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Clinopodium nepeta (L.) Kuntze from Bosnia and Herzegovina: Chemical Characterisation of Headspace and Essential Oil of Fresh and Dried Samples

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Abstract: The samples of *Clinopodium nepeta* (L.) Kuntze (Lamiaceae) aerial parts were collected from four different localities in sub-Mediterranean area of Bosnia and Herzegovina and subjected to phytochemical profiling. Shade-dried and fresh samples were used to determine their headspace volatile organic composition by solid-phase microextraction on two differently coated fibers. Corresponding essential oils were obtained by hydrodistillation. Both headspace and essential oil were analysed by gas chromatography-mass spectrometry Among detected compounds, piperitenone oxide and pulegone were dominant in the headspace of shade-dried and fresh *C. nepeta* samples. The essential oils contained 42 compounds including pulegone (44.8%), piperitenone (48.8%) and piperitenone oxide (60.2%) as the major compounds, followed by limonene, *cis*-piperitone oxide, *p*-menthone and dihydrocarvyl acetate. In this work, a wide range of volatile compounds present in *C. nepeta* samples from Bosnia and Herzegovina was determined. The obtained data provides detail phytochemical analysis of the volatiles of *C. nepeta* and therefore completes earlier researches of this plant from the other geographical areas.

Keywords: *Clinopodium nepeta* (L.) Kuntze; Lamiaceae; headspace solid-phase microextraction; hydrodistillation; essential oil.

1. Introduction

There is a growing interest in studying plant volatile compounds from the essential oils, extracts or headspace. They have been the focus of study for decades with one of the goals to find the

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compounds that possess different biological properties with their possible contribution in various applications in order to improve human health [1]. The aim of this research is to determine the volatile organic compounds of *Clinopodium nepeta* (L.) Kuntze from Bosnia and Herzegovina. *Clinopodium nepeta* (L.) Kuntze (Lamiaceae) was classified in different genera (*Calamintha, Satureja, Thymus*), but eventually into genus *Clinopodium* which was accepted by EMPB [2] on the basis of IPNI [3] and WCSP [4]. The most common synonym is *Calamintha nepeta* (L.) Savi subsp. *nepeta*. It is widespread throughout South, South-Central and West Europe [5]. Throughout Bosnia and Herzegovina's territory, it can be found in sub-Mediterranean area. It is traditionally used in tea form, for treating gastrointestinal illnesses, reducing gasses and cramps [6, 7]. The EOs are used to relieve headache, insomnia, respiratory illnesses [5, 8] or as compresses for external use in treating hips pain [5], while Italian household uses it as a spice [5, 6]. A wide phytochemical diversity *of C. nepeta* consisting of terpenes, alkaloids, saponins, flavonoids, tannins, sterols, and glycosides have been presented in scientific papers [9-13]. The most abundant constituents of *C. nepeta* EO, according to several literature data [5, 12, 14], are monoterpenes and sesquiterpenes such as pulegone, menthone, isomenthone, piperitone, piperitenone and its oxides as the main ones. However, some other research results do not confirm the mentioned compounds as dominant [6, 8, 12]. The results mainly differ depending on the origin of *C. nepeta*. For example, oxygenated monoterpenes, pulegone, neo-menthol and isomenthone, represent the major components of *C. nepeta* EO from Algeria [15]. Besides monoterpenes, *trans*-menthone and pulegone were found [13]. In Moroccan samples, predominance of 1,8-cineole followed by pinocamphene and *cis*-isopulegone was reported [16]. The samples from Serbia were rich in pulegone, piperitenone oxide, menthone, and menthol [17]. The chemical profile of *C. nepeta* EOs from Italy contained pulegone accompanied by menthone, piperitenone and piperitone [18]. Thymol was detected as the major component of the samples from Azerbaijan [19], while Turkish samples were rich in *trans*-piperitone oxide, piperitenone oxide, pulegone, and menthone [8].
C. nepeta EOs showed the following: a) excellent antimicrobial activity on different microorganisms which is associated with the presence of menthone [15] and pulegone [17]; b) antifungal activity that may be related to pulegone [14]; c) effectiveness in preventing rotting of several fruit trees after harvest [18]; d) bioherbicide activity – it shows an inhibitory potency on the processes of germination and root growth of several vegetables and weed species [20]; e) strong activity against toxic agents such as Aflatoxin 1 (AFB1) [21]; f) effectiveness as the insect repellent [22, 23].

Analyzing the literature data on the composition of *C. nepeta* EO, the diversity in the quality and quantity of the components present in relation to chemotype diversity is noticeable. This may be the reason for the diversity in the type and intensity of biological activities shown by the EOs [24]. Most likely, the component with the highest content as well as its interaction with other present compounds exhibited dominant influence on the biological activity of *C. nepeta* EO. In most studies, distillation, as a method that has been constantly modified and improved during its application to date [24, 25], is the first choice for the isolation of EOs from all parts of the plant [24]. For the phytochemical profiling of headspace, solvent-free solid phase microextraction (HS-SPME) has been increasingly used. This environmentally friendly method allows the extraction of low molecular weight compounds and the most volatile compounds from the plant surface [26].

The aim of this research is to obtain more complete chemical composition of volatiles (including headspace) of *C. nepeta* from Bosnia and Herzegovina, which is why a two-way approach was used: hydrodistillation (HD) and headspace-solid phase microextraction (HS-SPME). Due to expected complex headspace composition, two adsorption fibers, DVB/CAR/PDMS (F1) and PDMS/DVB (F2) for HS-SPME, with different polarities were applied in order to provide a more detailed insight into the aromatic profile of *C. nepeta*. To the best of our knowledge, the application of HS-SPME in phytochemical profiling of fresh and shade-dried *C. nepeta* was performed for the first time in this work.

2. Materials and Methods

2.1. Plant Materials

Chemical characterisation of fresh and dried samples of *Clinopodium nepeta* (L.) Kuntze

The samples of *C. nepeta* aerial parts (flowers, leaves and steam) were collected during the flowering period in August 2019 in southern Bosnia and Herzegovina: Pirići-A (altitude 586 m; 42°59′42″ N, 18°00′46″ E), Blagaj-B (altitude 142 m; 43°15′56″ N, 17°53′16″ E), Lokve-C (altitude 286 m; 43°08′22″ N, 17°52′21″ E), Počitelj-D (altitude 70 m; 43°08′05″ N, 17°43′55″ E).

Taxonomical determination of the plants was done at the University of Sarajevo-Faculty of Science on the Department of Biology by Prof. Senka Barudanović Research group. Specimens vouchers were deposited in the Herbarium of National Museum of Bosnia and Herzegovina, Herbarium code SARA (Table 1), as well as in the Herbarium of the University of Sarajevo - Faculty of Pharmacy (voucher no. FFOH/1010-13). Fresh samples were kept in the freezer until the analysis. *C. nepeta* was dried in room temperature with natural airflow without sun exposure for a week. Shade-dried material was stored in paper bags. Prior to the extraction (Paragraph *2.2.*) fresh and dry samples were chopped up manually into small pieces, and for HD dried samples were powdered in the mill.

2.2. Headspace Solid-Phase Microextraction

Two types of fiber were used for HS-SPME: DVB/CAR/PDMS (F1) and PDMS/DVB (F2) purchased from Supelco Co. (Bellefonte, PA, USA). They were conditioned by the manufacturer's protocol instructions prior to the extraction. The plant material (2 g) was put into glass vials (15 mL) and hermetically closed by PTFE/silicon septa. The content was heated in water bath (37 °C, 15 min) to induce volatiles from the plant material in the headspace. After HS-SPME extraction (45 min) SPME fiber was returned into the needle and inserted into injector (250 °C, 7 min) of the gas chromatograph where the extracted volatile compounds were thermally desorbed to the gas chromatograph column.

2.3. Hydrodistillation of Plant Materials

Hydrodistillation (HD) was performed with a modified Clevenger apparatus for 2 h from 50 g of the plant material. Pentane was used as trap (1 mL). After the completion of HD, the collected EO dissolved in pentane was dried using anhydrous sodium sulphate. Each sample was aliquoted, weighed and kept at 4 °C in proper dark glass containers, until the analysis. For gas chromatography and mass spectrometry (GC/MS) analysis 1 µL of each sample was used.

2.4. Gas Chromatography and Mass Spectrometry Analysis of Volatiles

GC/MS analysis was performed on gas chromatograph (GC) by Agilent 7820A model (Palo Alto, CA, USA) using mass spectrometer (MS) Agilent 5977E model using HP-5MS capillary column (5% phenyl-methylpolysiloxane: 30 m × 0.25 mm x 0.25 µm, Agilent J and W).

The GC conditions were set up 2 min isothermally at 70 °C, then raised up to 200 °C at a rate of 3 °C/min; injector temperature was 250 °C; split ratio was 1:50; gas carrier was He (1 mL/min). The MS conditions were: EI mode at 70 eV; ion source temperature was 280 °C; the mass range was 30-350 amu. The identification of compounds was performed using GC peaks by comparing its retention indices (RI) relative to C_9-C_{25} *n*-alkanes for HP-5MS with RI data from El-Sayed [27] and by comparing their mass spectra with those from the Wiley 275 (Wiley, NY, USA) and NIST02 [28] (Gaithersburg, MD, SAD) libraries. The quantification (%) of volatile components was calculated from the GC peaks areas (average of duplicate analyses) applying the normalization method with no correction factors.

3. Results and Discussion

Four different samples of *C. nepeta* from Bosnia and Herzegovina (geographic area of Pirići, Blagaj, Lokve, Počitelj) were subjected to HD and obtained yields of EOs are presented in Table 1.

Table 1. The yields of *C. nepeta* essential oils

Plant material	Code	Location	Yield (%)
Clinopodium nepeta (L.) Kuntze	SARA 53047	Pirići	1.99
	SARA 53048	Blagaj	2.3
	SARA 53049	Lokve	1.76
	SARA 53050	Počitelj	1.14

The yield of every sample of *C. nepeta* essential oil is calculated using the equation:

$$\% \text{ Yield of oil} = (\text{Weight of oil}/\text{Weight of dried plant}) \times 100$$

These values are appreciable and comparable to those found by other research groups as shown below.

Table 2. The yields of *C. nepeta* essential oils with respect to the literature data

Plant	Country	Dry material (g)	Yield (%)	Reference / Year
Clinopodium nepeta (L.) Kuntze	Greece	500	2.0	[29] / 1987.
	France	-	0.17 to 0.76	[7] / 1996.
	Italia	100	0.22	[30] /1998.
	France		0.4 to 1.2	[31] / 2000.
	France	1300	0.6	[32] / 2000.
	Türkiye	-	0.1 to 1.5	[8] / 2011.
	Italia	-	1.15±0.26 to 3.04±0.62	[33] / 2013.
	Türkiye	-	1.98	[21] / 2013.
	Algeria	100	1.4	[15] / 2013.
	Algeria	100	1.48	[34] / 2015.
	Algeria	100	0.54±0.11	[12] / 2018.
	Algeria	-	1.18	[13] / 2018.
	Azerbaijan	-	0.8-1.2	[19] / 2018.
	Portugal	-	0.61 ± 0.18	[1] / 2019.
	Tunisia	-	1.21	[22] / 2020.

The HS measurement composition was carried out from fresh and shade-dried *C. nepeta*. Collected fresh samples were stored in the freezer until the analysis. Used fibers DVB/CAR/PDMS (F1) and PDMS/DVB (F2) were suitable for the detection of headspace compounds [35]. Volatile organic compounds of HS and EOs identified by GC/MS are shown in Table 3, while several selected chromatograms and chemical structures are presented in Figures 1 and 2.

3.1. Headspace Composition of Fresh C. nepeta

In four fresh samples of *C. nepeta*, a total of 65 compounds were identified in HS (by both fibers) using GC-MS. Fiber F1 extracted 32 to 50 compounds making up 94.3% to 98.4% of the total chromatogram area. In the same samples, 32 to 38 compounds were found using Fiber F2, representing 93.5% to 100.0% of the total ion chromatogram. All identified compounds with a content of less than 0.1% are written as t (trace) in Table 3. The chromatograms of both fibers showed qualitatively similar chemical profiles with differences in the proportion of extracted compounds.

Table 3. Chemical composition of the headspace and essential oils volatiles of *Clinopodium nepeta* (L.) Kuntze from Bosnia and Herzegovina

No.	Compound	RI*	RI	Area percentages (%)																			
				Sample A					Sample B					Sample C					Sample D				
				(Fresh)		(Shade-dried)			(Fresh)		(Shade-dried)			(Fresh)		(Shade-dried)			(Fresh)		(Shade-dried)		
				HS-SPME		HS-SPME		HD	HS-SPME		HS-SPME		HD	HS-SPME		HS-SPME		HD	HS-SPME		HS-SPME		HD
				F1	F2	F1	F2		F1	F2	F1	F2		F1	F2	F1	F2		F1	F2	F1	F2	
1.	Ethanol	445	<900	0.1	t	-	-	-	t	-	-	-	-	0.1	-	-	-	-	0.1	-	-	-	-
2.	(*E*)-Pent-2-enal	748	<900	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.	Hexanal	800	<900	0.1	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-
4.	Ethyl 2-methylbutanoate	895	<900	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	-	-	-
5.	(*E*)-Hex-2-enal	855	<900	1.4	0.1	-	-	-	0.1	0.1	-	-	-	0.4	0.5	-	-	-	1.2	0.2	-	-	-
6.	α-Thujene	933	935	-	0.1	-	-	-	0.1	0.1	-	-	-	0.1	-	-	-	-	0.1	0.1	-	-	-
7.	α-Pinene	940	940	0.4	0.4	0.2	0.2	0.8	0.2	0.5	0.1	0.2	0.7	0.2	0.4	0.1	0.3	0.9	0.2	0.6	0.1	0.2	0.7
8.	Camphene	955	957	t	t	-	-	0.1	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1
9.	Benzaldehyde	966	967	t	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.	Sabinene	980	981	0.2	0.4	0.1	0.1	0.4	0.2	0.3	0.1	0.1	0.3	0.2	0.2	-	0.2	0.4	0.2	0.4		0.2	0.3
11.	β-Pinene	982	985	0.4	0.5	0.2	0.2	0.7	0.3	0.5	0.1	0.2	0.6	0.2	0.3	-	0.3	0.8	0.2	0.6	0.1	0.2	0.6
12.	Octan-3-one	985	990	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13.	β-Myrcene	993	994	0.6	0.5	0.3	0.2	0.6	0.3	0.6	0.2	0.3	0.4	0.3	0.4	0.2	0.4	0.7	0.3	0.7	0.3	0.4	0.6
14.	Octan-3-ol	996	998	1.1	0.8	1.1	0.7	1.2	1.1	0.5	0.8	0.6	0.7	1.0	0.9	1.0	0.7	0.9	1.0	0.6	1.2	0.7	0.9
15.	α-Terpinene	1024	1023	-	-	0.1	-	0.1	0.4	-	-	-	-	0.2	-	-	-	0.1	0.2	-	-	-	0.1
16.	*p*-Cymene	1030	1030	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17.	**Limonene**	**1035**	**1035**	**6.2**	**6.2**	**3.5**	**3.2**	**6.3**	**6.1**	**6.3**	**2.2**	**2.9**	**4.8**	**3.6**	**3.1**	**3.5**	**7.8**	**10.6**	**2.8**	**14.8**	**4.6**	**5.7**	**9.8**
18.	1,8-Cineole	1038	1039	-	0.1	-	0.1	0.1	-	0.1	-	0.1	-	0.2	0.1	-	-	0.2	-	0.3	-	0.1	0.2
19.	(*Z*)-β-ocimene	1044	1043	0.1	0.1	0.1	0.1	0.1	0.2	0.2	-	0.1	-	0.1	0.3	-	0.1	-	-	0.1	-	-	-
20.	(*E*)-β-ocimene	1054	1054	0.1	t	-	-	t	-	t	-	-	-	-	0.1	-	-	-	-	-	-	-	-
21.	γ-Terpinene	1064	1065	t	0.1	0.1	-	0.2	0.7	0.1	0.1	-	0.2	0.2	-	0.2	-	0.2	0.4	0.1	-	-	0.2
22.	*cis*-Sabinene hydrate	1074	1074	0.2	2.0	0.5	0.9	0.6	1.0	0.5	0.5	0.9	0.6	0.5	1.5	0.5	0.7	0.6	0.5	0.3	0.3	0.6	0.4
23.	α-Terpinolene	1097	1092	-	0.1	-	-	0.1	0.2	0.1	-	-	-	0.1	0.1	-	-	-	0.1	0.1	-	-	0.4
24.	*trans*-Sabinene hydrate	1101	1102	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25.	Linalool	1102	1103	0.1	0.2	0.2	0.2	0.4	-	0.1	0.2	0.3	-	0.3	-	0.3	0.4	0.4	0.2	0.1	0.6	0.4	-
26.	2-Methylbutyl 2-methylbutanoate	1104	1104	0.1	0.1	-	-	0.1	-	0.1	-	0.1	-	0.1	-	-	0.1	-	-	0.2	-	-	-
27.	***p*-Menthone**	**1163**	**1160**	**1.5**	**6.1**	**6.1**	**6.3**	**9.7**	**2.0**	**4.8**	**7.3**	**11.2**	**10.3**	-	-	**0.5**	**0.3**	**0.4**	**0.3**	**0.1**	**0.6**	**0.9**	**1.1**
28.	Isomenthone	1165	1168	0.1	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29.	Menthofuran	1169	1170	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-
30.	Neomenthol	1167	1171	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-
31.	Borneol	1172	1172	-	-	-	-	0.3	-	-	-	0.2	-	-	0.2	-	0.1	-	-	-	-	-	0.2
32.	*trans*-Isopulegone	1177	1179	1.2	1.8	-	0.7	0.6	0.3	1.9	0.4	1.0	0.7	-	-	-	0.0	-	-	-	-	0.1	0.1
33.	Menthol	1181	1181	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-
34.	Terpinen-4-ol	1182	1182	-	-	-	-	0.7	0.8	-	-	0.1	0.7	-	-	-	-	0.5	0.3	0.3	-	-	0.5
35.	α-Terpineol	1194	1194	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	-	0.2	-	0.2	0.2	0.3	0.2	0.1	0.2	0.2	0.3
36.	*cis*-Dihydrocarveol	1194	1198	-	-	-	-	0.2	0.2	0.1	-	-	-	0.4	-	-	0.1	-	0.9	0.2	-	-	0.2
37.	*cis*-Dihydrocarvone	1195	1199	0.1	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-
38.	(Z)-Hex-3-en-1-yl 2-methylbutanoate	1232	1236	0.7	0.4	-	0.1	0.1	0.1	0.4	-	0.1	-	0.4	0.1	-	0.1	-	1.2	0.9	-	0.2	0.1
39.	**Pulegone**	**1244**	**1244**	**40.6**	**44.8**	**24.8**	**23.2**	**23.2**	**23.9**	**39.7**	**34.5**	**30.1**	**43.9**	**0.2**	**0.3**	**2.9**	**2.4**	**2.2**	**3.4**	**0.2**	**7.8**	**5.7**	**8.5**

40.	***cis*-Piperitone oxide**	**1257**	**1258**	**-**	**-**	**-**	**12.6**	**14.5**	**-**	**-**	**-**	**-**	**-**	**21.1**	**36.6**	**18.4**	**12.1**	**15.2**	**14.1**	**0.4**	**15.4**	**14.1**	**15.1**
41.	**Piperitone**	**1259**	**1259**	**0.4**	**1.2**	**11.5**	**-**	**-**	**1.3**	**2.7**	**6.6**	**7.9**	**6.0**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
42.	*cis*-Isopiperitenone	1272	1275	0.3	0.6	0.9	0.7	0.5	1.9	0.6	0.7	0.7	0.3	1.4	0.2	1.1	0.9	0.7	0.4	1.1	1.0	0.7	0.6
43.	Isopulegyl acetate	1285	1287	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44.	Neodihydrocarveol	1220	1226	7.9	2.9	-	-	0.2	0.3	3.0	-	0.1	-	1.3	-	-	0.2	-	1.8	-	-	0.2	-
45.	**Dihydrocarvyl acetate**	**1328**	**1333**	**14.4**	**3.5**	**1.2**	**1.8**	**0.4**	**4.3**	**10.0**	**0.9**	**0.8**	**-**	**10.2**	**2.1**	**1.1**	**1.5**	**0.3**	**12.1**	**14.9**	**1.2**	**2.7**	**0.5**
46.	*trans*-Carvyl acetate	1342	1342	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47.	**Piperitenone**	**1347**	**1345**	**9.3**	**13.7**	**24.6**	**17.9**	**14.8**	**48.8**	**20.7**	**36.3**	**32.9**	**27.6**	**1.5**	**0.4**	**4.9**	**2.7**	**2.0**	**1.6**	**1.7**	**7.9**	**3.3**	**3.2**
48.	Eugenol	1362	1362	-	-	-	-	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49.	2-Methoxy-3-(2-propenyl)-phenol	1362	1365	-	-	-	-	0.1	-	-	-	-	-	0.2	-	-	-	-	0.2	-	-	-	0.1
50.	*cis*-Carvyl acetate	1365	1367	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51.	**Piperitenone oxide**	**1371**	**1371**	**4.1**	**5.2**	**20.4**	**25.0**	**16.1**	**0.2**	**0.1**	**5.6**	**4.3**	**1.1**	**47.7**	**39.5**	**60.2**	**59.8**	**60.0**	**48.2**	**48.3**	**55.6**	**56.4**	**51.7**
52.	α-Copaene	1378	1379	0.2	0.2	0.1	0.2	0.1	0.1	0.1	-	0.1	-	0.2	0.3	0.2	0.4	-	0.3	0.3	-	0.3	0.2
53.	β-Bourbonene	1386	1386	0.3	0.2	0.1	0.3	0.1	0.1	0.1	0.1	0.2	-	0.2	0.5	0.2	0.3	-	0.2	0.3	-	0.2	0.1
54.	β-Cubebene	1391	1391	0.1	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-
55.	β-Elemene	1394	1393	0.1	0.1	-	-	0.1	-	-	-	-	-	-	0.1	-	0.1	-	-	0.1	-	-	-
56.	*trans*-Caryophyllene	1423	1422	1.5	1.5	0.5	0.8	0.5		0.7	0.5	1.2	0.3	0.1	2.6	0.6	1.2	0.4		1.4	0.5	1.0	0.5
57.	*trans*-α-Bergamotene	1438	1438	0.2	0.1	-	-	-	0.1	0.1	-	-	-	-	0.2	-	-	-	0.3	0.1	-	-	-
58.	Aromadendrene	1444	1442	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-
59.	α-Humulene	1456	1456	0.1	0.1	-	-	0.1	-	-	-	0.1	-	-	0.2	-	0.1	-	-	-	-	0.1	-
60.	(*E*)-β-Farnesene	1460	1460	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1	-
61.	Epi-bicyclosesquiphellandrene	1464	1465	t	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
62.	**Germacrene D**	**1482**	**1483**	**3.3**	**5.3**	**-**	**2.7**	**1.3**	**1.5**	**2.7**	**0.3**	**1.8**	**0.7**	**1.4**	**4.6**	**0.6**	**3.7**	**1.3**	**1.3**	**4.0**	**0.6**	**3.0**	**1.5**
63.	Bicyclogermacrene	1499	1497	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-
64.	α-Muurolene	1505	1502	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65.	Ledene	1497	1504	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66.	γ-Cadinene	1508	1505	0.1	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67.	δ-Cadinene	1524	1526	0.2	0.1	0.1	0.1	t	0.3	-	-	0.1	-	0.3	0.1	-	0.1	-	0.4	0.1	-	0.1	-
68	Caryophyllene oxide	1583	1584	-	-	-	0.1	t	-	-	-	0.2	-	-	-	-	0.1	-	-	-	-	0.1	-
	Total number of identified compounds in each sample			**50**	**38**	**23**	**26**	**41**	**38**	**33**	**22**	**31**	**18**	**34**	**32**	**19**	**32**	**22**	**32**	**36**	**17**	**29**	**30**
	Total identified (%)			**98.3**	**100.0**	**97.0**	**98.5**	**95.8**	**98.4**	**97.9**	**98.1**	**98.9**	**100.0**	**94.4**	**96.2**	**96.6**	**97.4**	**98.9**	**94.3**	**93.5**	**97.8**	**98.0**	**98.4**
	Monoterpene hydrocarbons (MH)			8.2	8.4	4.6	3.9	9.6	8.7	8.7	2.9	3.8	7.08	5.1	5.0	4.0	9.2	13.5	4.5	17.3	5.1	6.7	12.7
	Oxygenated monoterpenes (MO)			80.5	82.7	90.4	89.4	82.6	85.4	84.4	93.5	90.6	91.2	84.8	80.7	90.0	81.5	82.7	83.8	68.0	90.5	85.6	82.5
	Sesquiterpene hydrocarbons (SH)			6.2	7.5	0.9	4.2	2.1	2.1	3.7	0.9	3.6	1.0	2.3	8.8	1.6	5.9	1.7	2.5	6.3	1.0	4.7	2.3
	Oxygenated sesquiterpenes (SO)			-	-	-	0.1	-	-	-	-	0.2	-	-	-	-	0.1	-	-	-	-	0.1	-
	Others (O)			3.5	1.4	1.1	0.8	1.5	2.1	1.1	0.8	0.8	0.7	2.2	1.6	1.0	0.9	0.9	3.6	1.9	1.2	0.9	1.0

Locations: A – Pirići; B – Blagaj; C – Lokve; D – Počitelj;
HD – hydrodistillation; HS-SPME – headspace solid-phase microextraction;
Extraction fibers: F1 – Divinylbenzene/Carboxen/Polydimethylsiloxane fiber (DVB/CAR/PDMS); F2 – Polydimethylsiloxane/Divinylbenzene fiber (PDMS/DVB);
RI – retention indices relative to C_8-C_{28} *n*-alkanes;
t - compounds content lower than 0.1%;
"-" – not detected.

The HS-GC-MS data of *C. nepeta* (sample A) is dominated by pulegone (44.8% - F2; 40.6% - F1) followed by dihydrocarvyl acetate (3.5%; 14.4%), piperitenone (13.7%; 9.3%), neodihydrocarveol (2.9%; 7.9%), limonene (6.2%; 6.2%), *p*-menthone (6.1%; 1.5%), piperitenone oxide (5.2%; 4.1%), germacrene D (5.3%; 3.3%), *trans*-isopulegone (1.8%; 1.2%), (*E*)-hex-2-enal (0.1%; 1.4%), and octan-3-ol (0.8%; 1.1%). However, the most abundant compounds of sample B were determined as piperitenone 48.8% (F1) and 20.7% (F2) by HS-GC-MS, and pulegone (23.9%; 39.7%) was found to be as another abundant compound as well. Limonene (6.1%; 6.3%), dihydrocarvyl acetate (4.3%; 10.0%), *p*-menthone (2.0%; 4.8%), neodihydrocarveol (0.3%; 3.0%), piperitone (1.3%; 2.7%), *cis*-isopiperitenone (1.9%; 0.6%), octan-3-ol (1.1%; 0.5%), *cis*-sabinene hydrate (1.0%; 0.5%), and *trans*-isopulegone (0.3%; 1.9%) were also identified accurately. On the other hand, piperitenone oxide was determined as the major constituent of sample C with the values of 47.7% (F1) and 39.5% (F2), and it followed by *cis*-piperitone oxide (21.1%; 36.6%), dihydrocarvyl acetate (10.2%; 2.1%), limonene (3.6%; 3.1%), piperitenone (1.5%; 0.4%), *cis*-isopiperitenone (1.4%; 0.2%), neodihydrocarveole (1.3%; nd), and octan-3-ol (1.0%; 0.9%). Piperitenone oxide was also dominant in HS of sample D with percentages of 48.3% (F2) and 48.2% (F1), followed by dihydrocarvyl acetate (14.9%; 12.1%), and *cis*-piperitone oxide (0.4%; 14.1%). *trans*-Caryophyllene and germacrene D were found to be the most abundant sesquiterpenes in HS of fresh *C. nepeta*. A higher number of sesquiterpenes were found in fresh *C. nepeta* HS compared to shade-dried samples. In addition, numerous sesquiterpenes present in HS of fresh *C. nepeta* were absent in HS of shade-dried samples. Among these sesquiterpenes, several of them act as repellents, herbivores or attractants, such as germacrene D [23, 26]. Low molecular weight compounds present in HS of fresh samples, were not detected in HS of shade-dried samples nor in EOs samples. It is most likely that they evaporated during the process of drying or HD of the plant material.

3.2. Headspace Composition of Shade-Dried C. nepeta

A total of 39 compounds were found in shade-dried *C. nepeta* using both fibers. F2 extracted more compounds, 26 to 32 (97.4% to 98.9% of the total ion chromatogram (TIC)) than F1 that extracted 17 to 23 compounds that constituted 96.6% to 98.1%. In shade-dried samples, less compounds (both individually and in total) were identified compared to the fresh samples. Piperitenone oxide was found as the dominant compound in the samples C and D. Its abundance was determined as 60.2%; 55.6% (F1) and 59.8%, 56.4% (F2), respectively. The mentioned compound was found in significantly lower abundance in sample B, where piperitenone was the major one (36.3% and 32.9%). HS of *C. nepeta* sample A is dominated by pulegone 24.8% and 23.2% (F1 and F2), followed by piperitenone (24.6%; 17.9%), piperitenone oxide (20.4% and 25.0%), *cis*-piperitone oxide (nd; 12.6%), piperitone (11.5%; nd), *p*-menthone (6.1%; 6.3%), limonene (3.5%; 3.2%), dihydrocarvyl acetate (1.2%; 1.8%), octan-3-ol (1.1%; 0.7%), and germacrene D (nd; 2.7%). HS of sample B mainly consisted of pulegone 34.5% (F1) and 30.1% (F2), *p*-menthone (7.3%; 11.2%), piperitone (6.6%; 7.9%), piperitenone oxide (5.6%; 4.3%), limonene (2.2%; 2.9%), *trans*-caryophyllene (0.5%; 1.2%), and germacrene D (0.3%; 1.8%). The compounds found in moderate percentage of *C. nepeta* HS from sample C were the following: *cis*-piperitone oxide (18.4%; 12.1%), limonene (3.5%; 7.8%), piperitenone (4.9%; 2.7%), germacrene D (0.6%; 3.7%), pulegone (2.9%, 2.4%), dihydrocarvyl acetate (1.1%; 1.5%), *cis*-isopiperitenone (1.1%; 0.9%), *trans*-caryophyllene (0.6%; 1.2%), and octan-3-ol (1.0%; 0.7%). Several compounds, such as *cis*-piperitone oxide (15.4%; 14.1%), pulegone (7.8%; 5.7%), piperitenone (7.9%; 3.3%), limonene (4.6%; 5.7%), germacrene D (0.6%; 3.0%), dihydrocarvyl acetate (1.2%; 2.7%), and octan-3-ol (1.2%; 0.7%) were found in sample D among major constituents.

Variation in qualitative composition of HS extracted on two different coated fibres, besides the fibres polarity, might be influenced by the drying process [36]. Prolonged air exposure of plant material probably could affect the evaporation of several volatile organic compounds [37]. When evaluating HS composition of *C. nepeta*, the presence of several monoterpene hydrocarbons (e.g. α-thujene, camphene, *p*-cymene, sabinene, and *trans*-sabinene hydrate), alcohols (neomenthol, menthol, and terpinene-4-ol), ketone (isomenthone), and few sesquiterpens (β-cubebene, *trans*-α-bergamotene, aromadendrene, and ledene) in fresh samples was observed, but they were absent in shade-dried samples. Numerous compounds of fresh samples were present in higher percentages compared to

shade-dried ones. The drying process or certain chemical reactions might contribute to reduction or absence of certain compounds [36, 37].

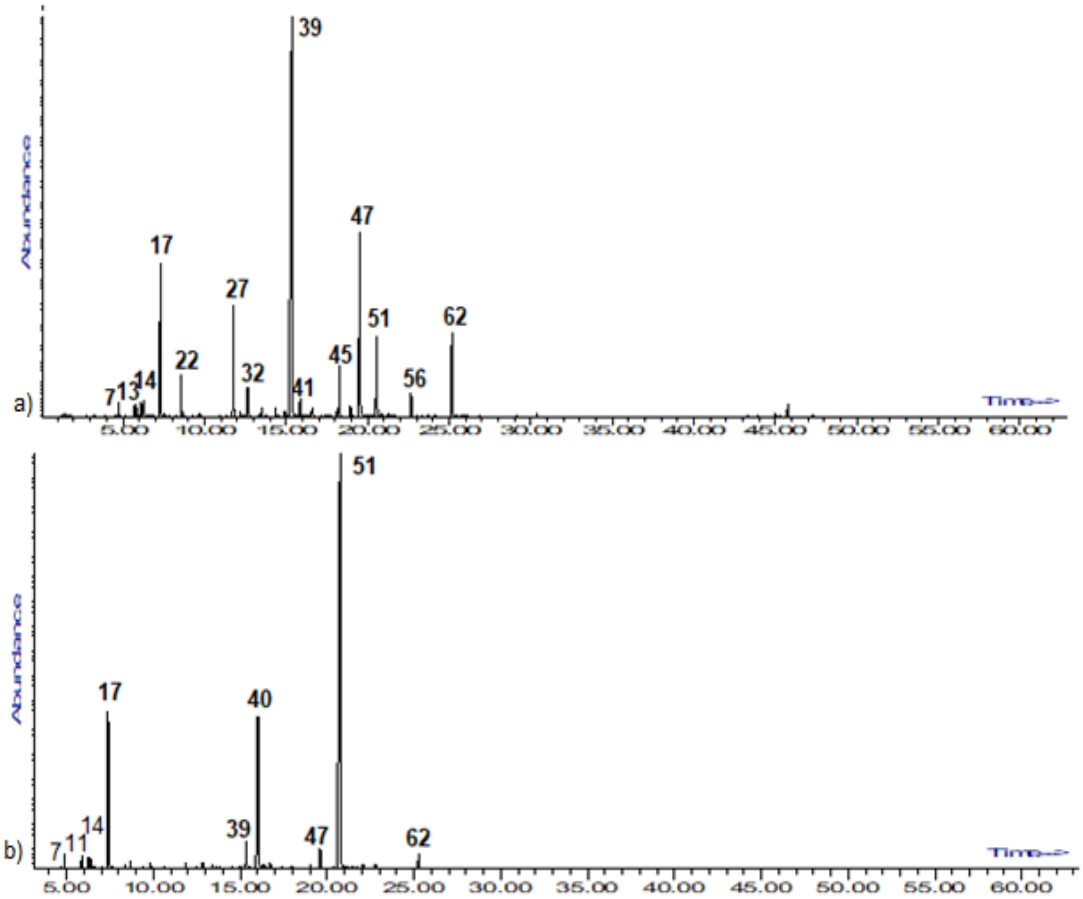

Figure 1. Exemplary chromatograms (GC-MS) of *Clinopodium nepeta* (L.) Kuntze aerial parts: **a**) HS volatiles fractions of fresh sample extracted on fiber F1 (DVB/CAR/PDMS) from location A-Pirići;**b**) EOs volatiles from location C-Lokve. The numbers are related to the numeration used in Table 3

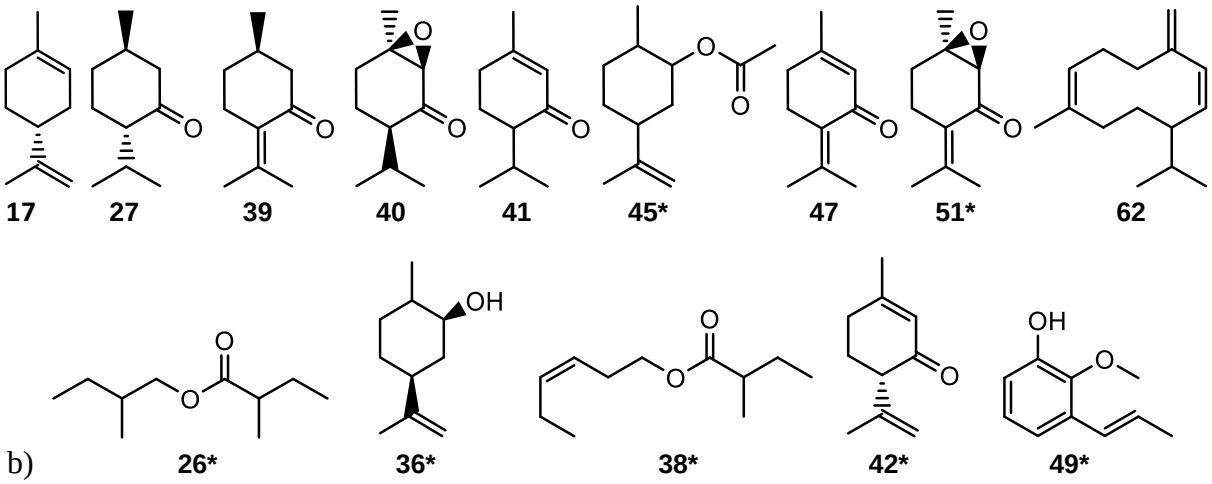

Figure 2. Characteristic chemical structures of *Clinopodium nepeta* (L.) Kuntze HS and EOs volatiles: a) main compounds; b) minor compounds; (*-first time detected compounds in *C. nepeta*). The numbers are related to the numeration used in Table 3

3.3. Chemical Characterization of C. nepeta Essential Oil

The results show 41 compounds in EOs with total chromatogram area percentage 95.8% - 100.0% depending on the origin of *C. nepeta*. HD required high temperatures and longer time, which might have affected the quality and quantity of identified compounds. EOs chemical profile mainly consisted of monoterpene hydrocarbons and oxygenated monoterpenes. According to presented results, pulegone is dominant in the samples A and B (23.2% and 43.9%). Piperitenone oxide (16.1%), piperitenone (14.8%), *cis*-piperitone oxide (14.5%), *p*-menthone (9.7%) and limonene (6.3%) were the main components in the sample A. The sample B also contained piperitenone (27.6%), *p*-menthone (10.3%), piperitone (6.0%), and limonene (4.8%) as the main compounds. Piperitone occurred only in the sample B. Piperitenone oxide was significantly lower in the sample B (1.1%) than in the others. Previous literature data show pulegone as the most common dominant compound of *C. nepeta* EOs [13, 14, 15, 16, 18, 20, 38], which is identical to our samples A and B. However, piperitenone oxide is the most dominant compound in the samples C and D (60.0% and 51.7%), followed by *cis*-piperitone oxide (15.2% and 15.1%), pulegone (2.2% and 8.5%), and piperitenone (2.0% and 3.2%). Sesquiterpenes, as *trans*-caryophyllene and germacrene D, were present in all the samples, but the abundance of germacrene D in sample B (0.7%) was twice as low as in three other samples. Some other sesquiterpenes are detected too, but with minor to negligible presence. It is interesting to note that piperitone is not present at all in either HS or EOs of the samples from locations C and D, although it was detected in the samples from locations A and B. The sample D contains a few compounds that were not found in other samples. Those compounds are mainly present in lower percentages (<1%), e.g. α-thujene, *p*-cymene, (*Z*)-β-ocimene, (*E*)-β-ocimene, 2-methylbutyl 2-methylbutanoate.

The organic volatiles composition of *C. nepeta* EOs from Bosnia and Herzegovina is similar to that in previous researches from different origins. The similarity is reflected in the presence of a variety of components belonging to mono- and sesquiterpenes [13, 14, 18] and also occurs with respect to dominant monoterpene ketone pulegone [14, 18, 21]. But for the first time in the EOs from Bosnia and Herzegovina several components were detected such as: 2-methylbutyl 2-methylbutanoate, *cis*-dihydrocarveole, (Z)-hex-3-en-1-yl 2-methylbutanoate, *cis*-isopiperitenone, dihydrocarvyl acetate, and 2-methoxy-3-(2-propenyl)-phenol. Most of these compounds appeared as minor ones. In all tested samples (in the HS and EOs), oxygenated monoterpenes were predominant (68.0-93.5%), compared to monoterpene hydrocarbons or sesquiterpene hydrocarbons.

Four samples of *C. nepeta* from different locations in Bosnia and Herzegovina showed diversity and variations in the total number and content of identified compounds in HS as well in EOs. Based on the results of the chemical composition of the samples, two chemotypes can be recognized: pulegone/piperitenone chemotype in two samples (A and B) and piperitenone oxide chemotype in other two samples (C and D). Compared to other researcher's reports given in the Reference list, this study provides a more complete chemical profile of *C. nepeta*. Furthermore, the existing data is reinforced by the results of the HS-SPME analysis, which, in addition to others, also detected compounds of small molecular masses. Several of the major identified volatile compounds such as pulegone, piperitone, piperitenone and piperitenone oxide are related to several biological activities. They exhibit antibacterial [39], antifungal activity and sedative effect; they act as pesticides and repellents [5], and have insecticidal properties against insects in their different life stages [5, 39-42].

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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