Analysis of Polar Antioxidants in Heartsease (*Viola tricolor* L.) and Garden Pansy (*Viola x wittrockiana* Gams.)

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Abstract

Heartsease (*Viola tricolor* L.) is a well-known medicinal plant. Its biological activities are supposed to be related to its antioxidant capacity. Garden pansies (*Viola x wittrockiana* Gams.) have been crossbred from heartsease and are applied as ornamental plants only. In this study, the mother and the daughter species are compared from a phytochemical point of view. Their flavonoid and anthocyanidin contents are determined by spectroscopic methods recommended by the European Pharmacopoeia 5.0. The compositions of the samples (heartsease and garden pansy varietas of several petal color) are analyzed by high-performance liquid chromatography with UV detection and their antioxidant capacity is determined by trolox equivalent antioxidant capacity assay. Our results suggest that garden pansy, especially its flower, is a promising source of natural antioxidants. In addition, a significant correlation is found between the flavonoid content and antioxidant activity.

Introduction

Antioxidants are molecules capable of quenching harmful free radicals; thus they are useful in the prophylaxis and treatment of more than eighty types of illnesses including cardiovascular problems, complications of diabetes, inflammations, immune disorders, and liver problems, just to list a few (1). These illnesses occur if the body's oxidative balance is disturbed. Although natural antioxidants are present in the daily diet in moderate amount, there are cases when additional supplements are necessary to support the body's own antioxidant system. In recent years, considerable attention has been devoted to natural antioxidants in order to replace synthetic ones. Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. Heartsease herb has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic (2). Due to its flavonoid and other phenolic compound content, heartsease may represent a promising source for natural antioxidants (5). Garden pansies (*V. x wittrockiana* Gams.) are plants of complex hybrid origin involving at least three species: *V. tricolor*, *V. altaica*, and *V. lutea*. They have several colorful varieties and are widely cultivated as ornamental plants. Since garden pansies have been crossbred from *V. tricolor*, they are believed to possess similar good antioxidant activity.

The antioxidant capacity can be determined by means of in vitro and in vivo assays. The in vitro techniques are classified according to the reaction mechanisms involved. Hydrogen atom transfer (HAT) based methods and single electron transfer (SET) based methods are both widely utilized (6). The so-called trolox equivalent antioxidant capacity (TEAC) assay characterizes the electron-donor (7) property of the samples. Due to its advantages in ease of use and reproducibility, this technique is applied in many laboratories (6). Although, due to the complex reaction paths involved, this assay does not always allow exact quantitative evaluation of the antioxidant capacity, it is still a proper method to provide the ranking order of antioxidants (8).

Besides the antioxidant capacity and phenolic content, their chemical compositions are characteristic of the samples of interest from the phytochemical point of view. Because of the unknown composition and complexity of plant extracts, high-performance liquid chromatography (HPLC)-based quantitation of the samples is apparently not an option. For the determination of the approximate anthocyanidin and flavonoid content, spectroscopic methods are being suggested by the European Pharmacopoeia 5.0 (9). The qualitative analysis of the flavonoid composition, however, is usually carried out by reversed-phase HPLC (C8 or C18) (10). The most common detection methods are UV and photodiode array based, although recently mass spectrometry has been rapidly gaining ground (11). In spite of the fact that for the separation of reference molecule mixtures proper methods have been developed (12,13), the qualitative analysis of plant extracts can not be standardized. For each plant sample, therefore, an identical HPLC method must be developed to be able to obtain the so-called fingerprint chromatograms. In phytomedicine, these chromatograms are important indicators of plant material quality. In some cases they reveal the absence of certain components (e.g., pesticide residues) (14,15), or prove that the components responsible for the biological activity are present in appropriate quantities (16–18).

Albeit, garden pansies have been crossbred from *V. tricolor*,

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their secondary metabolite composition has neither been studied nor compared with heartsease. In this paper, we report on a comparative study including HPLC and spectroscopic methods of the polar components of heartsease and garden pansy extracts. These compounds are believed to contribute to the antioxidant properties as well as to the biological activities of heartsease.

Materials and Methods

Chemicals and plant materials

Heartsease herb (*Viola tricolor* L., Violaceae) (SN = 28-56-05-VI/24) was purchased from Fitopharma Ltd. (Budapest, Hungary). Garden pansies (*Viola* x *wittrockiana* Gams., Violaceae) were cultivated in Nagyrécse (Hungary). They were selected by petal color: violet, violet-white, white and yellow, and collected as herbs, flowers and leaves. Ginkgo folium (*Ginkgo biloba* L.) was collected in the botanical garden of the Roland Eotvos University, Budapest and identified together with the pansy varietas in the Department of Pharmacognosy, Semmelweis University (Budapest, Hungary), where voucher specimens were deposited (n = 2).

All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Sample preparation

0.50 g air-dried plant material was sonicated with 20 mL of 70% methanol for 20 minutes in an ultrasonic bath at room temperature. The filtered extract was evaporated to dryness in vacuum at 60°C. The dry residue was re-dissolved in 1.5 mL 70% of methanol and separated by solid-phase extraction. The SPE cartridge (Supelclean LC-18, 500 mg/3 mL, Sigma-Aldrich) was activated with 3 mL methanol followed by 3 mL 2% acetic acid. After sample introduction, the cartridge was washed with 1.5 mL of 70% methanol. The loading and washing solvents were collected and combined for downstream analysis.

HPLC and LC–MS analysis

HPLC separation of the heartsease herb and garden pansy samples were performed by means of an ABLE-E & Jasco (Tokyo, Japan) apparatus consisting of the following components: ERC-3113 degasser, LG-980-02 solvent mixer, PU-980 pump, 20 µL Rheodyne 7725 injector, and UV-975 UV–vis detector. For the separation, gradient elution from 13% to 18% acetonitrile in 20 min (A = 2.5% of acetic acid) was performed at a flow rate of 1.0 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm, Sigma-Aldrich) column.

The LC–MS analysis of the heartsease herb sample was performed on a Model 1100 HPLC–MSD SL system (Agilent Technologies, Waldbronn, Germany), which was comprised of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the chromatographic separation, gradient elution from 10% to 40% ACN in 30 min (A = 2.5% acetic acid) was performed at a flow rate of 0.5 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm) (Sigma-Aldrich) column. The eluate was monitored with both the diode array (at 340 nm) and the mass selective detectors. MSD scanning was performed from *m/z* 100 to 1000 in 0.2 min intervals. The mass selective detector was equipped with a normal-flow electrospray ionization source (ESI). Electrospray conditions were as follows: drying gas flow, 13 L/min; drying gas temperature, 350°C; nebulizer pressure, 35 psi; capillary voltage, 3000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

Determination of the anthocyanidin and flavonoid content and their correlation to the antioxidant activity of the samples

The anthocyanidin and flavonoid content of the dried samples were determined by applying the methods of the European Pharmacopoeia 5.0, paragraph "Bilberry fruit, fresh" *(Myrtilli fructus recens)* and "Goldenrod" *(Solidaginis herba)* (9).

To be able to characterize the samples applied in the TEAC assay, we also determined their flavonoid content. Accordingly,

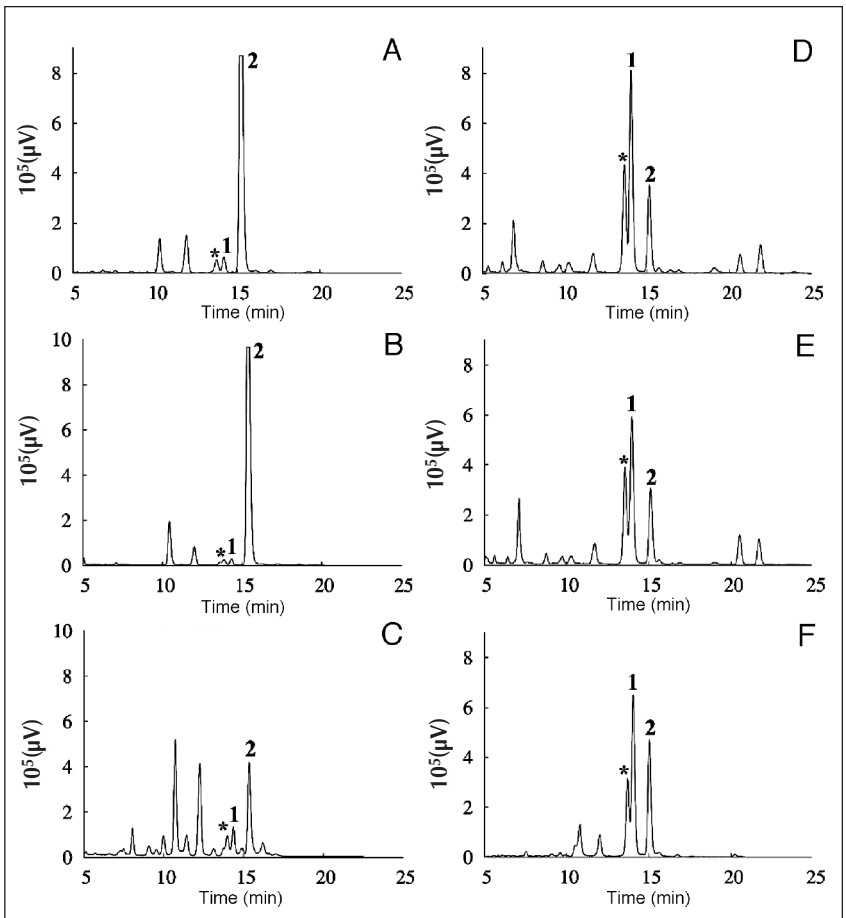

Figure 1. HPLC chromatograms of violet-white pansy flower (A), white pansy flower (B), violet pansy flower (C), yellow pansy herb (D), white pansy leaf (E), and heartsease herb (F). Gradient elution from 13% to 18% acetonitrile in 20 min (A = 2.5% acetic acid) was performed at a flow rate of 1.0 mL/min on a Hypersil ODS (250 x 4.6 mm, 5 µm) column.

0.5 mL of the extracts (see the TEAC assay section) was diluted and treated as described in the original method of the European Pharmacopoeia 5.0. The results were then used for calculating the correlation between the flavonoid content and antioxidant capacity. Data were imported into the Origin 6.0 software (Microcal Inc., Northampton, MA, x = flavonoid content / anthocyanidin content, y = IC_{50} value (g/mL)) and the "Correlation" statistical test was applied. The software calculated the correlation coefficient (r) and the statistical significance (p).

TEAC assay

1.0 g of dried and freshly powdered plant material was sonicated with 20 mL of 70% methanol for 20 min in an ultrasonic bath at room temperature. Five mL water and 5 mL CCl_4 were added to the filtered extract to get rid off the chlorophyll. After centrifugation (9000/min, 10 min) the chlorophyll-free supernatant was evaporated to dryness in vacuum at 60°C. The residue was re-dissolved in methanol for further analysis.

For the TEAC assay, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) was dissolved in water in 7mM concentration. Its radical mono-cation was produced by reacting the ABTS solution with 2.45mM (final concentration) potassium persulfate, and the mixture was stored in the dark at room temperature for at least 12 h before use. The $ABTS^{\bullet+}$ stock solution was diluted with spectroscopic grade ethanol to 0.9 absorbance unit at 734 nm. Five different volumes of the diluted sample were added to 2.5 mL ABTS solution resulting in different final concentrations and producing inhibition of the blank solvent between 20–80%. Absorbance values were measured at 734 nm after 0.0, 0.50, 0.66, 0.83, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00, and 6.00 min. To determine the so-called inhibition percentage, we extrapolated the final absorbance by numerically solving the simplest possible reaction kinetics model. The inhibition percentage produced by a given sample concentration was calculated as $(100 - A_t/A_0) \times 100$ (A_t = extrapolated final absorbance; A_0 = absorbance of the blank solvent). The antioxidant activity was characterized by plotting the inhibition percentage of the samples as a function of concentration and performing linear regression (data not shown). The concentration resulting in 50% inhibition is referred to as IC_{50} value.

Results and Discussion

Qualitative and quantitative analysis of heartsease and garden pansy polar extracts

Because the characteristics of the anticipated antioxidant components of anthocyanidins, flavonoids, and other phenoloids in heartsease and garden pansy are mostly polar, we have chosen to use methanol extracts of the plant materials for our studies. Extracts were prepared from herbs, flowers, and leaves of heartsease and several garden pansy varieties of violet, violet-white, white, and yellow petal color. All samples were analyzed by reversed-phase HPLC and representative chromatograms are shown in Figure 1. The chromatograms exhibit very similar peak distribution of all the samples with differences mostly observed in peak ratios. As most panels in Figure 1 exhibit, the two major components were eluted at retention times of 14.0 (Peak 1) and 15.0 min (Peak 2). Their UV spectra was typical of flavonoids (19), with absorption maxima at 274 and 336 nm, as well as 266 and 361 nm for the two peaks, respectively. Comparing the retention times, UV spectra, molecular masses (MW = 610.2) and fragmentation patterns with reference standard molecules, as a first approximation we consider Peak 2 to be rutin (see structure in Figure 2B). Peak 1 of Figure 1 was identified as violanthin (see structure in Figure 2A) by NMR (data not shown) and mass spectrometry. As this study was focused on rutin and violanthin, this publication did not aim to obtain any structural information about the rest of the peaks in Figure 1. However, according to their chromatographic retention data and absorption spectra profile, these components are most probably flavonoid glycosides. The peak marked by the asterix in Figure 1 with the retention time of 13.7 min (Peak*) might be isoviolanthin, based on UV spectroscopic considerations (absorption maxima at 274 and 335 nm).

By the different intensity of the individual components in the chromatogram, the samples were classified into the following two major groups of flower and herb/leaf samples. The main component of the flower samples (Peak 2 in Figure 1D and 1E) is proposed to be rutin, according to the HPLC and LC–MS experiments described earler. The second group (Figure 1A–1C) comprised the herb and leaf samples, apparently contained much lower quantities of rutin (Peak 2), but was rich in violanthin (Peak 1). The similarity between the herb and leaf samples can be associated with the fact that pansy herb samples consisted mostly of leaf (80%, determined by the

Table I. The Anthocyanidin and Flavonoid Contents, as well as the Antioxidant Capacity of Heartsease and Garden Pansies of Different Petal Color

Sample		Antocyanidin content (g cyanidin-3-glucoside per 100 g sample)	Flavonoid content (g rutin per 100 g sample)	Antioxidant activity IC50 value (g/mL)
Violet pansy	flower	1.52 ± 0.06	1.21 ± 0.07	$(1.57 \pm 0.05) \times 10^{-5}$
	leaf	0.05 ± 0.002	0.10 ± 0.006	$(3.86 \pm 0.18) \times 10^{-5}$
	herb	0.31 ± 0.01	0.38 ± 0.02	$(2.92 \pm 0.04) \times 10^{-5}$
Violet-white pansy	flower	0.19 ± 0.01	2.58 ± 0.15	$(8.59 \pm 0.25) \times 10^{-6}$
	leaf	0.04 ± 0.002	0.03 ± 0.002	$(3.18 \pm 0.09) \times 10^{-5}$
	herb	0.05 ± 0.004	0.62 ± 0.03	$(1.99 \pm 0.10) \times 10^{-5}$
White pansy	flower	0.09 ± 0.004	2.01 ± 0.12	$(6.35 \pm 0.08) \times 10^{-6}$
	leaf	0.06 ± 0.002	0.16 ± 0.01	$(5.32 \pm 0.12) \times 10^{-5}$
	herb	0.08 ± 0.003	0.59 ± 0.03	$(1.42 \pm 0.06) \times 10^{-5}$
Yellow pansy	flower	0.31 ± 0.01	2.93 ± 0.18	$(6.98 \pm 0.28) \times 10^{-6}$
	leaf	0.11 ± 0.004	0.11 ± 0.007	$(4.00 \pm 0.09) \times 10^{-5}$
	herb	0.11 ± 0.006	0.62 ± 0.04	$(1.87 \pm 0.05) \times 10^{-5}$
Heartsease	herb	0.02 ± 0.0008	0.50 ± 0.03	$(4.17 \pm 0.10) \times 10^{-5}$

weight of the fresh species) rather than flowers (20%, determined by the weight of the fresh species). The weight of the stem was negligible. The violet flower sample did not fit into this classification as it only possessed low amounts of rutin and violanthin, as depicted in Figure 1F, thus in the violet flower samples the minor components gained more significance in the HPLC peak pattern.

In addition to their flavonoid content distribution, the actual anthocyanidin and flavonoid contents are distinctive of the samples. Anthocyanidins are natural pigments in plants; consequently, they are present in flowers (especially with colorful petals) in larger quantities than in leaves. The quantity of anthocyanidins and flavonoids were determined by applying the registered methods of the European Pharmacopoeia 5.0 (Table I, Figure 3). The highest anthocyanidin content was measured in violet pansy. Colorless varieties (yellow, white and white with a little violet) possessed only low amounts of anthocyanidins. On the other hand, in regard to the flavonoid content, species with pale petals showed elevated concentration. The highest amount was measured in yellow pansy flower. As Table I and Figure 3 depict, flower samples had higher flavonoid content than leaf samples, while herb samples were in between.

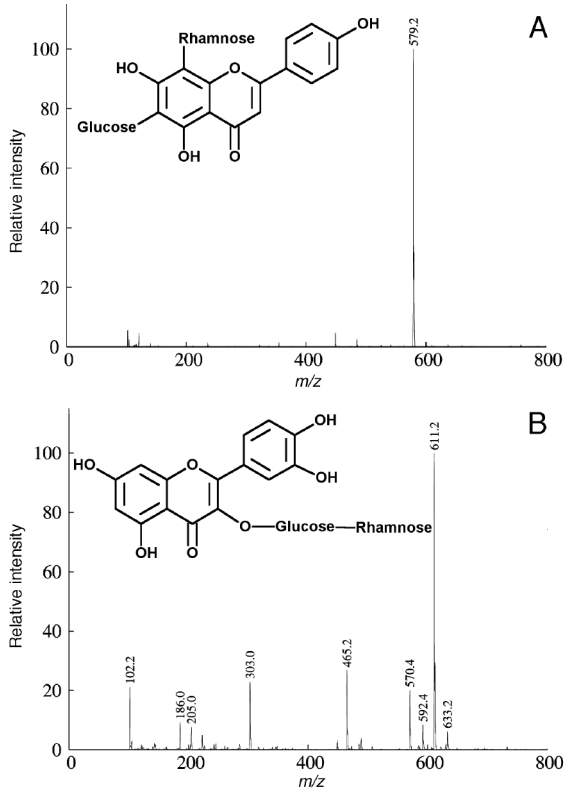

Figure 2. Chemical structures and MS spectra of component 1 (A) and component 2 (B).

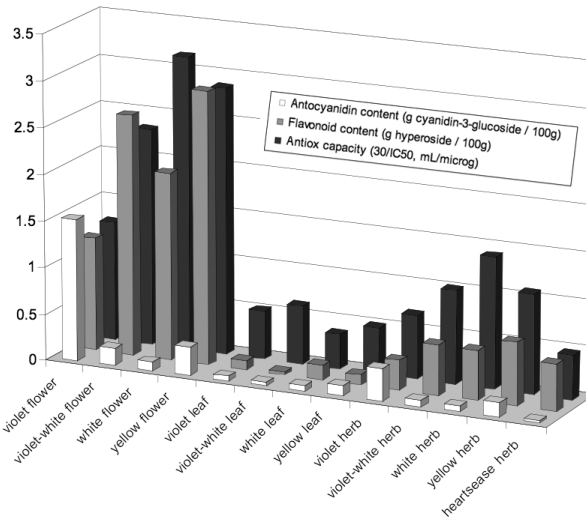

Figure 3. Anthocyanidin and flavonoid contents, as well as the antioxidant capacity of heartsease and garden pansies of different petal color.

Antioxidant activity of the samples

The antioxidant activity of the samples was determined by TEAC decolorization assay, in which colorful artificial free radicals ($ABTS^{\bullet+}$) were generated and their concentration was determined before and after sample introduction. Antioxidant components in the sample quenched the free radicals, whose concentration decreased proportionally to their antioxidant capacity. The oxidized and reduced forms of the radical absorbed at different wavelengths, allowing quantitative spectroscopic determination of the free radical concentration in the samples. The antioxidant capacity was characterized by calculating the IC_{50} value: the concentration, which produces 50% quenching of the free radicals (Table I, Figure 3). The lower the IC_{50} value, the better the antioxidant activity of the sample. Our experiments revealed that in this test system the heartsease and pansy samples both showed good antioxidant activities. Apparently, their antioxidant capacity was at the same level as the well-known antioxidant ginkgo leaf of $IC_{50} = 1.82\ (\pm\ 0.07) \times 10^{-5}$ mg/mL. In addition, the crossbred garden pansy possessed better antioxidant properties than its mother species, heartsease. Similarly to the flavonoid content, flower samples showed the highest antioxidant activity, whereas herb and leaf samples possessed inferior antioxidant properties according to the IC_{50} values attained (Table I, Figure 3). Based on this similarity, we examined the relationship between the antioxidant activity and the flavonoid content of the samples, and found significant correlation between the antioxidant capacity and the flavonoid content ($R = -0.6375$, $p = 0.02$). On the other hand, no correlation was found between the antioxidant capacity and the anthocyanidin content ($R = -0.2798$, $p = 0.35$).

Conclusion

In this paper we report on the study of polar antioxidants of heartsease (*Viola tricolor* L.) and its hybrid, garden pansy (*Viola* x *wittrockiana* Gams.). The flavonoid compositions of the extracts prepared from heartsease herbs, flowers, and leaves of several garden pansy varieties of violet, violet-white, white, and yellow petal color were analyzed by HPLC. Apparently similar major components were found in herb, leaf, and flower samples; however, differences were observed in their component ratios. According to the chromatographic peak distribution, the sam-

ples were classified into two major groups: flower and herb/leaf samples. Comparing the retention times, the UV spectra, the molecular masses, and fragmentation patterns with reference standard molecules, as a first approximation we consider the main component of the flower samples as rutin. The main component of the herb and leaf samples were identified as violanthin using NMR and MS.

The anthocyanidin and flavonoid contents of the samples were quantitated by spectroscopic methods registered in the European Pharmacopoeia 5.0. While the highest anthocyanidin content was measured in the violet flower sample, the white and yellow pansy samples showed the highest flavonoid content.

The antioxidant capacity of the samples was determined by TEAC assay. Our data revealed that in this test system the heartsease and pansy samples were as good antioxidants as the well-known antioxidant ginkgo leaf. In addition, significant correlation was found between the flavonoid content and the antioxidant capacity of the samples, whereas no correlation was observed between the antioxidant capacity and the anthocyanidin content.

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