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GC-MS and SPME-GC/MS Analysis and Bioactive Potential Evaluation of Essential Oils from Two *Viola* Species Belonging to the *V. calcarata* Complex

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Abstract: *Viola calcarata* L. and *Viola dubyana* Burnat ex Gremli belong to the *V. calcarata* complex in *Viola* section *Melanium* (Violaceae family). For the first time, the essential oils (EOS) obtained by hydrodistillation from dried flowers, were analyzed by GC/MS and SPME-GC/MS to describe their volatile chemical profile. Differences in the qualitative and quantitative composition between the two violets have been found. A total of 43 compounds were identified among which methyl salicylate was the most abundant (from 45.5 to 68.0%) both in the vapor and liquid phase. The performed bioactivity tests pointed out the greater effect of *V. dubyana* EO compared to that obtained from *V. calcarata.* Nevertheless, both EOs proved to be good scavengers, especially toward the ABTS·+ radical. They also showed a dose-dependent phytotoxic action against *Sinapis alba* and *Lolium multiflorum*. Their seed germination was inhibited up to 100% and 25%, respectively, in response to the highest used dose (100 µL) of each EO. Furthermore, a significant decrease in root and shoot length was observed. The resulting seedling vigor index was reduced by 15–100% and 8–82% for *S. alba* and by 11–91% and 4–91% for *L. multiflorum* by *V. dubyana* and *V. calcarata* EOS, respectively.

Keywords: *Viola calcarata*; *Viola dubyana*; alpine species; separation; chemical analysis; volatile compounds; antiradical activity; ABTS; DPPH; phytotoxicity

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1. Introduction

Essential oils (EOs) are a more or less complex mixture of natural organic substances of the secondary metabolism of plants where they play different roles as elements of interaction with the environment. For example, EOs are able to defend plants from pests and predators or to attract pollinators and disseminators [1]. Furthermore, men exploit the EO properties for food and therapeutic purposes, also applying them in perfumery, cosmetics, pharmaceutical, and agriculture field [2]. The bioactivity of EOs was widely demonstrated. Among others, they have shown remarkable antimicrobial, anti-inflammatory, antioxidant, cytotoxic, and anticancer effects both in in vitro and in vivo models [3–6]. Some EOs with allelopathic potential have also been identified [7–9].

EOs obtained from *Viola* species have previously been the subject of study for their chemical composition and biological activity. Many compounds including aliphatics, shikimic acid derivatives, sesquiterpenes, and monoterpenes have been identified in different relative quantities based on species and its origin. Antibacterial, antifungal, and antiplasmodial properties have been documented with mixed results [10–17].

Only a recent work has covered these aspects of *Viola calcarata* L. [16], however, no reports have dealt with documenting the non-volatile chemical composition of *V. dubyana* Burnat ex Gremli and *Viola calcarata* L. These are two rare *Viola* species belonging to the *V. calcarata* complex in *Viola* section *Melanium* (Violaceae) and growing on the Italian Alps and Prealps, linked together by a common aspect and similar ecology, but with constant botanical characteristics well correlated to the geographical distribution [18]. *V. calcarata* is typical of meadows, pastures, and screes, mostly above 1500 m, while *V. dubyana* grows on calcareous dry pastures and rocky places between 900 and 2100 m in altitude [19].

The aim of this work was to investigate, for the first time, the volatile chemical fraction of the EOs of the two violets by solid-phase micro-extraction (SPME) and gas chromatography-mass spectrometry (GC-MS) techniques to identify, quantify, and compare their content. In addition to this, the antiradical and phytotoxic activities were also evaluated to make initial assessments on their bioactive potential.

2. Materials and Methods

2.1. Materials

Ethanol, methanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2S_2O_8$) were from Merck (Darmstadt, Germany).

Plant Material

Undamaged flowers of *V. calcarata* and *V. dubyana* were collected in full bloom during summer 2018 in the Graian Alps (45°70′84.06″ N 6°92′21.14″ E at the Little St. Bernard Pass, Aosta, Italy) and in the Garda Prealps (45°79′62.92″ N 10°63′61.91″ E at Monte Caplone, Brescia, Italy), at 2100 and 1500 m above sea level, respectively. They were air dried away from light and heat sources. Herbarium samples (No. VC-PPSB-VO18 and No. VD-VS-BS18) were deposited at the Department of Agricultural and Environmental Sciences of the Milan State University (Milan, Italy), after the plant identification according to Flora d'Italia [18].

Seeds of *Sinapis alba* L. were purchased from the company "Padana Sementi" (Padua, Italy) while those of *Lolium multiflorum* Lam. were provided by the organic farm "Terre di Lomellina" (Pavia, Italy). Both types of seeds were sterilized for 10 min in a 1% sodium hypochlorite solution, then repeatedly rinsed with distilled water before use.

2.2. Distillation of Essential Oils

Dried flowers of *V. calcarata* and *V. dubyana* (25 g and 27 g, respectively) were subjected to hydrodistillation for 3 h using a clevenger-type apparatus. Both EOs were dried over anhydrous sodium sulfate and kept in the fridge (4 °C) until use.

2.3. SPME Sampling

To describe the chemical profile of the headspace from two EOs, a SPME fiber was used for the sampling. About 1.0 mL of each EO was individually placed into a 20 mL glass vial with PTFE-coated silicone septum. In order to reach thermal equilibrium, a thermostatic bath for 15 min with constant magnetic stirring was utilized. For the extraction of volatile compounds, a SPME device from Supelco (Bellefonte, PA, USA) with 1 cm fiber coated with 50/30 µm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) was chosen. Before use, the fiber was conditioned at 270 °C for 30 min. After conditioning, the fiber was inserted into the vials and exposed to the headspace for 30 min at 60 °C to adsorb the volatiles. Lastly, the SPME fiber was taken to the GC injector port maintained at 250 °C in split mode for the desorption phase.

2.4. GC-MS Analisys of EOs

To better describe the volatile chemical profile of the two oils, headspace analysis by SPME and the liquid phase analysis for both the oils were performed by direct injection. In both cases the analyses were performed by the use of a Clarus 500 model Perkin Elmer (Waltham, MA, USA) gas chromatograph coupled with a mass spectrometer and equipped with a FID (flame detector ionization). For the separation of compounds, a Varian Factor Four VF-1 capillary column was housed in the GC oven. The chromatographic conditions was followed as in [20] with some modifications. Briefly, the oven GC temperature program was: isothermal at 60 °C for 5 min, then ramped to 220 °C at a rate of 6 °C min^{-1}, and finally isothermal at 220 °C for 20 min. Carrier gas was He at flow rate of 1.0 mL min^{-1} in constant mode. The mass spectra were obtained in the electron impact mode (EI), at 70 eV in scan mode in the range 35–400 m/z. The identification of volatile compounds was performed by the matching their mass spectra with those stored in the Wiley 2.2 and Nist 02 mass spectra libraries database and by comparison of their linear retention indices (LRIs), relative to C8–C25 n-alkanes analyzed under the same conditions, with those available in the literature. Relative amounts of compounds, expressed as percentage, were calculated in relation to the total area of the chromatogram by normalizing the peak area without the use of an internal standard and any factor correction. All analyses were carried out in triplicate.

2.5. DPPH Test

The radical-scavenging capacity of EOs against DPPH· was assessed following Iriti et al. [21] with some modifications. Briefly, the DPPH· solution was diluted with methanol to obtain 1.00 ± 0.03 absorbance units at 517 nm. Then, 10 µL of each EO was added to 1990 µL of this solution. After vortexing and a reaction time of 30 min in the dark, at room temperature, the decrease in absorbance was spectrophotometrically measured and the obtained results are expressed as µM eq Trolox mL^{-1} EO. A DPPH· solution without EO was used as control. Test was performed in triplicate.

2.6. ABTS Test

The ABTS·+ radical cation-scavenging activity was determined following Iriti et al. [21]. The ABTS·+ radical cation was produced by reacting ABTS 7 mM with potassium persulfate 2.45 mM (final concentration) and keeping the mixture in the dark at room temperature for at least 6 h before use. The ABTS·+ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm and equilibrated at 30 °C. Then, 1 mL of this solution was mixed for 30 s with 10 µL of each EO. Ethanol and a standard solution of the synthetic antioxidant 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) were used as negative and positive controls, respectively. Their absorbance was read at 734 nm, at room temperature, 20 s after the end of the mixing. The inhibition percentage was calculated and the results reported as µM eq Trolox mL^{-1} EO. Test was performed in triplicate.

2.7. Phytotoxicity Test

A dose-response assay was performed following Vitalini et al. [22], with some modifications. EOs were tested at 2, 20, 50, and 100 µL by placing them in a small handmade aluminum container inside each Petri dish (9 cm in diameter). In this way, it is possible to avoid direct contact with the target seeds distributed evenly on a layer of filter paper moistened with 4 mL of deionized water. Petri dishes were set up in a vertical laminar flow hood using sterile materials and sealed with a double layer of parafilm to prevent leakage of the volatile compounds. Afterwards, they were transferred for 7 days in a growth chamber settled with a 16/8 h light/dark photoperiod and a corresponding temperature of 25/18 °C. For each combination of "target species × amount of EO", the experimental design was as follows: 15 seeds of *L. multiflorum* or *S. alba* × 4 levels of EO concentration plus distilled water as control × 3 replicates × 2 runs.

The germination of the seeds (when their radicle exceeded 1 mm) was recorded daily, while root and shoot lengths of all seedlings developed in each Petri dish were measured

on the seventh day, at the end of each run. The collected data were used to calculate some indices such as (i) Germination percentage [G% = (germinated seed number)/(seed total number) × 100]; (ii) Coefficient of Velocity of Germination (CVG = N1 + N2 + ... + Ni/100 × N1T1 + ... + NiTi, where N is the number of seeds germinated every day while T is the number of days from seeding corresponding to N) [23]; (iii) Mean Germination Time (MGT = ∑D × Germinated seed number/∑Germinated seed number, where D is the number of days from the beginning of germination) [24]; (iv) Seedling Vigor Index [SVI = (mean root length + mean shoot lenght) × germination percentage] [25].

2.8. Statistical Analysis

All data were expressed as means ± standard deviation (SD). Statistical analysis was preformed using one-way ANOVA test with a Stat-Plus software (AnalystSoft VC 2009) with the threshold of significance set at $p < 0.05$.

3. Results

3.1. EO Chemical Composition

For both *Viola* species, the analysis of the vapor phase and that of the liquid phase was carried out to obtain a more accurate description of the volatile chemical composition. The respective GC chromatograms were reported (Figures 1–4). A total of 43 compounds were identified and listed in the Table 1, among which methyl salicylate (MeSA), was the most abundant in both EOs but with higher percentage values in liquid and vapor phase of *V. dubjana* EO (67.3% and 68.0%) with respect to *V. calcarata* EO (45.5% and 46.3%). Differences in the qualitative composition between the two violets were found. For example, coumaran (2.9% and 0.2%), coumarin (6.2% and 5.8%), and epimanool (2.2% and 4.0%) were present only in the *V. calcarata*, as were the two monoterpenes terpinen-4-ol (0.7%; 1.1%) and linalol (0.5%; 0.7%). On the contrary, 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, a terpenic compound, reached major percentage values in *V. dubjana* (6.6%; 12.8%) compared to *V. calcarata* (4.2%; 3.0%) EO. The sesquiterpene content was higher in *V. calcarata* than in *V. dubyana*. Indeed, several sesquiterpenes such as sesquicineole (0.4%; 0.4%), nerolidol (0.7%; 0.8%), caryophyllene oxide (0.7%, 0.6%), *β*-eudesmol (1.3%; 1.3%), and *α*-bisabolol (1.2%; 1.6%) were characteristic only of *V. calcarata* sample. On the contrary, *δ*-cadinol (0.3%; 1.0%) and *α*-cadinol (0.3%; 0.4%) were characteristic only of *V. dubjana* sample. The only two sesquiterpene compounds common to both violets were spathulenol (from 0.2 to 1.1%) and hexahydrofarnesyl acetone (from 2.9 to 5.4%). Fatty acids were also detected in both samples except for dodecanoic (1.4% vapor phase; 3.2% liquid phase) and tetradecanoic (1.2%; 1.5%) acids which were present only in *V. dubjana* while hexadecenoic acid was only in *V. calcarata* vapor phase (0.3%).

Table 1. Chemical composition (peak area percentage) of *Viola dubyana* and *Viola calcarata* EOs.

N°	COMPONENT [1]	LRI [2]	*V.d.* [3] (%)	*V.d.* [4] (%)	*V.c.* [5] (%)	*V.c.* [6] (%)
1	hexanal	770	0.3 ± 0.02	-	-	-
2	furfural	832	0.3 ± 0.02	-	-	-
3	heptanal	874	0.1 ± 0.01	-	-	-
4	hexanoic acid	976	-	-	tr	0.3 ± 0.02
5	octanal	1005	0.1 ± 0.00	-	-	-
6	linalol	1078	-	-	0.5 ± 0.02	0.7 ± 0.02
7	nonanal	1091	0.6 ± 0.02	0.1 ± 0.02	-	0.2 ± 0.02
8	camphor	1157	0.4 ± 0.01	0.2 ± 0.01	0.5 ± 0.03	0.4 ± 0.01
9	trans-sabinene hydrate	1162	0.2 ± 0.01	-	-	-
10	octanoic acid	1172	0.2 ± 0.01	0.5 ± 0.02	0.3 ± 0.01	0.6 ± 0.01

Table 1. *Cont.*

N°	COMPONENT [1]	LRI [2]	*V.d.* [3] (%)	*V.d.* [4] (%)	*V.c.* [5] (%)	*V.c.* [6] (%)
11	terpinen-4-ol	1182	-	-	0.7 ± 0.02	1.1 ± 0.02
12	β-cyclocitral	1200	0.8 ± 0.02	-	-	-
13	methyl salicylate	1218	67.3 ± 0.02	68.0 ± 0.02	45.5 ± 0.02	46.3 ± 0.02
14	coumaran	1225	-	-	2.9 ± 0.02	0.2 ± 0.02
15	nonanoic acid	1263	-	0.9 ± 0.01	1.2 ± 0.01	1.4 ± 0.02
16	*p*-vinylguaiacol	1279	0.6 ± 0.02	0.5 ± 0.02	tr	0.4 ± 0.02
17	bornyl acetate	1280	0.4 ± 0.01	0.3 ± 0.02	-	-
18	decanoic acid	1349	1.4 ± 0.02	2.6 ± 0.02	1.6 ± 0.02	2.8 ± 0.01
19	2-(4-methoxyphenyl)-ethanol	1360	-	-	1.6 ± 0.02	2.0 ± 0.02
20	β-copaen-4α-ol	1400	0.7 ± 0.02	tr	-	-
21	coumarin	1430	-		6.2 ± 0.02	5.8 ± 0.02
22	trans-geranyl acetone	1437	0.2 ± 0.02	0.3 ± 0.02	-	-
23	trans-β-ionone	1463	1.8 ± 0.00	2.2 ± 0.02	-	-
24	sesquicineole	1500	-	-	0.4 ± 0.02	0.4 ± 0.02
25	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	1530	6.6 ± 0.01	12.8 ± 0.01	4.2 ± 0.03	3.0 ± 0.02
26	nerolidol	1537	-	-	0.7 ± 0.01	0.8 ± 0.02
27	dodecanoic acid	1555	3.2 ± 0.02	1.4 ± 0.02	-	-
28	spathulenol	1572	0.2 ± 0.02	0.5 ± 0.02	1.1 ± 0.02	1.1 ± 0.02
29	caryophyllene oxide	1578	-	-	0.7 ± 0.01	0.6 ± 0.04
30	humulene epoxide II	1598	-	-	1.3 ± 0.02	-
31	δ-cadinol	1621	0.3 ± 0.02	1.0 ± 0.02	-	-
32	bisabolol oxide B	1660	-	-	6.0 ± 0.03	5.9 ± 0.02
33	β-eudesmol	1668	-	-	1.3 ± 0.02	1.3 ± 0.02
34	α-cadinol	1672	0.3 ± 0.02	0.4 ± 0.02	-	-
35	α-bisabolol	1681	-	-	1.2 ± 0.02	1.6 ± 0.02
36	bisabolone oxide A	1700	-	-	1.2 ± 0.01	1.3 ± 0.02
37	bisabolol oxide A	1710	-	-	5.8 ± 0.02	5.7 ± 0.03
38	tetradecanoic acid	1755	1.5 ± 0.01	1.1 ± 0.01	-	-
39	hexahydrofarnesyl acetone	1844	4.2 ± 0.02	2.9 ± 0.02	5.4 ± 0.04	4.7 ± 0.02
40	diisobutyl phthalate	1868	0.9 ± 0.02	0.7 ± 0.02	1.1 ± 0.03	2.2 ± 0.04
41	dibutyl phthalate	1911	1.3 ± 0.02	0.2 ± 0.02	2.5 ± 0.01	3.3 ± 0.02
42	hexadecanoic acid	1975	6.1 ± 0.01	3.0 ± 0.01	1.6 ± 0.03	1.9 ± 0.04
43	epimanool	2061	-	-	2.2 ± 0.02	4.0 ± 0.02
	SUM		100.0	99.6	97.7	100.0
	Terpenoids		8.2	13.3	5.9	5.2
	Sesquiterpenoids		5.7	4.8	10.8	10.5
	Fatty acids		12.4	9.5	4.7	7.0
	Other		73.7	72.0	76.3	77.3

[1] The components are reported according to their elution order on apolar column; [2] linear retention indices measured on apolar column; *V.d.* [3]: peak area percentage of *Viola dubyana* liquid phase components (%); *V.d.* [4]: peak area percentage of *Viola dubyana* vapor phase components; *V.c.* [5]: peak area percentage of *Viola calcarata* liquid phase components (%); *V.c.* [6]: peak area percentage of *Viola calcarata* vapor phase components; -: not detected; tr: traces (mean value < 0.1%).

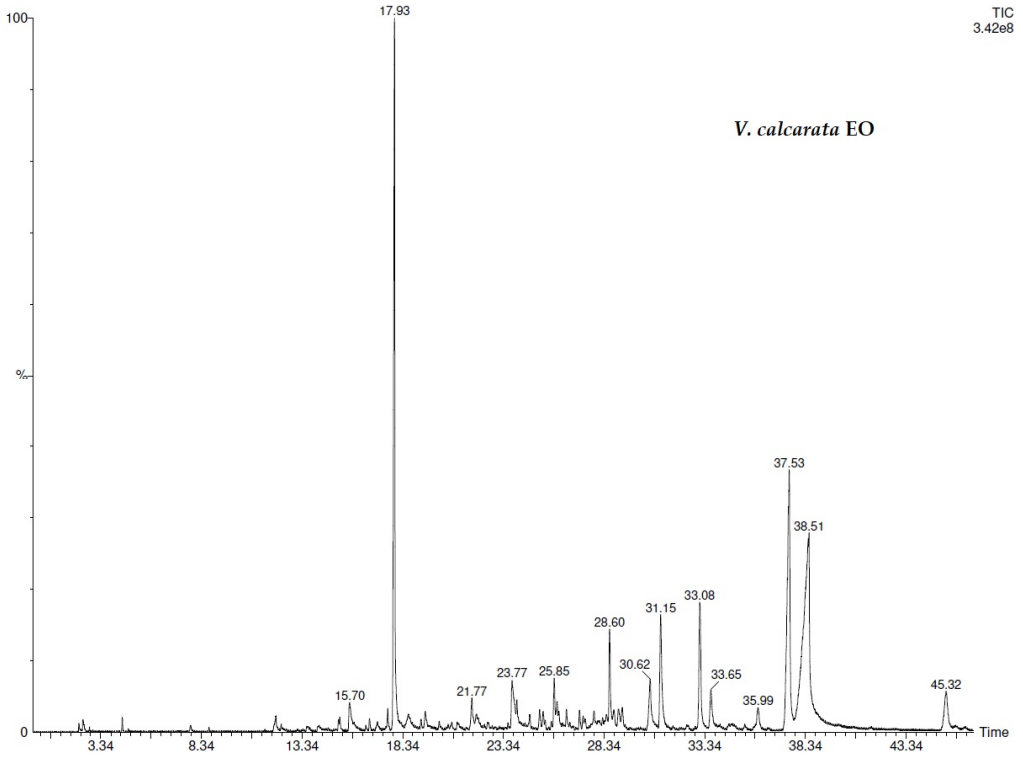

Figure 1. GC/GC-FID chromatograms of *V. calcarata* EO.

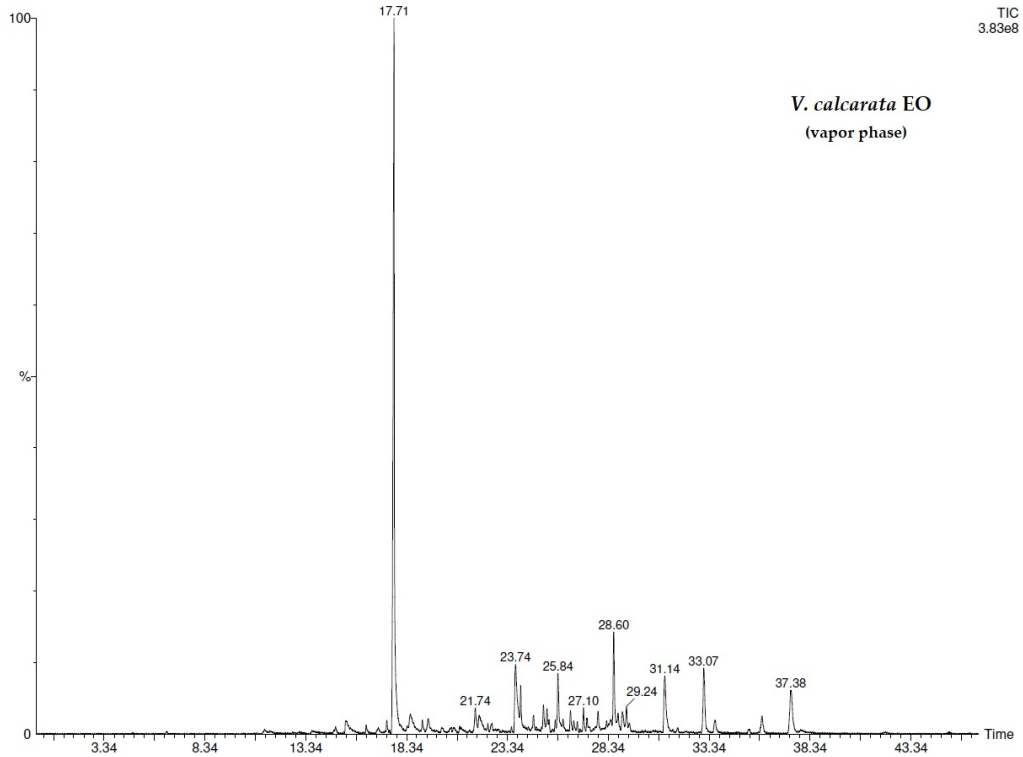

Figure 2. SPME/GC-FID chromatograms of *V. calcarata* EO.

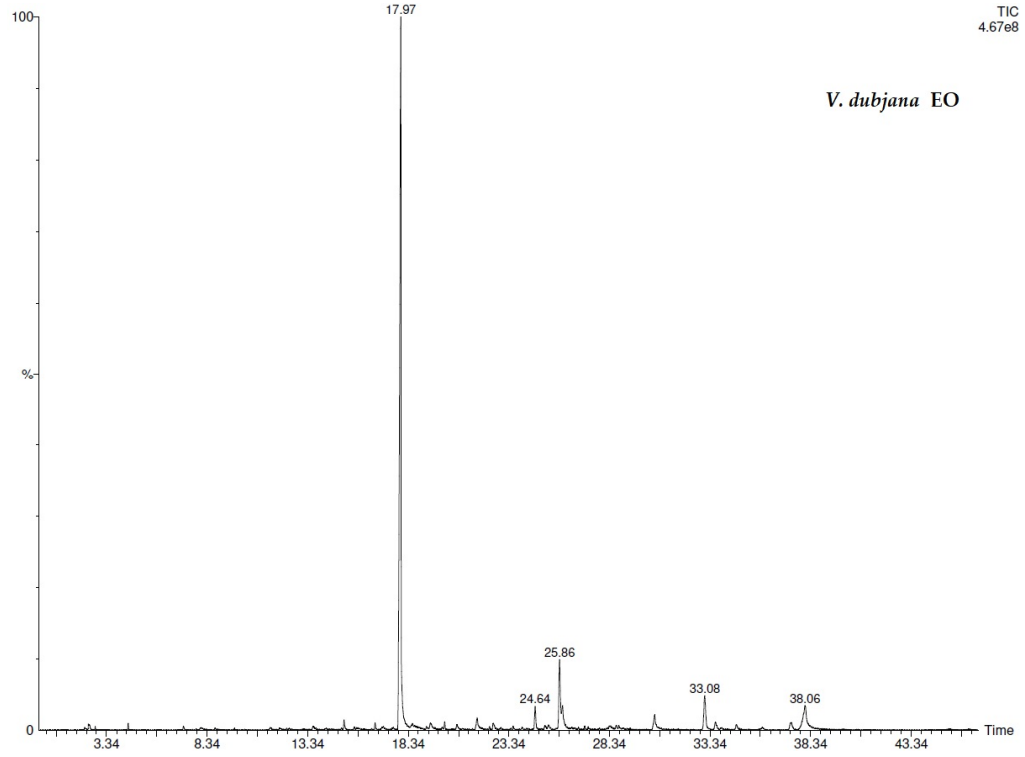

Figure 3. GC/GC-FID chromatograms of *V. dubjana* EO.

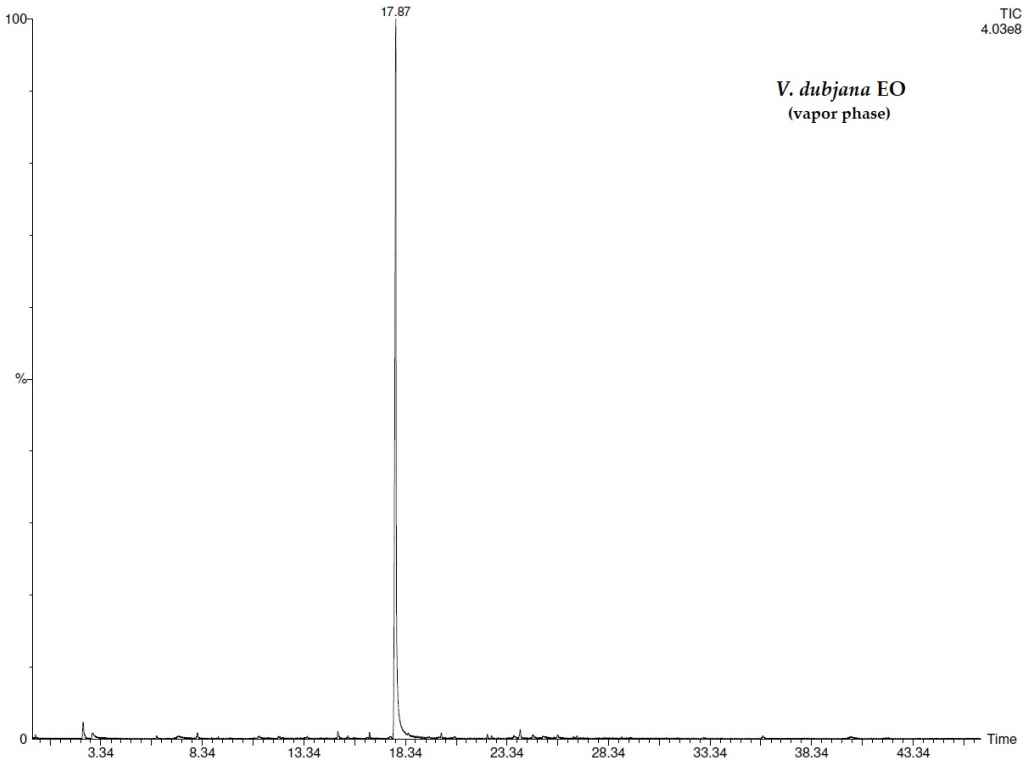

Figure 4. SPME/GC-FID chromatograms of *V. dubjana* EO.

3.2. EO Antiradical Activity

In terms of antiradical activity, both EOs showed different reactions with the two used radicals and the *V. dubyana* EO proved to be the most effective as a scavenger. EOs were

able to appreciably inhibit ABTS·+ cation radical while they were weakly active in the removal of the stable radical DPPH· (Table 2).

Table 2. Antiradical power of EOs from *Viola dubyana* and *Viola calcarata*.

EO	ABTS (μM Trolox eq/mL)	DPPH (μM Trolox eq/mL)
Viola calcarata	1.03 ± 0.01	0.29 ± 0.01
Viola dubyana	1.32 ± 0.03	0.37 ± 0.01

3.3. EO Phytotoxic Activity

As for the phytotoxic potential, the EOs of the two violets were found active, however revealing a greater efficacy of *V. dubyana* compared to *V. calcarata*, especially against the dicotyledon *S. alba*. EOs decreased, in a dose dependent manner, both the seed germination and seedling growth parameters (Tables 3 and 4). In detail, the germination percentage of the monocot *L. multiflorum*, the most resistant target species, was similarly reduced by 5–25% and by 10–20%, respectively, by all used doses (2–100 μL) of *V. calcarata* and *V. dubyana* EOs. Alike, its CVG values dropped 4–60% and 3–57% while MGT was only affected by the two highest doses of 50 and 100 μL (+4% to +15% and +8% to +23%) of both EOs. The same doses (50 and 100 μL) also significantly inhibited the development of *L. multiflorum* roots (−44% to −92% and 50% to −90%) and shoots (−13% to −82% and −25% to −87%) lowering the corresponding SVI values (−42% to −91% and 52% to −91%).

More marked differences between the effects of the two EOs were identified on *S. alba.* In detail, *V. dubyana* EO, in the highest dose (100 μL), prevented its germination (0%) (Table 4), while *V. calcarata* EO was able to almost halve it (−46%) (Table 3). The lower doses of *V. dubyana* EO decreased the germination of *S. alba* by 5–77%, those of *V. calcarata* EO by 5–39% (Tables 3 and 4). Accordingly, CVG and MGT indices were also influenced with values reduced up to −100% and −60%. When it occurred, the growth of roots and shoots of *S. alba* exposed to *V. dubyana* EO (2, 20 and 50 μL) stopped from −14% to −85% and from −5% to −63%, respectively, with reductions in SVI values up to 95% (Table 4). On the other hand, the length of *S. alba* roots and shoots under the effects of *V. calcarata* EO (2–100 μL) was found to be shorter than the controls by 6–74% and 2–58%. Hence, the SVI values decreased 1 to 6 fold (Table 3).

Table 3. Germination and growth parameters of two target species (*Lolium multiflorum* and *Sinapis alba*) under the phytotoxic effects of different doses of *Viola calcarata* EO.

Viola calcarata							
Target Species	**EO Doses (μL)**	**G (%)**	**CVG**	**MGT**	**SVI**	**Root (mm)**	**Shoot (mm)**
Lolium multiflorum	2	92.0 ± 9.0	101.0 ± 8.0	4.8 ± 0.3	10,332 ± 954	62.3 ± 8.9	50.0 ± 5.3
	20	90.0 ± 7.0	99.0 ± 5.0	4.8 ± 0.2	10,368 ± 686	64.1 ± 22.0	51.1 ± 7.23
	50	80.0 ± 8.0	79.0 ± 4.0	5.0 ± 0.2	6240 ± 555	34.7 ± 7.4	43.3 ± 12.4
	100	73.0 ± 5.0	42.0 ± 6.0	5.5 ± 0.3	1022 ± 286	5.1 ± 1.4	8.9 ± 1.9
	CTRL	97.0 ± 5.0	105.0 ± 9.0	4.8 ± 0.1	10,796 ± 731	61.4 ± 7.9	49.9 ± 4.0
Sinapis alba	2	83.0 ± 5.0	115.0 ± 6.0	4.2 ± 0.2	4001 ± 301	26.1 ± 7.7	22.1 ± 5.5
	20	80.0 ± 8.0	108.0 ± 7.6	4.2 ± 0.3	3200 ± 143	21.7 ± 9.2	18.3 ± 4.0
	50	53.0 ± 5.0	60.0 ± 7.3	4.3 ± 0.3	912 ± 69	7.3 ± 3.9	9.9 ± 2.2
	100	47.0 ± 3.0	48.0 ± 3.3	4.5 ± 0.0	785 ± 98	7.3 ± 2.5	9.4 ± 1.4
	CTRL	87.0 ± 5.0	119.0 ± 9.8	4.1 ± 0.1	4359 ± 353	27.8 ± 3.4	22.3 ± 6.8

Values are mean ± standard deviation. G%, germination percentage; CVG, coefficient of velocity of germination; MGT, mean germination time, SVI, seedling vigor index.

Table 4. Germination and growth parameters of two target species (*Lolium multiflorum* and *Sinapis alba*) under the phytotoxic effects of different doses of *Viola dubyana* EO.

Viola dubyana							
Target Species	**EO Doses (µL)**	**G (%)**	**CVG**	**MGT**	**SVI**	**Root (mm)**	**Shoot (mm)**
Lolium multiflorum	2	90.0 ± 14.0	104.5 ± 9.3	4.9 ± 0.2	11,601 ± 877	74.9 ± 11.2	54.0 ± 9.4
	20	83.0 ± 9.0	101.0 ± 15.0	4.9 ± 0.1	10,234 ± 941	69.9 ± 12.8	53.4 ± 8.3
	50	80.0 ± 5.0	74.0 ± 2.8	5.2 ± 0.1	6320 ± 458	38.0 ± 6.6	41.0 ± 9.8
	100	80.0 ± 0.0	47.0 ± 4.5	5.9 ± 0.0	1192 ± 237	7.8 ± 2.6	7.1 ± 1.7
	CTRL	100.0 ± 5.0	108.0 ± 4.0	4.8 ± 0.1	13,100 ± 687	76.3 ± 10.6	54.7 ± 11.2
Sinapis alba	2	83.0 ± 8.0	102.0 ± 9.0	4.2 ± 0.1	4116 ± 301	29.4 ± 6.7	20.2 ± 4.5
	20	57.0 ± 5.0	63.0 ± 2.0	4.2 ± 0.0	1533 ± 143	12.8 ± 4.5	14.1 ± 2.6
	50	20.0 ± 5.0	20.0 ± 6.0	4.1 ± 0.1	258 ± 69	5.1 ± 1.1	7.8 ± 1.5
	100	0.0 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.
	CTRL	87.0 ± 5.0	112.0 ± 11.0	4.2 ± 0.1	4829 ± 353	34.3 ± 8.5	21.2 ± 5.9

Values are mean ± standard deviation. G%, germination percentage; CVG, coefficient of velocity of germination; MGT, mean germination time; SVI, seedling vigor index; n.d., not determined.

4. Discussion

In this work, the volatile profile of two *Viola* species was described owing to the use of SPME-GC/MS techniques. The chemical composition highlighted the presence of MeSA as the principal compound in both investigated samples with higher percentages in *V. dubyana* rather than in *V. calcarata*. Our results agree with previous studies on several populations of another rare Italian *Viola* species, namely *V. etrusca* Erben, reporting MeSA as the main component [10,26]. MeSA is a volatile compound and a constituent of floral fragrance of various plant species capable of acting as a defense agent against pathogens and some species of herbivores including aphids [27]. Furthermore, MeSA presents a good phytotoxic and allelopathic activity toward invasive plants [27,28], and plays a role as a pollinator attractor and in insect defense [29,30]. It has been referred to as a means of plant-to-plant aerial communication [31,32] and as the major constituent (96%) of *Gaultheria procumbens* L., whose EO also showed good antioxidant and antiradical activity [33]. Through brine shrimp lethality test, the toxicity of *Laportea aestuans* (Gaud) EOs was also demonstrated, the most abundant compound of which was MeSa (54.50%) [34]. Jayasekara et al. [35] also reported MeSA repellent and toxic properties against invertebrate pest adult *Sitophilus zeamais* and a dose-dependent fumigant effect against *S. zeamais, Rhyzopertha dominica*, and *Prostephanus truncates.* MeSA compound has even been described as an inducer of programmed cell death (PCD) [36]. Lastly, MeSA compound is used topically as a counter-irritant. Once absorbed it is rapidly hydrolyzed to salicylic acid, which has analgesic, antipyretic, and anti-inflammatory effects. In general, salicylates are used precisely for these beneficial effects, but cases of salicylate intoxication may occur following overdoses due to ingestion or skin absorption [37–39].

In the two violet EOs, we have found two other bioactive compounds such as 2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl and hexahydrofarnesyl acetone with relative high percentages. Both compounds were reported in the chemical composition of the EOs obtained from the leaves of *V. odorata* L. [40] and dried aerial parts of *V. tricolor* L. [41]. 2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl- was also identified in the dichloromethane extract obtained from *Alstonia scholaris* (L.) R. Br. [42] and in the methanolic fractions of *Azadirachta indica* A.Juss. and *Aegle marmelos* (L.) Corrêa [43,44] showing antioxidant and antibacterial activity. Hexahydrofarnesyl acetone is a sesquiterpenoid associated with allelopathic activity [45]. It has been reported several times as one of the agents responsible for the phytotoxicity of various EOs [46–49]. Although present in lower percentages in the EO of *V. calcarata* and *V. dubyana* than the previous compounds, decanoic and hexadecanoic acids have already been detected as constituents of samples

(EOs, exudates or extracts) capable of selectively reducing the growth of some plant species including weeds [50–52].

V. calcarata EO was characterized by the presence of bisabolone oxide A, bisabolol oxide A and B, and α-bisabolol. The first three compounds were also reported by Anca et al. [41] in their study on *V. tricolor.* Bisabolone oxide A and bisabolol oxide B were identified among the most abundant components of the *Chamomilla recutita* (L.) Rauschert EO, which, tested together with other EOs against the germination of some weeds, showed one of the lowest activities [53]. Otherwise, the phytotoxic potential of the EO of an Egyptian ecospecies of *Pulicaria undulata* (L.) C.A. Mey as well the allelopathic properties of the EO of *Prangos pabularia* Lindl. was ascribed to their major compounds including α-bisabolol [54,55]. *V. calcarata* also differed from the composition of *V. dubyana* for the presence of coumarin and coumaran. Coumarin is a natural compound known for its negative effects on weed germination [56]. Dodecanoic acid in *V. dubyana* EO showed phytotoxic effect toward some plant species, able to reduce their germination and root development [52]. The inhibitory effects of EOs can be due to the single component but also be related to the mixture of compounds [57]. For example, it is known that sesquiterpene allelochemicals including spathulenol and β-caryophyllene detected in our investigation presented phytotoxicity against various invasive plants [58]. In our EOs, the activity of these two compounds could be added to that of other components present in greater quantities. Furthermore, it was proved that the phytotoxic potential of an EO also depends on the used dose and the tested plant species [53].

Lastly, the presence of fatty acids recognized as effective antioxidants, with hexadecanoic acid reaching 6.1% in *V. dubyana*, may have contributed to the antiradical activity showed by both *Viola* species [59].

5. Conclusions

This is the first paper dealing with the volatile chemical composition of *V. dubjana* and *V. calcarata*. SPME-GC/MS techniques allowed the separation and identification of several volatile compounds highlighting a different profile, both from a qualitative and quantitative point of view, between the two species, however, both having MeSA as the dominant component. Despite this, the detected effectiveness of their EOs could also be due to the synergistic and/or additive action of the minor components, which, as has been widely reported, are able to play a significant role in various biological activities.

The findings, although to be further investigated, revealed an interesting bioactive potential of these violets, which could have various implications including ecological ones supporting the development of new products respectful of the environment and its resources.

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