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Determination of the total content of polyphenols, flavonoids and the antioxidant capacity of extracts from *Viola x wittrockiana* flowers

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Abstract

Study objectives: *were evaluation of the content in bioactive compounds with antioxidant properties of the species* Viola x wittrockiana *Gams flowers, cultivated in the Bihor region of Romania.*

Materials and Methods*:* Viola x wittrockiana *Gams flowers were harvested, dried, then prepared for to evaluate the phenolic and flavonoid contents and for determined antioxidant capacity by the following methods: DPPH and FRAP. Results – after determined the total polyphenolic compounds content the concentration found was contained between 2.5-4.52 mg GAE/100 g dry flowers and flavonoids were contained between 46.75-54.49 mg QE/100 g dry flowers garden pansies.*

Conclusions: *strong correlation between the polyphenols and flavonoids content and antioxidant properties which have been evaluated at the extracts obtained from the pansy flowers have shown that the plant has inhibition capacity on DPPH reagent contained between 62.644 – 92.241% and antioxidant capacity which varies between 494.588-889.882 made by FRAP method.*

Keywords: *Viola x wittrockiana*, antioxidant capacity, polyphenols, flavonoids

INTRODUCTION

Flavonoids represent a category of the polyphenolic compounds which can be isolated from a wide range of the medicinal herbs where has antioxidant, antimicrobial, photoreceptor effect. On the body studies claim that flavonoids have antioxidant effect due to their ability to eliminate and reduce the formation of harmful free radicals. Flavonoids can be uses in the treatment or prophylaxis of various diseases: cardiovascular, diabetes, inflammation, immune system disorders, liver etc. [1,2].

Because the consumption of antioxidants in the last decade it was growing the researchers focused on replacing synthetic antioxidants with natural ones [2].

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Of all sources of natural antioxidants, the garden pansy *Viola x wittrockiana* Gams has been analyzed in this paper as a source of antioxidants although it is not considered a medicinal herb. The flowers of Viola x wittrockiana Gams herb in present are used in food and especially in pastry for decorating food dishes [3].

The garden pansy is a hybrid plant which involves three Viola species: *Viola tricolor L., Viola altaica L. and Viola lutea L.* and the taxonomic hierarchy of the plant is: kingdom Plantae, subkingdom Cormobionta, division Magnoliophyta, class Magnoliatae, subclass Dilleniidae, order Violales, family Violaceae, genus Viola [4,5]. All varieties of pansies are grown widely as ornamental plants. Because garden pansies were crossed with the wild pansy – *Viola tricolor* it is considered that *Viola x wittrockiana* Gams would have antioxidant activity [2].

Wild pansy - *Viola tricolor L* is a widespread plant in Europe and western Asia and has been used over time for treating various skin desease (dermatoses and eczema), for treating children urticaria and internal for the treatment of superior respiratory tract disorders, as an expectorant, diuretic and depurative [2,4,6].

In Romania, there are over 30 species of Viola of which we set out to study the species of *Viola x wittrockiana* Gams with different color of the petals to observe the total content of polyphenols and flavonoids, as well as theis antioxidant capacity.

MATERIALS AND METHODS

1. Materials

Reagents: ethanol, sodium carbonate and sodium nitrite were purchased from Promochem, LGC Standards GmbH (Wesel, Germany), quercetin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl, aluminiu chloride and sodium hydroxide were purchased from Sigma Aldrich Co (Missouri, USA), Folin-Ciocâlteu reagent, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and potassium persulfate were purchased from Merck (Darmstadt, Germany), bidistilled water was obtained at Milli-Q system Millipore, Bedford (MA, USA). All reagents used in the research had analytical purity.

Equipment: UV-VIS spectrophotometer T70+ (PG Instruments Ltd, Lutterworth, Great Britain), analytical balance type Kern ABT 220-5DNM (Kern and Sohn GmbH, Balingen, Germany), IKA VORTEX stirrer 0-2500 rpm (Werke GmbH and Co. KG, Straufen, Germany), rotary evaporators Heidolph Hei-VAP Precision-Platinum 3 (Heidolph Instruments Gmbh &Co. KG, Schwabach, Germany).

2. Methods

2.1. Preparing the herbal alcoholic extracts from *Viola x wittrockiana* Gams

Plant material (dry flos) that was used in this study *Viola x wittrockiana* Gams was purchased from independent producers from Bihor County. Extraction of active principles was made with the Soxhlet device because it is an easy, convenient and very efficient process to extract the active principles from herb plants of pansy. Concentration of the extract obtained was performed using a rotavapor under the following working conditions: temperature 40oC, speed 80 rpm and pressure 200 mBar until a uniform and thin liquid film is obtained on the balloon walls. This film was then taken over with 4 mL ethanol so that the solution obtained has a concentration of 1 g/mL [7]. The extracts flowers samples were note with: E1-flowers of yellow color, E2-flowers of burgundy color, E3-flowers of purple color.

2.2. Determination of the active principles from ethanolic extract of *Viola x wittrockiana* Gams

2.2.1. Determination of the total polyphenolic compounds content

Total plyphenolic contents were determined by using the Folin-Ciocalteu reagent, method requiring an alkaline reaction medium. 0.1 mL plant extract was mixed with 1.75 mL bidistilled water, 0.2 mL Folin-Ciocalteu reagent (dilution 1:10, v/v), 1mL of 15% Na2CO3 solution and then the mixture was incubated in the dark and at room temperature for 120 minutes. The absorbance was read at 765 nm using a spectrophotometer UV-VIS.

The equation of the calibration curve for gallic acid is:

$$y = 0.0135x + 0.0832, R^2 = 0.9963 \quad (1)$$

where:

x - the gallic acid equivalent (mg GAE/mL)

y - the absorbance recorded at 765 nm

For the determination as accurate the experiment was performed in triplicate and the results were expressed as mean ± SD (standard deviation).

2.2.2. Determination of total flavonoids compounds content

The total flavonoids content was determined using a colorimetric method so: over 1 mL plant extract was added 4 mL bidistilled water and 0.3 mL of 5% NaNO2. The content of the volumetric flask was shaken and left to stand in rest, in the dark for 5 minutes, then were added 0.3 mL of 10% AlCl3 and was left to stend in rest for 6 minutes. So AlCl3 formed a complex compound with the carbonyl groups of flavonoids. Finally, 2 mL of the NaOH (1M) solution was added to the volumetric flask, then was completed with bidistilled water to the 10 mL and the content was energetically mixed. The mixture was hold at room temperature for 30 minutes, than was read the absorbance at 510 nm using a UV-VIS spectrophotometer. Also was prepared a blank to which no pansy extract has been added [8-10].

The total flavonoids content was determined after tracing the calibration curve for standard of quercetin:

$$y= 0.8259x - 0.0028 \quad (2)$$

where:

y – the quercetin absorbance recorded at 510 nm,

x – the quercetin concentration expressed as mg quercetin equivalent (mg QE/mL)

The experiment was performed in triplicate and the results were expressed as average value, mg quercetin equivalent/100 g pansy dry flos (mg QE/100g dried pansy flowers) ± SD (standard deviation). The results are presented in Table 2.

2.2.3. Assessing the antioxidant capacity of the ethanolic extracts of Viola wittrockiana Gams

2.2.3.1. DPPH method

DPPH technique, that uses 2,2-diphenyl-1 picrylhydrazyl as a reagent, is the method by which the capacity of active compounds, extracted from plant products can be measured, to fight free radicals or compounds that can release hydrogen ions. The reagent used is a radical reagent whose color changes from deep purple in solution, to pale yellow or colorless when neutralized by flavonoids contained in ethanolic plant extracts [8, 11]. 2.9 mL from blank was added in a test tube over 0.1 mL plant extract. The content of a test tube was mixed with the vortex, kept in the dark for 15 minutes at room temperature, then for to find the concentration of the remaining DPPH radical was measured the absorbance against blank at 517 nm using UV-VIS spectrophotometer. All determinations were performed in triplicate and the results were expressed as average ± SD.

The inhibition capacity on DPPH radicals is expressed as scavenging capacity (%) and was calculated by equation (3):

$$\text{inhibition capacity (\%)} = A_{blanc} - A_{sample} / A_{blanc} \cdot 100 \quad (3)$$

where:

A_{blanc} – the blanc absorbance (DPPH reagent dissolved in ethanol) read at 517 nm (t = 0 minutes);

A_{sample} – the sample absorbance (sample with DPPH) read at 517 nm (t = 15 minutes).

2.2.3.2. FRAP method (Ferric Reducing Antioxidant Power)

The FRAP assay was made in accordance with the procedure described by Vicas L. et al. with some modifications [12].

The FRAP assay is a method of determination antioxidant capacity of various compounds from plant products which is based on the reduction reaction of the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) complex. The reaction requires a reducing agent and an acidic pH.

0.1 mL of ethanolic pansy extract was added into a test tube and mixed with 0.5 mL FRAP solution freshly prepared and 2 mL bidistilled water. The content was mixed with the vortex and kept in the dark for 60 minutes. The absorbance was measured at 595 nm, using UV-VIS spectrophotometer and the Trolox was used as the standard solution. All determinations were performed in triplicate and the results were expressed as average ± SD.

Assessing the antioxidant capacity of the ethanolic pansy extracts by using the FRAP assay was calculated by equation (4):

$$y = 0.0017 x + 0.0872 \quad (4)$$

where:

y – the sample absorbance read at 595 nm, expressed as u.a.

x – the Trolox concentration expressed as µmol Trolox equivalent/mL (µmol TE/mL)

RESULTS AND DISCUSSIONS

1. Determination of the active principles in the ethanolic extract of *Viola x wittrockiana* Gams

In identifying the plant product of *Viola x wittrockiana* Gams, the literature was consulted [5,13]. The flowers of pansy of color burgundy, yellow and purple were harvested in June 2021, on a clear and warm day, at noon, when the plant was in the flowering period. After harvesting, the vegetable pansy product was washed with water several times, its impurities were removed, and then it was removed on a paper to dry. The plant product dried in a POL-EKO drying chamber at a temperature of 30-35°C for 24 hours. After drying, it was atomized, packed in a paper bag and stored in a dry place, away from dust and insects. Dried Viola x wittrockiana flowers can be stored in this way for a maximum of 1-2 years.

In order to obtain the alcoholic extract on which the subsequent determinations will be made, 4 grams of dried pansies flowers were weighed using an analytical balance. As an extraction solvent, ethanol was used because this solvent causes good yields to the extraction of polyphenols, that emerge from the difference in polarity and the power of the eluent, comparing ethanol with methanol and water [14].

1.1. Determination of the total polyphenol content

The Folin-Ciocâlteu method is a colorimetric method widely used to identify the content of total polyphenols. This method uses Folin-Ciocâlteu reagent, which is a mixture of phosphomolybdate and phosphotungstenate that is added over the vegetable product previously treated with sodium carbonate. Initially, the phenolic hydroxyl groups react with sodium carbonate forming sodium phenolates, which then react with the Folin-Ciocâlteu reagent and form compounds that color the solution blue. The intensity of the blue color is even greater as the amount of sodium phenolate groups is greater [13,15]. The content of total polyphenols is expressed in mg GAE/g vegetable product using the calibration curve for gallic acid. Using equation (1) we have calculated the total content of polyphenols existing in the three types of pansy flowers and the results are shown in Table 1.

From the analysis of the obtained data, it can be concluded that the ethanolic extract of *Viola x wittrockiana* Gams has an appreciable content of total polyphenols, the descending order being E2, E3 and E1. The results obtained are consistent with determinations by other researchers, who obtained the extracts by maceration method for 24 hours at 4°C, then centrifugation, concentration by evaporation and lyophilization [16].

TABLE 1. Average total polyphenol content of pansy flower extracts

Extract	Total polyphenol content (mg GAE/g dried pansy flowers)	Average of the total polyphenol content (mg GAE/g dried pansy flowers)
E1	2.63	2.65 ± 0.02
	2.67	
	2.66	
E2	4.49	4.52 ± 0.03
	4.52	
	4.55	
E3	4.48	4.50 ± 0.02
	4.51	
	4.52	

E1-flowers of yellow color, E2-flowers of burgundy, E3-flowers of purple color

1.1.1. Determination of total flavonoid content

The method used to determine the total flavonoid content of pansy flowers is a colorimetric method based on the acid reaction of AlCl3 with the ketone group at position 4 of the heterocyclic ring C of quercetin or with the hydroxylic groups in the C3 positions of the heterocyclic ring C or the C5 of the aromatic ring A of quercetin, resulting colored complexes which can be determined in colorimetric (Figure 1) [17].

The calibration curve was obtained by plotting the absorbance of the standard quercetin solution against the concentration of the quercetin solutions and it was

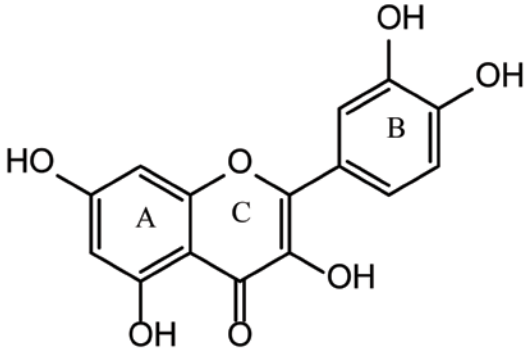

FIGURE 1. Chemical structure of quercetin

TABLE 2. Total flavonoid content of alcoholic extracts of pansy flowers

Extract	Flavonoid content (mg EQ/100 g dried pansy flowers)	Average flavonoid content (mg EQ/100 g dried pansy flowers)
E1	47.52	46.75 ± 0.91
	46.88	
	45.84	
E2	53.58	54.49 ± 0.91
	55.05	
	54.85	
E3	51.37	51.01 ± 1.11
	49.55	
	52.12	

E1-flowers of yellow color, E2-flowers of burgundy, E3-flowers of purple color

found that the variation was linear within the concentration range used. From the calibration curve we obtained the regression equation (2) for the standard solution of quercetin according to which we calculated the total concentration of flavonoids existing in the pansy extract (expressed as quercetin equivalent).

From the analysis of the data, we can conclude that the alcoholic extracts from the flowers of *Viola x wittrockiana* Gams have a high concentration of flavonoids (46.75 – 54.49 mg QE / 100 g dried pansies flowers), the order being E2, E3 and E1. The obtained data are consistent with those provided by current studies of some researchers, 46.11 mg QE/g lyophilized extract of yellow pansies flowers, 45.35 mg QE/g lyophilized extract of violet pansies flowers and 35.79 mg QE/g lyophilized extract of yellow pansies flowers [16].

2. Determination of the antioxidant capacity of the ethanolic extract of *Viola x wittrockiana* Gams

Polyphenolic compounds are among the most widespread secondary metabolites present in higher plants where they accumulate mainly in leaves, barks, flowers and fruits. The concentration in phenolic compounds varies depending on: plant product, variety, species, environmental factors, plant development, time of harvest, processing and storage of plant material [18]. Free radicals such as superoxide anionic radicals (O2-), hydroxyl radical (HO-), alkoxyl radical (RO-), peroxyl radical (ROO-) play an indispensable role in the normal metabolism of an organism at the cellular level [19]. The loss of balance between the levels of antioxidants and free radicals is characterized by oxidative stress that leads to the generation of various chronic diseases [20].

According to studies polyphenols generally possess a high antioxidant effect and are considered the most abundant compounds in the human diet protecting the body from the appearance of cardiovascular diseases, osteoporosis, diabetes, cancer etc. However, the antioxidant effect on herbal extracts has been observed to be influenced by the type and nature of the solvent used for extraction. This can be explained by the fact that most phytoconstituents, because they have different physicochemical properties and polarities, also have different solubility in certain types of solvents [21]. The antioxidant activity of extracts that is manifested by the ability to annihilate free radicals, for example O2- and Fe2+ ions, was highlighted in this paper using DPPH and FRAP methods [18,22-24].

2.1. Determination of antioxidant capacity by the DPPH method of *Viola X wittrockiana* Gams extracts

Flavonoids and polyphenolic compounds present in the extract of the pansy plant are compounds that have antioxidant capacity and can neutralize free radicals. The evaluation of the *in vitro* antioxidant capacity of the extract obtained from the plant can be determined using the DPPH technique, using the reagent 2,2-diphenyl-1-picrylhydrazyl. Reactive oxygen species are involved in many pathological diseases, which is why flavonoids are compounds with antioxidant capacity and can fight against free radicals [25].

The antioxidant capacity of samples obtained from the pansy plant extracts have been expressed in % of inhibition of the DPPH radical and the results obtained from the DPPH method are presented in Table 3.

The data provided in Table 3 shows that the ethanolic extracts of the three types of flowers of pansy has the inhibition capacity of the DPPH reagent, in ascending order: E1, E3, E2. All extracts from pansy flowers have a high percentage of inhibition of the reagent DPPH, which demonstrates a good ability to neutralize free radicals, as recorded by other existing studies, between 65.151 – 96.188% [16]. These results are due to the presence in the plant of the high content in polyphenolic compounds, which have antioxidant activity [26,27].

TABLE 3. DPPH results on ethanolic extracts from pansy flowers of different colors

Extract	Blank absorbance	Sample absorbance	Percentage of inhibition, %	Average of the percentage of inhibition ± SD, %
E1	**0.696**	0.265	61.925	62.644 ± 0.862
		0.254	63.506	
		0.261	62.500	
E2	**0.696**	0.059	91.523	92.241 ± 0.862
		0.048	93.103	
		0.055	92.098	
E3	**0.696**	0.068	90.230	89.751± 0.527
		0.071	89.799	
		0.075	89.224	

E1-flowers of yellow color, E2-flowers of burgundy, E3-flowers of purple colo

In other words, the capture capacity of radicals is directly related to the donation capacity of the hydrogen atom of a compound and is not correlated only with redox potentials [28].

2.2.Determination of the antioxidant capacity by the FRAP method of *Viola X wittrockiana* Gams extracts

FRAP assay is a simple spectophotometric method which is based on the ability of pansy extract to reduce Fe3+ at Fe2+. This happens in an acidic environment when in the presence of tripyridyltriazine (TPTZ) it takes place reduction of the ferric tripyridyltriazine complex at the ferrous form tripyridyltriazine [8,29].

Table 4 shows the results obtained for the determination of the antioxidant capacity of the ethanolic extracts from the pansy flowers by the FRAP method.

TABLE 4. Total flavonoid content of alcoholic extracts of pansy flowers

Extract	Sample concentration (µmol TE/100 mL extract)	Mean sample concentration (µmol TE/100 mL extract) ± SD
E1	489.882	494.588 ± 4.706
	496.353	
	497.529	
E2	891.647	889.882 ± 1.176
	888.706	
	889.294	
E3	686.941	689.882 ± 4.118
	688.706	
	694.000	

E1-flowers of yellow color, E2-flowers of burgundy, E3-flowers of purple color

The data provided in Table 4 shows that the ethanolic extracts from the flowers of *Viola x wittrockiana* Gams have antioxidant capacity that decreases in the order: E2, E3, E1. It can also be noted that, regardless of the method that is applied for the determination of antioxidant capacity (DPPH, FRAP), the descending order of ethanolic extracts from pansy flowers of different colors is the same: burgundy, purple, yellow. This conclusion was reached also by other researchers, even though they used other methods for the extraction of active principles. Reported results were 503.60 µmolTE/mL extract – yellow pansies flowers, 953.30 µmol TE/mL extract –burgundy pansy flowers and 758.66 µmol TE/mL extract –violet pansy flowers [16].

CONCLUSIONS

In this paper, *Viola x wittrockiana* Gams flowers of different colors were studied, both in terms of content in polyphenols, flavonoids, and antioxidant capacity, and it was observed that the ethanolic extracts of *Viola x wittrockiana* Gams have a high content of total polyphenols and flavonoids that vary depending on the color of the flowers. Thus, the largest amount of total polyphenols and the greatest amount of flavonoids are contained in the extracts obtained from the burgundy blossoms, followed by the purple ones and then the yellow ones. From the determination of the antioxidant capacity by the two methods DPPH and FRAP it could be observed that the greatest antioxidant capacity is held by the extracts from the burgundy flowers, followed by the purple ones and then the yellow ones.

Due to the antioxidant effect, polyphenols can be used as bioactive compounds with antioxidant effect, because they can be absorbed from consumed foods. Based on these characteristics of polyphenols and from the significant content in phenolic compounds of *Viola x wittrokiana* Gams flowers, we can consider that this plant product can be used as a source of micronutrients in the formulas of food supplements.

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