ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 15:5 (2021) 388-395

records of natural products

Characterization of *Nepeta viscida*, *N. nuda* subsp. *nuda* and the Putative Hybrid *N.* × *tmolea* Essential Oils

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(Received April 06, 2021; Revised April 30, 2021; Accepted May 01, 2021)

Abstract: *Nepeta viscida*, *N. nuda* subsp. *nuda*, and their putative hybrid *N.* × *tmolea* were evaluated for their essential oils with chemotaxonomical aspect. Hybrid of *N.* x *tmolea* individuals were observed in the regions where the distribution of *N. viscida* and *N. nuda* subsp. *nuda* taxa were present, namely Dursunbey (Balıkesir) and Ödemiş (İzmir) natural habitats, respectively. The aerial parts of the taxa were hydrodistilled for 4 h using a Clevenger-type apparatus. The essential oils were analyzed both by gas chromatography-mass spectrometry (GC/MS) and gas chromatography flame ionization detector (GC-FID). The main components of the oils for the species collected were spathulenol, *β*-elemene, and 1,8-cineole, supporting the hybridization proposition.

Keywords: Essential oil; *Nepeta viscida; Nepeta nuda* subsp. *nuda*; *Nepeta* × *tmolea*; chemotaxonomy.

1. Introduction

The Lamiaceae is an important medicinal and aromatic plant family, and its members are used in a wide range of applications in the world and in Turkey [1]. It is documented that the Lamaiaceae consists of 48 genera and 782 taxa (603 species, 179 subspecies and varieties), 346 taxa (271 species, 75 subspecies and varieties) of which are endemic (ca. 44%) in Turkey [2]. The genus *Nepeta* L. is a member of the Lamiaceae family, and one of the largest genera, consisting of approximately 300 species [3-5]. *Nepeta* is represented by 45 taxa, 19 of which are endemic in East Anatolia and the Taurus Mountains in Turkey [5].

Studies on the secondary metabolite composition of *Nepeta* species have revealed that terpenoids (iridoids and their glucosides, di-, and triterpenes), flavonoids, and phenolic compounds are the most abundant components. 1,8-cineole, citronellol, and iridoid monoterpene nepetalactones have frequently appeared as the main constituents of the essential oil composition [6,7], although composition of the oil varies in terms of region, genetics, climatic conditions, and vegetation period. Nepetalactones were reported to have insect repellent properties and are recorded as a major constituents of *N. cataria* L. (catnip), which is famous for its unusual effects on cat behavior [8-9]. Earlier studies conducted on the *Nepeta* extracts, essential oils, and their constituents showed analgesic, anti-Alzheimer, antibacterial, antifungal, repellent, cytotoxic, antioxidant, and urease activities among others [10-14].

N. nuda L. is one of the most common species of the genus and has four subspecies (KEW checklist, accessed on 18.02.2020) and *N. viscida* Boiss. is endemic to Turkey [5]. The essential oil of

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N. nuda subsp. *nuda* [9,15-17] and *N. viscida* [15] and fatty acid content [18] of both species were investigated in earlier studies. However, to the best of our knowledge this is the first report on the essential oil of the putative hybrid *N.* × *tmolea*. In this present study, the chemical compositions of essential oil of *N. nuda* subsp. *nuda*, *N. viscida* and their hybrid *N.* × *tmolea* collected from Ödemiş (İzmir) and Dursunbey (Balıkesir) districts were reported for the first time.

2. Materials and Methods

2.1. Plant Material

Aerial parts of *N. viscida*, *N. nuda* subsp. *nuda* and *N.* × *tmolea* were collected at flowering stage from their natural habitats in Balıkesir and İzmir provinces in 2017 (Table 1). The species were collected and identified by Prof. Dr. Tuncay Dirmenci and Assoc. Prof. Dr. Taner Ozcan. Voucher specimens were deposited in Necatibey Education Faculty Herbarium of Balıkesir University in Balıkesir, Turkey.

2.2. Essential Oil

The air-dried aerial parts of species (100 g of each) were cut into small pieces and subjected to hydrodistillation for 4 h, using a Clevenger-type apparatus to obtain the corresponding essential oil, the oil yields were listed in Table 1. The obtained essential oils were dried over anhydrous $CaCl_2$ and stored in amber vials at 4ºC until further work.

Table 1. List of the *Nepeta* species.

***Nepeta* Species**	**Oil Yield (v/w)**	**Code**	**Locality**
N. viscida	0.1	TD4759	Dursunbey, Alaçam Mount, Gölcük, around Karaveli Hill, 1515m
N. nuda subsp. *nuda*	0.1	TD4757	
N. × *tmolea*	0.05	TD4758	
N. viscida	0.03	TD4766	Ödemiş-Bozdağ, İzmir around Bozdağ ski resort
N. nuda subsp. *nuda*	0.1	TD4764	
N. × *tmolea*	0.1	TD4765	

2.3. Gas Chromatography-Mass Spectrometry Analysis

The essential oils were diluted with CH_2Cl_2 (1:10) and analyzed using a Trace 1310 GC series gas chromatograph (Thermo Scientific, Inc., ON, CA), which was connected to FID and a Thermo TSQ 9000 mass spectrometer. The separation was carried out in a DB5-fused silica column (60 m, 0.25 mm, ø with 0.5 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was kept at 100 °C for 5 min, programmed to 240 °C at a rate of 4 °C/min and kept constant at 240 °C for 5 min. The injection and source temperatures were 250 °C and 220 °C, respectively. The MS interface temperature was 240°C. The injection volume was 0.5 µL with a split ratio of 1:20. Injections of the samples were performed in triplicate. EI/MS was recorded at 70 eV ionization energy. The scan time was 0.5 sec. with 0.1 interscan delay. The mass range was *m/z* 35 to 650 amu [19].

Essential oil components were identified by comparing their retention indices (RI) obtained by a series of *n*-alkanes (C_5-C_{30}) and with mass spectra with those obtained from authentic samples and/or the National Institute of Standards and Technology (NIST) and Wiley spectra as well as the literature data [7, 19-28]. GC/MS analyses were performed in triplicate and listed in Table 2. as mean (RSD % <0.1).

2.4. Chemotaxonomic Evaluation

In this study, the hybrid *N.* × *tmolea* and its parents, *N. viscida* and *N. nuda* subsp. *nuda*, were examined in terms of chemotaxonomic characters. For this purpose, a total of 6 samples from two different regions and 36 characters were examined. Chemotaxonomic cladograms were constructed using PAUP* 4.0a165 [29] and a dendrogram was conducted using a dendroscope [30]. The data obtained according to 36 characters were first converted to a "nexus" file and the file in this format was converted to a dendrogram using the PAUP* program. Maximum Likelihood criterion was used to obtain a dendrogram according to 36 characters and the program was run with 10000 cycles to see bootstrap values.

3. Results and Discussion

3.1. Essential Oil Composition

The essential oils from aerial parts of *Nepeta* taxa and hybrid specimens were characterized by GC-FID and GC-MS. GC analysis of parents and hybrids of *Nepeta* oil samples showed that the oil content of all species was qualitatively similar but differed according to the abundance of their major components. Thirty-six compounds which accounted for 99.6–100% of the total composition of oils are listed in Table 2. The essential oil contents were grouped into five classes (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and hydrocarbons). The oil of five taxa consisted mainly of sesquiterpene hydrocarbons while *N.* × *tmolea* (Ödemiş) was oxygenated sesquiterpenes. Spathulenol (10.1-45.2%), *β*-elemene (1.2-76.8%) and 1,8-cineole (0.4-24.5%) were identified as the major components of the essential oil of *Nepeta* species.

When the essential oil result of *N. viscida* (Ödemiş) was examined, a total of 22 compounds accounting for 99.9% of total oil composition were identified, representing trace (t) to-19.5% of the oil. The oil was composed mainly of sesquiterpene hydrocarbons (53.9% of the total oil) and spathulenol was detected as a major compound (19.5%) together with *β*-caryophyllene (14.7%).

Nineteen compounds were identified in the oil of *N. viscida* (Dursunbey) representing 99.9% of the oil. It was composed of sesquiterpene hydrocarbons (37.4%) and oxygenated sesquiterpenes (30.1%). Unlike *N. viscida* (Ödemiş), an oxygenated monoterpene 1,8-cineole (24.5%) was determined as a major compound of the oil. On the other hand, spathulenol (21.8%) and *β*-caryophyllene (15.9%) were identified as the other main compounds as well. Both species were found to be rich in sesquiterpene hydrocarbons. Although they were collected from different localities, the qualitative contents of essential oils were found to be similar. However, in the former study on the essential oil of *N. viscida* from Muğla province α-terpineol (31.57%) was reported as the main compound together with *β*-caryophyllene (7.52%) [31].

N. nuda subsp. *nuda* (Ödemiş) and *N. nuda* subsp. *nuda* (Dursunbey) essential oils were characterized by 19 and 32 compounds accounting for 99.9% and 99.6% of total essential oil, respectively. *N. nuda* subsp. *nuda* (Ödemiş) was identified to be the richest one of the studied taxa in terms of sesquiterpene hydrocarbons (84.3%). Spathulenol (21.2%) was determined as the main compound of the *N. nuda* subsp. *nuda* (Dursunbey) followed by *β* -caryophyllene (18.2%). *N. nuda* subsp. *nuda* (Ödemiş) oil was composed of a high ratio of *β*-elemene (76.8 %). Following *β*-elemene, spathulenol (10.1%) was found in a high ratio in *N. nuda* subsp. *nuda* (Ödemiş) oil. The amount of other compounds was determined in the range of 0.1 to 3.2% of the total oil content. Although the oils of *Nepeta nuda* subsp. *nuda* species collected from different localities are highly similar as concerns sesquiterpene hydrocarbon, their main components differ from each other.

Table 2. Essential oil composition (relative %) of *Nepeta* species

			Ödemiş			Dursunbey		
RI[a]	**RI[L]**	**Compounds[b]**	***N. viscida***	***N. nuda* subsp. *nuda***	***N.* x *tmolea***	***N. viscida***	***N. nuda* subsp. *nuda***	***N.* x *tmolea***
		Monoterpene hydrocarbons	**1.1**	**0.1**	**17.8**	**5.6**	**1.9**	**3.9**
930	921-936	*α*-Thujene	t	-	0.5	-	t	0.1
939	931-942	*α* -Pinene	0.1	-	1.9	2.5	0.2	0.7
954	946-954	Camphene	-	-	-	-	-	-
975	956-978	Sabinene	0.8	-	13.3	3.1	1.4	2.0
979	974-981	*β*-Pinene	-	-	-	-	-	-
1003	1002-1006	*α*-Phellandrene	0.2	0.1	2.1	-	0.2	1.1
1037	1032-1041	(Z)- *β*-Ocimene	-	-	-	-	0.1	-
		Oxygenated monoterpenes	**14.6**	**2.8**	**8.9**	**25.6**	**2.8**	**9.4**
1031	1026-1035	1,8-Cineole	2.7	0.4	2.8	**24.5**	1.0	5.1
1060	1056-1061	*γ*-Terpinene	-	-	0.2	-	t	-
1070	1065-1067	*cis*- Sabinene hydrate	-	-	-	-	0.1	0.7
1143	1139-1143	*cis*-Sabinol	-	-	0.5	-	0.1	-
1162	1148-1161	Isoborneol	-	0.2	0.7	-	0.2	0.3
1221	1206	*cis*-Sabinene hydrate acetate	11.9	0.2	3.7	1.1	0.4	0.5
1229	1226-1230	*cis*- Carveol	-	0.6	-	-	0.7	0.5
1245	1244	Carvacrol methyl ether	-	-	-	-	t	-
1290	1290-1292	Thymol	-	-	-	-	0.3	2.2
1352	1350-1352	Thymol acetate	-	1.4	1.0	-	-	0.1
		Sesquiterpene hydrocarbons	**53.9**	**84.3**	**47.9**	**37.4**	**66.0**	**28.7**
1377	1363-1377	*α*-Copaene	3.6	0.7	0.7	0.5	6.0	2.0
1388	1369-1387	*β* -Bourbonene	1.9	-	-	0.8	0.9	2.2
1391	1377-1391	***β* -Elemene**	-	**76.8**	**43.2**	1.2	2.6	3.7
1412	1398-1414	Cedrene	6.8	-	-	-	0.4	-
1419	1405-1420	*β* -Caryophyllene	14.7	3.2	0.7	15.9	18.2	10.1
1434	1431-1432	*β*-Gurjunene	-	-	-	1.7	3.3	1.2
1460	1444-1458	Alloaromadendrene	8.2	2.2	1.4	13.8	8.9	3.8
1485	1474-1482	Germacrene-D	2.4	-	-	1.5	6.6	-
1500	1481-1500	Bicyclogermacrene	5.7	0.8	1.6	0.5	1.6	1.1
1523	1516-1525	*δ*-Cadinene	4.7	-	-	-	9.6	4.6
1539	1538-1539	*α*-Cadinene	5.3	-	-	1.5	7.0	-
1546	1538-1544	*α*-Calacorene	0.6	0.6	0.3	-	0.9	-
		Oxygenated Sesquiterpenes	**30.3**	**12.7**	**25.3**	**31.3**	**28.9**	**57.8**
1569	1565-1568	Ledol	1.2	0.1	0.8	-	0.2	-
1578	1577-1587	**Spathulenol**	**19.5**	10.1	18.9	21.8	**21.2**	**45.2**
1583	1581-1583	Caryophyllene oxide	0.9	0.1	0.5	1.9	-	1.0
1585	1584-1585	Globulol	3.3	0.8	2.8	1.5	3.3	3.7
1593	1593	Viridiflorol	2.1	0.3	-	1.1	1.4	1.3
1654	1652-1654	*α*-Cadinol	3.3	0.6	1.3	3.8	2.2	5.0
1563	1561-1563	Nerolidol	-	0.7	1.0	1.2	0.6	1.6
		Total	**99.9**	**99.9**	**99.9**	**99.9**	**99.6**	**99.8**

[a]RI Retention indices.
RI[L] Retention indices derived from literature [19-28] and NIST database
[b]The compounds are grouped into chemical families in elution order from DB-5 column (n=3, Mean RSD % value 0.05). t: trace.

Essential oil components of *N. nuda* subsp. *nuda* (Dursunbey) were found to be richer than *N. nuda* subsp. *nuda* (Ödemiş) essential oil. When compared to former studies, the main compound of essential oil of *N. nuda* subsp. *nuda* was determined as *β*-caryophyllene oxide (21.8%) [9], 1,8-cineole (14.9%) [15]. 4a*α*,7*α*,7a*β*-nepetalactone (24.7%) [16] and camphor (23.5%) [17] were determined in former studies, however in this study it was not possible to detect the nepetalactones in the essential oils. These different results in the oil contents and compositions could be explained by the location or the chemotype of the species.

The hybrid *N.* × *tmolea* (Ödemiş) was found to be rich in sesquiterpene hydrocarbon, like its ancestor. A total of 22 compounds were determined, representing 99.9% of the oil. *N.* × *tmolea* (Ödemiş) oil was characterized by the abundance of *β*-elemene (43.2%), spathulenol (18.9%) and sabinene (13.3%). The essential oil content of the hybrid was found to be very similar to that of the ancestor. *β*-Elemene was the main compound of ancestor *N. nuda* subsp. *nuda (*Ödemiş) and hybrid. Furthermore, spathulenol was the second major compound of the oil of *N.* × *tmolea* (Ödemiş), which was determined as the main component of another parent *N. viscida* (Ödemiş).

In the hybrid *N.* × *tmolea* (Dursunbey) 25 compounds were identified which represented 99.8 % of the oil. In the oil of *N.* × *tmolea* (Dursunbey) spathulenol (45.2%) and *β*-caryophyllene (10.1%) were found as major compounds. Unlike its parents *N. viscida* (Dursunbey) and *N. nuda* subsp. *nuda* (Dursunbey), hybrid *N.* × *tmolea* (Dursunbey) was found to be rich in oxygenated sesquiterpenes due to the high ratio of spathulenol. When other components of *N.* × *tmolea* (Dursunbey) essential oil were compared, it was associated to its parents.

Hybrids forms are more frequently formed among plant species than animal species in nature. In some genera (*Origanum* and *Thymus*) of the Lamiaceae family, hybrid taxa are frequently encountered [32-34]. However, the *Nepeta* genus, which has about 300 taxa, has only 4 hybrids; hybrids generally have morphological intermediate characters between parent species in plants [35,36]. Likewise, *N.* × *tmolea* has morphological intermediate characteristics such as indumentum, calyx, and corolla size between its parents. Although *N.* × *tmolea* individuals have morphologically intermediate forms between *N. viscida* and *N. nuda* subsp. *nuda*, some hybrid individuals are similar to *N. viscida* and some individuals are similar to *N. nuda* subsp. *nuda* as regards morphology [4]. In addition to this morphological evidence, the similarities and differences between *N.* × *tmolea* specimens and its parents have been genetically proven [5].

In the Lamiaceae family, morphological, molecular, and chemotaxonomic approaches can be useful to define the relationship between hybrids and their parents. In this study, the chemical data obtained using *N.* × *tmolea* individuals and their parents, *N. viscida* and *N. nuda* subsp. *nuda* taxa, support previous molecular and micromorphological studies [4,5]. As can be seen from the dendrogram (Figure 1), *N. viscida* individuals from two different regions (Ödemiş and (Dursunbey) are located on the outermost clade, and two *N. nuda* subsp. *nuda* (Ödemiş and Dursunbey) are on the inner clade. *N.* × *tmolea* (Dursunbey) and *N. nuda* subsp. *nuda* (Dursunbey) had similar chemical contents, and *N.* × *tmolea* (Ödemiş) and *N. viscida* (Dursunbey) were closer to each other. It was shown that; while the chemical contents of the *N. nuda* subsp. *nuda* samples in two different locations are similar, the contents of the *N. viscida* samples examined from different locations are also more similar. Hybrid taxa are similar to in terms of basic components of *N. nuda* subsp. *nuda*, in dendrogram; *N.* × *tmolea* (Dursunbey) shows similarity to *N. nuda* subsp. *nuda* (Dursunbey) while the other hybrid sample *N.* × *tmolea* (Ödemiş) seems closer to *N. viscida* (Ödemiş) in terms of overall content. As mentioned above, the morphological similarity of hybrid taxa to their parents may cause similarity of chemical contents. Therefore, it can be assumed that genetic factors as well as ecological conditions affect the similarity in terms of chemical content.

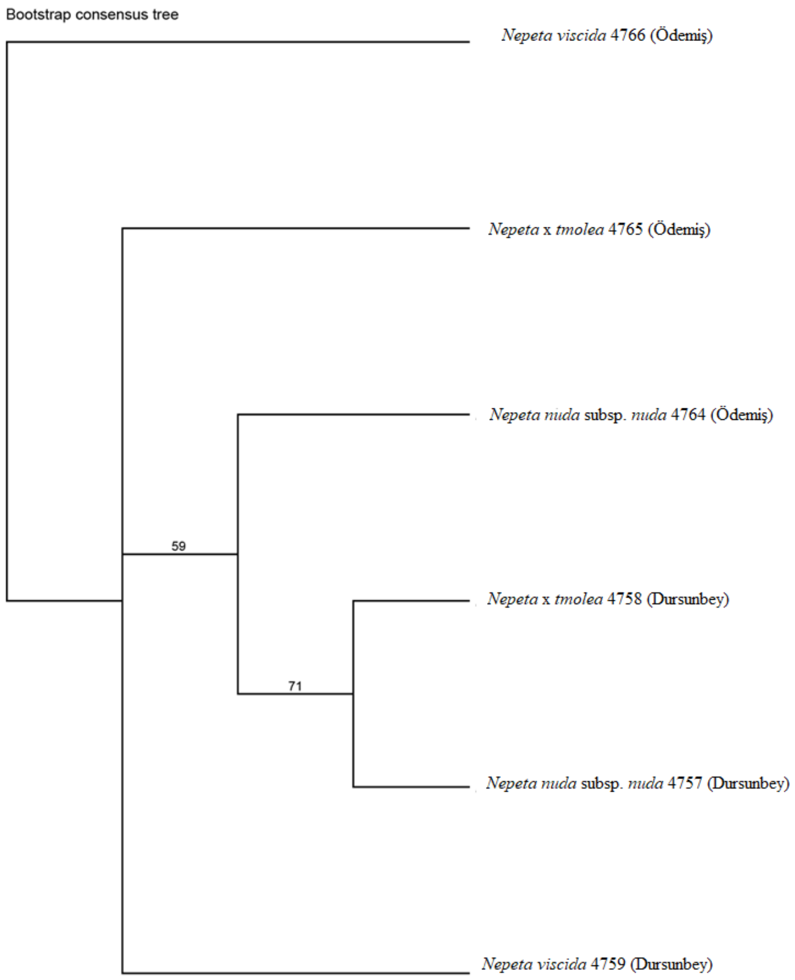

Figure 1. Dendrogram of cluster analysis constructed from the chemical contents of essential oil of *N. viscida*, *N. nuda* subsp. *nuda*, and their putative hybrids *N.* × *tmolea*

4. Conclusions

Essential oil compositions of two parents, *N. viscida*, *N. nuda* subsp. *nuda*, and their hybrid *N.* × *tmolea* were collected from two different localities, and their chemotaxonomic relationship are well defined in this study. Reported properties of six samples of three taxa belonging to the genus *Nepeta* are reported here for the first time. The composition of essential oils obtained from plants collected from different localities are similar, and hybrid species possess the characteristics of their parents. The hybrid shows a close similarity in the sesquiterpene profile, and essential oils are attributable to the relatively high concentrations.

Acknowledgments

The author is grateful to Prof. Dr. Tuncay Dirmenci, and Assoc. Prof. Dr Taner Ozcan (Balıkesir University, Necatibey Education Faculty, Department of Biology Education, Balıkesir, TURKEY) for the collection, identification and the photograph of the plant samples.

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