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Composition and antioxidant and antimicrobial activity of the essential oil and extracts of *Stachys inflata* Benth from Iran

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ABSTRACT

Composition and antioxidant and antimicrobial activities of essential oil and methanol extract polar and nonpolar subfractions of *Stachys inflata* were determined. GC and GC/MS analyse of the essential oil showed 45 constituents representing 95.46% of the oil, the major components linalool (28.55%), α-terpineol (9.45%), spathulenol (8.37%) and (2*E*)-hexenal (4.62%) constituted 50.99% of it. Essential oil and extracts were also tested for their antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene/linoleic acid assays. In the DPPH test, IC_{50} value for the polar subfraction was 89.50 μg/ml, indicating an antioxidant potency of about 22% of that of butylated hydroxytoluene (IC_{50} = 19.72 μg/ml) for this extract. In β-carotene/linoleic acid assay, the best inhibition belonged to the nonpolar subfraction (77.08%). Total phenolic content of the polar and nonpolar extract subfractions was 5.4 and 2.8% (w/w), respectively. The plant also showed a week antimicrobial activity against three strains of tested microorganisms. Linalool and α-terpineol were also tested as major components of the oil and showed no antioxidant but considerable antimicrobial activities.

1. Introduction

Antioxidants play important roles in the scavenging of free radicals and/or chain breaking of the oxidation reactions both *in vivo* and *in vitro*. The inhibition of oxidative reactions in food, pharmaceutical and cosmetic products and the prevention of oxidative stress related diseases in the human body are some of the useful potential functions of antioxidants (Moure et al., 2001). Application of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxytanisole (BHA), propyl gallate and citric acid in food processing has led to the appearance of remarkable side effects. For example, these substances can exhibit carcinogenic effects in living organism and enlarge the liver size and increase microsomal enzyme activity (Ames, 1983; Ito, Fukushima, & Hagiwara, 1983). Due to these limitations, there is an increasing interest in finding naturally and biologically produced antioxidants capable of inhibiting free radical reactions, retarding oxidative rancidity of lipids, protecting the human body from diseases, and preserving foods from spoiling (Pryor, 1991; Terao & Piskula, 1997). Microbial contamination is another important issue in the field of food, beverage, cosmetic, and pharmaceutical industries. Increased consumer demand on organic natural food stuff from one side and the observation of growing cases of microbial resistance to existing preservatives from the other has encouraged the world food research community toward the seeking of new natural antimicrobial substances. According to these facts, the plant kingdom with a remarkable diversity in producing natural compounds has attained a special interest and, today, accessing to plant materials with dual antioxidant and antimicrobial capabilities is an ideal goal in the filed of research on food additives.

The genus *Stachys* (family Lamiaceae) is represented by 270 species and distributed extensively in the tropical and subtropical countries. Thirty four species of this genus are found in Iran of which 13 species are endemic (Ghahreman, 1995; Rechinger, 1982; Zargari, 1992). Phytochemical studies on *Stachys* species has confirmed the presence of phenylethanoid glycosides (Miyase, Yamamoto, & Ueno, 1996; Nishimura, Sasaki, Inagaki, Chin, & Mitsuhashi, 1991), terpenoids and steroids (Ross & Zinchenko, 1975; Yamamoto, Miyase, & Ueno, 1994), diterpenes (Piozzi, Savona, & Hanson, 1980) and flavonoids (EL-Ansari, Barron, Abdalla, Saleh, & LE Quere, 1991; Zinchenko, 1970) in these plants. Also, biological studies have demonstrated considerable antibacterial, antiinflammatory, antitoxic, antinephritic, antihepatitis and antianoxia effects for some *Stachys* species (Hayashi, Nagamatsu, Ito, Hattori, & Suzuki, 1994a,b; Savchenko & Khvorostinka, 1978;

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Skaltsa, Lazari, Chinou, & Loukis, 1999; Yamahara, Kitani, Kobayashi, & Kawahara, 1990; Zinchenko, Voitenko, & Lipkan, 1981). *Stachys inflata* Benth is one of the endemic species of Iran (Persian names: poulk, ghole arghavan) and its aerial parts are orally used as herbal tea in the treatment of various infections, asthmatic, rheumatic and other inflammatory disorders (Zargari, 1992). Fresh flowering tops of the plant are also frequently used in jams and pickles as flavoring agents (Ghahreman, 1995). Potent antiinflammatory activity and remarkable reduction in the size of infracted heart muscle (myocardium) has been reported for its hydroalcoholic extract (Garjani, Maleki, & Nazemiyeh, 2004; Maleki et al., 2001).

To the best of our knowledge, reports on the chemical composition of the essential oil and antimicrobial profiles of *S. inflata* are scant and there is no report on its antioxidant potential in the literature. Thus, the present research reports (i) the chemical composition of the essential oil of *S. inflata* growing in the wild in Iran, (ii) *in vitro* antioxidant activity profiles of this plant products (essential oil and extracts) using two complementary methods, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and β-carotene/linoleic acid bleaching assay, (iii) total phenolic compounds content of the plant extracts as gallic acid equivalents, and (iv) antimicrobial potentials of the plant essential oil and extracts.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

Aerial parts of *S. inflata* were collected during June 2008 from Kashan area (Isfahan Province, Iran) at an altitude of ca. 1100 m. An authenticated specimen of the plant was deposited in the herbarium of the Kashan Research Botanical Garden, Research Institute of Forests and Rangelands, Kashan, Iran.

2.1.2. Solvents and chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH, 95%), β-carotene, linoleic acid, 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) and gallic acid were procured from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, dimethyl sulphoxide (DMSO), HPLC grade chloroform, linalool, α-terpineol, standard Folin–Ciocalteu's phenol reagent, anhydrous sodium sulphate, sodium carbonate, Tween 40, and all cultures media were obtained from Merck (Darmstadt, Germany). Ultra pure water was used for the experiments.

2.2. Preparation of the extracts

2.2.1. Isolation of the essential oil

Air-dried and ground (80 mesh) plant material was submitted to water distillation for 3.5 h using an all-glass Clevenger-type apparatus as recommended by the European Pharmacopoeia (Anonymous, 1996). The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored in an amber vial at low temperature (4 °C) prior to analysis.

2.2.2. Preparation of methanol extracts

Thirty grams of the powdered aerial parts of the plant were Soxhlet-extracted with 400 ml methanol for 8 h. Solvent removal by rotary evaporation (Buchi, Flawil, Switzerland) and drying the residue in a vacuum oven at 50 °C yielded 6.98 g (23.27% w/w) of dried extract. This extract was suspended in water (400 ml) and extracted with chloroform (4 × 100 ml) to obtain 5.60 g (18.67%, w/w) polar and 1.38 g (4.60%, w/w) nonpolar extracts. Extracts were concentrated, dried and kept in the dark at 4 °C until tested.

2.3. Chromatographic analysis

2.3.1. Gas chromatography (GC) analysis

Oil obtained from aerial parts of *S. inflata* was analysed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) equipped with an FID detector. Oven temperature was kept at 60 °C for 3 min initially, and then raised at the rate of 3 °C/min to 250 °C. Injector and detector temperatures were set at 220 and 290 °C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min, and diluted samples (1/1000 in *n*-pentane, v/v) of 1.0 μl were injected manually in the splitless mode. Peaks area percents were used for obtaining quantitative data.

2.3.2. Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis of the oil was carried out on an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (Ionization energy: 70 eV) operating under the same conditions as described above. Retention indices were calculated for all components using a homologous series of *n*-alkanes injected in conditions equal to samples ones. Identification of components of essential oil was based on retention indices (RI) relative to *n*-alkanes and computer matching with the Wiley275.L and Wiley7n.L libraries, as well as comparisons of the fragmentation pattern of the mass spectra with data published in the literature (Adams, 2001).

2.4. Antioxidant activity

2.4.1. DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay usually involves hydrogen atom transfer reaction, but, based on kinetic data, an electron transfer mechanism has also been suggested for this assay (Foti, Daquino, & Geraci, 2004; Huang, Ou, & Prior, 2005). Radical-scavenging activity (RSA) of essential oil, its major components (linalool, α-terpineol) and extracts of the plant was determined using a published DPPH radical scavenging activity assay method (Sarker, Latif, & Gray, 2006) with minor modifications. Briefly, stock solutions (10 mg/ml each) of the essential oil, linalool, α-terpineol, extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions are made to obtain concentrations ranging from 1 to 5×10^{-10} mg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of a freshly prepared 80 μg/ml DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbencies of these solutions were recorded using a spectrometer (Cintra 6, GBC, Dandenong, Australia) at 517 nm and using a blank containing the same concentration of oil or extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follow:

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

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

2.4.2. β-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari and

Shahidi (2008) was used with slight modifications. A stock solution of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 ml chloroform, 25 μl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of oxygenated distilled water were then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 μl of each sample solution were added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one contained BHT as a positive control and the other contained the same volume of DMSO instead of the samples. The test tube with BHT maintained its yellow colour during the incubation period. The absorbencies were measured at 470 nm on an ultraviolet spectrometer (Cintra 6, GBC, Dandenong, Australia). Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after2 hassay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$$

where $A_{\beta\text{-carotene after2 h assay}}$ is the absorbance of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial}\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.4.3. Assay for total phenolics

Many natural molecules, especially those produced in the plant kingdom, have at least one benzene ring with a hydroxyl functional group in their skeleton. Such compounds are collectively known as phenolic compounds and, due to their hydrogen or single electron donating potentials, usually play important rules in the antioxidant activity of the plant extracts. Total phenolic constituents of the polar and nonpolar subfractions of methanol extract of *S. inflata* were determined by literature methods involving Folin–Ciocalteu reagent and gallic acid standard (Slinkard & Singleton, 1977). Solutions of the extracts (0.1 ml each) containing 1000 μg of the extracts were taken individually in volumetric flasks, 46 ml of distilled water and 1 ml Folin–Ciocalteu reagent were added, and the flasks were thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixtures were allowed to stand for 2 h with intermittent shaking. Absorbencies were measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0–1000 mg/0.1 ml) and a standard curve obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid } (\mu g) + 0.0033$$

Total phenols of the extract, as gallic acid equivalents, was determined by using the absorbance of the extract measured at 760 nm as input to the standard curve and the equation. All tests were carried out in triplicate and phenolic contents as gallic acid equivalents were reported as means ± SD of triplicate determinations.

2.5. Antimicrobial activity

2.5.1. Microbial strains

The essential oil, its major components (linalool, α-terpineol) and the extracts of *S. inflata* were individually tested against a set of 11 microorganisms. Following microbial strains were provided by Iranian Research Organization for Science and Technology (IROST) and used in this research: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 10536), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737), *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus epidermidis* (ATCC 12228), *Shigella dysenteriae* (PTCC 1188), *Proteus vulgaris* (PTCC 1182), *Salmonella paratyphi-A serotype* (ATCC 5702), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). Bacterial strains were cultured overnight at 37 °C in nutrient agar (NA) and Fungi were cultured overnight at 30 °C in sabouraud dextrose agar (SDA).

2.5.2. Disk diffusion assay

Determination of antimicrobial activities of dried *S. inflata* extracts were accomplished by agar disk diffusion method (National Committee for Clinical Laboratory Standard, 1997). The dried plant extracts were dissolved in DMSO to a final concentration of 30 mg/ml and filtered by 0.45 μm Millipore filters for sterilization. Antimicrobial tests were carried out using the disk diffusion method reported by Murray, Baron, Pfaller, Tenover, and Yolke (1995) and employing 100 μl of suspension containing 10^8 CFU/ml of bacteria, 10^6 CFU/ml of yeast and 10^4 spore/ml of fungi spread on the nutrient agar (NA), sabouraud dextrose (SD) agar and potato dextrose (PD) agar mediums, respectively. The disks (6 mm in diameter) impregnated with 10 μl of the essential oil, its major components (linalool, α-terpineol) or the extracts solutions (300 μg/disk) and DMSO (as negative control) were placed on the inoculated agar. The inoculated plates were incubated for 24 h at 37 °C for bacterial strains and 48 h and 72 h at 30 °C for yeast and mold isolates, respectively. Gentamicin (10 μg/disk), and rifampin (5 μg/disk) were used as positive controls for bacteria and nystatin (100 I.U.) for fungi. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated twice.

2.5.3. Micro-well dilution assay

Bacterial strains and yeast sensitive to the essential oil, its major components (linalool, α-terpineol) and extracts of the plant in disk diffusion assay were studied for their minimal inhibition concentration (MIC) values using micro-well dilution assay method (Gulluce et al., 2004). The inocula of the microbial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oil, its major components (linalool, α-terpineol) and extracts of *S. inflata* dissolved in 10% DMSO were first diluted to the highest concentration (500 μg/ml) to be tested, and then serial twofold dilutions were made in a concentration range from 7.8 to 500 μg/ml in 10 ml sterile test tubes containing brain heart infusion (BHI) broth for bacterial strains and sabouraud dextrose (SD) broth for yeast. The 96-well plates were prepared by dispensing 95 μl of the cultures media and 5 μl of the inoculum into each well. A 100 μl aliquot from the stock solutions of the plant extracts initially prepared at the concentration of 500 μg/ml was added into the first well. Then, 100 μl from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μl of the cultures media without the test materials and 5 μl of the inoculum on each strip was used as the negative control. The final volume in each well was 200 μl. Gentamicin and rifampin for bacteria and nystatin for yeast were used as standard drugs for positive control in conditions identical to tests materials. The plates were covered with sterile plate sealers. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 μl samples from clear wells on NA medium. The MIC value was defined as the lowest concentration of the plant extracts required for inhibiting the growth of microorganisms. All tests were repeated two times.

2.5.4. MIC agar dilution assay

MIC values of linalool and α-terpineol for the fungus isolate sensitive to them were evaluated based on the agar dilution method described by Gul, Ojanen, and Hanninen (2002). Appropriate amounts of these compounds were added aseptically to sterile molted SDA medium containing Tween 20 (0.5%, v/v) to produce the concentration range of 7.8–500 μg/ml. The resulting SDA agar solutions were immediately mixed and poured into petri plates. The plates were spot inoculated with 5 μl (10^4 spore/ml) of fungus

isolate. Nystatin was used as reference antifungal drug and the inoculated plates were incubated at 30 °C for 72 h. At the end of incubation period, the plates were evaluated for the growth presence or absence. The MIC was defined as the lowest concentration of the compounds needed to inhibit the growth of microorganisms. Each test was repeated at least twice.

3. Results and discussion

3.1. Chemical composition of the essential oil

Air-dried herbal parts of the plant were subjected to hydrodistillation using a Clevenger-type apparatus and the yellow-coloured oil was obtained in the yield of 0.20% (w/w). Forty five compounds consisting up to 95.46% of the essential oil were identified by GC and GC/MS analysis (Table 1). The plant essential oil was consisted mostly of oxygenated monoterpenes and sesquiterpenes. Linalool (28.55%), α-terpineol (9.45%), spathulenol (8.37%) and (2*E*)-hexenal (4.62%) were the major components representing 50.99% of the total oil.

Linalool, the most abundant component of this oil, has also been reported by Norouzi-Arasi, Yavari, Kia-Rostami, Jabbari, and Ghasvari-Jahromi (2006) in the oil of this plant and also in the oil of other *stachys* species such as *S. iberica* growing in Turkey (Kaya, Demirci, & Baser, 2001), *S. acerosa* (Masoudi, Jamzad, Attari, & Rustaiyan, 2003) and *S. benthamiana* (Rustaiyan, Masoudi, Ameri, Samiee, & Monfared, 2006) both from Iran at the range of 3.5–23.5%. The second major component of the oil, α-terpineol, has also been found in the oils of *S. glutinosa* from Sardinia (Giorgio, Mario, Paola, Stefania, Antonella, & Bruno, 2006) and *S. iberica* growing in Turkey (Kaya et al., 2001) in about 5.3–18.5%. Spathulenol, the third major compound present, has also been found in the oil of *S. inflata* (3.2%), *S. pilifera* (15.8%), *S. obtusicrena* (11.5%) and *S. byzantina* (16.1%), all growing in Iran (Javidnia, Miri, Moein, Kamalinejad, & Sarkarzadeh, 2006; Javidnia, Rezai, Miri, & Jafari, 2006; Khanavi et al., 2004; Sajjadi & Somae, 2004). (2*E*)-Hexenal (5.11%), a non essential oil volatile component of the plant oil, was not reported in the oil of other *stachys* species. Some of the compounds reported in high concentrations in the oil of other *stachys* species were found in much lower amounts or did not exist at all in this plant. These compounds include cedrene (Giorgio et al., 2006), hexadecanoic acid, methyl linoleate and carvacrol (Khanavi et al., 2004), chrysanthenyl acetate (Javidnia, Miri, et al., 2006; Javidnia, Rezai, et al., 2006; Masoudi et al., 2003), α-pinene, β-phellandrene, germacrene D, bicyclogermacrene and 1,8-cineol (Meshkatalsadat, Sarabi, Moharramipour, Akbari, & Pirae, 2007; Morteza-Semnani, Akbarzadeh, & Changizi, 2006; Norouzi-Arasi et al., 2006; Rustaiyan et al., 2006; Sajjadi & Somae, 2004). It would also be noteworthy to point out that the constituents of the plants essential oils are normally influenced by several factors such as geographical, climatic, seasonal and experimental conditions.

Table 1
Chemical composition of the essential oil of *S. inflata*.

No	Compound[a]	Composition (%)	RI[b]	RI[c]
1	2-Furancarboxaldehyde	0.51	840	836
2	(2*E*)-Hexanal	4.62	850	855
3	*n*-Hexanol	1.12	866	871
4	Isocitrnelene	1.69	912	924
5	α-Pinene	0.34	932	939
6	1-Octene-3-ol	0.37	979	979
7	Dehydro-1,8-cineole	0.24	990	991
8	1,8-Cineole	1.24	1029	1031
9	Propyl tiglate	1.52	1340	1038
10	*trans*-Linalool oxide	0.99	1071	1073
11	*cis*-Linalool oxide	0.96	1088	1087
12	Linalool	28.55	1100	1097
13	*trans*-*para*-Menth-2-en-1-ol	0.83	1130	1141
14	*trans*-Pinocarveol	1.03	1137	1139
15	*cis*-Verbenol	1.36	1143	1141
16	Isopulegol	0.48	1150	1150
17	*delta*-Terpinenol	0.96	1165	1166
18	Terpinen-4-ol	3.22	1176	1177
19	*para*-Cymen-8-ol	1.11	1185	1183
20	α-Terpineol	9.45	1183	1189
21	Dihydrocarveol	1.63	1195	1194
22	Verbenone	0.74	1208	1205
23	*trans*-Polegol	0.52	1215	1215
24	*trans*-Carveol	0.41	1219	1217
25	*trans*-Chrysanthenyl acetate	0.59	1223	1238
26	Pulegone	0.42	1238	1237
27	*trans*-Geraniol	1.09	1258	1253
28	Carvacrol	2.27	1303	1299
29	Eugenol	4.00	1358	1359
30	*cis*-Muurola-4(14),5-diene	0.16	1470	1467
31	Germacrene D	0.57	1476	1485
32	γ-Cadinene	0.27	1509	1514
33	*delta*-Cadinene	0.46	1520	1523
34	Laciniata furanone F	1.06	1524	1533
35	Spathulenol	8.37	1573	1578
36	Globulol	1.20	1579	1585
37	Viridiflorol	1.31	1587	1593
38	Caryophylene oxide	0.43	1604	1583
39	*cis*-Cadin-4-en-7-ol	0.44	1625	1637
40	*epi*-α-Muurolol	0.83	1639	1642
41	α-Cadinol	3.32	1651	1654
42	Valeranone	0.56	1668	1675
43	Khusinol	3.10	1683	1680
44	*trans*-β-Santalol	0.26	1727	1716
45	*n*-Hexadecanol	0.86	1860	1876
Total		95.46		

[a] Compounds listed in order of elution from HP-5MS column.
[b] Relative retention indices to C_8–C_{24} *n*-alkanes on HP-5MS column.
[c] Literature retention indices.

3.2. Amount of total phenolic constituents

Total phenolic content of the plant extracts were determined using a colorimetric assay method based on Folin–Ciocalteu reagent reduction. Results, expressed as gallic acid equivalents, were 54.08 ± 1.62 μg/mg (5.4%, w/w) and 28.08 ± 0.87 μg/mg (2.8%, w/w) for polar and nonpolar subfractions of the plant extract, respectively.

3.3. Antioxidant activity

Essential oil, its major components (linalool, α-terpineol) and methanol extract subfractions of *S. inflata* were subjected to screening for their possible antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene/linoleic acid assay methods. DPPH is a stable free radical which can readily experience reduction in the presence of an antioxidant. It shows a maximum ultraviolet and visible (UV–Vis) absorbance at 517 nm. The reduction in the intensity of absorption at 517 nm of methanol solutions of DPPH radical in the presence of antioxidants is usually taken as a measure of their antioxidant activity. In this study, the ability of samples to scavenge DPPH radical was determined on the bases of their concentrations providing 50% inhibition (IC_{50}). Plant essential oil, its major components (linalool, α-terpineol), extract subfractions and positive control (BHT) IC_{50} values are given in Table 2. Polar subfraction of the plant extract showed the best radical scavenging activity with an IC_{50} value of 89.50 ± 0.65 μg/ml, about 22% of the potency of synthetic standard BHT. Plant essential oil, its major components (linalool, α-terpineol) and the extract nonpolar subfraction did not demonstrated considerable antioxidant activities in this test.

Polar subfraction of the plant extract was showed moderate values in both DPPH radical scavenging activity and total phenolic

Table 2
Antioxidant activity of the essential oil, its major components (linalool, α-terpineol) and methanol extract subfractions of *S. inflata* and BHT in DPPH free radical scavenging activity and β-carotene/linoleic acid bleaching assay methods.

Sample	DPPH	β-Carotene/linoleic acid
	IC_{50} (μg/ml)	Inhibition (%)
Polar subfraction	89.50 ± 0.65	26.05 ± 0.32
Nonpolar subfraction	422.4 ± 2.4	77.08 ± 0.73
Essential oil	ND[a]	25.01 ± 0.21
Linalool	ND[a]	2.70 ± 0.27
α-Terpineol	ND[a]	2.47 ± 0.33
BHT	19.72 ± 0.82	87.59 ± 0.93
Negative control	NA	2.35 ± 0.46

[a] Less than 40% inhibition for the essential oil and no inhibition for linalool and α-terpineol for concentrations up to 2 mg/ml, ND (Not determined), NA (Not applicable).

content tests. On the other hand, plant nonpolar subfraction activity in DPPH assay was about 20% of the polar one while its total phenolic content value was about half of that of polar subfraction. The relations reported between these two tests in the literature are somewhat confusing. Some investigators have proposed close correlations between antioxidant activity and phenolic compounds content of the extracts obtained from various natural sources (Liu et al., 2007; Verzelloni, Tagliazucchi, & Conte, 2007), while others did not correlate them merely to each other and contributed a wide range of compounds such as phenolics, peptides, organic acids and other components in antioxidant activity (Choi et al., 2002; Gallardo, Jimenez, & Garcia-Conesa, 2006; Meda, Lamien, Romito, Millogo, & Nacoulma, 2005).

Various results were obtained from radical scavenging activity evaluation of other *Stachys* species. Methanol extracts of seven *Stachys* taxa from Croatia showed considerable DPPH radical scavenging activity with IC_{50} values between 1.96 and 16.10 μg/ml, while their dichloromethane extracts were substantially weaker in this test with IC_{50} values in the range of 112 to 680 μg/ml (Vundac, Brantner, & Plazibat, 2007). Also, high anti-DPPH activities were reported for some *Stachys* taxa from Serbia (Kukic, Petrovic, & Niketic, 2006) and *S. byzantina* growing in Turkey (Erdemoglu, Turan, Cakici, Sener, & Aydin, 2006).

Literature review shows the presence of different hydroxyl containing and phenolic compounds such as polyphenols, tannins, phenolic acids, flavonoids, iridoids, loganic acid and phenylethanoid glycosides in the plants of the genus *Stachys* (EL-Ansari et al., 1991; Meremeti, Karioti, Skaltsa, Heilmann, & Sticher, 2004; Miyase et al., 1996; Nishimura et al., 1991; Vundac et al., 2007; Zinchenko, 1970). These compounds are commonly water soluble and the presence of them in the polar subfraction of *S. inflata* extract may be the main cause of its considerable radical-scavenging activity.

In β-carotene/linoleic acid test, the antioxidant transfers hydrogen atom(s) to the peroxyl ($R_1R_2CHOO^{\cdot}$) radicals formed from the oxidation of linoleic acid and converts them to hydroperoxides (R_1R_2CHOOH) leaving β-carotene molecules intact (Huang et al., 2005). Percent inhibition of linoleic acid oxidation of the essential oil and extracts of *S. inflata* are listed in Table 2. The effectiveness of the extract nonpolar subfraction (77.08%) was comparable to that of BHT (87.59%), but the inhibition values observed for the essential oil, its major components (linalool and α-terpineol) and polar subfraction of the extract were significantly lower than that of BHT.

Compounds containing hydrogen atoms in the allylic and/or benzylic positions may show better activity in this test because of relatively easy abstraction of hydrogen atom from these functional groups by peroxy radicals formed in the test circumstances (Larson, 1997). Occurrence of the compounds with allylic and/or benzylic hydrogens such as terpenoids and steroids were also reported in the *stachys* genus plants (Khanavi, Sharifzadeh, Hadjiakhoondi, & Shafiee, 2005; Piozzi, Paternostro, Servettaz, & Arnold, 2002; Piozzi et al., 1980; Ross & Zinchenko, 1975; Yamamoto et al., 1994). These compounds are commonly water insoluble and their presence in the nonpolar subfraction of *S. inflata* extract may be the main cause of its considerable antioxidant activity in this test.

Literature is poor about the antioxidant activity of linalool and α-terpineol as major components of the plant essential oil. Only two completely opposing reports have been appeared recently for linalool (Hussain, Anwar, Hussain Sherazi, & Przybylski, 2008; Tepe, Daferera, Tepe, Polissiou, & Sokmen, 2007) in both DPPH and β-carotene/linoleic acid tests and our results confirm the first one. Thus, week antioxidant activities observed for the plant essential oil (less than 40% and about 25% inhibitions in DPPH and β-carotene/linoleic acid tests, respectively) should be attributed to the remaining chemical components of the plant essential oil.

3.4. Antimicrobial activity

The antimicrobial activity of *S. inflata* essential oil and its major components (linalool, α-terpineol) and methanol extract subfractions were evaluated against a set of 11 microorganisms and their potency were assessed qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters and MIC values. The results are given in Table 3 and indicate that, at tested

Table 3
Antimicrobial activity of the essential oil, its major components (linalool, α-terpineol) and methanol extracts subfractions of *S. inflata*.

Test microorganisms	Essential oil		Linalool		α-Terpineol		Extracts				Antibiotics					
							$CHCl_3$		H_2O		Rifampin		Gentamicin		Nystatin	
	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
P. aeruginosa	–	–	30	62.5	35	15.6	–	–	–	–	–	–	23	500	NA	NA
B. subtilis	–	–	34	125	37	125	–	–	–	–	13	15.6	21	500	NA	NA
E. coli	–	–	27	125	32	500	–	–	11	500	11	500	20	500	NA	NA
S. aureus	–	–	35	500	31	500	–	–	–	–	10	250	21	500	NA	NA
K. pneumoniae	–	–	40	500	41	250	–	–	–	–	7	250	22	250	NA	NA
S. epidermidis	–	–	32	500	38	250	–	–	–	–	40	250	35	500	NA	NA
S. dysenteriae	–	–	35	500	37	250	–	–	12	7.8	8	250	18	500	NA	NA
P. vulgaris	–	–	33	500	29	500	–	–	–	–	10	125	23	500	NA	NA
S. paratyphi-A serotype	–	–	30	500	35	250	–	–	–	–	–	–	21	500	NA	NA
C. albicans	–	–	40	125	40	500	12	250	–	–	NA	NA	NA	NA	33	125
A. niger	–	–	25	500	30	250	–	–	–	–	NA	NA	NA	NA	27	31.2

A dash (–) indicate no antimicrobial activity.
[a] DD (Disk diffusion method), Inhibition zones in diameter (mm) around the impregnated disks.
[b] MIC (Minimal Inhibition concentrations as μg/ml), NA (Not applicable).

concentrations, the plant has no considerable antimicrobial activity against tested microorganisms. However, the polar subfraction of the plant methanol extract showed week antibacterial activities against *E. coli* and *S. dysenteriae* and the nonpolar extract subfraction exhibited low anticandidal activity. Saeedi, Morteza-semnani, Mahdavi, and Rahmani (2008) have also reported similar antimicrobial activities for this plant. Linalool and α-terpineol as major compounds of the plant essential oil were also tested for comparison and, in contrast to the oil itself, resulted in considerable antimicrobial activities. Our results about the antimicrobial activity of linalool and α-terpineol are similar to other reports about these compounds (Carson & Riley, 1995; Hussain et al., 2008; Vagionas, Graikou, Ngassapa, Runyoro, & Chinou, 2007). Inconsiderable antimicrobial activity of the whole essential oil of the plant despite the activity of its major components suggests a possible antagonistic relationship between these major components and the rest of oil components.

4. Conclusions

Plant secondary metabolites generally display remarkable biological activities such as antioxidant and antimicrobial properties which are useful for preserving foods from decay and contamination and/or preventing living tissues from various diseases. According to literature data, this is the first study on the antioxidant activity of the essential oil and extracts of *S. inflata* indicating good to moderate antioxidant activity for the plant. These results encourage complementary and more in-depth studies on the chemical composition of the plant extracts with the aim of separation and structure elucidation of their active components and evaluation of biological activity of each compound separately.

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