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The Leaf and the Gall Volatiles of *Salvia fruticosa* Miller from Turkey: Chemical Composition and Biological Activities

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Abstract: In this study two different extraction techniques namely, conventional hydrodistillation (HD) and micro-steam distillation-solid-phase microextraction (MSD-SPME), were used to analyze the volatile constituents from the leaves and the galls of *Salvia fruticosa* Mill. by gas chromatography (GC-FID) and gas chromatography coupled to mass spectrometry (GC-MS). The oxygenated monoterpenes (62.4-69.3%) were found to be predominating compound group with 1,8-cineole and camphor as the main constituents in all the tested samples with the exception of the gall oil in which oxygenated sesquiterpenes (25.6%) and diterpenes (17.3%) were detected in high percentages. Qualitative differences of the volatiles obtained by HD and MSD-SPME techniques from the leaf and the galls are discussed. The resulting leaf essential oils were evaluated for their in vitro acetylcholinesterase (AChE) inhibition potential. In vitro DPPH scavenging, and Cupric Reducing Antioxidant Capacity (CUPRAC) assays were used to evaluate the leaf essential oils. AChE inhibition 41.2% and antioxidant 36.8% assays results showed moderate levels of activity. In this present study, to the best of our knowledge comparative leaf and gall volatiles of *S. fruticosa* was reported for first time.

Keywords: *Salvia fruticosa*; galls; acetylcholinesterase, antioxidant; SPME; GC-MS.

1. Introduction

Turkey has a great variety of biotic and abiotic diversity, i.e. climatic, edaphic, geographic-geologic, and pollinator diversity. These factors lead Turkey to be one of the most important plant

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biodiversity centers in the world with over 10.000 plant species and ca. 35% endemism ratio. There are 100 species and 107 taxa of *Salvia* in the flora of Turkey. Rate of endemism on taxon basis is 54% Lamiaceae is the third largest family in Turkey with 46 genera, 782 taxa comprising 603 species and 179 subspecies and varieties. 346 taxa (271 species and 75 subspecies and varieties) are endemic. Endemism ratio is ca. 44% [1, 2]. The genus *Salvia* (sage) is one of the largest genera of Lamiaceae. Although *Salvia* has lower number of taxa in the Mediterranean phytogeographic region than the Irano-Turanian phytogeographic region in Turkey, endemism ratio is higher in the Mediterranean phytogeographic region [1]. In the Flora of Turkey, 100 *Salvia* species and 107 taxa are recorded, 58 of which are endemic taxa and endemism ratio 54% [1,3].

Sage has probably been used since 1400 B.C. because it was illustrated in the "blue bird fresco" in the House of Frecoes, Knossos [4]. Several *Salvia* species have important commercial value on the herbal market, *Salvia officinalis* L., *S. triloba* L. fil. (Syn. *S. fruticosa* Miller), *S. lavandulaefolia* Vahl., *S. verbenaca* L. and *S. sclarea* L. *S. tomentosa* Miller is another species with development potential. While *Salvia officinalis* is cultivated worldwide, and *S. sclarea* in Europe and North America, *S. fruticosa* oil is produced from wild plants as well as from cultivated ones [5]. Most *Salvia* species (69%) are moderately rich in oil (0.1–1.0%). Nine taxa (14%), which comprise commercial species, contained >1 % oil. *Salvia* taxa of Turkey were classified by Baser (2002) according to main components in their respective essential oils. Commercial *Salvia* species belong to the following groups: CiCa (1,8-cineole/camphor) group, *S. fruticosa* (syn. *S. triloba*); pinene (α/β) group, *S. tomentosa*; thujone (α/β) group, *S. officinalis, S. pomifera* (syn. *S. calycina*) [6]. The actual situation of sage oil production and export (mainly in Mediterranean countries) was reported by Baser. In Turkey, approximately 500 kg of the leaf oil from *S. triloba (S. fruticosa)* is annually produced and 600 tons of sage leaves worth more than 1.5 million US$ are exported in 1996. The sage oil is produced from *S. fruticosa* (wild) in Manisa and Alanya provinces of Turkey. The local people call the sage oil "elma yağı" (apple oil) due to resemblance of galls (apples) growing on its leaves and stems to small apples [7].

The literature survey revealed a number of ethnopharmacological reports about *S. fruticosa* usage in folk medicine: diarrhea [8], colds and abdominal pain [9], antiseptic, dyspepsia, tonsillitis [5, 10]. Also it is used for the treatment of burn by sowing the dried *S. fruticosa* leaves after being powdered [11].

Pharmacological properties and biological activities of the essential oils and extracts *S. fruticosa* have been reported such as anti-inflammatory [12], antioxidant [13, 14], antimicrobial [15], antifungal [16], anticancer [17], antilipolytic and hypotriglyceridemic [18], hypoglycemic [19], acetylcholinesterase inhibition [20], butyrylcholinesterase inhibition [21], pancreatic lipase inhibition [22].

Previous phytochemical investigations of *S. fruticosa* showed the occurrence of flavonoids [23-25], phenolic acids [25, 26], fatty acids [27], mono-, sesqui-, di- and triterpenoids, steroids [28-30]. There are number of essential oil reports of *S. fruticosa* from different regions of Turkey [30-33] however, detailed GC-MS analyses of the essential oils of the leaf and the galls have not been reported.

Aim of our work was to evaluate chemical composition of the oils obtained from the leaves and the galls of *S. fruticosa* growing in Turkey. In scope of the present work, we carried out the gas chromatographic analysis of the essential oils obtained by hydrodistillation (HD) and micro-steam distillation-solid-phase microextraction (MSD-SPME) techniques from the leaves and the galls of *S. fruticosa.*

Essential oil (EO) of *S. fruticosa* was evaluated *in vitro* for memory-enhancing effect via inhibition of the acetylcholinesterase (AChE) enzyme. Inhibition of AChE can be considered as a promising strategy for the treatment neurological diseases including Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, Parkinson's disease, amyotrophic lateral sclerosis etc. [34-38]. According to cholinergic hypothesis, AD is characterized by reduction in the activity of the cholinergic neurons [39]. Up to date, many attempts have been carried out to find effective anti-AChE drugs. For instance, galanthamine from *Galanthus nivalis* L. [40], eserin from *Physostigma venenosum* L. [41], huperzin A from *Huperzia serrata* (Thumb.) Trev. [42] possess pharmacologically proven anti-AChE activity. Galanthamine is applied clinically in USA and EU. However, eserin cannot be used in treatments because of side effects and huperzin A is still in phase-III clinical trials in China [43]. A comprehensive

review on natural anti-AChE agents has recently been published by Topcu [44]. There are reports about anti-AChE activity of essential oils obtained from *Citrus aurantifolia* Swingle, *C. aurantium* L., and *C. bergamia* Risso and Poit. Peels [45], *Origanum ehrenbergii* Boiss. and *O. syriacum* L. [46], *Acorus calamus* [47], *Salvia lavandulifolia* Spreng. [48]. The strong anti-cholinesterase potential of the extracts and essential oils from many *Salvia* species [39, 49, 50] prompted us to investigate the oil of *S. fruticosa* for memory enhancing potential.

2. Experimental

2.1. Plant Material

The leaf (L) and gall apple (G) of *Salvia fruticosa* Miller (Syn: *S. triloba* L.) (LI-GI, LII-GII and LIII-GIII) were collected from different localities of Turkey. The leaf and gall containing specimens of *S. fruticosa* are deposited at the Herbarium of the Faculty of Pharmaceutical Botany of the Istanbul University and Herbarium of the Faculty of Pharmaceutical Botany of the Marmara University, Turkey.

S. fruticosa LI-GI was collected in August 2007 from Muğla-Torba, Turkey. It was identified by Prof. Dr. Ertan Tuzlacı and has been deposited at the Herbarium of Faculty of Pharmacy, Marmara University with specimen number, MARE No: 11109a. *S. fruticosa* LII-GII was collected in May 2009 from Muğla, Marmaris-Bayır village, Turkey and also *S. fruticosa* LIII-GIII was collected in September 2009 from Tekirdağ, Gaziköy-Şarköy, Turkey. *S. fruticosa* LII-GII and *S. fruticosa* LIII-GIII were identified by Dr. Bahar Gürdal and have been deposited at the Herbarium of Faculty of Pharmacy, Istanbul University with specimen numbers, ISTE No: 91439 and ISTE No: 86111 respectively.

2.2. General

Agilent 5975 GC-MSD system (Agilent Technologies, Santa Clara, USA) equipped with the HP-Innowax FSC column (60 m × 0.25 mm id with 0.25 μm film thickness, Agilent, USA). The GC-FID analysis was carried out with capillary GC using an Agilent 6890N GC system (Agilent Technologies, Santa Clara, USA). Absorbance was recorded with Biotek Powerwave XS microplate reader. SPME fiber coated with PDMS-DVB (polydimethylsiloxane – divinylbenzene) 65 μm "blue type" was provided from Supelco (Supelco Park, Bellefonte, PA, USA). Before use, the fiber was reconditioned in accordance to manufacture recommendations.

2.3. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), anhydrous sodium sulphate (ACS-ISO, for analysis), sodium chloride, magnesium chloride hexahydrate, Tris(hydroxymethyl) aminomethane (ACS reagent), acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel, Type VI-S, 200-1,000 units/mg protein), bovine serum albumin (BSA), acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium phosphate, disodium phosphate, cupper chloride, ammonium acetate, neocuproine (Nc) and galanthamine hydrobromide from *Lycoris* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Hexane (ACS, for analysis) was purchased from Carlo Erba (Italy). Sodium chloride (extra pure) was obtained from Merck (Darmstadt, Germany). A C_9–C_{40} *n*-alkane standard solution was purchased from Fluka (Buchs, Switzerland).

2.4. Isolation of Essential Oil

2.4.1. Hydrodistillation

The essential oils were isolated separately from the dried and ground herb and the galls of *S. fruticosa* by hydrodistillation (HD) using a Clevenger type apparatus according to a procedure described in the European Pharmacopoeia [51]. The oil yield was calculated on a dry weight basis, dried over anhydrous sodium sulfate, and stored in sealed vials in refrigerator (4ºC), until GC-FID and GC-MS

analyses. The oil was dissolved in *n*-hexane (10%, v/v) to conduct chromatographic determination of its composition.

2.4.2. MSD-SPME Technique

Volatiles were isolated with MSD-SPME (micro-steam distillation-solid-phase microextraction) technique from the plant material using device described earlier [52]. The dried and ground plant material was placed in 25 mL round bottom flask used as refluxing vessel along with 5 mL of water. The flask was fitted with a Claisen distillation head with plug and a condenser set up for refluxing rather than distillation. MSD–SPME was performed on 0.5 g plant material with 1.0 min reflux. Heating was achieved using electric heater, and threaded plug was used for SPME fiber assembly. A manual SPME holder (57330-U, Supelco, Bellefonte, PA) and the PDMS-DVB 65 μm fiber "blue-type" were used for SPME procedure of volatiles. Fiber was conditioned at 250°C for 30 min before the experiment. After the SPME needle pierced the plug, the fiber was expressed through the needle and exposed to the headspace above a plant sample. After the trapping of volatile, the loaded SPME fiber was withdrawn into the needle, and then the needle was removed from the plug and subsequently used for GC-FID and GC-MS analyses. Desorption of the analytes from the fiber coating was performed by heating the fiber in the injection port to 250ºC for 5 min. The analytes were then transferred directly into the chromatographic column for analysis. Afterwards, the SPME fiber was reconditioned at 250°C for the next extraction experiment for 30 min. The fiber was subjected to a blank injection to ensure fiber integrity and the absence of any analytes after each reconditioning period. Experiments were performed in triplicate.

2.5. Chemical Composition of the Essential Oil

2.5.1. Gas Chromatography–Mass Spectrometry (GC-MS)

The volatiles were analyzed by GC-FID and GC-MS techniques. The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system (Agilent, Technologies, Santa Clara, USA). HP-Innowax FSC column (60m × 0.25mm, 0.25μm film thickness, Agilent, Walt & Jennings Scientific, Wilmington, DE, USA) was used with a helium carrier gas at 0.8 mL/min. GC oven temperature was kept at 60ºC for 10 min and programmed to 220°C at a rate of 4°C/min, kept constant for 10 min at 220ºC, and then programmed to increase at a rate of 1 ºC/min to 240ºC. The oil was analyzed with a split ratio of 40:1. The injector temperature was 250ºC. Mass spectra were taken at 70 eV and the mass range was from *m/z* 35 to 450. Experiments were performed in triplicate.

2.5.2. Gas Chromatography (GC)

The GC-FID analysis was carried out with capillary GC using an Agilent 6890N GC system (Agilent Technologies, Santa Clara, USA). Flame ionization detector (FID) temperature was set at 300ºC in order to obtain the same elution order with GC-MS. Simultaneous injection was performed using the same column and appropriate operational conditions. Experiments were performed in triplicate.

2.5.3. Identification and Quantification of Compounds

Identification of the compounds was performed according to the following: (i) comparison of the GC-MS Relative Retention Indices (RRI) of the compounds on polar column determined relative to the retention times of a series of *n*-alkanes (C_9-C_{40}), with those of authentic compounds or literature data; (ii) computer matching with commercial mass spectral libraries: MassFinder software 4.0, Adams Library, Wiley GC-MS Library (Wiley, New York, NY, USA) and NIST Library, and comparison of the recorded spectra with literature data [53-56]. Confirmation was also achieved using the in-house "Başer Library of Essential Oil Constituents" database, obtained from chromatographic runs of pure compounds performed

with the same equipment and conditions [57]. Percent composition was obtained for each constituent on the basis of GC-FID analyses of the oils.

2.6. Anti-Acetylcholinesterase Assay

Acetylcholinesterase (AChE) inhibition of the samples was evaluated using Ellman's method as previously reported [58]. Three buffers were used in the experiment: (**A**) 50 mM Tris-HCl (pH=8.0, in ultrapure water); (**B**) 0.1 % BSA in buffer A; (**C**) 0.1 M NaCl and 0.02 M $MgCl_2 \bullet 6H_2O$ in buffer A. In the 96-well flat bottom plates, 25 μL sample (EO/ reference compound), 50 μL buffer B and 25 μL AChE (0.22 U/mL in buffer A) solution were mixed and incubated for 15 min at 25ºC. Then, 125 μL Ellman's reagent DTNB (3.0 mM in buffer C) and 25 μL substrate ATCI (15 mM, in ultrapure water) were added. The mixture allowed to stand 15 min at 25ºC and the absorbances were recorded at 412 nm. Similarly, a blank (for eliminating the colors of the samples) was prepared by adding sample solution to all reaction reagents and 25 μL buffer instead of enzyme. The control wells contained all the reagents without the sample (the solvent of the sample instead was added). Galanthamine hydrobromide (0.1 mg/mL) was used as positive control. The percentage inhibition was calculated according to equation:

$$\%Inh = \left[\frac{(Abs_{control} - Abs_{control\ blank}) - (Abs_{sample} - Abs_{sample\ blank})}{Abs_{control} - Abs_{control\ blank}}\right] \times 100$$

where $Abs_{control}$ and $Abs_{control\ blank}$ are the absorbances of the control and its blank, Abs_{sample} and $Abs_{sample\ blank}$ are the absorbances of the sample and its blank.

2.7. Antioxidant Assays

2.7.1. Cupric Reducing Antioxidant Capacity (CUPRAC)

The cupric ion reducing capacity of EO was determined according to the method of Apak et al. [59] with slight modifications. The EO solution (3.75 mg/mL), gallic acid (0.01 mg/mL) and ascorbic acid (0.1 mg/mL) solutions were prepared in methanol. $CuCl_2$ solution (1.0×10^{-2} M) and ammonium acetate buffer (1.0 M, pH 7.0) were prepared in ultrapure water. Neocuproine (Nc) solution (7.5×10^{-3} M) was prepared in absolute ethanol. 55 μL sample solution (EO / reference compound), 50 μL $CuCl_2$ solution, 50 μL Nc solution and 50 μL ammonium acetate buffer were placed by multichannel automatic pipette (Eppendorf Research® plus, Germany) into 96-flat bottom well plate cells and allowed to stand in the dark for 30 min. The control well contained the same reagents except sample (methanol was added). After incubation at 25°C for 30 min, the absorbance at 450 nm of wells was measured using an ELISA microplate reader (Biotek Powerwave XS).

2.7.2. Free Radical Scavenging Assay (DPPH test)

The scavenging effect of the sample on DPPH free radical was determined using a modified method of Brand-Williams [60]. The solution of EO (3.75 mg/mL), gallic acid (0.01 mg/mL) and ascorbic acid (0.1 mg/mL) were prepared in methanol. 100 μL of the sample (EO / reference compound) solution was mixed with 100 μL DPPH solution (0.08 mg/mL in MeOH) in 96-flat bottom well plate cells. 100 μL methanol mixed with 100 μL of DPPH was used as control. The mixtures allowed to stand in the dark for 30 min. Decrease in the absorbance was recorded at 517 nm. Gallic acid (0.01 mg/mL) and ascorbic acid (0.1 mg/mL) were used as positive control. Experiments were performed in triplicate. The free radical scavenging activity of the sample was expressed as percentage of inhibition calculated according to equation:

$$Inh\ \% = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) x100$$

where $Abs_{control}$ is the absorbance of the control (containing all reagents except the test sample), Abs_{sample} is the absorbance of the sample with added DPPH.

3. Results and Discussion

3.1. Chemical Composition

Our work aimed to analyze the essential oil compositions of *S. fruticosa* leaves and galls and to investigate the biological proporties of its leaves' essential oil. Hydrodistillation of *S. fruticosa* yielded yellowish essential oils (EO)(2.3-3.0%) with a specific odor from the leaves and 0.10-0.12% from the gall. The GC-FID and GC-MS analyses allowed us to determine qualitative and quantitative profiles of the leaf and the gall volatiles of *S. fruticosa*. The list of detected compounds with their relative percentages, retention indices and method of identification is given in Table 1 in order of their elution on the HP-Innowax FSC column.

GC-FID and GC-MS analyses of the leaf oils obtained by hydrodistillation of *S. fruticosa* from three collection sites (I), (II) and (III), resulted in the characterization of 52 to 70 constituents corresponding to 96.0-98.9% of the oils. The essential oil composition is considered to be more complex than previous studies [30].

The gall oils analyses revealed 41-51 compounds constituting 92.1-98.1% of the oils. The volatile constituents detected in the leaf and the galls were classified as mono-, sesqui- and diterpenes, and fatty acids. As can be seen from Table 1, the leaf oils demonstrate differences in percentage of major constituents. The major oxygenated monoterpene, 1,8-cineole (27.2%) was detected in lower amount in L(I)-HD oil than in L(II)-HD and L(III)-HD EOs (47.1% and 46.7%, respectively). Meanwhile, camphor was found in higher amount (19.8%) than those of the other leaf oils (9.3%). Among the monoterpene hydrocarbons a quantitative difference was noted for camphene, which accounted for 10.7% in L(I)-HD oil while in the other leaf oils it was 3.7% and 3.8%. Topçu et al. reported higher amount of 1,8-cineole (58.9 %) [30].

Chemical composition of EOs obtained by hydrodistillation of the galls of *S. fruticosa* from three collection sites (I), (II) and (III) was found to differ significantly from the leaf oils. The big difference was detected in the amount of the main compound 1,8-cineole, whose percentage was down to 1.7% in G(I)-HD, and to 13.1% and 12.9% in G(II)-HD and G(III)-HD oils, respectively. Similarly, the percentage of camphor decreased to 3.2% in G(I)-HD oil, while in the G(II)-HD and G(III)-HD gall oils it was 21.3% and 21.0%. The sesquiterpenes were found in higher amount (up to 11.9%) in G(I)-HD oil than in other galls with β-caryophyllene (7.9%) and viridiflorol (8.6%) as major representatives. The gall oils were characterized with significant amount of diterpenes (5.2-17.2%) while in the leaf oils they were detected in scarce amount (0.8%). Manool (15.7%) was found to be the major constituent of the G(I)-HD oil. Another noteworthy fact is high percentage of fatty acids in the gall oil with hexadecanoic acid (14.8%) as the major compound in G(I)-HD EO.

According to literature data, climatic, and ecological differences as well as environmental conditions besides genetic variations could be responsible for the differences in the essential oil composition of *S. fruticosa* [61].

Distribution of the major compound groups in the leaf and the gall oils of *S. fruticosa* are represented in Figure S1. As can be seen, all the oils are rich with oxygenated monoterpenes with the exception of G(I)-HD oil in which oxygenated sesquiterpenes and diterpenes predominated over other compounds.

Table 1. Chemical constituents of essential oils obtained from the leaves and the galls of *Salvia fruticosa* with HD and MSD-SPME techniques [62-85]

RRI	RRI range (Ref.)	Compound	L(I)-HD	L(I)-MSD-SPME	L(II)-HD	L(III)-HD	G(I)-HD	G(I)-MSD-SPME	G(II)-HD	G(III)-HD	ID Method
1014	1012-1015[a]	Tricyclene	-	-	0.1	0.1	-	-	t	t	a,b,c
1032	1008-1039[a]	α-Pinene	**7.1**	**4.0**	**5.8**	**5.7**	**2.9**	**5.9**	**3.9**	3.8	a,b,c
1035	1012-1039[a]	α-Thujene	t	t	0.1	0.1	-	-	t	t	a,b,c
1076	1043-1086[a]	Camphene	**10.7**	**4.3**	**3.9**	**3.8**	**4.1**	**6.1**	**5.5**	5.4	a,b,c
1118	1085-1130[a]	β-Pinene	**5.7**	**1.8**	**5.8**	**5.8**	**1.7**	**1.4**	**5.1**	5.0	a,b,c
1174	1140-1175[a]	Myrcene	2.9	4.5	2.6	2.6	1.8	1.6	1.3	1.3	a,b,c
1188	1154-1195[a]	α-Terpinene	-	-	0.2	0.2	-	-	-	-	a,b,c
1195	1167-1197[a]	Dehydro-1,8-cineole	t	t	-	-	-	-	-	-	a,b,c
1203	1178-1219[a]	Limonene	1.8	t	1.3	1.3	0.9	0.9	2.1	2.0	a,b,c
1212	1177-1205[b,c]	Sylvestrene	-	-	t	-	-	-	-	-	a,b,c
1213	1186-1231[a]	1,8-Cineole	**27.2**	**34.1**	**47.1**	**46.7**	**1.7**	**32.4**	**13.1**	12.9	a,b,c
1218	1188-1233[a]	β-Phellandrene	-	-	-	-	-	-	t	-	a,b,c
1255	1222-1266[a]	γ-Terpinene	t	1.9	0.3	0.3	-	-	-	0.1	a,b,c
1266	1230-1280[a]	3-Octanone	-	-	0.1	0.1	-	-	-	-	a,b,c
1267	1232-1267[a]	*(E)*-β-Ocimene	t	0.2	-	-	-	-	-	0.1	a,b,c
1280	1246-1291[a]	*p*-Cymene	t	1.2	0.7	0.7	0.6	1.7	0.8	0.8	a,b,c
1290	1261-1300[a]	Terpinolene	0.1	0.1	0.2	0.2	-	-	t	0.1	a,b,c
1386	1386[d]	Octenyl acetate	0.1	t	-	-	-	-	-	-	a,b,c
1437	1435[e,f]	α-Thujone	**3.4**	**4.5**	**1.9**	**1.9**	**0.5**	**6.3**	**3.5**	3.4	a,b,c
1451	1456[g]	β-Thujone	2.6	4.2	1.3	1.3	0.4	4.0	1.4	1.4	a,b,c
1452	1411-1465[a]	1-Octen-3-ol	0.1	-	-	-	-	-	-	-	a,b,c
1474	1438[h]	*trans*-Sabinene hydrate	0.3	0.2	0.2	0.2	-	-	-	-	a,b,c
1494	1494[i]	(*Z*)-3-Hexenyl 3-methylbutyrate	-	-	t	t	-	-	-	-	b,c
1497	1462-1522[a]	α- Copaene	t	0.2	-	-	t	0.1	-	-	a,b,c
1532	1481-1537[a]	Camphor	**19.8**	**26.2**	**9.3**	**9.3**	**3.2**	**21.8**	**21.3**	21.0	a,b,c
1535	1496-1546[a]	β-Bourbonene	t	t	-	-	-	-	-	-	a,b,c
1553	1507-1564[a]	Linalool	0.6	0.3	0.4	0.5	0.3	0.3	1.8	1.8	a,b,c
1556	1526-1565[a]	*cis*-Sabinene hydrate	0.2	t	0.1	0.1	-	-	-	-	a,b,c
1565	1532-1570[a]	Linalyl acetate	0.6	0.5	0.5	0.5	0.3	0.7	2.4	2.4	a,b,c
1571	1557-1625[a]	*trans-p*-Menth-2-en-1-ol	t	-	-	-	-	-	-	-	a,b,c
1572	1589[j]	Acetoxy linalooloxide	t	-	-	-	-	-	-	-	b,c
1590	1570-1592[a]	Bornyl acetate	1.9	2.4	0.5	0.5	1.2	0.8	0.8	0.7	a,b,c
1592	1550-1603[a]	β-Copaene	-	-	-	-	t	-	-	-	a,b,c
1594	1518-1602[a]	Camphene hydrate	t	t	-	-	-	-	-	-	a,b,c

RRI	RRI range (Ref.)	Compound	L(I)-HD	L(I)-MSD-SPME	L(II)-HD	L(III)-HD	G(I)-HD	G(I)-MSD-SPME	G(II)-HD	G(III)-HD	ID Method
1610	1564-1618 [a]	Calarene (=β-Gurjunene)	-	-	0.1	-	-	-	-	-	a,b,c
1611	1564-1630 [a]	Terpinen-4-ol	0.5	0.9	0.9	0.1	0.1	0.1	-	-	a,b,c
1612	1569-1617 [a]	β-Caryophyllene	1.5	1.4	2.3	3.1	**7.9**	2.6	2.1	2.1	a,b,c
1628	1583-1668 [a]	Aromadendrene	0.1	0.2	0.2	0.2	0.9	0.2	t	0.2	a,b,c
1629	1632 [k]	2-Methyl-6-methylene-3,7-octadien-2-ol	t	-	-	-	-	-	-	-	c
1636	1694 [l]	Sylveterpineol	-	-	-	0.2	-	-	-	0.4	b,c
1638	1555-1645 [a]	*cis*-*p*-Menth-2-en-1-ol	t	t	-	-	-	-	-	-	a,b,c
1658	1660 [a]	Sabinyl acetate	0.1	0.1	-	-	-	-	-	-	a,b,c
1661	1624-1668 [a]	Alloaromadendrene	-	-	0.1	-	-	-	-	-	b,c
1670	1643-1671 [a]	*trans*-Pinocarveol	t	0.1	-	0.1	-	-	t	0.2	a,b,c
1682	1655-1687 [a]	δ-Terpineol	0.1	t	0.9	1.0	-	-	-	0.4	a,b,c
1687	1637-1689 [a]	α-Humulene	0.2	0.1	1.0	1.0	2.8	0.9	1.4	1.4	a,b,c
1700	1662-1717 [a]	*p*-Mentha-1,8-dien-4-ol	t	t	-	-	-	-	-	-	a,b,c
1704	1655-1714 [a]	γ-Muurolene	-	-	t	0.1	0.1	0.2	0.2	0.2	a,b,c
1706	1659-1724 [a]	α-Terpineol	0.2	0.3	2.8	3.0	-	-	0.6	0.8	a,b,c
1709	1672-1718 [a]	α-Terpinyl acetate	-	-	1.9	1.7	-	-	4.1	4.7	a,b,c
1710	1708 [n]	Ledene	-	-	-	-	t	0.1	-	-	a,b,c
1719	1653-1728 [a]	Borneol	4.4	3.6	1.4	1.5	2.2	2.7	2.2	3.0	a,b,c
1733	1693-1740 [a]	Neryl acetate	0.1	0.1	-	-	-	-	0.2	0.2	a,b,c
1740	1686–1753 [a]	α-Muurolene	-	-	t	t	-	-	-	-	a,b,c
1741	1688–1761 [a]	Valencene	t	0.1	-	-	-	-	-	-	a,b,c
1758	1668–1771 [a]	*cis*-Piperitol	t	-	-	-	-	-	-	-	a,b,c
1763	1708 [a]	Zonarene	-	-	-	0.1	-	-	-	-	b,c
1765	1728–1772 [a]	Geranyl acetate	0.1	0.1	0.1	t	-	-	0.5	0.5	a,b,c
1773	1722–1774 [a]	δ-Cadinene	0.1	0.3	0.1	0.1	0.2	0.1	0.1	0.1	a,b,c
1776	1735–1782 [a]	γ-Cadinene	t	0.1	t	t	t	0.1	0.1	0.2	a,b,c
1804	1743–1808 [a]	Myrtenol	0.1	0.1	-	-	-	-	0.2	-	a,b,c
1845	1805–1850 [a]	*trans*-Carveol	t	-	-	-	-	-	-	-	a,b,c
1853	1800–1853 [a]	*cis*-Calamenene	0.1	0.1	0.1	0.1	t	0.2	0.3	0.6	a,b,c
1864	1813–1865 [a]	*p*-Cymen-8-ol	t	t	t	-	-	-	-	-	a,b,c
1878	1826–1878 [a]	2,5-Dimethoxy-*p*-cymene	t	-	-	-	-	-	-	-	a,b,c
1882	1818–1882 [a]	*cis*-Carveol	t	-	-	-	-	-	-	-	a,b,c
1941	1893–1941 [a]	α-Calacorene	-	-	0.1	-	-	-	-	0.1	a,b,c
2008	1936–2023 [a]	Caryophyllene oxide	1.2	t	0.8	0.8	3.5	0.1	2.6	2.5	a,b,c
2045	2071[0]	Humulene epoxide-I	t	t	0.1	0.1	0.5	0.1	0.3	0.3	a,b,c

RRI	RRI range (Ref.)	Compound	L(I)-HD	L(I)-MSD-SPME	L(II)-HD	L(III)-HD	G(I)-HD	G(I)-MSD-SPME	G(II)-HD	G(III)-HD	ID Method
2050	1995–2055 [a]	(*E*)-Nerolidol	-	-	-	-	-	-	t	-	a,b,c
2071	2003–2071 [a]	Humulene epoxide-II	0.2	t	-	0.5	1.9	t	2.3	2.3	b,c
2081	2081 [p]	Humulene epoxide-III	-	-	-	t	0.3	t	0.1	0.1	b,c
2098	2049–2104 [a]	Globulol	t	t	0.1	0.1	0.2	0.4	-	-	a,b,c
2104	2041–2110 [a]	Viridiflorol	0.7	0.1	1.3	1.3	**8.6**	0.1	3.4	3.3	a,b,c
2131	2041–2110 [a]	Hexahydro farnesylacetone	-	-	-	-	-	-	-	0.1	a,b,c
2144	2074–2150 [a]	Spathulenol	0.1	t	0.1	0.1	0.4	t	t	-	a,b,c
2179	2123–2174 [a]	Tetradecanol	-	-	-	-	0.2	t	-	-	a,b,c
2180	2249 [q]	Pimara-8,15-diene	-	-	-	-	0.1	t	-	0.1	b,c
2187	2184 [r]	T-Cadinol	t	t	-	-	-	-	-	-	a,b,c
2192	2110–2196 [a]	Nonanoic acid	-	-	-	-	-	-	-	0.1	a,b,c
2198	2100–2205 [a]	Thymol	0.1	t	-	-	0.6	0.2	-	-	a,b,c
2217	2238 [s]	Clovenol	t	t	t	0.1	0.4	t	0.2	0.1	a,b,c
2239	2140–2246 [a]	Carvacrol	0.1	t	-	-	-	-	-	-	a,b,c
2255	2180–2255 [a]	α-Cadinol	t	t	-	t	-	-	-	-	a,b,c
2265	2231 [t]	Torilenol	t	t	-	-	-	-	-	-	a,b,c
2300	2289 [u]	Cinnamyl isovalerate	t	-	-	-	-	-	-	-	a,b,c
2316	2316 [o]	Caryophylla-2(12),6(13)-dien-5β-ol	t	t	-	0.2	0.2	t	-	-	a,b,c
2324	2324 [a]	Caryophylla-2(12),6(13)-dien-5α-ol	0.2	0.1	0.2	0.4	1.9	0.2	0.8	0.8	a,b,c
2389	2389 [v]	Caryophylla-2(12),6-dien-5α-ol	0.3	0.1	0.4	0.3	4.6	0.2	1.3	1.3	a,b,c
2389	2389 [t]	Eremophilone	-	-	-	-	-	-	-	0.1	b,c
2390	2351–2402 [a]	Eudesma-4(15),7-dien-1β-ol	t	-	-	-	-	-	-	-	a,b,c
2392	2392 [u]	Caryophylla-2(12),6-dien-5β-ol	0.3	t	0.3	-	3.5	0.1	1.1	1.0	a,b,c
2503	2442–2524 [a]	Dodecanoic acid	-	-	-	-	-	-	0.7	-	a,b,c
2524	2476–2530 [a]	Abietatriene	-	-	-	-	t	t	0.3	0.7	b,c
2681	2370–2628 [a]	Manool	0.8	t	0.7	0.7	**15.7**	0.1	4.9	5.1	b,c
2732	2735 [y]	Labda-7,14-dien-13-ol	t	-	0.1	0.1	1.4	-	t	0.5	a,b,c
2931	2862–2945 [a,z]	Hexadecanoic acid	-	-	-	-	**14.8**	-	3.8	2.4	a,b,c
		Total	**96.7**	**84.0**	**98.5**	**98.9**	**92.1**	**92.7**	**96.8**	**98.1**	
		Oil yield, %	3.0	-	2.3	2.1	0.10	-	0.12	0.12	
		Compounds detected	70	60	52	53	41	40	47	51	

L(I)-HD: Leaf volatiles (I) obtained by the hydrodistillation in Clevenger type apparatus; **L(I)-MSD-SPME**: Leaf volatiles (I) obtained by MSD-SPME technique; **G(I)-HD**: Gall apple volatiles (I) obtained by the hydrodistillation in Clevenger type apparatus; **G(I)-MSD-SPME**: Gall apple volatiles (I) obtained by MSD-SPME technique; **L(II)-HD**: Leaf volatiles (II) obtained by the hydrodistillation in Clevenger type apparatus; **G(II)-HD:** Gall apple volatiles (II) obtained by the hydrodistillation in Clevenger type apparatus; **RRI:** Relative retention indices calculated against *n*-alkanes; **%:** calculated from FID data; **t:** Trace (< 0.1 %); **a**) Identification based on retention index of genuine compounds on the HP-Innowax column; **b**) Identification on the basis of computer matching of the mass spectra and retention times from Başer Library, **c**) Identification on the basis of computer matching of the mass spectra from Adams, MassFinder, WileyNIST libraries. References in table: **a**: [62], **b**:[63], **c**:[64], **d**:[65], **e**:[66], **f**:[67], **g**:[68], **h**:[69], **i**:[70], **j**:[71], **k**:[72], **l**:[73], **m**:[74], **n**:[75], **o**:[76], **p**:[77], **q**:[78], **r**:[79], **s**:[80], **t**:[81], **u**:[82], **v**:[83], **y**:[84], **z**:[85]

In scope of the present study, together with conventional hydrodistillation procedure, a modern and rapid microscale technique MSD-SPME was applied to isolate volatiles from the leaves and the galls of *S. triloba.* MSD-SPME is known as a rapid, modern and trustworthy technique applied for isolation of volatiles from very small amount of plant material for the short time [86-88]. MSD-SPME can be attributed to new sampling and concentration techniques which involved concurrent solid-phase microextraction combined with continuous hydrodistillation of the volatiles. This technique combined with GC-FID and GC-MS methods provided important advantages in time saving for performing the quickly analysis of a number of samples. It is very important to notify that MSD-SPME is energy saving, solvent-less and non-toxic technique for the volatiles analysis at microscale level. In scope of the experiment three additional runs using different amounts of the plant material and different periods for reflux were performed for optimization of extraction process. As a result, the volatiles were isolated from 0.5 g plant material for 1.0 min. The chromatographic profiles of the leaf and the gall volatiles obtained with conventional HD and with MSD-SPME are compared and presented on Figure 2. As can be seen, MSD-SPME technique allowed to isolate leaf volatiles with profile similar to that obtained with conventional method. However, the oil hydrodistilled from the galls significantly differed from the volatiles isolated with MSD-SPME technique. Diterpenes and fatty acids were found in high amount in G(I)-HD oil while SPME procedure resulted with volatiles rich in oxygenated monoterpenes (1,8-cineole and camphor, Figure S2 and Table 1) Perhaps subjection to longer distillation procedure (3 h) resulted with diterpenes and fatty acid extraction in case of HD method.

3.2. Anti-acetylcholinesterase Activity of Essential Oil

Up to date, many attempts have been made to find effective sage EOs with anti-AChE property It has recently been reported that EO of *S. pseudeuphratica* Rech.f. possess significant inhibitory activity against AChE (IC_{50} 26.00 μg/mL) [21, 25, 48]. Topçu et al. reported that *S. fruticosa* EO inhibited AChE with 53.6% (at concentration 25 μg/mL) [44]. The oil of *S. fruticosa* oil collected from Cyprus demonstrated human anti-AChE activity with IC_{50} 0.05 mg/mL [21]. However, these EOs possess qualitative differences as compared to composition of L(I)-HD and L(II)-HD oils investigated in the present work.

In scope of our work the leaf oils L(I)-HD and L(II)-HD were investigated *in vitro* for memory vitalizing activity using Ellmans's method which uses an alternative substrate ATCI and chromogenic agent DTNB. The reaction results in production of 5-thio-2-nitrobenzoate that has yellow color due to the shift of electrons to the sulfur atom [89]. The percentage of the inhibition of AChE was determined by comparison of rates of reaction of test EOs relative to blank sample (methanol in phosphate buffer). The results of AChE inhibition test are presented in Table 2. As can be seen from the Table 2, the leaf oils inhibited AChE with 40-41 %.

Table 2. Biological activity results of the leaf EOs obtained from *S. fruticosa*

Test sample	Activity type		
	AChE enzyme inhibition (% ± SEM[a])	**Cupric reducing antioxidant capacity (% ± SEM)**	**Radical scavenging effect (% ± SEM)**
L(I)-HD[b]	41.2±3.4	36.8±7.4	12.9±4.2
L(II)-HD	40.7±3.8	26.3±0.4	14.1±0.4
Galanthamine[c]	70.0±1.0	-	-
Gallic acid[d]	-	100.0±0.2	69.0±0.5
Ascorbic acid[e]	-	100.0±0.1	67.4±0.5

[a] Standard error of the mean (n =3)
[b] the oils were tested in concentration 3.75 mg/mL
[c] reference for anti-AChE activity was tested at concentration 0.1 mg/mL
[d] reference for antioxidant activity was tested at concentration 0.01 mg/mL
[e] reference for antioxidant activity was tested at concentration 0.1 mg/mL

3.3. Antioxidant Activity of Essential Oil

It is known that antioxidants may act as free radical scavengers (DPPH˙ scavenging assay), as reducing agents (cupric ions) or as hydrogen atom donors (inhibition of linoleic acid oxidation) [90]. In scope of the present work, two different tests were applied to assess antioxidant properties of *S. fruticosa* EOs. The CUPRAC assay allowed us to measure the total antioxidant potential of EOs. This method is based on reduction of Cu(II) to Cu(I) by antioxidants present in the sample. A chromogenic reagent, neocuproine (2,9-dimethyl-1,10-phenanthroline), forms a complex with Cu(I), which has a maximum absorbance at 450 nm. Cupric reducing capacity of the tested EOs was found to be moderate (26-37%) in comparison to ascorbic and gallic acids which were used as reference reducing compounds.

Among the antioxidant activity measuring tests, DPPH free radical scavenging assay is the most extensive applied test. The reaction between antioxidant compounds and DPPH radicals behaves like an electron transfer reaction [91] . As represented in Table 2, the free radical scavenging potential (13-14%) of the both oils of *S. fruticosa* against DPPH radicals was found to be lower in comparison to ascorbic and gallic acids used as the reference compounds.

4. Conclusion

The oxygenated monoterpenes (62.4-69.3%) were found to be predominating compound group with 1,8-cineole and camphor as the main constituents in all the tested samples with exception of the gall oil in which oxygenated sesquiterpenes (25.6%) and diterpenes (17.3%) were detected in high percentages. Qualitative differences of the volatiles obtained by HD and MSD-SPME techniques from the leaf and the galls are reported here first. The major compounds were found to be similar in the composition of leaf and gall essential oil of *S. fruticosa* which were collected from three different regions of Turkey. The amounts of the major compounds in the essential oil differed according to region, essential oil processing methods (HD or MSD-SPME) and parts of the plant.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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