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Research Article

Chemical Composition and Antimicrobial Activity of the Volatile Oil of *Salvia santolinifolia* Boiss. From Southeast of Iran

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ABSTRACT

Background: *Salvia santolinifolia* is a medicinal plant, traditionally used for the treatment of inflammation, hypercholesterolemia, hemorrhoids and diarrhea. Discovery of new natural antimicrobial agents is necessary because of microorganism's resistance to common antibiotics.

Methods: Essential oil of *S. santolinifolia* was analyzed by GC-FID and GC-MS. Antibacterial, antifungal and general toxic activities of the essential oil were also evaluated.

Results: Chemical analysis of the oil revealed that α-pinene (49.3%), β-eudesmol (20.0%), camphene (7.8%) and limonene (7.7%) are the major components of the essential oil of *S. santolinifolia*. The inhibition zones ranged from 11.5 to 23.8 mm. Minimum inhibitory concentrations of the oil obtained from 200 to 800 µg/ml against several microbial strains.

Conclusion: Our results showed that the volatile oil of *S. santolinifolia* could be considered as a rich source of natural agents for several uses as antibiotics against human pathogenic microbes.

Introduction

Medicinal plants used in traditional medicine of various nations have the potential to provide biologically active natural products, extracts and essential oils which could be useful for the treatment of many disorders.[1-4] This could be obtained by taking advantage of information available from traditional medicine and also ethnobotanical knowledge.[5-8]

The genus *Salvia* is a member of Lamiaceae family and comprises over 1000 species around the world.[9] The genus is represented by 61 species in flora of Iran which 17 of them are endemic.[10] In folk medicine, *Salvia* is used for treatment of infections, hemostatic, aches, spasmolytic, colds, epilepsy, bronchitis, hemorrhage, tuberculosis, and menstrual disorders.[11-13] Various biological activities have been reported for this genus including antibacterial, antiplasmodial, antidiabetic, antifungal, antioxidant, anti-HIV, antimalarial, cytotoxicity, antitumor and cardiotonic properties.[14-16] Moreover, some of these species are used as ornamental plants.[17] Due to these properties, some *Salvia* species, such as *S. fruticosa*, *S. hispanica*, *S. miltiorrhiza*, *S. sclarea* and *S. officinalis* are cultivated in several countries for their uses as spices, food, perfume oils and medicinal herbs.[9,13]

Salvia santolinifolia is used traditionally in Iran, Afghanistan and Pakistan for the treatment of hemorrhoids, hypercholesterolemia, inflammatory and diarrhea.[18,19] Phytochemical studies proved the existence of terpenoids and lignans in *S. santolinifolia*.[19-21] Additionally, several biological properties such as antileishmanial,[21] butyrylcholinesterase inhibitory[19,21], neuroprotective,[22] antioxidant and anti-apoptotic[23] activities have been reported for different extracts or pure compounds, isolated from this plant.

Continuing our studies on Iranian *Salvia* species, we studied the chemical composition of the essential oil from the aerial parts of *S. santolinifolia*. Furthermore, antimicrobial activity of the EO was evaluated against four gram positive and gram negative bacteria and two fungi strains using two different methods. In addition, general toxicity of the oil was investigated against the brine shrimp larvae.

Materials and Methods

Chemicals

Podophyllotoxin, sea salt and sodium sulfate were purchased from Merck (Germany). Gentamicin, nystatin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Germany).

Plant material

The aerial parts of *S. santolinifolia* were collected during the flowering stage in April 2014 from wild natural environment (Zahedan, southeastern of Iran).

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The plant was identified by Mr. Shahram Bahadori, taxonomist, and a voucher specimen was deposited at the herbarium of Urmia School of Pharmacy.

Isolation of essential oil

The essential oil of the plant was extracted by hydrodistillation using a Clevenger type apparatus during 3 h based on the method described in British Pharmacopoeia. Resulted oil was dried using anhydrous sodium sulphate and stored at 4 °C in dark until analysis.

Essential oil analysis

GC-FID and GC-MS

The essential oil analysis was performed using an Agilent instrument (model 7890A) equipped with a flame ionization detector (FID) and a DB-5 capillary column, (30 m × 0.32 mm i.d., film thickness 0.25 µm). Helium was used as carrier gas (1.1 ml/min, in constant linear velocity mode). Temperatures of the injector and detector were set at 240 and 250 °C, respectively. The oven temperature was programed from 35 to 180 °C at the rate of 4 °C/min, then raised to 250 °C at 17 °C/min and held at this temperature for 10 min. The injection volume was 1 µl in split mode (1:100).

GC-MS analysis was carried out using a Thermoquest Finnigan instrument (model Trace GC, Trace MS) equipped with fused silica capillary DB-5 column. Essential oil was diluted in *n*-hexane (1/100, v/v) and 0.5 µl was injected manually. The temperature program of column was as mentioned above for the GC-FID. Spectra were obtained in the electron ionization (EI) mode.

Identification of volatile compounds

Identification of individual compounds was carried out by calculating of their retention indices (RI) using *n*-alkanes (C_6–C_{24}) under the same GC condition. The constituents of the oil were identified by comparison of their retention indices and mass spectra with those published in the literature[24] and with NIST, Wiley and Adams Mass Spectral libraries. Relative area percentages obtained from GC-FID were applied for quantification without consideration of calibration factors.

Antimicrobial activity assay

Antimicrobial activity of the essential oil of the plant was tested against following microorganisms: *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (PTCC 1399), *Aspergillus niger* (PTCC 5012) and *Candida albicans* (PTCC 5027). Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar and fungi were cultured overnight at 30 °C in Sabouraud dextrose agar. All strains were obtained from the Pasteur Institute of Iran (IPI).

Disc diffusion method

The agar disc diffusion method was employed for determination of antimicrobial activity of essential oil of the plant.[25] Briefly, about 100 µl of the tested microorganisms suspensions, adjusted to 10^6-10^8 CFU/ml were spread on the solid media plates. The plant essential oil was dissolved in dimethylsulfoxide (DMSO) at concentration of 400 µg/ml and filtered by 0.45 µm Millipore filters for sterilization. The paper discs (6 mm in diameter) impregnated with 10 µl of the essential oil solution, were placed on the inoculated agar. DMSO was used as negative control. These plates were incubated for 24 h at 37 °C for bacterial strains and 48 h at 30 °C for the yeasts. After the incubation period, the diameter of inhibition zone (IZ) was measured in mm. Gentamicin (10 µg/disc) and nystatin (50 IU) were used as positive controls for bacteria and fungi, respectively. All the assays were performed in triplicate and expressed as average values ± SEM.

Minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of the essential oil of the plant were determined through micro-well dilution assay method.[26] The inoculants of the microbial strains were prepared from freshly cultured bacteria that were adjusted to 0.5 McFarland standard turbidity. Serial dilutions of the essential oil were made in a concentration range from 25 to 1000 µg/ml in 96-well plates, containing Mueller-Hinton broth for bacterial strains and Sabouraud dextrose broth for yeast. Gentamicin (for bacteria) and nystatin (for fungi) were used as standard drugs for positive control in conditions identical to test materials. The plates were covered with sterile plate sealers and then incubated at 37 °C under normal atmospheric condition for 24 h for bacterial strains and at 30 °C for 48 h for yeasts. The MIC value was considered as the lowest concentration of the sample required for inhibiting the growth of microorganisms.

General toxicity assay

The experiments were carried out according to the method published previously with some modifications.[27] Brine shrimp (*Artemia salina*) eggs were allowed to hatch in a flask containing seawater (3.8% w/v salt in distilled water) for 48 h at 28 °C under constant aeration. The plant samples were prepared in DMSO. Ten larvae were collected with a pipette and added to the two fold serially diluted solutions (1000–15.6 µg/ml EO) in the test tubes. After 24 and 48 h, a magnifying glass was used to count the number of killed larvae and the mortality percentage was calculated. The triplicate mean of percentage mortality was plotted against the concentrations logarithm using Microsoft Excel 2013 software. Equation and regression appeared on the graph, and LC_{50} values were determined from the linear equation by taking the antilogarithm. Podophyllotoxin was used

as positive control, and pure DMSO as untreated control. Final DMSO concentration was 1%.

Results and Discussion

Chemical composition of the essential oil

The essential oil of *S. santolinifolia* was obtained as a light yellow liquid with a yield of 1.6% (v/w). GC/FID and GC/MS analysis (Figure 1) of the oil led to the identification and quantification of 16 compounds which represent 98.9% of total constituents as is presented in Table 1.

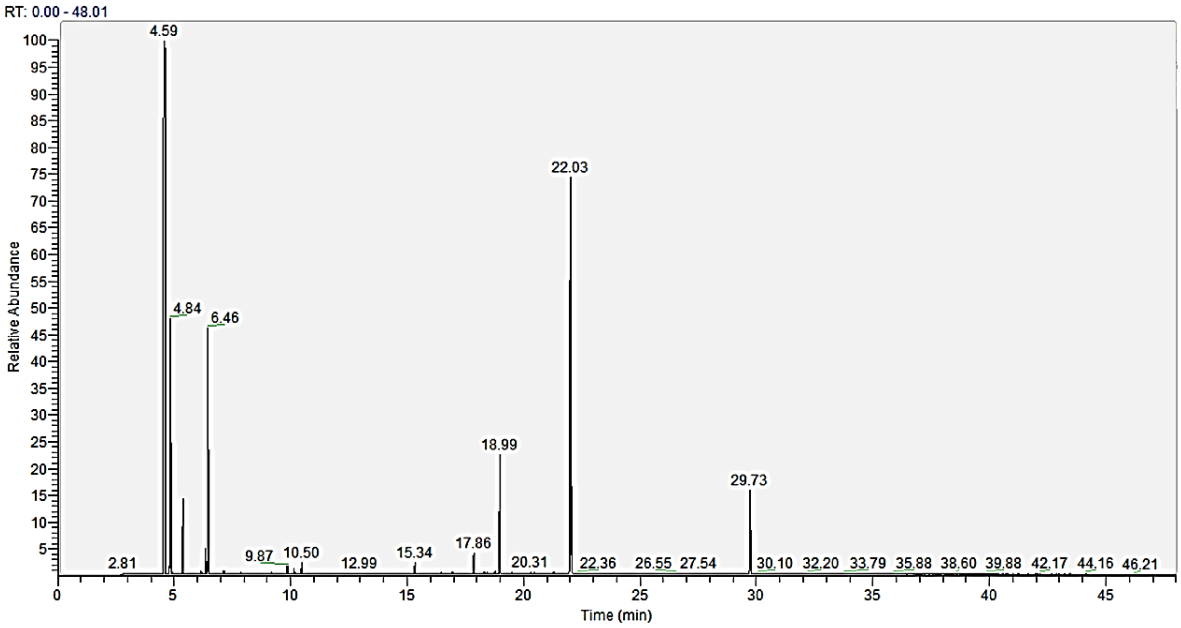

Figure 1. GC-MS chromatogram of *Salvia santolinifolia* essential oil.

Table 1. Chemical composition of the essential oil from the aerial parts of *Salvia santolinifolia*.

No	Compound	Percentage	RT	RI	Identification method
1	α-Pinene	49.3	4.59	935	RI, MS
2	Camphene	7.8	4.84	949	RI, MS
3	β-Pinene	1.9	5.37	977	RI, MS
4	O-Cymene	0.7	6.35	1024	RI, MS
5	Limonene	7.7	6.46	1029	RI, MS
6	γ-Terpinene	0.1	7.14	1058	RI, MS
7	Borneol	0.3	9.87	1170	RI, MS
8	Terpinene-4-ol	0.2	10.15	1181	RI, MS
9	α-Terpineol	0.4	10.5	1195	RI, MS
10	α-Copaene	0.4	15.34	1380	RI, MS
11	γ-Muurolene	0.7	17.86	1479	RI, MS
12	γ-Cadinene	0.1	18.78	1517	RI, MS
13	δ-Cadinene	3.8	18.99	1526	RI, MS
14	β-Eudesmol	20.0	22.03	1660	RI, MS
15	α-Eudesmol	3.0	22.17	1666	RI, MS
16	E,E-Geranyl linalool	2.5	29.73	2038	MS
Monoterpene hydrocarbons		67.5			
Oxygenated monoterpenes		0.9			
Sesquiterpene hydrocarbons		5.0			
Oxygenated sesquiterpenes		23.0			
Oxygenated diterpenoid		2.5			
Total identified		98.9			

The results showed that monoterpene hydrocarbons represented the major portion of the essential oil. Chemical profiling of the oil revealed that α-pinene (49.3%), β-eudesmol (20.0%), camphene (7.8%) and

limonene (7.7%) were the principle compounds in the volatile oil of *S. santolinifolia* (Figure 2). Several researchers studied the chemical composition of the essential oil of *S. santolinifolia* from different localities (Table 2). The monoterpene α-pinene is the principle compound in all of studies. Other monoterpene hydrocarbons like β-pinene, camphene and limonene are presented as major components in the most of previously studied oils. In comparison, there are two basic differences between present and former studies. The first, the yield of obtained oil in the present study (1.6%) is significantly higher than prior works (0.1-0.5%). It could be because of natural environment of the plant. The environment of the plant collected for this study is waterless with low humidity in comparison with the former studies. These conditions may be the reason of increasing of essential oil concentration in the plant body. Differences in chemical composition of essential oils could be utilized in chemotaxonomy studies for classification of the plants.[3] The second main difference is the occurrence of β-eudesmol (20%) as a major component in the essential oil in our work which is unprecedented. This oxygenated sesquiterpene is the second major compound in the oil composition. β-eudesmol is known for its antiproliferation and anti-inflammatory activities and unique effects on nervous system.[28] However, advanced pharmacological studies on the essential oil of *S. santolinifolia* are warranted.

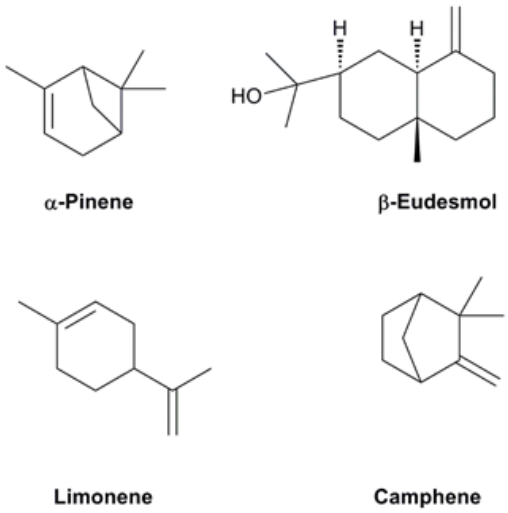

Figure 1. Structures of the major compounds from the essential oil of Salvia santolinifolia.

Table 2. Comparison of studies on the essential oil composition of *Salvia santolinifolia*.

Locality	Yield %	Major Compounds	Percentage	Reference
Iran, Hajiabad	0.5	α-pinene	72.4	32
		β-pinene	6.6	
		limonene	5.3	
		borneol	2.5	
Iran, Darab	0.5	α-pinene	35.7	33
		camphor	15.9	
		α-eudesmol	4.9	
		limonene	3.0	
Iran, Zabol	0.18	α-pinene	59.4	34
		β-pinene	12.4	
		(Z)-b-ocimene	3.8	
		camphene	2.9	
Iran, Darab and Fasa	0.1	α-pinene	52.5	35
		camphene	8.1	
		β-pinene	7.0	
		limonene	6.0	
Pakistan, Karachi	0.16	α-pinene	13.8	36
		caryophyllene oxide	4.3	
		β-caryophyllene	3.8	
		(E,E)-farnesol	3.8	
Iran, Zahedan	1.6	α-pinene	49.3	Present work
		β-eudesmol	20	
		camphene	7.8	
		limonene	7.7	

Antimicrobial activity

This is the first report on antimicrobial activities of *S. santolinifolia* essential oil against human pathogenic microorganisms. The antimicrobial activity of the essential oil of *S. santolinifolia* was evaluated against the following bacteria and fungi: Gram positive *Bacillus cereus* and *Staphylococcus aureus*, Gram negative *Escherichia coli* and *Pseudomonas aeruginosa*, fungi *Candida albicans* and *Aspergillus niger*. The diameters of inhibition zones ranged from 11.5 to 23.8 mm including the diameter of paper disc (6 mm). The results are summarized in Table 3. Findings

of the antimicrobial activity according to the disc diffusion method, demonstrated that the essential oil of *S. santolinifolia* has high antimicrobial potential against Gram positive bacteria. *Pseudomonas aeruginosa* was the most resistant microbe and *Bacillus cereus* was the most sensitive. Minimum inhibitory concentrations (MICs) were also determined and the MIC values ranged from 200 to 800 µg/ml (Table 3). Gentamicin and nystatin were used as standard antibiotics in antibacterial and antifungal assays, respectively. In comparison, essential oil of *S. santolinifolia* has similar or stronger antimicrobial activity than those reported in the literature.[29]

Table 3. Antimicrobial activity of the essential oil from *Salvia santolinifolia* against human pathogenic microorganisms.

Samples		Gram positive		Gram negative		Fungi	
		B. cereus	*S. aureus*	*P. aeruginosa*	*E. coli*	*A. niger*	*C. albicans*
EO	IZ	23.8 ± 1.6	18.5 ± 1.0	-	11.5 ± 1.6	13.8 ± 0.8	15.8 ± 1.5
	MIC	200	400	1000	1000	800	400
Gentamicin	IZ	34.7 ± 2.1	31.8 ± 1.1	28.6 ± 1.9	23.8 ± 1.3	-	-
	MIC	10	10	50	25	-	-
Nystatin	IZ	-	-	-	-	24.2 ± 1.2	27.0 ± 1.4
	MIC	-	-	-	-	50	50

Cytotoxicity

The toxicity value of the essential oil was determined against brine shrimp larvae (*Artemia salina*). The results are summarized in Table 4. The essential oil of this plant demonstrated moderate toxicity against *Artemia salina* larvae compared to the positive control, podophyllotoxin (LC_{50} = 40 µg/ml). The mortality rate of brine shrimp larva was found to be concentration dependent. In general, brine shrimp cytotoxicity activity of crude extracts with LC_{50} values less than 1000 µg/ml are considered to be active.[27] There are several studies on *Salvia* species which reported terpenoid compounds as cytotoxic agents.[10,30] Accordingly, the monoterpene and sesquiterpene rich essential oil of *S. santolinifolia* could have antiproliferative activities against human cancer cell lines. In comparison with previous studies (LC_{50} values ranging from 128 to 291 µg/ml for eight crude extract of five *Salvia* species)[31] essential oil of *S. santolinifolia* exhibited moderate brine shrimp toxicity. To the best of our knowledge, this is the first report on brine shrimp toxic activity of the essential oil from *S. santolinifolia.* However, more bioassays against human cancer cell lines are necessary for evaluation of cytotoxic activity of *S. santolinifolia*.

Table 4. Brine shrimp lethality assay (BSLA) of essential oil and extracts of Salvia santolinifolia.

Concentrations (µg/ml)	**Mortality percentage**
1000	76.6
500	43.3
250	26.6
125	16.6
62.5	6.6
31.25	3.3

Conclusion

The findings of the present work indicated that *S. santolinifolia* has a monoterpene rich essential oil. This essential oil exhibited remarkable antimicrobial activity and moderate toxicity. The results show this oil could be considered for discovery and formulation of new natural antibiotics for possible uses in food and pharmaceutical industries. Moreover, differences observed in the chemical composition and the oil yield, between this study and previous works, suggested that species collected from different regions may be are chemotypes of *S. santolinifolia*.

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Conflict of interests

The authors claim that there is no conflict of interest.

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