Journal of Medicinal Plants and By-products (2023) 4: 375-386
https://doi.org/10.22092/jmpb.2022.357895.1455

Original Article

Volatile Oil Composition of *Nigella sativa* L. Cultivars Collected from Iran

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Article History

Received: 20 February 2022
Accepted: 30 April 2022

Keywords
Black cumin
Geographical location
PCA
Thymoquinone
Volatile oil

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ABSTRACT

Nigella sativa L., belonging to the family of Ranunculaceae, is grown for its seeds, which are used in food and medicinal industries. In this study, the volatile oil compositions of 16 cultivars of *N. Sativa* collected from different parts of Iran were evaluated, and then the chemotaxonomic study was done. The analysis of volatile oils was done using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), which identified 22 components. Major constituents of the collected cultivars were *p*-cymene (27.7-38.1%), thymoquinone (19.5-40.9%), thymohydroquinone (5.8-9.6%), *α*-thujene (5.1-7.2%), and *trans*-4-methoxythujane (3.4-4.8%). Then, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used to assess differences in volatile oil composition. PCA displayed that different groups of cultivars were characterized by high contents of specific compounds. The results of chemometric analysis displayed that PCA was approximately in agreement with HCA, and allowed classification of the studied cultivars into four groups including Group-A (Ns-4, Ns-5, and Ns-16); Group-B (Ns-1), Group-C (Ns-2, Ns-3, Ns-7, Ns-8, and Ns-12), and Group-D (Ns-6, Ns-9, Ns-10, Ns-11, Ns-13, Ns-14, and Ns-15). This is the first study on *N. sativa* in a vast geographical context in Iran to this extent, using different chemometric techniques. The results of this study can offer scientific data for germplasm of *N. sativa* in Iran and for breeding programs to improve developed cultivars.

INTRODUCTION

Black cumin (*Nigella sativa* L.), belonging to the family of Ranunculaceae, is an herbal medicinal plant, which is cultivated in Southeastern and Western Asia, southern Europe, Northern Africa, and Australia [1]. This plant is generally cultivated in Iran in center and eastern parts in Khorasan, northwest parts in Tabriz, and south parts in Fars [2]. The seeds are used widely for flavoring and medicinal purpose. *N. sativa* seeds or extracts possess antibacterial [3], antioxidant [4], anti-inflammatory [5], antidiabetic [6], anticancer [7], and anti-nausea [8] activities. An authentic saying of the Prophet Muhammad (Peace Be Upon Him) about black seed is quoted in Al-Bukhari as: Abu Huraira (Allah be pleased with him) narrated that Allah's Apostle (peace be upon him) said 'Use the black seed, which is a healing for all diseases except Death' [9]. Many of the mentioned pharmacological activities have been attributed to the main active constituents of this plant such as thymoquinone and *p*-cymene [10,11]. Several researches have reported that *N. sativa* and its main bioactive component (thymoquinone) play a main role in cancer prevention through activation and deactivation of molecular pathways, and antimicrobial effects [12, 13]. However, the effects of other minor components cannot be neglected and may contribute to these observed effects. Previous researches hence indicated that monoterpenes including *p*-cymene, *α*-thujene, *γ*-terpinene, carvacrol, *α*-pinene, and *β*-pinene were major constituents of the volatile oils of *N. sativa* [14-16]. As far as we know, biological properties of medicinal plants have been found to be

directly linked to their phytochemical constituents, which are influenced by genetic and environmental factors [17-19]. Therefore, the availability of data describing the variations in beneficial components seems to be crucial and in turn, could allow us to better understand the quality of the herb material used [20]. Therefore, it would be really essential to develop a more reliable method to determine the phytochemical differences in *N. sativa* cultivars.

To best of our knowledge, several chromatographic approaches have been used for evaluation of phytochemical components of medicinal plants, of which GC-MS technique is prevalently applied to analyze volatile oil components. Previous studies have investigated the phytochemical diversity of medicinal plants with the help of chemometric techniques [21,22]. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are used to confirm phytochemical variability and interrelationships among cultivars and their similarities, regarding volatile oil components. These techniques are ordering methods, attempting to reduce data set dimension in order to simply describe behavior of the system based on phytochemical profile [23-25].

The aim of the present study was to investigate the variability in volative oils obtained from *N. sativa* cultivars, collected from 16 different geographical locations in Iran. The aim of this investigation was to analyse the volatile oil composition of 16 *N. sativa* cultivars, which were collected from different geographical zones, followed by performing different chemometric methods. The present study might provide a scientific data for germplasm of N. sativa in Iran.

MATERIALS AND METHODS

Chemicals

All solvents and chemical compounds which were used for the extraction of essential oils were purchased from Merck Co., Germany.

Plant Material

The sixteen cultivars of *N. sativa* from different provinces of Iran were collected during July-September, 2018. From each region, 80–100 samples were randomly gathered, and then their seeds were separated. The studied regions were located between the geographical coordinates from 31°02'47.59"N to 37°24'12.13"N and from 45°52'00.7"E to 60°17'22.4"E, and from -20 to 2303 m of height (Table 1). The identification of the plant material was done by one of the authors (Dr. Zohreh Ghanavi), and the voucher specimens (NS2018i, where i represents the provenance number varied from 1 to 16) of the plants were deposited at the Herbarium of Azarbaijan Shahid Madani University, Iran. Samples were allowed to dry in shade and were kept in refrigerator under 4 °C until analysis. Seeds were brought to the laboratory and their volatile oil compositions were analyzed using GC-FID and GC-MS.

Table 1 Locality information of the studied populations of *N. sativa*

Sample		Locality	Latitude (N)	Longitude (E)	Elevation (m)
Ns-1	NS20181	Gilan: Fuman	37°15'03.6"	49°20'34.7"	-3
Ns-2	NS20182	South Khorasan: Tabas: Esfandiar	33°02'14.7"	57°33'16.9"	960
Ns-3	NS20183	Kurdistan: Dehgolan: Qadermarz	35°08'41.44"	47°22'09.99"	1910
Ns-4	NS20184	Markazi: Shazand: Aliabad	33°59'24.4"	49°26'09.1"	1920
Ns-5	NS20185	Kerman: Ravar: Rihan	31° 16' 20.31"	56° 49' 53.78"	1919
Ns-6	NS20186	Razavi Khorasan: Quchan: Emamqoli	37°24'12.13"	58°31'00.28"	1680
Ns-7	NS20187	South Khorasan: Ferdows: Boshruyeh	33°53'01.11"	57°27'32.66"	873
Ns-8	NS20188	Khuzestan: Baghe malek	31°31'15.3"	49°52'45.9"	917
Ns-9	NS20189	Kermanshah: Rawansar: Elyasi	34°36'20.5"	45°52'00.7"	1336
Ns-10	NS201810	Mazandaran: Chalus	36°39'25.2"	51°22'42.3"	-20
Ns-11	NS201811	Golestan: Ramian: khanbin	37°00'57.10"	54°97'72.95"	41
Ns-12	NS201812	Fars: Abadeh	31°08'40.5"	52°37'36.1"	2030
Ns-13	NS201813	South Khorasan: Darmiyan: Rezeh	32°39'19.4"	60°17'22.4"	1290
Ns-14	NS201814	Isfahan:Semirom: Hana	31°12'03.75"	51°43'48.67"	2303
Ns-15	NS201815	Khuzestan: Dezful: Safiabad	32°22'38.7"	48°27'45.5"	1312
Ns-16	NS201816	Yazd: Abarkooh: Ardi	31°02'47.59"	53°20'23.55"	1487

Volatil Oil Extraction

For extraction of volatile oils from *N. sativa*, the method of Botnick and coworkers was followed with slight modifications [26]. Briefly, *N. sativa* seeds were frozen in liquid N_2 and crushed manually with a mortar and pestle. Extraction of the crude extract was performed via addition of a 3 to 1 ratio mixture (v/w) of tert-butyl methyl ether (purity: ≥99.8 %). After a short vortex (about 10 sec), the sample was shaken for 120 min/800 rpm in the refrigerator at 4 °C. Then, the mixture was centrifuged at 6000 rpm for 4 min/4 °C and the clear supernatant was collected. Then, the crude extract was subjected to the standard steam distillation, in order to isolate volatile oil components of the extract. The obtained volatile oil was dehydrated using anhydrous Na_2SO_4, and then was stored in a sealed dark vial (-20 °C in a freezer), before the next analysis.

Gas Chromatography (GC) Analyses

GC analysis was performed using a Varian CP-3800 gas chromatograph equipped with FID detector. The analysis was carried out on fused silica capillary DB5 column (60 m × 0.25 mm i.d.; film thickness of 0.25 µm). The injector and detector temperatures were kept at 250 and 300 ºC, respectively. N_2 was utilized as the carrier gas. The amount of injection was 1 µL with 1:50 ration. The initial oven temperature was set at 50 °C and temperature gradient was programmed to reach 190 °C by the ramp of 5 °C/min, and then 300 °C by the ramp of 15 °C/min, which was next held for 10 min. For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

Gas chromatography-mass spectrometry analyses (GC-MS)

GC-MS analysis was carried out on a GC/MS instrument (Agilent 6890, USA) equipped with an DB5 column (60 m×0.25 mm i.d.; 0.25 µm film thickness) in splitless mode. The injection volume was 1 µL. Helium was used as carrier gas at a flow rate of 0.8 ml/min. Oven temperature program was the same as mentioned above for GC. MS operating parameters were: ionization energy of 70 eV, ion source temperature of 230 °C, interface temperature of 250 °C, the scanning range of 41-350 m/z, and scanning time of 3.12 scan/sec.

The Kovats Indices (KI) of the components in volatile oils were calculated under temperature-programmed conditions for the homologous series of n-alkanes (C_6–C_{24}). Moreover, each component was identified via comparison of the corresponding mass spectra with those of internal reference mass spectra library or with those of authentic compounds, and subsequent confirmation was done by comparison of their retention indices with those of authentic compounds or reported retention indices in literature [27, 28].

Statistical Analysis

İn this study, each experiment was performed with at least four replication. Data were statistically analyzed using one-way ANOVA by SAS (version 9.4), and mean comparisons of the main constituents of essential oil were evaluated by applying Tukey's test at p <0.05 level. For multivariate analysis, the data were subjected to statistical software of XLSTAT (ver. 2014.2.03). In order to verify the relationships between volatile oil components, Pearson's correlation test was used. Estimation of correlation coefficients were carried out to evaluate the diferences among volatile oil constituents of each *N. sativa* sample using SAS version 9.4 software. The Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used to assess differences in volatile oil compositions. In this study, PCA was carried out based on the normalized relative peak areas of 25 constituents in volatile oils of *N. sativa* using XLSTAT software Version 2014.2.03 (STATCON, Witzenhausen, Germany). HCA using the un-weighted pair group method with arithmetic mean (UPGMA) was used to construct a dendrogram representing all cultivars. Concerning volatile oil constituents, the cultivars were classified in different groups, and cultivars with close similarities were categorized into the same cluster.

RESULTS

Volatile Oil Content of *N. sativa* Seeds

The essential oil yields of 16 cultivars of *N. Sativa* ranged from 0.12 mL/100 g to 0.49 mL/100 g in terms of dry weight (Fig. 1). The Ns-6 showed the highest yield (0.49 mL/100 g), followed by Ns-12 (0.42 mL/100 g). The Ns-11 indicated the lowest

yield (0.14 mL/100 g), and showed a significant difference with the other cultivars ($p < 0.05$).

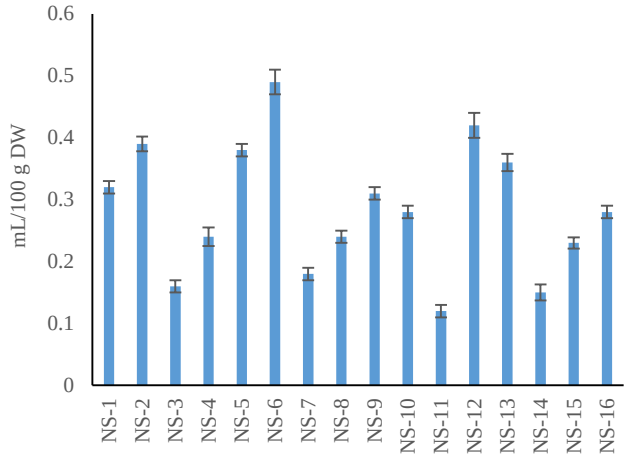

Fig. 1 Volatile oil yields of different cultivars of *N. sativa* (cultivars numbers are given in Table 1)

Then, the volatile oils were analyzed using GC-FID and GC-MS techniques. As can be seen in Table 2, a total of twenty-two compounds were identified, representing up to 95.5% of the total volatile oils. The identified compounds were found in all studied cultivars, but at different percentages. Bajalan *et al*. [29] studed the essential oil content and composition of different Iranian *Rosmarinus officinalis* accessions according to environmental conditions. Indeed, significant correlations were observed between essential oil composition and soil conditions in the growing sites. In another study, the variation of the essential oil compositions among 10 wild populations of *H. perforatum* growing in Iran was assessed, and it was concluded that the variability could be related to the effect of variables such as genetic factors, developmental stages, types of plant materials, methods of extraction, environmental conditions, etc. [30]. Morshedloo *et al*. [31] investigated the variation of the essential oil compositions at wild populations of *Rhus coriaria*, and significant variability was observed among the investigated populations. Therefore, according to previous researches and our results, the change in the percentage of volatile oil constituents of *N. sativa* cultivators colud be due to differences caused by the geographical location of plant growth, climate conditions, collection time, development stage, and other culture conditions, etc. [32, 33].

As can be seen in Table 2, *p*-cymene, thymoquinone, thymohydroquinone, *α*-thujene, and *trans*-4-methoxythujane were the major components of all collected cultivars, representing 94.5-96.4 % of monoterpene compounds. A high percentage of *p*-cymene showed that *N. sativa* seeds could be a good source of this compound, exhibiting percentages varying from 27.7 to 38.1%, with maximum values reported in Ns-9 (38.1 %), Ns-12 (38.0%), Ns-11 (37.7%), and Ns-8 (37.7%). The percentages of thymoquinone ranged from 19.5 to 40.9%, with maximum values obtained in Ns-16 (40.9%), Ns-5 (26.7%), Ns-4 (27.3%), Ns-6 (26.0%), and Ns-7 (26.2%). *p*-cymene is an alkyl-substituted aromatic compound, naturally occurring in essential oils of various aromatic plants. Numerous reports have demonstrated the pharmacological activites of *p*-cymene including antioxidant, antiparasitic, anti-inflammatory, antidiabetic, antitumor, antiviral, antibacterial, and antifungal activities. This compound has also been reported to act as an analgesic, immunomodulatory, antinociceptive, vasorelaxant, and neuroprotective agent [34]. The percentages of thymohydroquinone ranged from 5.8 to 9.6%, with maximum values obtained in Ns-11 (9.6%), Ns-3 (8.8%), and Ns-10 (8.6%). α-thujene was the other main compound in *N. sativa* seeds, and its percentages ranged from 5.1 to 7.2%, with maximum values recorded in Ns-2 (7.1%), and Ns-3 (7.2%). The percentages of *trans*-4-methoxythujane ranged from 3.4 to 4.8%, with maximum values reported in Ns-15 (4.8%) and Ns-14 (4.7%).

The identified compounds were categorized into seven groups including monoterpene hydrocarbons, monoterpene alcohols, monoterpene ethers, monoterpene ketones, monoterpene esters, aldehydes, and sesquiterpene hydrocarbons. The major group was monoterpene hydrocarbons at the percentages of 39.4-56.4 %, and this was in line with previous research done in the very area [26, 31]. Monoterpene ketones were the second group which were reported as the major group (22.2–31.9%). Among the studied cultivars, Ns-12 (94.7%) was in correspondence with reported monoterpene content for *N. sativa* samples collected from Naan (94 %) using the same extraction procedure [26]. This may account for the observed higher content of monoterpenes in *N. sativa* cultivars in present study compared to other terpenoids. *p*-cymene and thymoquinone in the present investigation were the most abundant constituents in volatile oil of *N. sativa* in all cultivars. These findings are in accordance with

report of Mahmoudvand and coworkers [35]. A previous study has indicated that thymoquinone content depends remarkably on the geographical location of growth [32]. These observed differences in chemical constituents of volatile oils might be influenced by various geographical factors, climate conditions, season at the time of collection, stage of development, occurrence of chemotypes, and other culture conditions, etc., which further lead to the quality differences in *N. sativa* samples [33]. These findings are in agreement with those of ours, in that volatile oil constituents in present study varied notably among different cultivars collected from various geographical locations. As an instance, the highest thymoquinone content was related to cultivar 16 (Table 2), which was similar to the reported content of Bam distinct, Kerman province, Iran (42.4 %) [35]. The amount of *trans*-4-methoxythujane in collected samples from cultivars 5 and 16 in present study was similar to that of Naan, Israel (4.0 %), whereas contents of thymohydroquinone and α-thujene in collected samples differed from those reported for samples of Naan (23.2 and 10.4 %) and Ein-Harod (16.2 and 9.7 %), which have used the same extraction method [26].

Correlation Analysis

Results of correlation analysis (Table 3) demonstrated that the highest positive correlations were observed between *β*-pinene and α-thujene (0.87) at 1 % probability level. Moreover, there were negative correlations between thymoquinone and *p*-cymene (0.89), limonene (0.82) and carvacrol (0.82) at 1 % probability level. When monoterpenes are synthesized, further modifications including oxidation, hydroxylation, methylation, acylation, and cleavage can occur so as to produce various monoterpene derivatives [36, 37]. In biosynthesis pathway of thymoquinone in *N. sativa*, *γ*-terpinene is aromatized into *p*-cymene, followed by hydroxylations to carvacrol and thymohydroquinone, and then oxidation to thymoquinone [26]. *p*-cymene is main precursor of the oxygenated monoterpenes [38] and as it is indicated in Table 2, this compound was the predominant constituent of volatile oils in the analyzed samples. However, in samples with the highest thymoquinone content, the levels of *p*-cymene were decreased (e.g. cultivar 16). This is in agreement with biosynthetic pathway of thymoquinone, in which *p*-cymene acts as the common precursor [39]. Thymoquinone is a valuable monoterpene ketone, having many potential medical applications for diabetes, cardiovascular diseases [40], stroke, neurodegenerative diseases [41], and different types of cancer [42].

Principal Component Analysis (PCA)

PCA was performed in order to reduce the number of variables (25 variables corresponding to the number of constituents in volatile oil from *N. sativa*) to a smaller number of new derived variables. Percentages of volatile oils in 16 samples were subjected to PCA for a better description of this flavoring and medicinal plant. Table 4 demonstrates eigenvalues, variance explained% and Cumulative variance. Eigenvalue accounts for explanation of variance in data set, and the higher the eigenvalue, the more it explains the variability in data set. Based on Kaiser's rule, eigenvalues higher than 1.0 could be considered as descriptors of variance. The first 2 axes (F1 and F2), having the highest eigenvalues (6.74 and 4.34) explained 26.95 % and 17.35 % of the variance, respectively; which were selected in order to construct a two-dimensional diagram. The first two axes jointly displayed 44.30 % of the total variance. Factor loading values higher than 0.5 are highlighted in bold in Table 4. As seen in this table, among volatile oil components, *α*-thujene, *α*-pinene, *β*-pinene, *α*-terpinene, *p*-cymene, limonene, *γ*-terpinene, carvacrol, *trans*-4-methoxythujane, and thymoquinone (with the highest loading value by 0.96) accounted for the variance in PC1, whereas terpinen-4-ol, *cis*-4-methoxythujane, *trans*-4-methoxythujane, 1,2-epoxy-menth-4-ene, *2E,4E*-decadienal, and *trans*-caryophyllene explained the variation in PC2 (Table 4).

As it is obvious from Fig. 2, the first axis accounted for the positive contributions of the contents of 1,2-epoxy-menth-4-ene, longifolene, *β*-myrcene, *trans*-2-caren-4-ol, thymoquinone, longipinene, terpinen-4-ol, and *2E,4E*-decadienal, and the negative contribution of the content of *trans*-caryophyllene, *trans*-4-methoxythujane, carvacrol, *2E,4Z*-decadienal, thymohydroquinone, *p*-cymene, limonene, *γ*-terpinene, terpinolene, bornyl acetate, *α*-terpinene, *β*-pinene, *α*-pinene, *α*-thujene, sabinene, carvone, and *cis*-4-methoxythujane.

Table 2 Phytochemical pattern analyses of variance and mean comparison of chemical constituents in volatile extract of *N. sativa* among collected samples from Iran.

No.	Component	IM	[a]RI	[b]RI	(*Ns*-1)	(*Ns*-2)	(*Ns*-3)	(*Ns*-4)	(*Ns*-5)	(*Ns*-6)	(*Ns*-7)	(*Ns*-8)
1	*α*-Thujene	RI, MS	924	914	5.5±0.1	7.1±0.1	7.2±0.1	6.1±0.1	5.8±0.1	5.6±0.3	6.4±0.0	6.9±0.0
2	*α*-Pinene	RI, MS, CoI	932	924	1.5±0.2	1.0±0.1	1.0±0.0	1.3±0.1	1.1±0.0	1.4±0.0	1.5±0.0	1.5±0.1
3	Sabinene	RI, MS	969	965	1.1±0.1	0.4±0.3	1.1±0.0	1.0±0.0	0.8±0.1	0.9±0.0	0.9±0.1	1.0±0.0
4	*β*-Pinene	RI, MS, CoI	974	971	2.1±0.1	2.2±0.1	2.2±0.0	1.9±0.0	1.7±0.2	1.8±0.2	1.9±0.1	2.1±0.0
5	*p*-Cymene	RI, MS	1020	1015	35.1±0.4	36.3±0.0	36.8±0.1	31.4±0.1	35.8±0.1	33.5±0.1	33.8±0.0	37.7±0.0
6	Limonene	RI, MS, CoI	1024	1025	1.6±0.1	1.0±0.0	1.7±0.1	1.4±0.1	1.3±0.1	1.5±0.0	1.5±0.0	1.7±0.0
7	*γ*-Terpinene	RI, MS	1054	1052	1.6±0.1	2.5±0.1	2.8±0.2	2.4±0.0	1.7±0.0	1.9±0.0	2.3±0.0	2.4±0.0
8	Terpinolene	RI, MS	1086	1091	0.6±0.1	0.6±0.1	2.2±2.1	0.7±0.1	0.7±0.1	0.6±0.1	0.7±0.0	0.8±0.1
	Total monoterpene hydrocarbons				49.8	51.5	56.4	46.7	49.3	47.6	49.4	54.6
9	Terpinen-4-ol	RI, MS	1174	1165	1.0±0.1	0.8±0.0	0.8±0.1	1.0±0.0	0.9±0.0	0.9±0.1	0.7±0.0	0.5±0.0
10	Carvacrol	RI, MS, CoI	1298	1280	2.2±0.1	2.5±0.0	3.0±0.0	2.6±0.0	3.7±0.1	3.1±0.0	3.1±0.0	3.9±0.1
11	Thymohydro quinone	RI, MS	1553	1513	7.8±0.1	7.0±0.1	8.8±0.1	6.8±0.1	5.8±0.1	8.5±0.1	7.5±0.0	7.2±0.1
	Total monoterpene alcohols				11.0	10.3	12.6	10.4	10.4	12.5	11.3	11.6
12	*cis*-4-Methoxythujane	MS	-	1099	0.2±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
13	*trans*-4-Methoxythujane	RI, MS	1120	1113	4.3±0.1	4.3±0.0	3.4±0.0	4.2±0.1	4.0±0.0	4.4±0.1	4.3±0.1	4.6±0.0
14	1,2-epoxy-menth-4-ene	MS	-	1188	0.5±0.2	0.8±0.0	0.9±0.0	0.8±0.1	0.9±0.0	1.0±0.0	1.0±0.0	1.0±0.0
	Total monoterpene ethers				5.0	5.2	4.3	5.1	5.0	5.5	5.4	5.7
15	Carvone	RI, MS, CoI	1239	1212	0.3±0.1	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.3±0.3	0.2±0.0	0.1±0.0
16	Thymoquinone	RI, MS, CoI	1248	1222	25.7±0.0	25.0±0.0	19.5±0.7	27.3±0.1	26.7±0.1	26.0±0.0	26.2±0.0	19.1±0.1
	Total monoterpene ketones				26.0	25.2	20.7	27.4	26.9	25.3	26.4	19.2
17	Bornyl acetate	RI, MS	1284	1244	1.4±0.0	0.6±0.0	0.7±0.1	1.0±0.0	0.6±0.0	0.5±0.0	0.3±0.0	0.8±0.0
	Total monoterpene ester				1.4	0.6	0.7	1.0	0.6	0.5	0.3	0.8
18	*2E,4Z*-Decadienal	RI, MS	1292	1272	0.3±0.1	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
19	*2E,4E*-Decadienal	RI, MS	1315	1293	1.3±0.0	0.4±0.0	0.2±0.0	1.0±0.0	0.4±0.0	0.1±0.0	0.2±0.0	0.1±0.0
	Total aldehydes				1.6	0.5	0.4	1.2	0.6	0.3	0.4	0.3
20	Longipinene	RI, MS	1350	1361	0.6±0.0	0.7±0.0	0.6±0.0	1.4±0.0	0.8±0.0	0.5±0.1	0.7±0.0	0.9±0.0
21	Longifolene	RI, MS	1407	1415	1.5±0.1	3.1±0.0	2.4±0.4	2.5±0.1	2.5±0.2	2.4±0.1	2.9±0.0	2.5±0.0
22	*trans*-Caryophyllene	RI, MS	1417	1457	0.1±0.0	0.4±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.3±0.0	0.4±0.0	0.5±0.1
	Total sesquiterpenes				2.2	4.2	3.1	4.1	3.4	3.2	4.0	4.0

IM: Identification method ; [a]RI: Retention indices relative to C_6–C_{24} *n*-alkanes on the DB5 column; [b]Calculated Kovats Retention Index; MS: Mass spectrum from computerized library; CoI: Coinjection with an authentic sample

Table 2 (Continue)

No.	Component	IM	[a]RI	[b]RI	(*Ns*-9)	(*Ns*-10)	(*Ns*-11)	(*Ns*-12)	(*Ns*-13)	(*Ns*-14)	(*Ns*-15)	(*Ns*-16)
1	*α*-Thujene	RI, MS	924	914	5.7±0.1	5.8±0.0	5.6±0.0	7.6±0.0	5.6±0.0	5.9±0.0	5.3±0.0	5.1±0.1
2	*α*-Pinene	RI, MS, CoI	932	924	1.5±0.0	1.5±0.0	1.6±0.0	1.2±0.0	1.7±0.0	1.4±0.0	1.3±0.0	1.2±0.1
3	Sabinene	RI, MS	969	965	1.2±0.0	1.2±0.0	1.2±0.0	0.7±0.0	1.2±0.1	1.5±0.0	1.4±0.0	1.2±0.0
4	*β*-Pinene	RI, MS, CoI	974	971	1.8±0.0	1.8±0.0	1.7±0.0	2.0±0.0	1.7±0.0	1.9±0.0	1.8±0.0	1.6±0.1
5	*p*-Cymene	RI, MS	1020	1015	38.1±0.1	34.4±0.0	37.7±0.0	38.0±0.0	35.0±0.0	36.9±0.0	34.5±0.0	27.7±0.0
6	Limonene	RI, MS, CoI	1024	1025	1.6±0.1	1.6±0.0	1.5±0.1	1.8±0.0	1.7±0.0	1.6±0.0	1.6±0.0	1.3±0.0
7	*γ*-Terpinene	RI, MS	1054	1052	1.8±0.0	2.5±0.0	1.5±0.0	1.7±0.0	1.7±0.0	1.9±0.0	1.1±0.0	0.7±0.1
8	Terpinolene	RI, MS	1086	1091	0.8±0.0	0.7±0.0	0.7±0.0	0.8±0.0	0.7±0.0	0.8±0.0	0.9±0.0	0.6±0.0
	Total monoterpene hydrocarbons				52.5	49.5	51.5	53.8	49.3	51.9	47.9	39.4
9	Terpinen-4-ol	RI, MS	1174	1165	0.5±0.1	0.4±0.0	0.6±0.0	0.6±0.0	1.0±0.0	0.8±0.0	0.5±0.0	0.8±0.1
10	Carvacrol	RI, MS, CoI	1298	1280	2.5±0.0	3.8±0.0	4.1±0.0	4.0±0.0	3.9±0.0	3.2±0.0	3.0±0.2	0.9±0.1
11	Thymohydro quinone	RI, MS	1553	1513	7.5±0.0	8.6±0.1	9.6±0.0	8.0±0.0	8.0±0.0	8.1±0.0	7.6±0.0	7.0±0.0
	Total monoterpene alcohols				10.5	12.8	14.3	12.6	12.9	12.1	11.1	8.7
12	*cis*-4-Methoxythujane	MS	-	1099	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0
13	*trans*-4-Methoxythujane	RI, MS	1120	1113	4.6±0.0	4.5±0.1	4.5±0.0	4.5±0.0	4.6±0.0	4.7±0.0	4.8±0.0	4.0±0.0
14	1,2-epoxy-menth-4-ene	MS	-	1188	1.0±0.0	1.0±0.0	0.9±0.0	0.9±0.0	1.0±0.0	0.9±0.0	1.1±0.0	1.2±0.0
	Total monoterpene ethers				5.7	5.6	5.5	5.5	5.7	5.6	6.0	5.3
15	Carvone	RI, MS, CoI	1239	1212	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.1	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0
16	Thymoquinone	RI, MS, CoI	1248	1222	24.3±0.0	24.1±0.0	21.8±0.0	21.1±0.1	23.8±0.2	23.2±0.0	28.4±0.0	40.9±0.0
	Total monoterpene ketones				24.5	24.3	21.9	21.2	24.0	23.3	28.5	41.0
17	Bornyl acetate	RI, MS	1284	1244	0.8±0.0	1.2±0.0	0.8±0.0	0.8±0.0	0.8±0.0	1.0±0.0	1.3±0.1	0.6±0.0
	Total monoterpene ester				0.8	1.2	0.8	0.8	0.8	1.0	1.3	0.3
18	*2E,4Z*-Decadienal	RI, MS	1292	1272	0.3±0.0	1.0±0.0	0.2±0.0	0.6±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.1±0.0
19	*2E,4E*-Decadienal	RI, MS	1315	1293	0.2±0.0	0.3±0.0	0.1±0.0	0.2±0.0	0.3±0.0	0.5±0.0	0.2±0.0	0.3±0.0
	Total aldehydes				0.5	1.3	0.3	0.8	0.7	0.9	0.6	0.4
20	Longipinene	RI, MS	1350	1361	0.6±0.2	0.9±0.16	0.9±0.0	0.6±0.1	0.7±0.0	0.7±0.0	0.7±0.0	0.6±0.0
21	Longifolene	RI, MS	1407	1415	1.8±0.0	2.6±0.0	2.4±0.0	2.2±0.0	3.0±0.0	2.1±0.0	1.7±0.0	1.9±0.1
22	*trans*-Caryophyllene	RI, MS	1417	1457	0.2±0.0	0.5±0.0	0.3±0.0	0.3±0.0	0.5±0.0	0.3±0.0	0.2±0.0	0.1±0.0
	Total sesquiterpenes				2.6	4.0	3.6	3.1	4.2	3.1	2.6	2.6

IM: Identification method ; [a]RI: Retention indices relative to C_6–C_{24} *n*-alkanes on the DB5 column; [b]Calculated Kovats Retention Index; MS: Mass spectrum from computerized library; CoI: Coinjection with an authentic sample

Table 3 Correlation coefficients among volatile extract components of *N. sativa* (collected samples)

Components	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	.775**	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	.168	.303	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	.870**	.745**	.322	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-.405*	-.029	.169	-.279	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	.344	-.031	-.177	.437*	-.243	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	.456**	.277	.076	.502**	-.300	.360*	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	.537**	.496**	.189	.692**	-.093	.510**	.687**	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	.667**	.569**	-.017	.561**	-.289	.396*	.491**	.558**	1	-	-	-	-	-	-	-	-	-	-	-	-	-
10	.339	.393*	.276	.314	-.143	-.260	.145	.286	.144	1	-	-	-	-	-	-	-	-	-	-	-	-
11	-.319	-.187	.015	-.429*	.333	-.073	-.242	-.018	-.023	-.124	1	-	-	-	-	-	-	-	-	-	-	-
12	.230	.104	.203	.173	.060	.164	-.238	-.118	.022	.127	.086	1	-	-	-	-	-	-	-	-	-	-
13	.148	.063	.064	.243	-.150	.360*	.776**	.620**	.571**	.042	-.008	-.414*	1	-	-	-	-	-	-	-	-	-
14	-.098	.112	.285	.073	.066	.102	.218	.310	.129	.231	-.034	-.119	.343	1	-	-	-	-	-	-	-	-
15	.071	-.034	.266	.170	.113	.435*	.044	.129	-.099	-.293	.218	.337	-.039	-.010	1	-	-	-	-	-	-	-
16	-.024	.143	.297	.254	.192	.135	.556**	.651**	.156	.091	.010	-.474**	.675**	.488**	-.086	1	-	-	-	-	-	-
17	-.503**	-.249	-.333	-.485**	.210	-.421*	-.266	-.180	-.253	-.048	.004	-.688**	.013	-.015	-.626**	.185	1	-	-	-	-	-
18	.006	.065	.135	-.061	.162	.019	.051	.111	-.120	.043	.631**	.093	-.102	-.069	.489**	-.054	-.374*	1	-	-	-	-
19	-.556**	-.469**	-.260	-.657**	.215	-.432*	-.888**	-.823**	-.693**	-.288	.188	.132	-.818**	-.448*	-.051	-.636**	.330	.001	1	-	-	-
20	-.112	-.079	.414*	.213	.248	.245	.075	.176	-.159	-.101	-.177	.051	.200	.244	.299	.375*	-.261	.031	-.228	1	-	-
21	-.127	-.175	.076	.091	-.097	.297	.163	.313	.157	-.028	-.080	-.428*	.437*	.423*	-.101	.402*	.159	-.076	-.342	.506**	1	-
22	.109	.006	.352*	.201	.043	.255	-.251	-.150	-.120	-.133	.002	.677**	-.355*	-.154	.523**	-.299	-.716**	.232	.128	.537**	-.021	1

* Correlation is significant at the 0.05 level (1-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Table 4 PCs, eighenvalues, and variance explained (%) by each principle component

Component	PC1	PC2	PC3	PC4	PC5	PC6	PC7
α-Thujene	-0.716	-0.402	-0.518	0.022	0.008	0.083	-0.032
α-Pinene	-0.613	-0.257	-0.406	0.494	0.052	0.191	-0.002
Sabinene	-0.327	-0.363	0.397	0.562	0.335	-0.101	0.277
β-Pinene	-0.839	-0.347	-0.205	0.087	0.098	0.112	-0.244
β-Myrcene	0.315	0.070	0.463	0.636	0.035	0.326	-0.060
α-Terpinene	-0.518	-0.224	0.219	-0.596	-0.255	0.114	-0.209
p-Cymene	-0.808	0.156	-0.013	-0.129	-0.178	-0.138	0.008
Limonene	-0.888	0.107	0.094	0.145	-0.264	0.078	-0.199
γ-Terpinene	-0.725	0.056	-0.329	-0.137	-0.009	0.385	0.280
Terpinolene	-0.444	-0.065	-0.394	0.522	0.199	-0.475	0.067
trans-2-Caren-4-ol	0.278	0.005	0.287	0.251	-0.570	0.195	0.521
Terpinen-4-ol	0.096	-0.809	-0.070	0.074	0.087	0.186	0.023
Carvacrol	-0.711	0.472	0.250	-0.218	-0.046	0.047	0.294
Thymohydroquinone	-0.379	0.224	0.365	0.221	0.134	-0.359	0.069
cis-4-Methoxythujane	-0.099	-0.608	0.507	-0.135	-0.372	0.153	0.023
trans-4-Methoxythujane	-0.579	0.505	0.443	0.274	0.022	-0.019	-0.093
1,2-epoxy-menth-4-ene	0.391	0.851	-0.099	0.147	-0.017	-0.056	-0.102
Carvone	-0.018	-0.488	0.264	0.226	-0.630	-0.225	0.137
Thymoquinone	0.962	-0.110	-0.083	0.007	0.005	0.015	-0.072
Bornyl acetate	-0.233	-0.200	0.746	-0.020	0.446	-0.063	-0.229
2E,4Z-Decadienal	-0.332	0.370	0.498	-0.228	0.168	-0.127	-0.168
2E,4E-Decadienal	0.060	-0.829	0.358	-0.137	0.267	0.178	-0.071
Longipinene	0.083	-0.023	0.021	-0.277	0.767	0.275	0.469
Longifolene	0.273	0.168	-0.046	0.416	-0.014	0.622	-0.435
trans-Caryophyllene	-0.316	0.584	0.106	0.010	-0.025	0.655	0.133
Eigenvalue	6.738	4.339	2.937	2.308	2.107	1.809	1.226
Variance explained (%)	26.952	17.354	11.747	9.234	8.430	7.237	4.904
Cumulative (%)	26.952	44.306	56.053	65.287	73.716	80.953	85.858

Factor loading values higher than 0.5 are given in bold.

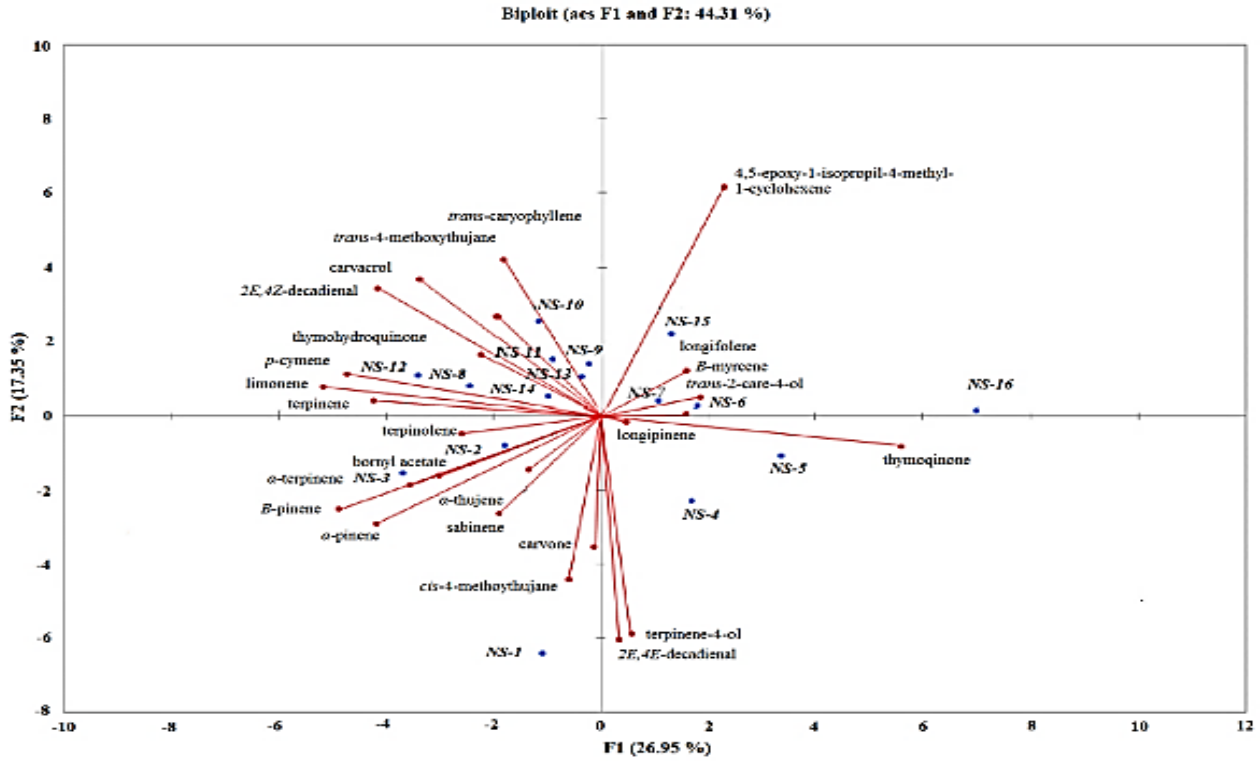

Fig. 2 Principal component analysis of the volatile oil components obtained from different cultivars of *N. sativa* (cultivars numbers are given in Table 1)

The second axis accounted for the positive contribution of *trans*-2-caren-4-ol, *β*-myrcene, longifolene, 1,2-epoxy-menth-4-ene, *trans*-caryophyllene, *trans*-4-methoxythujane, carvacrol, *2E,4Z*-decadienal, thymohydroquinone, *p*-cymene, limonene, and *γ*-terpinene, and the negative contributions of the contents of thymoquinone, longipinene, terpinen-4-ol, *2E,4E*-decadienal, carvone, *cis*-4-methoxythujane, sabinene, *α*-thujene, *α*-pinene, *β*-pinene, *α*-terpinene, bornyl acetate, and terpinolene. As it is illustrated from Figure 2, three cultivars including Ns-4, Ns-5, and Ns-16 were placed at right half of F1-F2 plot, having the highest thymoquinone content. Ns-1 was separated from the rest of the cultivars by having the highest percentage of some of the oxygenated monoterpenes including terpinen-4-ol, *cis*-4-methoxythujane, carvone, and *2E,4E*-decadienal. Ns-2, Ns-3, Ns-7, Ns-8, and Ns-12 were characterized by the highest levels of hydrocarbon monoterpenes; however, Ns-7 had approximately higher percentage of thymoquinone and lower percentage of *p*-cymene in this group, and because of this, it was placed far from the rest cultivars of its ilk. Moreover, the remaining cultivars were located in upper half of F1-F2 plot very close to each other, suggesting that there is approximately limited variability among them, likely showing comparable profiles including *trans*-2-caren-4-ol, carvacrol, thymohydroquinone, etc.

Hierarchical Cluster Analysis (HCA)

HCA was also performed as a technique for classification of *N. sativa* seed samples based on volatile oil compositions collected from cultivars of different geographical locations in Iran. Based on percentage values of 25 constituents in volatile oils from 16 samples, a matrix of 16×25 was formed. According to the relative percentages of each constituent in Table 2, HCA allowed subdivision of 16 accessions into four main groups (Fig. 3): Group-A consisted of three cultivars, including Ns-4, Ns-5, and Ns-16. Group-B comprised just one cultivar, Ns-1. Group-C consisted of five cultivars including Ns-2, Ns-3, Ns-7, Ns-8, and Ns-12. Group-D consisted of seven cultivars, encompassing Ns-6, Ns-9, Ns-10, Ns-11, Ns-13, Ns-14, and Ns-15. Results of classification using chyemometric techniques indicated that the results of PCA were approximately in accordance with the findings of HCA, suggesting that the cultivars belonging to the

same group have similar quality features from the phytochemical point of view. These results from two unsupervised multivariate methods including PCA and HCA suggest that clustering of studied *N. sativa* cultivars was independent of their geographical growth region.

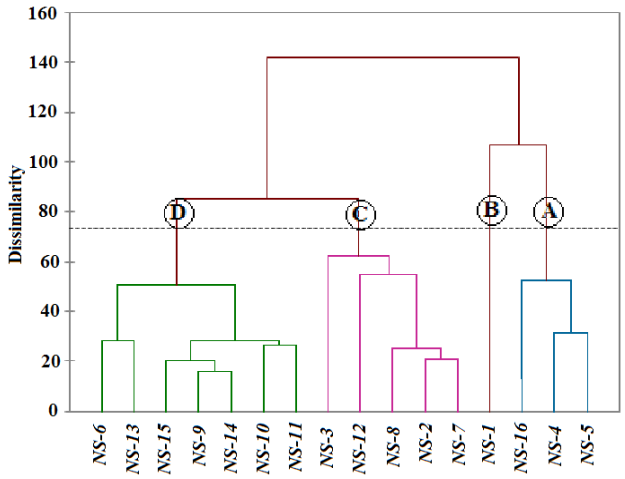

Fig. 3 Hierarchical cluster analysis of the studied *N. sativa* populations using Ward method (cultivars numbers are shown in Table 1)

CONCLUSION

In this study, the volatile oil compositions of 16 cultivars of *N. Sativa* collected from different parts of Iran were evaluated, and then the chemotaxonomic study was done. Main constituents of *N. sativa* volatile oils were found to be *p*-cymene, thymoquinone, thymohydroquinone, *α*-thujene, and *trans*-4-methoxythujane. Application of chemometric techniques using the data from volatile oil profiles allowed us to reliably categorize cultivars based on volatile oil components and hence, this technique is recommended as the method of choice for determination of black cumin quality. Results of chemometric analyses indicated that findings of HCA were in agreement with the results of PCA based on qualitative features of volatile oil, which classified *N. sativa* cultivars into four main groups. Some of the cultivars were characterized by high contents of specific compounds, which can be further exploited and utilized for future applications. In general, results of this study could provid useful information for preservation and selection of cross parents in breeding programs and industrial applications of *N. sativa* and selection of the cultivars with desired phytochemical traits, aromatic and/or therapeutic qualities.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from Islamic Azad University and Shahid Beheshti University of Medical Sciences.

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