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Impacts of diurnal variation on essential oil properties and antioxidant activity of endemic *Origanum dubium* Boiss

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Abstract
Purpose: This study aimed to investigate the impact of diurnal variation on the essential oil (EO) yield, chemical composition, and antioxidant activity of *Origanum dubium* Boiss., an endemic and carvacrol-rich species naturally distributed in Türkiye.
Method: The aerial parts of *O. dubium* were collected at five different harvest times during the flowering stage (06:00, 09:00, 12:00, 15:00, and 18:00) in Ermenek (Türkiye) in 2020. Essential oils were obtained by hydrodistillation using a Clevenger-type apparatus and analyzed by GC-FID/MS. Antioxidant activity was evaluated by the DPPH radical scavenging method.
Findings: The EO content ranged between 6.4% (12:00) and 8.6% (18:00). Oxygenated monoterpenes (79.9–88.0%) were the dominant group, with carvacrol (72.0–83.4%) as the main compound. The highest carvacrol concentration was recorded at 09:00, whereas the lowest was at 12:00. Other major constituents were α-thujene (1.3–1.5%), γ-terpinene (1.9–6.3%), and p-cymene (4.0–5.4%). Antioxidant activity varied significantly depending on the harvest time, with radical scavenging activity ranging from 32.21% (06:00) to 63.90% (18:00).
Conclusion: Diurnal variation significantly influences the EO yield, composition, and antioxidant activity of *O. dubium.* The results suggest that harvesting between 06:00–12:00 in the morning and 15:00–18:00 in the afternoon is optimal for obtaining higher oil yield, carvacrol content, and antioxidant potential.

Keywords: Origanum dubium, diurnal variation, essential oil, carvacrol, antioxidant activity, DPPH

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Diurnal varyasyonun endemik Origanum dubium Boiss'in uçucu yağ özellikleri ve antioksidan aktivitesi üzerine etkileri

Özet
Amaç: Bu çalışmada, endemik ve karvakrolce zengin bir tür olan *Origanum dubium* Boiss.’un uçucu yağ (UY) verimi, kimyasal bileşimi ve antioksidan aktivitesi üzerine günlük değişimlerin etkisinin araştırılması amaçlanmıştır.

Yöntem: *O. dubium* bitkisinin toprak üstü kısımları, 2020 yılında Türkiye’nin Ermenek (Karaman) ilçesinde, çiçeklenme döneminde beş farklı hasat zamanında (06:00, 09:00, 12:00, 15:00 ve 18:00) toplanmıştır. Uçucu yağlar, Clevenger tipi aparat ile su distilasyonu yöntemiyle elde edilmiş ve GC-FID/MS cihazında analiz edilmiştir. Antioksidan aktivite, DPPH serbest radikal süpürme yöntemiyle belirlenmiştir.

Bulgular: UY oranı %6.4 (12:00) ile %8.6 (18:00) arasında değişmiştir. Oksijenli monoterpenler (%79.9–88.0) baskın grup olup, karvakrol (%72.0–83.4) ana bileşen olarak tespit edilmiştir. En yüksek karvakrol oranı 09:00’da, en düşük değer ise 12:00’da kaydedilmiştir. Diğer önemli bileşenler α-tujene (%1.3–1.5), γ-terpinen (%1.9–6.3) ve p-simen (%4.0–5.4) olmuştur. Antioksidan aktivite hasat zamanına göre önemli farklılık göstermiştir ve serbest radikal süpürme aktivitesi %32.21 (06:00) ile %63.90 (18:00) arasında değişmiştir.

Sonuç: Günlük değişimlerin *O. dubium*’un UY verimi, bileşimi ve antioksidan aktivitesi üzerinde önemli etkileri olduğu belirlenmiştir. Sonuçlar, daha yüksek yağ verimi, karvakrol içeriği ve antioksidan potansiyel elde etmek için sabah 06:00–12:00 ile öğleden sonra 15:00–18:00 saatleri arasında hasadın uygun olduğunu göstermektedir.

Anahtar kelimeler: *Origanum dubium*, diurnal varyasyon, uçucu yağ, karvakrol, antioksidan aktivite, DPPH

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1. Introduction

Türkiye harbors a remarkably rich flora with 11,707 plant taxa, 3,649 of which are endemic, corresponding to an endemism rate of 31.82% [1]. Among these, the Lamiaceae family represents one of the most diverse and economically valuable plant groups. Members of this family are widely utilized in pharmaceuticals, food processing, cosmetics, perfumery, and traditional medicine. A large proportion of phytotherapeutic products are also derived from Lamiaceae species. Within this family, plants whose essential oils (EOs) are dominated by thymol and carvacrol are commonly referred to as "thyme." These taxa, mainly belonging to the genera *Origanum*, *Thymus*, *Thymbra*, and *Satureja*, are extensively used throughout Türkiye. Of the 23 *Origanum* species distributed across the country, 14 are endemic [2].

O. dubium is naturally grown in Türkiye, Greece and Cyprus as an economic species [3]. A recent report about this species provided a new basis for the taxonomic uncertainties about section "Majorana". The taxonomic status of *O. onites*, *O. syriacum*, *O. dubium*, and *O. majorana* was assessed with respect to their evolutionary relationships in section majorana using molecular, morphological, and phytochemical data. It was even claimed that "cymyl" chemotype of *O. majarona* could be classified as *O. dubium*. In terms of typical consumption and organ used (herba Origani, folia Origani, and oleum Origani), *O. dubium* is in the spice group [4]. This *O. dubium*'s high EO content (6-8%) is its most notable feature. Within the natural populations, there are different genotypes and chemotypes, like high linalool and high carvacrol types. In nature, the density of *O. dubium* populations decreases gradually and is in danger of extinction. Hence, the cultivation of *O. dubium* is a sustainable way to conserve wild genetic resources and produce its standard products [5, 6].

While secondary metabolite profiles are under genetic control, they are strongly influenced by environmental factors, cultivation practices, harvest timing, and post-harvest handling [7,8]. Numerous studies have demonstrated that EO yield and composition can fluctuate diurnally in response to changing environmental conditions. Key factors such as temperature, light quality, intensity, and duration, along with soil and water availability, substantially affect both the quantity and quality of secondary metabolites. In particular, temperature variation during the day plays a critical role in EO biosynthesis and accumulation. Consequently, the production and bioactivity of Origanum species may vary depending on the time of day and developmental stage [8]. In parallel, consumer demand for natural antioxidants has been steadily increasing. Synthetic antioxidants, though widely used, are subject to safety concerns and regulatory restrictions due to their potential side effects [9–11]. This has stimulated intensive research into plant-derived antioxidants, which represent safe and effective alternatives [12].

The present study aimed to investigate the diurnal variation in EO yield, chemical composition, and antioxidant activity of O. dubium collected from natural populations in the Ermenek district of Karaman, Türkiye. To date, limited information is available regarding the impact of harvesting time within a single day on the biochemical characteristics of this species. Understanding these dynamics is important not only for optimizing EO production and bioactivity but also for supporting the sustainable use of O. dubium, which is under threat in its natural habitats. The outcomes of this research are expected to provide valuable insights for growers, herbal industries, and conservation strategies by identifying the most suitable harvesting period for maximizing both yield and functional quality.

2. Materials and methods

In this study, *O. dubium* seedlings collected from the flora of Ermenek district of Karaman in 2014 were used as plant material (36°37′20″ N, 33°01′40″ E; 1096 m altitude). The cuttings were rooted and transplanted to the research farm of Karamanoğlu Mehmetbey University, Ermenek Vocational School (36°37′30″ N, 32°55′11″ E; 1303 m altitude). The plants were harvested in 2020 (at 6 years old) to obtain essential oil.

2.1. Climate characteristics of the trial area

Ermenek district, located between the Tarsus Mountains, represents a transition zone between the Mediterranean and the Central Anatolian continental climate. Summers are hot and dry, while winters are relatively cold, with higher precipitation in winter than in summer. The annual average temperature ranges between 0.5-24.6 ºC, with August and January being the hottest and coldest months, respectively. The mean annual precipitation is 564 mm. In 2020, when the harvest was conducted, the total annual precipitation was 273 mm, and the mean temperature was 13.5 °C. Meteorological data for the trial area between 2014 and 2020 are presented in Table 1.

2.2. Plant cultivation and harvest

The locations of *O. dubium* plants in the Ermenek flora were identified using Flora of Turkey [13]. In March 2014, cuttings were taken from the previously determined locations in summer. The plants rooted in viols were transplanted to the experimental field in Ermenek on 14.04.2014. Mechanical methods were employed to control weeds in the trial during the research. Plants were harvested at five different times at three-hour intervals (6:00 and 9:00 in the morning, 12:00 at noon, 3:00 and 6:00 in the afternoon) during the flowering period in 2020 (17.06.2020). The aerial parts of plants were dried in the shade under room temperature conditions.

Table 1. Climate data for 2014-2020

		Months												
Climate factors	**Years**	**Jan.**	**Feb.**	**March**	**April**	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**	**Nov.**	**Dec.**	**Av.**
Average Temp. °C	2014	4.8	5.4	7.5	11.6	14.6	19.0	23.8	24.6	18.6	13.1	7.2	6.0	13.0
	2015	1.8	2.0	6.0	8.7	15.7	17.9	23.0	23.4	22.3	15.1	9.7	4.1	12.5
	2016	0.5	6.5	7.2	14.0	14.5	21.2	24.5	24.6	19.9	13.4	9.0	4.0	13.3
	2017	-0.3	1.8	6.5	10.5	14.8	19.9	24.8	23.5	22.3	13.1	7.3	6.0	12.5
	2018	2.8	6.0	9.4	13.5	16.4	19.5	23.2	23.4	20.7	14.2	9.0	3.5	13.5
	2019	1.1	3.4	5.6	8.4	17.3	20.1	22.2	22.8	19.6	15.8	10.3	4.4	12.6
	2020	0.7	2.2	6.4	10.2	15.9	18.9							9.05
Precipitation mm	2014	71.8	9.7	15.9	15.6	82.8	35.5	14.6	5.0	9.6	48.7	55.2	66.7	431
	2015	25.6	108	77.6	2.9	17.4	38.5	2.2	29.6	4.2	48.1	11.8	9.6	376
	2016	78.3	22.9	53.5	20.6	63.3	5.5	0.0	2.1	7.4	7.2	37.5	172	470
	2017	91.1	0.7	107.1	33.9	55.6	11.2	0.0	4.1	0.0	53.8	58.7	28.9	445
	2018	141.1	36.6	54.5	13.6	36.6	49.9	0.0	0.0	6.1	45.8	57.4	126.7	568
	2019	208.6	96.2	68.9	25.4	1.8	63.5	0.0	27.7	0.4	18.9	41.8	181.1	734
	2020	151.9	43.7	51.1	14.8	10.0	1.7							273
Regional Directorate of State Meteorology														

2.3. Isolation of essential oils

Dried aerial parts were used to extract the essential oil. A neo-Clevenger type apparatus was used to hydro-distillate 25 g of samples for three hours in two replications using distilled water (1:10 v/v) to achieve isolation. The oils were kept in dark vials at 4°C after being dried over anhydrous sodium sulfate [14].

2.4. GC-MS analyses

Essential oil composition was determined using a GC system (Agilent 7890B) equipped with a flame ionization detector (FID) and coupled to a mass spectrometer detector (MSD, Agilent 5977A). Separation was performed on an HP-Innowax column (60 m × 0.32 mm i.d., 0.25 µm film thickness). Helium (99.999%) was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. Injection volume was 1 µL (20 µL EO diluted in 1 mL n-hexane) in split mode (40:1). The solvent delay was set to 8.20 min. The oven program was: 70 °C for 5 min, then increased to 160 °C at 3 °C min^{-1}, held for 5 min, then increased to 250 °C at 6 °C min^{-1}, and held for 5 min. Mass spectra were recorded at 70 eV with a scan range of 50–550 amu.

Retention indices (RI) were determined under the same chromatographic conditions by co-injection with a homologous series of C7–C30 n-alkanes (Sigma-Aldrich). Compound identification was based on comparison of RI values, Wiley and NIST library spectra, published data [15], and an in-house database. Relative percentages were calculated from FID peak areas without applying correction factors [4].

2.5. DPPH radical scavenging assay

Antioxidant activity of the EOs was evaluated using the DPPH radical scavenging method with minor modifications [16,17]. Briefly, 20 µL of EO solution (diluted in methanol to 10 mL) was mixed with 100 µL of 0.002 M DPPH in methanol and adjusted to 1 mL with methanol. After incubation in the dark for 90 min, absorbance was measured at 517 nm using a UV/Vis microplate spectrophotometer (Thermo Scientific Multiskan Go). The control contained 0.1 mL DPPH solution and 0.9 mL methanol. Methanol alone served as the blank. Butylated hydroxytoluene (BHT) was used as a positive control. The radical scavenging activity (%) was calculated as:

RSA(%)= ($A_{control}$ - A_{sample} / $A_{control}$) X 100

2.6. Assay for total phenolics

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method [18,19]. Gallic acid was used as a standard (0.2–1.0 mg mL^{-1}). Briefly, 20 µL of EO extract (diluted in 99.9% methanol) was mixed with 680 µL distilled water, 400 µL Folin–Ciocalteu reagent (0.5 N), and 400 µL Na_2CO_3 (10%). After 30 min incubation, absorbance was measured at 760 nm. The calibration curve (R^2 = 0.968) was used to calculate phenolic content, expressed as mg gallic acid equivalents (GAE) per g dry weight of sample. All experiments were performed in triplicate.

3. Results

3.1. Essential oil yield and components

The essential oil (EO) content of *O. dubium* varied significantly depending on the harvest time (Table 2). The highest yield (8.60%) was recorded at 18:00, while the lowest (6.40%) occurred at midday. Other yields were 7.40% at 06:00, 8.12% at 09:00, and 6.80% at 15:00. These findings indicate that EO accumulation was greater during cooler parts of the day.

The oils were dominated by oxygenated monoterpenes (81.3–89.0%), followed by monoterpene hydrocarbons (10.4–17.6%). Minor groups included oxygenated sesquiterpenes (0.08–0.34%), ketones (0.07–0.11%), and esters (0–1.12%). The proportion of oxygenated monoterpenes peaked at 09:00 (89.04%), whereas the highest monoterpene hydrocarbon content was observed at 15:00 (17.64%).

A total of 25 compounds were identified across all harvests. Carvacrol (72.0–83.4%) was the dominant constituent, reaching its maximum at 09:00 (83.40%) and minimum at midday (72.02%). Notably, the early morning (06:00) and late afternoon (18:00) harvests also contained high carvacrol levels (79.32% and 78.34%, respectively). γ-Terpinene (1.9–6.4%) and p-cymene (4.0–5.4%) were the other major constituents. Interestingly, the lowest levels of γ-terpinene and p-cymene coincided with the maximum carvacrol content (09:00). As both compounds are biogenetic precursors of carvacrol and thymol, this suggests active conversion under higher temperatures [20,21].

In addition, linalool was detected at appreciable levels only at midday (7.77%), when carvacrol levels were lowest. Other identified compounds included α-thujene (1.3–1.5%), β-myrcene (1.0–1.2%), α-terpinene (0.7–1.5%), and thymol (0.9–1.7%) (Table 2).

Table 2. Diurnal variation in essential oil yield and composition of *O. dubium*

No	RT	RI	RI_L	Compounds	Harvest Time				
					06:00 a.m. (%)	**09:00 a.m. (%)**	**12:00 Midday (%)**	**3:00 p.m. (%)**	**6:00 p.m. (%)**
1	8.688	1026	1026*	α-Thujene	1.38	1.43	1.42	1.59	1.39
2	9.666	1071	1068*	Camphene	0.11	0.11	0.12	0.16	0.12
3	10.742	1114	1110*	β-Pinene	0.11	0.13	0.12	0.13	0.12
4	12.103	1158	1146*	δ-3-Carene	nd	0.07	0.07	0.07	0.07
5	12.207	1161	1160*	β-Myrcene	1.03	1.11	1.13	1.27	1.14
6	12.418	1168	1167*	α-Phellandrene	0.19	0.20	0.21	0.24	0.21
7	12.899	1183	1181*	α-Terpinene	1.07	0.74	1.28	1.53	1.30
8	13.551	1203	1198*	Limonene	0.14	0.14	0.18	0.18	0.18
9	13.929	1213	1215*	β-Phellandrene	0.32	0.40	0.33	0.43	0.34
10	14.701	1234	1232*	Cis-ocimene	nd	nd	nd	0.08	nd
11	15.239	1248	1246*	γ-Terpinene	4.46	1.99	5.58	6.40	5.83
12	16.235	1274	1272*	p-Cymene	4.96	4.04	4.97	5.47	5.30
13	16.675	1286	1282*	α-Terpinolene	nd	0.09	0.08	0.10	0.08
14	23.158	1446	1444*	1-Octen-3-ol	0.08	nd	0.10	0.11	0.11
15	23.891	1464	1463*	Cis-sabinene hydrate	0.97	1.10	0.97	1.17	0.95
16	27.129	1544	1544*	Linalool	0.20	0.19	7.77	0.27	0.16
17	29.458	1603	1601*	Terpinen-4-ol	0.84	1.01	0.93	1.35	0.87
18	33.006	1696	1695*	α-Terpineol	0.52	1.25	0.70	0.81	0.70
19	33.2	1701	1702*	Borneol	0.38	0.33	0.39	0.63	0.39
20	34.557	1737	1736*	Carvone	0.12	0.19	0.12	0.15	0.10
21	44.501	1989	1990*	Caryophyllene oxide	0.16	nd	0.08	0.17	nd
22	48.352	2125	2124*	Spathulenol	0.18	0.08	0.17	0.17	0.20
23	49.657	2181	2183*	Thymol	0.97	1.57	1.02	1.78	1.49
24	50.332	2211	2210*	Carvacrol	79.32	83.40	72.02	75.18	78.34
25	50.893	2239	2237*	Isopropyl palmitate	1.12	nd	nd	nd	nd
Chemical grouped compounds (%)									
				Oxygenatedmonoterpenes	83.32	89.04	83.91	81.34	82.98
				Monoterpenehydrocarbons	13.76	10.45	15.47	17.64	16.08
				Oxygenatedsesquiterpenes	0.346	0.081	0.25	0.338	0.202
				Ketone	0.076	-	0.097	0.111	0.106
				Ester	1.12	-	-	-	-
				Total (%)	98.63	99.57	99.76	99.44	99.39
				Number of identified compounds	22	21	23	24	22
				Essential oil content (%)	7.40	8.12	6.40	6.80	8.60

RT: Retention time; RI: Retention indices; RI_L: Retention indices from different literatures; *: Babushok et al ., (21); nd: Not detected.

3.2. Antioxidant activity

The antioxidant activity of the EOs, determined by the DPPH assay, varied significantly across harvest times (Table 3). The highest radical scavenging activity (63.91%) was observed at 18:00, whereas the lowest (32.22%) occurred at 06:00. Intermediate values were recorded at 15:00 (44.54%), 09:00 (40.32%), and midday (39.44%). The IC_{50} value of the most active sample (18:00) was 0.14 µL mL^{-1}, indicating strong antioxidant potential.

Previous studies reported similar findings, with *O. dubium* showing high scavenging activity and low IC_{50} values compared to other Lamiaceae members [12,22]. Several terpenes, including γ-terpinene, α-terpinene, caryophyllene, p-cymene, α-pinene, and β-pinene, have been linked to antioxidant properties [14,22]. In the present study, the strong antioxidant capacity is likely attributable to the high proportion of carvacrol.

Table 3. Radical scavenging activity (%) of *O. dubium* essential oils at different harvest times

Harvesting Time	Absorption values at 517 nm	Radicalscavengingactivity $=\frac{A_{Control}-A_{sample}}{A_{Control}}\times 100$
06:00 a.m.	0.0385	32.2183098
09:00 a.m.	0.0339	40.3169014
Noon Midday	0.0344	39.4366197
3:00 p.m.	0.0315	44.5422535
6:00 p.m.	0.0205	63.9084507

3.3. Total phenolic content

The total phenolic content (TPC), expressed as gallic acid equivalents (GAE), also differed among harvest times (Table 4). The maximum phenolic content (1.20 mg GAE mL^{-1}) was obtained at 18:00, followed by midday (0.97 mg GAE mL^{-1}), 09:00 (0.81 mg GAE mL^{-1}), 06:00 (0.58 mg GAE mL^{-1}), and 15:00 (0.56 mg GAE mL^{-1}). These results are consistent with the DPPH assay, suggesting a strong relationship between carvacrol content, phenolic levels, and antioxidant capacity.

Table 4. Total phenolic content of *O. dubium* essential oils (mg GAE mL^{-1}) at different harvest times

Harvest Time	Absorption	Phenolic equivalents in gallic acid
06:00 a.m.	0.090	0.576
09:00 a.m.	0.124	0.806
12:00 Midday	0.147	0.967
3:00 p.m.	0.088	0.562
6:00 p.m.	0.181	1.203

4. Conclusions and discussion

The present study demonstrates that diurnal variation markedly influences the essential oil (EO) yield, composition, and antioxidant activity of *O. dubium*. EO accumulation was greater in the late afternoon (18:00), whereas the lowest content was recorded at midday. These findings are consistent with earlier reports showing that environmental factors such as light intensity, temperature, and relative humidity strongly affect secondary metabolite biosynthesis in Lamiaceae plants [3,15]. For instance, higher oil yields in the cooler hours of the day have been reported for *Origanum vulgare* and *Thymbra spicata* [16,18].

Carvacrol was identified as the dominant constituent across all harvest times (72.0–83.4%). This observation aligns with previous studies reporting that *O. dubium* is a carvacrol-rich chemotype [7,12]. Interestingly, carvacrol levels peaked in the morning (09:00) and declined towards midday, when linalool content increased sharply. This inverse relationship suggests a temperature-dependent modulation of monoterpene biosynthesis, likely mediated through the biogenetic pathway from p-cymene and γ-terpinene to carvacrol and thymol [19,20]. The observed reduction of precursor compounds (γ-terpinene and p-cymene) during the period of maximum carvacrol accumulation supports this pathway. Similar conversions have been documented in *O. onites* and *O. vulgare* [21,22].

Antioxidant activity, measured by the DPPH assay, also exhibited diurnal variation, with the highest radical scavenging activity (63.91%; IC_{50} = 0.14 µL mL^{-1}) at 18:00. These results corroborate earlier findings that carvacrol and other phenolic monoterpenes are the major contributors to the antioxidant capacity of oregano oils [22–24]. The parallel trend between total phenolic content (TPC) and DPPH activity observed in this study further supports the strong link between phenolic compounds and antioxidant potential. Similar correlations have been reported in oregano and thyme species [24,25].

From an applied perspective, the results indicate that harvesting *O. dubium* either in the early morning (06:00–12:00) or late afternoon (15:00–18:00) is optimal for obtaining high EO yields and enhanced antioxidant activity. These time windows coincide with periods of moderate temperature and reduced solar radiation, which appear to favor both EO biosynthesis and stabilization of phenolic compounds.

In conclusion, this study highlights the critical role of harvest timing in shaping the chemical profile and bioactivity of *O. dubium*. By aligning harvesting practices with natural diurnal fluctuations, growers and processors can maximize both the yield and functional value of essential oils. These insights contribute to the sustainable utilization of this endemic species and provide a scientific basis for improving the quality of oregano-based products. Future research should investigate molecular-level regulatory mechanisms of carvacrol biosynthesis and validate these patterns under different ecological and agronomic conditions.

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