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# Preliminary Observations on *Viola calcarata* as a Source of Bioactive Compounds: Antioxidant Activity and Phytochemical Profile of Two Alpine Subspecies

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**Abstract:** *Viola* L. is a botanical genus with approximately 525 to 620 species, spread worldwide. Several violets are traditionally used as edible flowers and have been recently proved to be a source of bioactive compounds, including flavonols, flavanols, benzoic acids, and cinnamic acids. However, no information is available about the phytochemical profile of the *Viola calcarata* complex, which is found in the Alpine environment. Thus, the present research aimed to assess the antioxidant activity and the presence of bioactive compounds (anthocyanins and phenolic compounds) in *V. calcarata* subspecies, to promote their biodiversity and use in the agrifood sector. Two *V. calcarata* subspecies were chosen, with different colors: *V. calcarata* subspecies *calcarata* L., with white (CW), yellow (CY), and violet flowers (CV); and *V. calcarata* subspecies *villarsiana* (Roem & Schult.) Merxm., with bicolor (violet and yellow—VB) flowers. CY showed a significantly higher phenolic content (1116.43 mg GAE 100 $g^{-1}$ FW) than the other subspecies, while CV showed higher values in anthocyanins content (44.73 mg C3G 100 $g^{-1}$ FW). Regarding the antioxidant activity, CW (215.07 mmol $Fe^{2+}$ $kg^{-1}$ FW, 99.53 µmol TE $g^{-1}$ FW, and 32.30 µmol TE $g^{-1}$ FW for FRAP, DPPH, and ABTS, respectively) and VB (217.33 mmol $Fe^{2+}$ $kg^{-1}$ FW, 90.97 µmol TE $g^{-1}$ FW, and 29.17 µmol TE $g^{-1}$ FW for FRAP, DPPH, and ABTS, respectively) showed the highest values. Through HPLC, a total of eight phenolic compounds were quantitatively identified among the two subspecies, including flavonols, cinnamic acids, benzoic acids, catechins, and vitamin C. Though different in their composition, the two subspecies are rich in phenolic compounds, highlighting the importance of preserving their biodiversity and their potential use in the agrifood sector.

**Keywords:** *Viola calcarata* subsp. *calcarata*; *Viola calcarata* subsp. *villarsiana*; antioxidant activity; phenolic compounds; anthocyanins; vitamin C; local biodiversity



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## 1. Introduction

Wild flowers can be an effective source of phytochemicals with antioxidant activity important for human health [1–4]. Since the third millennium, several studies assessed their chemical composition [3], increasing the knowledge on their edible flowers properties, thereby globally increasing the demand for this kind of product [2,5,6].

*Viola* L. is a botanical genus with approximately 525 to 620 species, spread throughout the temperate regions and montane zones of the tropics worldwide [7]. Several studies have been conducted on some of these species [1,2,8–10], investigating their potential as a source of bioactive compounds, including flavonols, flavanols, benzoic acids, and cinnamic acids [1,11].

Violet flowers have been used as food product since ancient times [5]. *Viola tricolor* L., having a refreshing and delicate taste, and many color combinations, is one of the most used often in sweets, salads, drinks, etc. [1]. Other species, such as *Viola cornuta* L., *Viola*

*odorata* L., and *Viola* × *wittrockiana* Gams ex Nauenb. & Buttler (pansy), also have been assessed as edible flowers [1,2,5,10–12].

Koike and colleagues [1] analyzed the phenolic profile, antioxidant activity, and shelf life of *V. tricolor*, offering solutions to enhance its shelf life without affecting its phytochemical composition. Demasi and colleagues [2] highlighted that *V. cornuta* is suitable for edible flower production, having a long shelf life and preserving its phytochemical characteristics. Benvenuti and colleagues [10] and Rop and colleagues [12] showed that *V.* × *wittrockiana* has high nutritional value, antioxidant capacity, and an attractive appearance, being also appreciated for its taste [10,12].

A wide variety of *Viola* species are found in the Alpine environment, which are potentially usable. These include the *Viola calcarata* L. complex, for which only one study tried to characterize it morphologically [7], while no information about its phytochemical profile are available. However, popular medicine indicates *V. calcarata* as an emollient, expectorant, sudorific, and with laxative properties [13], as its wild flowers have been used for a long time in folk medicine to cure diseases [6,8,14]. Viola [15] suggested that *V. calcarata* could have the same properties as *V. tricolor*, namely, bechic, expectorant, diuretic, depurative, laxative, and tonic. Similarly, Maugini [16] also indicated that *V. calcarata* could be similar to *V. odorata*, as the flowers contain similar essential oils, salicylic acid, mucilage, and anthocyanins. The author [16] underlined that both *Viola* species have the same bechic, emollient, hypotensive, and expectorant properties, and their roots contain saponosides and alkaloids (odoratine).

*Viola calcarata* has four subspecies: *calcarata*, *villarsiana*, *cavillieri*, and *zoysii*, of which only the first three can be found in the Italian Western Alps [17]. *Viola calcarata* subsp. *calcarata* (L.), the Spurred violet, is a subendemic entity (thus, it can also be present in rare external locations close to the boundaries of the territory considered) of the Western Alps [17,18]. It is a perennial, hemicryptophyte species, 3 to 10 cm tall. The flower, in bloom from May to August, has free, or nearly free, petals, is 2–4 cm in diameter [18], and can be violet, white, or yellow in color [17]. It is found mainly in rocky environments and in furrowed fields, but also in mesophilic, more or less eutrophic meadows and pastures, in orchards or artificial grasslands, in heaths, and in lavender stands [18]. *Viola calcarata* subsp. *calcarata* has an altitudinal distribution of alpine and subalpine type, from 1400 to 2800 m a.s.l. [17], and prefers substrates of the calcareous and dolomitic type and siliceous rocks, but also intermediate substrates, such as siliceous limestone or sandstone. This subspecies requires average soil moisture; it can also be found more poorly in soils that are drier or wetter than the optimum level. As far as pH is concerned, a neutral, mesotrophic substrate is optimal for the development of this plant, although it can also be found in slightly more acidic or more basic, oligotrophic soils [18].

*Viola calcarata* subsp. *villarsiana* (Roem. & Schult.) is an endemic species that can be found in a limited area of the Western Alps, including the provinces of Cuneo, Turin, Vercelli and Biella in Italy, and in the Hautes-Alpes in France. It is a perennial, hemicryptophyte species, 3 to 15 cm tall, whose flowers, bicolor violet with a yellow center, are similar to those of the previous species regarding petals and size. Its phytosociology and ecology are quite the same as in the previous species, but it is not found in mesophilic meadows and pastures [18].

The link between *V. calcarata* and the Alp territory is highlighted by the Italian historical cheese Plaisentif. It is a semi-hard, handmade cheese typically found in the Piedmont valleys in northwest Italy. The main special and traditional characteristic of this cheese is that the milk used to produce it is obtained from cows from pastures in the mountains, only in June–July, i.e., the months when the *V. calcarata* flowers are in bloom. Thus, Plaisentif is known as the "antique violet cheese", and is considered a niche product, as only small manufacturers of the region produce it [19].

Besides the abovementioned notation concerning *V. calcarata*, traditional uses of violets in texts of phytomedicine and ethnobotany are generically referred to mountain violets as a traditional remedy against colds or to fight skin infections in the form of infusions,

decoctions, and syrups. Violet flowers are reported also to be useful for stomach ache, inflammation, pain, and cough, as they cause sweating, thus purifying the blood [20]. It could be important to conserve the diversity of *V. calcarata* subspecies for possible commercial exploitation, not only as ornamental flowers, but also as edible flowers or a source of interesting bioactive compounds.

The aim of this study was to explore the presence of bioactive compounds in *V. calcarata* species, in order to promote the local biodiversity and its use in the agrifood sector. We investigated the phenolic profile and the antioxidant activity (FRAP, DPPH, and ABTS) in two *V. calcarata* subspecies, with different colors: *V. calcarata* subspecies *calcarata*, with white, yellow, and violet flowers; and *V. calcarata* subspecies *villarsiana*, with bicolor (violet and yellow) flowers.

## 2. Materials and Methods

### *2.1. Plant Material*

Fresh *V. calcarata* flowers were collected in 2017 around the Lake of Moncenisio, in the locality Plan des Fontainettes (Val-Cenis, France), at an altitude of 2090–2100 m.a.s.l. (Long. 338655.6; Lat. 5012069). The weather data of the sampling period (May), namely rainfall (83.4 mm) and maximum (14.5 °C) and minimum (7.2 °C) average temperatures, were not substantially dissimilar across the 10-year (2010–2020) average data of May in the same area: 93.4 ± 41.5 mm (mean value ± standard deviation), 13.5 ± 1.6 °C, and 5.9 ± 1.4 °C [21]. Each subspecies was associated with its phytosociological optimum: *Elyno seslerieteae variae* class for *V. calcarata* subsp. *calcarata* and *Juncetea trifidi* class for *V. calcarata* subsp. *villarsiana* [18]. *V. calcarata* subsp. *calcarata* was collected in three different colors: white, yellow, and violet (abbr. CW, CY, and CV, respectively) (Figure 1A–C); *V. calcarata* subsp. *villarsiana* was bicolor (violet and yellow—abbr. VB) (Figure 1D).

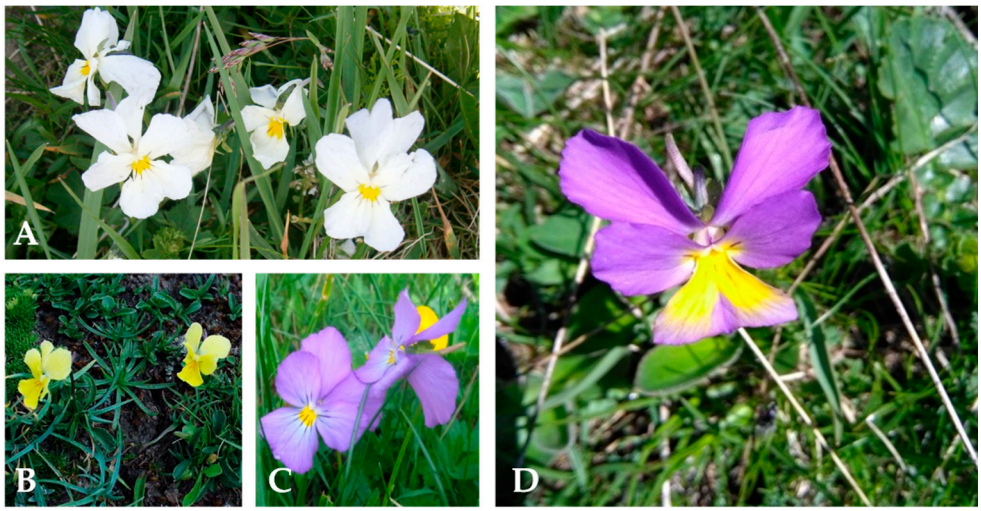


**Figure 1.** Flowers of *V. calcarata* subsp. *calcarata*: (**A**) white, (**B**) yellow, and (**C**) violet (picture by S. Ghirardi). (**D**) Flower of *V. calcarata* subsp. *villarsiana* bicolor (violet and yellow—picture by S. Ghirardi).

Approximately 100 g of flowers were collected per subspecies in summer 2017 at the optimal phenological stage (i.e., in full flowering), placed in sealed polyethylene bags, immediately stored at 4 °C in a portable refrigerator and brought to the laboratory for analysis.

Part of the material was weighed fresh and then oven-dried at 50 °C until a constant weight was reached, to determine the dry matter.

### *2.2. Extract Preparation*

Samples of fresh flowers were grinded with a mortar and pestle into a fine powder using liquid nitrogen, and then stored at −80 °C until ultrasound extraction, performed to obtain the extracts. One gram of flower powder was extracted with 50 mL of a water:methanol solution (1:1), at room temperature with an ultrasound extractor (Sarl Reus, Drap, France) at 23 kHz for 15 min [11]. The obtained solution was filtered with one layer of filter paper (Whatman No. 1, Maidstone, UK), then with a 0.45 mm PVDF syringe filter (CPS Analitica, Milano, Italy). The extracts were stored at −20 °C until analyses, which were performed in three replicates for each subspecies.

### *2.3. Bioactive Compounds*

#### 2.3.1. Total Phenolic Compounds

The total phenolic content of the *V. calcarata* extracts was determined following the Folin–Ciocalteu method [11,22]. The analysis was performed as follows: 1000 µL of diluted 1:10 Folin–Ciocalteu reagent were mixed with 200 µL of flower extract in each plastic tube. The samples were left in the dark at room temperature for 10 min, and then 800 µL of $Na_2CO_3$ (7.5%) were added to each plastic tube. After 30 min in the dark at room temperature, absorbance was read at 765 nm by means of a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA), and the results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (mg GAE 100 $g^{-1}$ FW).

#### 2.3.2. Total Anthocyanins

The total anthocyanin content was estimated by the pH differential method using two buffer systems, as described in the literature [2,23,24]. The analysis was performed as follows: 1 mL of phytoextract was diluted in a 10 mL volumetric flask, and then made up to volume with an aqueous buffer solution at pH 1 (KCl and HCl—25 mM). The same was made in a second volumetric flask with an aqueous buffer solution at pH 4.5 ($C_2H_3NaO_2$ and $C_2H_4O_2$—0.4 M). Samples were left in the dark at room temperature for 20 min. Absorbance of both flasks was measured at 515 nm and 700 nm by means of a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA), and the results were expressed in milligrams of cyanidin3Oglucoside per 100 g of fresh weight (mg C3G 100 $g^{-1}$ FW).

#### 2.3.3. Antioxidant Activity

##### FRAP Assay

The first procedure adopted to evaluate the antioxidant activity in *V. calcarata* extracts was the ferric ion reducing antioxidant power (FRAP) method [11,25,26]. To obtain the FRAP solution, a buffer solution at pH 3.6 ($C_2H_3NaO_2 \cdot 3H_2O$ + $C_2H_4O_2$ in water), 2,4,6-tripyridyltriazine (TPTZ, 10 mM in HCl 40 mM), and $FeCl_3 \cdot 6H_2O$ (20 mM) were mixed. Afterwards, 30 µL of flower extract were mixed with 90 µL of deionized water and 900 µL of FRAP reagent. The samples were then placed at 37 °C for 30 min and absorbance was measured at 595 nm by means of a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). Results were expressed as millimoles of ferrous iron equivalents per kilogram of fresh weight (mmol $Fe^{2+}$ $kg^{-1}$ FW).

##### DPPH Assay

The second procedure was the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [27].

The working solution of DPPH radical cations (DPPH, 100 µM) was obtained by dissolving 2 mg of DPPH in 50 mL of MeOH. The solution must have an absorbance of 1000 (±0.05) at 515 nm. The samples were prepared by mixing 40 µL of flower extract with 3 mL of the DPPH radical solution. The samples were then left in the dark at room temperature for 30 min. Absorbance was measured at 515 nm by means of a spectrophotometer (Cary

60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). The DPPH radical-scavenging activity was calculated as

$$[(\text{Abs0} - \text{Abs1})/\text{Abs0}] \times 100$$

where Abs0 is the absorbance of the control (extraction solution without sample) and Abs1 is the absorbance of the sample. The antioxidant capacity was plotted against a Trolox calibration curve and results were expressed as μmol of Trolox equivalents per gram of fresh weight (μmol TE $g^{-1}$ FW).

ABTS Assay

The third procedure was the 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method [11,26,28].

The working solution of ABTS radical cation (ABTS) was obtained by the reaction of 7.0 mM ABTS stock solution with a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution. After an incubation time of 12–16 h in the dark at room temperature, distilled water was used to dilute the working solution, thus obtaining an absorbance of 0.7 (±0.02) at 734 nm. The antioxidant activity of the *V. calcarata* samples was assessed by mixing 30 μL of flower extract with 2 mL of ABTS radical solution. The samples were then left in the dark at room temperature for 10 min, and absorbance was measured at 734 nm by means of a spectrophotometer (Cary 60 UVVis, Agilent Technologies, Santa Clara, CA, USA). Th ABTS radical-scavenging activity was calculated as

$$[(\text{Abs0} - \text{Abs1})/\text{Abs0}] \times 100$$

where Abs0 is the absorbance of the control (extraction solution without sample) and Abs1 is the absorbance of the sample. The antioxidant capacity was plotted against a Trolox calibration curve and results were expressed as μmol of Trolox equivalents per gram of fresh weight (μmol TE $g^{-1}$ FW).

#### 2.3.4. Phenolic Profile and Vitamin C

The bioactive compounds present in the extracts of *V. calcarata* flowers were determined using High-Performance Liquid Chromatography (HPLC) with Diode Array Detection (DAD) (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA) [29]. The separation of compounds was obtained with a Kinetex C18 column (4.6 × 150 mm, 5 mm, Phenomenex, Torrance, CA, USA) and different mobile phases, according to a previously validated methodology (Table 1) [30,31]. The identification of the compounds was made by comparison with retention times and UV spectra of the analytical standards (purity ≥ 95%; Sigma Aldrich, St. Louis, MO, USA) and the quantification was achieved using calibration curves at the same chromatographic conditions. The following bioactive compounds were determined: phenolic acids (cinnamic acids: caffeic, chlorogenic, coumaric, and ferulic acid; benzoic acids: ellagic and gallic acid); flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin); flavanols (catechin and epicatechin); and vitamin C. The results are expressed as mg 100 $g^{-1}$ of fresh flower.

### *2.4. Statistical Analysis*

All data were subjected to statistical analysis of the normality and homoscedasticity through a Shapiro–Wilk test and Levene test, respectively. Mean comparisons were computed using the one-way ANOVA test by means of SPSS 25 software (version 25.0; SPSS Inc., Chicago, IL, USA). A nonparametric Kruskal–Wallis test with stepwise comparison was performed on data groups where the variances were not homogeneous, as per the Levene test. Correlations among the bioactive compounds of the *V. calcarata* subspecies were calculated by Pearson's correlation coefficient test by means of PAST 4.03 software. A principal coordinate analysis (PCA) biplot was performed for the single phenolic compounds and categories of compounds (total phenolic and total anthocyanin) using the same

software. Eigenvalues were calculated using a covariance matrix among the 240 traits used as input, and the two-dimensional PCA biplot was constructed.

**Table 1.** Mobile phases, elution conditions, and wavelength used to detect the five classes of compounds with HPLC analysis.

| Classes of Compounds | Mobile Phase | Elution Conditions | Wavelength (nm) |
|---|---|---|---|
| Cinnamic acids<br>Flavonols | A: 10 mM $KH_2PO_4/H_3PO_4$, pH = 2.8<br>B: $CH_3CN$ | 5%B to 21%B in 17 min + 21%B in 3 min (2 min conditioning time); flow: 1.5 mL $min^{-1}$ | 330 |
| Benzoic acids<br>Flavanols | A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 *v/v/v*), pH = 2.5<br>B: $CH_3OH/HCOOH$ (100:0.1 *v/v*) | 3%B to 85%B in 22 min + 85%B in 1 min (2 min conditioning time); flow: 0.6 mL $min^{-1}$ | 280 |
| Vitamin C | A: 5 mM $C_{16}H_{33}N(CH_3)_3Br$/50 mM $KH_2PO_4$, pH = 2.5<br>B: $CH_3OH$ | Isocratic, ratio of phase A and B: 95:5 in 10 min (5 min conditioning time); flow: 0.9 mL $min^{-1}$ | 261, 348 |

## 3. Results and Discussion

The dry matter, total phenolic content, total anthocyanin content, and antioxidant activity (FRAP, DPPH, ABTS) of the four examined *V. calcarata* samples are reported in Table 2.

**Table 2.** Dry matter, total phenolic compounds, total anthocyanins, and antioxidant activity in *V. calcarata* subsp. *calcarata* white (CW), *V. calcarata* subsp. *calcarata* yellow (CY), *V. calcarata* subsp. *calcarata* violet (CV), and *V. calcarata* subsp. *villarsiana* (VB—*Viola* bicolor). Data are based on fresh weight (FW) and are presented as the mean value ± standard deviation.

| *Viola calcarata* Subspecies | Dry Matter % | Total Phenolic Compounds (mg GAE/100 g FW) | | Total Anthocyanins (mg C3G/100 g FW) | | Antioxidant Activity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | FRAP (mmol $Fe^{2+}$/kg) | DPPH (μmol TE/g FW) | | ABTS (μmol TE/g FW) | |
| CW | 17.60 ± 0.95 | 887.63 ± 94.93 | b | nd | | 215.07 ± 25.00 | 99.53 ± 7.4 | a | 32.30 ± 1.76 | a |
| CY | 17.47 ± 1.02 | 1116.43 ± 29.63 | a | 7.47 ± 4.89 | b | 223.57 ± 45.72 | 75.20 ± 7.62 | b | 22.43 ± 1.86 | b |
| CV | 18.13 ± 0.25 | 719.30 ± 30.45 | b | 44.73 ± 23.02 | a | 168.07 ± 6.73 | 64.27 ± 3.87 | b | 24.33 ± 3.17 | ab |
| VB | 17.33 ± 0.47 | 845.63 ± 106.08 | b | 10.67 ± 1.85 | b | 217.33 ± 26.43 | 90.97 ± 1.22 | a | 29.17 ± 5.94 | ab |
| *p* | ns | ** | | * | | Ns | *** | | * | |

Data are expressed on a fresh-weight basis. The statistical relevance is provided (*** = $p < 0.001$; ** $p < 0.005$; * $p < 0.05$; ns = not significant). Different letters inside a column indicate significant differences between the subspecies according to Tukey's post-hoc test ($p < 0.05$); nd = not detected.

No previous studies have reported on the bioactive compounds of *V. calcarata*; thus, comparisons were only possible with congeneric species [1,2,5,10–12].

No significant differences were found in dry matter percentage, ranging between 17.33% and 18.13% in VB and CV, respectively. The total phenolic content varied from 719.30 to 1116.43 mg GAE 100 $g^{-1}$, being significantly higher in CY. This result is higher than the values found by studies on other species: da Silva and colleagues (2020) found in yellow *V.* × *wittrockiana* flowers a total phenolic content of 725.50 mg GAE 100 $g^{-1}$ FW, similar to data found by Demasi et al. (2020) in *V. cornuta* fresh flowers (767.26 mg GAE 100 $g^{-1}$ FW). The CV and VB values (719.30 mg GAE 100 $g^{-1}$ FW and 845.63 mg GAE 100 $g^{-1}$ FW, respectively) are in agreement with data regarding the blue *V.* × *wittrockiana* flowers (716.50 mg GAE 100 $g^{-1}$ FW) [32] and the *V. cornuta* flowers [2]. Conversely, CW showed a higher phenolic content than *V.* × *wittrockiana* white flowers (73.00 mg GAE 100 $g^{-1}$ FW) [32]. All the four *V. calcarata* subspecies showed a higher phenolic content than *V. odorata* (428.40 mg GAE 100 $g^{-1}$ FW) [11].

Anthocyanins were significantly higher in CV, were similarly present in CY and VB, but not detected in CW. The values ranged from 7.47 to 44.73 mg C3G 100 $g^{-1}$ FW, being

in agreement with those found by Demasi et al. (2020) in *V. cornuta* flowers (27.76 mg C3G 100 $g^{-1}$ FW). Benvenuti and colleagues (2016) found significant differences between anthocyanins values for blue, yellow, and white *V.* × *wittrockiana* flowers (13.6, 2.93, and 0.35 mg C3G 100 $g^{-1}$ FW, respectively), confirming the higher value of blue flowers.

Despite showing slight differences in antioxidant activity, depending on the assay used (Table 2), the results showed that CW had the highest antioxidant activity (215.07 mmol $Fe^{2+}$ $kg^{-1}$ FW, 99.53 µmol TE $g^{-1}$ FW, and 32.30 µmol TE $g^{-1}$ FW for FRAP, DPPH, and ABTS, respectively), together with VB (217.33 mmol $Fe^{2+}$ $kg^{-1}$ FW, 90.97 µmol TE $g^{-1}$ FW, and 29.17 µmol TE $g^{-1}$ FW, for FRAP, DPPH, and ABTS, respectively), whereas CY and CV had a lower antioxidant activity..The antioxidant activity is mainly exerted by flavonoids and phenolic acids [33]. It is noteworthy that although CY has the highest phenolic content, its antioxidant activity is not the major one. A positive correlation between antioxidant activity and total phenol content was also not found in other crops such as apricot [34] and strawberry [35]. This might be due to the interaction of the Folin–Ciocalteu reagent with other compounds besides phenols, e.g., some inorganic ions, thiols, or proteins [36], suggesting the importance of deepening the analysis with the identification of single compounds by means of chromatographic methods. No significant differences were found in the FRAP assay. These values are higher than those obtained from fresh *V. odorata* flowers [11], collected as wild species in their natural habitat too; however, regarding the FRAP assay, Demasi and colleagues [2] found a higher result in *V. cornuta*. González-Barrìo et al. [37] found similar levels of antioxidant activity in *V.* × *wittrockiana*, with FRAP results ranging from 96.87 to 206.37 mmol $Fe^{2+}$ 100 $g^{-1}$ DW, compared to *V. calcarata* results expressed in dry weight (CW: 122.2 mmol $Fe^{2+}$ 100 $g^{-1}$ DW; CY: 127.97 mmol $Fe^{2+}$ 100 $g^{-1}$ DW; CV: 92.7 mmol $Fe^{2+}$ 100 $g^{-1}$ DW; and VB: 125.41 mmol $Fe^{2+}$ 100 $g^{-1}$ DW).

HPLC analysis was performed to determine the compounds mainly contributing to the phenolic profile of the four *V. calcarata* subspecies (Table 3). Six phenolic acids (four cinnamic and two benzoic acids), five flavonols, and two flavanols (catechins) together with vitamin C were evaluated. The results showed that each subspecies had a specific phenolic composition. Among the 14 compounds investigated, five were found in CW (quercitrin, ferulic acid, ellagic acid, epicatechin, and vitamin C) and six were found in the other three subspecies (CY, CV, and VB), although each one had a different combination of them.

**Table 3.** Bioactive compounds in *Viola calcarata* subsp. *calcarata* white (CW), *V. calcarata* subsp. *calcarata* yellow (CY), *V. calcarata* subsp. *calcarata* violet (CV), and *V. calcarata* subsp. *villarsiana* (VB). The values are given in mg 100 $g^{-1}$ of fresh weight (FW). Data are presented as the mean value ± standard deviation.

| ***Viola calcarata* Subspecies** | **Flavonols** | | | | | |
|---|---|---|---|---|---|---|
| | **Hyperoside** | | **Isoquercitrin** | **Quercitrin** | | **Rutin** |
| CW | nd | | nd | 41.73 ± 14.2 | b | nd |
| CY | 93.70 ± 30.25 | a | nd | 15.50 ± 3.22 | c | nd |
| CV | 13.70 ± 2.92 | b | 5.40 ± 0.89 | nd | | 329.63 ± 44.10 |
| VB | 12.10 ± 4.48 | b | nd | 136.85 ± 40.75 | a | 16.73 ± 0.64 |
| *p* | ** | | - | * | | *** |
| ***Viola calcarata* subspecies** | **Cinnamic acids** | | **Benzoic acids** | **Catechins** | | **Vitamin C** |
| | **Ferulic acid** | | **Ellagic acid** | **Epicatechin** | | |
| CW | 144.50 ± 13.86 | c | 27.03 ± 0.75 | 22.33 ± 3.07 | b | 4.20 ± 0.60 |
| CY | 288.63 ± 21.95 | a | 25.80 ± 3.60 | 21.00 ± 1.31 | b | 3.90 ± 1.31 |
| CV | 227.27 ± 33.07 | b | 28.27 ± 1.37 | nd | | 3.37 ± 0.59 |
| VB | nd | | 26.33 ± 3.57 | 176.83 ± 53.08 | a | 3.80 ± 0.46 |
| *p* | ** | | ns | *** | | ns |

The statistical relevance is provided (*** = $p < 0.001$; ** $p < 0.005$; * $p < 0.05$; ns = not significant). Different letters inside a column indicate significant differences between the subspecies according to Tukey's post-hoc test ($p < 0.05$); nd: not detected.

Despite six compounds not being detected in any of the four subspecies, all classes of phenolic compounds were found to be present (flavonols, cinnamic acids, benzoic acids, catechins), and vitamin C. Four out of five flavonols were detected, thus being the most abundant class in *V. calcarata* subspecies, followed by benzoic acids and catechins (one out of two compounds detected), and lastly cinnamic acids (only one out of four compounds detected).

Regarding the ellagic acid and vitamin C content, no significant differences were found among the four *Viola* subspecies.

Conversely, hyperoside was found in CY, CV, and VB, ranging from 12.10 to 93.70 mg 100 $g^{-1}$ FW, being less abundant in CV and VB, and showing a significantly higher value in CY. Similarly, quercitrin was found in CW, CY, and VB, ranging from 15.50 to 136.85 mg 100 $g^{-1}$ FW, being less abundant in CY and showing a significantly higher content in VB.

Rutin was found in CV and VB, ranging from 16.73 to 329.63 mg 100 $g^{-1}$ FW, being more abundant in CV; ferulic acid was found in CW, CY, and CV, ranging from 144.50 to 288.63 mg 100 $g^{-1}$ FW, being more abundant in CY.

Epicatechin was found in three samples (CW, CY, and VB), showing a significantly higher value in VB (176.83 mg 100 $g^{-1}$ FW), and similar lower values in CW and CY (22.33 and 21.00 mg 100 $g^{-1}$ FW, respectively). Lastly, isoquercitrin was only detected in CV (5.40 mg 100 $g^{-1}$ FW).

Some authors, such as Fernandes et al. [5], González-Barrio et al. [37], and Pires et al. [6], did not analyze these compounds in the *Viola* flowers they studied; however *V. wittrockiana* showed compounds such as quercetin, delphinidin, petunidin, kaempferol, luteolin, peonidin, malvidin, violanthin, and pelargonidin [5,37], *V. tricolor* showed violanthin, quercetin, isorhamnetin, apigenin, and kaempferol [5,6], while in *V. odorata*, violanin, quercetin, kaempferol, and other compounds were detected [38].

The correlation analysis highlighted that the total phenolic content was positively correlated ($p < 0.05$) with the FRAP assay (Figure 2), and the three methods for the evaluation of the antioxidant activity positively correlated with each other, thus confirming the positive link between phenolic compounds and antioxidant activity [3,39], as already highlighted in several edible flowers by Demasi et al. [11], who obtained the same correlation. The anthocyanins content was negatively correlated ($p < 0.05$) with the DPPH assay; however, it had a positive correlation ($p < 0.05$) with isoquercitrin and rutin. These two latter compounds were both negatively correlated with antioxidant activity, as were ferulic acid and hyperoside (Figure 2). The latter were positively correlated with each other. Isoquercitrin and rutin were positively correlated ($p < 0.05$) with each other, as were quercitrin and epicatechin.

The relationship between single compounds and categories of compounds were evaluated through a two-dimensional PCA scatterplot (based on the first two principal components (PCs)) (Figure 3). The first two PCs explained 92.5% of total variation. The first PC accounted for 56.4%, while the second PC accounted for 36.1%. The scatterplot showed that the four samples of *V. calcarata* are clearly distinguished.

For the positive values of both PCs, CY samples are found to be positively correlated mainly with phenolic compounds and hyperoside. For the positive PC1 values and the negative PC2 values, CW was found to be mainly correlated with quercitrin and epicatechin, as well as VB. For the negative PC1 values and the positive PC2 values, CV was mainly correlated with rutin.

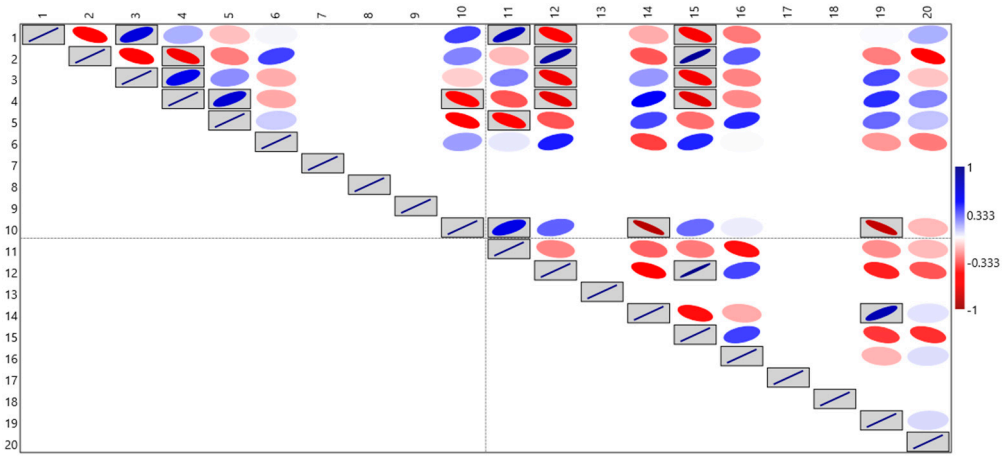


**Figure 2.** Pearson correlation among the bioactive compounds of *V. calcarata* subsp. *calcarata* white (CW), *V. calcarata* subsp. *calcarata* yellow (CY), *V. calcarata* subsp. *calcarata* violet (CV), and *V. calcarata* subsp. *villarsiana* (VB), calculated and drawn using PAST 4.03 software. Boxed ellipses are significantly correlated ($p < 0.05$). Blue indicates a positive correlation and red indicates a negative correlation. The more the ellipse is compressed, the stronger the correlation. 1. phenolic compounds; 2. anthocyanins; 3. FRAP; 4. DPPH; 5. ABTS; 6. dry matter; 7. caffeic acid; 8. chlorogenic acid; 9. coumaric acid; 10. ferulic acid; 11. hyperoside; 12. isoquercitrin; 13. quercetin; 14. quercitrin; 15. rutin; 16. ellagic acid; 17. gallic acid; 18. catechin; 19. epicatechin; 20. vitamin C.

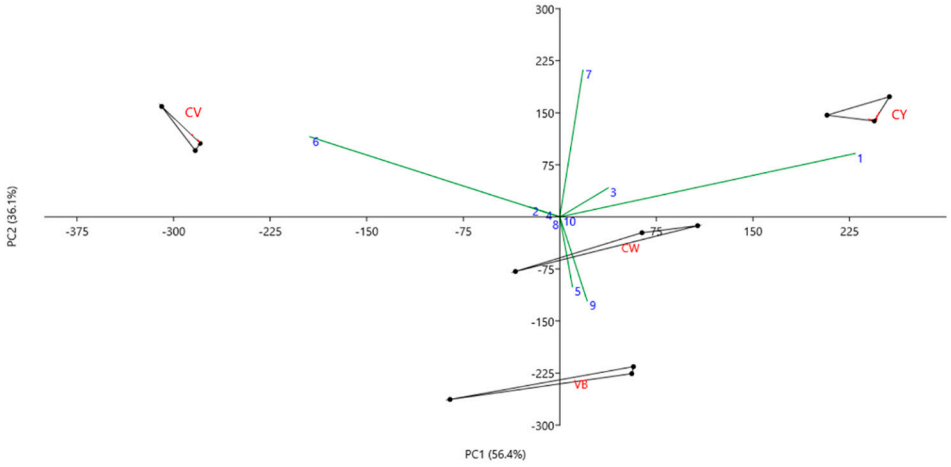


**Figure 3.** Principal component analysis (PCA)-biplot of *V. calcarata* subsp. *calcarata* white (CW), *V. calcarata* subsp. *calcarata* yellow (CY), *V. calcarata* subsp. *calcarata* violet (CV), and *V. calcarata* subsp. *villarsiana* (VB), calculated and drawn using PAST 4.03 software. 1. phenolic compounds; 2. anthocyanins; 3. hyperoside; 4. isoquercitrin; 5. quercitrin; 6. rutin; 7. ferulic acid; 8. ellagic acid; 9. epicatechin; 10. vitamin C.

## 4. Conclusions

Promoting the biodiversity of the Alp territory could help the development of new supply chains, based on local genetic resources, for the sustainable development of mountain territories. In this study, the flowers of *V. calcarata* subspecies *calcarata* and *villarsiana*, with different colors, were interesting not only for their ornamental value but also for their antioxidant activities and phytochemical profiles.

The two subspecies showed the same amount of vitamin C, but different phenolic compositions, mainly consisting of flavonols. This is the reason why it should be important to preserve the biodiversity of these two subspecies since they can be a valuable source of different bioactive compounds.

Their exploitation as edible flowers could be positive for the alpine economy and in particular for plant growers. However, currently there is no information on the proper cultivation techniques to grow *V. calcarata* subsp. *calcarata* and *villarsiana*, this then being an opportunity for future study. Future studies could also focus on the evaluation of seed viability, germinability in a controlled environment and in the field, adaptation to transplanting, crop viability, and establishing crop cycles and cultivation parameters.

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