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Research on biological parameters of some species of the genus *Viola* L.: bioresources with complex potential for valorisation

PhD Thesis Summary

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Summary

Introduction

Species of the genus *Viola* – *violet, pansy* – are spread across all continents, from temperate to tropical zones. Of the total of 550-600 species of this genus, 28 identified species grow spontaneously in Romania (Sârbu *et al.*, 2013; Toma and Ivănescu, 2013; Marcussen *et al.*, 2022).

The interest in using species of the genus *Viola* L. in the pharmaceutical industry stems from traditional medicine practices.

Internationally, studies on the biochemistry of the genus *Viola* have highlighted their pharmaceutical potential (Hammami *et al.*, 2011, 2012; Hellinger *et al.*, 2014; Saint-Lary *et al.*, 2014).

From an ecological point of view, these plants play an important role in maintaining biodiversity. (Knudsen *et al.*, 2006). Species of the subgenus *Melanium* are known to be metallophytes with a role in soil decontamination and ecosystem restoration. (Karatoteva *et al.*, 2014).

The main objective of this study is to supplement current scientific information with new data on the biology of some taxa belonging to the genus *Viola* existing in our spontaneous flora, collected from the North-Eastern Region of the country.

Three well-known and extensively studied species were examined (*V. odorata* L., *V. tricolor* L. and *V. arvensis* Murr.) in order to complete some data regarding their biology. These species are to be used as a benchmark in comparative research with other lesser-known species belonging to the Romanian plant heritage. *V. declinata* W. et K. and *V. dacica* Borb. have a special status, considered to be endemic to the Wooded Carpathians, Eastern and Southern Carpathian Mountains (Hurdu et al., 2012; Alexiu, 2013).

Taxonomic complexity and large-scale intraspecific hybridization cause confusion regarding the systematic classification of species (Marcussen *et al.*, 2022), which is why this paper also aims to address some aspects regarding taxonomic variability through comparative research of some biological parameters. Nine species of the *genus* Viola L. were studied: *V. arvensis* Murr., *V. canina* L., *V. dacica* Borb., *V. declinata* W. et K., *V. kitaibeliana* Schult., *V. odorata* L., V. *suavis* Bieb. and *V. tricolor* L.

In this context, the present work aims to conduct a comparative study of some biological and biochemical parameters of the respective taxa, in order to complete the state of knowledge regarding elements of the variability of the genus *Viola* L.

Preliminary research on the variability of the genus *Viola* L. led to the crystallization of the following main objectives that are the subject of the thesis:

- comparative research of some **morpho-anatomical parameters**, with emphasis on species that present phenotypic plasticity, in order to complete data from current scientific literature on some elements regarding their variability;
- research into **phytochemical parameters** with potential for pharmaceutical use, in order to complete data from current scientific literature;
- research on the **potential for valorisation** of species as bioresources.

1. History of research on Viola L. genus

The interest in using plants of this genus in the pharmaceutical industry stems from traditional medicine practices. Currently, several species are recognized as exhibiting antimicrobial and antifungal, antiplasmodial and anthelmintic activity (Hammami *et al.*, 2011, 2012). Also, different species have antihypertensive, antidyslipidemic, anticancer, analgesic, antipyretic, anti-inflammatory, diuretic, anti-HIV and antiasthmatic activity. (Muhammad *et al.*, 2012).

In Romania, research on *Viola* species has highlighted the presence of volatile oils in *V. tricolor* and *V. arvensis* (Toiu *et al.*, 2006), salicylic acid content in *V. tricolor*, *V. arvensis* and *V. declinata* (Toiu *et al.*, 2008), phenolic compounds content in *V. declinata*, (Toiu *et al.*, 2009), flavonoids content in *V. arvensis* and *V. declinata* (Toiu *et al.*, 2017), as well as analyses of methanolic extracts for saponins, mucilage and carotenoid pigments (Toiu *et al.*, 2009).

The latest studies and technologies use bioactive compounds specific to the *Viola* genus in modern treatments against neurodegenerative diseases and cancer (Pränting *et al.*, 2010; Chandra *et al.*, 2015; Moliner *et al.*, 2019; Dayani *et al.*, 2022). Cyclotides and flavonoid compounds isolated from *Viola sp.* extracts are used as antibacterial active principles (Pränting *et al.*, 2010; Zarrabi *et al.*, 2013). Flavonoids, such as violantine, are integrated into fabrics through modern encapsulation technologies, for patients who need clothing with ultraviolet protection or treatments for skin conditions (e.g., to treat eczema) (Zimniewska, 2019).

2. The taxonomic variability of the studied species

Genus *Viola* L. (*Violaceae* Batsch.) is divided into 16 subgenera (Sârbu *et al.*, 2013; Marcussen *et al.*, 2015). Plants of this genus grow in almost all ecological zones, except Antarctica, but mainly in the temperate zones of both hemispheres. The taxonomic epicentre is in the Mediterranean area, East Asia and North America. (Marcussen *et al.*, 2022).

The species studied fall into:

- subgenus *Viola* s.lat.;
    - section *Viola* - *V. odorata* L., *V. suavis* Bieb. and *V. alba* Bess.
    - section *Rostratae* - *V. canina* L.
- subgenus *Melanium* Ging., section *Bracteolatae* – *V. declinata* W. et. K., *V. dacica* Borbás, *V. arvensis* Murray, *V. kitaibeliana* Shult. and *V. tricolor* L.

2.1. Subgenus *Viola* s.lat.

■***Viola odorata*** L.

Figure. 1. V. odorata L. *(Bârnova, Iași, Romania, 2020, original foto)*

The plant is annual (flowers in March-April), but can also be biannual, spreading throughout the country. It is characterized by a dense articulated rhizome, long lateral stolons, leaves arranged in a rosette, ovate-lanceolate stipules (Ciocîrlan, 1988; Ballard et al., 1999; Jonsell *et al.*, 2009; Chifu *et al.*, 2001, 2006). Due to the diverse habitat conditions, the species has a great morphological variability. The color of the petals is generally purple, but sometimes it can be lilac, white, pale pink, sulphur, blue, reddish, variegated (Ciocîrlan, 1988; Jonsell *et al.*, 2009).

■ ***Viola suavis*** **Bieb.**

Figure. 2. V. suavis Bieb. *(Bârnova, Iași, Romania, 2019, original foto)*

It is a perennial plant (blooms in March-April) spread from the plains (steppe zone) to the mountain area (beech zone), and prefers nutrient-rich soils (Ciocârlan, 1988; Marcussen and Nordal, 1998; Jonsell *et al.*, 2009).

In general, the species is characterized by short, densely jointed rhizomes, 4-5 mm thick, aboveground stolons 15 cm long, approximately 2 mm thick, sometimes underground stolons, long fimbriated stipules and bracteoles inserted on the lower half of the flowering peduncle (Mereďa Jr *et al.*, 2008; Jonsell *et al.*, 2009; Sârbu *et al.*, 2013). Due to the different types of habitats, the taxon has high phenotypic plasticity, just like *V. odorata* L. and *V. alba* Bess.

■ ***Viola alba* Bess.**

It is a perennial plant (blooms in March-April), widespread up to 700 m altitude. It is common in the steppe zone and up to the beech forest area (Beldie *et al.*, 1955; Ciocârlan, 1988; Jonsell *et al.*, 2009).

It is characterized by flowers with white petals, almost ovate and acute leaves, linear-lanceolate and long-fimbriated stipules, light green in colour, and fairly long stolons (30 cm) that do not root at the nodes. The rhizome is densely articulated (Jonsell *et al.*, 2009; Sârbu *et al.*, 2013).

Figure. 3. V. alba Bess. *(Bârnova, Iași, Romania, 2020, original foto)*

■ ***Viola canina* L.**

It is part of the subsection *Rostratae* Kupffer (W. Becker).

The plants are perennial (flowering in May-June). It is common in the area of oak forests up to the spruce layer (1200 – 1700 m altitude). It prefers sunny habitats with moderate shade (Chifu *et al.*, 2001, 2006; Jonsell *et al.*, 2009).

The species is characterized by its herbaceous stem, free and long stipules, reaching up to the middle of the petiole. The style has a cylindrical shape and the stigma is rostrate.

Figure. 4. V. canina L. *(Bârnova, Iași, Romania, 2020, original foto)*

Due to the high variety of habitats inhabited, plants may have stipules and leaves of different sizes and general shapes, or flowers of various colours (Beldie *et al.*, 1955; Jonsell *et al.*, 2009; Espeut, 2020).

2.2 Subgenus *Melanium* Ging.

■***Viola declinata*** **W. et. K.**

This plant is perennial and blooms in July-August. The species is characterized by the elliptic-lanceolate middle leaves and the linear-lanceolate upper ones. The stipules are finely sectioned up to the midrib (Sârbu *et al.*, 2013).

Figure. 5. V. declinata W. et. K. *(Pietrosul Călimanilor Peak, Romania, 2020, original foto)*

It is common in the spruce to juniper layer, in meadows (Tomescu and Harasemciuc, 2018). It is characteristic for pastures with *Nardus stricta*. It grows in juniper areas, forest clearings, sunny places, on rocks and scree. It is also found on secondary meadows established after the clearing of spruce trees (Chifu *et al.*, 2006; Togor and Burescu, 2013).

This species is endemic to the Wooded Carpathians, Eastern, and Southern Carpathians (Ciocârlan, 1988; Coldea *et al.*, 2009; Hurdu *et al.*, 2012).

In Romania, as a result of <u>personal observations</u> carried out in the field between 2015-2022, we can report the presence of this species in alpine and subalpine meadows from different subdivisions of the Carpathian Mountains, such as: Călimani Mountains, trails to 12 Apostoli and Pietrosul Călimanilor Peak; Rodnei Mountains at Gârgălău Saddle, Știol plateau, Iezerul Pietrosului and in the vicinity of Craiu Peak; Bucegi Mountains at Țigănești Saddle and Scara Peak plateau; Piatra Craiului Mountains, trail to Măgura Mare Peak.

■ ***Viola dacica* Borbás**

Perennial plant that blooms in July-August. It is common in the spruce and juniper areas. It is endemic to Europe (Coldea *et al.*, 2009; Hurdu *et al.*, 2012).

The species is characterized by its middle leaves being broad-ovate and the upper ones being oblong-ovate. The stipules are less divided, 1/2-1/3 of their width, the undivided part being wider than the length of the segments. (Sârbu *et al.*, 2013).

Figure. 6. V. dacica Borbás *(Sângeorz Hillock, Rodnei Mountains, Romania, 2020, original foto)*

Personal observations from 2015-2022, in Romania, highlight the presence of this species in meadows and pastures (alpine and subalpine) in: Călimani Mountains, from Neagra Șarului to 12 Apostoli, and Pietrosul Călimanilor Peak, Rodnei Mountains on the peaks of Lucurel and Craia, Sângeorz Hillock, Obârşia Rebrii Saddle; Giumalău Mountains, Ceahlău Mountains, Făgăraș Mountains in the areas of Fereastra Mare, Urlea Peak, Dara Peak and Hârtopul Darei Peak.

■ ***Viola arvensis* Murray**

It is cosmopolitan, annual, blooms in the months of May-August, the plant having a distribution throughout Romania. The species is frequent in the steppe area up to the beech zone, widespread in the hill area, to the limit of cereal crops and up to the subalpine zone (Beldie *et al.*, 1955; Ciocârlan, 1988; Sârbu *et al.*, 2013).

Characteristic for this species is the length of the corolla equal to or shorter than the calyx, with flowers of 1-1.5 cm long. The middle leaves are elongated and ovate-elliptic (Sârbu *et al.*, 2013).

Figure. 7. V. arvensis Murray *(Cioatele, Vaslui, Romania, 2018, original foto)*

The species is morphologically similar to *V.*

tricolor L. and *V. kitaibeliana* Shult. which is why identification errors may occur (Jonsell *et al.*, 2009; Espeut, 2020).

■ ***Viola kitaibeliana* Shult.**

It is annual and blooms in the months of April-July. Generally, we find it on rocky or sandy coasts, meadows and bushes, it prefers arid places. It is sporadic in the steppe area and up to the Quercus zone (up to 600-700 m altitude) (Beldie et al., 1955; Magrini and Scoppola, 2015).

Figure. 8. V. kitaibeliana Shult. *(Târgu Neamț, Neamț, Romania, 2019, original foto)*

Characteristic of the species are the flower length of 0.4-0.8 cm and broad-ovate middle leaves (Sârbu *et al.*, 2013).

Phenotypic plasticity causes species identification problems because it has morphological characteristics similar to *V. arvensis* Murray and *V. tricolor* L. (Espeut, 2020).

This species is considered endangered in Italy, which is why it has a special status (Magrini and Zucconi, 2020).

■ ***Viola tricolor* L.**

It is a cosmopolitan species, the plant is spread throughout the entire territory of Romania, and almost throughout the entire European territory. It blooms in the months of May-August. It is annual, sometimes biannual. This species is widespread in the hilly area (200-300 m minimum altitude), up to the subalpine level (Chifu *et al.*, 2006; Tomescu and Harasemciuc, 2018; Espeut, 2020).

Figure. 9. V. tricolor L. *(Fălticeni, Suceava, Romania, 2019, original foto)*

The species is characterized by the length of the corolla which is longer than the calyx and the pinnate-lobed stipules with the terminal segment larger than the lateral ones (Sârbu *et al.*, 2013).

Due to habitats with different ecological requirements, some morphological characteristics show phenotypic varieties influenced by environmental conditions, mainly by the soil qualities (Jonsell *et al.*, 2009; Słomka *et al.*, 2008, 2011; Bezlova *et al.*, 2012).

3. Material, methods and working techniques

1. The plant material investigated

The investigated species were collected from different spontaneous flora habitats located in four counties from Romania: Iaşi, Neamţ Suceava and Vaslui.

The plant material belonging to the selected species was harvested during the flowering phenophase, from three different populations, over a period of two consecutive years.

The plant material was determined taxonomically according to Sârbu *et al.*, 2013 and verified by Biologist Ph.D. Irimia Irina, de la Herbarium of the Faculty of Biology, University „Alexandru Ioan Cuza" of Iași, were vouchers of the taxa taken into study are also deposited.

2. Techniques for morphological analyses

The morphometry was performed on herborized specimens. Measurements were performed according to the methods described in the specialized literature (Marcussen and Nordal, 1988; Marcussen *et al.*, 2001; Marcussen, 2003; Hodálová *et al.*, 2008).

3. Methods and techniques for plant histoanatomy

The plant material, preserved in 70% ethyl alcohol, was sectioned with a hand microtome and a botanical razor. The samples thus obtained were studied and photographed with an optical microscope (Niță *et al.*, 1997; Toma, 2003; Cutler and Botha 2007).

4. Determination of water and dry matter content

The classic method of removing water by repeatedly drying the plant material was used (Boldor *et al.*, 1982; Hodgson *et al.*, 2011).

5. Quantitative analyses of assimilatory pigments

The assimilatory pigments were extracted using the Mayer–Bertenrath method (Boldor *et al.*, 1982).

6. Quantitative analyses of anthocyanin pigments

For the quantitative determination of anthocyanin pigments, the pH differential method was used (Fuleki and Francis, 1968).

7. Plant extracts

The dried plant material containing the aerial part of the plants, without flowers, was mechanically ground. The solvents used to obtain extracts were distilled water and 50% ethanol. The plant extracts were made for four concentrations: 0.5%, 1%, 2.5% and 5% w/v. (dry material weight/volume).

8. Quantitative analyses of phenols

For the quantitative evaluation of the phenol content, we used the reagent Folin-Ciocâlteu method (Herald *et al.*, 2012).

9. Quantitative analyses of flavonoids

To evaluate the total amount of flavonoids, we used the classic analysis method taken from Jia *et al.*, 1999, and Herald *et al.*, 2012.

10. Determination of antioxidant capacity

Estimation of antioxidant activity was performed using the stable free radical 2,2-diphenyl-1-picrylhydrazyl, based on a colorimetric reaction (Blois, 1985; Molyneux, 2003; Siatka and Kašparová, 2010).

11. Statistical data processing

Statistical data processing was carried out using Microsoft Excel 2015, XLSTAT and OriginLab Pro.

12. PCR analyses

PCR analysis was performed within the Molecular Biology and Metagenomics Laboratory, University „Ştefan cel Mare" of Suceava.

a) Genomic DNA extraction

Genomic DNA was extracted from dried plant material using the Wizard Genomic DNA Purification Kit (Promega).

b) Genomic region selection and primer design

Specific primers for each species were selected based on sequences available in genetic databases and specialized literature (Ballard *et al.*, 1998; Hildebrandt *et al.*, 2006; Malécot *et al.*, 2007; Mered'a *et al.*, 2011; Pornon *et al.*, 2016; Tamura *et al.*, 2021).

c) PCR Protocol

It was used GoTaq Green Master Mix (Promega) on the equipment Applied Biosystems SimpliAmp Thermal Cycler.

4. Morpho-anatomical research on some species of subgenus Viola

4.1. Subsection *Viola* (= *Uncinatae* Kupffer, *Scapigerae* W.Becker, *Curvato-pedunculatae* W.Becker)

4.1.1. Morphometric analyses of taxa of *V. odorata* L., *V. suavis* Bieb., *V. alba* Bess.

Table 4.1. Morphometric indices analyzed for V. odorata L., *V. suavis* Bieb., *V. alba* Bess. *(mm, average values ± standard deviation)*

Morphometry	*V.odorata*	*V. suavis*	*V.alba*
length (L) petiole	26.54±0.97	36.6±0.32	27.33±0.42
length (L) stipule	10±0	10.73±0.16	11±0.67
width (l) stipule	2.53±0.17	3.13±0.37	1±0
ratio L/l stipule	4.11±0.2	3.62±0.3	11.00±0.5
leaf area (A)	956.43±26.74	684.67±61.93	488.33±22.93
length (L) leaf	59.29±2.53	60.63±2.68	49.04±4.24
width (l) leaf	34.59±0.97	26.99±1.05	24.46±1.69
leaf perimeter (P)	210.44±3.57	166.37±9.13	147.85±2.26
ratio L/l leaf	1.70±0.1	2.38±0.2	2.13±0.3
length (L) peduncle	61.07±0.82	32.47±0.39	53.4±1.91
length (L) bracts	3.87±0.2	4.73±0.5	3.47±0.1
width (l) bracts	0.65±0.1	1.00±0	0.50±0.0
ratio L/l bracts	6.53±0.6	4.73±0.5	6.93±0.3
length (L) flower	12.6±0.51	10.84±0.1	15±0
length (L) sepal	5.13±0.13	6±0	4.2±0.08
width (l) sepal	2.52±0.08	2±0	1±0
ratio L/l sepal	2.08±0.1	3.00±0.0	4.20±0.1

4.1.2. Anatomy analyses of taxa of *V. odorata* L., *V. suavis* Bieb., *V. alba* Bess.

From an anatomical point of view, the differences between these three species were as follows:

• *root*: thickness of the xylem, presence or absence of xylem parenchyma cells, presence or absence of periderm; only in *V. odorata* the structure remains primary, with visible absorbent hairs at the rhizoderm level;

• *rhizome*: the degree of thickening of the xylem vessels, respectively, the presence and frequency of cellulosic parenchyma xylem cells;

• *scapus type stem*: presence (*V. alba*), respectively absence in the bark of air cavities; number of conductive fascicles: 4 at *V. odorata*, 2 at *V. suavis*, 2 at *V. alba*;

• *leaf blade*: bicellular tector bristles are present at the level of the leaf blade veins, with the terminal cell very long at *V. odorata*, stomata protrude above the external level of the epidermis at *V. odorata* and *V. suavis*, at *V. alba* the cuticle is thick.

4.2. Subsection *Rostratae* Kupffer (W. Becker)

4.2.1. Morphometric analyses of taxa of *V. canina* L

A number of 10 morphometric indices associated with the leaf (petiole, stipule and leaf blade) and aerial stems were analysed. A number of 8 morphometric indices were considered for the flower.

4.2.2. Anatomy analysis of taxa of *Viola canina* L.

■ *root*: the structure, typically secondary, is the result of the activity of both lateral meristems: the cambium and the phellogen;

■ *rhizome*: the structure is of secondary origin, but with a thin periderm and a very thick central cylinder. The pith, extremely thin, parenchymatic-cellulosic, is penetrated by short rows of primary xylem;

■ *aerial stem:* the outline is semicircular, with two slightly prominent adaxial ridges and a flat adaxial face. The central cylinder is thick, comprising 7-8 conductive fascicles of different sizes;

■ ***leaf:***

<u>The petiole</u> - the outline is approximately elliptical modified by two strongly divergent latero-adaxial ridges.

<u>Lamina</u> – amphistomatic, the midrib protrudes visibly on both sides and the lateral ones of order I, only on the lower side of the leaf blade.

5. Morpho-anatomical research on some species of subgenus *Melanium*

5.1. Morphometric analyses of taxa of *V. declinata* W. et. K., *V. dacica* Borbás, *V. arvensis* Murray, *V. kitaibeliana* Shult. and *V. tricolor* L.

Table 5.2. Morphometric indices analyzed for V. declinata W. et. K., *V. dacica* Borbás, *V. arvensis* Murray, *V. kitaibeliana* Shult. *and V. tricolor* L. *(mm, average values ± standard deviation)*

Morphometry	*V. dacica*	*V. declinata*	*V. arvensis*	*V. kitaibeliana*	*V. tricolor*
length (L) petiole	3.60± 0.2	2.40± 0.2	5.20± 0.4	5.07± 0.6	8.33± 0.9
length (L) stipule	5.27± 0.1	4.80± 0.1	8.27± 0.4	9.07± 0.3	14.93± 1.0
width (l) of terminal lobe of the stipule	1.08± 0.0	1.00± 0.0	1.47± 0.1	1.53± 0.1	2.70± 0.3
leaf area (A)	149.45± 12.7	90.20± 4.2	178.93± 12.4	149.53± 12.2	180.07± 14.2
length (L) leaf	29.11± 1.1	22.62± 1.7	30.13± 1.6	31.83± 1.0	28.77± 1.5
width (l) leaf	10.24± 0.6	7.16± 0.5	10.31± 0.5	9.89± 0.6	11.46± 0.3
leaf perimeter (P)	58.14± 4.3	47.71± 2.0	69.08± 3.2	57.95± 1.5	66.06± 3.1
Ratio L/l lamina	3± 0.2	3.72± 0.6	3.07± 0.3	3.31± 0.1	2.50± 0.1

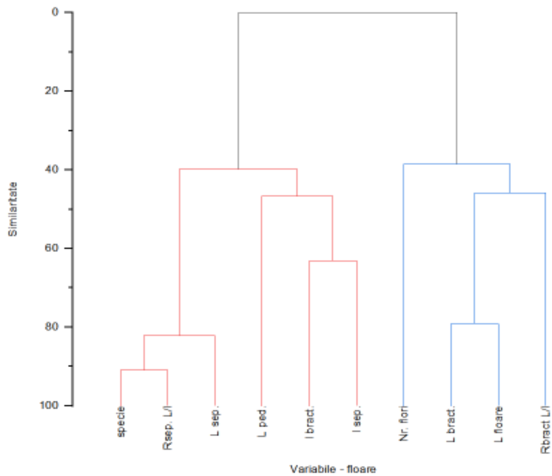

Figure 5. 1. Multivariate cluster analysis for flower morphometric indices for V. declinata W. et. K., *V. dacica* Borbás, *V. arvensis* Murray, *V. kitaibeliana* Shult. *and V. tricolor* L.

5.2. Morphometric analyses of taxa of the *V. declinata* W. et. K., *V. dacica* Borbás, *V. arvensis* Murray, *V. kitaibeliana* Shult. and *V. tricolor* L.

From an anatomical point of view, the main distinguishing features between these are the following:

- *root*: elliptical outline at *V. tricolor,* circular to other species;
- *rhizome:* is present only at *V. dacica* and *V. declinata*;
- *stem*: number of conductive fascicles - 10 for *V. dacica*, 7 for *V. declinata*, 10 for *V. tricolor*, 8 for *V. arvensis*, 8 or 9 for *V. kitaibeliana*;
- *petiole*: *V. tricolor* has 7 conductive fascicles centrally located and 3 in each adaxial wing; *V. arvensis* and *V. kitaibeliana* have 3 conductive fascicles: one central and one in each wing; *V. dacica* has 7 centrally located and *V. declinata*, 3 conductive fascicles centrally located.

6. Quantitative analyses of assimilatory pigments

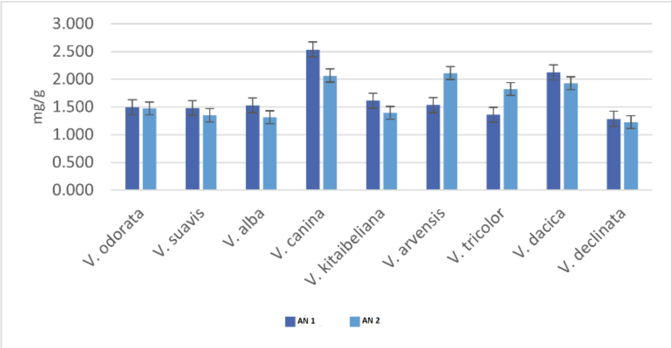

Figure 6.2. Total quantitative content of assimilatory pigments (chlorophyll a + chlorophyll b + carotenoid pigments) for the two consecutive years (mg/g), in the analysed species.

7. Total quantitative analyses of phenols

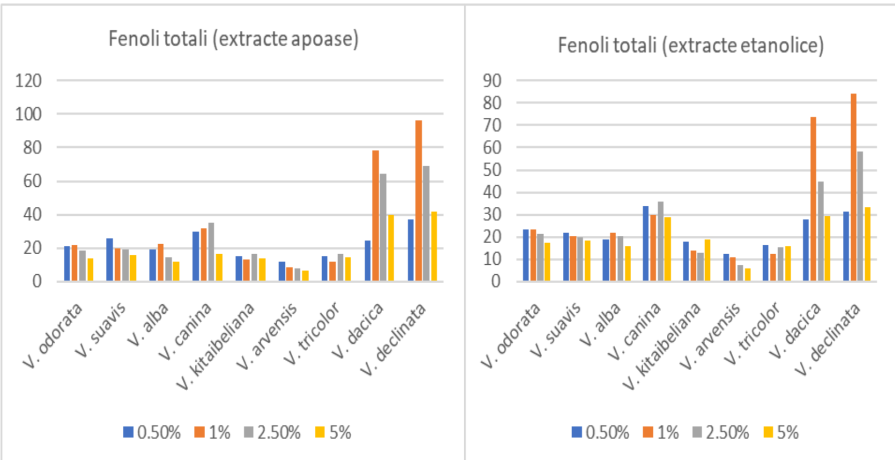

Figure 7.9. The total amount of polyphenols determined for aqueous and ethanolic extracts for concentrations of 0,5%, 1%, 2,5%, respectively 5%, for nine species of the genus Viola L. *(mg/g GAE/dry wt.)*

8. Total quantitative analyses of flavonoid compounds

Two types of extracts were analysed, aqueous and ethanolic (50%), with four concentrations being made for each: 0,5% (w/v), 1% (w/v), 2,5% (w/v) and 5% (w/v). The highest values were recorded for *V. canina* and *V. kitaibeliana*.

Comparing the two types of extracts, it is observed that the ethanolic extracts have a higher extraction efficiency of total flavonoid compounds. The highest values were recorded for the alcoholic extracts made for *V. canina*: 101.12±2.40 mg/g QE/dry wt. (0,5%), 87.17±0.73 mg/g QE/dry wt. (1%), 97.61±0. 31 mg/g QE/dry wt. (2,5%) and 80.78±0.62 mg/g QE/dry wt. (5%).

The lowest values, for all concentrations in both types of extracts, were observed mainly in the *V. arvensis*.

9. Total quantitative analyses of anthocyanin pigments

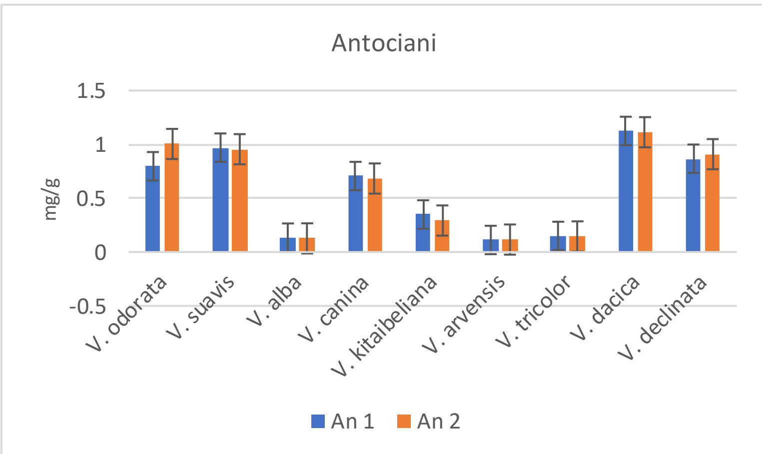

Figure 9.3. Comparative graph of the average values obtained for anthocyanin compounds following analyses carried out for nine species of the genus Viola L.*, in two consecutive years (mg/g dry wt.)*

10. Determination of antioxidant capacity

Testing the antioxidant capacity of plant extracts revealed significant activity.

Low values of the inhibition rate were recorded in species harvested from agricultural areas, *V. arvensis* (~17%-24% for aqueous extracts and ~18%-25% for the ethanolic extracts), and *V. tricolor* (~19-22% for aqueous extracts and ~19%-29% for the ethanolic extracts).

The highest values of the inhibition rate were ~30%-47% for aqueous extracts and ~46%-54% for the ethanolic extracts (*V. dacica*) and ~35%-63% for aqueous extracts and ~44%-55% for the ethanolic extracts (*V. declinata*).

11. Taxa of the genus *Viola* L., bioresources with complex potential for valorisation

11.1. Bioresources of valuable compounds in the pharmaceutical and cosmetic industry

The total content of polyphenols recorded by analysing the experimentally prepared aqueous extracts presented the highest values in species collected from mountain areas. (*V. declinata*, *V. dacica*), with values between 78-84 mg/g GAE/dry wt. (1% w/v). The lowest values recorded for aqueous extracts were 8-13 mg/g GAE/dry wt. (1%), for *V. arvensis* and *V. kitaibeliana*. Comparatively, the quantitative analysis of total polyphenols for alcoholic extracts revealed that the highest values were 94-95 mg/g GAE/dry wt. (1%) (*V. declinata*, *V. dacica*) and the lowest values were10-19 mg/g GAE/dry wt. (1%) (*V. arvensis*, *V. kitaibeliana*).

The class of polyphenolic compounds is recognized as having bactericidal, antifungal, anti-inflammatory and, very importantly, antioxidant properties.

Based on the results obtained, an overall analysis can be undertaken on the potential for valorisation of certain species of *Viola*.

11.2. Bioresources with ecological impact

This genus contains taxa that develops in very different biocenoses, from cosmopolitan species with diverse growth area, to endemic species with strict requirements or adapted to difficult environmental conditions. The adaptability of some taxa, such as *V. odorata* and *V. suavis*, allows them to develop in semi-natural habitats or habitats modified by human activity, gardens or parks (Ciocîrlan, 1988; Chifu *et al.*, 2001, 2006). Resistance to polluted soils and especially the accumulation of these pollutants in the plant body, gives species of the subgenus *Melanium* a major role in ecosystem restoration activities. (Hermann *et al.*, 2013).

12. Discussions

12.1. Species discrimination based on morphometric indices

The morphological analyses of the investigated species revealed common characters between the species, but also discriminatory elements between them. Our observations are, for the most part, in accordance with the specialized literature. The differences recorded are due to the fact that the species under study present a high phenotypic variability.

12.2. Species discrimination based on anatomical structure

The general anatomical structure is consistent with the description made for the genus *Viola*, in specialized literature. The observed anatomical differences may be useful in supplementing the specialized literature for identifying taxa by microscopic means.

12.3.The taxonomic confirmation of the species

Confirming that the taxa analysed were correctly identified, a molecular analysis of nuclear ribosomal DNA was performed.

12.4. The content of assimilatory pigments

The content of assimilatory pigments, as well as the ratio between chlorophylls a and b, are influenced by the light incidence on the leaf surface, which is why the highest values were recorded in species collected from alpine and subalpine meadows (*V. canina* and *V. dacica*).

12.5. Total phenols content

The total content of polyphenols recorded, analysing the experimentally prepared alcoholic and aqueous extracts, was highest in species collected from mountain areas *V. declinata* and *V. dacica,* closely followed by *V. canina*. Species collected from agricultural areas and semi-natural pastures presented the lowest values (*V. arvensis, V. tricolor* and *V. kitaibeliana*).

In general, the experimentally obtained data is similar to those presented in the specialized literature.

12.6. Total flavonoid content

The quantitative analysis of total flavonoids from aqueous and alcoholic extracts prepared from the tested *Viola* plants revealed that the maximum values were recorded for *V. canina,* and the minimum values for *V. arvensis*.

Analysing the similarity of the results obtained with those in the specialized literature, the values obtained by us partially correlate with the published data.

12.7. Total anthocyanin pigments content

The highest value obtained were in species with intensely purple petals (*V. dacica, V. declinata, V. canina*) and blue violet (*V. odorata, V. suavis*). The results obtained are partially correlated with published data, the fluctuation of quantitative values of anthocyanin compounds both between species and between populations of the same species may be due to harvesting from different habitats, soil quality or water stress.

12.8. Assessment of the antioxidant potential

Species of the genus *Viola* contain a significant number of compounds from the polyphenol class, which is why the extracts produced have a high antioxidant capacity. The inhibition rate values, for aqueous and ethanolic extracts, were high for mountain species, and lower for species harvested from agricultural areas (*V. kitaibeliana*, *V. tricolor* and *V. arvensis*).

13.9. Assessment of the complex potential for the valorisation of Viola species

Polyphenolic compounds are recognized in modern medicine for their anti-inflammatory and antibacterial effects, as well as their strong antioxidant capacity, qualities that have led to their use as adjuvants in the treatments of multiple conditions.

From an ecological point of view, the species have significant importance, both as cosmopolitan plants, growing on various substrates, including anthropized soils, and as taxa that occupy habitats with difficult and polluted environmental conditions.

Conclusions

The main objective of the research carried out was to analyse the taxa belonging to a number of nine species of the genus *Viola*, subgenres *Viola* and *Melanium.* Starting from these species, the aim of the work was to enrich the specialized literature with new data on the biology of some taxa belonging to this genus, present in the spontaneous flora of the geographical area of the North-Eastern Region of Romania.

• The analysis of **phytochemical parameters** was based on the premise that species of the genus *Viola* are appreciated in traditional medicine at an international level, which is why aqueous and ethanolic extracts were made with four concentrations (0.5%, 1%, 2.5% and 5%) using dried, mechanically ground plant material. Due to the applied working methodology, the efficiency of the extraction of biochemical compounds was limited by the type of solvent and the chosen concentration. The difference between the values obtained practically and those reported in the specialized literature may be due either to the difference in environmental factors in the habitat areas, to the harvesting period, or to the combination of these two premises.

- Comparative analyses of some **morphometric** and **anatomical parameters** have highlighted new elements that can be taken into account for species discrimination, the data obtained having a pronounced novelty character.
- **The phytochemical parameters** analysed were the *content of polyphenols, flavonoids, anthocyanin pigments* and *determination of antioxidant capacity*. The results obtained identified significant quantities of these classes of compounds in the analysed plant material. For the total amount of phenols and flavonoids, low values were determined in species collected from agricultural areas: *V. arvensis, V. tricolor* and *V. kitaibeliana,* and high values in species harvested from mountainous areas: *V. declinata*, *V. dacica, V. canina*. Quantitative analysis of anthocyanin pigments revealed low values in species with white, cream and pale-yellow flowers: *V. alba* and *V. arvensis*, and high values were identified in species with purple flowers: *V. declinata*, *V. dacica, V. canina, V. odorata* and *V. suavis*. The evaluation of antioxidant potential highlighted the antioxidant capacity of both aqueous and ethanolic plant extracts, with the latter recording higher values compared to the aqueous ones. The results obtained identified in the analysed plant material significant quantities of these compounds with **potential for pharmaceutical use**.
- Personal results, as well as those presented in the specialized literature, highlight the complex **potential for valorisation** of species of the genus *Viola*.
- From an **ecological** point of view, species of the genus *Viola* occupy a diverse number of habitats, including niche ones, such as alpine screes, to anthropized habitats, reality that contributes directly and/or indirectly to the health of ecosystems, soil quality, increasing the quality of the vegetation cover and the production of biomass available for insects and

herbivores. The resistance to polluted soils and especially the accumulation of heavy metals in the vegetative organs give species of the genus *Viola* a major role in soil decontamination and ecosystem restoration. All these attributes prove the special importance of these species, as well as the need for their conservation.

- **Morphometric** research on endemic species (*V. declinata* and *V. dacica*) and **anatomy** (*V. suavis*, *V. canina*), along with the identification of morphometric indices applicable for discriminating the analysed species, as well as research on the chemical composition of taxa belonging to the genus *Viola* collected from the North-East Region of Romania have a pronounced **novelty character**; these data enrich the national specialized literature with valuable information for completing the taxonomic determination keys of the studied taxa, for phytochemists and phytopharmacists, as well as for specialists working in the direction of environmental protection, engaged in the effort of its conservation and biological restoration.

The results obtained can be used as premises for new **research directions** in order to deepen knowledge regarding the biology of the *Viola* genus and to obtain a more detailed picture of how its taxa interact with the habitats they inhabit. Biochemical testing of extracts obtained from the analysed plant material reveals the importance of developing more complex experimental models to streamline the extraction and isolation of biologically active compounds of interest. Also, expanding the analyses to investigate a larger number of biocompounds may provide information necessary for new therapeutic applications of the analysed taxa. The evaluation of new species of the genus *Viola*, as well as their hybrids, can contribute to the identification of new bioresources with complex potential for valorisation and can highlight relevant information regarding the conservation status of the Romanian plant gene pool.

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I honour the memory of Mr. Academician Constantin TOMA (1935-2020), and I express my gratitude for the support and guidance provided in the research activity that formed the basis for the completion of this doctoral thesis.

Appendix – selection

Exhibit no. 7. *V. canina* L.: - **a./c.** Giumalău Mountain-deciduous Forest edge; **b./d.** Giumalău Mountain - short grass meadow; 29.04.2021 - **e.** Rarău Mountain – coniferous forest, 30.04.2021 (original photo)

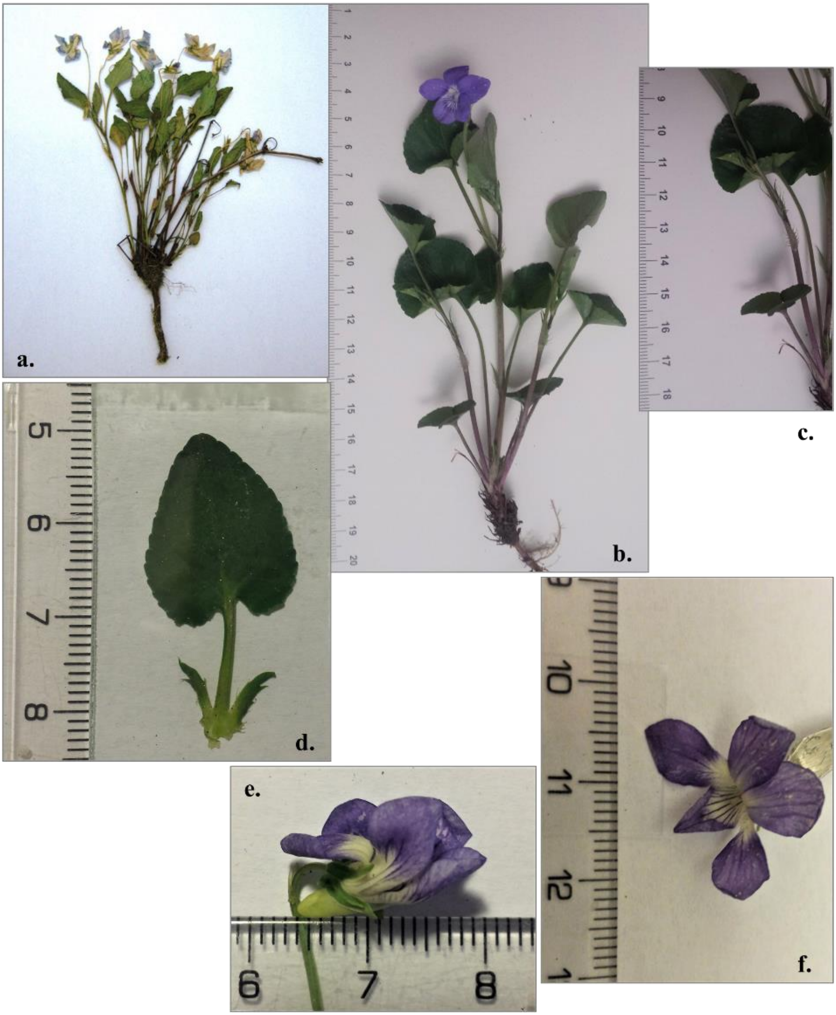

Exhibit no. 8. V. canina L. - a. general image, plant with multiple aerial stems, herborized; - b. general image; - c. detail showing the stipules; - d. leaf from the upper part of the aerial stem, with stipules; - e./f. flower (original photo)

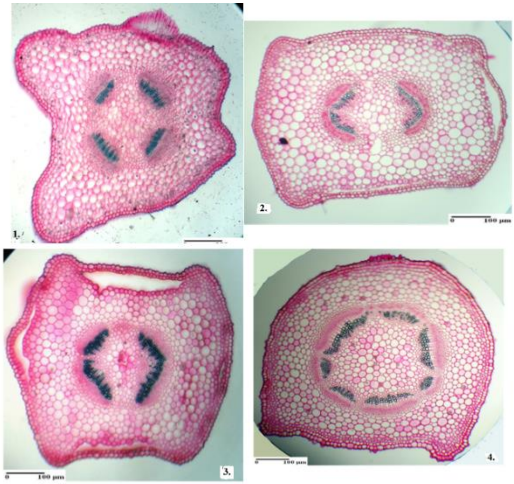

Figure. 10. Cross section through flower peduncle: 1. V. odorata L.*, 2. V. suavis* Bieb.*, 3. V. alba* Bess.*/ 4. Cross section through the stem V. canina* L.

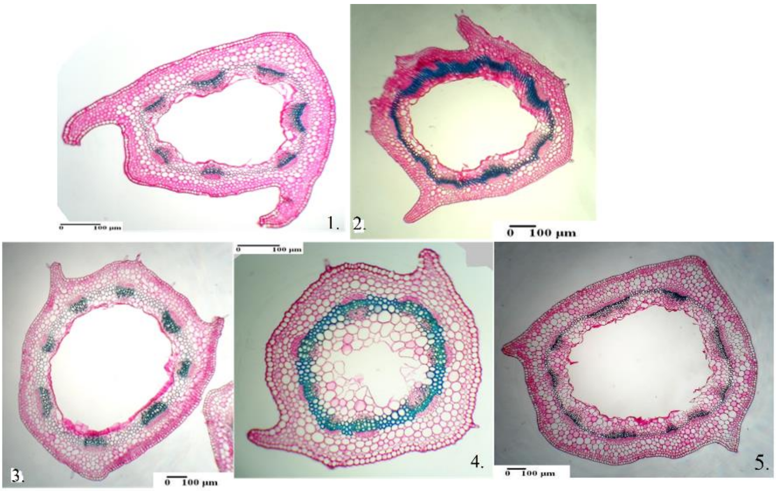

Figure. 11. Cross section through stem: 1. V. declinata Waldst. et Kit*; 2. V. dacica* Borbás*; 3. V. arvensis* Murr.*; 4. V. kitaibeliana* Schult.*; 5. V. tricolor* L.

Appendix 2

List of species of the genus *Viola* L. in Romania

The presence of a number of 28 species belonging to the genus *Viol*a L. was reported on the territory of Romania (Sârbu *et al.*, 2013):

1. *V. alba* Bess. [2 ssp] – white pansies
2. *V. alpina* Jacq.
3. *V. ambigua* W. et K.
4. *V. arvensis* Murray (include *V. banatica* Kit. Ex Roem. et Schult.)
5. *V. biflora* L. – yellow pansies
6. *V. canina* L. [3 ssp]
7. *V. collina* Bess.
8. *V. dacica* Borb. – bird's claw
9. *V. declinata* W. et K. – bird's claw
10. *V. elatior* Fr.
11. *V. epipsila* Ledeb.
12. *V. hymettia* Boiss. et Heldr.
13. *V. hirta* L.
14. *V. joói* Janka.
15. *V. jordanii* Hanry
16. *V. kitaibeliana* Schult.
17. *V. mirabilis* L. – pansies
18. *V. odorata* L. - pansies
19. *V. palustris* L. [1 ssp]
20. *V. perscifolia* Schreb. (=*V. stagnina* Kit.; *V. lactea* Auct.)
21. *V. pumila* Chaix
22. *V. reichenbachiana* Jord. Ex Boreau (=*V. sylvestris* Lam.)
23. *V. riviniana* Rchb
24. *V. rupestris* F. W. Schm.
25. *V. sieheana* W. Beckr
26. *V. suavis* Bieb. (= *V. cyanea* Čelak, *V. pontica* W. Becker, *V. ignobilis* Grinţ.) – pansies
27. *V. tricolor* L. [2 subsp.] – three spotted brothers
28. *V. uliginósa* Bess. (presence is not certain, reconfirmation required)

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