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Composition, antifungal and antioxidant properties of *Hyssopus officinalis* L. subsp. *pilifer* (Pant.) Murb. essential oil and deodorized extracts

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ABSTRACT

The aim of the study was to investigate the chemical composition of *Hyssopus officinalis* L. subsp. *pilifer* (Pant.) Murb. essential oil (EO) and deodorized extracts (DE) and to evaluate their potential antifungal and antioxidant activities. EO was analyzed by GC–FID and GC–MS. Among the 30 compounds identified in the oil, the main were 1.8-cineole (36.43%), β-pinene (19.55%), isopinocamphone (15.32%) and *trans*-pinocamphone (6.39%). Deodorized aqueous extract (DAE) was analyzed by LC-DAD/ESI-TOF MS. The most significant phenolic acids detected in DAE were syringic, caffeoylquinic acids, feruloylquinic and rosmarinic acid. *Aspergillus niger*, *A. ochraceus*, *A. versicolor*, *A. fumigatus*, *Cladosporium cladosporioides*, *C. fulvum*, *Penicillium funiculosum*, *P. ochrochloron*, *Trichoderma viride* and *Candida albicans* yeast were used for antifungal assay. Minimum inhibitory concentrations (MIC_s) and minimum fungicidal concentrations (MFC_s) of the tested EO and DEs were recorded by the microdilution method. The antioxidant potential of the extracts was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method. The deodorized aqueous extract (DAE) possessed the highest scavenging activity (EC_{50} = 0.54 mg/ml) followed by the deodorized methanol extract (DME) (EC_{50} = 0.82 mg/ml) and deodorized ethyl acetate extract (DEE) (EC_{50} = 2.97 mg/ml). EO possessed the lowest activities compared to other extracts and control substances. Total phenolic content of DEs was determined by Folin–Ciocalteau (FC) assay and gallic acid was used as the standard. DAE of *H. officinalis* subsp. *pilifer* exhibited the highest phenolic content 96.47 GAE.

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1. Introduction

Hyssopus officinalis L. (Lamiaceae), commonly known as hyssop, is a widespread species in the Mediterranean region. *H. officinalis* L. subsp. *pilifer* (Pant.) Murb. (=*H. officinalis* L. subsp. *aristatus* (Godr.) Nyman) (Govaerts, 2003) is wild growing taxon in Eastern Serbia. This is a perennial herb, growing in sunny habitats, mainly in depleted pastures on limestone (Diklić, 1974). The range of this subspecies extends from Bulgaria and N. Greece, north-westwards to Croatia (Čarneva, 1997). Hyssop is commonly used as fragrance. As a food ingredient, it is used for its flavor and in sauce formulations (Kazazi et al., 2007). Despite having a slightly bitter taste, *H. officinalis* is often used as a minty flavor and condiment in the food industry (Fathiazad and Hamedeyazdan, 2011). For pharmaceutical purposes, this plant is used as an antiseptic, stomachic, against chronic bronchitis and for asthma treatment (Tucakov, 1986). The plant is also applied in the treatment of rheumatic pains, bruises, wounds, blood pressure regulation, states of anxiety and hysteria (Lawles, 2001) and has a muscle-relaxing activity (Lu et al., 2002). The EO obtained by steam distillation of top shoots in flowers contains pinocamphone (42.66–46.70%) and isopinocamphone (30.88%), α-pinene (0.74–7.30%), β-pinene (5.30–8.80%) and myrtenil-methyl (3.97%) as dominant compounds (Jančić et al., 1995). Recently, some authors (De Martino et al., 2009; Kizil et al., 2010) demonstrated the antimicrobial activity of hyssop EO, and the antifungal effect of hyssop oil was also confirmed (Letessier et al., 2001; Fraternale et al., 2004; Moro et al., 2013). Previous study suggested that, in addition to the oil, hyssop extracts also inhibit lipid oxidation and degradation of heme pigments caused by cooking and storage, and may be a useful additive for meat

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processing to prevent lipid oxidation and discoloration (Fernandez-Lopez et al., 2003).

It was recently published that ethanolic extract from *H. officinalis* possesses gastro protective properties estimated by gastric adhesion mucus content and has considerable antiulcer and antioxidant potential demonstrated by decreased generation of ROS (Saini and Sharma, 2012). However, Dapkevicius et al. (1998) found that the antioxidant activity of hyssop DE was lower than that of rosmary, thyme, majoran and sage. Also, in an earlier report, Djarmati et al. (1991) focused on isolating rosmanol-9-ethyl ether from an alcoholic extract of the hyssop, which was characterized as a potent antioxidant compound.

This study reports on the composition, antifungal and antioxidant activities of EO and DEs obtained from the subspecies *pilifer* (*H. officinalis* ssp. *pilifer*) wild growing in eastern Serbia. The molds tested were soil-borne pathogens, food storage spoilage fungi, mycotoxin producers, as well as plant, fungi, animal and human pathogens.

2. Materials and methods

2.1. Plant material

The aerial parts of wild-growing plants were collected in Sicevo gorge (East Serbia), coordinates N 43°16′49.1″, E 022°13′23.5″, altitude 281 m, in July 2008. The plant was identified as *Hyssopus officinalis* L. subsp. *pilifer* (Pant.) Murb. by P.D. Marin. Voucher specimens where deposited in the Herbarium of the Institute of Botany and "Jevremovac" Botanical Garden (16701 BEOU).

2.2. Chemical reagents

Organic solvents were purchased from "Zorka pharma" Serbia. 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT) and 2,2-dyphenyl-1-picrylhydrazyl (DPPH), gallic, syringic, chlorogenic and rosmarinic acids were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin–Ciocalteu reagent was purchased from Merck, Darmstadt, Germany. Sodium carbonate anhydrous (Na_2CO_3) was obtained from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven, Belgium.

2.3. Essential oil isolation

The air-dried plant material of *Hyssopus officinalis* subsp. *pilifer* (200 g) was subjected to 3 h of hydrodistillation using a Clevenger type apparatus (European Pharmacopoeia, 2004). The oil was preserved in sealed vials at 4 °C prior to further analysis. The oil yield was 0.6% (w/w-dry bases).

2.4. Extract preparation

DEs were obtained after completing a distillation process using a modified extraction method (Dapkevicius et al., 1998; Tepe et al., 2005). After the removal of EO, the water waste was filtrated, frozen and stored for later lyophilization. Aqueous extract was lyophilized to produce the crude dry extract (DAE). The residual dried plant material (10 g) was milled and then extracted with methanol or ethyl acetate in the dark for 24 h. Ultrasonic bath was used during the first and the last hour of extraction. Afterwards, the filtrated extracts were evaporated by a vacuum evaporator to produce the crude dry methanol (DEM) and ethyl acetate extract (DEE).

2.5. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

Qualitative and quantitative analyses of the EO were performed using GC and GC–MS. The GC analysis of the oil was carried out on a GC HP-5890 II apparatus, equipped with a split–splitless injector, attached to a HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to FID. Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while the column temperature was linearly programmed from 40 to 240 °C (at rate of 4°/min). The same analytical conditions were employed for GC–MS analysis, where HP G 1800C Series II GCD system, equipped with a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The transfer line was heated to 260 °C. The mass spectra were acquired in EI mode (70 eV), in *m*/*z* range 40–400. Identification of individual EO components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with those held in Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software and literature (Adams, 2007). Area percentages obtained by FID were used as a base for the purpose of quantitative analysis.

2.6. HPLC–DAD–ESI–TOF-MS analyses of antioxidant fractions

The sample was dissolved in methanol to an approximate concentration of 5 mg/ml. The LC/DAD/MS analyses were carried out by an Agilent 1200 HPLC instrument (Agilent Technologies, Waldbronn, Germany) with a binary pump, an autosampler, a column compartment equipped with a Zorbax Eclipse Plus C18 column (1.8 μm, 4.6 mm × 150 mm, Agilent Technologies) and a diode-array detector coupled with a 6210 time-of-flight LC–MS system (Agilent Technologies). The mobile phase consisted of water containing 0.2% formic acid (A) and acetonitrile (B). A gradient program was used as follows: 0–1.5 min 5% B, 1.5–26 min, 5–95% B, 26–35 min, 95% B. The mobile phase flow rate was 1.4 ml/min, the column temperature was 40 °C and the injection volume was 5 μl. Spectral data from all the peaks was accumulated in the range of 190–450 nm and chromatograms were recorded at 280 nm. MS data was collected by applying the following parameters: ionization, negative ESI capillary voltage 4000 V, gas temperature 350 °C, drying gas 12 l/min, nebulizer pressure 45 psi, fragmentor voltage 140 V, mass range 100–2000 *m*/*z*. A personal computer system running MassHunter Workstation software was used for data acquisition and processing. Phenolic compounds were detected as $[M-H]^-$ or $[2M-H]^-$ signals using these parameters. Compounds were characterized by their retention times (t_r), mass spectra and UV spectra, and were tentatively identified based on previous data published by other authors. Their complete identification was not possible since the full scan mass spectra of the chromatographically separated compounds gave only deprotonated $[M-H]^-$ ions, and MS/MS experiments were not possible with the instrumentation used.

2.7. Antifungal activity tests

The fungi used in this study were: *Aspergillus niger* (ATCC 6275), *A. ochraceus* (ATCC 12066), *A. versicolor* (ATCC 11730), *A. fumigatus* (ATCC 9197), *Cladosporium cladosporioides* (ATCC 13276), *C. fulvum* (TK 5318), *Penicillium funiculosum* (ATCC 10509), *P. ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061) and *Candida albicans* yeast. The molds were obtained from the Mycotheca of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade. The fungi were maintained on malt agar (MA) (Booth, 1971). The cultures were stored at 4 °C and subcultured once a month.

2.8. Antifungal assay

Modified microdilution technique was used to investigate the antifungal activity of EO and DAE, DME, DEE extracts (Hanel and

Raether, 1988; Daouk et al., 1995; Espinel-Ingrof, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). Spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^6 in a final volume of 100 μl per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. The minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtitre plates. The investigated EO was dissolved in malt broth with fungal inoculum in a concentration of 10.44–104.40 mg/ml. The extracts (DAE, DME, DEE) were dissolved in a 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/ml) and added in broth Malt medium with inoculum (4–16 mg/ml). The lowest concentrations without visible growth (under a binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μl of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 μl of broth per well and further incubation for 72 h at 28 C. The lowest concentration with no visible growth was defined as MFC indicating a 99.5% killing of the original inoculum. DMSO (5%) was used as a negative control, and commercial fungicide, bifonazole (Srbolek, Belgrade, Serbia) was used as a positive control (1–2500 μg/ml).

2.9. Antioxidant activity

2.9.1. DPPH assay

The antioxidant activity of EO was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method. This spectrophotometric assay uses stable DPPH radical as reagent (Blos, 1958). The methanolic solution of the investigated EO (200 μl) (with starting concentrations of 200, 300, 400, 500 μl/ml of solution) was added to a 1800 μl methanolic solution of DPPH radical (concentration of 0.04 mg/ml) and after shaking, the reaction mixture was left to react in the dark for 30 min at room temperature. The absorbance of the remaining DPPH radical was measured at 517 nm after that time (A_1) on an Agilent GBC Cintra 40 UV–Visible spectrophotometer. Every concentration was done in triplicate and the same was done with Trolox and BHT, known antioxidants. The same procedure was used for extracts. Blank probes were done in the same way, using methanol instead of the investigated solution (A_0). The decrease in the absorption of DPPH solution is calculated by the following equation:

$$I(\%) = \frac{(A_0 - A_1) \times 100}{A_0}$$

Concentrations which reduce the absorption of DPPH solution by 50% (EC_{50}) were obtained from the curve dependence of absorption of DPPH solution on 517 nm from the concentration for each extract and standard antioxidant. Origin 7.0 software was used to calculate these values. Tests were carried out in triplicate.

2.9.2. Total phenol content

Total phenol content of the plant extracts was determined as described (Singleton et al., 1999) with Folin–Ciocalteau reagent and gallic acid was used as a standard. A volume of 200 μl of diluted extract was mixed with 1000 μl of FC-reagent diluted with distilled water in a 1:10 ratio. After spending 6 min in the dark, 800 μl of sodium carbonate solution (7.5%) was added. Absorbance at 740 nm was measured after shaking and spending an additional 2 h in the dark. Distilled water was used as blank. All samples were measured in triplicate. Four dilutions of each of the extracts were used and the results were averaged. Appropriate dilutions of each sample were experimentally found to give an absorbance between 0.2 and 0.7 at 740 nm. The same procedure was used for four concentrations of gallic acid standards (10, 25, 50 and 100 mg/l) and a calibration curve was calculated. The total phenolic content was expressed in gallic acid equivalents (GAE), as concentration of gallic acid in mg/l that corresponds to the dilution of extracts with the same value of absorbance at 740 nm.

Table 1
Composition of *Hyssopus officinalis* subsp. *pilifer* essential oil.

Constituents	KIE	KIL	%
α-Thujene	929.4	924	0.22
α-Pinene	934.9	932	2.57
Camphene	949.6	946	0.10
Sabinene	975.4	969	2.90
β-Pinene	977.7	974	**19.55**
Myrcene	996.0	988	1.95
α-Terpinene	1019.7	1014	0.35
p-Cymene	1028.0	1020	0.11
α-Phellandrene	1031.1	1025	3.74
1,8-Cineole	1033.2	1026	**36.43**
trans-β-Ocimene	1053.3	1044	1.03
γ-Terpinene	1062.0	1054	0.63
α-Terpinolene	1092.0	1086	0.36
Linalool	1107.0	1095	0.28
trans-Pinocarveol	1141.1	1135	0.44
n.i. (*M* = 166)[a]	1159.6	–	1.20
Pinocamphone	1163.7	1158	6.39
Isopinocamphone	1177.1	1172	**15.32**
Terpinen-4-ol	1180.6	1174	0.33
Myrtenal	1199.8	1195	0.59
β-Bourbonene	1388.4	1387	0.55
β-Caryophyllene	1422.9	1417	1.09
β-Copaene	1431.4	1430	0.06
Aromadendrene	1446.4	1439	0.04
α-Humulene	1457.7	1452	0.24
allo-Aromadendrene	1463.8	1458	0.10
Germacrene D	1483.1	1484	1.65
Bicyclogermacrene	1498.4	1500	0.81
Spathulenol	1580.6	1577	0.37
Caryophyllene oxide	1584.9	1582	0.43
Isospathulenol	1630.8	1625	0.07
Grouped constituents			
a. Monoterpene hydrocarbons			33.51
b. Oxygenated monoterpenes			59.78
c. Sesquiterpene hydrocarbons			4.54
d. Oxygenated sesquiterpenes			0.87
Sum of contents			**98.73**

[a] n.i., not identified.

3. Results and discussion

3.1. Chemical composition of the EO

The results of the chemical analysis of *H. officinalis* subsp. *pilifer* EO are listed in Table 1. GC–FID and GC–MS analysis of hyssop EO revealed thirty constituents categorized as oxygenated monoterpenes (59.78%), monoterpene hydrocarbons (33.51%), sesquiterpene hydrocarbons (4.54%) and oxygenated sesquiterpenes (0.87%) (Table 1). The preponderance of the oxygenated monoterpenes is obvious in the oil composition as reflected in its major constituents, 1.8-cineole (36.43%), isopinocamphone (15.32%) and pinocamphone (6.39%), while β-pinene (19.55%) was the only prominent monoterpene hydrocarbon. In commercial hyssop samples, isopinocamphone was mostly the dominant component in the oil, followed by pinocamphone, pinocarvone and 1.8-cineole, compounds connected with biosynthetical pathways (Lawrence, 1992; Soković et al., 2008; De Martino et al., 2009; Kizil et al., 2010). Isopinocamphone (43.3%) and limonene (12.2%) were dominant in the oil from Italy (Mazzanti et al., 1998), pinocarvone in *H. officinalis* oil from Turkey (Ozer et al., 2005), while thymol (18.95%), β-bisabolol (16.62%) and carvacrol characterized the EO from Iran (Dehghanzadeh et al., 2012). Cultivated hyssop EO from

Serbia contains isopinocamphone (44.7%) and pinocarvone (14.1%) (Mitić and Djordjević, 2000). Hyssop samples from India were rich in isopinocamphone (38.1%), pinocarvone (20.3%), 1.8-cineole (12.2%) and β-pinene (10.2%) (Jankovski and Landa, 2002). The EO of *H. officinalis* subsp. *aristatus* (=*H. officinalis* subsp. *pilifer*) from Bulgaria was characterized by the presence of 1.8-cineole (48.2 and 39.6%), isopinocamphone (16.3 and 28%) and β-pinene (11.4 and 9.4%) (Jankovski and Landa, 2002). Methyl eugenol (38.3%), limonene (37.4%) and β-pinene (9.6%) were major constituents in *H. officinalis* subsp. *aristatus* EO from Montenegro (Gorunović et al., 1995). In comparison with our sample, high similarity was evident with EOs from Bulgaria, which is geographically close to East Serbia, the place where the plants were collected. These data confirm the fact that EO composition can vary between harvesting seasons, extraction methods, geographical sources and soil. EOs from different parts of the same plant can also differ (Burt, 2004).

3.2. Chemical composition of the deodorized extract

In this study, deodorized aqueous extract (DAE) was analyzed by HPLC–DAD–ESI–TOF-MS analyses. A total of 9 phenolic acids were characterized in the DAE. Three of them were unambiguously identified by comparing retention times (t_r), UV and MS data with those of the reference standards. The possible structures of another 6 peaks in the chromatogram were tentatively characterized on the basis of multiple reaction mode (MRM), transition mass spectra and literature data. The HPLC–DAD chromatograms and total ion chromatograms (TIC) in the negative mode of the deodorised aqueous extract (DAE) of the investigated species are shown in Fig. 1.

Peak **1** at retention time 3.782 shows molecular ions at *m*/*z* 197.0482 $[M-H]^-$ and 395.1 $[2M-H]^-$ (Fig. 1). This compound was identified as syringic acid by comparing the retention time and the mass spectra with an authentic standard. Three isomers were detected in the full scan experiment showing molecular ion *m*/*z* 353 at the retention times *ca* 4.602, 5.542 and 5.727 min (Fig. 2). These isomers were identified as 3-caffeoylquinic (**2**), 5-caffeoylquinic (**3**) and 4-caffeoylquinic acid (**4**), respectively. The major peak **3**, is identified as 5-caffeoylquinic acid according to the retention time as compared with an authentic standard. 4-Caffeoylquinic acid can be differentiated from 5-caffeoylquinic acid isomer by its MS^2 fragmentation. 4-Caffeoylquinic acid gives an MS^2 base peak at *m*/*z* 173 due to the loss of caffeic acid and a water molecule, while 3-caffeoylquinic acid gave MS^2 at *m*/*z* 191 (deprotonated quinic acid) as a result of the loss of a caffeic acid unit (Table 2) (Clifford et al., 2003).

Two peaks (**5**, **7**) were found in the TIC, detected by the negative ion ESI-MS scan mode, and UV at 326 nm shows molecular ions at *m*/*z* 3,671,055 $[M-H]^-$ and 7,352,120 $[M-H]^-$ (Fig. 1). Based on the MS^2 base peak *m*/*z* 193.3, a peak (**5**) and *m*/*z* 173.3, a peak (**7**) were identified as 4-*O* feruloylquinic acid and 3-*O*-feruloylquinic acid by MRM spectral data and by comparing the UV and MS spectra data with those reported in literature (Clifford et al., 2003).

The presence of a peak **8** at retention time 7.64 with transition *m*/*z* 311 → 193 could be assigned to 1-(3-methylbenzoate) β-D-glucopyranuronic acid. Rosmarinic acid (**9**) was detected at retention time 9.04 and identification was performed by comparing retention time and characteristic MS spectroscopic data with those of an authentic standard. Also, 5-*O*-*p*-hydroxybenzoylquinic acid was tentatively identified using *m*/*z* ions 311, 191 (peak **6**).

These results are consistent with those described by Zgórka and Głowniak (2001) who found chlorogenic, protocatechuic, ferulic, syringic, *p*-hydroxybenzoic and caffeic acids followed by vanillic, *p*-coumaric, rosmarinic and gentisic acids in a hyssop methanolic extract. Proestos et al. (2005) believed the most abundant phenolic acids in *H. officinalis* to be ferulic and caffeic acids, followed by syringic, gentisic and *p*-hydroxybenzoic acids, along with two flavonoids (+)-catechin and apigenin also detected in the genus *H. officinalis*.

Acording to Hossain et al. (2010), aqueous methanol extracts obtained from rosemary, oregano, sage, basil and thyme using LC–ESI-MS/MS contained major categories, namely hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives, flavonoids and phenolic terpenes. Rosmarinic acid was found to be the dominant phenolic acid in sage, rosemary, oregano, mint, marjoram, and thyme (Lu and Foo, 2002; Fecka and Turek, 2008; Dorman et al., 2004; Kivilompolo et al., 2007; Lee and Scagel, 2009; Baj et al., 2011). Also, *Salvia* radix (Hu et al., 2005) and aqueous extracts from *Orthosiphon grandiflorus* (Nuengchamnong et al., 2011) mainly contain caffeic acid derivates. Apigenin, quercetin, diosmin, luteolin and their glycosides were the major flavonoids followed by phenolic, chlorogenic, protocatechuic, ferulic, syringic, *p*-hydroxybenzoic and caffeic acids in the hydromethanolic extract of the aerial parts of *Hyssopus officinalis* (Fathiazad et al., 2011; Babović et al., 2010).

3.3. Antifungal activity

Antifungal activity of EO and DEs was evaluated against ten micromycetes by the microdilution method (Table 3). *A. niger* was the most resistant fungus, so the concentration of *H. officinalis* oil had to be increased to 104 mg/ml to inhibit its growth. *Cladosporium* species proved the most sensitive, where inhibition was achieved at 4 mg/ml and fungicidal concentration at 6 mg/ml. All tested extracts showed activity in the range of 4–16 mg/ml. DAE was efficient in the range from 7 to 16 mg/ml. For DME, MICs ranged from 4 to 10 mg/ml and MFC (6–14 mg/ml). DEE was active in the range between 7 and 14 mg/ml. We could note that deodorized methanol extract possessed better antifungal activity than aqueous and ethyl acetate extracts. The commercial drug bifonazole was active in the concentration of 0.15–0.2 mg/ml, which was lower than the tested EO and DEs. Generally, if MICs were used like the MFCs obtained by the microdilution method, than concentrations which stopped fungal growth, would also kill fungi. An earlier report showed that *H. officinalis* EO affects *A. niger* growth, causing protein and uronic acid reduction and increasing the percentage of lipids and amino sugars in *A. niger* walls (Ghfir et al., 1997). According to the results given by Mazzanti et al. (1998), the growth of *Candida* species was inhibited by *H. officinalis* EO originating from Italy, but generally, in all antimicrobial tests *in vitro* the EO of *H. officinalis* var. *decumbens* was more effective. Glamočlija et al. (2005) tested hyssop oil as an antifungal agent, using the microatmosphere method. The EO characterized by isopinocamphone (43%), pinocamphone (16.79%) and β-pinene (16.31%) exhibited moderate antifungal activity. Also, mycelial growth of plant pathogenic fungi *Pyrenophora avenae* and *Pyricularia oryzae* was completely inhibited by 0.4% hyssop oil, as well as by individual components of the oil, L-bornyl acetate, isopinocampheol and pinocamphone (Letessier et al., 2001). Weak antimicrobial activity with bacteriostatic effects of the hyssop oil was reported by Marino et al. (2001), which contradicts the results of Mazzanti et al. (1998) where EO was effective, especially *H. officinalis* var. *decumbens*. Furthermore, Renzini et al. (1999) reported antibacterial and cytotoxic activity of *H. officinalis* and *H. officinalis* var. *decumbens* EOs where the latter one again proved more active. Also, Di Pasqua et al. (2005) found that hyssop EO was effective only against three bacterial strains in concentrations above 1%. De Martino et al. (2009) published that hyssop oil, using the disk diffusion method, was much more efficient against micromycetes than against bacteria. Letessier et al. (2001) explained that in their assay, further increase in oil concentration *in vivo* was not possible due to cytotoxicity of the oil and this phenomenon is taken as a possible reason for the increase in plant infections: the oil weakens the cuticle allowing an easier penetration of the pathogen.

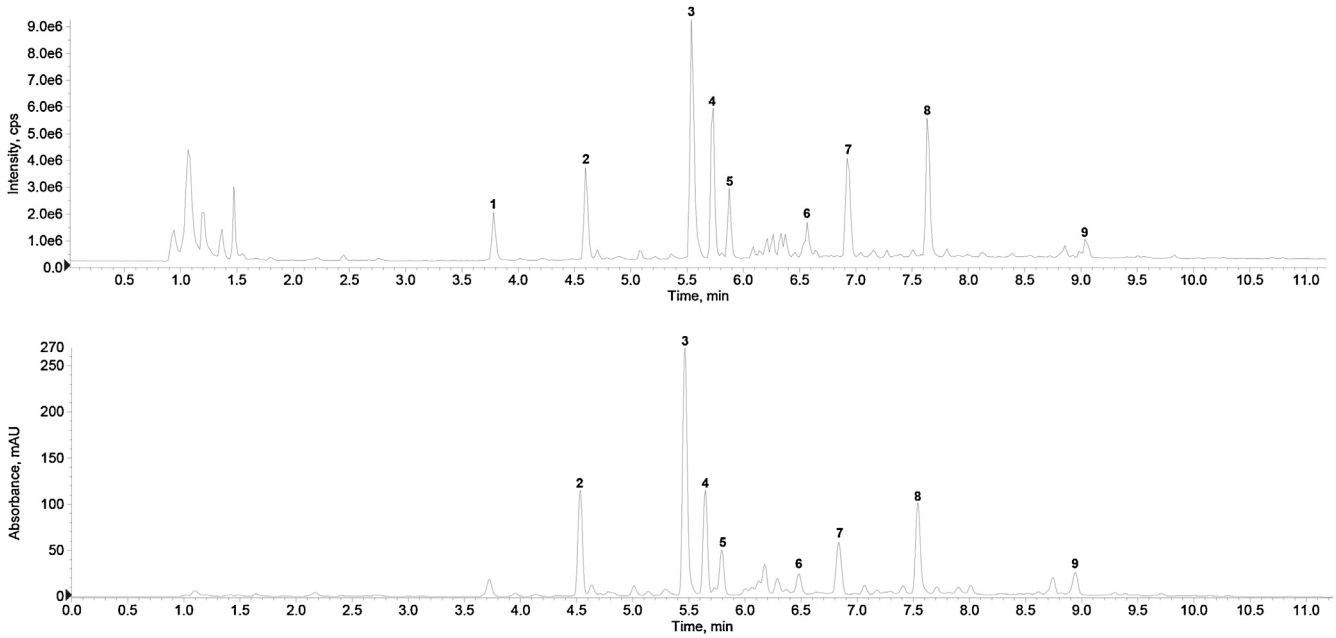

Fig. 1. Chromatographic profiles obtained for hyssop deodorized aqueous extract (DAE), ESI negative signal top, DAD signal at 280 nm bottom.

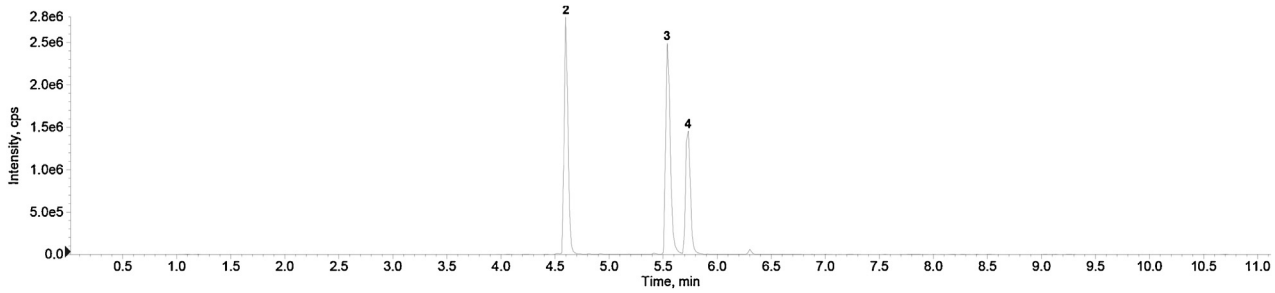

Fig. 2. Extracted ion current chromatogram of *m*/*z* 353.

Recently, it was published that the polar (methanol) extract of species *Thymus pectinatus* from the same family (Lamiaceae) did not exhibit antimicrobial activity, while the non-polar (chloroform) extract was efficient in preventing the growth of *C. albicans*, *C. krusei* and *Streptococcus pneumoniae* bacteria (Vardar-Unlu et al., 2003). Also, Tepe et al. (2005) found that water-soluble extracts had no antimicrobial activity, while extracts that are insoluble in water, displayed a moderate activity on the growth of tested bacteria and yeasts. Shinwari et al. (2009) reported a potent activity of methanol, ethanol, chloroform, diethyl-ether and aqueous crude extracts of *H. officinalis* against five respiratory bacteria.

3.4. Antioxidant activity

The antioxidant activity of EO and DE was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical assay (Table 4). The EO of the investigated species was able to reduce DPPH radicals into the DPPH-H form, and this activity was dose-dependent. The oil exhibited an antioxidant activity with EC_{50} = 156.6 mg/ml, much lower than BHT, artificial antioxidant (EC_{50} = 0.33 mg/ml). DAE (EC_{50} = 0.54 mg/ml) showed better antioxidant potential than methanol and ethyl acetate DEs (EC_{50} = 0.82 and 2.97 mg/ml), but lower than BHT. Trolox, a water-soluble analog to vitamin E, exhibited the most powerful activity, as it was expected.

Table 2
Composition of *Hyssopus officinalis* subsp. *pilifer* deodorized aqueous extract (DAE) analyzed by HPLC-DAD/ESI-ToF.

No.	t_r (min)	Acc. mass	$[M-H]^-$ $[2M-H]^-$ (*m*/*z*)	MF	λ_{max} (nm)	Compound	Identification
1	3.782	198.0528	197.0482; 395.0998	$C_9H_{10}O_5$	198, 218, 280	Syringic acid	Standard
2	4.601	354.0951	353.0902; 707.1823	$C_{16}H_{18}O_9$	220, 234sh, 300sh, 324	3-*O*-Caffeoylquinic acid	353 → 191
3	5.542	354.0951	353.0899; 707.1823	$C_{16}H_{18}O_9$	220, 240, 298sh, 326	5-*O*-Caffeoylquinic acid	Standard
4	5.727	354.3104	353.0899; 707.1833	$C_{16}H_{18}O_9$	222, 238, 296sh, 326	4-*O*-Caffeoylquinic acid	353 → 173
5	5.875	368.1107	367.1055; 735.2120	$C_{17}H_{20}O_9$	236,298sh, 324	3-*O*-Feruloylquinic acid	367 → 193
6	6.569	312.0845	311.0797; 623.1622	$C_{14}H_{16}O_8$	238, 302sh, 328	5-*O*-*p*-Hydroxybenzoylquinic acid	311 → 191
7	6.927	368.1107	367.1060	$C_{17}H_{20}O_9$	240, 300sh, 326	4-*O*-Feruloylquinic acid	367 → 173
8	7.639	312.0845	311.0800; 623.1621	$C_{14}H_{16}O_8$	240, 296, 300sh, 326	1-(3-Methylbenzoate) β-D-glucopyranuronic acid	311 → 193
9	9.038	360.0845	359.0794; 719.1607	$C_{18}H_{16}O_8$	244, 288, 328	Rosmarinic acid	Standard

t_r, retention time in minute; MF, postulated molecular formula. λ_{max}, wavelength at which maximum absorption occurs; identification for syringic, chlorogenic and rosmarinic acids were performed using standards.

Table 3
Antifungal activity of *H. officinalis* subsp. *pilifer* essential oil and extracts (DAE, DME and DEE).

Fungi	*H. officinalis* subsp. *pilifer*								Bifonazole	
	Essential oil[a]		Deodorized aqueous extract (DAE)		Deodorized methanolic extract (DME)		Deodorized ethyl acetate extract (DEE)			
	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
Aspergillus niger	52.20	104.40	10.00	16.00	7.00	10.00	10.00	14.00	0.15	0.20
Aspergillus fumigatus	–	–	10.00	14.00	6.00	7.00	10.00	12.00	0.15	0.20
Aspergillus ochraceus	26.10	52.20	10.00	16.00	6.00	10.00	9.00	12.00	0.15	0.20
Aspergillus versicolor	10.44	26.10	7.00	16.00	4.00	6.00	7.00	9.00	0.15	0.20
Cladosporium cladosporioides	10.44	26.10	7.00	7.00	4.00	6.00	7.00	9.00	0.15	0.20
Cladosporium fulvum	26.10	26.10	7.00	7.00	4.00	6.00	7.00	9.00	0.15	0.20
Penicillium funiculosum	52.20	52.20	10.00	16.00	4.00	6.00	9.00	12.00	0.20	0.25
Penicilium ochrochloron	26.10	52.20	10.00	16.00	4.00	6.00	9.00	12.00	0.15	0.20
Trichoderma viride	10.44	26.10	10.00	16.00	7.00	12.00	6.00	7.00	0.20	0.25
Candida albicans	–	–	7.00	10.00	10.00	14.00	7.00	10.00	0.15	0.20

[a] Specific weight of hyssop oil $\rho = 1.044\ g/cm^3$.

Table 4
EC_{50} values and total phenolic content of *H. officinalis* subsp. *pilifer*.

	DPPH (EC_{50}) (mg/ml)	Total phenol contents (mg GAE/l)
Essential oil	156.60	Ns[a]
Deodorized aqueous extract (DAE)	0.54	96.47
Deodorized methanolic extract (DME)	0.82	71.18
Deodorized ethyl acetate extract (DEE)	2.97	15.06
BHT	0.33	Ns[a]
Trolox	0.06	Ns[a]

[a] Not studied.

The DPPH-radical-scavenging activity decreased in the following order: trolox > BHT > DAE > DME > DEE > EO. The majority of natural antioxidants are phenolic compounds or polyphenols and the antioxidant activity of many natural extracts is due to such phenolic compounds. The tested EO was rich in oxygenated monoterpenes which are known to have low radical scavenging activity (Pizzale et al., 2000; Lopez et al., 2007).

3.5. Total phenol content

Total phenol content of the extracts was determined spectrophotometrically according to the Folin–Ciocalteu method and calculated as gallic acid equivalents (GAE). Total phenols found in the plant extracts are shown in Table 4. The total phenol content of the water, methanol and ethyl acetate extracts was 96.47 GAE, 71.18 GAE and 15.06 GAE respectively. The results indicated that the water extract has a higher total phenolic content and antioxidant ability than the methanol extract, and both are much better than the ethyl acetate extract. Soleimani et al. (2011) examined the antioxidant activity of leaf extracts obtained from Iranian *H. officinalis* using three methods: DPPH, ABTS and beta carotene bleaching tests. In DPPH test, EC_{50} value was 35.6 ± 4.7 ppm. The total phenolic content of *H. officinalis* was 200 mg/g GAE. The antioxidant activity of the extracts improved with the increase in concentration and the extract was able to reduce the oxidation rate of soybean oil under conditions of the oven test at 70 °C. Also, Ebrehimzadeh et al. (2010) noted a high total phenol and flavonoid content in crude methanolic extracts of *H. officinalis* and using that to explained their high reducing properties.

Moreover, *n*-butanol extract possessed the best antioxidant activity among the various *H. officinalis* extracts from Iran, because it had the highest content of total phenolic compounds (Fathiazad et al., 2011). High DPPH free radical activity corelated with high content of phenolic acids, such as rosmarinic acid and caffeic acid derivates. This correlation was confirmed with oregano extracts (Doroman et al., 2003; Lattanzio et al., 2009). The radical scavenging and antioxidant properties of the caffeic acid derivatives might have potential applications in food as antioxidants as well as in the healthcare industry (Nuengchamnong et al., 2011). It has been recorded that utilization of synthetic antioxidants is limited because consumers are increasingly demanding additive-free or natural products (Ahn et al., 2002).

4. Conclusion

The findings of the study revealed that essential oil and extracts from wild growing *Hyssopus officinalis* subsp. *pilifer* possess valuable biological potential. Determination of the chemical composition of essential oil, which is rich in 1.8-cineole and deodorized extracts, from which aqueous was abundant with phenolic compounds, could help to develop new drug candidate for antifungal and antioxidant activity. The results of present work indicated that antioxidant activity of deodorized aqueous extract of the analyzed hyssop subspecies was nearly as high as BHT. Tested extracts may be considered as an alternative additive in foods and cosmetic preparations instead of synthetic antioxidants. In addition, possible medicinal use of this subspecies, characterized by specific EO composition, could be of interest.

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