Qualitative and Quantitative Changes in the Essential Oil of Origanum vulgare L. ssp. gracile as Affected by Different Harvesting Times

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ABSTRACT

Qualitative and quantitative variations in the essential oil of wild growing Origanum vulgare L. ssp. gracile plants were studied in response to different phenological stages (pre, full and post-flowering). The essential oil of air-dried leaves was isolated by water distillation using a Clevenger-type apparatus and was analyzed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC/MS). The highest (1.87%) and the lowest (1.01%) essential oil content were obtained from post-flowering and preflowering stages, respectively. In total, 24 components were identified and quantified in three phenological stages representing 96.75, 97.63, and 98.59% of the oil, respectively. Carvacrol (46.62, 46.5 and 27.6%), ρ -cymene (7.76, 13.54 and 37.08%) and γ -terpinene (21.54, 13.91 and 6.82%) were the main constituents of essential oils in pre, full, and postflowering stages, respectively. Oxygenated monoterpenes (43.35-61.32%) and monoterpene hydrocarbons (30.81-48.02%) were the main classes of identified compounds in three essential oils. According to the findings of this research, the postflowering stage can be considered as the most appropriate time for obtaining the highest essential oil content, but to achieve the highest rate of phenolic compounds, the preflowering and full-flowering stages can be recommended.

Keywords: Carvacrol, Oregano, ρ -Cymene, Phenological stage, γ -Terpinene.

INTRODUCTION

The genus *Origanum* (Lamiaceae family), which includes about 38 species and 6 subspecies, is native to the Mediterranean Region and it is widely distributed in many parts of the world in West, East and Central Asia, South Europe, and North Africa (Ietswaart, 1980; Vokou *et al.*, 1993). The *Origanum* species, due to richness in essential oils, have been used for centuries as spices and as local medicines in traditional medicine (Fleisher and Fleisher, 1988).

O. vulgare L. is one of the most important species of this genus, which is widely distributed in the Mediterranean, Euro-Siberian and Irano-Turanian Regions (Spada Perrino, 1997). The Iranian flora comprises three subspecies of O. vulgare L. (ssp. viride, ssp. vulgare and ssp. gracile), which are distributed in the north and northwest of the country (Mozaffarian, 1996; La Gow, 2004). O. vulgare ssp. gracileis a perennial herb with white flowers, the leaf is nearly elliptical, covered with glandular hairs, which are more numerous on the lower leaf side. This subspecies is native to Turkey, Afghanistan, Iran, north of Iraq, northwest of

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Pakistan, and south and center of Russia. In Iran, it grows wild in north and west provinces (Ietswaart, 1982).

Since ancient times, the *Origanum* leaves have been used as a spice for flavoring of food, and it is also used for medicinal carminative, diaphoretic, purposes, as expectorant, sedative, stimulant, stomachic, antineuralgic, diuretic, antitussive, antirheumatic (Skoula and Kamenopoulos, 1996; Afsharypour et al., 1997; Kintzios, 2002; Dundar et al., 2008). In addition, due to the presence of phenolic compounds (mainly carvacrol and occasionally thymol) in the oil, its antimicrobial, antifungal, insecticidal, and antioxidative effects have received considerable attention in recent years (Kokkini et al., 1997; Kulisic et al., 2004; Bakkali et al., 2008; Hassani et al., 2012).

The biosynthesis of secondary metabolites in plants is not only controlled by interaction between the genotype and environment, but also is strongly influenced by growth stages (harvest time), method of distillation, part of plant utilized, and postharvest drying and storage (Figueiredo et al., 2008; Nejad Ebrahimi et al., 2008; Ozkan et al., 2010; Rostaefar et al., 2017). Like in any other essential oil bearing plants, one of the most important characteristics of oil accumulation in Origanum species is its dependence on the developmental (ontogenetic) stage (Ozkan et al., 2010; Toncer et al., 2009). In Thymus vulgaris, it was reported that oil qualitative changes induced by ontogenetically variation were more than changes caused environmental conditions (Ozguven Tansi, 1998).

The essential oil composition of different species of *Origanum* has been studied in many researches (Sezik *et al.*, 1993; Arnold *et al.*, 1993; Vokou *et al.*, 1993; Baser *et al.*, 1994; Afsharypour *et al.*, 1997; Toncer *et al.*, 2009; Ozkan *et al.*, 2010), but there are only a few reports on the essential oil of *O. vulgare* ssp. *gracile* from France (Chalchat and Bernad, 1999) and Turkey (Kilic and Bagci, 2008). Since there is no bibliographic data reporting the qualitative and quantitative variations in the essential oil of *O.*

vulgaressp. gracile plants in response to different growth stages, this study was conducted to investigate the effects of three phenological stages on essential oil content and composition of *O. vulgare* ssp. gracile plants growing wild in Iran.

MATERIALS AND METHODS

Plant Material

The areal parts of *O. vulgare* ssp. *gracile* were harvested at three phenological stages: pre-flowering or vegetative, just before the flower buds appeared (June 1), full flowering (July 1), and post-flowering or beginning of the seed set (August 10) stages, in 2012, from its wild habitat in the Saral Region (mountains between Sanandaj and Divandareh) (35° 24′ to 35° 54′ N; 46° 36′ to 47° 00′ E; 2,300 meter above sea level) in Kurdistan Province, Iran. A voucher specimen (No. 9428) was deposited in the herbarium of the Agriculture and Natural Resources Center of Kurdistan Province.

Essential Oil Extraction

Plant samples from each harvest time were air dried in shade at room temperature. Essential oils were extracted by water distillation of dried and powdered leaves (25 g) for 3 hours, using Clevenger type apparatus. The essential oil content was expressed as volume per weight (v/w) based on plants material oven-dried weight. The essential oil extraction was repeated three times and the average of three replications was reported as essential oil content for each harvesting time.

GC and GC/MS Analysis

The analysis of oils was carried out using a Shimadzu 9A gas chromatograph equipped with a Ph-5 column (30 m \times 0.1 mm, film thickness 0.25 μ m). Oven temperature was

held at 60°C for 5 minutes and then programmed to 250°C at a rate of 3°C min⁻¹ and kept constant at 250°C for 10 minutes. Injector and detector (FID) temperature were 260°C and Helium (with 99.999% purity) was used as carrier gas with a linear velocity of 32 cm s⁻¹. Data were calculated by electronic integration of FID peak area without using response correction factor. GC/MS analysis was also carried out on a Varian 3400 GC/MS system equipped with a DB-5 fused silica column (30 m×0.25 mm, film thickness 0.25 μm). Oven temperature program was 50-250°C at a rate of 4°C/min, transfer line temperature was 260°C, carrier gas was Helium (with 99.999% purity) with a linear velocity of 31.5 cm s⁻¹, split ratio 1/60, with ionization energy of 70eV.

The essential oil components were identified by comparison of their mass spectra with those of a computer library or with authentic compounds, and confirmed by comparison of their retention indices either with those of authentic compounds or with data reported in the literature (Davies, 1990; Adams, 2017).

RESULTS AND DISCUSSION

Essential Oil Content

The essential oil content of *O. vulgare* ssp. *gracile* differed among the three harvesting times. The highest essential oil content was obtained in post-flowering stage (1.87%) followed by full flowering (1.44%) and preflowering (1.01%) stages, respectively (Table 1).

Our results are in agreement with the findings of other researchers who indicated

Table 1. Essential oil components of *O. vulgare* ssp. *gracile* leaves in three phenological stages.

No	Compounds	RI ^a	RI ^b	Pre-flowering	Full-flowering	Post- flowering	
	_			_	_		
1	α-Thujene	928	930	0.80	0.72	0.73	
2	α-Pinene	937	939	0.70	0.47	0.81	
3	Octen-3-ol	983	979	0.49	0.50	0.86	
4	3-Octanone	991	984	3.51	3.50	5.37	
5	Myrcene	999	991	-	-	0.71	
6	α-Phellanderene	1005	1003	0.24	0.24	0.49	
7	α-Terpinene	1015	1017	2.06	1.23	0.70	
8	ρ-Cymene	1025	1025	7.76	13.54	37.08	
9	1,8-Cineole	1033	1031	2.44	2.76	3.75	
10	(Z)-β-ocimene	1044	1037	0.43	0.56	0.51	
11	γ-Terpinene	1058	1060	21.54	13.91	6.82	
12	Terpinolene	1081	1089	0.21	0.14	0.17	
13	Cis-ρ-menth-2-en-1-ol	1118	1122	0.51	0.47	0.44	
14	Terpinene-4-ol	1161	1177	0.56	0.94	0.86	
15	α-Terpineol	1172	1189	0.15	1.07	0.61	
16	Thymol methyl ether	1225	1235	0.13	0.15	-	
17	Carvacrol methyl ether	1236	1245	4.82	7.19	8.54	
18	Thymol	1291	1290	2.61	2.24	1.55	
19	Carvacrol	1304	1299	46.62	46.50	27.60	
20	E-caryophyllene	1408	1419	0.48	0.85	0.45	
21	Germacrene D	1497	1485	0.28	0.24	0.18	
22	Germacrene A	1505	1509	0.20	0.19	-	
23	Spathulenol	1555	1578	0.21	0.22	0.24	
24	Caryophyllene oxide	1560	1583	-	-	0.12	
	Total			96.75	97.63	98.59	
	Essential oil content (%)			1.01	1.44	1.87	

^a Retention Indices, ^b RI from Adams (2017).



that the essential oil content of O. onites was highest at the end of flowering and beginning of seed formation stage (Yaldiz et al., 2005; Ozkan et al., 2010). Similarly, the highest essential oil content of O. vulgare ssp. Hirtum (Baranauskiene et al. 2013; Krol et al. 2019), O. vulgare ssp. vulgare (Baranauskiene et al., 2013), and Thymus vulgaris (McGimpsey et al., 1994; Ozguven and Tansi, 1998) plants were obtained at full flowering and post-flowering (after full blooming) stages. According to Sellami et al. (2009), the low rate of volatile compounds biosynthesis during vegetative stage may be due to partial inactivation of enzymes necessary for the biosynthesis of certain compounds.

The oil content in this study was higher than the values reported in France (0.7%) (Chalchat and Bernad, 1999) and Turkey (0.9-1.2%) (Kilic and Bagci, 2008). These differences may be due to the different environmental and genetic factors, harvesting time, as well as different essential oil extraction procedures.

Essential Oil Composition

The essential oils of *O. vulgare* ssp. *gracile* were analyzed by GC and GC/MS. The identified essential oil components as well as the main classes and subclasses of oil constituents are shown in Tables 1 and 2, respectively. In total, 24 components were identified and quantified in three

phenological stages representing 96.75, 97.63, and 98.59% of the oil, respectively. The main constituents of leaf oils in preflowering (vegetative) and full flowering stages were carvacrol (46.62 and 46.5%), γ terpinene (21.54 and 13.91%), ρ -cymene (7.76 and 13.54%), carvacrol methyl ether (4.82 and 7.19%), and 3-octanone (3.51 and 3.5%), respectively. In the post-flowering stage (beginning of seed set), ρ -cymene (37.08%), carvacrol (27.6%), carvacrol methyl ether (8.54%) and 3-octanone (5.37%) were the main components of essential oil. There are a few published reports on the essential oil analysis of this subspecies from different origins. The main components of O. vulgare ssp. gracile growing in Turkey were β -ocimene, β caryophyllene and germacrene (Sezik et al., 1993), β -caryophyllene and thymol (Baser, and thymol, γ -terpinene, α terpinolene, carvacrol, ρ -cymene, carvacrol methyl ether and thymol methyl ether (Kilic and Bagci, 2008). Investigation on essential oil composition of O. vulgare ssp. gracile from France showed that oils had high levels of sabinene (Chalchat and Bernad, 1999). In another study, the main components of O. vulgare ssp. gracile oil from Italy were thymol, carvacrol and ρ -cymene (De Mastro, 1996). Qualitative differences observed in the chemical composition of the oil samples were not so remarkable. The essential oils from pre-flowering and full flowering stages were found to have a completely similar composition, but in the oil of post-flowering

Table 2. Main classes and subclasses of essential oil constituents of O. vulgare ssp. gracile.

Class and subclass of compounds			(%)			
		Pre-	Full-	Post-		
		flowering	flowering	flowering		
Monoterpenes		91.58	92.13	91.37		
-	Oxygenated monoterpenes	57.84	61.32	43.35		
	Monoterpene hydrocarbons	33.74	30.81	48.02		
Sesquiterpenes		1.17	1.49	0.99		
	Oxygenated sesquiterpenes	0.21	0.22	0.36		
	Sesquiterpene hydrocarbons	0.96	1.27	0.63		
Others		4.00	4.00	6.23		
Total identified		96.75	97.63	98.59		

stage, two components (thymol methyl ether and germacrene A) were not identified. In addition, myrcene and caryophyllene oxide were present only in the post-flowering stage oil. However, from a quantitative point of view, remarkable differences existed among the essential oils. The greatest changes occurred in carvacrol, ρ -cymene and γ -terpinene contents. These components are biosynthetically related (Müller-Riebau et al., 1997). According to a proposed biogenetic pathway, γ-terpinene and ρcymene are the precursors of thymol and carvacrol (Ozguven and Tansi, 1998). The occurrence of the highest amounts of thymol and carvacrol and the lowest values of their precursors (γ -terpinene+ ρ -cymene) in the pre-flowering stage confirms this claim. At the post-flowering stage, the amount of carvacrol+thymol was decreased, but the amount of their precursors increased.

In total, the amounts of octen-3-ol, ρ -cymene, 1,8-cineole, carvacrol methyl ether and spathulenol increased during plant development to reach a maximum during the post-flowering stage. On the other hand, the highest content of α -terpinene, γ -terpinene, Cis- ρ -menth-2-en-1-ol, thymol, carvacrol (as major component), and germacrene D was observed in pre-flowering stage and decreased during plant development to reach its minimum percentage during the post-flowering stage.

The main classes of constituents in three oil samples were monoterpenes (91.58, 92.13 and 91.37%) and sesquiterpenes (1.17, 1.49, and 0.99%), respectively. Oxygenated monoterpenes were the dominant subclass of identified compounds in pre-flowering (57.84%) and full-flowering (61.32%) stages, with carvacrol, carvacrol methyl ether, 1,8-cineole and thymol as major components. Monoterpene hydrocarbons were the main subclass (48.2%) at the postflowering stage due to the presence of ρ cymene and y-terpinene. It was also found the number and amounts sesquiterpenes were low in oils (Table 2).

The results of this study showed that a close relationship exists between the

phenological stages and the production of essential oil and phenolic compounds (mainly carvacrol, carvacrol methyl ether, and thymol) in the *Origanum* plants. In agreement with the results of this study, Krol *et al* (2019) reported that the beginning of blooming stage should be chosen when raw material with the highest flavonoids and monoterpenoid phenols (carvacrol and thymol) content is expected.

In the essential oil bearing plants, the content of the essential oil is mostly variable and many factors such as genetic variation, geographical origin, climatic conditions, plant growth phase, and postharvest processes (drying, distillation etc.) play an important role in yield and composition of essential oils (Figueiredo et al., 2008; Nejad Ebrahimi et al., 2008; Ozkan et al., 2010; Rostaefar et al., 2017). According to Grevsen et al. (2009), the development stage has a significant impact on the content and composition both terpenes of polyphenols in O. vulgare ssp. hirtum, and the optimal harvest time depends on the compounds of interest. Hence, optimization of cultivation conditions and harvesting time to obtain higher quantity and quality of essential oils that fit market requirements is crucial. Essential oils rich in phenolic compounds, such as carvacrol and thymol, are widely reported to possess high levels of antimicrobial, antifungal, insecticidal, and antioxidative activity (Kokkini et al., 1997; Kulisic et al., 2004; Bakkali et al., 2008; Nejad Ebrahimi et al., 2008; Hassani et al., 2012).

CONCLUSIONS

The essential oils obtained from different harvesting times showed considerable variation in content and composition. Postflowering stage could be considered the best harvesting time for the maximum essential oil content. On the other hand, the oil of this subspecies was found to be rich in the active monoterpene phenols (carvacrol and thymol) and their corresponding monoterpene

hydrocarbon precursors (ρ -cymene and γ -terpinene). The highest carvacrol content as the main phenolic compound was obtained at pre- and full-flowering stages. Based on the content and biological activity (antimicrobial, antifungal, antioxidant etc.) of essential oil, this subspecies should be harvested in full-flowering stage.

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تغییرات کمی و کیفی اسانس مرزنجوش بخارایی .(Origanum vulgare ssp. تغییرات کمی و کیفی اسانس مرزنجوش بخارایی gracile)

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چکیده

تغییرات کمی و کیفی اسانس گیاهان خودروی مرزنجوش بخارایی .gracile) تحت تاثیر مراحل فنولوژیکی مختلف (قبل از گلدهی، گلدهی کامل و پس از گلدهی) مورد مطالعه قرار گرفت. برای این منظور اسانس برگهای خشک شده به روش تقطیر با آب و با استفاده از دستگاه کلونجر استخراج شده و به وسیلهی دستگاههای کروماتو گرافی گازی (GC) و کروماتو گرافی گازی (GC) و (GC/MS) مورد آنالیز قرار گرفتند. بیشترین (۱/۱۸) کروماتو گرافی گازی متصل به طیفسنج جرمی (GC/MS) مورد آنالیز قرار گرفتند. بیشترین (۱/۱۸) کروماتو گرافی گازی محتوی اسانس به ترتیب در مراحل پس از گلدهی و قبل از گلدهی بدست آمد. در مجموع، ۲۴ ترکیب که به ترتیب ۱۹۶۷۵ کر ۹۶/۷۶ و ۹۸/۵۹ کر اسانس سه مرحله فنولوژیکی اتشکیل میدادند شناسایی گردید. کارواکرول (۴۶/۶۲ گره ۴۶/۵ گروههای عمده ترکیبات مهم اسانس به ترتیب در مراحل قبل از گلدهی، گلدهی کامل و پس از گلدهی بودند. مونو ترپنهای اکسیژندار (۴۱/۳ گره ۴۱/۳۵ کر و ۱۳/۸۵ کر و ۱۳ ترکیبات شناسایی شده مرحله بودند. طبق یافتههای این تحقیق، برای بدست آوردن بالاترین محتوی اسانس، مرحله ی س از گلدهی مناسب ترین زمان برداشت است اما برای حصول بالاترین مقادیر ترکیبات فنلی مرحله ی پس از گلدهی مناسب ترین زمان برداشت است اما برای حصول بالاترین مقادیر ترکیبات فنلی در اسانس، برداشت در مراحل قبل از گلدهی و گلدهی کامل قابل توصیه میباشد.