



Variations of essential oil characteristics of *Clinopodium pulegium* (Lamiaceae) depending on phenological stage

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ABSTRACT: The variability of essential oil characteristics in different phenological stages of *Clinopodium pulegium* from its natural habitat (Svrljiški Timok gorge, Serbia) and from cultivated plants (Niš, Serbia) was determined.

The essential oils were obtained from aerial parts of the plants by hydrodistillation and analyzed by GC-MS. These are the first data on the essential oil characteristics of plants of the population from the Svrljiški Timok gorge.

Samples originating from both natural and cultivated populations were characterized by high amounts of essential oils in all stages of development (0.8% - 1.4%). Twenty-one compounds were identified, representing 95.3-99.6% of the total oils.

Differences in the essential oil composition were more quantitative than qualitative. The quality of the essential oil was stable and did not vary with differences in environmental conditions.

Dominance of the main components was modified by phenological stage. Pulegone was dominant in the vegetative (76.1% wild population, 62.7% cultivated population) and the flowering (49.5%, 64.6%) stages, while menthone (48.5%, 65.3%) displaced pulegone (34.7%, 18.4%) at the fruiting stage.

Cultivated plants in the vegetative and flowering stages can be considered a significant source of pulegone, and in the fruiting stage a significant source of menthone. Careful selection of the developmental stage of the plant is a potential tool which could be employed to obtain the preferred chemical composition of *C. pulegium* for commercial use.

KEY WORDS: *Clinopodium pulegium*, essential oil, pulegone, menthone, phenological stage

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INTRODUCTION

Clinopodium pulegium [syn: *Micromeria pulegium* (Rochel) Benth.] is a species recently transferred from the genus *Micromeria* sect. *Pseudomelissa* (BRÄUCHLER *et al.* 2006).

Numerous *Pseudomelissa* taxa of genus *Micromeria* have been transferred to genus *Clinopodium*, after morphological and molecular studies (BRÄUCHLER *et al.* 2008).

C. pulegium is an endemic S. Carpathian species distributed in S.W. Romania and E. Serbia, inhabiting

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rocky ground, mainly in gorges, at 1000 to 1200 m above sea level. This species is a perennial herb with erect straight stems and ovate, obtuse to acute, more or less crenate-dentate leaves. The leaves are densely punctate on the abaxial side. The bracts are linear. The verticillasters have 10-40 flowers. The calyx is 13-veined, shortly pubescent, sparsely hairy in the throat, the teeth are 1/3-1/2 the length of the tube, linear-lanceolate, equal. The corolla is white or lilac in color (CHATER & GUINEA 1972).

Previous studies on essential oil characteristics of the related species (VLADIMIR-KNEZEVIĆ *et al.* 2001; MEDINE *et al.* 2004; DURU *et al.* 2004; SLAVKOVSKA *et al.* 2005; TELCI & CEYLAN 2007; ŠAVIKIN *et al.* 2010; RADULOVIĆ & BLAGOJEVIĆ 2012; KAROUSOU *et al.* 2012) have determined that they are rich in essential oil (over 0.5%) dominated by oxygenated monoterpenes of the menthane class. Pulegone, isomenthone and menthone are the most frequently present essential oil components.

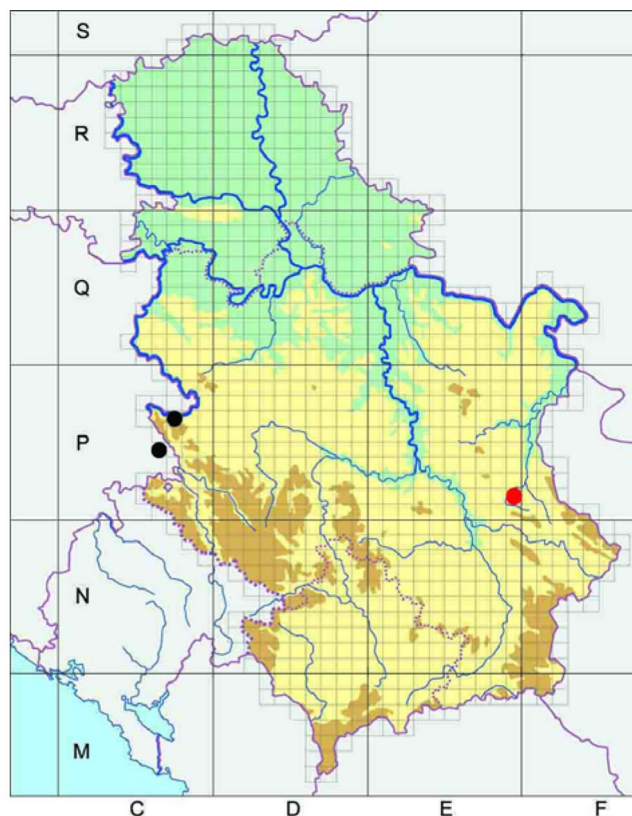
Several *Clinopodium* species have considerable antimicrobial (MEDINE *et al.* 2004; DURU *et al.* 2004; CASTILHO *et al.* 2007; ŠAVIKIN *et al.* 2010; ARSLAN & DERVIS, 2010) and antioxidant (ÖZTÜRK *et al.* 2011; NUR HERKEN *et al.* 2012) activities.

The variability of the amount and composition of the essential oils depending on the season and phenological stages has been studied in many aromatic species of the Lamiaceae family (SANTOS-GOMES & FERNANDES-FERREIRA 2001; MILOŠ *et al.* 2001, MASTELIĆ & JERKOVIĆ 2003; CHORIANOPULOS *et al.* 2006, MARIĆ *et al.* 2006; LAKUŠIĆ *et al.* 2011; NURZYŃSKA-WIERDAK *et al.* 2012; RODRIGUES *et al.* 2013; LAKUŠIĆ *et al.* 2013). There are few publications related to the essential oils of *Clinopodium* species (PUTIEVSKY *et al.* 1995; DUDAI *et al.* 2001; VLADIMIR-KNEŽEVIĆ *et al.* 2001). Pulegone was found to be the major component in oils with only small seasonal and phenological variation in composition.

In this paper we report the content and chemical composition of essential oil of *C. pulegium* collected from the recently recorded population from Svrlijski Timok gorge within the territory of Eastern Serbia (BOGOSAVLJEVIĆ *et al.* 2007). The variability of essential oil characteristics in different phenological stages of both plants growing in their natural habitat and cultivated plants of *C. pulegium* was also studied.

MATERIAL AND METHODS

Plant material: The material was collected from the Svrlijski Timok gorge, Serbia (Map 1). A representative specimen of the sampled population was deposited at the Herbarium of the Institute of Botany, Faculty of Pharmacy, University of Belgrade (HFF, specimen number: 3203). The plant material was collected during the vegetative



Map 1. Distribution of *Clinopodium pulegium* in Serbia [black spots – data from literature (Šilić, 1979; Bogosavljević *et al.*, 2007); red spot – population from Svrlijski Timok gorge]

(June, 2009), flowering (July, 2009) and fruiting stage (September, 2009).

The plants were transferred from their natural habitat into a private garden in Niš. Voucher specimen has been deposited in the Herbarium of the University of Belgrade (BEOU, specimen number: 16404). The aerial parts of these cultivated plants were collected at different developmental stages: vegetative (May, 2010), flowering (August, 2010) and fruiting (beginning of October, 2010).

The material was dried at room temperature.

Isolation of the Essential Oil. The essential oils from the aerial parts of the plants were obtained by hydrodistillation for 3 h in a Clevenger-type apparatus (EUR. PHAR. 6, 2007). The essential oils were dissolved using *n*-hexane and dried over anhydrous sodium sulphate and kept at -40°C until they were analyzed. The oil yield was expressed as ml/100g of dry weight of the plant material.

GC-MS Analysis: GC-MS analysis was carried out using a Hewlett Packard 5973-6890 GC-MS system operating in EI mode at 70 eV, equipped with a HP 5MS capillary column (30 m x 0.25 mm; film thickness 0.25 mm). The initial temperature of the column was 60°C and was heated to 280°C with a rate of 3°C/min. Carrier

gas He; flow rate, 1 ml/min; split ratio, 1:10. Based on the estimated obtained volume of essential oil, the corresponding amount of capillary GC grade pentane was added to afford an appropriate concentration of 10 $\mu\text{l/ml}$. The injection volume was 1 μl . Relative percentage amounts were calculated from total ion chromatograms (TICs).

Identification of the compounds was based on comparison of their retention indices (RI), their retention times (RT) and mass spectra with those obtained from authentic samples and/or the MS library (ADAMS 2001).

RESULTS AND DISCUSSION

Oil content. All samples were characterised by a high essential oil content (0.8% - 1.4%).

The plants from the natural habitat yielded the least amount of oil in the vegetative stage, 1% (v/w), the amount was higher in flowering stage, 1.4% (v/w), while it was slightly lower in the fruiting stage, 1.2% (v/w) (Fig. 1).

The cultivated plants in the vegetative stage contained 1.2% (v/w) of oil. The highest oil content was determined in the flowering stage, 1.3% (v/w), and the lowest in the full fruiting stage 0.8% (v/w).

Some studies have shown that the essential oil content depends on environmental factors (KOKKINI *et al.* 2004; LAKUŠIĆ *et al.* 2011). However, in our studies, the oil content was approximately equal in plants from natural habitats and cultivated plants. The highest amount of essential oil at the flowering stage could be associated with the biological role of essential oils. It is known that the essential oils of many *Clinopodium* species have antimicrobial and antifungal activities (MEDINE *et al.* 2004; DURU *et al.* 2004; CASTILHO *et al.* 2007; ŠAVIKIN *et al.* 2010; ARSLAN & DERVIS 2010), and consequently protect the plant in this very important stage of development from pathogen attacks.

Composition of essential oil. Twenty-one compounds were identified, representing 95.3-99.6% of the total oils (Table 1). Differences in the essential oil composition were more quantitative than qualitative.

Monoterpenoids dominated in the oils (in concentrations higher than even 94%). Among monoterpenoids, oxygenated monoterpenes of the menthane type were present in a very high percentage (90.6%-94.7%). Sesquiterpene components were found in only small quantities (0.5%-2.5%).

Monoterpenoids also dominated in the essential oils of related *Clinopodium* species according to previous reports (STOJANOVIĆ *et al.* 1999; VLADIMIR-KNEZEVIĆ *et al.* 2001; DURU *et al.* 2004; SLAVKOVSKA *et al.* 2005; CASTILHO *et al.* 2007; RADULOVIĆ & BLAGOJEVIĆ 2012).

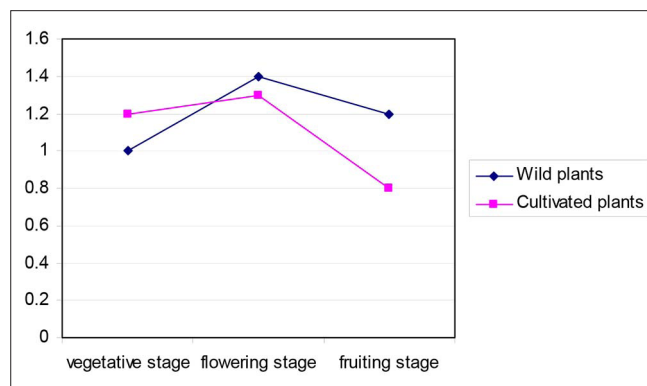


Fig. 1. Oil content (% v/w) of *Clinopodium pulegium*

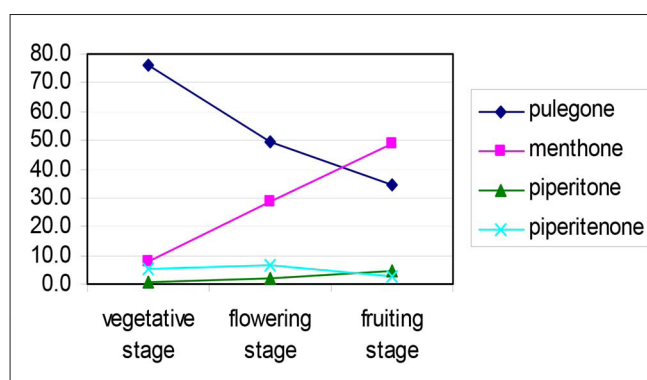


Fig. 2. The dominant constituents (%) of essential oils of wild *Clinopodium pulegium*

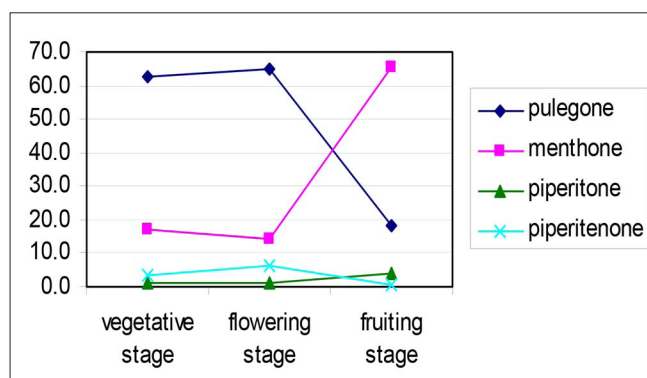


Fig. 3. The dominant constituents (%) of essential oils of cultivated *Clinopodium pulegium*

Based on our results we can conclude that the monoterpene and sesquiterpene ratio is a stable character in the oil of *C. pulegium* and largely independent of environmental conditions and phenological phases.

Dominant compounds in the essential oils. The oils were characterised by a large quantity of C-3 monoterpene

Table 1. Content and chemical composition of the essential oil of *Clinopodium pulegium*

		Wild plants			Cultivated plants			
		June	July	September	May	August	October	
		vegetative stage	flowering stage	fruiting stage	vegetative stage	flowering stage	fruiting stage	
Oil content (% v/w)		1.0	1.4	1.2	1.2	1.3	0.8	
Constituents	RI							
1.	α -pinene	937	0.3	0.9	tr	tr	tr	0.4
2.	sabinene	974	0.2	0.4	tr	tr	tr	0.3
3.	β -pinene	977	0.7	1.2	tr	tr	tr	1.0
4.	myrcene	988	0.1	0.5	tr	tr	tr	0.3
5.	3-octanol	993	0.2	-	-	-	-	-
6.	limonene	1027	1.7	4.1	3.3	4.2	3.5	2.4
7.	(E)- β -ocimene	1041	-	tr	tr	0.1	0.1	tr
8.	menthone	1148	7.8	28.4	48.5	16.9	14.5	65.3
9.	iso-menthone	1157	0.6	1.7	2.8	0.9	0.9	4.1
10.	isopulegone*	1176	0.9	2.1	1.6	3.5	3.5	1.0
11.	α -terpineol	1181	-	tr	tr	tr	0.3	tr
12.	pulegone	1232	76.1	49.5	34.7	62.7	64.6	18.4
13.	piperitone	1247	0.5	2.1	4.5	0.9	1.2	3.8
14.	piperitenone	1337	5.4	6.3	2.4	3.3	6.2	0.6
15.	piperitenone oxide	1360	0.3	0.5	0.2	3.9	2.3	0.2
16.	β -bourbonene	1380	-	tr	0.3	tr	tr	0.3
17.	(E)-caryophyllene	1412	0.1	0.4	0.5	0.5	0.4	0.5
18.	germacrene D	1479	-	1.1	0.3	0.8	1.4	0.4
19.	bicyclogermacrene	1492		0.4	tr	0.1	0.3	tr
20.	spathulenol	1571	0.2	tr	0.4	1.1	0.2	0.3
21.	caryophyllene oxide	1583	0.2	-	-	-	-	-
Monoterpenoids			94.6	97.7	98.0	96.4	97.1	97.8
Hydrocarbons			3.0	7.1	3.3	4.3	3.6	4.4
Oxygenated monoterpenes			91.6	90.6	94.7	92.1	93.5	93.4
Sesquiterpenoids			0.5	1.9	1.5	2.5	2.3	1.5
Hydrocarbons			0.1	1.9	1.1	1.4	2.1	1.2
Oxygenated sesquiterpens			0.4	-	0.4	1.1	0.2	0.3
Other compounds			0.2	-	-	-	-	-
Total			95.3	99.6	99.5	98.9	99.4	99.3

* Correct isomer not identified; RI, Retention Indices relative to C₉-C₂₄ n-alkanes on HP-5MS; %, Relative percentage obtained from peak area; tr, trace (< 0.1%)

ketones such as: pulegone (18.4%-76.1%), menthone (7.8%-65.3%), piperitone (0.5%-4.5%), and piperitenone (0.6%-6.3%).

The main compounds of the oils from wild plants and cultivated plants were pulegone and menthone.

The concentration of pulegone in plants from the natural population (Fig. 2) was the highest (76.1%) in the vegetative stage, and declined over the flowering (49.5%) to the fruiting stage (34.7%). In contrast, the concentration of menthone was lowest in the vegetative stage (7.8%) and then linearly increased over the flowering (28.7%) to the fruiting stage (48.5%).

Quantitatively the oils of cultivated plants were slightly different (Fig. 3). Pulegone was consistently high in both the vegetative (62.7%) and flowering stage (64.6%), while its concentration decreased in the fruiting stage (18.4%). Menthone exhibited a completely opposite tendency. Its concentration was low in the vegetative (16.9%) and flowering stage (14.5%) and high in the fruiting stage (65.3%).

The contents of dominant components in the oils of wild and cultivated plants were characterised by a gradual decrease of pulegone and increase of menthone during plant development under natural conditions (Fig. 2). In the oil of cultivated plants an acute decrease of pulegone and increase of menthone from the flowering to the fruiting phase we observed (Fig. 3). The differences may have resulted from the fact that the wild and cultivated plants were gathered in different months.

The concentration of piperitone in the oil of the wild plants increased from the vegetative stage (0.5%), through the flowering stage (2.1%) to the fruiting stage (4.5%). Cultivated plants also exhibited an upward trend in the concentration of piperitone from the vegetative stage, through the flowering to the fruiting stage (0.9%, 1.2%, 3.8%, respectively).

The oil of wild and cultivated plants in the flowering stage exhibited a significant presence of piperitenone (6.3% and 6.2%, respectively). This compound appeared in lower concentrations in the oil of other phenological stages (from 0.6% to 5.4%).

Very often, pulegone also appears in high concentrations in other species of this genus, for example: *C. thymifolium* (Scop.) Kuntze (MARINKOVIĆ *et al.* 2002; SLAVKOVSKA *et al.* 2005; ŠAVIKIN *et al.* 2010), *C. dalmaticum* (Benth.) Bräuchler & Heubel (KOSTADINOVA *et al.* 2006; ŠAVIKIN *et al.* 2010; RADULOVIĆ & BLAGOJEVIĆ 2012; KAROUSOU *et al.* 2012), *C. fruticosum* (L.) Kuntze (DUDAI *et al.* 2001; BASER 2002), *C. cilicicum* (Hauskn. ex P.H.Davis) Bräuchler & Heubel (DURU *et al.* 2004). Also, pulegone can be found as a dominant component in the essential oils of other species of the Lamiaceae family, for example in the species of genus *Mentha* (RODRIGUES *et al.* 2013) *Ziziphora* and

Cyclotrichium (BASER 2002).

High concentrations of pulegone in the oil of plants in the vegetative and flowering stage can be explained by its biological role. Pulegone in the essential oils may act as part of the plant's protective biochemical response to the actions of other organisms, plants, insects, herbivores, parasites or pathogens (FRANZIOS *et al.* 1997; PAVLIDOU *et al.* 2004; KOUL *et al.* 2008). For example, pulegone is shown to be effective against *Musca domestica*, *Diabrotica virgifera*, *Peridroma saucia*, *Spodoptera litura*. Research done on *Spodoptera eridania* has shown that the larvae treated with pulegone develop into adults with lowered fecundity. Pulegone has also been observed to be toxic against the European corn borer, *Ostrinia nubilalis*. Pulegone is known as an effective fumigant against the rice weevil, *Sitophilus oryzae* (KOUL *et al.* 2008). Moreover, research has shown that pure pulegone has a higher inhibitory activity on the growth of bacteria than the essential oil in total (STOJANOVIĆ *et al.* 2000).

Pulegone has a significant role in allelopathic relations. The inhibitory activity of pulegone on seed germination and sprout growth of *Raphanus sativus* in *in vitro* conditions has been shown to be four times stronger than that of HCl (JANČIĆ 1995).

The essential oil of *Clinopodium* species and its main component pulegone have also been shown to prevent seed germination and plant development of other species (DUDAI *et al.* 1993; DUDAI *et al.* 1999; DUDAI *et al.* 2000).

POTIEVSKY *et al.* (1995), DUDAI *et al.* (2001) and RODRIGUES *et al.* (2013) have shown that the concentration of some essential oil components increases or decreases at the expense of other components during the plant life cycle. The amounts of pulegone and menthone in the essential oil of *Mentha pulegium* L. (RODRIGUES *et al.* 2013), and *C. pulegium* studied here follow the same pattern.

FRANZIOS *et al.* (1997) demonstrated that the synergistic/antagonistic relationships between essential oil components can alter the toxicity of the entire essential oil. Pulegone was found to be significantly more effective as an insecticide, while menthone was less effective, when used in their authentic forms. A mixture of authentic pulegone and menthone, in relative quantities resembling their content in the oil of *M. pulegium* showed that the strong toxicity of pulegone was suppressed in the presence of menthone. Thus, we conclude that the essential oil obtained in the fruiting stage in our experiment would be less toxic, with a lower insecticidal activity than oils obtained in the vegetative and flowering stages. The use of *C. pulegium* essential oil as a bio-pesticide should be possible only if the essential oil is isolated in the vegetative or flowering stage of development.

CONCLUSION

C. pulegium originating from both natural and cultivated populations was characterized by high amounts of essential oils in all stages of development. However, this species had the highest yield in the flowering stage.

The quality of the essential oil was stable and did not vary with the change of environmental conditions.

The dominance of the main components was modified with phenological stages. Pulegone was dominant in the vegetative and the flowering stage, while menthone displaced pulegone in the fruiting stage.

Cultivated plants in the vegetative and flowering stage could be considered a significant source of pulegone, and in the fruiting stage a significant source of menthone. Careful selection of the developmental stage of the plant is a potential tool which could be employed to obtain the preferred chemical composition of *C. pulegium* for commercial use.

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REZIME

Promenljivost osobina etarskog ulja vrste *Clinopodium pulegium* (Lamiaceae) u zavisnosti od fenološke faze

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U radu je praćena promenljivost kolićine i sastava etarkog ulja *Clinopodium pulegium* kroz različite stadijume razvoja biljke (vegetativni, stadijum cvetanja i plodonošenja).

Materijal je sakupljan iz samonikle (klisura Svrljiškog Timoka) i gajene (Niš) populacije. Etarsko ulje je izolovano iz nadzemnih delova biljaka destilacijom vodenom parom i analizirano GC-MS metodom. Ovo su prvi podaci o osobinama etarskog ulja biljaka populacije iz klisure Svrljiškog Timoka.

Svi uzorci kako iz samonikle tako i iz gajene populacije su se odlikovali velikim sadržajem ulja (0.8% - 1.4%) u svim stadijumima razvoja. Dvadeset jedna komponenta je identifikovana što predstavlja 95.3-99.6% ukupnog etarskog ulja.

Razlike u sastavu ulja su bile više kvantitativne nego kvalitativne. Kvalitet etarskog ulja je bio stabilan a variranja uslovljena promenom sredine relativno mala.

Dominacija glavnih komponenata je bila uslovljena promenama fenoloških faza. Pulegon je bio dominantan u vegetativnoj (76.1% u ulju biljaka samonikle populacije, 62.7% u ulju biljaka gajene populacije) i fazi cvetanja (49.5%, 64.6%), dok je menton bio dominantan (48.5%, 65.3%) u fazi plodonošenja.

Rezultati su pokazali da se biljake u vegetativnoj i fazi cvetanja mogu smatrati značajnim izvorom pulegona, a u fazi plodonošenja značajnim izvorom mentona. Pažljivim odabirom razvojnog stadijuma biljke moguće je dobiti etarsko ulje željenog sastava koje bi se moglo upotrebiti u komercijalne svrhe.

Ključne reći: *Clinopodium pulegium*, etarsko ulje, pulegon, menton, fenološke faze