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Essential Oil Composition, Antimicrobial and Antioxidant Activities of *Salvia staminea*

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Abstract: Volatile constituents of the essential oil which was obtained by hydrodistillation (HD) in a Clevenger-type apparatus from the air-dried *Salvia staminea* Montbret & Aucher ex Bentham (Lamiaceae) collected from Bayburt (North East Part of Black Sea Reagan of Turkey), were analyzed by GC-FID and GC-MS. Additionally, volatiles for the whole part of *S. staminea* were analyzed by two different methods which are headspace (HS)-GC-FID/MS and headspace solid-phase micro extraction (HS-SPME)-GC-FID/MS. As results of this study, thirty compounds from hydrodistillation, fifteen constituents from HS-SPME and two components from headspace of *S. staminea* were identified with GC-FID/MS. The major compounds identified in the essential oil, SPME and HS of *S. staminea* were linalyl acetate (23.30%, 85.07%, and 87.55%) and linalool (22.05%, 9.02%, and 12.44%), respectively. The antimicrobial activities of the essential oil of *S. staminea* were screened against seven microorganisms (*Escherichia coli, Yersinia pseudotuberculosis*, *Staphylococcus aureus*, *Enterococcus faecalis*, Bacillus *cereus*, *Listeria monocytogenes*, and *Candida albican*) and showed good antimicrobial activity against Gram-positive bacteria which is consistent with the literature with the other types of *Salvia* species. The essential oil of *S. staminea* showed good antioxidant activity with IC_{50} 60.4 µg/mL.

Keywords: Headspace; SPME; GC-MS; PDMS; linalool; antimicrobial activity; antioxidant activity.

1. Introduction

The genus *Salvia* L., represented by 900 species, is one of the most widespread members of the Lamiaceae and is well known its uses in folk medicine [1-3]. Recently, many medicinal plants have appeared on markets shelves as antioxidants resources for the food industry. *Salvia* is represented with 89 species, 50 of them are endemic to Turkey [4]. Several species such as *S. officinalis* L., *S. fruticosa* Miller, *S. tomentosa* Miller and *S. virgata* Jacq. are used in Anatolian folk medicine [5,6]. *S. staminea* is a perennial herb, distributed in Northern East of Turkey [7]. Natural compounds such as carvacrol, *α*-

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pinene, linalool and camphene were identified form other *Salvia* species [8]. The essential oil from the genus of *Salvia* had significant antioxidant activity [9].

Salvia plants are commonly known as sages which mean "to be in good health" or "to be well" [10, 11]. Sages are among the best-known classes for various biological activities [12-14]. A great number of the aromatic taxa make Lamiaceae commercially significant, owing to their aromas, infusions, tinctures, and flavors that are used as resources of herbal products [15]. The genus *Salvia* is known around the world for their profitable, pharmaceutical, and cultural importance, due to useful essential oils [11,15,16]. Sages for medical applications vary in terms of pharmacological activity. Some of them are used as nutritive tract stimulators and digestion regulators with additional antiseptic features and some of them are known for their antipyretic, analgesic, and expectorant properties [11].

The chemical composition of the essential oils of some *Salvia* species have been reported [17, 18]. Essential oils investigated parts of the *Salvia* species include flowers, leaves, stems, roots, aerial parts, and whole plants.

The target of this research was to investigate and compare the effect of extraction methods on the chemical composition variation for the volatile constituents of the essential oils, HS and SPME of *S. staminea* collected from natural source. In fact, previous works [11, 15, 17-19] on the essential oils of *S. staminea* have quite different variation with the present work for the volatile organic compounds.

2. Materials and Methods

2.1. Plant Collection and Identification

Samples of *S. staminea* were collected at the full flowering stage from Kopuz Köyü (Kopuz Village), Bayburt-Turkey (A8), alpine meadows at heights of ~1740 m in June 2010 and identified by Prof. Dr. Kamil Coşkunçelebi. Voucher specimens were deposited in KTUB (Herbarium of the Department of Biology at Karadeniz Technical University, Turkey; Coşkunçelebi 726). Plant materials were air-dried under the shadow at room temperature for later analysis.

2.2 Isolation of the Essential Oils

The air-dried whole plants (~1040 g) of *S. staminea* were hydrodistilled in a Clevenger-type apparatus using a cooling bath (-15°C) system (3 h). The obtained oil was extracted in HPLC grade *n*-hexane (0.5 mL) and kept at 4°C in a sealed brown vial. The percentage yields of the oils from *S. staminea* calculated on a moisture-free basis were 0.15% (v/w).

2.3. Identification of Components by GC-MS

For all analyses, a Shimadzu QP2010 ultra GC–MS, fitted with a PAL AOC-5000 plus auto sampler, and Shimadzu 2010 plus FID and Shimadzu Class-5000 Chromatography Workstation software was used. The gas chromatographic conditions were as followed: GC oven fitted with a RTX-5M 30 m capillary column (0.25 mm I.D., 0.25 μm film thickness, Restek, USA) with carrier gas helium at flow rate of 1.0 mL min^{-1}, operating under an initial temperature at 60°C (2 min) to 240°C (5 min) at 5°C/min, with He as carrier gas (1 mL/min), the temperature of the injector and detector were 250°C and 230°C, respectively. The ionization voltage was 70 eV. Identification of compounds was based on a comparison of their mass spectra with standards [20-22]. Confirmation of compound identities was obtained using retention index [22]. The datum analysis was performed on a National Institute of Standards and Technology (NIST) library (Shimadzu, Kyoto, Japan) and Wiley libraries of mass spectra and literature comparison [22,23-25]. After analysis on a Restek RTX-5M column, quantification was performed as peak area using integration data.

Essential oils are a mixed combination of natural compounds. The constituents of the oils are mainly terpenes or oxygenated compounds derived from these hydrocarbons. The chemical composition of essential oils differs in each species or subspecies. The compositional studies of essential oils have been carried out extensively by using gas chromatography-mass spectrometry (GC-MS), which is based on the comparison of the relative retention times/indices and mass spectra of the specific natural

compounds found in an essential oil [22, 26-28]. Identification of individual components was made by comparison of their retention times with those of available analytical standards (*α*-pinene, camphene, linalool, geraniol, *n*-tetradecane, *n*-pentadecane, *n*-heptadecane, and *n*-octadecane), and by computer searching, matching mass spectral data with those held in the National Institute of Standards and Technology (NIST) and Wiley libraries of mass spectra and literature comparison [22,23].

2.4. Headspace Solid-Phase Micro Extraction

2.4.1. Sampling Preparation

For SPME sampling, *S. staminea* puree was prepared by blending 100 g plant for 3 min without water in a Waring blender. The plant puree (1 g) was placed into a 10 mL headspace vial, and the flask was closed with a septum. The SPME headspace volatiles were collected using a Supelco 57348 2 cm, 50/30 μm DVB/Carboxen/PDMS Stable-Flex fiber (precondition of the fiber at 250 °C in the injection port of a GC for 10 min) for 30 min (the highest concentration of aroma compounds without qualitative change of the composition; tested by 10 min extraction steps up to 30 min). After sampling, the SPME device was placed into the injector of the GC and the GC-MS instruments through the whole GC analysis time of 62 min using RTX-5M column. The identified compounds are listed in Table I.

2.5. Headspace Gas Chromatography–Mass Spectrometry

The headspace gas chromatography–mass spectrometry (HS–GC–MS) analyses were carried out with use of a Shimadzu QP2010 ultra GC–MS, fitted with the Shimadzu AOC-5000 plus autosampler, and Shimadzu 2010 plus FID used in the headspace mode. Temperature and time of the headspace desorption were, respectively, 70ºC and 15 min. 0.5 mL of the headspace phase was introduced on to the RTX-5M 30 m capillary column (0.25 mm I.D., 0.25 μm film thickness, Restek, USA). Helium was used as carrier gas. Gradient analysis was run using the above. The temperature of the injector was kept constant at 250ºC. The mass spectrometer was fitted with an EI source operated at 70 eV. Identification of individual compounds was based on a comparison of the obtained mass spectra of the individual chromatographic peaks with those valid for the standards and available from the National Institute of Standards and Technology (Gaithersburg, MD) software library. The identified compounds are listed in Table 1.

2.6. Antimicrobial Activity Assessment

Test microorganisms obtained from the Hifzissihha Institute of Refik Saydam (Ankara, Turkey) are as followings; *Escherichia coli* (*E. coli*) ATCC 25922, *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) ATCC 911, *Staphylococcus aureus (S. aureus*) ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis* (E. faecalis) ATCC 29212, *Listeria monocytogenes* ATCC 43251, *Bacillus cereus (B. cereus)* 709 ROMA, *Candida albicans (C. albicans)* ATCC 60193 and *Saccharomyces cerevisiae (S. cerevisiae)* RSKK 251. Essential oil obtained by hydrodistillationfrom *S. staminea* was dissolved in acetone to prepare sample stock solutions of 38.27 μg/mL.

2.7. Agar Well Diffusion Method

Simple susceptibility screening test using agar-well diffusion method [29] as adapted earlier [30] was used. Each bacterium was suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth. The yeast like fungi was suspended in Yeast extracts broth. Then the microorganisms were diluted approximately 106 colonies forming unit (cfu) per mL. For yeast like fungi, Sabouraud Dextrose Agar (SDA) (Difco, Detriot, MI) were used. Brain Heart Infusion Agar (BHI) (Difco, Detriot, MI) was used for *M. smegmatis*. They were "flood-inoculated" onto the surface of MH, BHI and SD agars and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 50 μL of the stock extract substances were delivered into the wells. The plates were incubated for 18 h at 35°C. The

Mycobacterium smegmatis was grown for 3 days on BHI agar plates at 35°C [31]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Ampicillin (10 µg) was standard drug. Hexane was used as a solved control.

Table 1. Identified components in the HD, SPME, and HS of *S. staminea*

No	Compounds	HD[a]		SPME[b]		HS[c]		Exp.RI[d]	Lit. RI[e]
		Q[f] (%)	% Area	Q[f] (%)	% Area	Q[f] (%)	% Area		
1	Hexanal	96	0.44					807	801[33]
2	Cyclohexanone	98	1.24					902	895[30]
3	Myrcene	90	2.01	95	0.81			992	997[33]
4	Dehydroxy-(*Z*)-Linalool oxide	95	1.83					997	991[33]
5	Limonene	95	0.19	92	0.26			1027	1029[33]
6	*δ-3*-Carene	81	0.21					1035	1031[33]
7	*cis*-Ocimene	97	0.66	94	0.17			1042	1037[33]
8	*E-β*-Ocimene	95	1.49	96	0.22			1045	1050[33]
9	*trans*-Linalool oxide			99	0.11			1076	1073[33]
10	Terpinolene	91	0.17					1082	1089[20]
11	*cis*-Linalool oxide			95	0.12			1096	1087[20]
12	Linalool	91	22.05	98	9.02	96	12.44	1102	1097[20]
13	*α*- Terpineol	91	4.98					1193	1189[20]
14	Linalyl formate			97	1.72			1215	1216[20]
15	Linalyl acetate	91	23.30	90	85.07	97	87.55	1257	1257[20]
16	Geraniol	91	1.04					1260	1253[20]
17	Geranyl Formate	83	0.58					1287	1298[20]
18	*α*-Cubebene	98	2.28					1342	1351[20]
19	8-Acetoxylinalool			85	0.34			1342	-
20	*α*-Terpenylacetate			85	0.24			1355	1349[20]
21	Geranyl acetate	95	1.16	85	0.25			1383	1379[20]
22	*α*-Copaene	98	1.61	94	0.60			1386	1374[20]
23	*β*-Bourbonene			93	0.11			1395	1388[20]
24	*β*-Caryophyllene	99	0.80	95	0.17			1411	1419[20]
25	*α*-Humulene	90	0.25					1461	1454[20]
26	Geranyl Propanoate	90	1.70					1470	1478[20]
27	Valencene	95	2.36					1490	1496[20]
28	*δ* -Cadinene	99	0.86					1523	1523[20]
29	*α*-Calacorene	89	0.75					1549	1546[20]
30	Spathulenol	99	10.02					1585	1578[20]
31	Caryophyllene oxide	93	5.45					1595	1582[20]
32	Sclareoloxide	91	2.89					1903	1906[23]
33	*β*-Springene	95	2.45					1920	1922[22]
34	Sclarene	96	1.05					1986	1975[20]
35	Manoyl oxide	99	0.56					2008	1998[20]
36	Sclareol	85	4.85					2233	2223[20]
	Total		99.23		99.21		99.99		

[a]HD: Hydrodistillation, [b]SPME: Solid phase microextraction, [c]HS: Head space, [d] Experimental RI, retention index, [e]LRI, literature retention index, [f]Q: Quantities.

2.8. Antioxidant Activity Assessment

2,2-Diphenyl-2-hydrazyl picryl (DPPH) is a fast and convenient method [32-34], commonly used for detection of radical scavenging activity, evaluation of the antioxidant properties components. DPPH stable free radical can accept an electron or hydrogen radical and thereby turns into a stable diamagnetic molecule. DPPH has one electron; therefore, it has a strong absorption band at 517 nm. Treatment with antioxidants causes to decrease the intensity of the violet DPPH absorbance stemming of DPPH. The mixtures were incubated for 30 min at room temperature in the dark. After incubation,

the absorbance of the essential oil was measured at 517 nm. Assay mixture without essential oil was used as a control. The inhibition percentage was calculated using the equation y =ax+ b, it can be determined the concentration of sample which reduces DPPH concentration to half-dominated mg/mL. This concentration is expressed as the IC_{50} value. Resulting extract and ascorbic acid as a standard were prepared at different concentrations. After the samples are pipetted into tubes needed primarily sample solution onto an equal volume (750µL) solution of DPPH adding, vortex and allows incubating at room temperature for 50 minutes. At the end of that time, absorbance was read at 517 nm and IC_{50} values were calculated from the graph.

3. Results and Discussion

Hydrodistillation of the dried whole plant material of *S. staminea* produced light orange oil with a yield of 0.15% (v/w). HD, SPME, and HS of *S. staminea* were investigated by GC-FID/MS and a total of 30, 15, and 2 compounds, representing 99.23%, 99.21% and 99.99% were identified, respectively. The identity, retention time, and the percentage composition of the HD, SPME, and HS of *S. staminea* are presented in Table 1.

The major constituents of the essential oil of *S. staminea* were linalyl acetate (23.30%), linalool (22.05%), spathulenol (10.02%), caryophyllene oxide (5.45%), *α*-terpineol (4.89%), sclareol (4.85%), and sclareoloxide (2.89%). SPME and HS GC-FID/MS analysis of *S. staminea* gave the linalyl acetate (85.07% and 87.55%), linalool (9.02% and 12.44%) as the main compounds, respectively. Since the linalyl acetate and linalool was the major constituent in all three methods, linalyl acetate was found to be greatest amount in HS sample. Whereas, HD of *S. staminea* had the linalool in the highest amount.

In the essential oil of *S. staminea*,thirty components were classified as 7 monoterpenes, 6 monoterpenoids, 8 sesquiterpenes, 3 diterpenes, 2 sesquiterpenoids, 2 diterpenoids, and 2 others type compounds. SPME analysis of *S. staminea* revealed 5 monoterpenes, 7 monoterpenoids, and 3 sesquiterpene type of compounds, whereas HS analysis of *S. staminea* gave only 2 monoterpenoids.

Table 2. The chemical class distribution of the HD, SPME and HS components of *S. staminea*

Compound class	HD		SPME		HS	
	Area%	NC[a]	Area%	NC[a]	Area%	NC[a]
Monoterpenes	4.73	6	1.57	5	-	-
Monoterpenoids	58.47	8	96.76	7	99.99	2
Sesquiterpenes	8.91	7	0.88	3	-	-
Sesquiterpenoids	18.36	3	-	-	-	-
Diterpene	3.50	2	-	-	-	-
Diterpenoids	5.41	2	-	-	-	-
Others	1.68	2	-	-	-	-
The common compounds	45.35	2	94.09	2	99.99	2

[a]NC: Number of compounds

SPME rendered possible the analysis of all the plant material under the same conditions even though its comprehensive composition. This is not always possible with other extraction methods. Using a nonpolar poly (dimethylsiloxane) (PDMS) phase, many terpenoid hydrocarbons, together with alcohols, cyclic ethers, and esters, were extracted. The ease of use and the high resolution of the chromatographic profiles obtained make HS-SPME well suited to the rapid characterization of the main components of the volatile fraction of plants [35].

The chemical class of the volatile constituents from HD, SPME and HS of *S. staminea* grouped into seven classes which are named monoterpenes (4.73% HD, 1.57% SPME), monoterpenoids (58.47% HD, 96.76% SPME, 99.99% HS), sesquiterpenes (8.91% HD, 0.88% SPME), sesquiterpenoids (18.36% HD), diterpenes (3.50% HD), diterpenoids (5.41% HD), and others (1.68% HD) were given in Table 2, respectively.

The major compounds in the classified group (monoterpenes, monoterpenoids, sesquiterpenes, sesquiterpenoids, diterpenes, diterpenoids, and others) of volatiles for the HD, SPME and HS of *S.*

staminea listed in Table 3. Myrcene, linalyl acetate, valencene, spathulenol, *β*-springene, sclareol, and cyclohexanone in HD, myrcene, linalyl acetate, and *E*-caryophyllene in SPME and linalyl acetate in HS were found to be major compounds.

Table 3. The major components in the chemical class distribution of the HD, SPME, and HS constituents of *S. staminea*

Compound class	HD			SPME			HS		
	Major compound	**Area %**	**RI**	**Major compound**	**Area %**	**RI**	**Major compound**	**Area %**	**RI**
Monotertene	Myrcene	2.01	995	Myrcene	0.81	995	-	-	-
Monoterpenoid	Linalyl Acetate	23.30	1257	Linalyl Acetate	85.07	1257	Linalyl Acetate	87.55	1251
Sesquiterpene	Valencene	2.36	1490	*E*-Caryophyllene	0.17	1411	-	-	-
Sesquiterpenoid	Spathulenol	10.02	1585	-	-	-	-	-	-
Diterpene	*β*-Springene	2.45	1920	-	-	-	-	-	-
Diterpenoid	Sclareol	4.85	2233	-	-	-	-	-	-
Others	Cyclohexanone	1.24	902	-	-	-	-	-	-

In the previous studies, the volatile composition and antimicrobial activity of the essential oils of *S. stamenia* collected from the botanical garden have been studied [11,15] and the major components of the essential oil for *S.staminea* were mentioned to be *α*-pinene, camphene, *β*-pinene, limonene, eucalyptol and *β*-chamigrene [11,15].

In the literature, volatile organic compounds from dried aerial parts (flowers, leafs, leafy branches and stems) of forty-five *Salvia* species have been identified using thermal desorption technique coupled to GC-MS (TD-GC-MS) and sesquiterpene hydrocarbons (0.90-45.02%) were recorded to be the major class of volatile constituents and chemical composition [17]. The essential oil *S. staminea* growing in Iran from different populations, had been reported and *β*-caryophyllene (15.5-19.62 %), ledol (2.5-18.81 %), pentamethyl-1, 3 cyclopantadien (5-12.69 %), *p*-cresol (9.3-12.91%) and pentamethylcyclopentadien (4.66-12.29 %) were mentioned to be major constituents. [18].

Various experimental techniques were used for the isolation and identification of volatile organic compounds of the plants which are; low-temperature TLC-MS of the essential oils from five different *Salvia* species (*S. lavandulifolia, S. staminea, S. hians, S. triloba,* and *S. nemorosa*) [36,37]; the volatile compounds of five different *Salvia* species (*S. lavandulifolia, S. staminea, S. hians, S. triloba,* and *S. nemorosa*) using four different techniques (head-space, vapor distilation, and accelerated solvent extractions) [11]; the essential oils of five different *Salvia* species (*S. lavandulifolia, S. staminea, S. hians, S. triloba,* and *S. nemorosa*) using low temperature planar chromatography-densitometry attached with gas chromatography [38] have been reported. Also, the essential oils of twenty species of *Salvia* natural or cultivated in Poland have been investigated by headspace GC-MS and the major compounds of the volatile fraction of these *Salvia* species were reported as *α*-pinene, camphene, *β*-pinene, thujol, camphor, *β*-chamigrene, and cadina-3,9-diene. Thujenone was mentioned to be as chemotaxonomic marker in *S. staminea*. In the literature, *β*-caryophyllene (15.5-19.62 %) and α-copaene (7.0 %) were major constituent of the esential oil of *S. staminea* [18]. In our case, they were minor compounds which were less than 1.61%. Also, thujenone mentioned to be as chemotaxonomic marker for the *S. staminea* [11], however it was not observed it in this work.

In the present study, different chemical composition of the plant that was harvested from the natural environment was obtained. Essential oil composition and antioxidant activity of various extracts of *S. staminea* were reported [18]. When the structures of the most abundant components reported in literature for the HD, SPME, and HS of *S. staminea* were examined, quite different variation was observed.

S. staminea plant contains linalyl acetate which is the main component of the plant. However, the other types of *Salvia* plants grown in Turkey do not contain any linalyl acetate. Additionally, *Salvia* plants except for *S. staminea* comprise a very small amount of linalool which is the second main component of these plants. Linalool contents of *S. candidissima* Vahl., *S. chionantha*and *S.*

potentillifolia were 0.31 %, 0.42 %, and, 0.43 %, respectively. The main volatile component of *S. staminea* and *S. candidissi* Vahl. plants, collected from the pharmacy garden, is *β*-pinene [18]. The major components of *S. staminea* were germacrene D (36.3 %) followed by hexahydrofarnesylacetone (11.2 %) and α-copaene (7.0 %) [18].

Our study showed that chemical differences of the composition of the HD, SPME, and HS could be attributed to the geographical source, time of collection of the plant and the specific climate there. In addition, stress-induced alteration of the chemical structure, since the plant grows in the botanical gardens away from the natural environment, may cause these differences [37-40]. The reason for the variation in essential oil contents can be caused by the ecosystem of the plants, the solvent used during extraction, and harvest season. However, separation of plants from their natural environment causes stress factors, which can be biotic or abiotic, and give rise to losses in quality and quantity of the product. Furthermore, the content of the plant may change. Physical conditions (snow, the wind, and mechanical effects) and chemical conditions (such as pesticides, etc.) can be effective on the content according to the level of adaptation of the plants. Light, due to the formation mechanism of photosynthesis, can produce stress effects on the plant directly, resulting in reduced production of photosynthesis of carbohydrates produced by the plant which directly reduces the number of nutrients and hence causes a slowing of plant growth.

The antimicrobial activities of the essential oils of *S. staminea* were tested against seven bacterai *in vitro* using the agar-well diffusion method [33, 34, 39] with the microorganisms listed in Table 4. Only the essential oil of *S. staminea* showed good antibacterial activity against Gram-positive bacteria and the fungi with 6 mm to 23 mm inhibition zone, respectively.

Table 4. Antimicrobial activity of the essential oil of *S. staminea*

Sample	**Stoc. (**µg/ml)	**Microorganisms and Inhibition zone (mm)**						
		Ec	Yp	Sa	Ef	Bc	Li	Ca
Essential oil	38.27	-	-	20	15	23	-	6
Ampicillin	10	10	18	35	10	15	10	-

Ec*: Escherichia coli* ATCC 25922, Yp: *Yersinia pseudotuberculosis* ATCC 911, Sa: *Staphylococcus aureus* ATCC 25923, Ef: *Enterococcus faecalis* ATCC 29212, Bc: *Bacillus cereus* 702 Roma, Li: *Listeria monocytogenes* ATCC 43251, Ca: *Candida albicans* ATCC 60193, (-): no activity.

The essential oil of S. *steminea* was found to exhibit significant antioxidant activity by scavenging DPPH. The order of IC_{50} values for DPPH for the essential oil was found 60.4 µg/mL references to ascorbic acid (28.2 µg/mL) [33, 34,38].

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