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ORIGINAL PAPER

Phytochemical and sensorial characterization of *Hyssopus officinalis* subsp. *aristatus* (godr.) Nyman (*Lamiaceae*) by GC–MS, HPLC–UV–DAD, spectrophotometric assays and e-nose with aid of chemometric techniques

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Abstract
The aim of this study was to evaluate the potential compositional differences among different populations of *H. officinalis* subsp. *aristatus* (Godr.) Nyman. The plant specimens were collected in different locations in Western Balkans (Kosovo and Albania) and subjected to phytochemical profiling (GC–MS for their essential oils and HPLC-UV-DAD for fingerprinting of their solvent extractable phytochemicals). Antioxidant capacity, total flavonoid and phenol contents were measured using different assays. Out of the five location considered, the specimen from one location displayed significant differences both in terms of essential oil composition and of polyphenolic total content. The electronic nose measurements used to characterize their aromatic profile, was able to clearly discriminate the accessions, indicating a good correlation, in particular, with the marked chemotypic difference established by essential oil profiling (1,8-cineol vs. isopinocamphone/camphone). *H. officinalis* subspp. *aristatus* (Godr.) Nyman may constitute an interesting subject for further studies on the effect of genetic and environmental factors, or of their combinations, on its chemotypic expression and sensorial properties.

Keywords *Hyssopus officinalis* subsp. *aristatus* · Essential oil · Volatile compounds · Chemotype · Chemometric analysis · e-Nose

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Introduction

Hyssopus officinalis L., commonly known as hyssop, is a polymorphous species belonging to the family of Lamiaceae, well known for its pleasant aromatic scent, as an ornamental and bee-attracting plant, and in food industry as a condiment and spice or as a minty flavor [1].

Since ancient times it has been used to treat rheumatic pains, bruises, wounds, states of anxiety and hysteria, and for blood pressure regulation.

The hyssop essential oil (EO) is used also as ingredient for the production of liqueurs, cosmetic products and perfumes as well as in phytotherapy, even if caution in its dosage is needed due to the presence of the epileptogenic substances pinocamphone and isopinocamphone [2].

Several reports concerning the *H. officinalis* phytochemical profile as well as the antioxidant and the antimicrobial activities of its extracts and/or EO, are available in the current literature [3].

The results of these studies are consistent with the chemotypic profile of cultivated *H. officinalis L.* populations from different areas (Poland, Spain, Turkeu, Italy, Serbia, India) of the Eurasian continent [4–10] and for *H. officinalis* subsp. *angustifolius* grown in Turkey [11], in which the monoterpenes pinocamphone/isopinocamphone are the dominant EO components, while significant differences have been observed for the subspecies *H. officinalis* subsp. *officinalis* grown in Poland [12], *H. officinalis L.* var. *decumbens* from France [10] and *H. officinalis* L. subsp. *aristatus* grown in Central Italy [13, 14].

Similar degrees of similarity/dissimilarity have been found also for the phenolic profile of *H. officinalis* L. [15–17], *H. officinalis* L. subsp. *aristatus* [13], and for *H. officinalis* L. subsp. *pilifer* (Pant.) Murb. [18], which share a composition based on chlorogenic acid and its quinic isomeric derivatives, while *H. officinalis* subsp. *angustifolius* grown in Turkey displayed the presence of a composition dominated by p-coumaric acid, benzoic acid, o-coumaric acid, ferulic acid and quercetin [11].

Hence, in the present work, we evaluated the phytochemical profile of hyssop accessions growing wild in different geographical areas between Albania and Kosovo (Western Balkans, Southeastern Europe), and identified as *H. officinalis* L. subsp. *aristatus* (Godr) Nyman.

Beside the phytochemical profile of their EO and volatile organic substances (VOCs) components determined by gas-chromatography coupled to mass spectrometry (GC–MS), the samples were subjected to the determination of (1) their volatile fingerprint by electronic nose (e-nose), of (2) their non volatile (semi-polar extractable) profile by high-performance liquid chromatography coupled to diode array ultraviolet detection (HPLC–UV–DAD), of (3) their antioxidant and antiradical capacity using different colorimetric assays. The differences among the EO profiles were evaluated by chemometric analysis.

Materials and methods

Sampling area

Aerial parts of wild populations of *H. officinalis* L. were collected from July to September 2014, in five different locations in Kosovo [Prizren, Novoberdë, Pejë and Pashtrik and Albania (Valbonë) (Table 1)]. In Prizren, plant material were collected in the city of Prizren, an urban historical centre in Southern Kosovo, on upslope of the hill under its ancient fortress. In Novobërdë, (Eastern Kosovo) plant material were collected in hilly areas characterized by a typical continental climate and very diverse geological composition surrounding the plant population. The plant populations in Pashtrik (south-west) and Peja (Western Kosovo; above Kaliqan village) were located in mountainous area in Pashtrik, respectively, on Albanian Alps. In Albania, the plant material was collected in Valbona valley—Northern Albania (Valbona National Park). Geographical coordinates of the location

Table 1 Basic characteristics of the sites from where the plant materials of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman populations were collected

Location	North	South	Elev. m a.s.l.	Substrate	Climate	Herbarium accession no.
Prizren	42°12′38″	20°44′47″	446	Limestone	Continental, modified by Mediterranean	EO/05/17
Novoberdë	42°37′02″	21°26′61″	1083	Mix of marbles and recrystallized limestone	Continental	EO/02/17
Pejë	42°47′10″	20°21′24″	1257	Limestone	Continental, modified by subalpine	EO/01/17
Valbonë	42°26′56″	19°52′46″	1012	Limestone	Continental, modified by Mediterranean and subalpine	EO/04/17
Pashtrik	42°12′00″	20°31′40″	1440	Limestone	Continental, modified by Mediterranean and subalpine	EO/03/17

where plant materials were collected, its climate condition, type of substrate and elevation are given in Table 1.

Plant material

For each of the above-mentioned populations, $n = 4$ samples, constituted by $n = 5$ individual plants pooled together, were collected. These samples were separately subjected to distillation for GC–MS of their EO, to solvent extraction for HPLC–UV–DAD and analysed separately. For E-nose and HS-SPME GC–MS analyses, powdered samples were used. Voucher specimens of each population were identified by Prof. A. Hajdari as *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman and deposited at the Herbarium of the Department of Biology, University of Prishtina with the accession number reported in Table 1.

EO hydrodistillation

Plant material was air dried in the shade at room temperature and cut in small pieces (> 0.5 cm). EO was obtained by hydrodistillation (50 g of cut tissue in 0.6 Ls of water contained in a 1 L flask) at a distillation rate of 3 mL/min in a Clevenger apparatus for 3 h. The samples were stored in the dark at − 18 °C in a freezer until further analysis. The yield of EO is expressed as the volume percentage of air-dried plant material dry mass.

GC–FID and GC–MS analyses

GC–FID analyses were performed using an Agilent 7890A GC system equipped with an FID detector and a 5975C MSD (Agilent Technologies). The separation was conducted on a HP-5MS column (30 m × 0.25 mm with a 0.25 µm film thickness). Helium was used as the carrier gas with an initial flow rate of 0.6 mL/min and then at a constant pressure of 50.0 psi. The front inlet was maintained at 250 °C in a split ratio of 50:1. The GC oven temperature was increased from 60 to 260 °C at a rate of 5°C/min, and the FID was operated at 250 °C with an air flow of 350 mL/min and a hydrogen flow of 35 mL/min. The injection volume was 1.0 µL.

For GC/MS, the ionisation energy was 70 eV with a mass range of 40–400 m/z. The identification of each of the components of the EO was performed by comparing their Kovats retention indices with those in the literature [19]. The Kovats index was calculated based on a linear interpolation of the retention times of a homologous series of *n*-alkanes (C9–C28) under the same operating conditions. The components were also identified by comparing the mass spectra of each constituent with those stored in the NIST 08.L and WILEY MS 9th databases and with mass spectra from the literature [19]. Furthermore, some of the main peaks were identified by comparing the retention times and mass spectra with those of authentic constituents. The percentage composition of the oils was computed using the normalization method from the GC peak areas, calculated as the mean of four samples, without correction factors.

Headspace-solid phase microextraction GC–MS (HS-SPME GC–MS)

Aliquots of 0.5 g of powdered sample were analysed by HS-SPME-GC–MS to determine their volatile profile (VOCs).

Samples were placed in 20 mL screw-top vials with polytetrafluoroethylene/silicone septum (Supelco) and located in the cycle composer Pal sampler (CTC Analytics) of the SPME at 15 °C.

A carboxen/polydimethylsiloxane/divinylbenzene StableFlex™ fiber (Supelco) was employed to extract the headspace volatiles (3 h extraction) and extracts were afterward injected into the Rtx-Wax column (30 m; 0.25 mm i.d.; 0.25 µm film thickness, Restek) of a Thermo GC Trace Ultra-MS using a gas carrier of Helium at a constant flow of 1.0 mL/min. The injector temperature was held at 220 °C in splitless mode (8 min). The oven temperature program: 40 °C, hold 3 min, to 160 °C at 3°C/min and then to 200 °C at 10°C/min. Transfer line temperature 210 °C, ion source temperature 250 °C. Electron ionization energy 70 eV, spectral collectioin rate of 1 scan/s over the m/z range 40–500 Da. Structural identification was done based on retention times and by mass spectral comparison with the NIST database. Each sample was analysed in duplicate and, to prevent possible contaminations, blank samples were run after each sample by desorption of the fiber in the GC injector for 5 min at 250 °C.

Electronic nose (e-nose) analysis

E-nose analyses were performed with a portable PEN3 e-nose from Win Muster Airsense (WMA) Analytics Inc. (Schwerin, Germany). It consists of a sampling apparatus, a detector unit containing the sensor array, and a pattern recognition software (Win Muster v.1.6) for data recording and elaboration. The sensor array is composed of 10 Metal Oxide Semiconductor (MOS) sensors: W1C (aromatic compounds), W5S (broadrange compounds, polar compounds, nitrogen oxides and ozone), W3C (ammonia, aromatic compounds, aldehydes and chetones), W6S (hydrogen), W5C (aromatic and aliphatic compounds, less polar compounds), W1S (methane, broad-range compounds), W1W (sulphur-organic), W2S (broad-alcohol), W2W (sulph-chlor), W3S (methane-aliph). The sensor response is expressed as resistivity (Ohm).

Powdered samples (0.5 g) were placed in 30 mL Pyrex® vials fitted with a pierceable Silicon/Teflon disk in the cap. After 20 min equilibration at room temperature, the

measurement was started. The headspace was pumped over the sensor surfaces for 60 s (injection time) at a flow rate of 300 mL/min, and during this time the sensor signals were recorded. After sample analysis, the system was purged for 180 s with filtered air prior to the next sample injection to allow reestablishment of the instrument baseline. The sensor drift was evaluated considering the sensors responses to a 1% ethanol aqueous standard solution included in each measurement cycle. Since all samples were analysed in few days, no significant sensors drift was detected during the analytical period. Three samples for each different location were independently analyzed and the average of sensor responses was used for the subsequent statistical analysis.

HPLC–UV–DAD: sample preparation and instrumentation

Powdered aerial parts (1.0 g) were extracted with methanol (10 mL). After sonication (30 min), the insoluble residue was separated by centrifugation and the solvent layer filtered on paper (0.2 µm, Whatman®) and this solution was submitted to HPLC–UV–DAD analysis.

Analyses were done using a Varian LC-940 analytical/semipreparative HPLC system (Varian, Turin, Italy) equipped with binary pump, autosampler, fraction collector, a UV–DAD detector operating in the 200–400 nm range. Column for analytical separations: Kinetex™ C18, particle size 2.6 µm, pore size 100 Å, 100 × 4.6 mm column. Solvent system: $A = 0.1\%$ formic acid in H_2O and $B = 0.1\%$ formic acid in acetonitrile, flow rate = 0.4 mL/min. Gradient: 0–3 min, from $B = 5\%$; 3–17 min, from $B = 5$–30%; 17–20 min, $B = 30\%$; 20–21 min, from 30 to 60%. Injection volume 5 µL. The concentration of polyphenols was estimated by comparison of the total chromatogram area (adjusted for minor peaks) with a calibration curve built using pure caffeic acid (98%) as standard.

Total phenols content (TPC) and total flavonoids (TF)

For the analysis of TPC and TF, 150 mg of dried leaves and inflorescences were ground and extracted with 25 mL of methanol (50%) in a shaking water bath for 90 min at 75 °C and stored at − 20 °C until further analyses.

TF were determined using a previously reported photometric method [20]. Catechin (0–10 mg/mL) was used as a standard to build the calibration curve. Absorbance was measured at $\lambda = 510$ nm. TF was expressed as mg catechin equivalent/g plant dry weight.

The TPC was determined using the Folin–Ciocalteu method in alkaline environment [21]. Caffeic acid (0–25 µg/mL) was used as a standard for the calibration curve construction, absorbance was measured at 725 nm against the blank and results were expressed as mg caffeic acid equivalent/g plant dry weight.

Fast Blue BB method for the alternative determination of the TPC (FBBB) was performed as described by Medina et al. [22] with minor modifications. Briefly, 1.0 g of plant material was extracted with 10 mL of CH_3OH by sonication ($t = 30$ min) and then centrifuged to remove the insoluble residue (14.000 rpm, $r = 10$ cm, $t = 10$ min). A 100 µL aliquot of the methanolic solutions was diluted with 900 µL of CH_3OH and then added to 100 µL of Fast-Blue BB reagent (0.1% w/v in CH_3OH) and to 100 µL of aqueous NaOH (5% w/vol). The samples were left incubating in the dark for 90 min and then absorbance $\lambda = 420$ nm was determined using a Varian Cary UV spectrophotometer against a blank sample in which the extract was replaced by the same volume of pure methanol. FBBB was expressed as gallic acid equivalent (GAE) by comparison with a calibration curve constructed starting from a methanolic GA stock solution (1 mg/mL) diluted from 10 to 500 µg/mL and treated as described for the samples. The calibration line ($y = 0.0078x + 0.0094$) was linear ($R^2 = 0.9966$) in the concentration range considered.

2.2-Diphenyl-l-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays

DPPH radical scavenging assay was used as reference substances according to the protocol of Chizzola et al. [21]. Trolox (0–50 µg/mL) was used to construct the calibration curve starting from a 2.5 mM stock solution in methanol. After 30 min of incubation, the absorbance was measured at $\lambda = 515$ nm against a blank made by 500 µL Trolox (2.5 mM) and 1500 µL of methanol. The results were expressed as DPPH scavenging percent calculated using the following equation: % DPPH radical scavenging = [(absorbance of control − absorbance of test sample)/(absorbance of control)] × 100.

The FRAP assay measures the ability of antioxidants to reduce the ferric $[Fe(2,4,6\text{-tripyridyl-s-triazine})]^{3+}$ complex to the intensely blue-coloured ferrous complex $[Fe(2,4,6\text{-tripyridyl-s-triazine})]^{2+}$ in acidic medium [23]. The calibration curve was constructed using calibration standards of trolox dissolved in ethanol (from 0 to 400 µg/mL), and the absorbance measured at $\lambda = 593$ nm. The results were expressed as mg Trolox equivalent/g plant dry weight.

All spectrophotometric measurements were performed using a UV–Vis spectrophotometer (Thermo Spectronic GENESYS 10S UV–Vis spectrophotometer, ThermoFisher Scientific, New York, USA). In all cases, the results represent the average ± standard deviations (SD) of $n = 5$ independent measurements.

Statistical and chemometric analysis

Hierarchical cluster analysis (HCA) and principal component analyses (PCA) were used to evaluate whether the identified EO components can be useful for reflecting the chemotaxonomy of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman specimens. PCA and HCA were performed using the statistical analysis software XLSTAT Version 2014.2.03 (STATCON, Witzenhausen, Germany). The oil components with concentrations higher than 1% (Table 2) of the total oil were subjected to statistical analyses. E-nose data were elaborated by PCA in combination with HS-SPME GC–MS data to evaluate the aromatic profile of powered samples and their relationship with VOCs profile. For this elaboration, Minitab Statistical software (v. 17 1.0) was applied.

One-way analysis of variance (ANOVA) was used to determine the differences of total phenolics and total flavonoids and the antioxidant activities (DPPH and FRAP) among the localities. Statistical data analyses were performed using SPSS for Windows, version 15.0.

Results

EO yield, GC–MS profile and chemometric analysis

The phytochemical profile of the EO from the $n=5$ different populations of *H. officinalis* L. subsp. *aristatus* analysed in this study are reported in Table 2.

Hydrodistillation of the aerial parts yielded light-yellowish EO. The yield of EO differed depending on the origin of the populations and ranged from 0.24 to 2.0% based on plant dry weight (Table 2). The highest EO content was found in the accession from locality 3 (Valbonë, 1.7–2.0% v/w), whereas the lowest contents were found in the populations from locality 2 (Novobërdë, 0.24–0.56% v/w).

A total of $n=70$ different substances were identified.

The GC–MS profiles showed significant differences. The presence of 1,8-cineol as dominant constituent (45.27%) of the EO from *H. officinalis* L. subsp. *aristatus* collected in location 1 (Prizren) indicated a chemotypic identity different from the other four accessions. Samples from localities 2–5 shared similar compositions with the main presence of *cis*-pinocamphone (30.44%-57.73%), followed by β-pinene, caryophyllene oxide (12.66%) in that from locality 2 and *trans*-pinocamphone (14.76%).

Interestingly, PCA results revealed a chemotaxonomical discrimination of different accession based also on the variation of EO minor constituents. The two-dimensional axis system of the PCA indicated the existence of three main clusters, corresponding to the chemical compositions of EO originating from different plant populations (Fig. 1).

H. officinalis L. subsp. *aristatus* samples from location 2 clustered in the negative quadrant of the PC1–PC2 space (cumulative explained variance > 98%) with *cis*-pinocamphone, *trans*-pinocamphone, *trans*-pinocarveol and E-anethol as main variables contributing to their separation from the others (cluster 1, Fig. 1).

Locations 3, 4 and 5 were discriminated along the positive direction of PC2 based on *Z*-β-ocimene, borneol, β-pinene, *E*-caryophyllene and limonene (cluster 2, Fig. 1).

As expected, the samples from location 1 were well separated from all the others based on 1,8-cineol along the PC1 positive direction (cluster 3). However, in this case, also several other minor constituents contributed to their separation (i.e. myrtenol, α-pinene, caryophyllene oxide, etc. see Fig. 1 right side).

HCA confirmed the clustering found by PCA. The general structure of the corresponding dendrogram reported in Fig. 2 indicated the existence of three main clusters, reflecting the chemical compositions of the EO originating from the different plant populations.

Of note, EO from location 5 (Pashtrik) showed the highest degree of dissimilarity with that from location 1 (Prizren) (see next paragraph).

VOCs

The profiles of the *H. officinalis* spp. *aristatus* accessions are shown in Table 3. A total of 110 compounds. Terpenes were the dominant class of VOCs in all accessions, reaching around the 80% of total chromatogram areas. In good accordance with the phytochemical profile of their EO, the main representative constituents were limonene, isopinocamphone (0.38–13.32%) and pinocamphone (31.15–46.22%) in all accessions with the exception of that from Prizren, dominated by eucalyptol (19.84%) and linalool (45.07%).

Volatile fingerprint by e-nose

E-nose data in combination with those from VOCs analysis were elaborated by PCA. In Fig. 3 are reported the relative score plot (a) and loading plot (b), in the plane defined by the first two PCs accounting for 71.8% of the total variance.

From the score plot (Fig. 3a), it can be noticed that samples were distributed and discriminated along the two PCs: the samples from Valbona and Peja, located in the negative upper-left part of the score plot, were characterized by a similar aromatic character perceived by the W3S sensor.

The WC sensors (WC1, WC3 and WC5), discriminated the Pashtrik sample in the lower-left quadrant.

These samples clustered together due to VOCs profiles differentiated from the others based on the combination of the dominating component pinocamphone, followed by limonene,

Table 2 Composition (%) of EO from the aerial part of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman collected in different locations

RT	KI	Compound	Prizren 1	Novoberdë 2	Pejë 3	Valbonë 4	Pashtrik 5
4.84	930	α-Thujene	0.09	0.12	0.07	0.33	0.12
5.00	939	**α Pinene**	**1.82**	**0.15**	**0.98**	**0.50**	**1.12**
5.34	953	Camphene	0.03	0.09	0.04	0.04	0.09
5.90	975	**Sabinene**	**1.33**	**0.29**	**1.22**	**1.10**	**1.54**
6.00	979	**β-Pinene**	**3.30**	**3.41**	**23.31**	**13.66**	**11.23**
6.19	989	1-Decene	0.00	0.07	0.00	0.01	0.01
6.28	990	**Myrcene**	**1.39**	**0.08**	**1.03**	**0.85**	**1.02**
6.38	991	3-Octanol	0.38	0.23	0.04	0.05	0.80
6.66	1005	α-Phellandrene	0.00	0.05	0.00	0.00	0.80
7.00	1017	α Terpinene	0.13	0.11	0.01	0.07	0.08
7.22	1026	o-Cymene	0.10	0.36	0.00	0.04	0.09
7.36	1029	**Limonene**	**3.36**	**0.00**	**14.47**	**7.75**	**3.92**
7.45	1031	**Eucalyptol**	**45.27**	**7.15**	**0.95**	**12.29**	**16.67**
7.58	1037	***Z*-β-Ocimene**	**0.63**	**0.00**	**1.93**	**4.43**	**9.04**
7.86	1050	*E*-β-Ocimene	0.85	0.14	0.02	0.18	0.76
8.20	1059	γ-Terpinene	0.15	0.19	0.02	0.11	0.12
8.46	1070	*cis*-Sabinene hydrate	0.27	0.00	0.04	0.10	0.24
9.10	1088	Terpinolene	0.00	0.33	0.00	0.03	0.20
9.34	1103	Perillene	0.54	0.08	0.25	0.39	0.27
9.42	1096	Linalool	0.07	0.06	0.00	0.05	0.37
9.57	1102	*cis*-Thujone	0.00	0.19	0.24	0.08	0.52
10.31	1126	α-Campholenal	0.00	0.10	0.06	0.01	0.40
10.36	1130	*allo*-Ocimene	0.08	0.10	0.00	0.11	0.04
10.73	1139	***trans*-Pinocarveol**	**0.35**	**2.19**	**0.46**	**0.37**	**0.99**
10.93	1144	*trans*-Verbenol	0.34	0.22	0.05	0.05	0.02
11.37	1169	**Borneol**	**0.38**	**0.15**	**1.17**	**3.81**	**4.11**
11.45	1162	**Pinocamphone < trans> isopinocamphone**	**0.33**	**14.76**	**1.01**	**0.61**	**1.46**
11.52	1164	Pinocarvone	0.19	0.00	0.00	0.29	0.13
11.65	1165	delta-Terpineol	0.64	0.16	0.00	0.06	0.02
11.91	1175	**Pinocamphone < cis>**	**0.00**	**57.73**	**33.81**	**37.86**	**30.44**
12.00	1177	Terpene-4-ol	0.51	0.13	0.00	0.12	0.06
12.45	1188	α-Terpineol	0.19	0.21	0.05	0.27	0.29
12.65	1195	**Myrtenol**	**1.55**	**2.36**	**1.46**	**0.67**	**0.69**
13.38	1216	*trans*-Carveol	0.09	0.05	0.04	0.01	1.11
14.11	1229	*cis*-Carveol	0.00	0.09	0.00	0.01	0.12
14.24	1243	Carvone	0.07	0.06	0.00	0.01	0.13
14.96	1267	Geranial	0.15	0.08	0.04	0.06	0.10
15.32	1282	Methyl nerolate	0.00	0.25	0.00	0.05	0.15
15.65	1284	***E*-anethenol**	**0.21**	**4.83**	**0.00**	**0.20**	**0.06**
16.07	1294	Methyl myrtenate	0.00	0.09	0.00	0.05	0.13
17.11	1326	Myrtenyl acetate	0.00	0.11	0.00	0.01	0.29
19.03	1388	β-Bourbonene	0.35	0.77	0.19	0.16	0.26
19.11	1390	Iso-Longifolene	0.01	0.29	0.04	0.05	0.24
19.25	1389	β-Elemene	0.00	0.12	0.00	0.04	0.09
19.43	1400	Tetradecane	0.59	0.79	0.31	0.22	0.03
19.84	1409	α-Gurjunene	0.69	0.00	0.01	0.09	0.00
20.00	1411	α-Cedrene	0.00	0.00	0.00	0.00	0.00
20.17	1419	***E*-Caryophyllene**	**2.15**	**0.02**	**13.73**	**9.04**	**4.98**
20.48	1432	β-Copaene	0.13	0.00	0.45	0.05	0.37
20.85	1441	Aromadendrene	0.33	0.00	0.03	0.03	0.62

Table 2 (continued)

RT	KI	Compound	Prizren 1	Novoberdë 2	Pejë 3	Valbonë 4	Pashtrik 5
21.28	1454	α-Humulene	0.40	0.00	0.05	0.08	0.33
21.51	1460	allo-Aromadendrene	0.25	0.06	0.00	0.02	0.00
22.17	1466	***cis*-Muurola-4(14),5-diene**	**1.76**	**0.07**	**0.61**	**0.27**	**0.02**
22.66	1500	**Bicyclogermacrene**	**2.16**	**0.04**	**0.91**	**1.13**	**0.01**
22.94	1500	α-Muurolene	0.50	0.00	0.00	0.04	0.23
23.20	1511	γ-Amorphene	0.14	0.05	0.00	0.01	0.46
23.47	1523	δ-Cadinene	0.27	0.17	0.08	0.18	0.04
24.00	1531	Citronellyl butanoate	0.75	0.32	0.06	0.10	0.26
24.10	1545	**α-Calacorene**	**1.69**	**0.27**	**0.49**	**0.65**	**0.77**
24.42	**1581**	**Caryophyllene oxide**	**12.66**	**0.00**	**0.00**	**0.00**	**0.15**
25.69	1600	**Guaiol**	**7.26**	**0.00**	**0.00**	**0.24**	**0.33**
25.98	1608	Atlantol β	0.34	0.00	0.03	0.06	0.21
26.15	1619	Junenol	0.42	0.00	0.00	0.03	0.19
26.55	1626	*trans*-Isolongifolanone	0.24	0.00	0.02	0.06	0.04
26.98	1646	α-Muurolol	0.21	0.00	0.00	0.02	0.26
27.06	1646	**Cubenol**	**1.09**	**0.00**	**0.00**	**0.38**	**0.07**
27.47	1663	7-epi-α-Eudesmol	0.28	0.06	0.04	0.07	0.03
27.57	1667	*Z Z*-14-hydroxy-Caryophyllene	0.33	0.11	0.10	0.21	0.22
27.71	1669	*E*-14-hydroxy-9-epi-Caryophyllene	0.20	0.11	0.07	0.09	0.05
		Yield %v/w (min and max. values)	0.42–0.56	0.24–0.56	0.60–0.90	1.70-2.00	0.80–1.06

The percentage for each population represents the mean values of *n* calculated samples ($n = 3$ samples). Compounds marked in boldface (with concentrations higher than 1%) were chosen for HCA and PCA statistical analyses. tr = trace < 0.1%. Id. = peak identification mode: 1: constituent identified by comparison of mass spectra, 2: constituent identified by retention index matching and 3: constituent identified by comparing the retention times with those of authentic constituents

[a]Kovats indices calculated against a mixture of C9- C28 n-alkanes on the HP-5MS column

[b]Compounds are listed in order of elution from a HP-5MS column

6-isoprenyl-3-methoxymethoxy-3-methyl-cyclohexene, linalool, spathulenol and trans-caryophyllene.

On the other side of the plot, samples from the localities Novoberde and Prizren were characterized by WW sensors (W1W and W2W) and WS sensors (W1S, W2S, W5S and W6S).

These two samples were clearly mutually discriminated along PC2, with the sample from Novoberde located in its positive part, and characterized by β-pinone, anethol, D-fenchone and p-cymene. By contrast, the sample from Prizren was located in the PC2 negative part due to several components, among which stands out eucalyptol, the species found as major component in both the EO and VOCs profile of *H. officinalis* L. subsp. *aristatus* from this location.

Non-volatile constituents: phytochemical profile and antioxidant activity

The HPLC–UV–DAD profile of the methanol extracts of the *H. officinalis* L. subsp. *aristatus* examined accessions are reported in Fig. 4.

In the RT range between 1 min and 10 min, minor peaks generated by small amounts of gallic acid and caffeic acid were barely detectable. In good accordance with previous results obtained for other *H. officinalis* sub-species [3, 18], starting from RT at around 11 min, appeared several peaks characterised by the typical UV spectral absorption of caffeic acid: chlorogenic acid (RT = 11.5 min, dominating the chromatographic profile of all accessions with exception of that from E).

The chromatographic profiles of the different *H. officinalis* L. subsp. *aristatus* accessions differed mainly in the relative proportions between chlorogenic acid (3-O-caffeoylquinic acid), 4-O-caffeoylquinic acid (RT ~ 12.2 min), 5-O-caffeoylquinic acid (RT ~ 13.5 min) and 4-O-feruloylquinic acid (RT ~ 18 min).

Interestingly, in the chromatographic profile of the accession from location 5 (Pashtrik mountain) the quantitative ratio of the caffeoylquinic derivatives was around 1:1:1, while in all the others chlorogenic acid was the major represented isomer.

Polyphenols and antiradical activity

In Table 4 are reported the phenolic content of the different *H. officinalis* L. subsp. *aristatus* accessions measured using

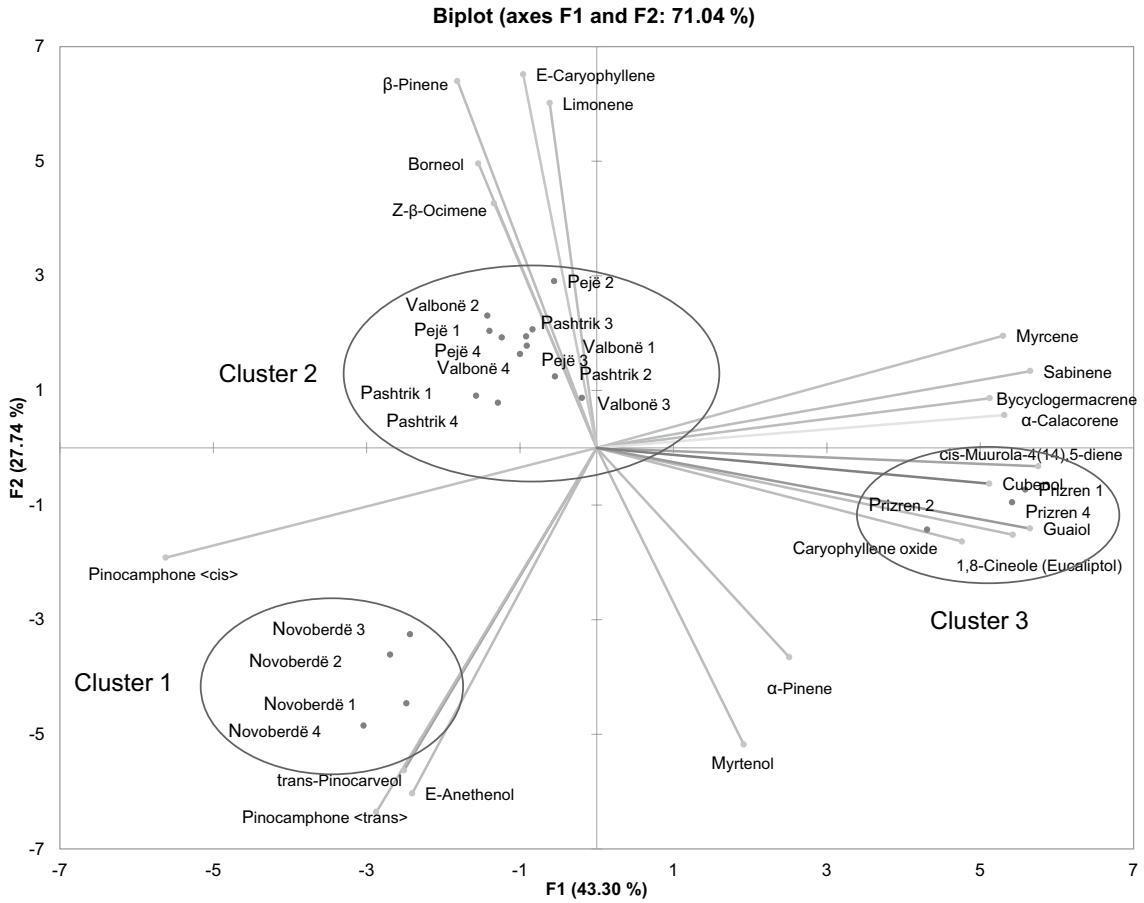

Fig. 1 Principal component analysis (PCA) of the EO components obtained from five populations of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman

two different assays (Folin–Ciocalteu and FBBB), their total flavonidic contents and FRAP and DPPH values as indexes of antioxidant activity.

In all cases, the highest values were found for the solvent extracts of accession 1 (Prizren), and the lowest for the samples from accession 2 (Pejë), indicating a consistent correlation between the equivalent content of caffeic acid (mg_{CAE}/g) measured by HPLC–UV–DAD (around 95% of the total chromatogram area/DAD 3D map volume was due to caffeic acid derivatives, not shown) with the TF content and with the indexes of antioxidant/antiradical capacity (TPC, FBBB, FRAP and DPPH).

Discussion

Previous studies on the phytochemical profile of hyssop have been focused mainly on hyssop of the species *H. officinalis* L., with only few describing its sub-species. More commonly, the investigations have been limited to the determination of the composition of their EO.

Only recently, Džami et al. [18] working with HPLC-MS, reported the phenolic composition of wild *H. officinalis* L. subsp. *pilifer* (Pant.) Murb. from East Serbia (a synonym of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman), which was found dominated by caffeoyl and feruloyl derivatives, among which chlorogenic acid and its positional isomers 4-O- and 5-O-caffeoylquinic acid derivatives were the major ones.

This phenolic profile was in good accordance with that reported by Venditti et al. [13] for *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman growing wild in central Italy.

The chromatographic profiles found for these *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman accession was highly similar to those reported in the present work.

However, although not differing enough to be considered as suitable markers of chemotaxonomic identity, it is worth noting that the overall concentration of the polyphenolic derivatives in the accession from locality 1 (Prizren) was significantly higher compared to all the others.

Its EO composition differed from those of all the other accessions, with the unusual dominant presence of

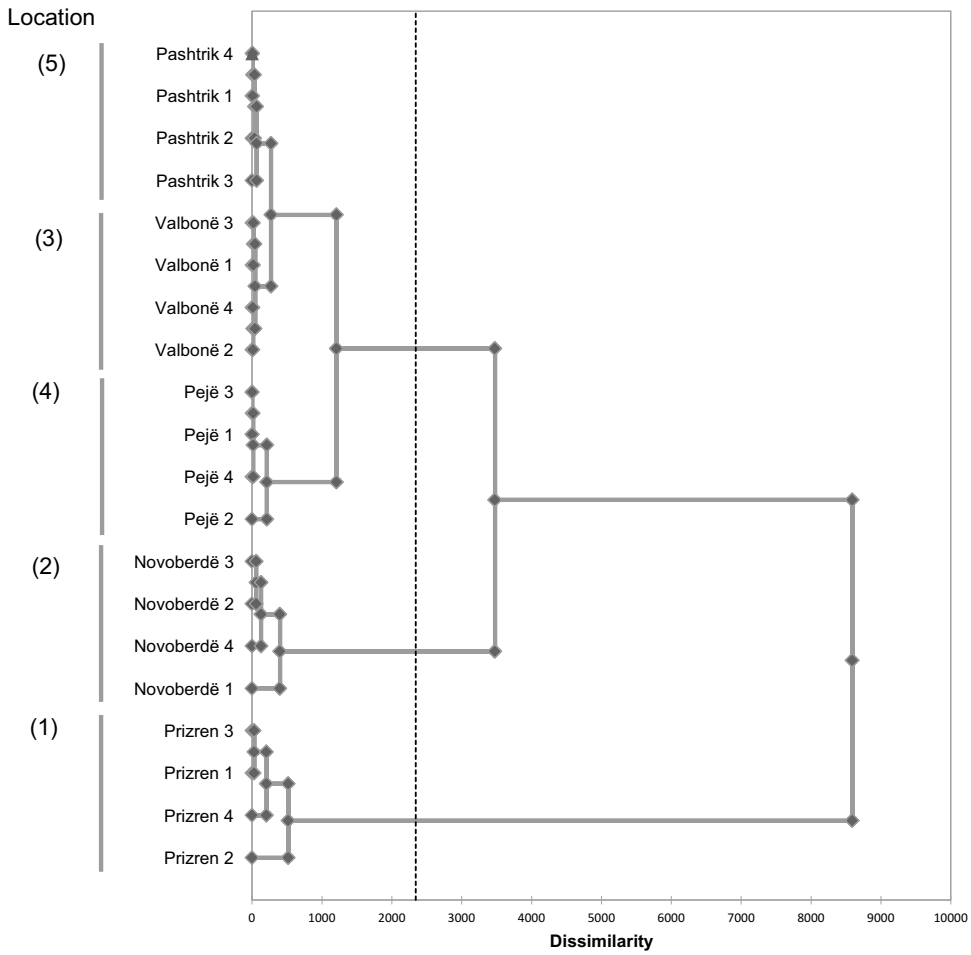

Fig. 2 Two-dimensional dendrogram obtained by the cluster analysis of the essential oils (EO) from five populations (quadruplicate) of *H. officinalis* based on the unweighted pair-group method (square Euclidean distance)

eucalyptol instead of *trans*-pinocamphone and *cis*-pinocamphone, the most commonly found and abundant components found in Hyssop species.

The remarkable phytochemical difference found for this accession, in spite of its morphological indistinguishability from all the others, interpreted with the presence of *H. officinalis* subsp. *aristatus* genetic chemotype lacking the putative enzyme pinocamphone/isopinocamphone synthase(s), directing the plant metabolism toward the formation 1,8-cineol by action of cineol synthase [24]. Interestingly, this particular chemotypic profile resembles that previously reported for *H. officinalis* L. grown in Spain, which presented 1,8-cineole (52.89%) and β-pinene (16.82%) as the main components [25]. On the other hand, the vast majority of studies on *H. officinalis* L., *H. officinalis* subsp. *aristatus* as well as *H. officinalis* subsp. *officinalis* indicated for these species a dominant isopinocamphone/pinochamphone chemotype.

By contrast, for the EO of *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman growing wild in Central Italy, different chemotaxonomic characteristics have been reported by Venditti et al. [13] with high percentages of linalool (35.3–51.2%), a composition similar to that of *H. officinalis* subsp. *canescens* typical of Western Europe (Spain, France) reported by Mazzanti et al. [26].

The EO from *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman specimens collected at different altitudes and analysed by Piccaglia et al. [14], were found containing different compositions: methyl-eugenol > limonene > 1,8-cineole (260 m), myrtenol > β-pinene > isopinocamphone (697 m), β-pinene > 1,8-cineole > methyl-eugenol (1040 m). These results suggested a potential influence of the climatic/geographical conditions on the chemotypic profile of *H. officinalis* subsp. *aristatus* (Godr.) Nyman.

In addition, evaluation of the sensory properties of the *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman samples allowed to observe the association of particular groups of VOCs with the overall capacity of the e-nose selective sensors to generate their individual responses. In this groups were represented the few major constituents determining the accession chemotype (i.e. pinocamphone and eucalyptol), as well as several additional minor constituents belonging to different classes of phytochemicals (mainly hydrocarbon and oxygenated terpenes). Due to the relatively scarce availability of literature data in these promising field, further work is

Table 3 Composition (%) of volatiles from the aerial part of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman collected in different locations and analysed by HS-SPME GC–MS

RT	RI	Compound	Prizren	Novoberde	Peja	Valbona	Pashtrik
2.05	550	Dimethyl sulfide	0.04	0.01	0.02	0.01	0.02
5.37	707	Pentanal	0.09	0.03	0.02	0.01	0.03
6.61	939	α-Pinene	0.51	0.17	0.20	0.06	0.03
6.99	932	α-Thujene	0.00	0.03	0.04	0.03	0.00
10.04	785	Hexanal	0.69	0.58	0.16	0.09	0.23
10.69	989	2-β-Pinene	0.33	0.10	2.13	0.95	0.17
11.63	985	Sabinene	0.23	0.04	0.27	0.17	0.00
13.35	820	5-Methyl, 5-Hexen-2-one	0.00	0.13	0.00	0.00	0.00
14.14	991	β-Myrcene	0.23	0.04	0.80	0.35	0.05
14.46	1026	α-Terpinene	0.24	0.05	0.00	0.04	0.00
15.26	1038	Limonene	4.05	0.38	13.32	4.67	0.70
15.45	1045	Eucalyptol	19.84	1.85	0.29	0.20	0.93
15.57	946	β-Phellandrene	0.87	0.19	0.74	0.35	0.05
16.20	814	2-Hexenal	0.56	0.18	0.12	0.06	0.07
17.02	976	*cis*-Ocimene	2.65	0.06	2.00	0.81	0.66
17.12	1066	γ-Terpinene	1.28	0.37	0.22	0.24	0.07
17.55	1029	β-ocimene	0.86	0.00	0.39	0.14	0.03
17.57	952	3-Octanone	0.17	0.21	0.32	0.00	0.00
17.99	1034	p-Cymene	1.37	2.08	0.13	0.14	0.16
18.10	1141	Ketone	0.00	0.50	0.04	0.00	0.00
18.36	1327	p-Menth-1-en-4-ol, acetate	0.33	0.00	0.12	0.06	0.02
19.72	769	2-Penten-1-ol	0.50	0.24	0.00	0.00	0.08
20.15	987	6-Metil-5-epten-2-one	1.26	0.65	0.10	0.09	0.12
20.41	1041	1-(1,2-Dimethyl-2-cyclopenten-1-yl)ethanone	0.00	0.10	0.02	0.00	0.00
20.60	831	1-Hexanol	0.19	0.26	0.03	0.00	0.00
21.16	1255	6-Isopropenyl-3-methoxymethoxy-3-methyl-cyclohexene	2.07	1.83	2.33	5.23	3.03
21.49	1100	D-Fenchone	0.60	1.07	0.00	0.00	0.00
21.69	979	3-Octanol	0.43	0.27	0.51	0.21	0.00
22.19	1118	α-Thujone	0.04	0.74	0.19	0.35	0.11
22.78	1077	Linalool oxide	0.96	0.56	0.06	0.06	0.10
23.09	961	1-Octen-3-ol	0.84	0.30	0.65	0.73	0.24
23.36	1041	*cis* 4-Thujanol	5.51	1.70	0.35	1.64	0.88
23.56	852	Methyl, 4-methyl-4-Pentenoate	0.00	0.51	0.00	0.00	0.00
23.94	1393	α-Copaene	0.00	0.00	0.17	0.00	0.23
24.04	921	2,4-Heptadienal	0.51	0.25	0.07	0.12	0.04
24.48	1109	isopinocamphone	0.63	10.00	2.62	4.09	8.23
24.6	1406	β-Bourbonene	1.20	1.72	1.26	0.83	2.40
24.68	968	3,5-Octadiene-2-one	0.53	0.74	0.19	0.00	0.16
25.21	1177	pinocamphone	7.07	39.67	31.15	46.22	37.25
25.28	1082	Linalool	1.17	0.31	0.69	0.00	1.16
25.64	1114	Pinocarvone	2.43	2.06	1.45	2.30	2.59
25.74	1500	Sesquiterpene	0.00	0.14	0.13	0.11	0.27
25.83	1047	β-Pinone	1.13	2.23	0.82	0.47	1.11
25.91	1232	α-Fenchyl acetate	0.09	0.00	0.14	0.00	0.08
26.12	1339	β-Cubebene	0.13	0.00	0.14	0.11	0.40
26.22	1109	6-Methyl-3,5-heptadienN-2-one	1.12	0.21	0.00	0.12	0.00
26.24	1494	*trans*-Caryophyllene	0.00	0.00	1.20	0.00	2.67
26.33	1088	Pinanol	1.40	0.32	0.38	0.15	0.58
26.39	1209	4-Terpineol	0.47	0.48	0.00	0.15	0.21

Table 3 (continued)

RT	RI	Compound	Prizren	Novoberde	Peja	Valbona	Pashtrik
26.77	1204	β-Cyclocitral	0.31	0.25	0.07	0.05	0.14
26.93	1215	Myrtenal	2.71	1.87	2.07	1.13	2.97
26.98	1001	Sabina ketone	0.51	0.26	0.11	0.00	0.00
27.23	1386	Aromadendrene	0.00	0.13	0.44	0.63	1.12
27.36	1343	Myrtenylacetate	0.00	0.04	0.00	0.00	0.16
27.48	1157	trans-Pinocarveol	3.71	1.67	1.75	0.81	3.94
27.59	1085	Terpene	0.13	0.46	0.05	0.00	0.20
27.82	1172	Estragole	2.38	1.40	0.42	0.51	0.95
27.93	1136	Verbenol	1.68	1.00	0.22	0.16	1.02
28.18	1435	Methyl 4-(1-hydroxyethyl)benzoate	0.21	1.41	0.23	0.66	1.02
28.31	1209	α-Terpineol	2.83	0.12	0.06	0.18	1.81
28.38	1189	Borneol	0.31	0.16	0.24	0.28	0.28
28.45	1119	2-Pinen-4-one	0.69	0.17	0.11	0.00	0.36
28.81	1134	2,3-Pinanediol	0.36	0.05	0.11	0.06	0.00
29.03	1268	Carvone	0.83	0.21	0.67	0.42	1.43
29.11	1255	Epoxylinalool	0.08	0.06	0.04	0.00	0.00
29.27	–	*cis*-Limoneneoxide	0.00	0.00	0.07	0.26	0.00
29.39	1206	*trans*-Carveol	0.07	0.00	0.09	0.06	0.13
29.49	1435	γ-Cadinene	0.00	0.00	0.00	0.14	0.00
29.79	1191	Butanoic acid, 3-hexenyl ester	0.08	0.00	0.19	0.31	0.11
29.96	1230	Cumaldehyde	0.40	0.11	0.00	0.00	0.26
30.13	1212	Myrtenol	2.15	0.47	2.21	0.80	2.25
30.23	1131	Terpene	0.13	0.00	0.44	0.43	0.23
30.41		2,6-Dimethyl-3,5,7-octatriene-2-ol	0.02	0.00	0.12	0.15	0.50
30.49	1148	Ipsdienol	0.00	0.00	0.01	0.00	0.05
30.69	1160	Ketone	0.02	0.00	0.02	0.03	0.06
30.81	1190	Anethole	4.95	7.00	2.81	2.83	1.38
30.91	1206	*cis*-Carveol	0.50	0.00	0.39	0.15	0.59
31.41	1207	Terpene	0.09	0.00	0.12	0.00	0.23
31.73	1200	*p*-Mentha-1(7),8-dien-2-ol	0.06	0.00	0.28	0.30	0.06
32.04	1136	Benzeneethanol	0.08	0.07	0.06	0.00	0.05
32.21	1338	*cis*-Jasmone	0.06	0.00	0.02	0.03	0.00
32.27	1138	Benzyl nitrile	0.08	0.00	0.01	0.08	0.00
32.37	1072	3,7-Dimethyl-1,5-octadien-3,7-diol	0.08	0.00	0.11	0.09	0.00
32.56	1276	2,3-Pinanediol	0.59	6.93	4.96	4.23	5.67
32.76	937	6-Octen-1-ol, 3,7-dimethyl-, formate	0.03	0.66	0.39	0.45	0.86
32.84	1617	Caryophyllene oxide	0.88	0.21	0.58	0.00	2.32
32.91	1454	β-Ionone epoxide	0.11	0.06	0.05	0.00	0.12
33.02	1326	Perilla alcohol	0.05	0.03	0.05	0.00	0.12
33.15	1361	Methyl eugenol	1.00	0.00	11.31	12.21	0.00
33.29	1171	4-methoxy, benzaldehyde	0.28	0.38	0.23	0.21	0.03
33.42	1643	Humulene oxide	0.07	0.00	0.03	0.00	0.31
33.76	–	Unknown	0.00	0.14	0.11	0.10	0.09
33.89	1530	Veridiflorol	2.81	0.00	0.00	0.00	0.00
34.05	–	Unknown	0.07	0.03	0.04	0.00	0.04
34.19	1599	Alcohol	0.04	0.00	0.04	0.00	0.07
34.3	1536	Spathulenol	1.43	0.15	1.72	1.26	2.26
34.83	1392	p-Eugenol	0.00	0.00	0.05	0.14	0.07
35.00	1379	Methyl-isoeugenol 2	0.00	0.00	0.11	0.13	0.00
36.18	1195	Methylethylmaleimide	0.19	0.04	0.16	0.06	0.15

Table 3 (continued)

RT	RI	Compound	Prizren	Novoberde	Peja	Valbona	Pashtrik
37.01	–	Unknown	0.01	0.00	0.02	0.02	0.06
37.56	1426	Dihydroactinidiolide	0.32	0.17	0.28	0.11	0.16
37.99	–	Unknown	0.07	0.01	0.07	0.00	0.36
38.4	1360	3,4-Dimethoxy, benzaldehyde	0.00	0.00	0.07	0.03	0.00

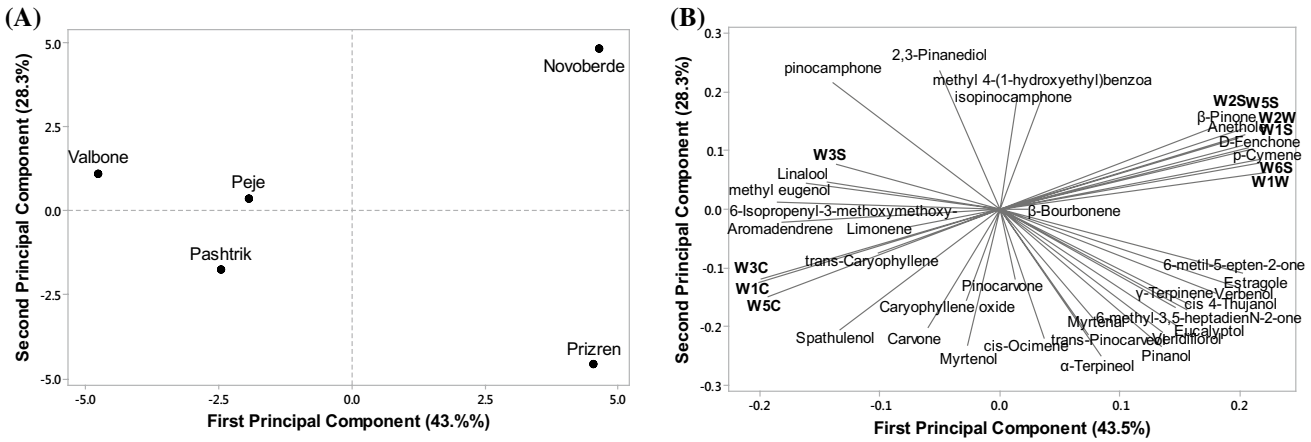

Fig. 3 PCA **s**core plot (**a**) and loading plot (**b**) of e-nose sensorial properties and VOCs composition of five populations of *Hyssopus officinalis subsp. aris*tatus (Godr.) Nyman

needed to better understand the potentiality of such approach (e-nose/VOCs analysis) for the characterisation of medicinal plants sensorial properties [27].

The metabolic and or genetic factors (gene deletions/genetic mutations) underlying these compositional differences are currently unknown.

An additional intriguing interpretation for the different chemotype found in location 1, is the potential foreign origin of the sub-population of *H. officinalis* subsp. *aristatus* (Godr.) Nyman collected nearby the historical urban town of Prizren in Southern Kosovo, close to the borders with Albania and with the Republic of Macedonia. Due to its favourable geographic position, especially under the period of the Ottoman empire domination (1389–1912 a.C.), Prizren was a prosperous trade center, so it is possible in principle that the sub-population presenting the peculiar chemotypic profile may have originated from imported *H. officinalis* subsp. *aristatus* accidentally or intentionally seeded during those times, and surviving until today in that sampling site, on the wild upslope of the hill guesting the ancient fortress dominating the surrounding area.

In conclusion, the present study shows that remarkable chemotaxonomic differences may exist among *H. officinalis* subspp. *aristatus* (Godr.) Nyman from different populations, which may influence the suitability for their use in the food and beverage industries.

Hence, the reported results, showing clearly different sensors responses to pinocamphone or eucalyptol *H. officinalis* subspp. *aristatus* (Godr.) Nyman chemotype samples, provide strong support to the effective utility of E-nose screening for the quick and robust discrimination of the plant material endowed with the best desirable properties for further processing (e.g. as aromatizer in food preparation or as ingredient for liquors production).

The extractable polyphenolic components seem to less affect these differences, with the EO and VOCs profiles more markedly sensitive to these differences. Comparing to other results reported in the literature, in the case here described, the observed compositional differences can be more likely explained on the basis of genetic variations rather than and environmental factors in determining the composition of its EO.

Taken all together, these results suggest that the case of *H. officinalis* subspp. *aristatus* (Godr.) Nyman is an interesting subject for further studies aimed at the understanding of the effect of genetic and environmental factors, or of their combinations, on the chemotypic expression and on the organoleptic properties of this interesting plant species.

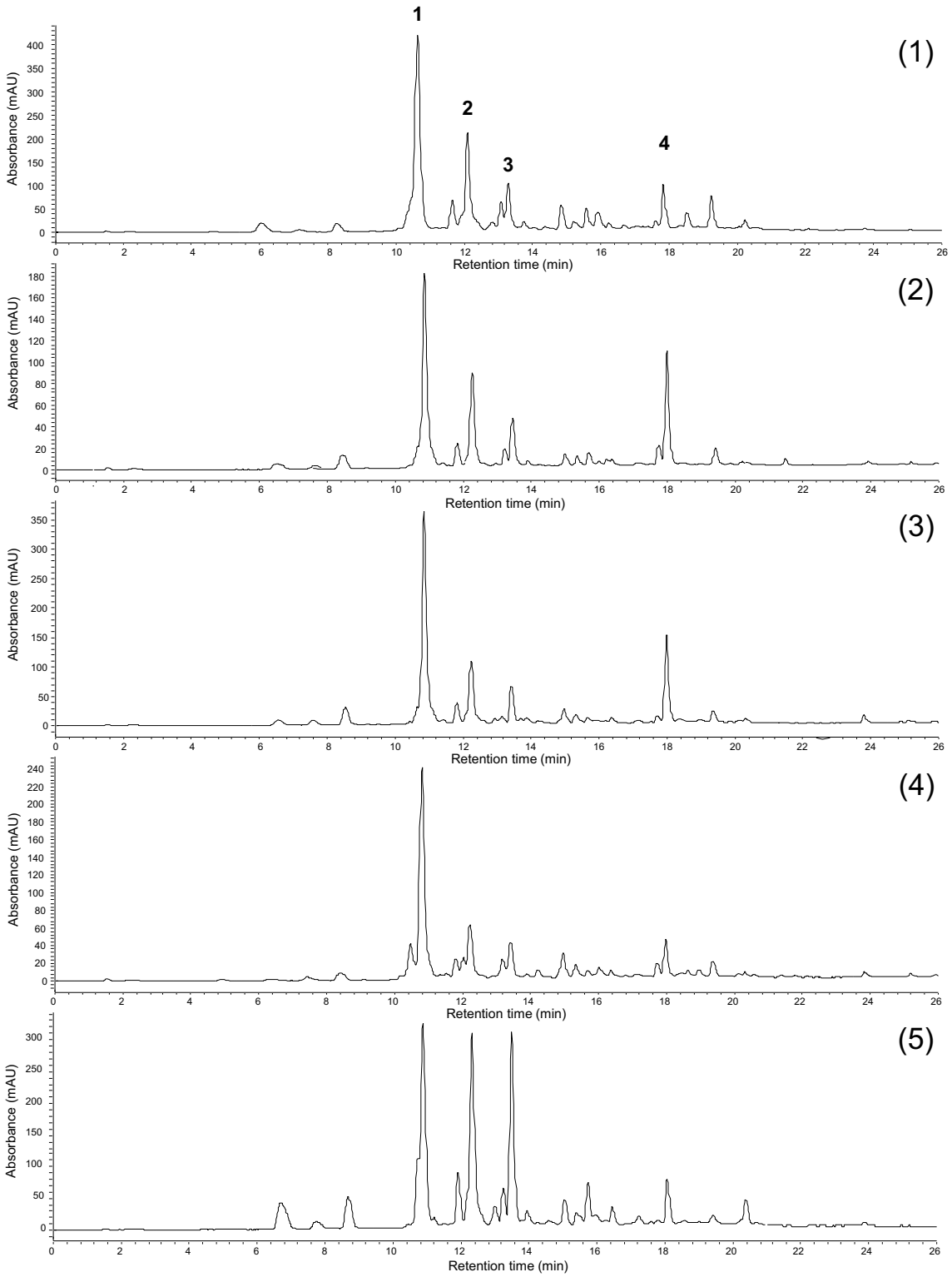

Fig. 4 Representative HPLC-UV-DAD profiles (methanol extracts) of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman, accessions collected in different geographical areas. Main peaks assignments: 1: chlorogenic acid (3-O-caffeoylquinic acid), 2: 5-O-caffeoylquinic acid, 3: 4-O-caffeoylquinic acid, 4: 4-O-feruloylquinic acid. Numbers at right-hand side indicate the individual sampling location according to Table 1

Table 4 Total flavonoids (TF), Folin–Ciocalteu total phenol content (TPC), total phenols content measured by fast blue BB method (FBBB), DPPH and FRAP antioxidant activity in *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman aerial parts

Location	TPC (mg_{CAE} /g)	FBBB (mg_{GAE}/g)	TF (mg_{CE}/g)	FRAP (mg_{TE}/g)	DPPH (mg_{TE}g)	DPPH (%)	TPC HPLC (mg_{CAE}/g)
1 Prizren	84.28 ± 20.18^{a}	2.69 ± 0.09^{a}	43.60 ± 6.58^{a}	1242.20 ± 293.66^{a}	73.10 ± 3.97^{a}	95.57 ± 5.30^{a}	14.66 ± 0.61^{a}
2 Novobërdë	50.25 ± 2.78^{b}	1.29 ± 0.06^{b}	20.49 ± 1.36^{bc}	812.10 ± 38.23^{b}	64.96 ± 1.13^{c}	84.73 ± 1.5^{c}	5.67 ± 0.50^{c}
3 Valbonë	41.43 ± 1.69^{b}	1.27 ± 0.06^{b}	23.37 ± 1.83^{b}	743.00 ± 44.83^{b}	59.17 ± 0.40^{b}	77.02 ± 0.54^{b}	9.05 ± 0.32^{b}
4 Pejë	32.65 ± 2.53^{b}	0.82 ± 0.02^{c}	15.04 ± 0.57^{c}	572.17 ± 36.35^{b}	54.92 ± 0.76^{d}	71.34 ± 1.01^{d}	5.28 ± 0.26^{c}
5 Pashtrik	42.23 ± 2.53^{b}	1.66 ± 0.07^{b}	19.26 ± 0.80^{bc}	792.20 ± 62.03^{b}	63.79 ± 0.65^{ec}	83.17 ± 0.86^{ec}	11.27 ± 0.24^{b}

Different subscript letters within the same column indicate significant differences ($p < 0.05$ according to Tukey's HDS test) of means between localities

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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