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NEPETALACTONES AS CHEMOTAXONOMIC MARKERS IN THE ESSENTIAL OILS OF *Nepeta* SPECIES

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The genus *Nepeta* L. comprises 75 species in Iran and is the largest genus of the Lamiaceae family found in the area. Furthermore, the area is one of two throughout the entire world for which the genus is endemic [1, 2]. From a taxonomic point of view, it is a complicated genus, and the range of morphological variation within *Nepeta* can lead to difficulties of specific classification and identification [3]. In Flora Iranica [4], the *Nepeta* species has been divided into 13 sections based on morphological characters. In a recent ITS sequence analysis, two clades were informally recognized for 34 studied Iranian species, where one clade was further divided into four new clades [1].

Nepetalactone isomers are monoterpene-iridoids that exhibit peculiar physiological activity in cats. These isomers are of great interest owing to their potential application as aphid sex pheromones and insect repellents [5]. Nepetalactones are characteristic constituents of the oil of many *Nepeta* species. In many cases, they represent the dominant compounds in the oil of investigated taxa. However, in some instances these structures were identified in negligible amounts or not at all. Therefore, *Nepeta* species can be divided into two groups: nepetalactone-containing and nepetalactone-less species [6].

The aim of this study was to evaluate the potential of nepetalactones as chemotaxonomic indicators in the oil of *Nepeta*, as well as to investigate the oil composition of two taxa including *N. laxiflora* Benth and *N. pungens* Benth. The latter findings are reported in this article for the first time.

Plant Material and Isolation Procedure. Twelve samples of 8 species from 3 sections, all of them at full flowering stage, were collected from different localities (Table 1). Voucher specimens were deposited in the Herbarium of Shiraz University, Department of Botany. The aerial parts of species were air-dried. The oil samples were obtained by hydrodistillation using a Clevenger-type apparatus for 4 h. The composition of oil samples are shown in Table 2.

The compounds were identified by comparison of retention indices (RRI, HP-5) with those reported in the literature and by comparison of their mass spectra with the Wiley and Mass finder 3 libraries or with the published mass spectra [7].

Data on the oil composition of the studied taxa are presented in Table 2. According to Table 2, a high quantity of nepetalactone was only detected in the oil of NK ($4a\alpha,7\alpha,7a\alpha$-nepetalactone, 31.5%); previously, research on the oil of NK had revealed the presence of $4a\beta,7\alpha,7a\alpha$-nepetalactone [8], and NO1 ($4a\alpha,7\beta,7a\alpha$-nepetalactone, 69.9%). $4a\alpha,7\alpha,7a\alpha$-nepetalactone was also identified in the oil of ND3 and NO1. Similarly, 3.8% $4a\alpha,7\alpha,7a\beta$-nepetalactone was detected in the oil of ND2; trace quantities of this compound were detected in the oil of NL and NK.

NO1 is the only studied *Nepeta oxyodonta* species that contains nepetalactones. This taxa is also different from NO2 and NO3 based on morphological characteristics, but, because of feature overlap, it cannot be regarded as another species. ITS sequence analysis might be useful for establishing its identity.

In previous works on Iranian species, nepetalactones were not detected in the oil of plants from the Psilonepeta section [9–27]. Here, we report for the first time the presence of nepetalactones in NO1, ND2, and ND3 members of this section.

High quantities of nepetalactones have been identified in section *Stenostegiae* and *Micranthae*: all investigated species contain at least one of the isomers. Nepetalactones are uncommon in the oil of species from sections *Spicatae, Schizocalyx*, *Psilonepeta*, *Spartonepeta*, and *Oxynepeta*; all are suffroticose species. Nepetalactones are also uncommon in species from section XII: dwarf, annual, and herbaceous plants.

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TABLE 1. Plant Materials

Scientific name	Locality	Date	Abbreviation
N. depauperata Benth.	Fars-Northern mountains of Shiraz	May 17, 2005	ND1
	Fars-Sarvestan-Post-e Chenar	June 2, 2005	ND2
	Fars-South of Firooz Abad	April 27, 2006	ND3
N. oxyodonta Boiss.	Fars-SW of Shiraz-Derak mountain	May 6, 2005	NO1
	Fars-Northern mountains of Shiraz	May 17, 2005	NO2
	Fars-Northern mountains of Abadeh Tashk	May 28, 2005	NO3
N. laxiflora Benth.	Kohkilouyeh-Sisakht-Dena mountain	June 20, 2005	NL
New species	Fars-Northern mountains of Abadeh Tashk	May 28, 2005	NN
N. bracteata Benth.	Fars-Northern mountains of Abadeh Tashk	May 29, 2005	NB
N. daenensis Boiss.	Fars-Neyriz-Meshkan mountain	2007	NDa
N. pungens Benth.	Fars-Neyriz-Meshkan mountain	2007	NP
N. Kotschyi Boiss.	Fars-Northern mountains of Shiraz	May 17, 2005	NK

Further investigation of other members of the *Nepeta* genus is necessary. Research is required regarding the effects of season, locality, growth stage, and environmental factors on essential oil composition, especially nepetalactone content. Nepetalactone may represent a defining component of this genus, and studying the possible relation between its presence or absence and morphological characters is recommended.

A feature selection was performed to identify more relevant chemicals for cluster analysis and pattern recognition. Therefore, a supervised classification method (counter-propagation neural network) was used. Through the process discussed in the experimental section, the most relevant chemicals were selected. All populations are classified correctly according to their sections, as determined by Rechinger′s classification. Finally, we selected six chemicals (including 4aα,7α,7aα-nepetalactone, 3 caryophyllene skeleton-type compounds, germacrene D, and hexanal) detected in quantities conducive to classification (see Table 3).

Then, the selected chemicals of 12 samples were subjected to cluster analysis using the Pearson correlation coefficient and between-group linkage methods. Aside from *N. kotschyi* (NK) and *N. pungens* (NP), which did not cluster with the others, the figure revealed four clusters: 1) *N. depauperata* (ND1) and N. *oxyodonta* (NO3); 2) N. *oxyodonta* (NO2), *N. depauperata* (ND3), and *N. oxyodonta* (NO1); 3) *N. pungens* (NP); 4) *N. laxiflora* (NL), new species (NN), *N. depauperata* (ND3), *N. daenensis* (NDa), and *N. bracteata* (NB). This pattern is supported by other findings [1]. This plot clearly shows the difference between *N. kotschyi* and other species, which is in agreement with the previous taxonomical classification [4]. The cluster also shows clear separation between Micronepeta and other species.

For further investigations, we performed PCA; this analysis was performed for all 12 populations and six selected chemicals. Three principal components were extracted, which explains the 94.2% of the data variance. This analysis showed the clear distinction of NK (*N. kotschyi*) from other species. PCA also showed that *N. pungens* (NP) is placed far from other species, accomplished by the second PC. In the next step, when the third PC was incorporated into analysis, another group became clearer, distinguishing *N. oxyodonta* (NO2 and NO3) from *N. depauperata*. Overall these analyses suggest hybridization between *N. oxyodonta* and *N. depauperata.* In addition, new species may result from this hybridization; however, gene-sequence analysis is necessary for a definite conclusion.

Classification and Clustering. In order to classify the population based on the chemical composition of essential oils, we used the counter propagation neural network (CPNN) [28]. CPNN is a supervised method of classification that has two active layers, a Kohonen layer and an output layer, where the Kohonen network performs competitive learning. Thus, it can be assumed that CPNN is a combination of both supervised and unsupervised learning methods. First, sections based on the classification by Rechinger were used (Psilonepeta: 1, Micronepeta: 2, and Stenostegiae: 3). Then, Wilk′s lambda was calculated for all chemicals based on their assumed classification [29]. Chemicals were ranked based on the decreasing Wilk values, then used for designing the classification networks. CPNN was optimized in a definite structure (epoch = 75, nodes of hidden layer = 15). The identified chemicals in the previous section were included in the network in a stepwise manner, i.e., if one variable's inclusion caused decreased error of the model, it was retained; otherwise, it was removed. This procedure was repeated for all variables. This process facilitated removal of chemicals that were not relevant to the classification of species. The selected chemicals were used for final network modeling, yielding a map of species distribution.

Afterwards, variables selected by CPNN were used in cluster analysis and principal component analysis (PCA). Cluster analysis was performed using the Pearson correlation coefficient and between-group linkage methods.

TABLE 2. The Chemical Composition of the Essential Oils of 12 *Nepeta* Samples

Compound	RI	NO3	NO2	NO1	ND1	ND2	NK	NB	NL	NN	NDa	NP	ND3
Hexanal	802	–	–	0.1	–	–	0.3	–	–	–	–	–	0.1
2-(*E*)-Hexenal	852	0.5	0.1	0.2	–	–	0.8	–	–	–	1.0	–	0.3
Tricyclene	927	0.1	–	–	–	8.2	–	–	–	0.1	–	–	–
α-Thujene	930	–	–	–	–	–	–	–	–	–	3.0	–	0.5
α-Pinene	939	0.8	0.1	0.8	2.1	41.0	–	0.1	1.3	5.4	1.0	–	3.9
Thuja-2,4(10)-diene	960	0.1	–	–	–	0.4	–	–	–	0.1	–	–	0.1
Sabinene	975	0.2	–	–	–	1	–	–	–	–	1.0	0.4	0.3
β-Pinene	979	1	–	0.1	0.7	1.8	–	0.1	0.1	0.3	3.0	–	0.4
3-Octanone	984	–	–	–	–	–	1.1	–	–	–	–	–	0.3
Dehydro-1,8-cineol	991	0.4	0.3	–		–	–	–	–	0.1	1.0	–	1.4
3,5-Dimethyl-cyclohexanone	996	–	–	–	–	–	1.3	–	–	–	–	–	–
Decane	1000	–	–	–	–	1	–	–	–	–	–	–	–
1-Acetyl-1-cyclohexene	1008	–	–	–	–	–	2.2	–	–	–	–	–	–
α-Terpinene	1017	0.1	–	–	–	0.5	–	–	0.1	–	1.6	–	0.1
p-Cymene	1025	0.1	–	–	–	3.8	–	0.1	–	–	2.7	–	0.4
Limonene	1029	–	–	–	–	0.8	–	0.5	–	–	–	–	–
1,8-Cineole	1031	9.4	1.2	0.8	4.0	7.3	–	1.1	–	37.1	9.9	0.6	0.8
γ-Terpinene	1060	0.1	–	–	–	0.7	–	–	0.2	0.1	1.6	–	0.4
trans-Linalool oxide	1073	–	–	–	0.1	–	–	–	–	0.2	1.9	–	0.2
cis-Linalool oxide	1087	–	–	–	–	–	–	–	–	–	1.6	–	0.3
Linalool	1097	0.2	–	–	2	–	0.2	0.5	2.5	0.2	3.6	0.4	1.5
α-Campholenal	1126	0.1	–	–	–	–	–	–	0.1	0.6	–	–	0.4
trans-Pinocarveol	1139	–	–	–	0.2	–	–	–	0.2	0.7	–	–	–
trans-Verbenol	1145	0.6	–	0.1	0.2	–	–	–	–	–	–	–	–
Camphor	1146	–	–	–	–	–	–	–	–	–	–	–	0.7
Borneol	1169	–	–	–	0.1	–	–	–	–	–	–	–	0.7
Nonanal	1169	–	6.1	–	–	–	0.2	0.5	–	–	–	–	–
Terpinen-4-ol	1177	0.3	–	–	0.1	–	–	0.1	0.9	0.2	7.4	0.3	0.9
α-Terpineol	1189	0.4	0.3	–	1.0	–	–	0.1	0.5	–	5.7	0.6	1.1
2-Methyl-2,3-dihydroindole	1199	–	–	–	–	–	1.2	–	–	–	–	–	–
Dodecane	1200	–	–	–	–	2	–	–	–	–	–	–	–
Decanal	1202	–	0.6	–	0.2	–	–	–	–	–	–	–	2.9
Verbenone	1205	0.3	0.4	–	0.1	–	–	–	0.1	1.0	–	–	0.3
Thymol methyl ether	1235	–	–	–	–	–	–	–	–	–	–	0.8	–
cis-Chrysanthenyl acetate	1265	–	–	–	–	–	–	–	–	–	–	–	0.8
Decanol	1270	–	–	–	–	–	–	–	–	–	–	–	0.6
Isobornyl acetate	1286	–	–	–	0.1	–	–	–	–	0.1	–	–	2.4
Carvacrol	1299	–	0.2	–	–	–	0.2	–	–	–	–	–	18
(2*E*,4*E*)-Decadienal	1317	–	0.3	–	–	–	1.1	–	–	–	–	–	–
Eugenol	1359	–	1.5	–	0.2	–	–	–	–	–	1.5	–	–
4aα,7α,7aα-Nepetalactone	1360	–	–	0.3	–	–	40.9	–	–	–	–	–	2.5
α-Copaene	1377	0.2	0.3	0.2	–	–	–	0.1	–	–	–	0.7	–
4aα,7α,7aβ-Nepetalactone	1387	–	–	–	–	3.8	0.3	–	0.3	–	–	–	–
β-Bourbonene	1388	0.2	0.3	0.3	–	0.5	1.0	1.0	0.1	–	1.2	2.0	2.6
β-Elemene	1391	2	0.5	–	0.2	–	–	1.7	–	–	–	1.9	0.3
4aα,7β,7aα-Nepetalactone	1392	–	–	69.9	–	–	–	–	–	–	–	–	–
Tetradecane	1400	–	0.8	–	–	2.9	0.4	–	–	–	–	–	–
β-Longipinene	1401	–	–	–	–	0.7	–	–	–	–	–	–	–
cis-Caryophyllene	1406	–	–	–	0.2	–	–	0.2	–	–	–	–	–
Dodecanal	1409	–	0.3	–	–	–	–	–	–	–	–	–	1.9
α-Gurjunene	1410	–	–	–	–	–	–	–	0.6	–	–	–	–
β-Caryophyllene	1418	17.8	3.7	–	23.4	3.1	1.3	4.8	7.2	0.5	5.4	20.0	7.8
(*Z*)-β-Farnesene	1443	–	–	–	–	–	–	–	–	–	–	–	0.7
2-Pentadecanone	1451	–	3.7	0.2	–	0.5		–	–	–	–	–	–
α-Humulene	1453	0.8	0.5	0.1	1.5	–	0.1	0.5	0.8	–	–	1.2	0.3

TABLE 2 (Continued)

Compound	RI	NO3	NO2	NO1	ND1	ND2	NK	NB	NL	NN	NDa	NP	ND3
(*E*)-β-Farnesene	1457	0.4	1.2	3.2	–	1.2	11.4	0.7	–	–	–	–	–
Germacrene D	1485	0.1	2	0.2	0.6	–	–	4.1	–	–	6.5	32.7	4.2
(*E*)-β-Ionone	1489	0.1	0.8	–	–	–	0.1	–	–	–	1.2	0.7	–
β-Selinene	1490	–	1.9	–	0.1	–	–	–	–	–	–	–	–
Pentadecane	1500	–	0.7	–	–	–	0.1	–	–	–	–	–	–
Bicyclogermacrene	1502	1.1	1.4	–	0.3	–	–	5.7	7.1	–	2.3	6.6	2.4
α-Muurolene	1504	–	–	–	–	–	–	–	12.4	–	–	–	–
γ-Cadinene	1514	–	–	0.1	0.1	1.5	–	–	1.8	0.7	–	0.4	0.3
1-Endo-bourbonanol	1520	–	–	–	–	–	0.6	–	–	–	–	–	–
δ-Cadinene	1523	0.2	0.5	0.1	–	–	0.2	0.4	–	0.1	–	1.4	0.5
β-Caryophyllene oxide	1546	1.6	1.5	–	0.5	–	–	0.7	0.2	–	–	–	–
Elemol	1550	–	–	–	–	–	–	–	–	–	–	1.3	–
Germacrene B	1561	–	1.1	–	–	–	–	–	–	–	–	–	–
1-Nor-bourbonanone	1563	–	–	–	–	–	1.2	–	–	–	–	–	–
(*E*)-Nerolidol	1563	–	0.4	–	–	–	–	0.7	–	–	–	–	–
3-(*Z*)-Hexenyl benzoate	1567	–	1.1	–	–	–	–	–	–	–	–	–	–
Caryophyllenol	1572	–	3.0	–	–	–	–	–	–	–	–	–	–
α-Cedrene epoxide	1575									8.2			
Spathulenol	1578	–	–	–	–	–	–	32.2	45.3	8.7	–	–	5.8
Caryophyllene oxide	1583	42.6	34.5	13.1	37.4	3.8	7.3	0.6	–	3.8	27.1	16.2	0.9
Salvial-4(14)-en-1-one	1595	–	–	–	0.7	–	0.6	–	–	–	–	–	0.5
Hexadecane	1600	–	0.8	–	–	2.3	–	–	–	–	–	–	0.5
Humulene epoxide II	1608	0.7	0.8	0.4	1.3	–	–	0.8	–	–	–	–	–
Tetradecanal	1613	–	–	–	–	–	–	–	–	–	–	–	1.2
Dillapiole	1621	–	0.3	–	–	–	1.2	–	–	–	1.4	1.5	–
β-Cedrene epoxide	1623	–	–	–	–	–	–	–	–	3.7	–	–	–
epi-Cubenol	1630	–	–	–	–	–	–	–	0.8	–	–	–	–
γ-Eudesmol	1632	–	–	–	–	–	–	–	1.0	–	–	–	–
Isospathulenol	1637	–	–	–	–	–	–	1.2	–	–	–	–	–
Caryophylla-4(14), 8(15)-diene-5-α-ol	1641	1.7	1.4	–	1.3	–	–	–	–	–	–	–	0.8
allo-Aromadendrene	1641	–	–	–	0.1	–	–	–	0.8	2.6	–	0.6	–
α-Muurolol	1646	–	–	0.5	–	–	–	–	–	3.2	–	–	–
β-Eudesmol	1651	0.2	0.5	–	2.2	–	–	–	–	3.3	–	–	1.4
Selin-11-en-4α-ol	1660	–	–	–	5.5	–	–	–	–	–	–	–	–
cis-Calamene-10-ol	1661	–	–	–	–	–	–	–	–	1.0	–	–	–
ar-Tumerone	1669	–	–	–	–	–	–	1.3	–	–	–	–	–
14-Hydroxy-9-epi-(*E*)-caryophyllene	1670	2.5	0.6	–	2.1	–	–	–	–	–	–	–	–
Eudesma-4(15),7-dien-1-β-ol	1688	–	–	–	0.9	–	–	–	–	–	–	–	–
Heptadecane	1700	–	0.9	–	–	–	–	–	–	–	–	–	–
Benzyl benzoate	1760	2	–	–	0.1	–	0.2	–	0.1	–	–	–	–
Octadecane	1800	–	0.3	–	–	0.8	–	–	–	–	–	–	0.2
6,10,14-Trimethyl 2-pentadecanone	1845	0.2	–	–	0.2	–	0.9	2.8	0.1	0.2	–	0.8	0.5
Nonadecane	1900	–	0.6	–	–	0.5	–	–	–	–	–	–	0.2
Farnesyl acetone	1917	0.1	0.7	–	0.1	–	0.2	–	–	–	–	–	–
Methyl hexadecanoate	1922	0.1	–	–	–	0.8	–	–	–	–	–	–	–
Phytol	1943	0.2	0.2	0.2	–	–	–	3.8	–	–	–	–	–
Pimaradiene	1950	–	–	–	–	–	–	–	–	–	–	–	0.6
Sclarene	1975	–	–	–	0.2	–	–	–	–	–	–	–	–
Hexadecanoic acid	1976	0.2	–	0.4	–	–	–	9.1	0.1	–	–	–	0.6
Geranyl linalool	2032	–	–	–	0.6	–	–	–	–	–	–	–	0.1
Abietatriene	2057	–	–	–	–	0.8	–	–	–	–	–	–	–
Heneicosane	2100	–	5.1	–	–	–	–	0.5	–	–	–	–	0.2
Tricosane	2300	–	0.5	–	–	–	–	0.8	–	–	–	–	0.2
Tetracosane	2400	–	0.3	–	–	–	–	0.8	–	–	–	–	–
Pentacosane	2500	–	–	–	–	–	–	1.7	–	–	–	–	–
Hexacosane	2600	–	–	–	–	–	–	12.8	–	–	–	–	–
Heptacosane	2700	–	–	0.1	–	–	–	2.1	–	–	–	–	–
Total		89.7	84.3	91.4	90.7	91.7	76.6	94.2	84.7	82.2	92.6	91.1	77

TABLE 3. Factor loadings for PCA of 12 population of *Nepeta*

Chemicals	PC1	PC2	PC3
4aα,7α,7aα-Nepetalactone	–0.150	0.988	–0.032
Hexanal	–0.001	0.007	0.000
Germacrene D	–0.622	–0.120	–0.770
Caryophylla-4(14),8(15)-diene-5-α-ol	–0.029	–0.003	0.077
β-Caryophyllene	–0.767	–0.095	0.623
14-Hydroxy-9-*epi*-*E*-caryophyllene	–0.036	–0.006	0.112

CPNN was performed using the CPNN toolbox in MATLAB environment (version 1.0, Milano Chemometrics and QSAR Research Group http://www.disat.unimib.it). Cluster and PCA analyses were performed by SPSS (SPSS version 11.5).

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