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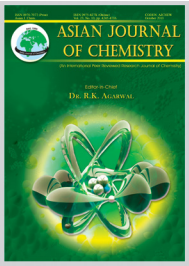

Evaluation of Essential Oils and Phenolic Compounds of Some *Origanum* (Labiatae/Lamiaceae) Taxonomy

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In present study, essential oils and phenolic compounds of five *Origanum* species, namely, *Origanum onites, Origanum vulgare* subsp. *hirtum, Origanum minutiflorum, Origanum majorana* and *Origanum syriacum* var. *bevanii* were analyzed and compared for their taxonomical importance. The data obtained from essential oil and phenolic compounds analyses were also addressed to find any correspondence with the taxonomic order of the five species based on morphological data. The relationships between *Origanum* taxa were analyzed by using T-rex programme. Reticulograms produced from both phenolics and essential oil data indicated that chemotaxonomic order of *Origanum* taxa based on essential oils data is more congruent with the taxonomic order of the same taxa based on Iestwaart's classification. In both reticulogram, *Origanum vulgare* subsp. *hirtum* was placed distantly from other *Origanum* species, this may be because this species is herbaceous while others are semi-shrubs.

Key Words: Essential oil, *Origanum*, Phenolic compounds, Taxonomy.

INTRODUCTION

The genus *Origanum* L. (Labiatae) is an annual, perennial and shrubby herb growing on stony slopes and in rocky mountains up to 4000 m altitude[1]. This genus comprises 38 species native to the Mediterranean, Euro-Siberian and Iran-Siberian regions[2] but most *Origanum* species (*ca.* 75 %) are found exclusively in the East Mediterranean sub-region[3]. Since the revision of genus *Origanum* by Ietswaart for the flora of Turkey[1], three new species have also been described from Turkey and the East Aegean Islands, namely *Origanum symes* A. Carlström, *Origanum munzurense* Kit Tan & Sorger[4] and *Origanum husnucan-baseri* H. Duman, Aytaç & A. Duran[5]. The total number of *Origanum* taxa known from Turkey is 27. The *Origanum* genus is regarded as taxonomically complicated due to naturally occurring hybrids between species[6].

Origanum species contain an essential oil with monoterpenes and sesquiterpenes responsible for their use as aromatic plants. *Origanum* is widely consumed in agricultural, pharmaceutical and cosmetic industries as a culinary herb, flavouring substances in food products, alcoholic beverages and perfumery for its spicy fragrance[2]. It has also been used as a folk medicine to treat various ailments[7].

The information gathered from essential oil studies has proved to be of value in the taxonomic and evolutionary investigations of plants[8-10]. Phenolic compounds have also been used frequently in chemotaxonomic studies, because these compounds can be easily extracted from plant material and readily identified by reagents after separation by chromatography[11]. The essential oils of several *Origanum* species growing in Turkey have been studied to varying extents[12-14], however, their taxonomic significance have not been considered. First chemosystematic investigation in the genus *Origanum* was undertaken by Skoula *et al.*[15] and it was shown that most *Origanum* species are rich either in sabinyl compounds or cymyl compounds. By using essential oil components and RAPD markers, Gounaris *et al.*[3] also discriminated two *Origanum* taxa *i.e.*, *Origanum onites* and *Origanum vulgare* and their putative hybrid *Origanum* × *intercedens*. The objectives of this study are to compare the ability of essential oils and phenolic compounds in determination of chemotaxonomic relationships between five *Origanum* species, namely *Origanum minutiflorum* Origanum Schwarz & P.H. Davis, *Origanum majorana* L., *Origanum onites* L., *Origanum syriacum* L. subsp. *bevanii* (Holmes) Ietswaart and *Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart and to trace any correspondence with species taxonomic order based on morphological data.

EXPERIMENTAL

The *Origanum* taxa studied here represent three section of the genus[6]. *Origanum minutiflorum* is a member of Chilocalyx section. *Origanum majorana, Origanum onites* and

Origanum syriacum subsp. *bevanii* are members of Majorana section and *Origanum vulgare* subsp *hirtum* is a member of *Origanum* section.

Origanum minutiflorum is a sub shrub up to 35 cm with white flowers that bloom in July and August. This plant prefers rocky limestone slopes, from 1500-1800 m above sea level. It is endemic to Anatolia, where it grows in Antalya and Isparta province only[4]. *Origanum majorana* is a sub shrub up to 80 cm with white flowers. This plant prefers dry slopes and rocky places, sometimes in partial shade, from 400-1500 m above sea level. Flowering time of this plant covers the period from May to September[4]. *Origanum onites* is also a sub shrub up to 65 cm with white flowers. This plant prefers stony hills and rocky slopes, usually grows on limestone and sometimes in partial shade from sea level to 970 m. Flowering time of this plant is from April to August. It is common in west and southwest Anatolia[4]. *Origanum syriacum* subsp. *bevanii* is a sub shrub up to 90 cm with white flowers. This plant prefers calcareous rocks and slopes, often in partial shade, from 400-2700 m above sea level. This plant flowers from May to November. It grows in Mersin, Adana, Hatay and Antalya provinces of Turkey[4]. *Origanum vulgare* subsp. *hirtum* is a perennial herb up to 100 cm with white, purple or pink flowers. This plant prefers dry hills and rocky slopes, on calcareous and non calcareous soils, often in partial shade of coniferous or mixed, maquis, from sea level to 970 m. This plant also flowers from May to November[4]. The most important morphological differences between these species appear in Table-1.

The flowering aerial parts were sampled from natural populations of *Origanum onites, Origanum vulgare* subsp. *hirtum*, *Origanum minutiflorum, Origanum majorana, Origanum syriacum* var. *bevanii* in 2005 in various regions of Turkey (Table-2). Herbarium specimens were also collected from each locality, taxonomically characterized and deposited at the Herbarium of Akdeniz University (7427-AKDU).

Analysis of essential oil composition

Isolation of essential oil: Two hundred grams of air dried leaves were cut in small pieces and the essential oils were obtained by steam distillation in 3000 mL H_2O for 3 h by Clevenger apparatus. The oils dried over anhydrous sodium sulphate and after filtration, stored at -20 °C until tested and analyzed.

GC-MS analysis of volatile compounds: The composition of the volatile constituents was established by GC-MS/Quadropole detector analyses, using a Shimadzu QP 5050 system, fitted with an FFAP (50 m × 0.32 mm (i.d.), film thickness: 0.25 μm) capillary column. Detector and injector temperature were set at 230 °C. The temperature program for FFAP column was from 120 °C (1 min) to 230 °C at a rate of 6 °C/min and than held at 200 ºC for 35 min. Helium was used as a carrier gas at a flow 14 psi. (Split 1:10) and injection volume of each sample was 1 μL. The identification of the components was based on comparison of their mass spectra with those of Wiley and Nist, Tutore Libraries. The ionization energy was set at 70 eV.

Analysis of phenolic compounds

Preparation of the extract: Dried herb leaves at room temperature were ground to fine powder with a grinder. Then the powdered herb material (15 g) was extracted with 200 mL of methanol for 2 h, using an ultrasonic bath. The extract was concentrated by using rotary evaporator (Rotavator, T < 40 ºC) under vacuum to get crude extract and dried extract was stored in desiccators until use.

RP-HPLC analysis of phenolic compounds: The procedure for quantization of the phenolic compounds has previously been described by Capanio *et al.*[16]. The reversed phase-high performance liquid chromatography (RP-HPLC) was used. Detection and quantification was carried out with a SCL-10 Avp System controller, a SIL-10AD vp Autosampler, a LC-10AD vp pump, a DGU-14a degasser, a CTO-10 A vp column heater and a diode array detector set at 278 nm. The 250 × 4.6 mm i.d. C18 column used was filled with Agilent Eclipse XDB C-18 (250 mm × 4.6 mm), 5 μ. The flow rate was 0.8 mL/min, injection volume was 10 mL and the column temperature was set at 30 ºC. Gradient elution of two solvents was used. Solvent A consisted of acetic-water (2:98, v/v) and solvent B consisted

TABLE-1
MORPHOLOGICAL DIFFERENCES OF TAXONOMIC IMPORTANCE IN *O. minutiflorum* (sect. *Chilocalyx), O. majorana, O. onites, O. syriacum* subsp. *bevanii* (sect. *Majorana*) AND *O. vulgare (*sect. *Origanum)*

	O. minutiflorum	*O. majorana*	*O. onites*	*O. syriacum*	*O. vulgare*
Habitus	subshrub	subshrub	subshrub	subshrub	perennial herb
Stem	hirtello-pubescent	tomentellous	hirsute	hirsute-tomentose	adpressed-pilose
Bract (mm)	1-3 × 0.5-1.5	2-4 × 1-3	2-5 × 1,5-4	2-5 × 1,5-3,5	2-10 × 1-7
Calyx (mm)	2	2-3,5	2-3	2,5	2-4
Calyx	1-or 2 lipped	1-lipped	1-lipped	1-lipped	subequal 5-toothed
Corolla (mm)	2,5-4	3-7	3-7	4,5-7,5	3-10
Stamens	Included in corolla	Shortly exerted from corolla	Shortly exerted from corolla	Shortly exerted from corolla	Shortly exerted from corolla

TABLE-2
PROVENANCE OF THE FIVE *Origanum* SPECIES COLLECTED IN THIS STUDY

Name	Form	Part	Harvest time
O. onites	Wild	Leaves + Flowers	Isparta, Yenisarbademli, in August 2005 at altitudes of 1400–1600 m
O. vulgare subsp. *hirtum*	Wild	Leaves + Flowers	Çanakkale, University campus, in June 2005 at altitudes of 10–100 m
O. minutiflorum	Wild	Leaves + Flowers	Isparta, Yenisarbademli, in August 2005 at altitudes of 1400–1600 m
O. majorana	Wild	Leaves + Flowers	Antalya and Isparta, in June 2005, at altitudes 1400–1600 m
O. syriacum var. bevanii	Wild	Leaves + Flowers	Mersin, in June 2005, at altitudes 1400–1600 m

TABLE-3
SOLVENT GRADIENT CONDITIONS WITH LINEAR GRADIENT

Final time	3	20	28	35	45	60	62	70	75	80
A %	95	75	72	70	65	63	55	50	20	0
B %	5	25	28	30	35	37	45	50	80	100

TABLE-4
CHEMICAL COMPOSITION (%) OF *O. minutiflorum*, *O. majorana*, *O. onites*, *O. syriacum*, *O. vulgare* ESSENTIAL OILS

Constituents	*O. minutiflorum*	*O. majorana*	*O. onites*	*O. syriacum*	*O. vulgare*
α-Thujen	0,73	0.76	0.00	0.55	1.91
α-Pinen	0.70	0.54	0.93	0.67	0.00
Sabinen	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Myrcen	1.33	1.64	1.07	1.92	2.09
α-Terpinen	0.60	0.90	0.98	**2.12**	2.06
Limonen	0.00	0.00	0.22	0.34	0.38
γ-Terpinen	2.65	2.54	3.20	**9.33**	**13.51**
p-Cymen	3.25	3.30	6.00	7.37	9.29
Linalool	0.00	0.32	**5.11**	0.87	1.17
Terpinen-4-ol	0.71	0.63	0.97	1.77	2.01
β-Caryophyllen	1.03	0.27	0.87	1.50	1.25
Borneol	**2.01**	0.27	1.69	0.21	0.24
Thymol	0.69	0.57	0.36	**6.60**	**22.18**
Carvacrol	82.88	86.67	75.61	60.48	**39.11**

of methanol (Table-3). The data were integrated and analyzed using the Shimadzu Class-VP Chromatography Laboratory Automated Software System (Chiyoda-ku, Tokyo, Japan). The extract samples, standard solutions and mobile phases were filtered by a 0.45 μm pore size membrane filter (Vivascience AG, Hannover, Germany). The amount of phenolic compounds in the extract was calculated as mg 100 g^{-1} herb using external calibration curves, constructed for each phenolic standard.

Data analysis: Statistical analyses were carried out separately based on components of phenolic compounds and essential oils present in different taxa. Distance matrices were generated Bray-Curtis formula[17] and reticulograms were constructed by using T-REX online software[18].

RESULTS AND DISCUSSION

The five different species of *Origanum* showed variation in their essential oil composition (Table-4). The major essential oil components carvacrol, thymol, γ-terpinen, *p*-cymene were detected in each species. *Origanum majorana, Origanum minutiflorum, Origanum onites*, yielded essential oil with high carvacrol content (86.67, 82.88 and 75.61 %, respectively), whereas *Origanum syriacum* and *Origanum vulgare* yielded less carvacrol in their essential oil (60.48 and 39.11 %, respectively). On the other hand, *Origanum syriacum* and *Origanum vulgare* produced high thymol (6.60 and 22.18 %) in their essential oil, but the amount of thymol was very low in essential oils of *Origanum onites, Origanum majorana* and *Origanum minutiflorum* (0.36, 0.57, 0.69 %, respectively).

The distance matrix and the additive distance matrix were calculated for five *Origanum* species based on essential oil content by using Bray-Curtis formula[17] (Tables 5 and 6). In a reticulogram based on essential oil composition (Fig. 1), *Origanum vulgare* was placed distantly from all other taxa that were followed by *Origanum syriacum*. Like *Origanum vulgare* this species also contains relatively high amount of

TABLE-5
ESSENTIAL OIL DISTANCE VALUES OF THE FIVE *Origanum* SPECIES CALCULATED BY BRAY-CURTIS FORMULA

	O. munituflorum	*O. majorana*	*O. onites*	*O. syriacum*
O. majorana	0.039848			
O. onites	0.095924	0.121789		
O. syriacum	0.241553	0.251067	0.204152	
O. vulgare	0.475023	0.480605	0.436138	0.244376

TABLE-6
ADDITIVE DISTANCE (TREE METRIC) MATRIX BASED ON ESSENTIAL OIL DISTANCE VALUES

	O. minituflorum	*O. majorana*	*O. onites*	*O. syriacum*
O. majorana	0.03985			
O. onites	0.10203	0.11568		
O. syriacum	0.23940	0.25306	0.20431	
O. vulgare	0.47107	0.48472	0.43598	0.24438

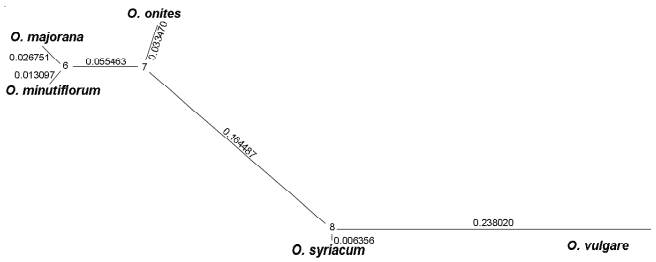

Fig. 1. Reticulation network inferred from the distance matrix based on essential oils of five *Origanum* species

thymol, γ-terpinene, *p*-cymene in the essential oil when compared with remaining taxa (Table-4). *Origanum minutiflorum* and *Origanum majorana* were closely located on reticulogram. These two species are distinguished by their high carvacrol content in their essential oil (Table-4). *Origanum onites* was also placed closely to *Origanum minutiflorum* due to its relatively high carvacrol content. Therefore, the similarity

TABLE-7
PHENOLICS OF *O. minutiflorum*, *O. majorana*, *O. onites*, *O. syriacum*, *O. vulgare*

Sample (in extract %)	*O. minutiflorum*	*O. majorona*	*O. onites*	*O. syriacum*	*O. vulgare*
Gallic acid	0.004352	0.008668	0.005440	0.009406	0.006257
Catechin	0.035580	0.074839	0.005660	0.032003	0.153606
Caffeic acid	0.042435	0	0.020094	0.028033	0.012307
Epicatechin	0.044189	0.035511	0.083068	0	0
Vitexin	0	0.070516	0	0	0
Rutin	0.030562	0.056152	0.363929	0	0.0283690
Naringin	0	0	0.010067	0	0
Hesperidin	0.010596	0	0	0	0.015666
Rosmarinic acid	2.255157	2.918196	2.441454	2.283034	4.016564
Eriodiktiol	0.243328	0.156270	0.061356	0.213956	0.431773
Naringenin	0.012300	0.158989	0.079181	0.113496	0.163014
Luteolin	0.024651	0.077460	0.084401	0.069283	0.047457
Apigenin	0.063495	0.425673	0.067934	0.079350	0.031800

sequence of five *Origanum* species based on essential oil variables is *Origanum majorana, Origanum minutiflorum, Origanum onites, Origanum syriacum* and *Origanum vulgare*.

The phenolic profiles in five *Origanum* species were identified by RP-HPLC analysis. Thirteen phenolic metabolites were found and rosmarinic acid was a major phenolic in each species studied (Table-7). Rosmarinic acid content was the highest in *Origanum vulgare* (4.01 mg/g dw) and lowest in *Origanum minutiflorum* (2.25 mg/g dw). Other phenolics were generally found in lower concentrations in each *Origanum* species and some of them were species specific. For instance, vitexin was only detected in *Origanum majorana* and naringin was only found in *Origanum onites*.

Distance values of five *Origanum* species based on phenolic compounds were calculated by using Bray-Curtis formula[17] (Table-8) and then these values were used to calculate additive distance matrix (Table-9) to construct a reticulogram. In this reticulogram (Fig. 2), *Origanum vulgare* was distantly placed from other species again, probably due to its high rosmarinic acid content (Table-7). It is followed by *Origanum majorana* and *Origanum onites* that are also contain fairly high amount of rosmarinic acid. *Origanum minutiflorum* and *Origanum syriacum* was closely placed on the reticulogram since their rosmarinic acid contents were similar. Therefore, according to the reticulogram based on the phenolic variables

TABLE-8
PHENOLIC DISTANCE VALUES OF THE FIVE *Origanum* SPECIES CALCULATED BY BRAY-CURTIS FORMULA

	O. minituflorum	*O. majorana*	*O. onites*	*O. syriacum*
O. majorana	0.224208			
O. onites	0.157883	0.214378		
O. syriacum	0.058499	0.194782	0.143309	
O. vulgare	0.307108	0.230027	0.33254	0.291486

TABLE-9
ADDITIVE DISTANCE (TREE METRIC) MATRIX CALCULATED FROM PHENOLIC DISTANCE VALUES

	O. minituflorum	*O. majorana*	*O. onites*	*O. syriacum*
O. majorana	0.21471			
O. onites	0.16053	0.22383		
O. syriacum	0.05850	0.19483	0.14066	
O. vulgare	0.31396	0.23003	0.32309	0.29409

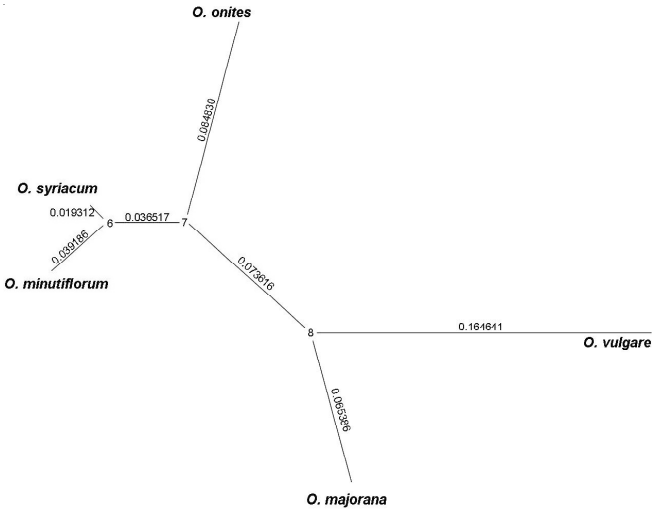

Fig. 2. Reticulation network inferred from the distance matrix based on phenolic compounds of five *Origanum* species

the similarity sequence of five *Origanum* species can be either *Origanum onites, Origanum minutiflorum, Origanum syriacum, Origanum majorana, Origanum vulgare* or *Origanum minutiflorum, Origanum syriacum, Origanum onites, Origanum majorana* and *Origanum vulgare.*

The genus *Origanum* consists of three groups, 10 sections, 43 species, six subspecies and 18 hybrids[6]. In this study, chemotaxonomic relationships between five *Origanum* species, namely *Origanum minutiflorum, Origanum majorana, Origanum onites, Origanum syriacum* subsp. *bevanii* and *Origanum vulgare* subsp. *hirtum* was studied based on composition of their essential oils and phenolic compounds. The main components of essential oils of *Origanum* species were carvacrol, thymol, *p*-cymene and γ-terpinene. The essential oils of *Origanum calcaratum, Origanum dictamnus, Origanum microphyllum, Origanum onites* and *Origanum vulgare* have been analyzed and it was shown that most *Origanum* species are rich either in sabinyl compounds or cymyl compounds but never both[15]. Our result also showed that *Origanum* species we studied did not contain sabinyl compounds, therefore supported the findings of Skoula *et al.*[15].

The essential oil and phenolic contents of five *Origanum* species were used to calculate distance matrices and then reticulograms were constructed (Figs. 1 and 2). The similarity sequence based on essential oil content was found as *Origanum*

majorana, Origanum minutiflorum, Origanum onites, Origanum syriacum subsp. *bevanii* and *Origanum vulgare* subsp. *hirtum*, respectively. However, the similarity sequence based on phenolics was slightly different; the species order was obtained as *Origanum minutiflorum, Origanum syriacum* subsp. *bevanii*, *Origanum onites*, *Origanum majorana* and *Origanum vulgare* subsp. *hirtum* or *Origanum onites, Origanum minutiflorum, Origanum syriacum* subsp. *bevanii*, *Origanum majorana* and *Origanum vulgare*, respectively. Based on morphological data[6], the phylogenetic order of these five *Origanum* species was arranged as *Origanum minutiflorum, Origanum majorana, Origanum onites, Origanum syriacum* subsp. *bevanii* and *Origanum vulgare* subsp. *hirtum*, respectively. In this case, the most suitable similarity sequence to the Ietswaart's classification was achieved by the essential oil data. The only difference was the orders of *Origanum minutiflorum* and *Origanum majorana*. This could be explained by effects of environmental and genetic factors. Several studies reported that both genetic and environmental factors play important role in determination of the essential oil compositions of, for example, *Leptospertum*[19], *Juniperus*[20], *Lomatium*[21], *Croton*[22] and *Hypericum* L. species[23]. Effect of harvest season on the composition and quantity of essential oils was also shown for *Origanum onites*[24] and *Origanum minutiflorum*[25]. In both reticulogram, *Origanum vulgare* was placed distantly from other *Origanum* species studied (Figs. 1 and 2). This could be because *Origanum vulgare* subsp. *hirtum* is an herb species whereas others are semi shrub[6].

In conclusion, essential oil data seems much powerful than phenolic data as chemical characters to study *Origanum* taxonomy. However, it is obvious that combination of several markers *e.g.* morphological, chemical and molecular can help to obtain much better results.

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