ORIGINAL ARTICLE

Biological Activities of the Essential Oil and Total Extract of *Salvia macrosiphon* Boiss.

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ABSTRACT

Introduction: *Salvia macrosiphon* Boiss. (Lamiaceae) is an aromatic perennial herb that its phytochemical analysis has shown presence of flavonoid and phenolic compounds. **Material and Methods:** Essential oil of the aerial parts of *S. macrosiphon* collected from south of Iran was obtained by hydrodistillation and analyzed using gas chromatography-mass spectrometry (GC/MS). Cytotoxic activity of the essential oil and the total extract of this herb were investigated by using MTT assay on three cell lines. **Results:** Twenty six components, representing 100% of the total oil, were identified. The main components of the essential oil were Linalool (19%), β-Cedrene (14.64%) and β-Elemene (13.33%). Both of the total extract and the essential oil showed remarkable cytotoxic activity against all tested cell lines. Moreover, the volatile oil exhibited significant antioxidant and anti-acetylcholinesterase activities; however the total extract did not exhibit any effect on inhibition of acetylcholinesterase. **Conclusion:** According to achieved results, this herb could be considered as a valuable source of antioxidant, cytotoxic and anti-AChE compounds.

Key words: Acetylcholinesterase, antioxidant, cytotoxic, essential oil, *Salvia macrosiphon*, total extract

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INTRODUCTION

Salvia is a genus of Lamiaceae family with 58 known species widespread around Iran. Generally, this genus is known as Maryam-Goli[1] Traditionally, some species of this genus are used for medicinal applications in curing diverse ailments, e.g., infection, rheumatoid, chronic pains, inflammatory, cardiovascular and cerebrovascular disease, as well as its common use as a nutritional spice[2-5] Up to now, many researches have been conducted on biological effects of various species of the genus such as antioxidant, acetylcholinesterase inhibition, anti-nociceptive, anti-inflammatory, antidepressant, anxiolytic, antitumor and cytotoxic activities.[6-11]

Salvia macrosiphon is an aromatic perennial herb whose phytochemical analysis has shown presence of flavonoid and phenolic compounds such as apigenin, luteolin, salvigenin, eupatorin and rosmarinic acid.[12-15] On the other hand, rosmarinic acid, apigenin and luteolin as the main compounds of the plant show significant biological effects such as cytotoxic, anti-acetyl cholinesterase, anti-inflammatory and antioxidant effects.[16-20] Linalool also as the identified major component of *S. macrosiphon* essential oil, exhibits pharmacological effects such as cytotoxic, anti-inflammatory and antioxidant activities.[21]

According to previous studies on Alzheimer's disease (AD), several different factors play role in treatment of this disease such as AChE inhibitors, anti-inflammatory and antioxidant agents.[22] Based on the identified components of *S. macrosiphon* and their biological background, it seems that the AChE inhibitory and antioxidant activities of the plant compounds could be considerable factors in control or treatment of Alzheimer's symptoms.

There are few reports on biological effects of *S. macrosiphon* extract.[23-25] However to the best of our knowledge, there is no study on biological effects of essential oil of *S. macrosiphon;* consequently no data present for comparison of biological effects of the essential oil and extract of the herb.

The current study investigates the total phenol content and antioxidant activity of the essential oil and total extract of *S. macrosiphon*. Also, the aim of this research is to determine anticholinesterase inhibitory and cytotoxic activity of the oil and extract for the first time. Moreover, for more information on the major compounds of the essential oil, it analyses by gas chromatography-mass spectrophotometry.

MATERIALS AND METHODS

Plant collection

The flowering aerial parts of *S. macrosiphon* were collected from Nurabad Mamassani, located in Fars province, Iran, on May 2014. The specimen of the plant was identified and authenticated by Professor G. Amin and deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences (No.6762-TEH).

Preparation of the total extract

The aerial parts of flowering plant were air-dried in shade, grounded and extracted by hydro alcoholic solution (methanol/water 80:20 v/v). The extract was filtered and concentrated under vacuum, in a rotary evaporator and finally lyophilized. The extract was kept in fridge for afterward tests.[26]

Isolation of the essential oil

Dried powder of the plant was extracted with hydro-distillation, using a Clevenger type apparatus for 5 h. the obtained essential oil was dried

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over anhydrous sodium sulfate then stored in the sealed amber vial at 4°C until succeeding tests.[27]

Gas chromatography-Mass spectroscopy

Analysis of the essential oil sample was performed on an Agilent gas chromatography, carrier gas, He; flow rate 1 ml/min; split ratio, 1:25, using a 30 m length capillary column (DB-5) and equipped with flame ionization detector (FID). The column was held at 50°C for 5 min then increased to 280°C at a rate of 3°C min^{-1} held for 10 min before injection. 1.0 µL of the essential oil was injected to the column with 280°C and detected in 300°C.

GC-Mass was carried out using an Agilent GC with a quadruple detector, on capillary column DB-5 (GC), carrier gas, He; flow rate 1 ml/min; The column was programmed at 50°C for 5 min then heated to 280°C at a rate of 3°C/min and kept constant at 280°C for 10 min. Mass spectra were measured at 70 eV ionization energy. Retention indices were determined relative to the retention time of n-alkanes that were injected after the essential oil.

The volatile compounds were identified using their retention times, Kovats retention indices (KI) and mass spectra and comparison with data in Wiley library and those published in the literature.[28,29]

BIOLOGICAL ACTIVITIES

Antioxidant activity assay

Free radical scavenging capacity of the essential oil and the total extract were examined by DPPH (2, 2- Diphenyl-1-Picrylhydrazyl) assay.[30,31] 1 ml of the sample solution (essential oil or total extract) at different concentrations was prepared in methanol and added to 2 ml of DPPH solution (0.1 mM in methanol) (Merck, Germany). A test tube contains 2 ml DPPH solution diluted in 1 ml of methanol and used as blank tube. All of the mixtures were shaken and then incubated for 30 min at room temperature. Finally, the absorbance was measured at 517 nm using UV spectrophotometer. The percentage of inhibition was calculated using equation 1.

$$\text{Inhibition } (\%)=((A_b-A_s)/A_b) \times 100 \quad (1)$$

A_b and A_s were the absorbance of blank and sample respectively. The free radical scavenging ability of the sample was expressed as IC_{50} which was defined as the required concentration (µg/ml) of sample for 50% inhibition of the free radical DPPH. Vitamin E was used as positive controls. All tests were repeated for three times.

Determination of total phenolic content

Total phenolic content was measured using the Folin-Ciocalteu colorimetric method.[32,33] Nine ml distilled water was added to 1 ml of the Folin-Ciocalteu reagent. One ml of sample solution was mixed with 1.5 ml of Folin-Ciocalteu reagent and incubated at 25°C for 10 min. Then, 1.5 mL of $NaHCO_3$ (7.5%) solution was added to the mixtures and incubated for 30 min at room temperature. This reaction produced a blue color which measured at 765 nm. Gallic acid was used as a standard solution to plot a calibration curve. The results were calculated as mg Gallic acid equivalents (GAE) per gram of each sample. All tests were conducted triplicate.

Determination of anticholinesterase activity

The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) by the essential oil and the total extract were measured by the Ellman spectrophotometric method with slight modification.[34,35]

Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as Ellman's reagent was used for the measurement of the cholinesterase activity. At first, 50 µl of a 100 mM sodium phosphate buffer (pH 8.0), 25 µl of sample solutions and 25 µl of AChE or BChE solution (0.22 U/ml) were mixed and incubated for 15 min at room temperature. Then, 125 µl of a 3 mM DTNB was added. Finally, the substrates added and the absorbance was measured at 412 nm using a micro plate reader (ELX800, BioTek, USA) after 15 min. The solution of all ingredients except substrates was used as negative control, and tacrine was applied as positive control. Both of the essential oil and the extract were prepared in different concentrations and measured three times. The inhibitory effect of each sample was calculated using equation 1.

The IC_{50} value (concentration of sample which inhibits 50% of AChE and BuChE), was calculated by a liner regression analysis.

Cytotoxic activity by MTT assay

Three human breast cancer cell lines, MCF-7, MDA-MB-231 and T47D were prepared from Pasture Institute of Iran, Tehran, Iran. The cells were maintained in RPMI 1640 medium (Biosera, England) contains sodium bicarbonate and N-Hydroxyethylpiperazone-n-2-Ethanesulfonic Acid (HEPES, Biosera, England) supplemented with 10% fetal bovine serum (FBS; Biosera, England) and 1% antibiotics including streptomycin (100 µg/ml) and penicillin (100 U/ml). All the cell lines were incubated at 37°C in air atmosphere with 5% carbon dioxide. The cell lines were passaged by trypsinization and then incubated. The viable cells were counted using trypan blue dye exclusion method and finally the cytotoxic activity of the samples were measured by the colorimetric method of MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) assay with slight modification.[36]

Two hundred µL of the cells suspension in growth media were seeded into the wells of 96-well plates and incubated at 37°C in air atmosphere with 5% CO_2 for 24 h. Five µL of various concentrations of samples were added per well in triplicate and re-incubated overnight. Then twenty µL of MTT reagent (5 mg/ml) and 180 µL of the media were added to each well and incubated for 4 h. Finally, the medium of each well was replaced with 200 µl of pure DMSO and the absorbance was measured at 545 nm using a microplate reader (ELX800, BioTek, USA). Three wells containing the cells, the medium and DMSO was used as negative control while etoposide was used as positive control. The final concentration of DMSO did not exceed 0.1% in all of the tests.

Cytotoxic activity, calculated by regression analysis, was expressed as the concentration of sample which inhibits 50% of cell growth (IC_{50} ± SD).

Statistical analysis

All Data are reported as the mean ± SD of triplicate tests and statistical analysis was conducted using Microsoft Excel 2010.

RESULTS

Chemical composition of the essential oil

The aerial parts of *S. macrosiphon* yielded 0.2% v/w essential oil using hydro-distillation method (Density=0.69 g/cm^3). The result of GC-MS analysis of the essential oil was presented in Table 1.

This study showed that the volatile oil of *S. macrosiphon* aerial parts was rich in sesquiterpene compounds (51.05%). The monoterpenoids (37.69%), aliphatic esters (8.5%) and miscellaneous (2.76%) were the other constituents in the oil. Twenty six components were characterized constituting 100% of the total oil with linalool (19%), β-cedrene (14.64%) and β-elemene (13.33%) as major components.

Antioxidant activity

Total phenolic content of the essential oil and the total extract of *S. macrosiphon* were determined using Folin–Ciocalteu reagent and compared with Gallic acid as a standard phenolic compound. As seen in Table 2, the total phenolic content (TPC) of the essential oil and

Table 1: Chemical composition of *Salvia macrosiphon* essential oil

No	Compound	Percentage	KI	RT
1	Linalool	19.00	1097	7.012
2	Hexyl isobutyrate	0.96	1147	7.606
3	Hexyl2-Methyl butanoate	2.48	1236	8.414
4	Hexyl isovalerate	5.06	1244	8.454
5	Thymol	8.73	1290	8.805
6	Carvacrol	9.96	1299	8.850
7	δ-Elemene	4.02	1338	9.194
8	Isocomene	1.15	1388	9.535
9	β-Elemene	13.33	1391	9.582
10	β-Cedrene	14.64	1421	9.770
11	γ-Elemene	0.6	1437	9.994
12	α-Guaiene	1.66	1440	10.145
13	Germacrene-D	0.93	1485	10.176
14	β-Selinene	3.18	1490	10.236
15	Germacrene-A	0.91	1509	10.269
16	α-Cadinene	1.04	1539	10.300
17	Elemicin	1.49	1557	10.420
18	Germacrene-B	0.73	1561	10.638
19	β-Copaen-4-α-Ol	1.92	1591	10.773
20	Khusimone	0.81	1604	10.804
21	α-Eudesmol	0.82	1654	11.193
22	Caryophyllene acetate	0.41	1701	11.266
23	Occidentalol acetate	0.53	1787	12.443
24	Lanceol acetate-Z	4.37	1856	12.769
25	Manool	0.67	2057	12.978
26	Sclareol	0.6	2223	14.006
	Total	100		

KI: Kovats retention Index (KI) on DB-5, RT: Retention times

Monoterpene hydrocarbons	0
Oxygenated monoterpene	37.69
Sesquiterpene hydrocarbons	42.19
Oxygenated sesquiterpene	8.86
Diterpenoid	1.27
Aliphatic ester	8.5
Others	1.49

Table 2: Antioxidant and total phenolic content of the essential oil and total extract of *Salvia macrosiphon*

Sample	DPPH IC_{50} (μg/ml)	Total phenol content GAE/g Sample
Essential oil	1.83 ± 0.03	119.85 ± 0.06
Total extract	55.07 ± 0.018	111.9 ± 0.023
Vit. E	14 ± 0.03	---

the total extract were estimated to be 119.85 and 111.9 mg GAE/g sample respectively. Antioxidant activities of the essential oil and the total extract were examined using DPPH assay. The IC_{50} value for the essential oil and the total extract were reported 1.83 μg/ml and 55.07 μg/ml respectively.

Anticholinesterase activity

Acetylcholinesterase inhibition activity of *S. macrosiphon* essential oil and total extract were investigated for the first time. According to results shown in Table 3, the oil inhibited significantly AChE activity (IC_{50}=0.169 ± 0.045) while its anti-BuChE activity was >0.5 μg/ml. Total extract of *S. macrosiphon* did not exhibit AChE and BChE inhibitory effects at concentrations up to 500 μg/ml.

Cytotoxic activity

The effects of the essential oil and the total extract of the plant on proliferation of cell lines MCF-7, MDA-MB-231 and T47D was assessed by treating the cells with different concentrations of the samples. The essential oil of *S. macrosiphon* revealed a potent cytotoxic activity on all of the cell lines (IC_{50}<0.15 μg/ml), while the total extract exhibited moderate cytotoxic activity (IC_{50}<100 μg/ml) [Table 4].

DISCUSSION

According to traditional background and extensive pharmacological studies of different Salvia species, it can be noted that this genus is a valuable source of natural bioactive agents.[4] Similar to our study, sesquiterpenes were the main components in essential oil obtained from stem bark of *S. macrosiphon* collected from Tehran province, Iran.[37] The major constituents of the oil obtained from Tehran were identified to be α-gurjunene (11%), β-cubebene (10.6%) and germacrene B (7%); whereas in the present study, α-gurjunene and β-cubebene were not detected in the essential oil at all. In another study conducted on this species collected from Fars province, Iran, the main components of the oil were linalool (21.02%), hexyl isovalerate (14.83%) and hexyl 2-methylbutyrate (10.64%) which showed minor similarity to our results.[38] Sefidkon *et al.* also reported linalool (21.84%), sclareol (15.76%), hexyl 3-methyl butanoate (9.39%) and hexyl octanoate (8.94%) as major components of the oil. As it can be observed, different samples collected from diverse geographic areas have represented different composition quantitatively or even qualitatively which could be due to the various ecological areas, different collection time or different investigated parts of the plant in each study.[37-39]

The present study reported the considerable antioxidant activity of the essential oil of *S. macrosiphon* for the first time. Agree with our data, previous study showed antioxidant activity of *S. macrosiphon* extract.[24] There were several reports on cytotoxic, anti- AChE and anti-BuChE activities of some species of *Salvia* such as *S. lavandulaefolia, S. miltiorrhiza* and *S. potentillifolia*;[7,40] Whereas no data was reported on cytotoxic and anti-AChE effects of *S. macrosiphon* up to now.

In the present study, for the first time, the essential oil of the plant showed significant biological effects such as antioxidant, cytotoxic and anti-cholinesterase activities. It is noticeable that thymol and carvacrol which were identified as ingredients of the essential oil of the plant, have shown considerable anti-AChE activity.[41] Among all other major components of *S. macrosiphon* essential oil, linalool has been investigated previously for acetylcholinesterase inhibitory effect which disclosed poor activity.[41,42] Further studies should be conducted on acetylcholinesterase inhibitory effect of β-elemene and β-cedrene as the other major compounds. Also, several researches have confirmed antioxidant and cytotoxic effectiveness of β-elemene and linalool which both compounds were identified as major constituents of *S. macrosiphon* oil.[43-47]

Hence, the potent antioxidant and cytotoxic effects of the oil could be due to the characteristics of the first three major components; however its anti-AChE activity could not be justified with these components. The significant anti-AChE activity of the oil may be due to the activity of other constitutes such as thymol and carvacrol or their synergism effects.

Several researchers reported antioxidant, anti- AChE and cytotoxic activities of some natural phenolic compounds such as apigenin, luteolin and rosmarinic acid which previously identified as major compounds of the total extract of S. *macrosiphon*.[12,17-19,48] The total extract of *S. macrosiphon* exhibited significant antioxidant and cytotoxic activities; nevertheless no inhibitory effect was exhibited on AChE and BuChE. The total extract of *S. macrosiphon* showed more potent cytotoxic

Table 3: Acetylcholinesterase and butyrylcholinesterase of the oil and total extract of *Salvia macrosiphon*

Sample	Acetylcholinesterase IC_{50} (µg/ml)	Butyrylcholinesterase IC_{50} (µg/ml)
Essential oil	0.169 ± 0.045	>0.5*
Total extract	>500*	>500*
Tacrine	0.0095 ± 0.0022	0.0020 ± 0.0008

Table 4: Cytotoxic activity of the volatile oil and total extract of *Salvia macrosiphon*

Sample	MCF-7 IC_{50} µg/ml	T-47D IC_{50} µg/ml	MDA-MB-231 IC_{50} µg/ml
Essential oil	0.155 ± 0.064	0.093 ± 0.012	0.145 ± 0.036
Total extract	46.559 ± 0.008	54.304 ± 0.04	75.251 ± 0.032
Etoposide	16.082 ± 0.095	18.286 ± 0.064	19.639 ± 0.149
Vinca rosea[a]		195.78 ± 17.96	

[a]The positive control [49]

activity compared to *Vinca rosea* methanol extract which was used as positive control in cytotoxic activity assay in identical conditions of the present study.[49]

CONCLUSION

Based on the findings in this study, it could be concluded that *S. macrosiphon* could be a valuable source of antioxidant, cytotoxic and anti-AChE compounds. Complementary phytochemical and biological studies are suggested for detection and isolation of pharmaceutically active ingredients of the herb.

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