

## Research article

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### Antioxidant Capacity of *Origanum heracleoticum* L. Flower and Leaf Extracts and Their Essential Oil Profiles of Plants from Micropropagation and Collection from Natural Habitats

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#### Abstract

##### Keywords

*Origanum heracleoticum* L.;  
essential oils;  
enzyme and non-enzyme  
plant antioxidant capacity;  
micropropagation

The composition of the essential oils and the antioxidant properties of *Origanum heracleoticum* L. leaves and flowers collected from four different natural populations in Bulgaria (two locations in Kresna Gorge and two locations in Rhodopes Mountain) were studied and compared with those of micropropagated and field-adapted plants. Explants for micropropagation from wild-growing plants from Gorna Breznitsa (Kresna Gorge) were used. The enzyme antioxidant potential characterised by the activity of superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase as well as the non-enzyme antioxidant potential characterized by the content of phenols, flavonoids, water- and lipid-soluble antioxidant metabolites, were affected by environmental conditions and type of propagation (wild or micro). The highest enzyme antioxidant potential was observed in the flowers and leaves of the micropropagated plants, followed by the plants collected from Gorna Breznitsa. The variation in the content of metabolites with antioxidant potential in the Greek oregano collected from the two locations from each two areas of Bulgaria was detected. The higher content of phenols and flavonoids was detected in micropropagated plants as well as in wild-plants collected from two localities from Kresna Gorge, when compared with the wild-plants collected from Eastern Rhodopes. Forty-five compounds in the *O. heracleoticum* essential oil collected from the native populations were identified. Based on the essential oil composition, and especially on the carvacrol and thymol contents, the *O. heracleoticum* plants from all investigated natural populations in Bulgaria belonged to the carvacrol chemotype. Besides environmental conditions, another factor that affected the composition of *O. heracleoticum* essential oil was the existence of chemotypes.

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## 1. Introduction

*Origanum heracleoticum* L. (*Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart), also known as “Greek oregano”, is a perennial shrub that is endemic to the Mediterranean area, Balkan Peninsula and Southwest Asia [1]. It prefers mountainous areas with dry rocky soil and plenty of sun. Greek oregano is a good source of natural biologically active compounds can be used in the food, pharmaceutical and cosmetic industries. The type of components and the quantity of the essential oil of spices are highly dependent on the geographical location (local growing conditions) and harvest season [2, 3]. Analysis of the chemical compositions of the hexane and methanol extracts of *O. heracleoticum* L. by GC-MS revealed that Greek oregano populations contained high amounts of carvacrol, thymol, and *p*-cymene [4]. The total phenols and carvacrol (monoterpenoid phenol) contents of the methanol extract were higher than those of the hexane extract, which contributed significantly to its higher total antioxidant capacity. Baser *et al.* [5] detected 48 compounds in *O. heracleoticum* L. essential oil that had been collected from different wild populations in Turkey. The authors reported that carvacrol content was the highest in the oils, and it varied between 23.43%-78.73%. Furthermore, oil from Aydin, Nazilli, Tapan mountain, was the only oil that had thymol content higher than carvacrol. Kokkini and Vokou [6] found that Greek oregano of Greek origin was very rich in essential oil; the oil was found at up to 8%. Other studies showed that phenols, thymol and carvacrol were the dominant compounds in *O. vulgare* L. ssp. *hirtum* (Link) Ietswaart) oil [7-9]. The chemical components of the essential oil could be used for the characterization of different subgroups of Greek oregano. For example, based on the main components of the essential oils, Fleisher and Sneer [10] classified them into three chemotypes: thymol, carvacrol, and a combination of them in almost equal amounts. De Martino *et al.* [9], exploring the essential oils from different wild populations of Greek oregano in Southern Italy, identified three different chemotypes: two chemotypes characterized by high percentages of phenols: carvacrol/thymol, and thymol/ $\alpha$ -terpineol, and a third one with a prevalence of linalyl acetate/linalool. D'antuono *et al.* [11] reported that in Greek oregano plants collected from Levanto and Monterosso sites in Italy, the essential oil was characterized by low thymol and high carvacrol content. However, the group of plants collected from Finale, Savona, Recco, Sestri and Cerri sites in Italy had higher thymol and lower carvacrol content.

*In vitro* vegetative propagation with a high multiplication rate has several advantages over the traditional methods of propagation through seed. One advantage is that it makes the production of pathogen-free homogeneous plants possible in a relatively short period, irrespective of the season and weather. Nevertheless, there is a limited number of studies concerning the influence of micropropagation on the essential oil composition and antioxidant capacity of leaf extracts from Greek oregano.

Wild populations of *O. heracleoticum* L. from Bulgaria have been poorly investigated. Therefore, our research was focused on the geographical characterization of subspecies from different regions in Bulgaria as potential sources of phenolic antioxidant compounds based on essential oil chemical composition and leaf extract antioxidant potential and compared with micropropagated and cultivated plants.

## 2. Materials and Methods

The aerial parts of wild populations of *O. heracleoticum* L. during the full flowering stage were collected in 2018 from the following four natural habitats regions of Bulgaria, from the end of July to 4 August: 1) M - Madzharovo (GPS data: 41°38'36.4"N 25°52'11.9"E) and 2) G - Gugutka (GPS data: 41°25'32.4"N 25°54'55.8"E) are villages in the Eastern Rhodopes, Southeast Bulgaria; 3) R –

Rafting center situated in the banks of the river Struma (GPS data: 41°50'00.1"N 23°09'09.9"E) and 4) B – village Gorna Breznitsa (GPS data: 41°45'04.7"N 23°06'29.8"E) in Kresna Gorge, Southwest Bulgaria. The plants were harvested within a week, under the same weather conditions. The plants were authenticated by Dr. Inna Aneva, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Bulgaria. Voucher specimens were deposited at the Botany Department Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. The aerial parts of plants were cut and part of them was immediately weighed and frozen. The other part of the samples was air-dried in the shade under laboratory conditions.

A mature plant of *O. heracleoticum* L., collected from the natural population from village Gorna Breznitsa in Kresna Gorge, Bulgaria, was used as a source plant for micropropagation. The stem tip explants were cut and inoculated on Murashige and Skoog medium with 500 mg l<sup>-1</sup> CaCl<sub>2</sub>, 30 mg l<sup>-1</sup> sucrose, and 7 g l<sup>-1</sup> agar supplied with 1.0 mg l<sup>-1</sup> zeatin for shooting [12]. Well elongated plantlets were transferred to half-strength MS with 0.5 mg l<sup>-1</sup> IBA for rooting initiation. The rooted plants were carefully taken out from the tubes, washed under running tap water to remove the gelling agent, and transferred to 10 cm diameter pots filled with peat: perlite (2:1 v/v) for *ex vitro* adaptation. The pots were covered with porous polyethylene in order to maintain high humidity (90% RH). The polyethylene was removed after two weeks. After six weeks, the plants were transferred to the experimental field.

The plant tissues (0.5 g flowers and leaves) were ground with liquid nitrogen and 5 ml of 100 mM potassium phosphate buffer, pH 7.5 containing 0.5 mM EDTA, 1 % PVP and PMSF for enzyme extraction for the determination of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPO) activities [13]. After each extract was centrifuged at 12,000 g at 4°C for 30 min, the supernatant was used for the enzyme activity estimation. Total SOD (EC 1.15.1.1) activity was determined and expressed according to Giannopolitis and Ries [14]. The absorbance was recorded at 560 nm, and the volume of sample causing 50% inhibition in absorbance increase was taken as one unit of SOD activity and the activity was expressed as units per mg of protein. The CAT (EC 1.11.1.6) activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H<sub>2</sub>O<sub>2</sub> consumption and was expressed as mmol of H<sub>2</sub>O<sub>2</sub> decomposed per minute per mg of protein [15]. For the APX (EC 1.11.1.1) activity assay, the decrease in absorbance due to ascorbic acid at 290 nm was observed [16]. The specific activity of the enzyme was expressed as mmol ascorbate oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>. GPO (EC 1.11.1.7) activity was determined by Lagrimini *et al.* [17]. Enzyme specific activity was calculated as the increase in absorbance ( $\Delta E$ ) min<sup>-1</sup> mg<sup>-1</sup> protein. The soluble protein concentration was determined according to the method proposed by Bradford [18] using bovine serum albumin as standard protein.

For the determination of metabolites with antioxidant capacity, dry flower and leaf samples (1 g) were ground and extracted with 96% (v/v) methanol. The amount of total phenolic content in the *O. heracleoticum* methanolic extract was determined spectrophotometrically using the Folin-Ciocalteu reagent and calculated as caffeic acid equivalents [19]. The total flavonoid content in plant tissues was measured spectrophotometrically using a method based on the formation of the flavonoid–aluminium complex [20], and the standard curve of catechin. For the determination of water-soluble (WS-AOM) and lipid-soluble (LS-AOM) antioxidant metabolites expressed as equivalents of ascorbate and  $\alpha$ -tocopherol, respectively, plant samples were extracted with water and hexane. After centrifugation, the supernatants were used for spectrophotometric quantification of the metabolites through the formation of the phospho-molybdenum complex [21]. The free radical-scavenging activity was determined using a method based on the reduction of a methanolic solution of the purple coloured free radical DPPH<sup>•</sup> (1,1-diphenyl-2-picrylhydrazyl free radical) [22]. The antioxidant testing involved transition metal reduction that could be monitored by colourimetric-reduction of ferric ions: FRAP (ferric reducing antioxidant power) [23].

The investigated plants were collected between 9:00 and 10:00 am by randomized collection of 30 individual plants per treatment. The air-dried and finely ground inflorescence

samples were hydrodistilled in Likens–Nickerson apparatus with modification, for 2 h [24]. The hydrodistillates were analyzed by gas chromatography and gas chromatography-mass spectrometry to define essential oil profiles. For separation of the compounds and checking for overlapping peaks, two columns, Hp-1 and DB-5 were used. GC analyses were carried out on HP 5890 gas chromatograph/flame ionization detector. A fused silica capillary column HP-1 (30 m × 0.25 mm × 0.25 µm) with linear velocity 25 cm s<sup>-1</sup> was used. The carrier gas was nitrogen. The injector and detector temperatures were set at 260°C, column temperature was programmed from 50°C to 260°C at 4°C min<sup>-1</sup>, and 15 min at 260°C. The GC–MS spectra were recorded on a Hewlett Packard 6890 + MSD 5975 (Hewlett Packard, Palo Alto, CA, USA) operating in EI mode at 70 eV and filled with DB-5 MS column (30 m×0.25 mm×0.25 µm). The temperature programme used was: 50-230°C at 4°C, 15 min<sup>-1</sup>, 1 min hold at 180°C and 180-300°C at 5°C min<sup>-1</sup>, and 1 min hold at 300°C. The injector temperature was 260°C. The flow rate of carrier gas (Helium) was 0.8 ml min<sup>-1</sup>. The split ratio was 1:15. The identification of detected compounds was based on comparison mass spectra and Kovats retention indexes as in Adams [25] and literature data [26].

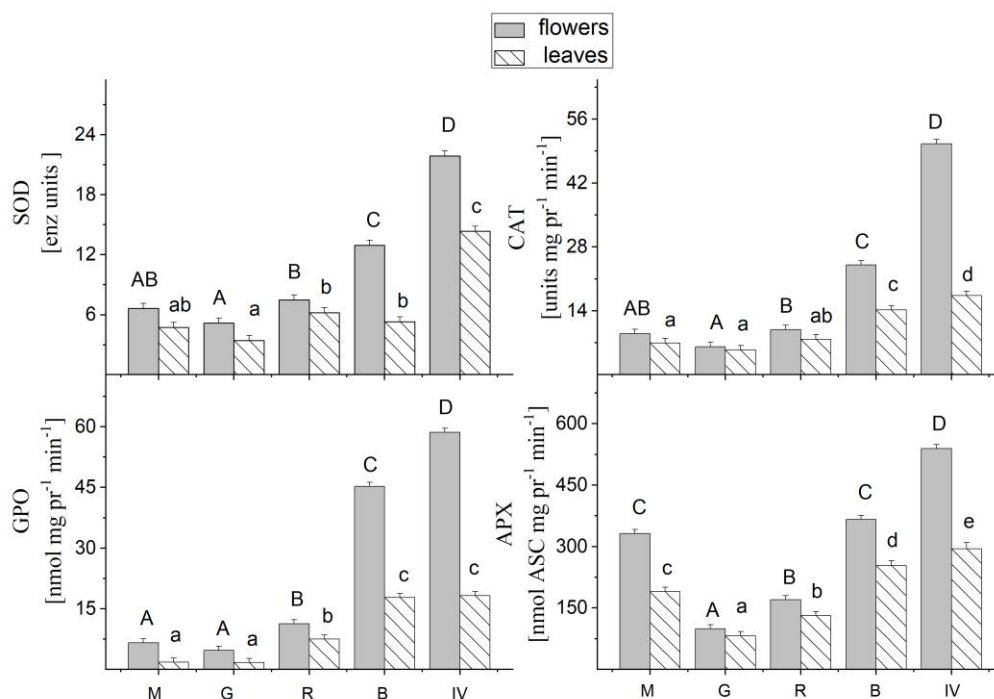
Statistical analysis was performed using a statistical software package (StatGraphics Plus, version 5.1 for Windows). The flower and leaf extractions and the antioxidant activity assays were carried out in triplicate. Data were presented as mean ± standard error and were compared with the Fisher least significant difference (LSD) test at  $P \leq 0.05$  after performing ANOVA.

### 3. Results and Discussion

The focus of the present study was to evaluate the flower and leaf antioxidant potential and the essential oil content of wild-grown *O. heracleoticum* L. plants harvested from four natural habitats in Bulgaria, and compared with *in vitro* propagated plants and adapted to field conditions. The screening of the enzyme activities (SOD, CAT, GPO and APX) characterizing the enzyme antioxidant potential in *O. heracleoticum* flowers and leaves showed that the highest enzyme activities were recorded in micropropagated and acclimatized-to-field condition plants (Figure 1). Regarding plants collected from natural habitats, the level of the enzyme activities was in the order Gugutka and Madzharovo (villages in the Eastern Rhodopes, Southeast Bulgaria), followed by Rafting center and village Gorna Breznitsa (Kresna Gorge, Southwest Bulgaria). The values of enzyme activities were higher in the flowers than in the leaves of Greek oregano.

In the *O. heracleoticum* L. leaves and flowers from plants collected from the four localities and in those propagated *in vitro* and field adapted, the total phenolic content and the WS- and LS-AOM were higher in the leaves than in the flowers, while the total flavonoid content was higher in the flowers (Figure 2). The total phenolic content, total flavonoid content, and WS-AOM and LS-AOM of wild-grown plants collected from the two natural habitats in Eastern Rhodopes Mountain (Madzharovo and Gugutka) had lower values than those from other locations and from micropropagated plants.

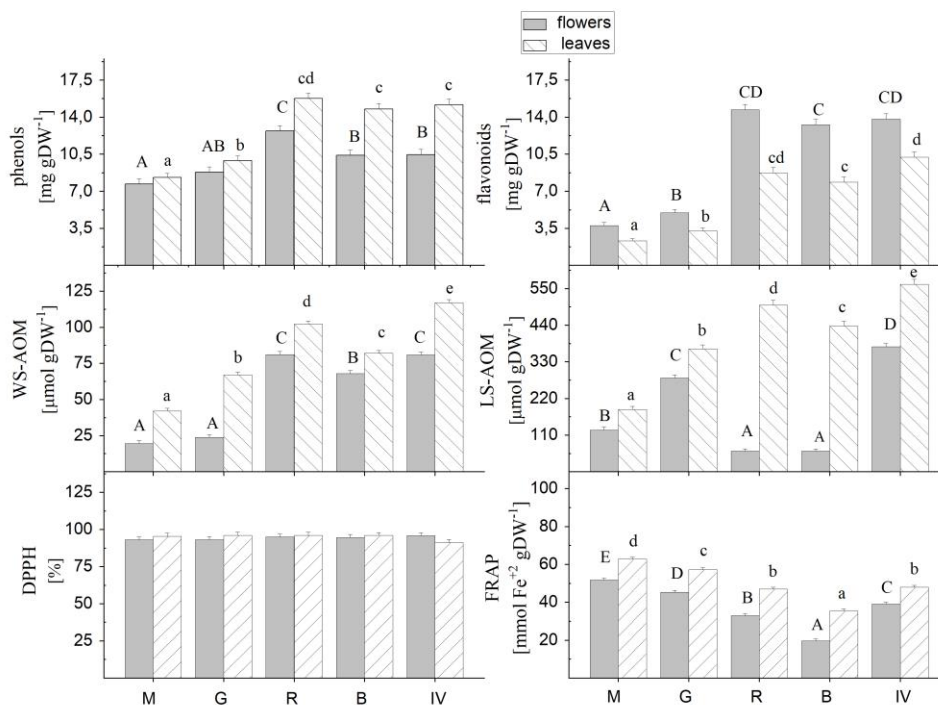
The highest measured values for WS-AOM and LS-AOM were found in micro propagated and acclimatized-to-field condition plants. Both contents from leaves and flowers were higher than in plants collected from the natural locations of Kresna Gorge (Rafting and Breznitsa). In the investigated methanol extracts, no significant difference in the levels of the free radical scavenging ability (DPPH test, Figure 2) were observed. In the plants from different natural habitats, specific results were obtained for antioxidant activity measured by ferric reducing antioxidant power (FRAP test). The highest antioxidant potential values were established in the flowers and leaves from plants collected from Madzharovo and Gugutka, followed by Rafting and Breznitsa. The antioxidant value of flowers and leaves of micropropagated and adapted plant (39.01 and 48.05 nmol Fe<sup>+2</sup> g DW<sup>-1</sup>)



**Figure 1.** The activity of antioxidant enzyme catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPO), and ascorbate peroxidase (APX) in the *in vitro* propagated *O. heracleoticum* L. and in plants collected from different natural habitats; (M) - Madzharovo - village in the Eastern Rhodopes, Bulgaria; (G) - Gugutka - a village in the Eastern Rhodopes, Bulgaria; (R) – Rafting centre situated in the Kresna Gorge; (B) – Gorna Breznitsa - a village in Kresna Gorge, Bulgaria. Values are means  $\pm$  SE,  $n=3$ ; different letters indicate significant differences assessed by Fisher LSD test ( $P \leq 0.05$ ) after performing ANOVA multi-factor analysis. The statistical analysis of flowers (uppercase) and leaves (lowercase) was performed separately.

were higher than those found for wild-grown plants in Gorna Breznitsa village in Kresna Gorge, Bulgaria ( $19.55$  and  $35.37$   $\text{nmol Fe}^{+2} \text{g DW}^{-1}$ ).

The screening of the total phenolic content, total flavonoid content, WS-AOM and LS-AOM content of the dried plant samples indicated that all of the plants from natural habitats and from micropropagation were good sources of antioxidants. These results were consistent with the results of previous studies which reported that *O. heracleoticum* was a potential source of natural antioxidants and could be further considered for commercial exploitation [27, 28]. The study of the content of metabolites with antioxidant power found in the Greek oregano flowers and leaves, collected from plants in the four natural habitats in Bulgaria, as well as from micropropagated and adapted plants, revealed that there was no correlation between the levels of antioxidant capacity as measured by the two different methods (DPPH and FRAP). This contradiction was probably due to the method of measurement. The ferric reducing antioxidant power (FRAP) mechanism is based on electron transfer rather than hydrogen atom transfer [29]. FRAP cannot detect compounds that act by radical quenching (hydrogen transfer), particularly thiols (as glutathione) and proteins. Other studies of the relationship between the plant antioxidant capacity and total phenolic and flavonoid content have shown that the antioxidant activity might not always correlate with the amount of



**Figure 2.** The content of metabolites with antioxidant potential and the level of antioxidant potential in the *in vitro* propagated *O. heracleoticum* L. and collected from different natural habitats; (M) - Madzharovo - village in the Eastern Rhodopes, Bulgaria; (G) - Gugutka - a village in the Eastern Rhodopes, Bulgaria; (R) – Rafting centre situated in the Kresna Gorge; (B) – Gorna Breznitsa - a village in Kresna Gorge, Bulgaria. Values are means  $\pm$  SE, n=3; different letters indicate significant differences assessed by Fisher LSD test ( $P \leq 0.05$ ) after performing ANOVA multi-factor analysis. The statistical analysis of flowers (uppercase) and leaves (lowercase) was performed separately.

phenols. Velioglu *et al.* [30] reported high correspondence between total phenolic content and antioxidant activity assayed by  $\beta$ -carotene bleaching method, while Kähkönen *et al.* [31] did not observe a correlation between total phenolic content and antioxidant activity examined by autoxidation of methyl linoleate.

After the analysis, 45 components of the recorded 60 components present in Greek oregano collected from the four natural populations and *in vitro* propagated and field acclimatized plants were identified (Table 1). Carvacrol (51.98-76.04%) was found to be the major component in the essential oils from plants collected from the two regions of Bulgaria (Southeast and Southwest) as well as in *in vitro* propagated and soil acclimatized plants. The highest carvacrol contents (73.58-76.04%) were recorded in *O. heracleoticum* plants collected from the Southwest part of Bulgaria (Rafting and Gorna Breznitsa). The values of carvacrol content in extracts obtained by hydrodistillation of micropropagated and field acclimatized plants (58.07%) are comparable with those collected from the southeastern areas of Bulgaria, Madzharovo and Gugutka (51.98-56.36%). Thymol, which is responsible for the phenolic character of the oils, was found to be present at a low level (less than 0.08%) in the essential oils of plants from all collected areas and in micropropagated plants. Based on the essential oil compositions of the Greek oregano plants collected from four

natural populations in Bulgaria and the micropropagated and field acclimatized, it was concluded that all plants belong to the carvacrol chemotype because the major component identified in the oils was carvacrol (51.98-76.04%). The same results were obtained for Greek oregano plants collected from Anatolia, Turkey, with the main component identified as carvacrol, followed by p-cymene [5]. Other investigators also studied the essential oil from *O. vulgare* L. ssp. *hirtum* (Link) Ietswaart that originated in Mt. Iti (Central Greece) and Campania (Southern Italy) and noted that phenols, thymol, and carvacrol were dominant compounds in the oil [7, 9]. Dzamic *et al.* [8] identified 24 compounds, which represented 97.16% of the total oil extracted from *O. heracleoticum* L., and the main compounds present were carvacrol (65.25%), followed by thymol (14.84%) and  $\beta$ -phellandrene (4.36%).

On the other hand, comparative studies on the essential oils from plants grown in different parts of Greece showed that Greek oregano collected from the northern part of Greece was rich in thymol (30.3-42.8% of total oil), whereas those from the southern part of the country were rich in carvacrol (57.4-69.6% of total oil) [32]. The authors suggest that the differences in oil compounds for the same aromatic plant species collected in different geographic areas in the world could be attributed to external factors such as climate, nature of the soil, age of the plants, and time of collection. We established that the essential oils isolated from micropropagated Greek oregano differed in the quantitative composition of the individual components, both from the plants used as starting material for their micropropagation and those collected from the other natural habitats. It would be speculative to conclude that the difference in the amounts of ingredients in essential oils is due only to the process of the plant *in vitro* propagation. The different growing conditions of micropropagated and acclimatized plants, and those from natural habitats must also be considered.

For the type of the major components (more than 1 percent) in the composition of the essential oils, it was found that there was a relationship with the location at which they were collected. The components over 1% in the essential oils of all investigated samples were  $\beta$ -caryophyllene <E>,  $\beta$ -bisabolene, p-cymene,  $\gamma$ -terpinene, carvacrol methyl ether, and caryophyllene oxide. The essential oils from micropropagated and field acclimatized plants contained the following major components, in order:  $\alpha$ -terpinene, terpinene-4-ol,  $\beta$ -pinene and  $\beta$ -Z-ocimene, borneol.

The content of the sesquiterpenes, germacrene D, and  $\beta$ -humulene was higher in the essential oil of plants derived from the southeastern part of Bulgaria (Gugutka and Madzharovo) compared with other localities. The other major components of the essential oils of the plants collected from the southwestern part of Bulgaria were borneol and terpinene-4-ol.

Considering monoterpenes such as hydrocarbon monoterpenes and oxygenated monoterpenes, quantitative differences were observed. The content of hydrocarbons monoterpenes was higher in *in vitro* propagated and field adapted Greek oregano plants than in wild plants collected from four natural population in Bulgaria including those from Gorna Breznitsa that had been used as the starting material for micropropagation. On the other hand, the oxygenated monoterpene content showed a different trend. Their content in micropropagated and field adapted *O. heracleoticum* L. was lower in comparison with collected plants from Kresna Gorge (source plants for micropropagation) and reached the content of wild plants collected from both populations from Eastern Rhodopes. Regarding Sesquiterpenes, their content in *in vitro* propagated and adapted plants had a higher value compared to the wild plants from the Kresna Gorge, Bulgaria but was lower than that of plants collected from the two habitats in Eastern Rhodopes.

Studies have shown that different natural monoterpenes and their synthetic derivatives possess various biologically active properties [33]. The effective concentrations of combinations of thymol and carvacrol exhibiting antimicrobial effects are significantly lower than concentration needed when the two structural isomers are used alone [33]. Cineole and carvacrol demonstrated a synergistic antibacterial effect [33]. In botanical medicine, there is an association of  $\beta$ -ocimene as one of the most common monoterpenes found in nature with anticonvulsant activity, antifungal

**Table 1.** Chemical composition of *Origanum heracleoticum* L. essential oil (Expressed as %) collected from different natural habitats (M) - Madzharovo - a village in the Eastern Rhodopes, Bulgaria; (G) - Gugutka - a village in the Eastern Rhodopes, Bulgaria; (R) – Rafting centre situated in the Kresna Gorge; (B) – Gorna Breznitsa - a village in Kresna Gorge, Bulgaria.

Components	RT	RI	<i>in vitro</i>	G	M	R	B
<b>Hydrocarbons monoterpenes</b>			<b>22,083</b>	<b>6,522</b>	<b>6,648</b>	<b>9,568</b>	<b>9,867</b>
unknown	7.11	912	0.006	0.015	0.015	tr	0.013
$\alpha$ -pinene	8.01	945	0.021	2.168	2.720	0.209	0.013
sabinene	8.17	965	0.156	0.110	0.157	0.096	0.051
$\beta$ -pinene	8.41	978	1.643	0.162	0.199	0.400	0.353
unknown	8.56	985	0.060	0.190	0.203	0.050	0.067
unknown	8.69	989	0.046	0.146	0.177	0.044	0.057
$\beta$ -myrcene	8.89	998	0.255	0.001	tr	0.062	0.063
unknown	8.96	1001	0.090	0.009	0.009	0.024	0.023
$\alpha$ -terpinene	9.18	1015	2.556	0.187	0.155	0.567	0.519
p-cymene	9.41	1023	5.964	1.302	0.938	5.159	6.658
$\beta$ -Z-ocimene	9.65	1034	1.608	1.185	0.971	0.320	0.041
$\beta$ -E-ocimene	9.91	1047	0.167	0.112	0.155	0.183	0.009
$\gamma$ -terpinene	10.32	1059	9.593	1.254	1.315	2.560	2.110
allo-ocimene	12.02	1130	0.120	0.042	0.038	0.013	0.049
<b>Unknown - total</b>			<b>0.216</b>	<b>0.360</b>	<b>0.404</b>	<b>0.118</b>	<b>0.161</b>
<b>Oxygenated monoterpenes</b>			<b>64.795</b>	<b>56.575</b>	<b>61.098</b>	<b>80.060</b>	<b>78.815</b>
1,8-cineole	9.51	1032	0.187	0.252	0.307	0.320	0.042
cis-sabinene hydrate	10.61	1072	0.612	0.118	0.148	0.357	0.009
terpinolene	10.96	1093	0.163	0.041	0.030	0.078	0.076
linalool	11,12	1094	0.701	0.816	0.781	0.298	0.429
trans-sabinene hydrate	11.22	1102	0.362	0.172	0.124	0.217	0.407

Table 1. Continued

Components	RT	RI	<i>in vitro</i>	G	M	R	B
borneol	13.26	1169	0.909	0.559	0.499	0.545	1.174
terpinen-4-ol	13.45	1175	1.770	0.710	0.942	0.815	1.232
$\alpha$ -terpineol	13.78	1188	0.081	0.526	0.639	0.147	0.069
$\gamma$ -terpineol	14.22	1196	0.176	0.034	0.021	0.383	0.235
trans-dihydrocarvone	14.38	1222	0.187	0.035	0.047	0.117	0.197
carvacrol methyl ether	14.81	1231	1.402	1.239	1.125	0.512	1.095
unknown	14.93	1240	0.045	0.035	0.030	0.395	0.602
thymol	15.79	1287	0.084	0.025	0.017	0.037	0.045
carvacrol	16.39	1299	58.067	51.979	56.359	76.045	73.579
carvacrol acetate	17.78	1365	0.094	0.070	0.060	0.191	0.227
<b>Unknown - total</b>			<b>0.045</b>	<b>0.035</b>	<b>0.029</b>	<b>0.395</b>	<b>0.602</b>
Components	RT	RI	<i>in vitro</i>	G	M	R	B
<b>Sesquiterpenes</b>			<b>12,248</b>	<b>35,379</b>	<b>30,744</b>	<b>8,610</b>	<b>9,914</b>
$\beta$ -bourbonene	18.28	1390	0.029	0.309	0.260	0.021	0.052
$\beta$ - caryophyllene <Z>	18.51	1401	0.033	0.206	0.111	0.031	0.037
$\beta$ - caryophyllene <E>	19.11	1429	4.274	9.920	8.175	2.144	2.205
unknown	19.28	1437	0.089	0.191	0.272	0.058	0.093
$\beta$ -humulene	19.47	1446	0.019	1.993	1.383	0.042	0.072
$\alpha$ -humulene	19.85	1460	0.936	0.115	0.126	0.328	0.353
cis-muurolo-4(15),5-diene	19.96	1470	0.188	0.136	0.126	0.035	0.026
$\gamma$ -muurolole/1480	20.21	1482	0.247	0.443	0.344	0.199	0.329
germacrene D	20.37	1490	0.455	14.654	13.140	0.091	0.331
$\alpha$ -muurolole	20.57	1499	0.127	0.371	0.339	0.088	0.156
$\alpha$ -farnesene	20.73	1507	0.060	0.728	0.597	0.126	0.183

**Table 1.** Continued

Components	RT	RI	<i>in vitro</i>	G	M	R	B
β-bisabolene	20.86	1513	3.942	3.702	3.023	2.309	3.684
sesquicineole	21.01	1520	0.077	0.128	0.159	0.026	0.253
cadina-1(10),4-diene	21.09	1524	0.395	0.231	0.587	0.396	0.600
sesquiphellandrene	21.18	1523	0.188	0.124	0.117	0.098	0.075
α-cadinene	21.49	1544	0.081	0.031	0.044	0.040	0.066
spathulenol	22.32	1583	0.032	0.311	0.422	0.356	0.172
caryophyllene oxide	22.45	1590	0.867	1.621	1.334	1.906	1.125
humulene epoxide II	23.01	1618	0.081	0.059	0.089	0.151	0.095
unknown	23.07	1622	0.133	0.141	0.106	0.050	0.041
α-cadinol	23.82	1662	0.101	0.156	0.193	0.093	0.045
unknown	24.10	1677	0.032	0.004	0.006	0.073	0.022
α-bisabolol	24.35	1690	0.119	0.143	0.176	0.133	0.058
<b>Unknown - total</b>			<b>0.254</b>	<b>0.337</b>	<b>0.384</b>	<b>0.181</b>	<b>0.156</b>
<b>Other compounds</b>			<b>0.024</b>	<b>0.094</b>	<b>0.071</b>	<b>0.034</b>	<b>0.007</b>
unknown	24.51	1699	0.014	0.180	0.193	0.038	0.052
hexahydrofarnesyl acetone	27.01	1820	0.024	0.094	0.071	0.034	0.007
unknown	27.42	1859	0.076	0.023	0.034	0.162	0.061
unknown	28.08	1848	0.155	0.025	0.058	0.396	0.163
unknown	28.49	1859	0.099	0.063	0.037	0.249	0.130
unknown	31.76	1943	tr	nd	nd	0.071	0.020
unknown	31.83	1955	tr	nd	nd	0.098	0.030
unknown	32.02	1961	tr	0.408	0.298	0.019	0.030
<b>Unknown - total</b>			<b>0.344</b>	<b>0.699</b>	<b>0.621</b>	<b>1.033</b>	<b>0.486</b>

RT- retention time; RI - retention indices; nd - not detected, tr - trace (<0.01%). Component percentages represent the mean values of 30 plants.

activity, antitumor activity, and pest resistance. Moreover, it is a volatile pheromone important in the social regulation of honeybee colonies [33]. On the other hand, excessive doses of terpinen-4-ol, the diuretic principle in the volatile oil may cause renal pain [33]. The two common isomers of terpineols,  $\alpha$ -terpinene and terpinen-4-ol, are unsaturated monocyclic mono-terpenoid alcohols that occur naturally in a large number of essential oils. Because of its pleasant odor, which is similar to lilac,  $\alpha$ -terpinene is widely used in the manufacturing of cosmetics, soaps, perfumes, antiseptic agents and is considered one of the most frequently used fragrance compounds. From the results discussed above, the composition of the essential oils of plants collected from different locations, a variation in the amounts of the phytochemical components was found. For the use in the pharmaceutical, cosmetics and food industries, it is very important to know the type and quantity of the components found in the different oils because different substances may have different biological activity.

#### 4. Conclusions

In this study, we demonstrated that the extracts of *O. heracleoticum* collected from the natural populations in the southwestern and southeastern parts of Bulgaria exhibited antioxidant activity. Plants from Southeast and Southwest of Bulgaria were characterized as having carvacrol chemotype based on their essential oil composition and content. *In vitro* propagation of *O. heracleoticum* L. did not lead to changes in the total content of monoterpenes and sesquiterpenes in the essential oil as a whole; it only produced changes in the ratio of hydrocarbon monoterpenes to oxygenated monoterpenes. Therefore, based on the chemical characteristics of the essential oil and the observed antioxidant properties of leaf extracts, Greek oregano is a potential source of natural antioxidants that can be used in food, cosmetics and pharmaceutical industries. The results suggest that the study of the variation of the phytochemical constituents of Greek oregano essential oil depending on the locality of plant collection will provide more evidence relating to the biosynthetic interconversion of essential oil constituents in the plants from different locations. The study also offers information that can be used for the better commercial exploitation of Greek oregano plants. This work, therefore, provides a scientific basis for further investigation of the effects of varied Greek oregano essential oil composition on the biological activities, which is essential if the plants and their extracts are to be used in the pharmaceutical, cosmetic and food industries.

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#### References

- [1] Letswaart, J.H., 1980. *A Taxonomic Revision of the Genus Origanum (Labiatae)*. The Hague: Leiden University Press.
- [2] Jerkovic, I., Mastelic, J. and Milos, M., 2001. The impact of both the season of collection and drying on the volatile constituents of *Origanum vulgare* L. ssp. *hirtum* grown wild in Croatia. *International Journal of Food Science and Technology*, 36, 649-654.

- [3] Esen, G., Azaz, A.D., Kurkcuoglu, M., Baser, K.H.C. and Tinnaz, A., 2007. Essential oil and antimicrobial activity of wild and cultivated *Origanum vulgare* L. subsp. *hirtum* (Link) Letswaart from the Marmara region, Turkey. *Flavour and Fragrance Journal*, 22, 371-376.
- [4] Lagouri, V., Guldaz, M. and Gurbuz, O., 2011. In vitro antioxidant/free radical scavenging and antibacterial properties of endemic oregano and thyme extracts from Greece. *Food Science and Biotechnology*, 20, 1487-1493.
- [5] Baser, K.H., Özec, T., Kürkçüoğlu, M. and Tümen, G., 1994. The essential oil of *Origanum vulgare* subsp. *hirtum* of Turkish origin. *Journal of Essential Oil Research*, 6, 31-36.
- [6] Kokkini, S. and Vokou, D., 1989. Carvacrol-rich plants in Greece. *Flavour and Fragrance Journal*, 4, 1-7.
- [7] Adam, K., Sivropoulou, A., Kokkini, S., Lanaras, T. and Arsenakis, M., 1998. Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. *Journal of Agriculture and Food Chemistry*, 46, 1739-1745.
- [8] Dzamic, A., Sokovic, M., Ristic, M.S., Grujic-Jovanovic, S., Vukojevic, J. and Marin, P.D., 2008. Chemical composition and antifungal activity of *Origanum heracleoticum* essential oil. *Chemistry of Natural Compounds*, 44(5), 659-660.
- [9] De Martino, L., De Feo, V., Formisano, C., Mignola, E. and Senatore, F., 2009. Chemical composition and antimicrobial activity of the essential oils from three chemotypes of *Origanum vulgare* L. ssp. *hirtum* (Link) Letswaart growing wild in Campania (Southern Italy). *Molecules*, 14, 2735-2746.
- [10] Fleisher, A. and Sneer, N., 1982. Oregano spices and origanum chemotypes. *Journal of Science of Food and Agriculture*, 33(5), 441-446.
- [11] D'antuono, L.F., Galletti, G.C. and Bocchini, P., 2000. Variability of essential oil content and composition of *Origanum vulgare* L. populations from a north Mediterranean area (Liguria Region, Northern Italy). *Annals of Botany*, 86, 471-478.
- [12] Zayova, E., Geneva, M., Miladinova-Georgieva, K., Hristozkova, M. and Stancheva, I., 2019. Impact of plant growth regulators on Greek oregano micropropagation and antioxidant activity. *Biosciences Biotechnology Research Asia*, 16(2), 297-305.
- [13] Hristozkova, M., Geneva, M., Stancheva, I., Iliev, I. and Azcon-Aguilar, C., 2017. Symbiotic association between golden berry (*Physalis peruviana*) and arbuscular mycorrhizal fungi in heavy metal-contaminated soil. *Journal of Plant Protection Research*, 57, 173-184.
- [14] Giannopolitis, C.N. and Ries, S.K., 1977. Superoxide dismutases I. Occurrence in higher plants. *Plant Physiology*, 59, 309-314.
- [15] Upadhyaya, A., Sankhla, D., Davis, T.D., Sankhla, N. and Smith, B.N., 1985. Effect of paclobutrazol on the activated oxygen metabolism and lipid peroxidation in senescing soybean leaves. *Journal of Plant Physiology*, 121(5), 453-461.
- [16] Nakano, Y. and Asada, K., 1987. Purification of ascorbate peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiology*, 28, 131-140.
- [17] Lagrimini, M., Gingas, V., Finger, F., Rothstein, S. and Liu, T.-T.Y., 1997. Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. *Plant Physiology*, 114(4), 1187-1196.
- [18] Bradford, M.M., 1976. A rapid and sensitive method for the estimation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
- [19] Pfeiffer, H., Dannel, F. and Römheld, V., 1998. Are there connection between phenol metabolism, ascorbate metabolism and membrane integrity in leaves of boron-deficient sunflower plants? *Physiologia Plantarum*, 104(3), 479-485.

- [20] Zhishen, J., Mengcheng, T. and Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64(4), 555-559.
- [21] Prieto, P., Pineda, M. and Aguilar, M., 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry*, 269(2), 337-341.
- [22] Tepe, B., Sokmen, M., Akpulat, H.A. and Sokmen, A., 2006. Screening of the antioxidant potentials of six *Salvia* species from Turkey. *Food Chemistry*, 95(2), 200-204.
- [23] Benzie, I. and Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, 239, 70-76.
- [24] Berkov, S., Bastida, J., Viladomat, F. and Codina, C., 2011. Development and validation of a GC-MS method for rapid determination of galanthamine in *Leucojum aestivum* and *Narcissus* ssp.: A metabolomic approach. *Talanta*, 83(5), 1455-1465.
- [25] Adams, R.P., 2001. *Identification of Essential Oil Components by GAS Chromatography/Mass Spectrometry*. 4<sup>th</sup> ed. Illinois: Allured Publishing Corporation.
- [26] Moro, A., Zalacain, A., De Mendoza, J.H. and Carmona, M., 2011. Effects of agronomic practices on volatile composition of *Hyssopus officinalis* L. essential oil. *Molecules*, 16, 4131-4139.
- [27] Exarchou, V., Nenadis, N., Tsimidou, M., Gerathanassis, I.P. and Trovani, A., 2002. Antioxidant activities and phenolic composition of extracts from greek oregano, greek sage, and summer savoury. *Journal of Agriculture and Food Chemistry*, 50, 5294-5299.
- [28] Tsimogiannis, D., Stavrakaki, M. and Oreopoulou, V., 2006. Isolation and characterisation of antioxidant components from oregano (*Origanum heracleoticum*). *International Journal of Food Science and Technology*, 41, 39-48.
- [29] Prior, R.L., Wu, X. and Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agriculture and Food Chemistry*, 53(10), 4290-4302.
- [30] Velioglu, Y.S., Mazza, G., Gao, L. and Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, 46(10), 4113-4117.
- [31] Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S. and Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agriculture and Food Chemistry*, 47, 3954-3968.
- [32] Kokkini, S., Karousou, R., Dardioti, A., Krigas, N. and Lanaras, T., 1997. Autumn essential oils of Greek oregano. *Phytochemistry*, 44, 883-886.
- [33] Koziół, A., Stryjewska, A., Librowski, T., Sałat, K., Gawęł, M., Moniczewski, A. and Lochyński, S., 2014. An overview of the pharmacological properties and potential applications of natural monoterpenes. *Mini-Reviews in Medicinal Chemistry*, 14, 1156-1168.