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Phytochemical Diversity in Essential Oil of *Vitex negundo* L. Populations from India

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Abstract: *Vitex negundo* L., commonly known as the 'Nirgundi' has a long history of medicinal use in traditional and folk medicines for various diseases. To explore the diversity of the essential oil yield and composition of *V. negundo*, 23 populations were collected during spring season from the western Himalayan region. The essential oil yields varied from 0.06 to 0.10% in different populations of *V. negundo*. GC-FID, GC-MS, and statistical analysis of the leaf volatile oils showed significant phytochemical diversity. The volatiles of *V. negundo* were complex mixtures of 61 constituents, with sabinene (2.8-40.8%), viridiflorol (10.7%-23.8%), β-caryophyllene (5.3-21.4%), terpinen-4-ol (0.1-7.2%), *epi*-laurenene (2.2-5.9%), humulene epoxide II (0.5-4.6%), and abietadiene (0.1%-4.3%) as major constituents. Based on the distribution of major constituents, four groups were noticed by the multidimensional scaling and hierarchical average linkage cluster analyses. In conclusion, the yield and composition of the essential oils isolated from *V. negundo* varied considerably, depending on the origin.

Keywords: *Vitex negundo*; essential oils; phytochemical diversity; sabinene; viridiflorol; β-caryophyllene.

1. Introduction

The genus *Vitex* of the family Verbenaceae is represented by 250 species of small trees and shrubs occurring in tropical and subtropical regions, mainly distributed throughout Asia and Southern Europe [1]. Most of the *Vitex* species have been used in folk medicines due to their analgesic, anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, antihistaminic, and antiasthmatic properties, while some species are utilized to produce good quality timber, and as ornamental-hedge plants in gardens [2,3].

Vitex negundo L. commonly known as 'Nirgundi', 'Sambhalu' and 'five-leaved chaste tree', is one of the most common *Vitex* species widely distributed from Afghanistan to Bhutan, India, China, South Asia, and East Africa up to an altitude of 2000 m. It is found abundantly throughout the tropical,

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semi-tropical and temperate regions of India, mainly near moist cultivated areas, wasteland, and is widely planted as a hedge plant along the roadsides and open fields [4-6]. It is a large deciduous shrub or slender small tree up to 4-6 m height with whitish hairy branches, digitate leaves with 3-5 leaflets, which are lanceolate, opposite, 5-10 cm long, hairy beneath and pointed at both ends. It has many small pale mauve flowers in branched clusters forming a long terminal branched pyramidal inflorescence. The fruit is succulent and black when ripe rounded and about 4.0 mm in diameter [7, 8]. All plant parts (leaves, roots and fruits/seeds) of the *V. negundo* are medicinally used for treatment of a wide range of health disorders in traditional and folk medicines. Leaves of *V. negundo* have been reported to possess antiinflammatory, antioxidant, antipyretic, analgesic, antibacterial, hepatoprotective, antihistaminic and insecticidal properties [9-11]. Leaves and seeds of *V. negundo* are used to control pain, inflammation and other related diseases in the Ayurveda and Unani system of medicines [3]. Decoctions of leaves are used for treatment of inflammation, fever, eye-disease, toothache, leucoderma, gonorrhea, ulcers, rheumatoid arthritis, and bronchitis, while roots are considered as tonic, febrifuge, expectorant, antihelmintic and diuretic [8,9,12]. Root decoction is also used in rheumatism, dyspepsia, dysentery, leprosy, piles, cough, malarial fever, urinary disorders, skin diseases, and as an antidote to snake venom, while flowers of the plant are used in fever, diarrhea and liver complaints [4,13,14]. The seeds of *V. negundo* are being used as a condiment and employed in indigenous medicine for antiinflammatory, analgesic, and antioxidant purposes [15]. Phytochemical analysis of *V. negundo* from diverse geographic regions showed a variety of potential bioactive classes of constituents, such as iridoids, iridoid glycosides, lignans, flavonoids, flavones glycosides, sterols, polyphenols, and terpenoids (essential oils with mono and sesquiterpenes, diterpenes, triterpenes) etc. [3-5,15]. Earlier reports on volatile constituents of the essential oil of *V. negundo* revealed intricate compositions with monoterpenoids, viz. α-pinene, sabinene, limonene, 1,8-cineole, camphene, γ-terpinene, terpinyl acetate; and sesquiterpenoids, viz. globulol, viridiflorol, β-caryophyllene, germacrene D, caryophyllene oxide, β-elemene, eremophilene, bicyclogermacrene as most common constituents distributed in the essential oil of different plants parts [11-23]. As per literature, the composition of *V. negundo* growing in south and north India have been explored; however, no systematic work has been carried out on *V. negundo* populations growing in foot and mid-hills of Western Himalaya, India. Considering the enormous medicinal potential of *V. negundo* and intricate compositional variability in *Vitex* species, in the present investigation leaf volatile oil composition of 23 populations of *V. negundo* collected from different locations of Western Himalaya have been compared by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analyses, and classified based on clustering pattern derived from statistical analysis.

2. Material and Methods

2.1. Plant Materials

To avoid variability in essential oil yield and composition due to season, fresh leaves of *V. negundo* growing in the wild were collected during spring season from 23 different locations of five districts of Uttarakhand state (India), covering Kumaon and Garhwal regions of Western Himalaya. One of the authors (AC) authenticated the studied populations of *V. negundo* and voucher specimen of the plant is kept in the departmental Herbarium of CIMAP Research Centre, Pantnagar, India. The origin (location sites, coordinates, and altitude) of investigated populations of *V. negundo* are summarized in Table 1.

2.2. Extraction of essential oils

The fresh leaves of *V. negundo* were subjected to hydro-distillation in a Clevenger type apparatus for 3 h for isolation of essential oil. Essential oil content (%) was calculated on fresh weight of plant materials distilled. The oils were collected and dehydrated by anhydrous Na_2SO_4 and stored in amber vials at a cool and dark place until analysed.

2.3. Gas Chromatography (GC-FID)

GC analyses of the essential oil samples were carried out on a Nucon Gas Chromatograph Model 5765 equipped with flame ionization detector (FID) and a DB-5 (60 m length × 0.32 mm internal diameter; 0.25 µm film coating) fused silica capillary column. The oven column temperature ranged from 60-230°C, programmed at 3°C/min, using H_2 as carrier gas at 1.0 mL/min. Injector and detector temperatures were 220°C and 230°C, respectively. Injection size was 0.02 µL neat (syringe: Hamilton 1.0 µL capacity, Alltech USA) with a split ratio 1:40. The relative content of individual components of the oil is expressed as percent peak area relative to total peak area from the GC-FID analyses of the whole essential oil by electronic integration without response factor correction.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses of the essential oils were performed on a Clarus 680 GC interfaced with a Clarus SQ 8C Mass Spectrometer of Perkin Elmer fitted with a Elite-5 MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; Supelco Bellefonte, PA, USA). The oven temperature program was from 60 to 240°C, at 3°C/min, and programmed to 270°C at 5°C/min; injector temperature was 250°C; transfer line and source temperatures were 220°C; injection size 0.03 µL neat; split ratio 1:50; carrier gas He at 1.0 mL/min; ionization energy 70 eV; mass scan range 40-450 amu.

2.5. Identification of constituents

Identification of the essential oil constituents was done on the basis of retention index (RI, determined with reference to a homologous series of *n*-alkanes, C_8-$C_{24;}$ Supelco Analytical, Bellefonte PA, USA), coinjection with known compounds, MS Library search (NIST and WILEY), by comparing with internal reference mass spectra library search (NIST/EPA/NIH version 2.1 and Wiley registry of mass spectral data 7^{th} edition), and by comparison with the mass spectra literature data [24]. Further, the retention times/indices of authentic compounds (Aldrich, Switzerland; and Fluka, St. Louis, USA), standards/marker constituents of known essential oils were also used to confirm the identities of the constituents. The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

2.6. Statistical Analysis

To examine the phytochemical diversity based on the content (%) of chemical constituents (total 35 constituents, content ≥ 1.0%, representing 84.8%-92.6% of total essential oil composition) of the studied 23 populations; these were subjected to statistical analysis based on Euclidean distance scaling model using SPSS statistics 17.0 software (SPSS, Inc.). The derived multidimensional scaling plot depicts the grouping of individual accessions as per their chemical constituents. The plot was further used for classifying the accessions as based on their major chemical components. Further, the percentage of essential oil constituents (total 35 constituents, content ≥ 1.0 %) were also used as a basis for hierarchical cluster analysis using average method to reflect the chemical relationships among the compositions of different populations of *V. negundo* [25]. This software computes the hierarchical clustering of a multivariate dataset and the derived dendrogram depicts the grouping of chemical compositions as per their chemical constituents.

3. Results and Discussion

Essential oil yield of the fresh leaves of the investigated 23 *V. negundo* populations collected from five districts of Uttarakhand, India are presented in Table 1. The essential oil yield varied from 0.06 to 0.10% in different populations of *V. negundo*. Highest essential oil yield was noticed in populations V3, V5, V10, and V18 (0.10%), followed by V21 (0.09%), and V1, V4, V6, V8, V11, V12, V17, V19, V20, V23 (0.08%). While, in populations V2, V13, V15, and V16 essential oil yield was noticed to be 0.07%; and in populations V7, V9, V14, and V22 the lowest oil yield was noticed (0.06%). Essential oil yield is known to depend considerably on various extrinsic and intrinsic factors, such as environmental, soil and climatic conditions, seasonal changes, extraction methods etc. Further, inter population variation in essential oil yield was reported earlier in several other aromatic plants from Himalayan regions [26], and these variations might be due to varied climatic conditions of the growing site and due to difference in the genetic makeup of the *V. negundo* populations. The essential oils of all 23 populations of *V. negundo* were analysed by GC-FID and GC-MS.

Table 1. Collection sites and oil yields of *Vitex negundo* populations from Uttarakhand, India.

S.No.	Accessions/ Abbreviations	Collection sites	Coordinates	Altitude (m) msl	Oil Yield[a] (%, v/w)
1	V1	Beriparav, Nainital	N 29° 07.493; E 79° 31.134	329	0.08
2	V2	Bindukhatta, Nainital	NR	340	0.07
3	V3	Haldwani, Nainital	N 29° 13.161; E 79° 30.518	440	0.10
4	V4	Kathgodam, Nainital,	N 29° 16.450; E 79° 32.724	568	0.08
5	V5	Lohali, Nainital	N 29° 29.790; E 79° 30.135	945	0.10
6	V6	Bhimtal, Nainital	N 29° 21.978; E 79° 32.826	1405	0.08
7	V7	Someshwar, Almora	N 29° 45.540; E79° 44.570	1230	0.06
8	V8	Manan, Almora	N 29° 43.419; E 79° 37.081	1294	0.08
9	V9	Tarikhet, Almora	NR	1550	0.06
10	V10	Balighat, Bageshwar	N 29° 52.523; E 79°46.931	905	0.10
11	V11	Gagrigole, Bageshwar	N 29° 53.546; E79° 39.755	938	0.08
12	V12	Dewalchoura, Bageshwar	N 29° 53.532; E 79° 47.706	960	0.08
13	V13	Ashon, Kapkot, Bageshwar	N 29° 55.855; E 79° 53.287	980	0.07
14	V14	Bharadi, Kapkot, Bageshwar	N 29 ° 57.077; E 79° 54.166	1072	0.06
15	V15	Lobanj, Bageshwar	N 29° 52.560; E 79° 35.511	1156	0.07
16	V16	Dungoli, Bageshwar	N 29° 55.538; E 79° 36.747	1207	0.07
17	V17	Kandhar, Bageshwar	N 29° 56.758; E 79° 35.124	1320	0.08
18	V18	Sirkot, Bageshwar	N 29° 57.956; E 79° 34.357	1630	0.10
19	V19	Ghingartola, Bageshwar	N 29° 50.023; E 79° 51.111	1670	0.08
20	V20	Chhati, Bageshwar	N 29° 49.514 E 79° 48.882	1675	0.08
21	V21	Pantnagar, U. S. Nagar	N 28° 59.685; E 79° 31.203	220	0.09
22	V22	CIMAP, field gene bank, U. S. Nagar	N 29° 01.438; E 79° 30.995	238	0.06
23	V23	Tharali , Chamoli	N 30° 04.299; E 79° 30.005	1250	0.08

[a]Calculated on fresh weight basis (v/w), NR: Not recorded

Table 2. Compositional variation in the essential oils of *Vitex negundo* populations (V1-V12) from Uttarakhand, India

Compounds	**RI_{Exp}**	**RI_{Lit}**	**Content (%)**											
			V1	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**V9**	**V10**	**V11**	**V12**
(*Z*)-2-Hexenol	862	859	-	0.4	-	0.3	-	0.4	-	-	-	-	0.3	-
α-Thujene	926	924	-	-	-	0.3	-	t	t	0.1	0.2	-	0.1	0.1
α-Pinene (1)	934	932	2.6	0.5	0.9	2.0	0.4	1.3	0.3	0.4	0.2	-	0.4	0.5
Sabinene (2)	973	969	**11.7**	**4.3**	**2.8**	**8.0**	**30.3**	**7.3**	**11.0**	**23.5**	**24.0**	**20.6**	**26.2**	**27.6**
β-Pinene	978	974	-	-	-	0.2	-	t	-	-	0.6	-	-	-
3-Octanone	983	979	-	-	-	0.3	-	0.4	-	-	t	-	0.6	-
Myrcene (3)	988	988	0.6	0.2	0.2	0.7	1.3	0.9	0.3	0.9	t	1.0	-	0.9
2-Octanol	994	994	0.4	-	-	0.1	-	-	-	-	t	-	1.0	-
α-Phellandrene	1005	1002	-	-	-	0.1	0.2	-	0.2	-	-	-	t	-
(*E*)-2-Hexenyl acetate	1014	1010	-	-	-	0.3	-	-	-	-	-	-	0.4	-
α-Terpinene	1020	1014	-	-	-	0.6	-	0.3	0.1	0.3	0.5	-	0.3	-
p-Cymene (4)	1023	1020	-	-	-	1.2	0.1	0.2	-	0.3	t	0.4	0.6	1.0
β-Phellandrene (5)	1024	1025	-	-	-	1.3	0. 8	0.1	0.1	0.5	0.1	0.2	0.4	0.7
1,8-Cineole (6)	1026	1026	3.1	1.8	1.2	0.1	0.4	2.3	1.0	0.4	0.5	-	t	0.5
(*E*)-β-Ocimene (7)	1044	1044	0.7	0.7	0.3	0.6	0.6	0.4	0.5	t	1.4	0.6	1.0	0.2
γ-Terpinene (8)	1054	1054	-	-	-	0.2	0.8	0.2	-	0.8	0.3	0.4	0.6	t
Terpinolene	1088	1086	-	-	-	0.1	0.2	0.1	0.1	0.2	0.4	t	0.3	0.3
Linalool (9)	1098	1095	0.7	0.9	0.7	0.4	0.7	0.5	0.1	0.5	0.1	0.4	0.5	0.4
Isopentyl-2-methyl butanoate (10)	1100	1100	-	0.2	0.2	1.5	t	0.1	t	0.1	0.1	0.2	t	0.1
2-Methylbutyl-2-methyl butanoate	1105	1100	-	t	-	0.2	0.2	0.1	t	t	t	-	0.2	t
Terpinen-4-ol (11)	1178	1174	1.3	2.6	0.1	1.9	3.6	1.4	0.5	3.2	2.3	4.5	3.3	2.0
α-Terpineol	1193	1186	-	0.4	-	0.2	0.4	0.2	-	0.3	0.2	0.5	0.4	0.2
δ-Terpinyl acetate	1318	1316	-	-	-	0.2	0.4	0.2	0.2	0.3	0.3	0.3	0.3	0.2
β-Elemene (12)	1395	1389	-	1.7	0.1	0.2	0.3	0.2	0.2	0.1	0.1	0.3	0.2	t
α-Gurjunene	1414	1409	-	-	-	0.2	-	0.2	-	0.2	0.2	0.3	0.2	0.1
β-Caryophyllene (13)	1425	1417	**5.3**	**6.7**	**6.1**	**7.4**	**10.7**	**7.0**	**21.4**	**13.8**	**11.1**	**12.1**	**14.6**	**11.6**
α-Guaiene	1445	1437	-	-	-	0.2	-	-	-	-	0.2	-	-	-
α-Humulene	1456	1452	-	-	-	0.4	-	0.1	-	-	t	-	-	-
(*E*)-β-Farnesene (14)	1459	1454	2.1	1.8	1.1	0.3	1.1	0.9	0.9	0.7	1.5	1.7	0.8	0.6
Dehydro aromadendrene	1466	1460	0.8	0.9	0.8	1.3	0.5	0.8	0.8	0.7	0.6	0.9	0.7	0.7
β-Selinene	1491	1489	-	-	0.5	0.1	0.3	0.5	0.1	0.2	0.2	0.6	-	0.3
(*Z*)-β-Guaiene	1494	1492	0.7	0.6	-	0.2	0.3	0.1	0.4	t	0.1	0.2	0.3	t
α-Selinene	1499	1498	-	0.5	-	1.0	-	0.1	0.2	0.1	0.1	-	0.1	0.1
δ-Cadinene	1526	1522	-	-	-	0.1	0.2	-	t	-	0.9	0.5	-	-
Elemol	1552	1548	-	-	-	1.1	t	-	0.3	1.1	0.1	-	0.8	-
(*E*)-Nerolidol (15)	1561	1561	1.4	1.4	1.1	0.2	1.1	1.2	0.1	0.1	0.2	1.0	t	1.1
Germacrene D-4-ol	1574	1574	-	-	-	t	0.6	0.2	0.3	t	0.1	t	t	0.2
Spathulenol	1578	1576	-	0.3	0.6	0.2	0.2	0.1	0.1	0.1	t	0.2	0.1	0.1
Caryophyllene oxide (16)	1589	1582	-	-	0.2	0.6	1.1	0.1	0.6	0.5	0.4	0.8	0.6	1.8
Viridiflorol (17)	1599	1592	**22.2**	**20.3**	**18.4**	**20.3**	**16.8**	**17.0**	**15.5**	**16.5**	**13.7**	**17.1**	**15.1**	**20.1**
Humulene epoxide II (18)	1610	1608	1.0	1.0	2.3	1.0	3.9	0.9	1.7	0.8	2.9	1.4	0.9	1.2
epi-α-Cadinol	1637	1638	0.3	0.4	0.2	0.1	0.2	0.1	0.2	0.2	0.4	0.2	0.2	0.2

β-Eudesmol (19)	1643	1649	-	0.3	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.2	0.1	0.2
α-Cadinol	1657	1652	-	0.4	0.2	0.1	0.6	0.2	0.2	t	0.5	0.4	0.1	0.2
Selin-11-en-4α-ol (20)	1660	1658	-	0.6	0.2	0.2	0.6	0.2	0.2	0.2	0.3	0.2	0.1	0.3
Diterpene (M^+: 272)[a] (21)	1871	-	**9.9**	**13.1**	**15.0**	**10.9**	**6.3**	**13.7**	**13.2**	**9.9**	**10.1**	**11.0**	**10.0**	**8.1**
Farnesyl acetone (22)	1889	1889	1.9	2.2	2.5	1.7	1.4	2.4	3.2	2.2	2.0	1.8	2.1	**2.1**
Rimuene	1896	1896	0.6	0.7	0.9	0.7	0.2	0.7	0.7	0.4	0.4	0.4	0.4	0.4
***epi*-Laurenene** (23)	1902	1901	**3.2**	**3.9**	**4.6**	**3.6**	**2.6**	**4.6**	**5.9**	**4.5**	**4.1**	**4.2**	**4.1**	**3.4**
Diterpene (M^+: 272)[b] (24)	1951	-	0.9	1.4	1.8	0.9	0.9	3.1	0.1	0.1	0.2	0.3	0.1	0.2
Diterpene (M^+: 286)[c] (25)	1956	-	3.9	3.4	3.2	2.8	0.3	1.4	1.8	1.3	1.3	1.2	1.3	1.0
(*Z*,*Z*)-Geranyl linalool (26)	1960	1960	1.4	1.7	1.9	1.6	0.8	1.9	0.3	0.6	0.4	0.5	0.4	0.9
(*E*,*Z*)-Geranyl linalool (27)	1972	1980	2.1	2.4	2.4	2.1	0.2	2.2	1.0	1.9	1.2	1.0	0.7	1.0
1-Eicosene (28)	1984	1987	2.7	2.7	3.0	1.6	0.2	2.6	1.4	0.1	1.5	1.3	1.0	0.2
(6*Z*,10*Z*)-Pseudo phytol (29)	1989	1988	3.0	2.4	3.2	2.9	0.3	2.5	0.2	t	0.3	0.3	0.1	0.4
(Z,*E*)-Geranyl linalool (30)	1996	1998	2.7	2.3	2.8	2.5	0.6	0.8	0.5	0.4	0.7	0.6	0.3	t
n-Eicosane (31)	2002	2000	0.7	0.6	2.2	1.1	t	0.6	1.9	1.5	1.7	1.6	1.5	1.3
Diterpene (M^+: 272)[d] (32)	2024	-	1.6	2.0	0.9	1.7	t	0.1	0.1	t	t	0.4	0.1	t
(6*E*,10*E*)-Pseudo phytol (33)	2060	2058	0.9	1.0	1.2	0.8	t	1.1	2.5	1.7	2.0	1.6	1.3	0.3
Abietadiene (34)	2081	2087	1.0	3.4	3.6	0.8	1.0	4.3	0.4	0.6	0.6	0.6	0.5	1.5
Methyl linoleate (35)	2097	2095	3.0	1.8	1.6	1.5	0.4	1.2	1.6	1.1	0.5	0.6	0.7	0.7
Class composition														
Monoterpene hydrocarbons			*15.6*	*5.7*	*4.2*	*15.3*	*33.9*	*10.8*	*12.6*	*27.0*	*27.7*	*23.2*	*29.9*	*31.3*
Oxygenated monoterpenes			*5.1*	*5.7*	*2.0*	*2.8*	*5.5*	*4.6*	*1.8*	*4.7*	*3.4*	*5.7*	*4.5*	*3.3*
Sesquiterpene hydrocarbons			*8.9*	*12.2*	*8.6*	*11.4*	*13.4*	*9.9*	*24.0*	*15.8*	*15.0*	*16.6*	*16.9*	*13.4*
Oxygenated sesquiterpenes			*24.9*	*24.7*	*23.4*	*23.9*	*25.3*	*20.1*	*19.4*	*19.7*	*18.7*	*21.5*	*18.0*	*25.4*
Diterpenoids			*33.1*	*39.9*	*44.0*	*33.0*	*14.6*	*38.8*	*29.9*	*23.6*	*23.3*	*23.9*	*21.4*	*19.3*
Aliphatic compounds			*6.8*	*5.7*	*7.0*	*6.9*	*0.8*	*5.4*	*4.9*	*2.8*	*3.8*	*3.7*	*5.7*	*2.3*
Total			**94.4**	**93.9**	**89.2**	**93.3**	**93.5**	**89.6**	**92.6**	**93.6**	**91.9**	**94.6**	**96.4**	**95.0**

RI_{Exp} : Retention index determined on DB-5 capillary column (60 m × 0.32 mm); RI_{Lit}: Retention index from literature [24]; t: trace (component <0.05%); Value in parenthesis (1-35) represent constituents (content ≥ 1.0%) used for statistical analysis viz. MDS and cluster analysis; for population abbreviations, see Table 1; Unidentified constituents: [a]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 201, 192, 191 (100%), 173, 161, 135, 119, 95, 69; [b]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 252, 207, 192, 191 (100%), 173, 149, 135, 119, 95, 69; [c]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 229, 215, 190, 175, 149, 134, 119 (100%), 107, 91, 79, 69; [d]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 204, 191, 147, 135, 109 (100%), 107, 91, 79, 69; Mass spectra of unidentified constituents are given in Figure S1 (Supplementary informations)

Table 3. Compositional variation in the essential oils of *Vitex negundo* populations (V13-V23) from Uttarakhand, India.

Compounds	**RI$_{Exp}$**	**RI$_{Lit}$**	**Content (%)**										
			V13	**V14**	**V15**	**V16**	**V17**	**V18**	**V19**	**V20**	**V21**	**V22**	**V23**
(Z)-2-Hexenol	862	859	0.2	-	-	-	-	-	-	-	-	-	-
α-Thujene	926	924	0.2	0.2	0.2	0.5	0.5	0.3	-	-	-	-	0.2
α-Pinene (1)	934	932	0.9	0.5	0.4	1.1	0.8	0.5	-	-	1.8	2.7	0.5
Sabinene (2)	973	969	**27.1**	**36.0**	**28.8**	**24.4**	**27.9**	**40.8**	**12.0**	**8.3**	**11.2**	**10.4**	**40.5**
β-Pinene	978	974	t	-	-	-	-	-	-	-	-	-	-
3-Octanone	983	979	0.8	t	-	-	-	-	-	-	-	0.8	-
Myrcene (3)	988	988	1.2	1.3	1.0	1.1	1.1	1.2	t	0.2	1.0	1.2	1.8
3-Octanol	994	994	0.1	-	-	0.7	-	-	0.2	-	-	0.1	-
α-Phellandrene	1005	1002	0.1	-	0.1	1.1	-	0.1	-	-	-	-	-
(E)-2-Hexenyl acetate	1014	1010	t	-	-	t	-	-	-	-	-	-	-
α-Terpinene	1020	1014	0.9	0.5	0.3	t	0.1	0.4	-	-	-	-	-
p-Cymene (4)	1023	1020	0.3	t	0.6	0.4	1.1	1.5	-	-	-	0.3	0.4
β-Phellandrene (5)	1024	1025	1.3	0.4	0.6	1.7	2.2	1.0	0.5	0.3	-	0.2	0.2
1,8-Cineole (6)	1026	1026	t	1.3	0.3	1.5	1.6	0.5	0.1	0.2	3.8	3.0	1.2
(*E*)-β-Ocimene (7)	1044	1044	2.2	1.2	1.0	1.1	2.9	1.1	0.3	0.3	-	0.8	1.0
γ-Terpinene (8)	1054	1054	0.3	0.5	0.4	0.5	1.9	-	-	-	-	-	-
Terpinolene	1088	1086	0.6	0.3	0.3	0.3	0.6	0.7	-	-	-	0.2	0.3
Linalool (9)	1098	1095	0.4	0.4	0.5	0.9	2.3	1.1	0.5	0.4	1.0	0.4	0.5
Isopentyl-2-methyl butanoate (10)	1100	1100	t	t	-	t	0.5	0.4	-	-	-	0.1	t
2-Methylbutyl-2-methyl butanoate	1105	1100	0.2	0.2	0.2	0.3	0.2	0.2	-	-	-	0.2	t
Terpinen-4-ol (11)	1178	1174	5.6	3.2	3.3	4.3	5.0	7.2	1.8	1.6	1.5	1.4	2.6
α-Terpineol	1193	1186	0.4	0.3	0.4	0.5	0.3	0.6	0.2	0.2	-	0.2	0.3
δ-Terpinyl acetate	1318	1316	0.3	0.4	0.3	1.8	0.5	1.0	0.3	0.4	-	-	0.5
β-Elemene (12)	1395	1389	0.2	t	0.2	t	t	t	0.1	-	-	0.2	-
α-Gurjunene	1414	1409	0.8	0.4	0.1	0.1	t	t	-	-	-	0.2	-
β-Caryophyllene (13)	1425	1417	**13.3**	**10.1**	**15.0**	**11.7**	**10.2**	**8.6**	**15.8**	**15.2**	**6.7**	**8.3**	**11.7**
α-Guaiene	1445	1437	t	-	-	0.1	-	-	-	-	-	-	-
α-Humulene	1456	1452	0.7	-	t	0.1	-	-	0.2	-	-	-	-
(*E*)-β-Farnesene (14)	1459	1454	0.6	0.7	0.8	1.0	0.6	0.5	0.8	1.0	1.5	2.6	0.7
Dehydro aromadendrene	1466	1460	0.3	0.6	0.7	0.6	0.6	0.4	0.6	0.8	1.0	1.1	0.6
β-Selinene	1491	1489	0.4	-	0.3	0.7	0.1	0.1	0.3	-	-	0.1	0.2
(*Z*)-β-Guaiene	1494	1492	0.1	0.1	0.1	0.4	0.1	t	0.1	0.3	0.7	0.8	-
α-Selinene	1499	1498	0.3	-	0.2	0.4	t	t	0.1	0.1	-	0.2	-
δ-Cadinene	1526	1522	t	-	t	0.4	-	t	-	-	-	0.2	-
Elemol	1552	1548	0.4	0.6	0.6	0.3	-	t	0.6	1.2	t	t	-
(*E*)-Nerolidol (15)	1561	1561	t	-	0.1	0.7	0.7	0.7	0.3	0.3	1.5	0.9	0.5
Germacrene D-4-ol	1574	1574	0.1	0.2	0.1	-	t	t	0.5	0.2	-	t	t

Spathulenol	1578	1576	0.1	0.2	t	0.1	0.2	0.2	0.3	0.2	-	-	0.1
Caryophyllene oxide (16)	1589	1582	0.7	0.5	0.7	0.6	1.4	1.3	3.2	2.2	-	0.3	0.5
Viridiflorol (17)	1599	1592	**10.7**	**12.8**	**14.0**	**14.0**	**15.0**	**11.2**	**20.1**	**22.1**	**23.8**	**23.8**	**11.1**
Humulene epoxide II (18)	1610	1608	0.5	3.3	1.3	0.6	0.8	0.5	3.8	1.0	4.6	1.2	0.5
epi-α-Cadinol	1637	1638	0.2	0.3	0.2	0.3	0.2	0.3	0.9	1.5	0.2	0.2	1.1
β-Eudesmol (19)	1643	1649	t	-	0.1	2.2	0.3	t	0.2	0.4	0.5	0.2	0.2
α-Cadinol	1657	1652	0.1	0.7	0.2	0.2	t	0.2	0.8	0.2	0.8	0.3	0.2
Selin-11-en-4α-ol (20)	1660	1658	0.2	0.3	0.2	0.2	t	0.2	1.6	0.3	0.5	0.3	0.5
Diterpene (M^+: 272)[a] (21)	1871	-	**10.2**	**8.6**	**9.0**	**6.6**	**6.5**	**5.5**	**7.1**	**10.8**	**11.0**	**8.7**	**8.8**
Farnesyl acetone (22)	1889	1889	2.2	1.7	2.2	1.5	1.7	1.2	2.6	2.8	1.8	1.5	1.9
Rimuene	1896	1896	0.4	0.3	0.4	0.4	0.3	0.2	0.4	0.4	0.4	0.4	0.4
***epi*-Laurenene** (23)	1902	1901	**3.9**	**3.6**	**3.7**	**2.9**	**2.8**	**2.2**	**3.6**	**4.7**	**3.5**	**2.8**	**3.4**
Diterpene (M^+: 272)[b] (24)	1951	-	0.1	1.0	0.1	0.2	t	0.1	0.3	0.4	0.7	0.4	0.2
Diterpene (M^+: 286)[c] (25)	1956	-	1.2	0.3	1.1	0.1	0.7	t	1.0	1.8	2.2	2.7	1.0
(*Z*,*Z*)-Geranyl linalool (26)	1960	1960	0.1	-	0.3	0.8	0.2	0.5	0.2	0.9	1.4	1.2	0.3
(*E*,*Z*)-Geranyl linalool (27)	1972	1980	1.4	0.5	0.6	0.2	0.5	0.2	0.2	1.6	1.6	1.9	0.2
1-Eicosene (28)	1984	1987	0.1	0.9	0.8	0.5	0.8	0.4	1.0	1.6	1.6	2.3	1.1
(6*Z*,10*Z*)-Pseudo phytol (29)	1989	1988	t	0.2	0.2	0.8	0.1	0.5	1.2	0.5	2.2	2.5	t
(Z,*E*)-Geranyl linalool (30)	1996	1998	0.2	t	0.3	0.1	0.2	0.2	0.3	0.9	1.7	2.1	t
n-Eicosane (31)	2002	2000	1.2	1.2	1.3	0.3	0.9	t	0.5	2.0	0.4	0.4	t
Diterpene (M^+: 272)[d] (32)	2024	-	-	t	0.2	1.1	0.2	0.7	1.0	0.1	1.6	1.4	t
(6*E*,10*E*)-Pseudo phytol (33)	2060	2058	t	0.9	1.4	1.4	0.8	0.6	0.1	3.4	t	0.5	0.7
Abietadiene (34)	2081	2087	1.0	0.4	0.4	0.2	0.1	0.1	2.1	0.1	0.6	1.9	0.4
Methyl linoleate (35)	2097	2095	0.5	0.2	0.1	0.8	0.3	0.1	1.7	1.2	2.2	1.1	0.1
Class composition													
Monoterpene hydrocarbons			*35.1*	*40.9*	*33.7*	*32.2*	*39.1*	*47.6*	*12.8*	*9.1*	*14.0*	*15.8*	*44.9*
Oxygenated monoterpenes			*6.7*	*5.6*	*4.8*	*9.0*	*9.7*	*10.4*	*2.9*	*2.8*	*6.3*	*5.0*	*5.1*
Sesquiterpene hydrocarbons			*16.7*	*11.9*	*17.4*	*15.5*	*11.6*	*9.6*	*18.0*	*17.4*	*9.9*	*13.7*	*13.2*
Oxygenated sesquiterpenes			*13.0*	*18.9*	*17.5*	*19.2*	*18.6*	*14.6*	*32.3*	*29.6*	*31.9*	*27.2*	*14.7*
Diterpenoids			*20.7*	*17.5*	*19.9*	*16.3*	*14.1*	*12.0*	*20.1*	*28.4*	*28.7*	*28.0*	*17.3*
Aliphatic compounds			*3.1*	*2.5*	*2.4*	*2.6*	*2.7*	*1.1*	*3.4*	*4.8*	*4.2*	*5.0*	*1.2*
Total			**95.3**	**97.3**	**95.7**	**94.8**	**95.8**	**95.3**	**89.5**	**92.1**	**95.0**	**94.7**	**96.4**

RI_{Exp}: Retention index determined on DB-5 capillary column (60 m × 0.32 mm); RI_{Lit}: Retention index from literature [24]; t: trace (component <0.05%); Value in parenthesis (1-35) represent constituents (content ≥ 1.0 %) used for statistical analysis viz. MDS and cluster analysis; for population abbreviations, see Table 1; Unidentified constituents: [a]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 201, 192, 191 (100%), 173, 161, 135, 119, 95, 69; [b]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 252, 207, 192, 191 (100%), 173, 149, 135, 119, 95, 69; [c]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 229, 215, 190, 175, 149, 134, 119 (100%), 107, 91, 79, 69; [d]Diterpene, MS (electron impact, EI, 70 eV), 272 (M^+; $C_{20}H_{32}$), 257, 204, 191, 147, 135, 109 (100%), 107, 91, 79, 69; Mass spectra of unidentified constituents are given in Figure S1 (Supplementary informations

Altogether, 61 constituents, representing 89.2-97.3% of total oil compositions were identified in different populations (Table 2 & 3). Monoterpenoids (6.2-58.0%), sesquiterpenoids (24.2-50.3%), and diterpenoids (12.0-44.0%) constituted the volatile oil compositions of the studied *V. negundo* populations. Among the terpenoids, monoterpenoids (>40.0%) contributed the major proportion of essential oil composition in populations V13, V14, V17, V18, and V23 (41.8-58.0%), while populations V7, V19, V20, V21, and V22 were dominated by > 40.0% of sesquiterpenoids (40.9-50.3%), and V3 was the only population which contained >40.0% of diterpenoids as the prevalent class of constituents in compositions. Other populations such as V1, V2, V4, and V6 contained >30.0% of both diterpenoids and sesquiterpenoids in their essential oils, while populations V5, V8, V9, V11, V12, V15 and V16 contained > 30.0% of both mono- and sesquiterpenoids. Moreover, the V10 population contained 28.9% monoterpenoids, 38.1% sesquiterpenoids, and 23.9% diterpenoids. The components belonging to other class, aliphatic class were detected in relatively low amounts (1.1-7.0%) in different populations. Major components of the essential oils were sabinene (2.8-40.8%), viridiflorol (10.7-23.8%), β-caryophyllene (5.3-21.4%), terpinen-4-ol (0.1-7.2%), *epi*-laurenene (2.2-5.9%), humulene epoxide II (0.5-4.6%), abietadiene (0.1-4.3%), 1,8-cineole (≤0.03-3.8%), (6*E*,10*E*)-pseudophytol (≤0.03-3.4%), (6Z,10Z)-pseudophytol (≤0.03-3.2%), farnesyl acetone (1.2-3.2%), methyl linoleate (0.1%-3.0%) and 1-eicosene (0.1-3.0%). The content of the major constituents showed considerable changes in different studied populations. The content of sabinene was found to be maximum in population V18 (40.8%), followed by V23 (40.5%), V14 (36.0%); and lowest in V3 (2.8%). β-Caryophyllene was found to be highest in V7 (21.4%), followed by V19 (15.8%) and V20 (15.2%) with lowest in population V1 (5.3%), while viridiflorol was recorded higher (23.8%) in V21 and V22, followed by V1 (22.2%) and V20 (22.1%), while its lowest content was noticed in population V13 (10.7%). The contents of others constituents also showed noteworthy inter population variations (Table 2-Table 3).

To evaluate whether the identified essential oil constituents may be useful in reflecting the phytochemical diversity within the investigated 23 populations of *V. negundo*, 35 components (amount ≥ 1.0%; representing 84.3% to 92.6% of total compositions, marked by 1-35 in parenthesis in Table 2 & 3) were subjected to statistical analysis based on Euclidean distance scaling model. The derived multidimensional scaling plot (MDS) depicting four clusters within 23 populations with individual compounds expressed as percentage of the total fraction, as shown in Figure 1. Further, to cross-examine the similarity and dissimilarity among the essential oil composition of studied populations, the same 35 constituents were subjected to hierarchical cluster analysis using average method to obtain the dendrogram as shown in Figure 2. Based on MDS plot and hierarchical cluster analysis (Figure 1 & 2) 23 populations could be divided into four clusters, first with seven populations (I; V6, V2, V3, V4 , V21, V22, and V1), second (II; V19, V20, and V7) and third (III; V18, V14 and V23) each with 3 populations, and fourth with 10 populations (IV; V5, V12, V16, V17, V9, V8, V10, V13, V11, and V15). Further, the cluster I was characterized by the presence of relatively high amounts of viridiflorol (17.0-23.8%), followed by small quantities of β-caryophyllene (5.3-8.3%) and sabinene (2.8-11.7%). Further, the populations V4, V21, V1, and V22 of cluster **I** were close to each other as they contain the highest viridiflorol (20.3%, 23.8%, 22.2%, 23.8%, respectively) content compared to other populations of the cluster. While, the cluster **II** was characterized by a high amount of β-caryophyllene (15.2-21.4%), followed by viridiflorol (15.5%-22.1%), sabinene (8.3-12.0%), and unidentified diterpene (7.1-13.2%; M^+ 272, RI_{Exp}=1871 see Table 2 –Table 3, and Figure S1). The populations belonging to cluster III were characterized by the highest percentage of sabinene (36.0-40.8%), followed by β-caryophyllene and viridiflorol; and the cluster IV also contain the notable amount of sabinene (23.5-30.3%), followed by viridiflorol (10.7-20.1%) and β-caryophyllene (10.2-15.3%). The major constituents of each population were also mentioned in the dendrogram (Figure 2) to explain the inter population similarity in compositions. Earlier, various studies on essential oil composition of *V. negundo* have been carried out to explore the variability of their volatile constituents under different geographical and ecological conditions. Earlier analysis of essential oil of *V. negundo* from Indian origin reported β-caryophyllene, caryophyllene oxide, cineole, sabinene, globulol and benzaldehyde as major constituents [19-21]. In another report from India, viridiflorol (19.5%), β-caryophyllene

(16.6%), sabinene (12.1%), and terpinen-4-ol (9.6%) were reported as the major constituents of leaf essential oil of *V. negundo* [5, 8]. Singh et al. (2000) reported viridiflorol (26.5%) and β-caryophyllene (13.2%) as the major constituents of flowering twigs of *V. negundo* growing at Dehradun, India [6]. Moreover, *epi*-globulol (30.3%), δ-iraldeine (10.3%), and terpinen-4-ol (9.4%) were reported as major constituents from leaf essential oil of *V. negundo* from north Indian plains [4]; while, in another report from Indian origin, α-copaene (25.3%), β-elemene (19.2%), and camphene (21.1%) were reported as the major constituents in leaf essential oil of *V. negundo* [14]. Khokra et al. (2008) reported ethyl-9-hexadecenoate (28.5%), *δ*-guaiene (18.0%), caryophyllene oxide (10.2%) in leaf oil; β-selinene (22.0%), β-cedrene (14.2%), germacrene D (8.0%) in fruit oil; and α-selinene (17.0%), caryophyllene oxide (15.2%), and germacrene D 4-ol (9.0%) in flower oil of *V. negundo* from northern, India [9].

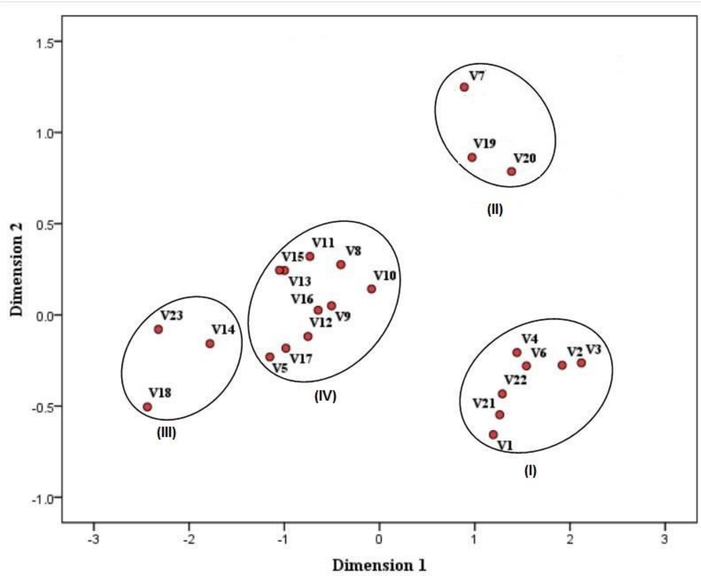

Figure 1. MDS plot based on Euclidean distance scaling depicting phytochemical proximities among the studied 23 *Vitex negundo* populations.

Further, β-caryophyllene (36.0%), bicyclogermacrene (20.5%), germacrene D (5.8%) were reported as major constituents of fruit essential oil of *V. negundo* [11]. The flower essential oil of *V. negundo* from south Indian origin was shown to be dominated by sabinene (20.3%), β-caryophyllene (14.1%) and globulol (19.2%) [18]. Moreover, viridiflorol (26.8%), drimenol (21.9%), β-caryophyllene (16.6%) and laurenene (8.0%) were reported as major constituents from seed bearing flowering twigs of *V. negundo* from Uttarakhand, India [23]. The volatile oil from leaves of *V. negundo* from Iran was characterized by 1,8-cineole (20.8%) and α-pinene (18.8%) [17]; α-pinene (18.8%) and α-guaiene (10.5%) [27]. Further, sabinene (19.0%), β-caryophyllene (18.3%), eremophilene (12.8%) were reported as major constituents of leaf oil of *V. negundo* from Taiwan [12]. On the contrary, the leaf volatile oil from *V. negundo* from Chinese origin was shown to be characterized by β-caryophyllene (26.3%), 1,8-cineole (11.8%), sabinene (7.8%) [28]. Further, in an another report from China, δ-guaiene (up to 50.0%) and β-caryophyllene (30.0-38.0%) were reported as major constituents in leaf oil [22]; whereas *n*-hexadecanoic acid (17.7%), eudesm-4(14)-en-11-ol (12.4%), caryophyllene oxide (10.8%) were reported as the major constituents in seed essential oil of *V. negundo* [16]. Camphene, β-caryophyllene, citral and α-pinene rich and β-eudesmol rich compositions of *V. negundo* essential oils were also reported from Philippines [8].

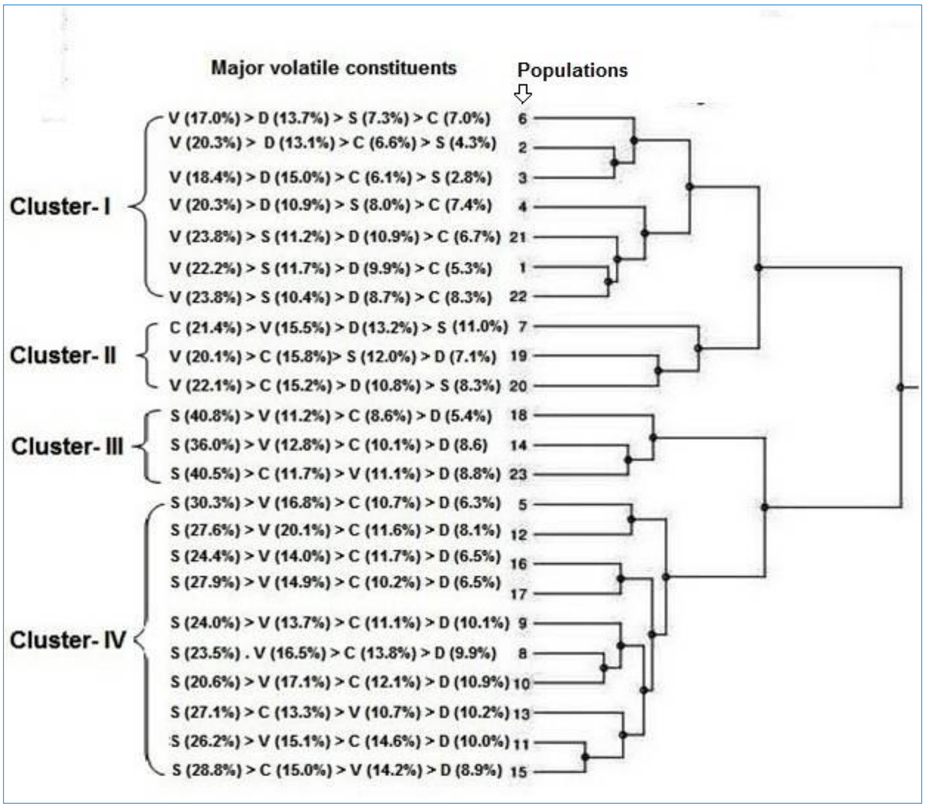

Figure 2. Hierarchical cluster analysis based on average method depicting compositional proximities in studied populations, Major constituents (V = viridiflorol; S = sabinene; C = β-caryophyllene, D = Diterpene (M^+ 272, RI= 1871 in DB-5, see Table 2 and 3, Figure S1), 1-23 in dendrogram represents V1 to V23 populations of *Vitex negundo* collected from different locations (see Table 1)

Literature survey revealed that the essential oil composition of *V. negundo* is mainly dominated by monoterpenoids and sesquiterpenoids; and from a phytochemical point of view α-pinene, sabinene, limonene, 1,8-cineole, globulol, viridiflorol, β-caryophyllene, germacrene D, bicyclogermacrene, caryophyllene oxide are the main characteristic compounds reported to be distributed in *V. negundo* from different countries. Further, the occurrence of chemical variations, both qualitative and quantitative, in *V. negundo* from different geographic regions, as well as among the presently studied *V. negundo* populations from diverse localities of Uttarakhand, Himalaya, seems to be due to the divergent climatological and geographical conditions. The existence of interpopulation chemical polymorphism within the botanical species is quite common and it has been observed earlier in different aromatic plant species growing in western Himalaya [26].

4. Conclusions

GC-FID and GC-MS analysis followed by statistical analyses of the leaf volatile oil constituents of 23 populations of *V. negundo* collected from different regions of foot and mid hills of western Himalaya, India showed significant phytochemical diversity. In conclusion, the yield and composition of the essential oils isolated from *V. negundo* varied considerably, depending on the origin of the plant material. β-Caryophyllene is a common sesquiterpene that is quite widely distributed in plants and is known due to its use as a cosmetic ingredient and a food flavoring additive. Besides, it also possesses antiinflammatory, anticarcinogenic, antibiotic, antioxidant and local anaesthetic properties and its derivatives are used in the plant defense system [29]. Sabinene and viridiflorol are also widely distributed in plants and have been used as flavour and fragrance additives [30]. Based on their volatile oil constitution four types of the *V. negundo* populations were characterised. These compositions were dominated by sabinene, β-caryophyllene, viridiflorol and mixed proportion of these constituents. Therefore, the genetic resources of this traditionally important medicinal plant that is growing wild in this region can be utilised for high value aroma chemicals, such as sabinene, β-caryophyllene, and viridiflorol.

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