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PHYTOCHEMICAL CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF *SALVIA NEMOROSA* L. OF IRAN

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Abstract

The essential oil of *Salvia nemorosa* L. was analyzed by GC-MS. The major constituents of the oil were E-caryophyllene (26.37%), phytol (16.92%), germacrene-D (15.34%), sabinene (12.86%), caryophyllene oxide (6.33%), bicyclogermacrene (4.71%) and n-tetradecane (2.45%). The total phenolic content and the antioxidant activity of methanolic extracts were determined with the Folin-Ciocalteau reagent and by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, respectively. The antimicrobial activity of the essential oil was individually evaluated against *Staphylococcus aureus, Staphylococcus epidermidis*, *Escherichia coli* and *Candida albicans* using disc diffusion and serial dilution methods. The oil showed good antimicrobial activity against four medically important pathogens.

Introduction

The use of traditional herbs and medicinal plants has become very popular because of the presence of natural products with biological properties and plants as one of the important sources of new pharmaceuticals and healthcare products (Mulabagal and Tsay 2004). The Lamiaceae family includes about 250 genera and 6700 species and plants spread in the warm and temperate region all over the world (Mabberley 1997). The genus *Salvia* the largest and one of the most important genera of the Lamiaceae family consists of about 900 species, out of which 58 species are distributed in Iranian flora (Rechinger 1982). Some members of this genus have been widely used as herbal tea and flavor component and in folk and traditional medicine, perfumery and pharmaceutical purposes (Kamatou *et al.* 2008 and Keller 1978). Previous reports on *Salvia* species revealed that they exhibit various biological activities, such as antimicrobial, antiviral, antioxidant, cardiovascular, anticancer, anti-inflammatory, antidiabetic, spasmolytic, antiseptic and sedative properties (Ryu *et al.* 1997, Haznedaroglua *et al.* 2001, Longaray Delamare *et al.* 2006, Kamatou *et al.* 2006, Loizzo *et al.* 2007, Kelen and Tepe 2008, Loizzo *et al.* 2008, Hayouni *et al.* 2008, Eidi and Eidi 2009). *Salvia nemorosa* is a drought-tolerant perennial herb (30 - 70 cm high) with an erect stem and violet, purple, or white to pink colored flowers and flowering occurs from middle spring to summer in flora of Iran (Rechinger 1982).

The information on the essential oil of *S. nemorosa* is very scarce. Meshkatalsadat and Norani (2015) reported that, β-caryophyllene (18.7%), isocaryophyllene (6.8%) and caryophyllene oxide (5.2%) were the major constituents of *S. nemorosa* essential oil. In the present continuing research on the essential oil composition and biological activities in Iranian medicinal and aromatic plants, phytochemical composition and biological activities of *S. nemorosa* have been evaluated. An attempt was made to use this plant as alternative products as natural antioxidant and antimicrobial agent for food and medicinal uses.

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Materials and Methods

The aerial parts of *Salvia nemorosa* were collected at full-flowering stage from its wild habitat in Estahban, Fars province, at an altitude of 1700 m. Voucher specimen was deposited at the herbarium of medicinal and aromatic plants of Estahban branch, Islamic Azad University, Estahban, Iran(voucher no 223). Dried aerial parts were ground into powder (mesh < 35), and 100 g of the powdered tissue was distillated with one litre of water for 3 hrs using a Clevenger-type apparatus according to the method recommended in the British Pharmacopoeia (British Pharmacopoeia 1988). The oils were dried over anhydrous sodium sulfate, weighed, and stored in dark glass vials at 4ºC prior to analysis and antimicrobial tests. The essential oil composition was determined by GC and GC-MS analysis. The analysis was performed using a gas chromatograph (Agilent Technologies 7890 GC) equipped with a FID detector, using HP-5MS 5% capillary column (30 m × 0.25 mm, 0.25 µm film thicknesses). The carrier gas was Helium at a flow of 1 ml/min. Initial column temperature was 60°C and was programmed to increase at 3°C/min to 280°C. The injector and detector temperatures were set at 280°C. The split ratio was 20 : 1. Oil samples (0.2 µl) were injected manually. The percentage compositions were obtained from electronic integration of peak areas without the use of correction factors. The GC-MS analysis was done on the Agilent Technologies 5975 Mass system. The EI-MS operating parameters were as follows: ionization voltage, 70 eV; ion source temperature, 200°C. The retention indices for all the components were determined according to the Van Den Doll method using n-alkanes as standard (Van Den Dool and Kratz 1963). The compounds were identified by comparison of retention indices (RRI- HP-5) with those reported in the literature and by comparison of their mass spectra with the Willey and mass finder 3 librariesor with the published mass spectra (Adams 2001).

For preparation of methanolic extracts of the *S. nemorosa* (7.5 g) were defatted with petroleum ether for 3 hrs and then, macerated twice for 24 hrs with 200 ml of methanol/water (90/10) at room temperature. After filtration through Whatman filter paper (Whatman, Little Chalfont, UK), supernatants were combined and the solvent was evaporated to a volume of about 1 ml using a rotary evaporator. The concentrated extracts were freeze-dried and weighed for yield determination, and kept at –20°C until used for antioxidant activity and total phenolic content tests.

Total phenolic content in *S. nemorosa* plant extracts was determined by the Folin-Ciocalteau colorimetric method, as described by the method of Singleton and Rossi, (1965). Different concentrations of gallic acid in methanol were tested in parallel to obtain a standard curve. Total phenolic contents were determined as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Radical scavenging activity of plant extracts against the stable free radical DPPH was measured according to the method employed by Brand-Williams *et al.* (1995). Different concentrations of the plant extract dissolved in methanol were incubated with a methanolic solution of DPPH (100 µM) in 96-well micro plates. After 30 min of incubation at room temperature, the absorbance was recorded at 517 nm. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. The amount of methanolic extract and essential oils necessary to decrease the absorbance of DPPH by 50% (IC 50) was calculated graphically and the percentage inhibition was calculated according to the equation:

Percentage inhibition (%I) = [(A blank – A sample)/ A blank] × 100

where, A blank is the absorbance of the control reaction (DPPH alone), and A sample is the absorbance of DPPH solution in the presence of the plant extract. The IC50 values were calculated as the concentration of extracts causing a 50% inhibition of DPPH radical, a

lower IC50 value corresponds to a higher antioxidant activity of plant extract sample. Quercetin was used as reference compound. Gallic acid, Quercetin and Vitamin E were used as standard antioxidant.

Standard strains of *Candida albicans* (ATCC 10231), the Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 1435), and the Gram-negative bacterium *Escherichia coli* (ATCC 25922) were all obtained from the Iranian Research Organization for Science and Technology. *In vitro* antimicrobial activity of the essential oil of *S. nemorosa* was evaluated by disc diffusion method, with determination of inhibition zones (IZ), using Mueller-Hinton agar for bacteria (MHA) and Sabouraud Dextrose Agar (SDA) for fungi, according to the National Committee for Clinical Laboratory Standards. The density of microorganism culture required for the test was adjusted to 0.5 McFarland standards, using 100 µl each suspension of the tested microorganisms containing 1.0 × 106 CFU/ml for bacteria and 1.0 × 105 CFU/ml (0.5 McFarland) spore for fungi strain using the spectrophotometer (LABOMED. INC. UV/VIS double beam PC.UVD.2980) using Mueller-Hinton agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungi (Baron and Finegold 1990). Disks containing different concentration of essential oil were used and inhibition zones diameter were measured in millimeters after 24 and 48 hrs of incubation at 37 and 24°C for bacteria and fungi, respectively. All the experiments were performed in triplicate. Blank disks containing 20 µl DMSO were used as negative controls. Tetracycline (30 µg/disk), amoxicillin (10 µg/disk), ketoconazol (20 µg/disk), gentamicine (30 µg/disk), were used as positive reference standards to determine the sensitivity of the microorganisms. A broth micro-dilution method was used to determine the minimum inhibitory concentration (MIC) according to the National Committee for Clinical Laboratory Standards (NCCLS 2001). Bacterial and fungi strains were cultured overnight in Muller Hinton Broth at 37 and 24°C, respectively. A serial doubling dilution of the oil was prepared in a 96-well micro titer plate over the range of 0.02 - 50.00 µl/ml. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The MBC and MFC were referred to the minimum concentration of the essential oils with no viable bacteria. All determinations were performed in triplicate.

All data were expressed as mean ± standard deviation. Analysis of variance was performed by ANOVA by the software SAS (version 9.2 for windows). Significant differences between means were determined by Duncan's new multiple-range test. IC50 values of the antioxidant activity were calculated by the software curve expert (version 1.3 for Windows).

Results and Discussion

Water-distilled essential oil from aerial parts of *Salvia nemorosa* was found to be pallid yellow liquid and was obtained in yields of 0.62% (w/w) based on dry weight. The results obtained by GC and GC-MS analysis of the essential oil are presented in Table I, where compounds are listed in order of their elution from a DB-5 column. Forty two components were identified, representing more than 98.24% of the total oil. The major components of the essential oil were found to be E-caryophyllene (26.37%), phytol (16.92%), germacrene-D (15.34%), sabinene (12.86%), caryophylleneoxide (6.33%), bicyclogermacrene (4.71%) and n-tetradecane (2.45%). Sesquiterpene hydrocarbons (51.91%), oxygenated sesquiterpenes (25.07%), monoterpene hydrocarbons (17.87%) and oxygenated monoterpenes (3.39%) constituted the most abundant fractions of the oil respectively (Table 1).

Comparing the present data with those previously reported for *S. nemorosa* collected from the Vienna area of Austria show a strong similarity with this report. Sabinene, germacrene D, caryophyllene and caryophyllene oxide were the main components in both reports; but phytol,

Table 1. Essential oil composition of *S. nemorosa* growing wild in Iran.

No.	Component	RI*	% of component
1	α-thujene	926	0.50 ± 0.06
2	α-pinene	933	0.31 ± 0.05
3	Sabinene	973	12.86 ± 1.12
4	1-octen-3-0l	977	1.88 ± 0.18
5	Myrcene	991	0.22 ± 0.05
6	α-terpinene	1017	0.12 ± 0.03
7	p-cymene	1024	0.11 ± 0.04
8	Limonene	1028	0.13 ± 0.02
9	(Z)-β- ocimene	1036	0.07 ± 0.02
10	Benzen acetaldehyde	1043	1.07 ± 0.14
11	(E)-β-ocimene	1046	0.18 ± 0.04
12	γ-terpinene	1057	0.33 ± 0.05
13	Cis-sabinene hydrate	1066	0.33 ± 0.08
14	Terpinolene	1088	0.09 ± 0.02
15	Linalool	1098	0.28 ± 0.05
16	Trans-sabinene hydrate	1099	0.17 ± 0.03
17	n-nonanal	1104	0.23 ± 0.04
18	Terpinene-4-ol	1177	0.46 ± 0.05
19	n-decanal	1206	0.19 ± 0.03
20	Thymol	1292	0.19 ± 0.02
21	Carvacrol	1300	0.11 ± 0.03
22	p-vinyl guaiacol	1313	0.41 ± 0.11
23	δ-elemene	1337	0.27 ± 0.03
24	Eugenol	1358	0.06 ± 0.02
25	α-copaene	1375	0.08 ± 0.03
26	(E)-β-damascenone	1384	0.30 ± 0.04
27	(Z)-jasmone	1392	0.31 ± 0.05
28	n-tetradecane	1399	2.45 ± 0.23
29	(E)-caryophyllene	1419	26.37 ± 2.25
30	Aromadendrene	1438	0.08 ± 0.03
31	α-humulene	1453	0.86 ± 0.18
32	(E)-β-farnesene	1457	0.39 ± 0.08
33	α-amorphene	1476	0.05 ± 0.02
34	Germacrene D	1480	15.34 ± 1.48
35	(E)-β-ionone	1486	0.49 ± 0.11
36	Bicyclogermacrene	1496	4.71 ± 0.57
37	(E,E)-α-farnesene	1509	0.18 ± 0.04
38	Myristicin	1522	0.21 ± 0.03
39	Spathulenol	1577	1.82 ± 0.25
40	Caryophyllene oxide	1582	6.33 ± 1.63
41	n-hexadecane	1599	0.78 ± 0.12
42	Phytol	2111	16.92 ± 1.87
	Monoterpene hydrocarbons		17.87
	Oxygenated monoterpens		3.39
	Sesquiterpene hydrocarbons		51.91
	Oxygenated sesquiterpenes		25.07
	Total		98.24
	Essential oil yield (%)		0.62

*RI, retention indices in elution order from HP-5 column. Each value in the table was obtained by calculating the average of three experiments ± standard deviation. Data expressed as percentage of total.

bicycloger-macrene and n-tetradecane were not present as major components in *S. nemorosa* oil in Austria (Chizzla 2012). Some differences were also found in respect of the Iranian *S. nemorosa* oil studied by Meshkatalsadat and Norani (2015): the main constituents of this oil were, β-caryophyllene (18.7%), isocaryophyllene (6.8%) and caryophyllene oxide (5.2%). These changes in the essential oil compositions might arise from several environmental (climatical, seasonal, geographical) and genetic differences (Alizadeh *et al.* 2011 a,b).

The total phenolic content and antioxidant activity of the methanolic extracts of *S. nemorosa* was measured by the Folin-Ciocalteu reagent and (2,2-diphenyl-1-picrylhydrazyl) DPPH free radical scavenging methods, respectively. The total phenolic content was 47.48 mg gallic acid equivalent/g dry weight. Results show that *S. nemorosa* has high phenolic content compared with a previous report by Firuzi *et al.* 2010 in *S. santolinifolia* (28.6), *S. eremophila* (16.1), *S. palestina* (14.6), *S. syriaca* (13.0), *S. multicaulis* (13.0), *S. hydrangea* (10.9), *S. reuterana* (10.1), *S. virgata* (7.5) and *S. macrosiphon* (7.1) mg catechin equivalent/g dry weight nine *Salvia* species in Iran.

The antioxidant activity of the methanolic extract of *S. nemorosa* was assessed by the DPPH free radical scavenging methods. The IC50 value was 40.22 µg/ml. The antioxidant activity of standard antioxidants was 18.22, 18.0 and 7.45 µg/ml for BHT, gallicacid and quercetin, respectively (Table 2). The lower IC50 value indicates a stronger ability of the extract to act as a DPPH scavenger while the higher IC50 value indicates a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction.

Table 2. Total phenolic content and radical scavenging activity of *S. nemorosa* and synthetic antioxidant.

Plant species	Total phenolic content[a] (mg GAE/g DW)	IC50[b] (µg/ml)
S. nemorosa	47.48 ± 0.78	40.22 ± 1.13
Synthetic antioxidant		
BHT	ND	18.22 ± 0.26
Gallic acid	ND	18.0 ± 0.45
Quercetin	ND	7.45 ± 0.48

Each value in the table was obtained by calculating the average of three experiments ± standard deviation. [a]Data expressed as mg of gallic acid equivalents per g dry weight (DW). [b]IC50 : Data expressed as µg per milliliter. Lower IC50 values indicated the highest radical scavenging activity. ND = not determined.

The present results show that *S. nemorosa* has high antioxidant activity compared with a previous report by Firuzi *et al.* 2010 in *S. santolinifolia* (115.7), *S. eremophila* (206.1*), S. palestina* (207.9), *S. syriaca* (371.7*), S. multicaulis* (246.6), *S. hydrangea* (301.9), *S. macrosiphon* (415.3), *S. reuterana* (459.1) and *S. virgata* (644.8) µg dry weight/ml growing wild in Iran. Tepe (2008) reported the antioxidant activity of methanolic extract of *S. virgate, S. virgate* and *S. verbenaceae* growing in Turkey were 65.70, 75.40 and 14.30 µg/ml, respectively. The antioxidant activity of essential oil of *S. aucheri* (18.8), *S. aramiensis* (12.2) and *S. pilifera* (24.1) µg/ml grown wild in Turkey was reported (Kelen and Tepe 2008). The methanolic extract of *S. nemorosa* showed high amount of phenolic content and moderate antioxidant activity compared to synthetic antioxidant.

The *in vitro* antimicrobial activity of the essential oils of *S. nemorosa* were assessed by the disc diffusion and serial dilution methods against microbial strains include *Staphylococcus aureus*,

Staphylococcus epidermidis, *Escherichia coli* and *Candida albicans*. Antimicrobial activity was expressed as diameter of the inhibition zones (DIZ), minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal (MBC /MFC) concentration values (Table 3).

Table 3. Antimicrobial activity of the essential oils of *S. nemorosa.*

Microorganism	Inhibition zone (mm)[a]	MIC[b]	MBC/ MFCc	Standard antibiotics			
				Tetracycline (30 µg/disk)	Amoxicillin (10 µg/disk)	Ketoconazol (20 µg/disk)	Gentamicine (30 µg/disk)
S. aureus	13	6.25	12.5	20	17	NA	NA
C. albicans	19	3.125	6.25	NA	NA	23	NA
S. epidermidis	13	6.25	12.5	26	16	16	NA
E. coli	9	12.5	25	NA	15	NA	23

[a]Diameter of inhibition zones (mm) including diameter of sterile disk (6 mm), essential oil was tested at 20 µl/disk for tested microorganism. [b]Minimum inhibitory concentration, values as mg/ml. Minimum Bactericidal/Fungicidal concentration, values as mg/ml. NA, not active.

The results show that *S. nemorosa* essential oils have moderate antimicrobial properties against all the four microorganisms tested, compared to the positive standard antibiotics (Table 3).

Generally, the antimicrobial activity of the essential oils of *S. nemorosa* against the studied microorganism was good and E. coli as gram-negative bacteria was more resistant to the essential oil compare Gram-positive bacteria and *Candida albicans* was more sensitive.

Recent studies on the antimicrobial activity of the essential oils show that, this activity is generally correlated to the chemical composition of the oil (Baratta *et al.* 1998). The antimicrobial activities of *S. nemorosa* can be attributed to the presence of high concentrations of E- caryophyllene, germacrene D, sabinene, bicyclogermacrene and caryophyllene oxide, with well documented antibacterial and antifungic potential (Azaz *et al.* 2002 and Ulubelen *et al.* 1994). Other than the major compounds, carvacrol, thymol and linallol, as well as other minor constituents of the essential oil of. *S. nemorosa* have antimicrobial activity (Juven *et al.* 1994 and Ultee *et al.* 1999). In fact, the synergistic effects of the diversity of major and minor constituents present in the essential oils should be taken into consideration to account for their biological activity (Alizadeh 2015). The *in vitro* antimicrobial activity may support the use of *Salvia* species in traditional medicine to treatmicrobial infections.

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