

## THE HISTO-ANATOMICAL INVESTIGATION AND THE POLYPHENOLIC PROFILE OF ANTIOXIDANT COMPLEX ACTIVE INGREDIENTS FROM THREE *VIOLA* SPECIES

TÜNDE JURCA<sup>1</sup>, ANNAMARIA PALLAG<sup>1\*</sup>, ELEONORA MARIAN<sup>1</sup>, MARIANA EUGENIA MUREȘAN<sup>1</sup>, ROXANA LIANA STAN<sup>2</sup>, LAURA GRAȚIELA VICAȘ<sup>1</sup>

<sup>1</sup>University of Oradea, Medicine and Pharmacy Faculty, Department of Pharmacy, 29 Nicolae Jiga Str., 410028, Oradea, Romania

<sup>2</sup>Department of Pharmaceutical Biochemistry and Clinical Laboratory, Faculty of Pharmacy, University of Medicine and Pharmacy „Iuliu-Hațieganu” Cluj-Napoca, 400012, Romania

\*corresponding author: [annamariapallag@gmail.com](mailto:annamariapallag@gmail.com)

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### Abstract

In the present study three edible species of the *Violaceae* family (*Viola odorata* L., *Viola tricolor* L. and *Viola wittrockiana* Gams.) were screened in order to assess their morphological and anatomical characteristics, their polyphenolic contents and their antioxidant activities. The dried herbal drugs were lyophilized and underwent pre-treatment for high pressure liquid chromatography. Thus 6 flavonoids and 6 phenolic acids were quantitatively determined. Antioxidant activity was evaluated using several methods: FRAP (ferric reducing antioxidant power), DPPH (2,2-diphenyl-1-picrylhydrazyl) and CUPRAC (cupric ion reducing antioxidant capacity) assay. The polyphenolic profile revealed the presence of common components between species such as rutin, myricetin, p-coumaric acid, ferulic acid, sinapic acid, vanillic acid and other compounds such as catechin, luteolin, epicatechin, naringenin, syringic acid and caffeic acid, specific only in some of the studied species.

### Rezumat

În acest studiu am caracterizat trei specii aparținând familiei *Violaceae* (*Viola odorata* L., *Viola tricolor* L. și *Viola wittrockiana* Gams.) din punct de vedere morfologic, anatomic, al conținutului de polifenoli și a capacității antioxidante. Florile uscate au fost supuse liofilizării, după care s-au pregătit extractele pentru analiza HPLC. Astfel, s-au determinat cantitativ 6 flavonoide și 6 acizi fenolici. Activitatea antioxidantă s-a studiat prin tehnicile FRAP, DPPH și CUPRAC. Dintre polifenoli s-au identificat câteva componente comune pentru toate trei speciile, respectiv rutinul, miricetina, acidul p-coumaric, acidul ferulic, acidul sinapic, acid vanilic, dar și compuși fenolici ce diferă de la o specie la alta, precum catechina, luteolina, epicatechina, naringenina, acidul siringic și acidul cafeic.

**Keywords:** *Viola odorata* L., *Viola tricolor* L., *Viola wittrockiana* Gams., phenolic acids, flavonoids, DPPH, CUPRAC, FRAP

### Introduction

Phenolic compounds, ubiquitous in plants, are of considerable interest and have received increased attention in recent years due to their bioactive functions [14]. Polyphenols are among the most desirable phytochemicals due to their antioxidant activity. These components are known as secondary plant metabolites and possess antimicrobial, antiviral and anti-inflammatory properties along with high antioxidant capacity [15, 29, 35].

Plants are well known for their capacity to produce a diverse array of secondary metabolites in order to interact with their environment [36, 40, 43]. The major classes of secondary metabolites are alkaloids, terpenes and polyphenols. The amount and type of polyphenols produced by plants vary considerably between species [5, 6, 13, 16, 18, 32].

Polyphenols are present throughout the plant kingdom as they offer plants protection against UV radiation. These compounds play an important role in plant

growth and reproduction induces antibacterial activity and modulates oxidative stress, inflammation and apoptosis in endothelial cell exposed to hyperosmotic stress, as well as contributing to the colour, sensory characteristics and nutritional properties of fruits and vegetables [17, 18]. Some polyphenols are proposed as therapeutic agents for a variety of diseases or to promote general health [16, 26].

The *Violaceae* family, consisting of 806 species, includes mostly flowering plants. It takes its name from its most known genus, *Viola*, which includes 450 species. The Romanian flora comprises around 30 species from the genus *Viola* [3].

Species from genus *Viola* present rich sources of phytochemical compounds, being involved in plant growth, metabolism and reproduction, as well as providing protection against pathogens and UV radiation [19, 37].

Studies have demonstrated that dietary polyphenols can act as free radical scavengers, by inducing, inhibiting

or modulating the transduction signal of antioxidant enzymes or by chelating metal ions [8-10, 12].

The purpose of this study was the morphological, histoanatomical, physico-chemical characterization, quantification of the polyphenolic content and antioxidant capacity of three *Viola* species flowers extracts: *Violae odoratae flos*, *Violae tricoloris flos* and *Violae wittrockianae flos*.

## Materials and Methods

### Plant materials

We conducted a comparative study on the species *Viola odorata* L., *Viola tricolor* L. and *Viola wittrockiana* Gams. *Viola tricolor* L. and *Viola odorata* L. are found as part of the north western Romanian spontaneous flora, but *Viola wittrockiana* Gams. is cultivated as an ornamental plant.

*Violae odoratae flos* and *Violae tricoloris flos* were collected from the spontaneous flora of Bihor County, Romania, from unpolluted areas in 2017. *Violae wittrockianae flos* was collected from a cultivated area of Bihor County, in 2017. Microscopic sections were made from the parts of the collected plants.

A specimen from each of the studied species was deposited in the Pharmaceutical Botany Herbarium of the Faculty of Medicine and Pharmacy Oradea, Romania.

Plant materials were dried at room temperature and grounded before extraction.

### Extract preparation

10 g dried flowers were extracted with 70% aqueous ethanol (100 mL), using a magnetic mixer for 45 min and further sonicated for 5 min. The alcohol was evaporated using a rotary evaporator and the dried extract was lyophilized.

#### Preparation of the plant extract (1)

50 mg dry (lyophilized) extract was weighed and dissolved in 2 mL 90% methanol and 0.5% acetic acid mixture. The sample was centrifuged on a rotary evaporator to dryness. The residue was dissolved in 1 mL DMSO and transferred into a test tube.

#### Preparation of the plant extract (2)

About 50 mg dry (lyophilized) extract was weighed and dissolved in 4 mL 25% methanol. After addition of 1 mL of 6 M HCl, the sample was sonicated for 2 min and mixed in a water bath at 85 - 90°C for 2 h, 2.5 mL ethylacetate was then added to each sample and this extract was vacuum evaporated to dryness on a rotary evaporator. The residue was dissolved in 1 mL DMSO and transferred into a test tube.

### Analysis of phenolic compounds by HPLC

Pure phenol standards including 6 phenolic acids (caffeic, syringic, sinapic, vanillic, p-coumaric, ferulic) and 6 flavonoids (rutin, naringenin, luteolin, quercetin, epicatechin, myricetin) were dissolved in the mobile phase (1 mg/mL). The solutions were freshly prepared, filtered through a 0.45 µm membrane filter and

immediately injected to HPLC column. Evaluation of each sample was repeated three times [20].

We used the Shimadzu SCL-POA reversed-phase high-performance liquid chromatography system in order to assess the phenolic compound content. The system consisted of a LC-10ADVP pump equipped with SPD-10AVP Diode Array (UV) detector. The column type was Kintex 5u RP C18 µm, 4.6 mm internal diameter × 250 mm. The mobile phase was composed of (A) 0.05% formic acid and (B) 0.05% formic acid-acetonitrile, 50:50 v/v. We performed the gradient elution process as follows: 0 min, 95:5; 10 min, 90:10; 40 min, 60:40; 55 min, 45:55; 60 min, 20:80; and 65 min, 0:100. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate was 1.5 mL/min. UV absorbance was measured at 260 - 380 nm. The operating temperature was maintained at room temperature. Identification of the phenolic compounds was achieved by comparison with the standards retention times, UV spectra and by calculating the UV absorbance ratios after simultaneous injection of samples and standards. Analyses were performed in triplicate [15, 20, 33, 44, 45].

### The bioactive compounds of plant extracts

#### Determination of polyphenols content

The Folin-Ciocalteu method was used in order to determine the total phenolic content. Using the Folin-Ciocalteu method the OH groups can be evaluated in an alkaline medium (adjusted with sodium carbonate). The absorbance at 765 nm, increases proportionally with the number of OH groups of the polyphenols. The extract solution (0.1 mL) containing 1000 µg of the extract was mixed with 46 mL distilled water in a volumetric flask and 1 mL Folin-Ciocalteu (Merck) reagent was added. After 3 min, 3 mL aqueous solution of 2% Na<sub>2</sub>CO<sub>3</sub> were added. After incubation for 2 h at room temperature, the absorbance of each mixture was measured at 765 nm in a Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer. Results were expressed as mg GAE/g DW (dried weight) [18, 23, 25, 33, 39].

#### The total flavonoid content

The total flavonoid content was determined using a colorimetric method described by Kim *et al.* [23]. 1 mL sample (containing 0.1 mg/mL dry substance) was mixed with 4 mL water and inserted in a 10 mL volumetric flask. Firstly, 3 mL 5% NaNO<sub>2</sub> solution were added, after 5 minutes - 0.3 mL 10% AlCl<sub>3</sub> and after 6 minutes - 2 mL 1 M NaOH. The solution was mixed and its absorbance was detected at 510 nm. Results were expressed as mg QE/g DW [23, 32, 34, 39, 44].

### The antioxidant capacity of flowers extracts

#### Reagents

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-

triazine (FRAP) were purchased from Sigma Aldrich, USA. Gallic acid and sodium carbonate were purchased from Fluka, Switzerland. All the chemicals used were of analytical grade.

#### DPPH method

We determined the free radical scavenging activity of the plant extracts against stable 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), using the slightly modified method of Brand-Williams *et al.* [2, 4, 30]. The change in colour (from deep violet to light yellow) was measured at 517 nm using a UV visible light spectrophotometer. The  $6 \times 10^{-5}$  methanolic DPPH solution was freshly prepared before each UV measurement. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100,$$

where, AB = absorption of the blank sample (t = 0 min), AA = absorption of the test extract solution (t = 15 min) [2, 18, 31].

#### FRAP method (ferric reducing antioxidant power) [1, 2]

The stock solutions included: 300 mM acetate buffer; 270 mg  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$  dissolved in 50 mL distilled water; 150 mg TPTZ and 150  $\mu\text{L}$  40 mM HCl, dissolved in 50 mL distilled water. We prepared the FRAP solution by mixing 50 mL acetate buffer, 5 mL  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$  solution and 5 mL TPTZ solution. We used Trolox as a standard solution. The results are expressed as  $\mu\text{mol}$  Trolox equivalents (TE)/100  $\mu\text{L}$  extract.

#### The CUPRAC assay

We used the method proposed by Karaman *et al.* with slight modifications [36] in order to determine the cupric ion ( $\text{Cu}^{2+}$ ) reducing antioxidant capacity. We added 0.25 mL  $\text{CuCl}_2$  solution (0.01 M), 0.25 mL

ethanolic neocuproine solution ( $7.5 \times 10^{-3}$  M) and 0.25 mL  $\text{CH}_3\text{COONH}_4$  buffer solution (1 M), and mixing the plants extracts. Then, we adjusted the total volume to 2 mL with distilled water, and mixed thoroughly. We sealed the tubes and kept them at room temperature. Absorbance was measured at 450 nm against a reagent blank 30 min later. Increased absorbance of the reaction mixture indicated increased reduction capacity [39, 41]. The calibration curve was achieved for different concentrations of Trolox.

## Results and Discussion

### Morphological and histoanatomical analysis

Microscopic sections were made from the collected stems, as shown in Figures 1-5.

Since *Viola wittrockiana* Gams. have been crossbred from *Viola tricolor* L., they are believed to possess similar antioxidant activity. Due to its carotenoids, anthocyanins and flavonols content, garden pansies may represent a promising source of natural antioxidants [8, 26, 42]. Attractive flowers and antioxidant capacity enable the garden pansies to become suitable candidates as functional food products [8, 15, 42].

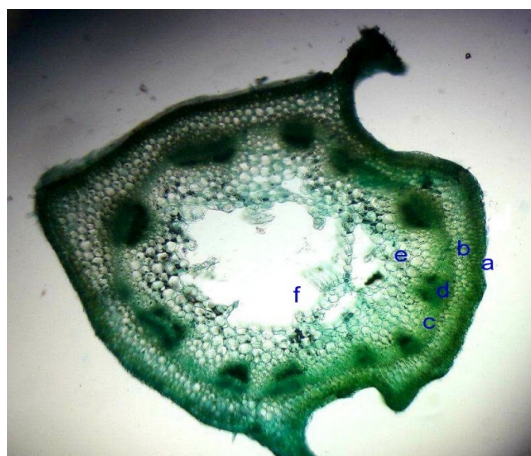
Regarding *Viola tricolor* L., the main stem has 5 edges, as shown in Figure 2. Throughout the edges, the collenchyma tissue is not well represented in comparison with *Viola wittrockiana* Gams (Figure 4 and 5), in which the main stem has 4 edges.

The central cylinder begins with a pericycle. In the fundamental parenchyma of the marrow there are numerous vascular bundles, all arranged in a circle. The central cylinder *Viola tricolor* L., there are more than 10 vascular bundles present. The central cylinder of *Viola wittrockiana* Gams. has 4 vascular bundles, placed symmetrically.



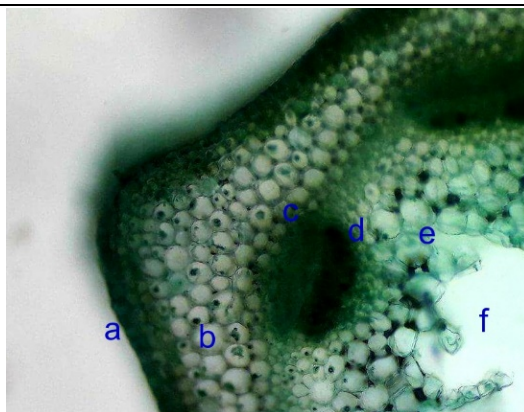
**Figure 1.**

Cross section through the *Viola odorata* L. main stem (100X). a - epidermis; b - cortical parenchyma; c - central cylinder parenchyma; d - vascular bundles; e - central marrow



**Figure 2.**

Cross section through the *Viola tricolor* L. main stem (100X). a - epidermis; b - cortical parenchyma; c - pericycle; d - vascular bundles; e - central cylinder parenchyma; f - main gap marrow

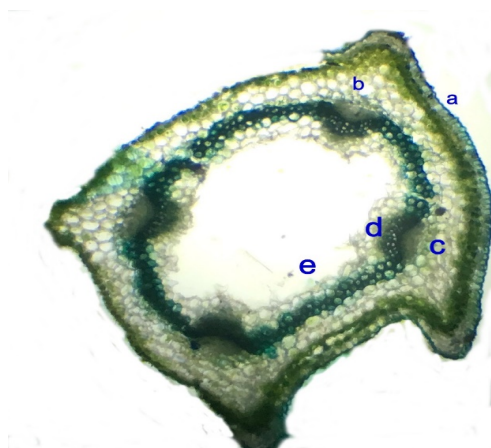


**Figure 3.**

Cross section through the *Viola tricolor* L. main stem (200X). a - epidermis; b - storage parenchyma; c - phloem tissue; d - xylem tissue; e - central cylinder parenchyma; f - main gap marrow

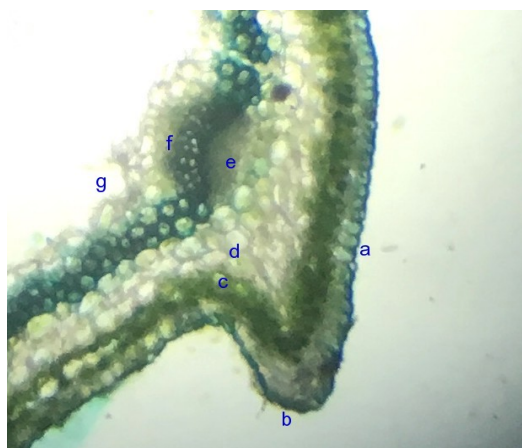
The main marrow gap is larger in *Viola wittrockiana* Gams., than in *Viola tricolor* L., in which the central marrow tissue is present around the main marrow

gap. In *Viola odorata* L., the central marrow tissue occupies the centre of the marrow (Figure 1).



**Figure 4.**

Cross section through the *Viola odorata* L. main stem (100X). a - epidermis; b - cortical parenchyma; c - central cylinder parenchyma; d - vascular bundles; e - central marrow



**Figure 5.**

Cross section through the *Viola wittrockiana* Gams. main stem (200X). a-epidermis; b - collenchyma; c - assimilated cortical parenchyma; d- storage parenchyma; e - phloem tissue; f - xilem tissue; g - main gap marrow

#### *Content of flavonoids and phenolic acids*

HPLC-RP with UV detection was used to identify and quantify the phenolic and flavonoid compounds from the *Viola* species lyophilized extract. Chromatograms of the *Viola* species were determined at 270, 310 and 360 nm.

Each sample was analysed after two different types of extractions were performed, in order to obtain exact data regarding flavonoids and phenolic acids, and to estimate the nature of the respective compounds. Total phenols generally correlate with redox and antioxidant capacities, as measured by the TEAC or DPPH methods.

The polyphenolic compounds identified in the analysed samples are presented in Table I.

Four glycosides (epicatechin, catechin, rutin and myricetin) were identified after the first extraction, and from the phenol derivatives, four compounds (sinapic acid, p-coumaric acid, ferulic acid, vanillic acid). Ferulic acid was the most abundant phenolic acid for *Viola wittrockiana* Gams. (129.89 mg/Kg) and the predominant flavonoid was myricetin (131.89 mg/Kg). Regarding flavonoids, the predominant compound in *Viola odorata* L. and *Viola tricolor* L. was myricetin and rutin (84.67 mg/Kg, 89.67 mg/Kg).

Table I

Content of flavonoids and phenolic acids determined in *Viola* species flowers

Bioactive compounds	$\lambda_{\max}$	$R_T$ (min)	<i>Viola odorata</i> L.		<i>Viola tricolor</i> L.		<i>Viola wittrockiana</i> Gams.	
	nm		Extract 1 (mg/kg)	Extract 2 (mg/kg)	Extract 1 (mg/kg)	Extract 2 (mg/kg)	Extract 1 (mg/kg)	Extract 2 (mg/kg)
Epicatechin	280	10.82	22.21 ± 0.32	11.34 ± 0.84	-	-	93.67 ± 3.35	87.56 ± 1.37
Luteolin	345	18.27	-	20.56 ± 0.52	-	80.98 ± 3.34	-	-
Catechin	280	9.46	23.12 ± 0.92	11.21 ± 0.34	-	-	-	-
Naringenin	285	19.99	-	-	-	149.12 ± 4.37	-	-
Rutin	260 - 270 360	13.2	87.34 ± 1.12	-	124.56 ± 4.54	-	67.89 ± 2.35	-
Myricetin	255; 370	15.78	84.67 ± 2.92	34.56 ± 1.67	89.67 ± 1.64	47.80 ± 1.26	131.89 ± 4.46	56.78 ± 3.44
Sinapic acid	235; 320	13.47	19.07 ± 0.72	-	99.12 ± 2.34	-	82.34 ± 3.45	-
p-coumaric acid	310	12.47	71.56 ± 1.97	64.34 ± 1.46	89.23 ± 1.24	76.89 ± 5.37	102.34 ± 6.37	29.07 ± 0.39
Caffeic acid	320	10.55	-	40.67 ± 2.35	-	72.67 ± 4.67	-	-
Ferulic acid	320	13.51	121.54 ± 5.36	87.65 ± 3.42	77.76 ± 2.35	45.78 ± 2.74	129.89 ± 5.31	100.78 ± 4.58
Vanillic acid	259; 292	10.4	14.67 ± 0.52	12.78 ± 0.39	-	-	-	-
Syringic acid	274	11.1	-	30.78 ± 1.93	-	-	-	-

\*Results are expressed as mean ± standard deviation

The presence of luteolin, syringic acid and caffeic acid only in the second extract is suggesting the release of these compounds from their corresponding glycosylated forms. The highest quantity of polyphenolic compounds can be found in *Viola wittrockiana* Gams., *Viola odorata* L. presents the largest variety of phenolic acids and flavonoids.

*The polyphenols, flavonoids content and the anti-oxidant capacity*

The total phenolic and flavonoid content and the antioxidant capacity of the samples, measured by different complementary assays are shown in Table II.

Table II

The total polyphenols, flavonoids and the antioxidant capacity of samples

No.	Methods	<i>Viola odorata</i> L.	<i>Viola tricolor</i> L.	<i>Viola wittrockiana</i> Gams.
1.	Total Polyphenols (mg GAE/100g DW)	434.14 ± 3.01	445.03 ± 0.12	485.48 ± 0.53
2.	Flavonoids (mg QE/mL)	1.40 ± 0.32	2.69 ± 0.17	3.96 ± 0.28
3.	DPPH %	14.49 ± 2.81	74.34 ± 2.64	44.58 ± 3.15
4.	FRAP (mg Trolox/mL)	17.14 ± 2.01	14.71 ± 1.41	214.09 ± 0.91
5.	CUPRAC (mmol Trolox/100 g)	1.72 ± 1.81	2.68 ± 1.74	5.63 ± 0.94

\*Results are expressed as mean ± standard deviation

The polyphenolic content of the three studied species was almost identical, being slightly increased for the *Viola wittrockiana* Gams. flowers (485.48 ± 0.53 mg GAE/100 g DW). The flavonoid content was three times higher for the *Viola wittrockiana* Gams. flowers (3.96 ± 0.28 mg QE/mL) compared to the *Viola odorata* L. flowers (1.40 ± 0.32 mg QE/mL).

The antioxidant capacity of *Viola* flowers was tested by measuring their free radical scavenging activity. The highest flavonoid content was recorded for *Viola wittrockiana* Gams. flowers extract, with similar polyphenolic content floral the samples.

The highest DPPH inhibition was observed for the *Viola tricolor* flowers extract. Therefore, increased activity for these tests also suggests that anthocyanins and phenols present in *Viola* species flowers play an important role as antioxidants [11, 24].

The highest antioxidant activity was measured for *Viola wittrockiana* Gams. through the CUPRAC and Trolox assays.

The comparative study complements the specialty literature so that the antioxidant activity of the three

*Viola* species offers the possibility of their use in therapy after a correct identification [7, 27, 28, 38].

## Conclusions

On a macroscopic level, especially at the beginning of the flowers period, the studied species may be easily confused, but microscopic analysis proved differences in the main stems. Such observations can contribute to the correct systematic classification of the species.

The physico-chemical characterization of the studied species indicated that *Viola* species has relatively high antioxidant activity in flowers.

The substances with antioxidant capacity were accumulated in large quantities in the studied flowers, compared with other plants [21, 22].

Our results have shown that the *Viola wittrockiana* Gams. flowers can be used as a source of natural antioxidants in pharmaceutical compounds, food processing and human and food medicine.

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