole learning phase (6 days), contrast analyses were conducted to elaborate the analyses on the progress of learning. In these contrast analyses, escape latencies from the first day was compared with each of the following days (i.e. 1st day vs. 2nd day, 1st day vs. 3rd day, 1st day vs. 4th day, 1st day vs. 5th day, 1st day vs. 6th day). Bonferroni correction was applied to control familywise error rate in these contrast analyses which involved multiple comparisons, i.e. P values are multiplied with 5 (the number of additional tests).

The platform latency (seconds) and number of platform crossing from the probe trial (7th day) were evaluated with univariate analyses. Normal distribution of platform latency was confirmed in each group by Shapiro-Wilk tests ($p>0.05$) and one-way ANOVA test was conducted to assess group differences. Tukey test was used for post-hoc tests (pair-wise group comparisons). Kruskal-Wallis test was conducted on number of platform crosses and Dunn-Bonferroni correction was applied on post-hoc comparisons. SPSS v. 23 software is used for all statistical analyses.

3. RESULTS

GC-MS analysis of different S. triloba samples and bioavailability

Phytochemical profiles of *S. triloba* essential oils from different geographical regions were evaluated via GC-MS analysis and the results were given in the Table I and Figure 1. In total 32 compounds representing the 86.76-98.09% of the total oils were identified. Results showed that 1,8-cineole amount of the samples varies between 13.22% to 40.48% which was measured 45.31% in commercial essential oil product. Contents of camphor, α and β-thujone are also very noteworthy for *S. triloba* essential oil. Results of the analysis also specified that proportion of these substances fluctuate considerably. α and β-thujone differ from 0.55 to 17.94% and 0.53 to 9.21% respectively. Likewise, camphor content varies between 4.02 to 19.40% in the studied samples.

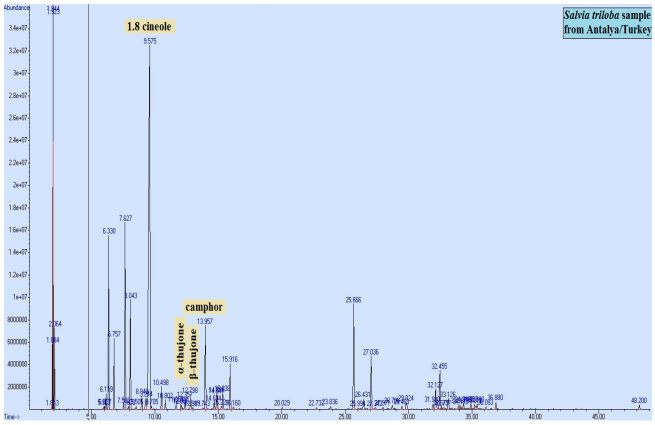

Figure 1. *GC/MS chromatogram of the S. triloba essential oil.*

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Table I. *Essential oil composition of aerial parts of various S. triloba samples*

RT (min)	RRI	Compounds	STA (%)	STY (%)	STI (%)	STB (%)	STC (%)
6.119	927	α-Thujene	0.58	6.60	0.55	0.38	0.75
6.330	933	α-pinene	7.40	8.15	5.39	5.40	6.03
6.757	948	Camphene	3.01	0.11	5.94	4.60	2.03
7.144	976	1-Octen-3-one	-	7.14	0.42	-	0.63
7.627	977	β-pinene	9.54	1.21	-	2.95	9.51
8.043	991	β-myrcene	5.05	0.38	2.18	1.87	2.83
8.940	1017	2-carene	0.66	0.16	0.56	0.52	0.65
9.244	1025	p-cymene	0.83	-	1.15	0.40	0.82
9.398	1029	D-Limonene	-	-	-	1.78	-
9.575	**1034**	**1,8-cineole**	**40.48**	**33.41**	**32.54**	**13.22**	**45.31**
10.498	1058	γ-Terpinene	1.10	0.67	0.80	0.93	1.24
10.802	1066	Sabinenehydrate	0.47	-	-	0.16	0.31
11.654	1088	α-Terpinolene	0.31	-	0.47	0.57	0.35
12.357	**1106**	**α-Thujone**	**0.55**	**0.82**	**3.84**	**17.94**	**2.49**
12.798	**1117**	**β-Thujone**	**0.80**	**0.53**	**3.98**	**9.21**	**4.50**
13.957	**1144**	**Camphor**	**5.15**	**19.40**	**18.53**	**14.55**	**4.02**
14.886	1166	Borneol	1.52	-	2.53	2.47	0.93
15.338	1177	Terpinene-4 ol	-	-	-	-	-
15.916	1191	α-Terpineol	2.73	3.89	-	0.39	1.24
20.016	1288	Isobornyl acetate	-	0.35	2.49	-	0.59
22.743	1348	D-Elemene	-	-	-	-	0.90
23.848	1375	α-Copaene	-	-	-	-	0.35
25.666	1420	Caryophlenne	7.32	4.79	2.33	4.14	6.89
26.431	1439	Aromadendrene	0.66	-	0.11	-	0.73
27.036	1454	α-Humulene	3.48	0.93	1.00	2.31	2.02
29.824	1524	D-Cadinene	0.59	0.81	0.17	-	0.50
31.910	1577	Spathulenol	0.35	-	0.98	-	-
32.127	1583	Caryophylleneoxide	1.56	-	-	0.30	-
32.455	1591	Viridiflorol	2.47	1.00	0.80	5.23	0.29
33.126	1609	Humulene epoxide II	0.76	-	-	-	-
34.316	1641	Cadinol	0.37	-	-	-	-
34.907	1657	Manool	0.35	-	-	-	-
		Total (%)	**98.09**	**90.35**	**86.76**	**89.32**	**95.91**

RT: Retention time, RRI: Relative Retention Index, STA: S. triloba samples from Antalya, STY: S.triloba samples from Yalova, STI: S.triloba samples from Izmir, STB: S.triloba samples from Balıkesir, STC: S. triloba commercial essential oil sample.

GC-MS-Head Space analyses were also conducted on the blood of essential oil inhaled rats for evaluating bioavailability of major principle of *S. triloba* oil, 1,8-cineole. Results of the analysis revealed that significant amount of 1,8-cineole was present in the blood stream of the rats and possible bioactivity of the essential oil inhalation may be attributed to bioavailable 1,8-cineole content (Figures 2 and 3).

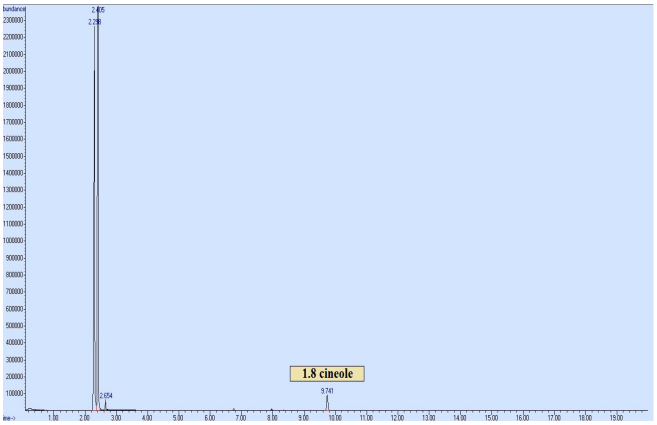

Figure 2. *GC/MS chromatogram of the S. triloba essential oil inhaled rat blood sample.*

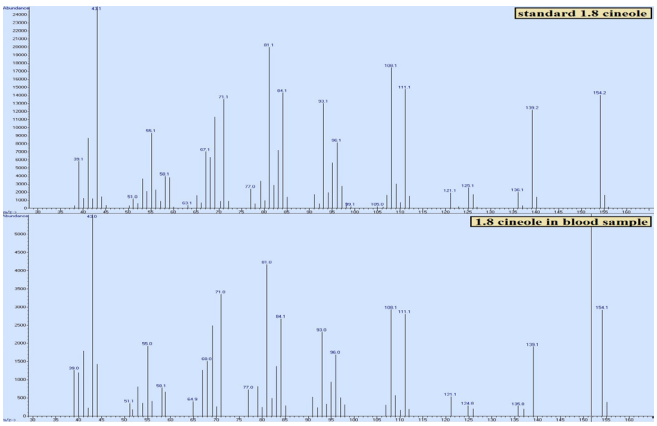

Figure 3. *Mass spectra of standard 1,8 cineole (upper pane) and 1,8 cineole in S. triloba essential oil inhaled rat blood sample (lower pane).*

In vitro evaluation of AChE and BChE inhibitory and antioxidant activities

AChE and BChE inhibitory activities of the STA essential oil are summarized in Table II. *S. triloba* essential oil possessed significant AChE and BChE inhibitory activities with respect to a strong anti-cholinesterase inhibitor, galanthamine. Results revealed that STA showed 841.47841.47 ± 15.57 mg GALAE/g AChE and 914.16 ± 9.58 mg GALAE/g BChE inhibition activity.

Table II. *In vitro AChE and BuChE inhibition of S. triloba essential oil (n=3)*

	AChE[#A] inhibition (mg GALAE /g)	BuChE[§A] inhibition (mg GALAE /g)
***S. triloba* essential oil (STA)**	841.47 ± 15.57	914.16 ± 9.58

[#] Acetylcholinesterase, [§] Butyrylcholinesterase, [A] Results were stated as the mean of triplicates and standard deviation (S.D.) and as mg galantamine equivalents (GALAE) in 1 g sample

In vitro antioxidant evaluation of STA was implemented with four different methods; DPPH radical scavenging activity (DPPH), Ferric reducing antioxidant power (FRAP), Cupric reducing antioxidant capacity (CUPRAC) and Total Antioxidant Capacity (TOAC). Result of the assays were given in Table III. It was determined that STA exhibite

d significant *in vitro* antioxidant activity.

Table III. *In vitro antioxidant activity of S. triloba essential oil (n=3)*

	DPPH[#A] (mg -BHTE/g)	FRAP[##A] (mg BHTE/g)	CUPRAC[§B] (mg AAE/g)	TOAC[§§B] (mg AAE/g)
***S. triloba* essential oil (STA)**	211.64 ± 9.88	71.31 ± 0.36	341.25 ± 12.39	578.94 ± 9.31

[A] Results were stated as the mean of triplicates § standard deviation (S.D.) and as mg butylated hydroxytoluene equivalents (BHTE) in 1 g sample. [B] Results were stated as the mean of triplicates § standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample. [#] 2,2-diphenyl-1-picrylhydrazyl. [##] Ferric reducing antioxidant power. [§]Cupric reducing antioxidant capacity. [§§] Total Antioxidant Capacity

Morris water maze

Animals in the *S. triloba* inhalation groups did not display any peripheral cholinergic side effects such as hypersalivation, vomiting or frequent micturition and/or defecation. The gradual decrease in daily escape latencies was indicative of learning the hidden platform in the water maze, shown in Figure 4. Escape latencies were significantly different between the groups [F (3,20) = 21.553; P<0.001].

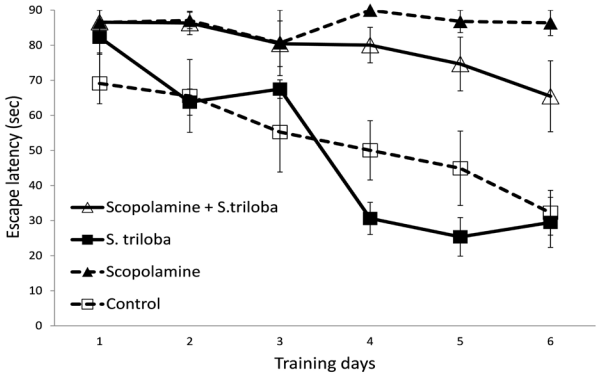

Figure 4. *Morris water maze acquisition (learning) performance, escape latencies during 6 training days.*

Post-hoc tests revealed that the escape latencies of Scopolamine group were significantly longer than *S. triloba* group and control group (P<0.001 for both comparisons). Scopolamine + *S. triloba* group also displayed significantly longer escape latencies compared with *S.triloba* group and control group (*S. triloba*) (P<0.001, P=0.001, respectively). In reference to the control group, learning was not observed in the two groups with scopolamine induced learning impairment. Pair-wise group comparisons also revealed no significant difference between Scopolamine group and Scopolamine + *S. triloba* group. Escape latencies were also comparable between control group and *S. triloba* group by the end of 6 day of learning phase.

In addition to the GROUP effects in the overall (6 days) escape latencies, GROUP x DAY interaction was also significant [F(15,100) = 3.948; P<0.001]. Contrast analyses comparing escape latencies from 1st day against the following days revealed significant group differences on 4th, 5th and 6th days (P<0.0001, P=0.001, P=0.006, respectively). In the *S. triloba* group and control group (i.e. group that displayed learning), GROUP x DAY interactions in the escape latencies were significant [F(5,50) = 2.861; P=0.031]. Contrast analyses comparing escape latencies against the 1st day revealed that *S. triloba* group displayed significantly shorter escape latency on 4st day compared with the control group (P=0.01, Bonferroni corrected, Figure 5).

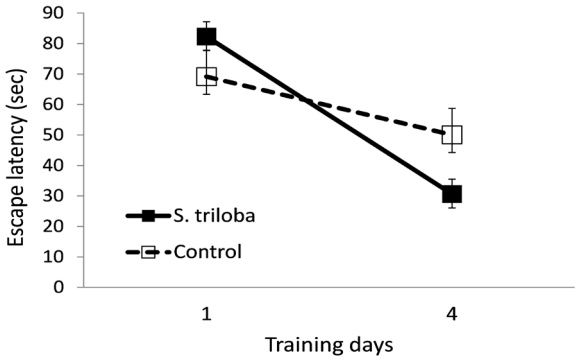

Figure 5. *Morris water maze test escape latencies of 1st and 4th days of learning comparing S. triloba vs. control group.*

On the probe day, the number of platform crossing was significantly different between 4 groups [$X^2(3)$ = 13.772, P= 0.003]. The control group and *S. triloba* group performed significantly more crosses than scopolamine group (P=0.02, P=0.005, respectively). Scopolamine + *S. triloba* group displayed few crosses on average and it was lower than that of *S. triloba* (P= 0.052). There was no significant difference between control group and *S. triloba* or Scopolamine + *S. triloba* groups. Results of post-hoc tests are listed in Table IV (also see Figure 6).

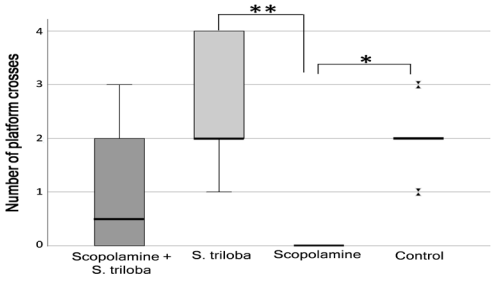

Figure 6. *Morris water maze test memory retention performance, boxplot chart with medians of number of platform crosses (*P< 0.05; **P< 0.01).*

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Platform latency was significantly different between 4 study groups [F (3,20) = 4.05; P<0.05]. Post-hoc tests revealed that, platform latency was significantly shorter in control and *S.triloba* groups compared with the Scopolamine group (P<0.05 for both comparisons). Results of post-hoc tests are listed in Table IV (also see Figure 7).

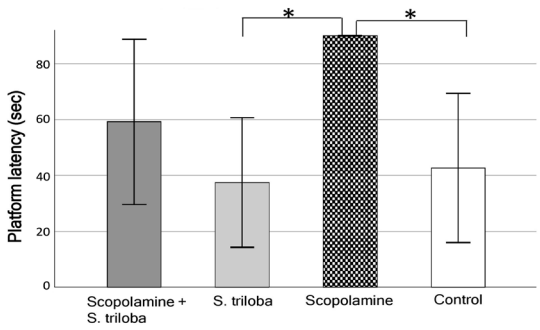

Figure 7. *Morris water maze test memory retention performance, bar graph of platform latency (*P < 0.05).*

Table IV. *Morris water maze pair-wise group comparison, p values for number of platform crosses and platform latency on probe trial*

Pair-wise group comparisons	Number of platform crosses	Platform latency
Control vs. *S. triloba*	0.9	0.989
Control vs. Scopolamine	0.02*	0.045*
Control vs. Scopolamine + *S. triloba*	0.837	0.755
Scopolamine vs. *S. triloba*	0.005**	0.024*
Scopolamine vs. Scopolamine + *S. triloba*	0.872	0.571
S. triloba vs. Scopolamine + *S. triloba*	0.375	0.278

**P < 0.05, **P< 0.01*

4. DISCUSSION

Phytochemical profiles of *S. triloba* essential oils from different geographical regions were evaluated via GC-MS analysis and the results were given in the Table 1 and Figure 1. Previous studies revealed that phytochemical profile of essential oil composition of *Salvia* species even *S. trioba* essential oils might be highly variable [26, 39]. In a recent study, phytochemical composition of *S. triloba* samples from two different localities showed variations [40]. 1,8-cineole content of the samples was measured up to 47.1% however; there are significant variation for the profile depending on the geographic differences and essential oil obtaining methods. In this study, phytochemical analysis was performed on the essential oils which were obtained from aerial parts of *S. triloba* from different locations of Turkey and essential oil purchased from market. Results revealed that phytochemical profiles of *S. triloba* essential oils are highly variable depending upon the geographical differences. 1,8-cineole is known as the major component of *S. triloba* essential oil and the responsible molecule for its various bioactivities especially for its memory-enhancing activity [28]. Nonetheless, previous studies demonstrated that *S. triloba* essential oil samples on the market might contain high amount of camphor, α-thujone and β-thujone ketones which are infamous for their toxic properties [41, 42]. Camphor, α-thujone and β-thujone contents were highly variable likewise 1,8-cineole content. For further *in vitro* and *in vivo* investigations on cognitive properties of *S. triloba* essential oil, appropriate sample selection is crucial. Thujone content is lowest in the essential oils from Yalova (STY) and Antalya (STA) however camphor content is overwhelmingly higher in STY when compared to STA (19.40% and 5.15%, respectively). In addition, 1,8-cineole content is highest in STA (40.48%) which is very close to commercial essential oil (STC) (45.31%). In the light of these data, although, STC contains the highest amount of 1,8-cineole content, STA was selected as the most suitable candidate for further bioactivity studies due to its high amount of 1,8-cineole content and lower amounts of camphor, α-thujone and β-thujone contents.

In the light of prior research pertaining to the significant anti-cholinesterase activities of various extracts and essential oil of *S. triloba*, this study is planned as an investigation on its enzyme inhibitory activity, phenolic profile and antioxidant potentials. Senol et al., revealed that essential oil of *S. triloba* samples have stronger anti-cholinesterase inhibition rather than dichloromethane, ethyl acetate, and ethanol extracts of the same plant [26]. Similar to this study the tested essential oil was dominated with 1,8-cineole (36.25%). The data obtained in this study were in accordance with the results of Savelev et al. [25]. In a recent study, AChE inhibitory activity of *S. triloba* essential oils from Turkey were evaluated [40]. Results demonstrated that both essential oil samples from diverse geographic locations contains high amount of 1,8-cineole, exhibited significant AChE inhibitory activity which are coherent with our findings. Our results and previous data are indubitably indicating that, *S. triloba* essential oil has significant cholinesterase inhibition potential which may lead to potential clinical effects.

It is well known that oxidation and ROS production is highly correlated with the progress of neurodegenerative diseases. Therefore, reducing oxidation and scavenging ROS might be beneficial for preventing such diseases and enhancing cognitive performance [32, 33, 43]. Thus, antioxidative properties of STA essential oil were determined via *in vitro* assays for revealing possible positive effects in our study. DPPH radical scavenging activity of *S. triloba* essential oil from Turkey was previously evaluated. Former results demonstrated that essential oil inhibited DPPH radical by 5.12±0.42% in 1 mg/mL concentration while gallic acid reference showed 92.57 ± 0.1% inhibition at the same concentration [26]. In addition, same study revealed that ferric reducing power of the sample showed 0.302 ± 0.01 absorbance at 1 mg/mL concentration while chlorogenic acid showed 3.618 ± 0.01 at the same concentration. In the present study, DPPH

radical scavenging activity of STA essential oil was measured as 211.64 ± 9.88 mg BHTE/g and FRAP activity was measured 71.31 ± 0.36 mg BHTE/g. Relatively higher antioxidant activity of present sample may be originated by its higher amount of 1,8-cineole and significantly lower amount of camphor when compared to aforementioned study. In a recent study, CUPRAC assay was also employed for evaluation of metal reducing capacity of *S. triloba* essential oils collected from different locations of Turkey [40]. Results showed that both essential oils had significant metal reducing activity, which are consistent with our findings. In our study, *S. triloba* essential oil showed 341.25 ± 12.39 mg AAE/g cupric reducing activity. In addition, TOAC assay was conducted for determining molybdenum reducing potential of *S. triloba* which is the first study of this subject in the literature to our knowledge. Results revealed that essential oil showed 578.94 ± 9.31 mg AAE/g total antioxidant capacity which is coherent with former results measuring metal reducing activity. Especially low molecular weight compounds in the essential oils of *Salvi*a species readily cross blood and blood-brain barrier due to their small molecular size and polarity. In order to verify the absorption of volatile components in *S. triloba* essential oil via inhalation, existence in the blood of rats were examined by comparison of GC-MS spectra of standard 1,8-cineole and blood. The presence of 1,8-cineole in whole *in vivo* test groups were verified for the reliability of the current study.

Control and *S. triloba* groups displayed learning effect in Morris water maze, i.e. gradual decrease in mean escape latency from 70-80 seconds to 30 secs by the end of the learning phase (6^{th} day). Besides, *S. triloba* group reached peak performance 2 days earlier than the control group, this effect was confirmed with contrast analyses. Scopolamine group and Scopolamine + *S. triloba* group displayed little or no learning. Escape latencies of Scopolamine + *S. triloba* group had a tendency to decrease in of 4^{th},5^{th} and 6^{th} days of learning phase. Nevertheless, this slight difference was non-significant, and restorative effect of *S. triloba* inhalation on scopolamine induced learning impairment was invalidated.

On the probe trial, Scopolamine group failed to cross platform zone, as it was expected due to lack of learning. Although, the statistical tests rejected a solid reversing effect of *S. triloba* inhalation, *S. triloba* treated amnesia group performed somewhere in between a drastic memory failure and normal memory performance, (i.e. in the probe trials they performed statistically indifferent from both control group and Scopolamine group due to high variance).

Our results are coherent with several previously reported findings obtained by *S. miltiorrhiza* [44], *S. officinalis* and *S. lavandulaefolia* extracts [45]. These results are also consistent with cognitive enhancer effects of ingesting *S. lavandulaefolia* essential oil capsules on healthy young volunteers [46]. In the present study and those previous studies, the behavioral results of *Salvia* species are mostly attributed to AChE and BChE inhibiting effects of 1,8-cineole. We assume these positive effects of *S. triloba* essential oil on rats' Morris water maze performance are due to cholinergic boosting that was applied preceding the maze sessions. The improved spatial learning due to cholinergic enhancement is mostly explained by facilitation cholinergic projections from septum to hippocampus, retrosplenial and posterior parietal cortices that are essential for spatial navigation and learning [47]. We used GC to confirm high amount of 1,8-cineole in the essential oil and its bioavailability by inhalation. *S. triloba* essential oil also has antioxidant activity that was also a favorable feature by cholinesterase inhibitors [24, 48].

In conclusion, samples of *S. triloba* which is a well-known Anatolian sage, were obtained from different geographical regions of Turkey. The hydro-distilled essential oil compositions of whole samples were identified by using GC-MS. The desired essential oil was selected according to its 1,8-cineole, camphor α-thujone and β-thujone amounts. Afterwards, the essential oil of *S. triloba* was examined for its inhibitory activity on cholinesterases and antioxidant potential. *In vitro* tests exhibited significant AChE and BChE inhibitory activities as well as antioxidant activities. Additionally, cognitive boosting effect was evaluated via Water maze test. Our results refuted a hypothetical reversing effect of *S. triloba* inhalation on the devastating scopolamine induced learning impairment. This negative behavioral result could have several grounds including insufficient *S.triloba* dose or a mild reversing effect could have failed to occur due to insufficient acquisition period. Depending on maze pool size, number of visual cues, or several animal features like age and strain, reaching stable minimum escape latency may take up to 10 days [49]. Nevertheless, *S. triloba* inhalation led to a significant improvement in learning performance compared with control group and an indistinct effect on memory retention in scopolamine induced impairment group. These preliminary results suggest that it is worth studying with variant doses. Also, future *S. triloba* inhalation studies on transgenic AD models can provide more sensitive evaluation of its supplemental effects on cognition and learning.

Compliance with the Ethical Standards

Ethics Approval: All protocols involving animals were approved by Animal Experiments Local Ethics Committee of Acibadem Mehmet Ali Aydinlar University (decision number 2020/13).

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Conflict of interest statement: All authors declare no conflict of interest.

Authors contributions: GSE: contributed to all processes of the study; ME: study design, statistics and interpretation of the behavioral study; THB: Gas Chromatography, *In vitro* studies, analysis and evaluation; HB: supervision and active involvement in plan gathering and essential oil extraction; GS: design, interpretation and supervision for behavioral study.

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