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Viola calcarata L. and *Viola dubyana* Burnat ex Gremli hydrolates: DI-SPME-GC-MS analysis and biological activity evaluation

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Abstract: The hydrolates obtained from the aerial parts of two indigenous *Viola* L. species (*V. calcarata* L. and *V. dubyana* Burnat ex Gremli) growing in the Italian Alps were studied for the first time. The direct immersion solid-phase microextraction (DI-SMPE) sampling and the GC–MS analysis revealed the presence of methyl salicylate as the main compound in both samples along with a different set of terpenes for each hydrolate and some other minor compounds. The most interesting results were achieved by evaluating their phytotoxic potential against two target species (*Sinapis alba* L. and *Lolium multiflorum* Lam.) through treatments without direct contact with the seeds. In general, the hydrolates showed species and dose-dependent effects. They were more active on growth (maximum reduction = -86.8% due to *V. dubyana* hydrolate on *S. alba*) than on germination (maximum inhibition = -45.9% due to *V. calcarata* hydrolate on *S. alba*). No significant results were obtained for the antiradical activity.

Keywords: *ABTS, chromatographic analyses, DPPH, phytotoxicity, Violaceae, volatiles*

INTRODUCTION

Hydrolates, also known as floral aromatic waters as well as hydrosols, are produced by hydro-distillation or steam distillation [Aćimović et al. 2020a]. During process, the fat-soluble and volatile compounds of the plants give rise to the essential oil, while the water-soluble components remain in the distilled water forming the hydrolate [Tavares et al. 2022]. The scientific literature on hydrolates is less common than that on essential oils. Nevertheless, it is known that hydrolates have specific aromatic constituents, often numerically and/or at lower concentrations than those of the corresponding essential oils [Šilha et al. 2020]. In 2008, Inouye with his collaborators [Inouye et al. 2008] found that 42% of investigated hydrolates have different main compounds than those of essential oils and, therefore, also a probable different bioactivity. This was lately confirmed by Aćimović and co-authors [2020b] studying the essential oil and hydrosol composition of *Artemisia annua* L. from Serbia.

After years, the use of hydrolates has appreciably increased due to the rediscovery of their properties. Currently, many hydrolates are widely exploited in aromatherapy, cosmetic formulations, food industry and organic agriculture [Aćimović et al. 2020a]. Several studies reported their antioxidant, anti-allergic, anti-inflammatory antimicrobial and fungicidal properties as well as cytotoxic or anti-enzymatic activity [Boyraz & Ozcan 2006; Ko et al. 2017; Shen et al. 2017; D'Amato et al. 2018; Mohamed Gameil et al. 2019; Ha et al. 2021; Pérez-Izquierdo et al. 2022; Yu et al. 2022]. Allelopathic effect was also studied to find alternative agricultural strategies, for example in weed control in order to reduce the use of traditional agrochemicals along with their side effects on the environment and human health. In this context, hydrolates obtained from different plant organs such as leaves, stems and flowers were found to inhibit both the seed germination and the shoot and root growth of some noxious species (e.g., *Amaranthus retroflexus* L.) as well as of some model species (e.g., *Lactuca sativa* L.) [Özkan & Tunçtürk, 2021; Politi et al. 2022]. In general, hydrolates are considered as an important source of phytochemicals with a significant bioactive potential. Data on the hydrolates from this work complemented the previous study both in terms of chemical composition and biological activity of *Viola calcarata* L. and *Viola dubyana* Burnat ex Gremli (complex *V. calcarata*, section *Viola* Melanium, Violaceae family) (Fig. 1) [Vitalini et al. 2022]. These are two indigenous Italian species present in the Alps where bloom between April-May and July-August, above 1000 meters s.l. [Tutin et al. 1981; Pignatti, 1982].

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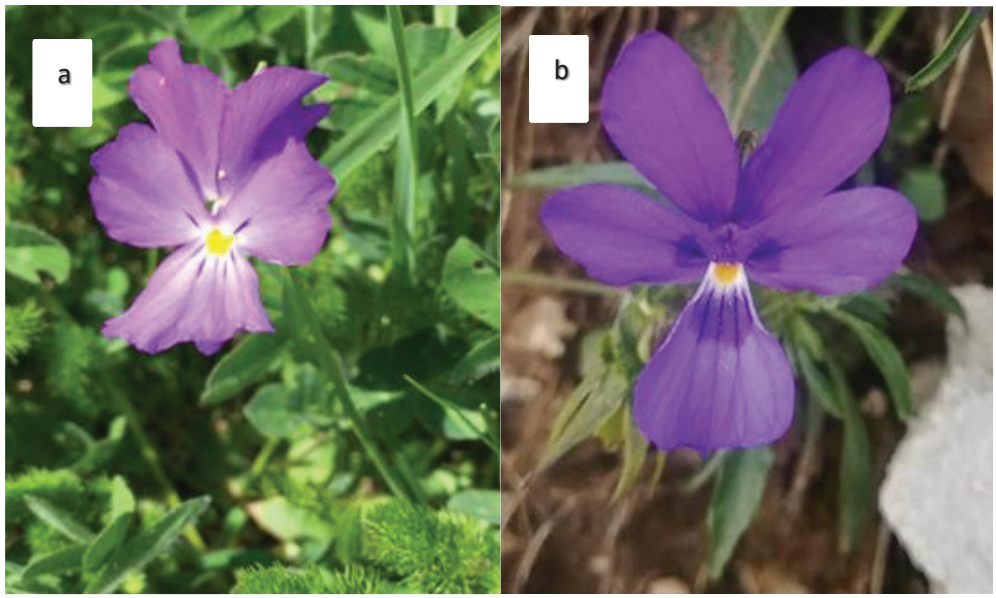

Figure 1. a. *Viola calcarata* L., b. *Viola dubyana* Burnat ex Gremli.

MATERIAL AND METHODS

Plant material. Flowers of *V. calcarata* and *V. dubyana* were collected in June 2018 in Italy at Colle del Piccolo San Bernardo (Valle d'Aosta, 2100 m a.s.l.) and at Monte Caplone (Lombardy, 1500 m a.s.l.), respectively (Fig. 1b). After the determination according to Flora d'Italia [Pignatti, 1982], the herbarium voucher specimens (No. VC-PPSB-VO18 and No. VD-VS-BS18) were kept at the Department of Agricultural and Environmental Sciences of the Milan State University (Milan, Italy). Seeds of *Sinapis alba* L. and *Lolium multiflorum* Lam. were purchased from the company "Padana Sementi" (Padova, Italy) and supplied by the organic farm "Terre di Lomellina" (Pavia, Italy), respectively. Before use, they were sterilized for 10 minutes in a sodium hypochlorite solution (1%) and then rinsed several times with distilled water.

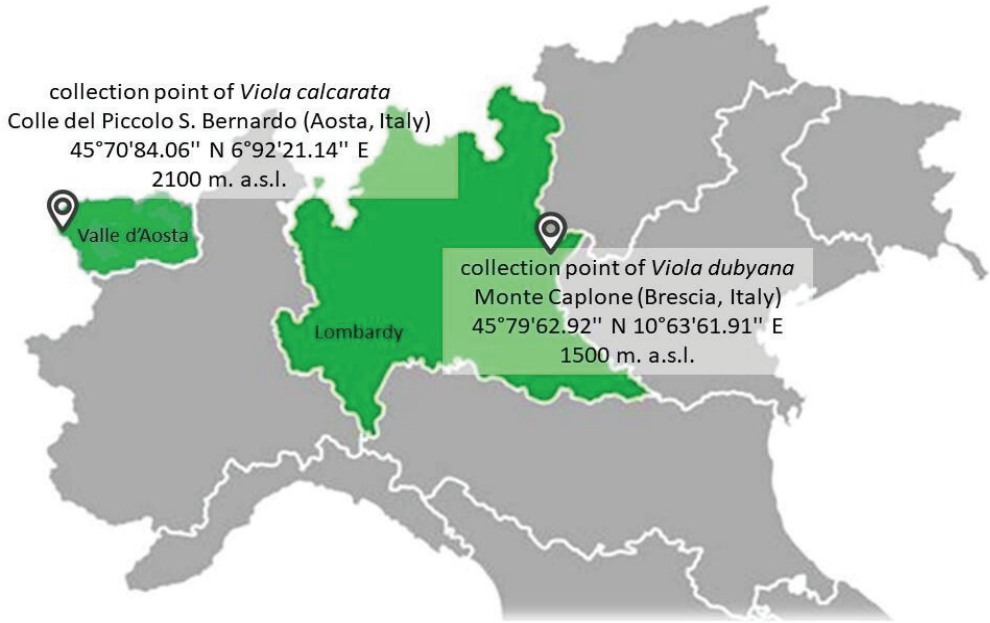

Figure 2. Collection points of *Viola calcarata* and *Viola dubyana* in Northern Italy.

Hydrolate preparation. After the hydrodistillation of the essential oil of *V. calcarata* and *V. dubyana* [Vitalini et al. 2022], the respective hydrolates were collected and placed in dark vials, then stored at 4 °C until use.

Direct Immersion Solid-Phase Microextraction (DI-SMPE) sampling. First the vial was heated to 50 °C using a thermostatic bath and then a holder equipped with 1 cm fiber coated with 50/30µm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane was immersed directly in the aqueous solution for 20 minutes for the extraction of volatile and semi-volatile compounds. For the extraction of the adsorbed compounds, the fiber was then inserted into the injector of the GC maintained at 250 °C.

Table 1. Chemical volatile composition of *Viola calcarata* and *Viola dubyana* hydrolates.

COMPONENT[1]	LRI[2]	LRI[3]	*V.c.*[4]*(%)*	*V.d.*[5]*(%)*
2-propen-1-ol	541	549	0.1±0.02	-
2-ethylaziridine	648	650	0.5±0.04	-
α-pinene	933	936	0.5±0.05	-
δ-2-carene	992	996	1.7±0.02	-
trans-sabinene hydrate	1050	*	-	3.2±0.02
methyl salicylate	1511	1215	96.1±0.02	82.2±0.06
thymol	1270	1272	1.0±0.02	-
β-damascenone	1358	1356	-	4.8±0.02
trans-geranyl acetone	1428	1434	-	2.3±0.02
trans-*β*-ionone	1462	1460	-	1.5±0.03
benzophenone	1618	1621	-	0.9±0.03
bisabolol oxide II	1649	1655	-	0.8±0.02
apiol	1679	*	0.1±0.02	-
diisobutyl phthalate			-	4.3±0.03
SUM			100.0	100.0
Terpenoids			3.2	12.6
Other			96.8	87.4

Note: [1]The components are reported according to their elution order on apolar column; [2]Linear Retention Indices measured on apolar column; [3] Linear Retention indices from literature; [*]LRI not available; V.c.[4]: Percentage mean values of *Viola calcarata* vapor phase components; V.d.[5]: Percentage mean values of *Viola dubyana* vapor phase components (%); -: Not detected.

Gas chromatography–mass spectrometry (GC-MS) analysis. A Clarus 500 model Perkin Elmer (Waltham, MA, USA) gas chromatograph coupled with a mass spectrometer and equipped with an FID (flame detector ionization) was used for the analyses. For the separation of compounds, a Varian Factor Four VF-1 capillary column was housed in the GC oven. The chromatographic conditions were used as reported in our previous study [Ovidi et al. 2021] with minor modifications. The was used as a gas carrier with the flow rate of 1.0 mL min^{-1}. The mass spectra were obtained in the electron impact mode (EI), at 70 eV in scan mode ranging 35-400 m/z. The separated compounds were identified by matching their mass spectra with the Wiley 2.2 and Nist 02 library database and comparing their calculated linear retention indices (LRIs) with those available in the literature. The relative amount of compounds, expressed as a percentage, was calculated in relation to the total area of the chromatogram by normalizing the peak area without the use of an internal standard and any other correction factor. The analyses were carried out in triplicate.

Phytotoxicity assay. A dose-response test was carried out by following S. Vitalini et al. [2022], with minor modifications. The phytotoxic potential of hydrolates was assessed at 2, 20, 50, 100 and 200 µL by placing them in a small handmade aluminum container inside the Petri dish (90 mm in diameter) to avoid direct contact with the target seeds (15) of *L. multiflorum* and *S. alba* distributed evenly on a filter paper layer moistened with 4 mL of deionized water. Petri dishes were set up in a vertical laminar flow hood and sealed with a double layer of parafilm to prevent the volatile compound spillage. Then, they were transferred in a growth chamber (16/8 h light/dark photoperiod and 25/18 °C) for 7 days. The experimental design involved 5 different doses of hydrolates or distilled water as a control × 3 replicates × 2 runs.

Measurement of the germination and seedling growth parameters. The germination of the seeds with radicle ≥ 1 mm was recorded daily, while root and shoot lengths of the seedlings were measured at the end of each run. The collected data were used to calculate the following indices:

(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) [Al-Mudaris, 1998];

(iii) Mean Germination Time (MGT) = ∑D × Germinated seed number/∑Germinated seed number, where D is the number of days from the beginning of germination) [Ellis & Roberts, 1981];

(iv) Seedling Vigor Index (SVI) = (mean root length + mean shoot lenght) × germination percentage [Abdul-Baki & Anderson, 1973].

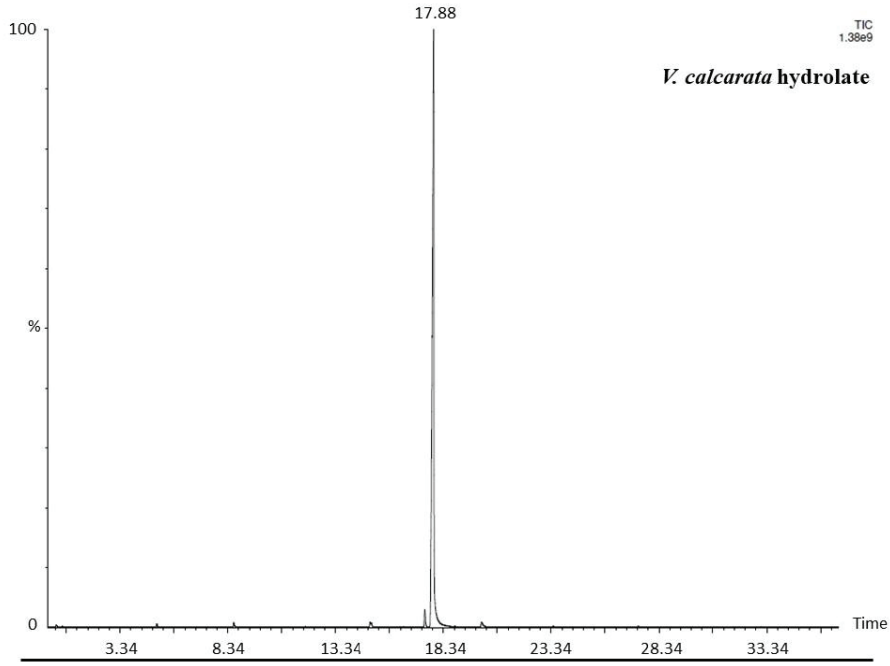

Figure 3. GC-FID chromatogram of *Viola calcarata* hydrolate.

Determination of total polyphenols. The total phenolic content was determined by the Folin-Ciocalteu method reported by S. Vitalini et al. [2021]. Briefly, a mixture with an adequate amount of each hydrolate and 50 µL of the Folin-Ciocalteu reagent placed in a 10 mL test tube was kept at room temperature for 3 min. A saturated solution of sodium carbonate (100 µL) was added and a final volume of 2.5 mL was achieved with distilled water. The reaction was allowed to continue in the dark for 60 min, after which absorbance was measured at 765 nm with respect to a blank using a Jenway 6310 spectrophotometer (Keison, Chelmsford, Essex, UK). The test was performed in triplicate, and the results were reported in micrograms of Gallic Acid Equivalents (GAE) per mL of hydrolate.

Determination of total flavonoids. The total flavonoid content was determined by the aluminum chloride colorimetric method as per S. Vitalini et al. [2021], with some modifications. Briefly, 100 µL of each hydrolate was separately mixed with 300 µL of methanol, 20 µL of 10% aluminum chloride, 20 µL of 1 M potassium acetate and 560 µL of distilled water. The final solution was incubated in the dark for 30 min at room temperature. Then, absorbance was measured at 420 nm using a Jenway 6310 spectrophotometer (Keison, Chelmsford, Essex, UK). The test was performed in triplicate, and the results were reported in micrograms of Quercetin Equivalents (QE) per mL of hydrolate.

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging assay. The antiradical activity of the two hydrolates against DPPH• was evaluated following S. Vitalini et al. [2022]. Briefly, a suitable amount of hydrolate was added to the DPPH• solution (1.00 ± 0.03 absorbance units at 517 nm), then vortexed and incubated for 30 min in the dark at room temperature. The decrease in absorbance was measured spectrophotometrically (Jenway 6310, Keison, Chelmsford, Essex, UK) and the obtained results were expressed as µM eq Trolox mL^{-1} hydrolate. A solution of DPPH• without hydrolate was used as a control. Test was performed in triplicate.

ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical cation-scavenging assay. The antiradical activity of the two hydrolates was determined following S. Vitalini et al. [2022]. 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 reach an absorbance of 0.7 (± 0.02) at 734 nm and equilibrated at room temperature. Then, 1 mL of this solution was mixed for 30 s with 10 µL of each hydrolate. The decrease in absorbance was read at 734 nm using a Jenway 6310 spectrophotometer, 20 s after the end of the mixing. Therefore, the obtained results were reported as µM eq Trolox mL^{-1} hydrolate. Ethanol and a standard solution of the synthetic antioxidant

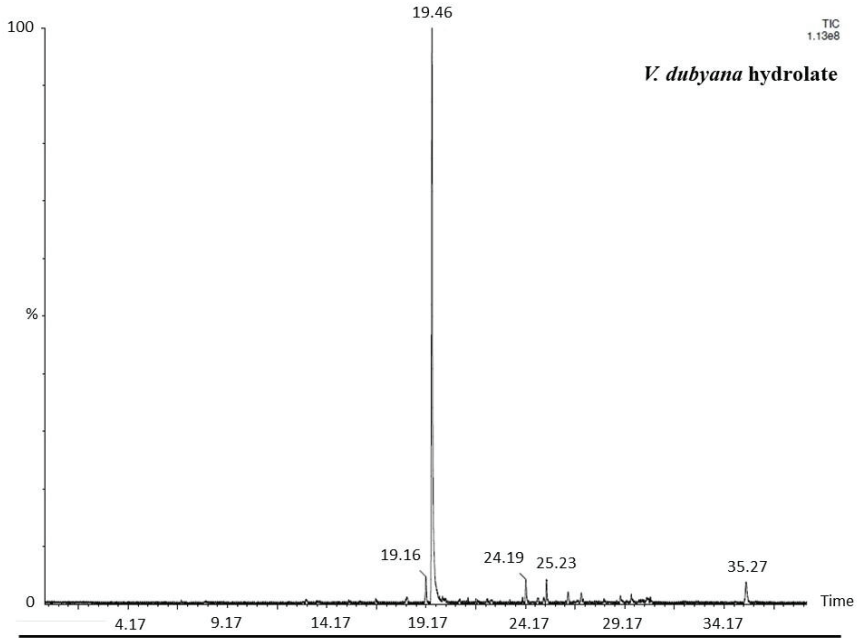

Figure 4. GC-FID chromatogram of *Viola dubyana* hydrolate.

6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) were used as negative and positive controls, respectively. Test was performed in triplicate.

RESULTS AND DISCUSSION

IGC-MS results. Thanks to the application of DI-SPME-GC-MS techniques, in total 15 components were detected and identified, 7 in *V. calcarata* and 8 in *V. dubyana*. The analyses showed that the main component in both hydrolates was methyl salicylate (> 90% and > 80%, respectively), in accordance with the data reported in our previous work conducted on the essential oils of the same *Viola* samples [Vitalini et al., 2022]. With the exception of this compound, the composition of the two hydrolates differed. Some monoterpenes such as trans-sabinene hydrate (3.2%), β-damascenone (4.8%), trans-geranyl acetone (2.3%), trans-β-ionone (1.5%), and the sesquiterpene bisabolol oxide II (0.8%)were detected only in the *V. dubyana* hydrolate. Otherwise, the monoterpenes α-pinene, δ-2-carene and thymol were (3.2% in total) exclusively present in the *V. calcarata* hydrolate.

Phytotoxicity. The phytotoxicity data obtained by testing five different quantities (2 to 200 µL) of the *V. calcarata* and *V. dubyana* hydrolates are shown in tables 2 and 3, respectively. In general, the most susceptible target species was *S. alba*, confirming the previous results of essential oils from the same plants. The effects were more evident when the dose of both hydrolates increased. At the highest tested dose, its germination decreased by 30.2% and 45.9% due to the action of *V. calcarata* and *V. dubyana*, respectively. Likewise, CVG shrank by 38.2% and 51.1%, while MGT increased by 10.0% and 2.4%. As for the development of the seedlings, *V. calcarata* reduced the length of the *S. alba* roots and shoots by 84.0% and 68.0% with only 2 µL of hydrolate as well as the corresponding SVI value by 82.9% (Table 2).

On the other hand, the same parameters were affected to a lesser extent by 2 µL of *V. dubyana* hydrolate (roots, -37.3%; shoots, -22.3%; SVI, -39.7%) still managing to drop to -73.1%, -53.6% and -81.0%, respectively, at the dose of 200 µL (Table 3).

Both hydrolates showed significant effects against the *L. multiflorum* growth. In detail, *V. calcarata* sample reduced root and shoot development up to 66.5% and 58.7%, lowering the SVI by 19.6%-69.8% (Table 2) as well as *V. dubyana* hydrosol decreased root and shoot length and thus SVI up to 44.9%, 29.7% and 42.6% (Table 3). Germination stopped at 77% (-23%) and 87% (-9.4%) compared to controls at 200 µL, CVG values diminished between 9.4% and 29.2% under the action of *V. calcarata*, and between 3.0% and 50.5% due to the effect of *V. dubyana*. In parallel, MGTs reached + 10% and +16.3%, respectively (Table 2 and 3).

The herbicidal efficacy of the two hydrolates is most likely to be ascribed to methyl salicylate, whose role in plant allelopathy has been previously documented [Bi et

Table 2. Germination and growth parameters of *Lolium multiflorum* and *Sinapis alba* after the treatments (no direct contact) with different doses of *Viola calcarata* hydrolate.

Target species	EO (µL)	G (%)	CVG	MGT	SVI	Root (mm)	Shoot (mm)
L. multiflorum	0	100±0.0	96±4.5	4.9±0.1	10757±215	69.2±11.4	46.5±3.2
	2	93.0±0.0	87±1.5	4.9±0.1	8644±242	55.1±2.6	44.8±4.7
	20	93.0±0.0	86±5.2	5.0±0.1	7566±256	46.5±5.7	40.3±2.7
	50	87.0±9.0	84±0.0	5.2±0.0	7168±319	39.2±3.8	37.9±2.0
	100	87.0±0.0	82±4.8	5.3±0.0	5247±132	25.7±6.0	26.8±3.4
	200	77.0±5.0	68±9.0	5.4±0.1	3245±676	23.2±2.6	19.2±1.9
S. alba	0	96.0±4.0	131±5.5	4.0±0.0	5406±262	31.2±4.9	24.2±3.7
	2	80.0±0.0	106±9.4	4.1±0.0	927±170	5.0±1.7	7.7±2.1
	20	77.0±5.0	100±3.0	4.1±0.1	895±403	4.7±1.1	7.4±2.6
	50	77.0±5.0	96±11.0	4.2±0.1	831±125	4.5±2.3	7.2±2.6
	100	70.0±5.0	92±6.0	4.2±0.0	824±85	4.3±2.4	6.6±2.1
	200	67.0±3.0	81±2.0	4.4±0.1	712±181	3.2±2.1	6.1±2.2

Note: Values are mean ± standard deviation. G (%), Germination percentage; CVG, Coefficient of Velocity of Germination; MGT, Mean Germination Time, SVI, Seedling Vigor Index.

al. 2007; Patni et al. 2019; Asif et al. 2021]. However, its interaction (additional or synergistic) with other components such as thymol [Azirak & Karaman, 2008; Nasrollahi et al. 2018; Mohammedi, 2021], α-pinene [Luiza Ishii-Iwamoto et al. 2012] and 2-propen-1-ol [Ye et al. 2016], capable of inhibiting the germination of weed seeds even at low concentrations, is not to be underestimated and could partially explain the different activity between the two hydrolates. Other compounds such as δ-2-carene, trans-sabinene hydrate, bisabolol oxide II and benzophenone were identified among the major constituents of essential oils with proven phytotoxic properties [Salomé Kpoviessi et al. 2009; Tilaki et al. 2013; Kinga et al. 2014; Sothearith et al. 2021].

Antiradical activity. Before the antiradical tests, *V. calcarata* and *V. dubyana* hydrolates were evaluated for their total polyphenol and flavonoid content. The greatest differences were found between the levels of phenolic compounds, higher in the hydrolate of *V. calcarata* (Table 4). The two samples showed similar scavenging action against the tested radicals. In particular, neither was able to appreciably inhibit the cationic radical ABTS•+ and

Table 3. Germination and growth parameters of Lolium multiflorum and Sinapis alba after the treatments (no direct contact) with different doses of *Viola calcarata* hydrolate.

Target species	EO (µL)	G (%)	CVG	MGT	SVI	Root (mm)	Shoot (mm)
L. multiflorum	0	96.0±4.0	101±11.0	4.9±0.0	10923±226	68.5±13.4	46.1±9.3
	2	96.0±4.0	98±6.0	4.9±0.1	8977±532	51.6±10.6	42.6±4.1
	20	93.0±0.0	95±7.0	5.1±0.1	8222±627	47.1±11.8	41.3±9.7
	50	93.0±0.0	66±5.0	5.2±0.1	6736±328	43.1±7.1	34.8±2.1
	100	87.0±0.0	59±4.5	5.6±0.0	6719±455	40.0±4.8	34.2±3.2
	200	87.0±0.0	50±5.2	5.7±0.2	6267±321	37.7±9.0	32.4±8.3
S. alba	0	98.0±4.0	135±6.2	4.1±0.1	5289±104	30.8±5.6	23.3±4.3
	2	91.0±8.0	124±6.1	4.1±0.1	3191±291	19.3±13.1	18.1±3.9
	20	84.0±10.0	115±1.5	4.2±0.1	3054±655	16.9±11.4	16.7±2.6
	50	82.0±4.0	106±6.0	4.2±0.1	2060±300	11.1±7.1	13.9±4.1
	100	82.0±14.0	103±11.0	4.2±0.1	2010±180	11.5±12.8	12.8±3.0
	200	53.0±6.0	66±8.0	4.2±0.0	1007±95	8.3±3.5	10.8±2.5

Note: Values are mean ± standard deviation. G (%), Germination percentage; CVG, Coefficient of Velocity of Germination; MGT, Mean Germination Time, SVI, Seedling Vigor Index.

Table 4. Total polyphenols and flavonoids and antiradical activity of hydrolates from *Viola calcarata* and *Viola dubyana*.

Hydrolates	Total polyphenols (mg GAE/mL hydrolate)	Total flavonoids (mg QE/mL hydrolate)	ABTS (μM Trolox eq/mL)	DPPH (μM Trolox eq/mL)
V. calcarata	282.5±16.3	12.5±1.1	0.26±0.00	0.28±0.01
V. dubyana	227.1±8.7	12.2±0.4	0.23±0.01	0.27±0.01

the stable radical DPPH• (Table 4). In the first case, the detected activity was about 4 and 6 times lower than that of the corresponding essential oils of *V. calcarata* and *V. dubyana*, while the action against DPPH was comparable [Vitalini et al. 2022].

To conclude, this first study on hydrolates of species belonging to the *Viola* genus confirmed that these by-products are biologically less active than their respective essential oils. Nevertheless, they can still be considered as natural sources of new eco-friendly alternative products, especially in the field of organic agricultural.

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Viola calcarata L. və *Viola dubyana* Burnat ex Gremli hidrolatları: DI-SPME-GC-MS analizi və bioloji aktivliyin qiymətləndirilməsi

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İtaliyanın Alp dağlarında bitən iki yerli *Viola* L. növünün (*V. calcarata* L. və *V. dubyana* Burnat ex Gremli) yerüstü hissələrindən alınan hidrolatlar ilk dəfə tədqiq edilmişdir. Birbaşa immersion bərk fazalı mikroekstraksiya (DI-SMPE) və GC-MS analizi ilə aparılmış, hər iki nümunədə əsas birləşmə kimi metil salisilat, hər bir hidrolat və bəzi digər ikinci dərəcəli birləşmələrdə isə fərqli terpenlər dəsti aşkar edilmişdir. Hər iki növə münasibətdə toxumları birbaşa işləmədən fitotoksiki potensialın qiymətləndirilməsi zamanı maraqlı nəticələr əldə edilmişdir. Ümumiyyətlə, hidrolizatların effekti növlərdən və dozadan asılı olmuşdur. Onlar cücərmə ilə müqayisədə (*S. alba* növündə *V. calcarata* hidrolatına görə maksimum ləngimə = -45,9%) böyümədə (maksimum azalma = -86,8% *S. alba* növündə *V. dubyana* hidrolatına görə) daha aktiv olmuşdur. Antiradikal aktivlik üçün əhəmiyyətli nəticələr əldə edilməmişdir.

Açar sözlər: *ABTS, xromatoqrafik analiz, DPPH, fitotoksiklik, Violaceae, uçucu maddələr*

Гидролаты *Viola calcarata* L. и *Viola dubyana* Burnat ex Gremli: анализ DI-SPME-GC-MS и оценка биологической активности

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Впервые были изучены гидролаты, полученные из надземных частей двух аборигенных видов *Viola* L. (*V. calcarata* L. и *V. dubyana* Burnat ex Gremli), произрастающих в Итальянских Альпах. Отбор проб проведенный твердофазной микроэкстракции прямым погружением (DI-SMPE) и ГХ–МС-анализами выявил присутствие метилсалицилата в качестве основного соединения в обоих образцах наряду с различным набором терпенов для каждого гидролата и некоторыми другими второстепенными соединениями. Наиболее интересные результаты были достигнуты при оценке их фитотоксического потенциала в отношении двух целевых видов (*Sinapis alba* L. и *Lolium multiflorum* Lam.) путем обработки без прямого контакта с семенами. В целом, гидролаты проявляли видозависимые эффекты и дозозависимую активность. Они были более активны в отношении роста (максимальное снижение = -86,8% из-за гидролата *V. dubyana* на *S. alba*), чем в отношении прорастания (максимальное ингибирование = -45,9% из-за гидролата *V. calcarata* на *S. alba*). Никаких существенных результатов в отношении антирадикальной активности получено не было.

Ключевые слова: *ABTS, хроматографический анализ, DPPH, фитотоксичность, Violaceae, летучие вещества*