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Original article

Compositions of Essential Oils and Some Biological Properties of *Stachys laxa* Boiss. & Buhse and *S. byzantina* K. Koch

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Abstract

Background and objectives: *Stachys* L. genus from the Lamiaceae family is distributed worldwide. It is used for medicinal purposes in traditional medicine. *Stachys laxa* as an endemic species and *S. byzantina* which grow in the north of Iran were selected in this study for analyzing the chemical compositions of the volatile oils and investigation of some biological activities. **Methods:** The chemical constituents of the oils from the aerial parts were analyzed by GC-MS. The antimicrobial activity of the essential oils was investigated by disc diffusion method and the MIC was determined. Toxicity and total phenolics content were surveyed by brine shrimp lethality and Folin-Ciocalteu assays, respectively. Two different methods (DPPH and FRAP) were conducted to assess the antioxidant activity of both extracts. **Results:** Sixty-one compounds were identified in the oils, whereas sesquiterpenes were the major components in both volatile oils. Hexadecanoic acid (16.65%) and hexahydrofarnesyl acetone (20.41%) were the main compounds in *S. laxa* and *S. byzantina*, respectively. The ethyl acetate fraction of *S. byzantina* showed the strongest antioxidant activity (DPPH IC_{50}: 18.3 µg/mL; FRAP: 687.4 $FeSO_4$.7 H_2O mg /g extract) and the highest total phenolics content (115.43 gallic acid mg /g extract) compared to other fractions. The volatile oil of *S. laxa* showed more potent antimicrobial activity on *Salmonella paratyphi* A (MIC: 5.62 µg/mL). **Conclusion:** Both species were safe and showed no toxicity. They demonstrated strong antioxidant properties. The essential oil of *S. laxa* showed potent activity against *Salmonella paratyphi* A.

Keywords: antimicrobial; antioxidant; essential oil; *Stachys byzantina; Stachys laxa*

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Introduction

Stachys L. is one of the largest genera in the world. It belongs to the family Lamiaceae (Labiatae). *Stachys* consists of about 300 species of annual and perennial herbs and also small shrubs [1].

Different species of *Stachys* spread in areas with high temperature like the Mediterranean reigon and southwest of Asia, South Africa, North and South America. In fact, *Stachys* is distributed worldwide for except New Zealand and Australia [2].

Stachys has a long-term history of use in

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traditional medicine. Numerous species have been used in decoctions and infusions to treat inflammatory diseases such as rheumatic disorder, ulcers, sclerosis of the spleen, migraine and headache, respiratory problems including asthma, cough and sore throat, fevers, diarrhea, genital tumors and liver disorders [1, 3-5]. Diverse natural compounds are biosynthesized by genus *Stachys* including flavonoids, phenolic acids, phenylethanoid and phenylpropanoid glycosides, diterpenoids, iridoids, saponins and steroids which are responsible for the biological properties [5,6]. In addition, the volatile oils of several species of *Stachys* are rich in sesquiterpenoids and monoterpenoids. In the literatures germacrene D, β-caryophyllene, caryophyllene oxide, spathulenol and α-cadinene are mentioned as prevailing constituents of the essential oils of *Stachys* [5,7,8]. Not only a multitude of effects of different species of *Stachys* such as antibacterial [9], anti-inflammatory [10], antioxidant and cytotoxic effects [9] have been documented, but also anti-tumor, anti-nociceptive, anti-pyretic [11], anti-anxiolytic [12], anti-spasmodic [13] and immunomodulatory [14] activities are reported in previous studies.

Nearly 34 of the whole species of *Stachys* are accessible in Iran of which 13 are endemic [15]. *Stachys laxa* Boiss. & Buhse is the endemic species in Iran [16]. *Stachys laxa* with the synonym name of *S. demavendica* Bornm. is distributed in the north of Iran. Even though, the volatile oil of S. laxa was analyzed twice in 2003 and 2006, the habitat of the plants was different from this study [17,18]. The present study was designed to compare the essential oil and some biological properties of *S. laxa* with a well-known species *S. byzantina*.

Materials and Methods

Ethical considerations

The research was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.PSRC.REC.1396.2594).

Plant material

The aerial parts of *S. laxa* and *S. byzantina* were respectively collected in summer 2016 from Kojur (altitude of 1038m) and Marzanabad (altitude of 1972m), Mazandaran province, North of Iran. The plants were identified by Dr Yousef Ajani, botanist. Voucher specimens 6507-THE and 6506-THE were deposited at the Herbarium of Tehran University of Medical Sciences for *S. laxa* and *S. byzantina*, respectively.

Extraction of essential oil

The aerial parts of the plants were dried in shade. The essential oils were obtained by hydro-distillation method using Clevenger-type apparatus. One hundred g of the aerial parts of each plant was extracted separately for 4 hours and the essential oils were kept at 4˚C for further analyzing by gas chromatography-mass spectrometry (GC-MS).

Extraction

The plants (200 g) were extracted by maceration method with 80 % methanol for 6 days. The solvent was evaporated by rotary evaporator (Heidolph, Germany) at 40 ˚C to yield total extracts. Finally, the total extracts were fractionated by different solvents such as hexane, chloroform, ethyl acetate and methanol using solid-liquid fractionation method. Four different fractions were used in further evaluations.

GC-MS analysis procedure

Gas chromatography of the essential oils was carried out on HP-5973 system coupled with a mass detector equipped with HP-5MS column (60 m × 0.32 mm × 0.5 µm). The initial temperature of oven was 80 ˚C and programmed to reach 230 ˚C with a rate of 3 ˚C /min (held for 5 min), finally reached 250˚C and held for 10 min. The temperature of injector and detector was 250 ˚C and 0.1 µL of sample was injected. Helium with 99.99% purity was used as the carrier gas with a flow rate 1.5 mL/min. Ionization voltage of detector was equal to 70 ev. Normal Alkanes (C_8 – C_{32}) were injected with the same condition [19].

Antimicrobial activity

Microbial strains

Antimicrobial activity of the essential oils were assessed against six Gram-negative bacterial strains including *Escherichia coli* (ATCC 10536), *Salmonella paratyphi* A (ATCC 5702), *Klebsiella pneumonia* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (PTCC 1182), *Shigella dysenteriae* (PTCC 1188), three Gram-positive bacterial strains including *Staphylococcus aureus* (ATCC 29737), *Staphylococcus epidermidis*

(ATCC 12228), *Bacillus subtilis* (ATCC 6633), and three fungi including one yeast, *Candida albicans* (ATCC 10231) and two molds, *Aspergillus brasiliensis* (PTCC 1015) and *Aspergillus niger* (ATCC 16404). All were provided from Iranian Research Organization for Science and Technology (IROST).

Disc diffusion assay

The antimicrobial activity was evaluated by methods described by National Committee for Clinical Laboratory Standards [20]. The disc diffusion method assessed the antimicrobial activity of *S. laxa* and *S. byzantina* essential oils. One hundred µL of bacterial suspension (turbidity equivalent to 0.5 McFarland) was cultured on the Mueller-Hilton Agar (MHA) medium as basal layer. The essential oils (1 mg/mL) in 10% Dimethyl sulfoxide (DMSO) were sterilized through Millipore filter (0.45 µm) then 10 µL of each sample was loaded over sterile filter paper discs (6 mm in diameter). Standard antibiotics, gentamicin, rifampin and nystatin were used as positive control [21].

Determination of minimum inhibitory concentration (MIC)

The MIC values of microbial strains, which were determined susceptible in disc diffusion assay, were estimated in sterilized 96-well microplates. Brain Heart Infusion (BHI) medium (95 µL) was added to microplates, then 5 µL of the bacterial suspension (0.5 McFarland) and 100 µL of different concentrations of the essential oils (7.8 to 500 µg/mL) were added. The Plates were shaken at 3000 rpm for 20 seconds and incubated at 37 ˚C for 24 h. The appearance of white spots at the bottom of the wells indicated the microbial growth. The lowest concentration of the essential oil which inhibited the microbial growth was reported as the MIC value [20]. Standard antibiotics, gentamicin, rifampin and nystatin were used as the positive control. For negative control, 195 µL of BHI medium and 5 µL of bacterial suspension with no essential oil was used. The tests were repeated three times for all microorganisms.

Brine Shrimp lethality test

The brine shrimp lethality assay was conducted to evaluate the general toxicity of the extracts. Artificial seawater was prepared by dissolving sea salt (38 g) in water (1 L) ad the pH was adjusted to 9.0 by Na_2CO_3. Brine shrimps (*Artemia salina* Leach) eggs hatched in sterile artificial seawater under constant aeriation at 30 ˚C for 48 h. The fractions of both plants were prepared by artificial seawater in different concentrations: 1000, 700, 500, 300, 100 and 10 µg/mL and DMSO (1% v/v) was used for better solubility. Five mL of each sample and 20 live nauplii were put in different tubes in triplicate. After incubation under light at 30 ˚C for 24 h, the number of survived nauplii was counted and recorded. A cytotoxic natural compound, podophyllotoxin, was used as the positive control. The percentage of brine shrimp lethality was calculated for each concentration. Finally, by the concentration-mortality curve, the median lethal dose (LD_{50}) value of each sample was determined and reported as means ± SD [22].

Total phenolics assay

Total phenolic content of different fractions was determined by Folin-Ciocaltue method with slight modifications [23]. Briefly, 200 µL of each fraction (20 µg/mL) was mixed with 1 mL, 1:10 diluted Folin-Ciocaltue reagent. After 5 min, 3 ml Na_2CO_3 (7.5% w/v) was added and incubated at room temperature in a dark place for 2 h. The absorbance was measured by spectrophotometer at 760 nm. Different concentrations of gallic acid (25-150 µg/mL) was used to obtain the calibration curve. Eventually, the results were expressed as milligrams of gallic acid equivalents per gram of dry extract and reported as means ± SD.

Antioxidant assay

DPPH method

To determine the antioxidant capacity of the fractions, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used according to Moradi-Afrapoli et al. with some modifications [23]. One mL of different concentrations (1000, 500, 250 125 µg/m) of each fraction was added to 2 mL of methanol DPPH solution (40 µg/mL). After incubation at 37 ˚C for 30 min, the absorbance was read at 517 nm. Butylated hydroxytoluene (BHT) was used at the same concentrations (1000, 500, 250 and 125 µg/mL) as the positive control. Inhibition percent which indicates the antioxidant activity was calculated by the following equation:

$$I\% = [(A_{Blank} - A_{Sample}) / A_{Blank}] \times 100$$

A_{blank} is the absorbance of the control reaction which contains all of the components without the tested sample and A_{sample} is the absorbance of the tested sample. The concentration that inhibited 50% of DPPH solution (IC_{50}) was calculated by plotting the inhibition percent against sample concentrations. The test was performed in triplicate and the IC_{50} values were reported as means ± SD.

FRAP method

The antioxidant potential of different fractions was also determined by the ferric reducing ability of plasma (FRAP) assay. Briefly, 25 mL of acetate buffer (0.3 mol/L, pH= 3.6) was added to 2.5 mL of $FeCl_3$ (20 mmol/L) and 2.5 mL of 2,4,6- tripyridy-*s*-triazine (TPTZ) solution (10 mmol/L in 40 mmol/L HCl) to prepare the FRAP reagent freshly. Three mL of reagent was incubated in different tubs at 37 ˚C for 5 min. Then 100 µL of fractions were added and incubated again for 10 min at 37 ˚C. Finally, the absorbance of mixtures was measured at 593 nm. A standard curve of different concentrations of $FeSO_4.7H_2O$ solution was used to determine the antioxidant capacity of the samples. FRAP values were expressed as the concentration of antioxidants showing ferric reducing ability equivalent to that of FeSO4 [24]. The test was performed in triplicate and results were reported as means ± SD.

Results and Discussion

Yellow oils in the yield of 0.15% and 0.21% (w/w) were obtained by hydro-distillation from *S. laxa* and *S. byzantine*, respectively. The chemical constituents of the essential oils of both common Iranian *Stachys* species were identified and showed in Table 1.

Sixty-one compounds were identified in the essential oils of which 16 were common in both species. Accordingly, 41 components were identified for *S. laxa* which exhibited 91.46% of the total essential oil component. Hexadecanoic acid (16.65%), germacrene D (9.99%) and α-pinene (9.44%) were reported as the main constituents in the essential oil of *S. laxa*. On the other hand, 36 components representing 92.49% of total essential oil were identified in *S. byzantina*. Hexahydrofarnesyl acetone (20.41%), 1-octen-3-ol (9.77%), benzaldehyde (7.57%) and α-bisabolol (6.3%) were reported as the major compounds in the volatile oil of *S. byzantina*. In line with previous studies on genus *Stachys*, sesquiterpenes were characterized as the prevailing constituents in both species [25]. Generally, 40.59% of *S. laxa* oil was consisted of sesquiterpene components, most of which contained sesquiterpene hydrocarbon (23.15%). On the contrary, *S. byzantina* oil was rich in oxygenated sesquiterpenes (37.55%). The amount of sesquiterpene compounds were 41.09%.

The essential oil composition of some *Stachys* species were analyzed and reported that is summarized in Table 2. Hexadecanoic acid was the major compound of *S. laxa* oil in our study while germacrene D was the main compound in previous studies [17,18]. Some studies reported germacrene D as the major compound of essential oils of *S. inflata* and *S. lavandulifolia* [26].

In the present study, hexadecanoic acid was absent in *S. byzantina* oil but Bahadori MS et al. reported the compound as the third abundant major constituent (10.9%) in *S. byzantina* collected from Khalkhal [26]. Additionally, Conforti et al. reported hexadecanoic acid as the dominant component of oil in four species of *Stachys* which were collected from Mediterranean area [27]. The prevailing constituents identified in *S. byzantina* volatile oil, hexahydrofarnesyl acetone and 1-octen-3-ol, were previously reported in *S. byzantina* [26].

There are several studies on different species of the *Stachys* genus with similar compositions but different major compounds in their essential oils. Usually, many factors affect the composition of essential the oils. In 2013 the effect of locality was investigated on the components of *S. lavandulifolia* essential oil. Two different populations of *S. lavandulifolia* were collected from Isfahan and Chaharmahal va Bakhtiary provinces in Iran. This study in agreement with some other studies confirmed that not only the geographical and environmental factors like climate and elevation, but also the genetic and experimental conditions such as the different parts of plant, collection time, drying conditions and extraction technique affect the quality and quantity of the oil composition [8,29,30].

Limit of the effective life span of antibiotics due to the microbial resistance, have persuaded researchers to find new antimicrobial agents. One of the most available resources for investigation are natural compounds.

Table 1. The constituents of essential oils of *Stachys laxa* and *S. byzantina*

No.	Compounds Name	*S. laxa* %	*S. byzantina* %	[1]RRI	[2]KI
1	α-Thujene	0.23	-	926	924
2	α-Pinene	**9.44**	3.09	930	932
3	Camphene	-	2.26	949	946
4	Sabinene	0.82	-	965	969
5	Benzaldehyde	-	**7.57**	969	970
6	β-Pinene	0.77	2.54	977	974
7	1-Octen-3-ol	**6.66**	**9.77**	982	979
8	β-Myrcene	0.37	-	985	988
9	Furan-2-pentyl	-	0.91	997	993
10	Hemimellitene	1.69	-	1019	1021
11	Limonene	1.26	0.12	1020	1024
12	β-Phellandrene	1.19	-	1026	1025
13	1,8-Cineol	-	0.73	1029	1026
14	cis-Ocimene	0.1	-	1035	1032
15	1-Octanol	1.44	-	1067	1063
16	Acetophenone	-	1.16	1074	1078
17	Linalool	1.14	**4.37**	1097	1095
18	Nonanal	0.28	1.47	1105	1100
19	α-Campholene aldehyde	0.16	-	1128	1125
20	trans-Pinocarveol	-	1.09	1136	1135
21	cis-Verbenol	0.4	0.32	1140	1137
22	Camphor	-	1.28	1142	1141
23	Nonenal	-	0.53	1147	1144
24	Nonanol	0.11	-	1162	1165
25	Borneol	-	0.75	1171	1173
26	3-Methyl acetophenone	-	0.52	1182	1179
27	Methyl salicylate	0.43	-	1194	1190
28	Verbenone	-	0.52	1205	1204
29	E-Geraniol	-	1.62	1252	1249
30	Bornyl acetate	-	0.93	1285	1281
31	2-Undecanone	0.2	-	1287	1288
32	trans-Anethol	-	1.11	1289	1290
33	2,4-Decadienal	-	0.51	1296	1292
34	Eugenol	0.52	2.09	1351	1356
35	α-Copaene	1.2	-	1369	1367
36	β-Bourbonene	0.94	-	1373	1375
37	β-Damacenone	-	0.57	1386	1380
38	β-Elemene	0.66	-	1393	1389
39	β-Caryophyllene E	3.19	-	1419	1417
40	β-Farnesene Z	1.47	-	1436	1440
41	trans-Geranylacetone	-	0.43	1446	1445
42	α-Humulene	0.41	-	1455	1452
43	α-Amorphene	0.48	-	1481	1483
44	Germacrene D	**9.99**	2.27	1488	1484
45	Bicyclogermacrene	1.28	-	1503	1500
46	δ-Cadinene	**3.53**	1.27	1525	1522
47	Spathulenol	**3.39**	1.93	1572	1577
48	Caryophyllene oxide	**3.57**	1.23	1586	1582
49	Salvial-4(14)-en-1-one	1.11	-	1591	1594
50	T-Muurolol	0.96	2.04	1648	1644
51	α-Cadinol	1.03	-	1656	1652
52	Valeranone	1.33	**4.64**	1677	1674
53	α-Bisabolol	-	**6.3**	1684	1685
54	2-Pentadecanone	0.64	-	1700	1697
55	Tetradecanoic acid	1.09	-	1772	1770
56	Octadecane	0.24		1803	1800
57	Hexahydrofarnesyl acetone	**6.05**	**20.41**	1840	1838
58	Hexadecanoic acid	**16.65**	-	1966	1959
59	Octadecadienoic acid methyl ester	-	**3.14**	2078	2078
60	Phytol	**5.04**	2.14	2128	2122
61	Tricosane	-	0.86	2307	2300
		Results	Results		
	Monoterpene hydrocarbon	14.18	8.01		
	Oxygenated monoterpene	2.65	14.81		
	Sesquiterpene hydrocarbon	**23.15**	3.54		
	Oxygenated sesquiterpene	17.44	**37.55**		
	Nonterpene	**29.00**	**26.44**		
	Diterpene	5.04	2.14		
	Unknown	8.54	7.51		
	Total identified	91.46	92.49		

[1]RRI: Relative Retention Indices as determined on a HP-5MS column using homologous n-alkanes; 2KI: Kovats Indices

Table 2. Comparison of essential oil constituents of some *Stachys* species from Iran

Species	Origin (city, province)	Main compound (%)	References
S. laxa	Kojur, Mazandaran	Hexadecanoic acid (16.65%)	[Present study]
		Germacrene D (9.99%)	
		α-Pinene (9.44%)	
	Behshahr, Mazandaran	Germacrene D (17.1)	[17]
		4-hydroxy-4-methyl-2-pentanone (12.3)	
		7-epi-α-selinene (8.3)	
	Charat, Mazandaran	Germacrene D (40.1)	[18]
		B-caryophyllene (16.7)	
		β-phellandrene (5.5)	
S. byzantina	Marzanabad, Mazandaran	Hexahydrofarnesyl acetone (20.41%)	[Present study]
		1-octen-3-ol (9.77%)	
		benzaldehyde (7.57%)	
		α-bisabolol (6.3%)	
	Khalkhal, Ardabil	Hexahydrofarnesyl acetone (25.7)	[26]
		Valeranone (17.1)	
		Hexadecanoic acid (10.9)	
		Phytol (6.9)	
		1-octen-3-ol (6.6)	
	Urmieh, western	1,8-cineole (14.8)	[31]
		Linalool (12.9)	
		Cubenol (9.9)	
		Germacrene D (9.6)	
	Behshahr, Mazandaran	Piperitenone (9.9)	[17]
		6,10,14-trimethyl pentadecan-2-0ne (6.4)	
		n-tricosane (6.4)	
S. inflata	Behshahr, Mazandaran	Hexadecanoic acid (9.1)	[17]
		Germacrene D (8.9)	
		α-pinene (5.8)	
		bicyclogermacrene (5.1)	
	Kermanshah, Kermanshah	Germacrene D (21.6)	[26]
		β-pinene (15.6)	
		β-phellandrene (9.8)	
		α-pinene (9.6)	
	Kashan, Isfahan	Linalool (28.55)	[32]
		α-terpineol (9.45)	
		spathulenol (8.37)	
		(2E)-hexenal (4.62)	
S. lavandulifolia	Behshahr, Mazandaran	4-hydroxy-4-methyl-2-pentanone (9.3)	[17]
		α-pinene (7.9)	
		Hexadecanoic acid (5.2)	
	Marand, East Azarbaijan	Germacrene D (22.5)	[26]
		α-pinene (15.5)	

Many studies were conducted on antimicrobial effect of plants or their metabolites. Based on literatures, terpenoids which are abundant in the essential oils are remarked as antibacterial, antifungal, antiviral and antiprotozoal agents [30].The antimicrobial effects of both essential oils of the present study were investigated against 12 different bacterial and fungal strains. According to the results of Table 3, the oils of *S. laxa* and *S. byzantina* showed strong to moderate antimicrobial effect. These two essential oils have shown more powerful effect in Gram-negative bacteria compared to Gram-positive ones. No effect was observed on fungi strains. Some bacterial strains such *Esherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus epidermidis* were resistant to the essential oils. Although both oils showed potent effects on Gram-negative bacteria such as *Klebsiella pneumonia* and *Shigella dysenteriae*. *Stachys laxa* demonstrated the most considerable effect on *Salmonella paratyphi* A with MIC = 5.62 µg/mL (Table 3).

There are a number of investigations on antibacterial and antifungal activities of *Stachys* species which relatively confirm the results of the present study. Manafi et al. investigated the antimicrobial effect of the essential oils from the leaves and stem of *S. byzantina*. They reported a moderate antibacterial activity while no effect was observed against *E. coli* [33]. In another study in 2005, Sonboli et al. found a moderate

antibacterial effect of *S. schtschegleevii*, while the oil was more effective against Gram-positive bacteria than Gram-negative bacteria which is in line with the findings of Grujic-Jovanovic et al. [34,35]. The volatile oil of *S. inflata* showed no considerable antimicrobial activity; however, some pure constituents of the oil such as linalool and α-terpineol were effective [32]. The essential oil of 22 species of *Stachys* showed moderate activity against bacteria and *C. albicans* while some pure components of the oil demonstrated strong activity. It is interesting that the non-oxygenated constituents were more effective than the oxygenated compounds of which β-caryophyllene showed the strongest effect [36]. Another report on antimicrobial effect of the essential oils of 8 species of Stachys genus from Greece, confirms our findings that the antibacterial effect of this genus is more potent than antifungal activity [37]. The only antimicrobial report of *S. laxa* is a conducted study in 2008. Saeedi et al. surveyed the antimicrobial effect of the methanol extract of four species including *S. laxa* that showed antibacterial but no antifungal effects [38].

To evaluate the general toxicity of different fractions of the extracts, brine shrimp lethality bioassay was conducted. The results are shown in Table 4 as LD_{50} value. In comparison to the positive control, podophyllotoxin (LD_{50} = 2.4 µg/mL), there was not any toxicity in the plants. Some limited studies which are in agreement with ours were conducted to investigate the toxicity of *Stachys* species [39,40].

Reactive oxygen species (ROS), as the harmful mediators, can be produced during biological processes. Accumulation of damage due to ROS in lipids, proteins and deoxyribonucleic acid (DNA), leads to oxidative stress [41]. Antioxidant compounds with two different mechanisms can prevent these problems: transfer of single electron or hydrogen atom. Because of the potential toxicity of chemicals which are used as antioxidants such as butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA), today, a lot of attention paid to natural antioxidant especially phenolic compounds [41,42].

Table 3. Antimicrobial activity of essential oils of *Stachys laxa* and *Stachys byzantina*

		MIC[a]				
Microorganism		***Stachys laxa***	***Stachys byzantina***	**Rifampin (5 µg/disc)**	**Gentamycin (10 µg/disc)**	**Nystatin (100 i.u./disc)**
Gram-negative	*E. coli*	-	-	500	500	NA[b]
	S. paratyphi A	5.62	-	-	500	NA
	K. pneumonia	<31.25	<31.25	250	250	NA
	P. aeruginosa	-	-	-	500	NA
	P. vulgaris	-	-	125	500	NA
	S. dysenteriae	<31.25	<31.25	250	500	NA
Gram-positive	*S. aureus*	<2000	1000	250	500	NA
	S. epidermidis	-	-	250	500	NA
	B. subtilis	<2000	<2000	15.6	500	NA
Fungi	*C. albicans*	250	500	NA	NA	125
	A. brasiliensis	500	500	NA	NA	2.31
	A.niger	500	500	NA	NA	2.31

a. MIC: Minimal Inhibition Concentration as µg/mL, b. NA: Not Applicable

Table 4. General toxicity, antioxidant effects and total phenolics content of *Stachys laxa* and *S. byzantina*

Samples		**Brine Shrimp Lethality LD_{50} (µg/ml)**	**DPPH assay IC_{50} (µg/ml)**	**FRAP value as $FeSO_4.7H_2O$ mg /g extract**	**Phenolics content as gallic acid mg /g extract**
S. laxa	Methanol	4508.3 ± 0.03	159.1 ± 0.96	514.7 ± 0.014	94.95 ± 0.007
	Ethyl acetate	313.2 ± 0.07	182.6 ± 0.78	526.7 ± 0.018	74.24 ± 0.009
	Chloroform	561.1 ± 0.08	293.0 ± 0.70	493.4 ± 0.008	34.95 ± 0.005
	Hexane	1270.9 ± 0.06	382.4 ± 0.72	490.7 ± 0.007	16.86 ± 0.001
S. byzantina	Methanol	3021.1 ± 0.07	173.6 ± 0.95	565.4 ± 0.022	51.68 ± 0.002
	Ethyl acetate	227.2 ± 0.07	18.3 ± 0.90	687.4 ± 0.016	115.43 ± 0.006
	Chloroform	498.2 ± 0.05	488.9 ± 0.67	524.0 ± 0.008	46.62 ± 0.002
	Hexane	702.5 ± 0.09	1782.3 ± 0.90	447.4 ± 0.007	39.71 ± 0.006
Positive control	Podophyllotoxin	2.4 ± 0.8	-	-	-
	BHA	-	14.3 ± 0.6	-	-

So, the antioxidant capacity of the extracts of both plants were investigated by two common antioxidant methods (DPPH and FRAP) based on transferring the electron. 2,2-diphenyl-1-picrylhydrazyl as a stable free radical (purple color) can react with reducing agents or antioxidants and convert to non-radical form, 1,1-diphenyl-2-picrylhydrazine (yellow color). In the ferric reducing antioxidant power method, the Fe^{3+} complex reduces to Fe^{2+} complex in an acidic medium by antioxidants and changes to blue color.

The results of both methods are measured spectrophotometrically [43]. Total phenolics content of the extracts was determined also by Folin-Ciocalteu assay. The phenolics compounds reduce Folin-Ciocalteu reagent and cause a blue-color complex that can be measured at 750 nm against a standard like gallic acid [44].

The methanol and ethyl acetate fractions exhibited the maximum phenolics contents and antioxidant activity (Table 4). The ethyl acetate extract of *S. byzantina* showed the most considerable results (total phenolics content: 51.68 gallic acid mg/g extract; FRAP: 687.4 $FeSO_4.7H_2O$ mg/g extract; DPPH IC_{50}: 18.3 µg/mL) compared with positive controls.

In previous studies the antioxidant activity and total phenolics contents of *Stachys* species have been measured which are in line with our results. In 2010, the antioxidant activity of the hexan, dichloromethane and methanol extracts of *S. byzantina* was investigated by DPPH method and the methanol extract showed the strongest activity (IC_{50}: 0.015 mg/mL) [16]. In addition, Morteza-Semnani K. et al. measured the antioxidant activity of four different *Stachys* species including *S. laxa* and *S. byzantina* and the methanol extract of *S. laxa* exhibited potent results. Since the method was different from the present study, the obtained results are not directly comparable [45]. No direct correlation has been reported between previously about the total phenol content and antioxidant activity of *Stachys* species [9

Conclusion

No antifungal effect for any of the *Stachys* species was observed but they showed considerable activity on Gram-negative bacteria. Additionally, the two plants seem safe and showed no toxicity against *Artemia salina*. Besides *S. byzantina* ethyl acetate fraction exhibited powerful antioxidant activity and can be suggested as a potent natural antioxidant agent.

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Author contributions

The whole parts were performed by Fatemeh Kiashi and also prepared the manuscript of article. Abbas Hadjiakhoondi, Zahra Tofighi and Mahnaz Khanavi were the adviser of project. Yousef Ajani as a botanist collected and identified the plants. Sheyda Ahmadi Koulaei cooperated in practical parts. Narguess Yassa was supervisor and designed the study.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

GC-MS: gas chromatography-mass spectrometry; IROST: Iranian research organization for science and technology; NCCLS: national committee for clinical laboratory standards; MHA: mueller-hilton agar; MIC: minimum inhibitory concentration; BHI: brain heart infusion; DMSO: dimethyl sulfoxide; LD_{50}: median lethal dose; DPPH: 2,2-diphenyl-1-picrylhydrazyl; BHT: butylated hydroxytoluene; IC_{50}: median Inhibitory concentration; FRAP: ferric reducing ability of plasma; TPTZ: 2,4,6- tripyridy-s-triazine; KI: Kovats indices; NA: not applicable; DNA: deoxyribonucleic acid; BHA: butylhydroxyanisole; ROS: reactive oxygen species