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Anthocyanins Profiling of *Prunella vulgaris* L. Grown in Ukraine

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By the method of microscopy, thin-layer chromatography, UV/VIS spectrophotometry and HPLC the quality composition and quantitative maintenance of anthocyanins of above-ground and underground organs of common self-heal (*Prunella vulgaris* L.) were established. The highest amount of anthocyanins was found in prunella inflorescences (spikes) and the lowest one – in leaves. The histochemical studies of *Prunella vulgaris* L. fresh inflorescences cup and bracts transverse sections found that cups accumulate anthocyanins and permeated by vascular passages. The results of spectrophotometric pH differential assay showed the highest monomeric anthocyanin pigment concentration, as paeonidin-3-glucoside, color density and percent of polymeric color for self-heal inflorescences. Chromatographic analysis has shown a large difference between qualitative and quantitative composition in different parts of self-heal. Paeonidin and cyanidin glycosides were the major anthocyanin derivatives identified in spikes and stems of self-heal, especially the 3-O-glucosides and 3-O-rutinoside. The most representative anthocyanin in prunella roots were delphinidin-3-O-galactoside. This is the first study regarding anthocyanin composition in *Prunella vulgaris* L.

Keyword: *Prunella vulgaris* L., Common Self-Heal, Anthocyanin, HPLC, Microscopy.

1. Introduction:

Anthocyanins (from greek: *anthos*-color, *cyanos* - azure) are plant glycosides containing as aglycone, also called anthocyanidin, hydroxy derivatives of 2–phenylchromene. Sugar (mostly the rest of glucose, rhamnose, galactose etc.) is attached to aglycone in the position 3, less often in 3 and 5 positions. Some anthocyanins have methylated or acylated hydroxyl groups. Anthocyanins are soluble in water pigments that have red, purple or blue colors. Three non-methylated glycosides of anthocyanidins (cyanidin, delphinidin and pelargonidin) are the most widespread in nature and are present in 80% of colored leaves, 69% fruits, 50% of the flower petals. At present, it was found more than 400 anthocyanins in the nature [1].

These pigments are often found in the cell sap (vacuoles), much less - in the plant walls. They can exist in different forms: as oxonium cation or carbonium cation [2].

In alpine plants anthocyanins, by absorbing excess of solar radiation, protect chlorophyll and hereditary apparatus of cells from damages. The bright colors of flowers and fruits play an important role in attracting of pollinators and distributing of fruits. It is interesting that the plants with a large amount of anthocyanins, have high resistance to air pollution and industrial acid gases.

In the human organism anthocyanins maintain normal blood pressure and blood vessel shape, preventing internal bleeding. They form complexes with radioactive elements and remove them from the organism [3].

Self-heal (*Prunella vulgaris* L.) also known as carpenter's herb or all-heal, has a long history of medical use in medicine of Europe, China and North America. This herb is especially helpful in treatment of inflammations, pains in the throat, fevers. It accelerates wound healing.

Fig 1: Common Self-heal (*Prunella vulgaris* L.)

The most useful are prunella's spikes. They have a tubular blue-violet, rarely pink or white lips clustered into cylindrical spikes. Their beautiful colors attract many insects and cover the grass land by the carpet of flowers. This perennial herb was widely used in the past by herbalists and now modern science has focused on its pharmaceutical potential [4].

Last ten years scientists have conducted many studies about this herb. They have approved its anti-cancer effects and anti-viral activity, especial against HIV and herpes viruses [5-8].

The aim of our study was to investigate total anthocyanin content, anthocyanin composition and anthocyanin localization in self-heal spikes, leaves, stems and roots and to compare anthocyanin content in different parts of common self-heal.

2. Materials and Methods:

2.1 Plant Material:

The inflorescences (spikes), leaves, stems and roots of self-heal were included in this study. *Prunella vulgaris* L. harvested during the plant's flowering was collected in July 2012 from Carpathian Mountains (near Otyniya village, Ivano-Frankivskiy region, Ukraine. The products were naturally dried in shadow and stored in controlled laboratory conditions. The species were identified and the voucher specimens were deposited at the Herbarium of the Faculty of Pharmaceutical Chemistry and Pharmacognosy of Medical University of Ukrainian Association of Folk Medicine (Kyiv, Ukraine).

2.2 Methods

a. Microscopical Study: The histochemical studies were performed using the microscope brands «Olympus» with a magnification of x40, x100 and x400 times. To study microscopic characters of *Prunella vulgaris* L. anthocyanins, transverse sections of fresh inflorescences cup and bracts were prepared with the help of double edge razor blade accordingly a free hand section method. Taking samples were made by micro digital camera Nikon D40.

b. Thin-Layer Chromatography: Pigments were extracted from the plant material by homogenizing 1 g of the dry material in 10 ml methanol-acetic acid 25% (9:1) mixture. The homogenizing was heated for 30 minutes. About 20 µg of the concentrated extract was spotted on each cellulose plate (20×20 cm). As eluent a solvent system of ethyl acetate: acetic acid: formic acid: water (100:11:11:26) was used. Detection was carried by visualization in day light.

c. Measurement of Anthocyanins by UV-Visible Spectroscopy:

The pH-differential method is based on the changes structural transformations of anthocyanin pigments that provide different pH. At pH 1.0 predominate the colored oxonium

form and at pH4.5 - the colorless hemiketal form. This method is also effective because it permits rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments [1].

For the measurement of anthocyanins by UV-visible spectroscopy two dilutions of the sample, one with potassium chloride buffer pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each by the previously determined dilution factor, were prepared.

A Hewlett Packard UV/VIS 8452A spectrophotometer and 1 cm pathlenght disposable cells were used for spectral measurements at 700 and 420 nm.

d. HPLC Analysis of Anthocyanins:

High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20 module system using diode array detection (DAD) [9].

Previously we have examined the bilberry extract, grape juice and cranberry extract to obtain optimum separation of anthocyanins. The excellent and reproducible separation was obtained for all 15 anthocyanins in bilberry extract by using of Luna C18 column at 25 °C and solvent system for elution: A: Acetonitrile and B: Acetic acid: acetonitrile: trifluoroacetic acid: water (10:2:0.2:87.8) in gradient elution. The program used a linear gradient from 0 to 20% solvent A in 20 min; then a linear gradient of solvent A from 20 to 40% in 5 min and from 40 to 0% solvent A in next 10 min. The flow rate was 1ml/min; injection volume was 10 µl.

Samples were prepared by heating of 2 g of dry matter in100 ml of 4% phosphoric acid aqueous solution. Prior to injection all samples were filtered through a 0.45 mm Millipore membrane filter.

3. Results and Discussions:

The histochemical study revealed the presence of anthocyanins and their localization in the tissues of different organs of prunella.

Self-heal inflorescences cup accumulate anthocyanins and permeated by vascular passages. On the edge they have pubescent multicellular hairs.

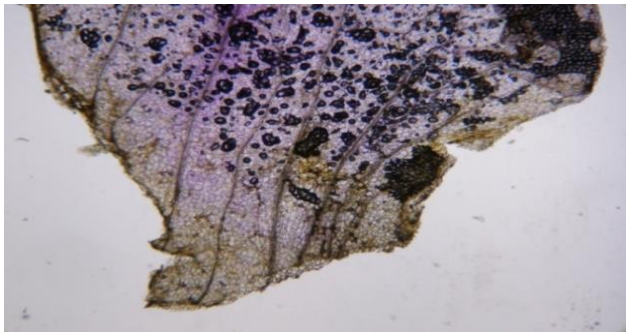


Fig 2: Anthocyanins on the surface of flower petal from corolla

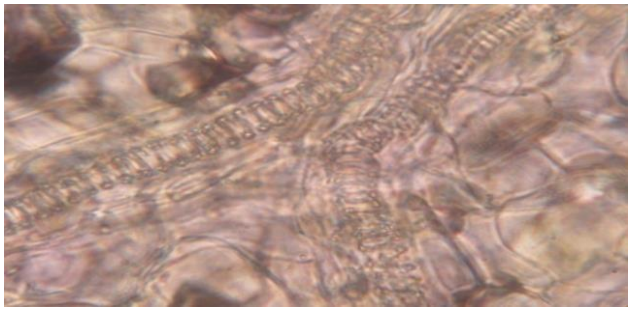


Fig 3: Transverse section of the flower petal vessels showing scalariform pitting

Vessels of flower petal from corolla form a cluster (Fig. 3), have scalariform pitting, numerous multiples of cells joint together.

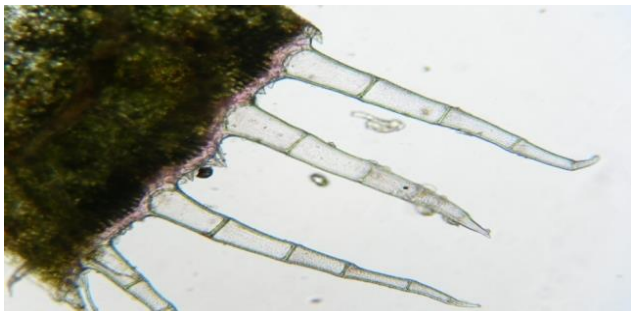


Fig 4: Lower epidermis of prunella bracts

In transversal section of the bracts, upper and lower epidermal cells are covered by a thin cuticle. An epidermal cell accumulates the anthocyanins.

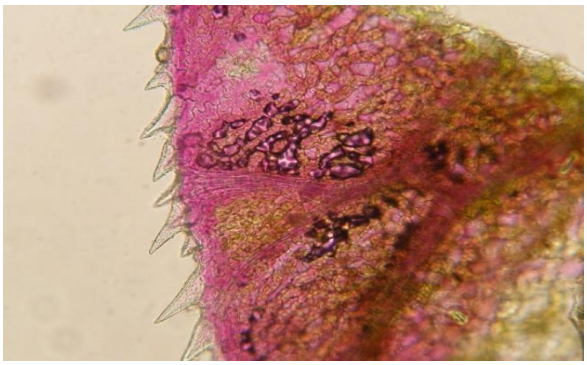


Fig 5: Upper epidermis of prunella bracts

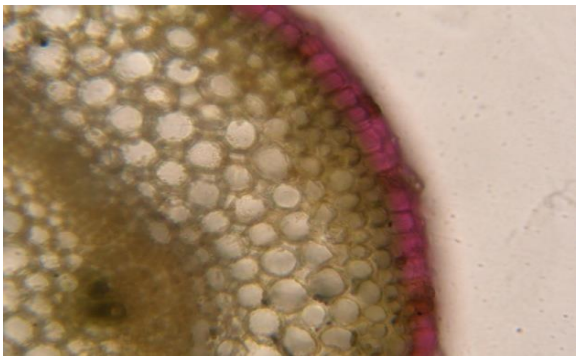


Fig 6: Cross section of the self-heal stem

Bracts are pubescent along the edge by single and multicellular hairs. On the edge of bracts are located cells that accumulate anthocyanins, as evidenced by their coloring in the purple color.
Anthocyanins of the stem are accumulated in the isodiametric epidermal cells and cuticule.

TLC separation of anthocyanin pigments in different organs of self-heal demonstrates the presence of yellow and violet pigments. Their identification is more difficult for prunella leaves because of the high presence of chlorophylls.
The values of Rf of different pigments are shown in the Table 1.

Table 1: TLC investigation of prunella different pigments

Rf	Spikes	Leaves	Stems	Roots	Color of spot	Pigment assignment
0.03	+	-	-	-	Violet	NI
0.04	-	+	-	-	Yellow	NI
0.05	+	+	+	-	Violet	NI
0.07	+	+	+	-	Violet	NI
0.09	+	-	+	+	Violet	Mvd-3,5-diglc
0.19	+	+	+	+	Violet	Pnd-3-glc
0.22	-	-	-	+	Yellow	Cyd-3-glc
0.27	-	+	+	-	Light yellow	NI
0.30	-	+	+	-	Light yellow	Mvd-3-glc
0.33	-	-	+	-	Light yellow	NI

"-" - negative; "+" - positive; NI – unknown

Indices for anthocyanin degradation of a self-heal aqueous extracts were derived from a few absorbance readings of a sample that has been treated with sodium bisulfate by forming a colorless sulfonic acid adduct. Polymerized colored anthocyanin-tannin complexes are resistant to bleaching by bisulfite, whereas the bleaching reaction of monomeric anthocyanins will rapidly go to completion. The absorbance at 420 nm of the bisulfite-treated sample serves as an index for browning. Color density is defined as the sum of absorbances at the Avis_max and at A420 nm. The ratio between polymerized color and color density is used to determine the percentage of the color that is contributed by polymerized material. The ratio between monomeric and total anthocyanin can be used to determine a degradation index. We have calculated the color density the polymeric color of the bisulfite bleached sample and the percent polymeric color.
The values obtained were compared with the literature value for the extinction coefficient for peonidin-3-glucoside of 11300, by using molecular weights of 463.2 g/mol–1 for peonidin-3-glucoside.

Table 2: Total anthocyanin content in self-heal

	Spikes	Leaves	Stems	Roots
Monomeric anthocyanin pigment concentration (as peonidin-3-glucoside), mg/l	0.065	-	0.032	0.027
Color density	0.041	-	0.025	0.012
% polymeric color, %	109.32	-	84.50	79.36

The monomeric anthocyanin pigment concentration, colour density and percent of polymeric colour as peonidin-3-glucoside in the original sample was calculated using molecular weight (MV), dilution factor and molar absorptivity (ε) founded in the literature.

Total anthocyanin content in self-heal, collected in Ukraine presented in Table 2.

The results of spectrophotometric pH differential assay, reported in Table 2, showed variations in total anthocyanin content among investigated parts. The highest Monomeric anthocyanin pigment concentration, as peonidin-3-arabinoside, was found for prunella spikes and the lowest – for roots. Color density and percent of polymeric color were also highest for inflorescences.

We made up an anthocyanin profile by using the HPLC-DAD technique. Anthocyanins identification and peak assignment are based on their retention times, UV-VIS spectra comparing with standardized extracts of blueberries, strawberries, grapes and chokeberry and the literature data.

Anthocyanins 3-O-glucoside derivatives eluted as follows: galactoside → glucoside → arabinoside → xyloside → rhamnoside (for sugars) and sophoroside → sambucoside → rutinoside (for disaccharides).

In our study we found 35 anthocyanins with different nature.

The chromatogram of prunella inflorescences anthocyanins is presented in Fig. 7. Anthocyanin composition of self-heal is given in Table 3.

Chromatographic analysis has shown a large difference between qualitative and quantitative composition in different parts of self-heal.

For confirmation of the presence of anthocyanins, spectra of peak were registered on-line (Fig. 7).

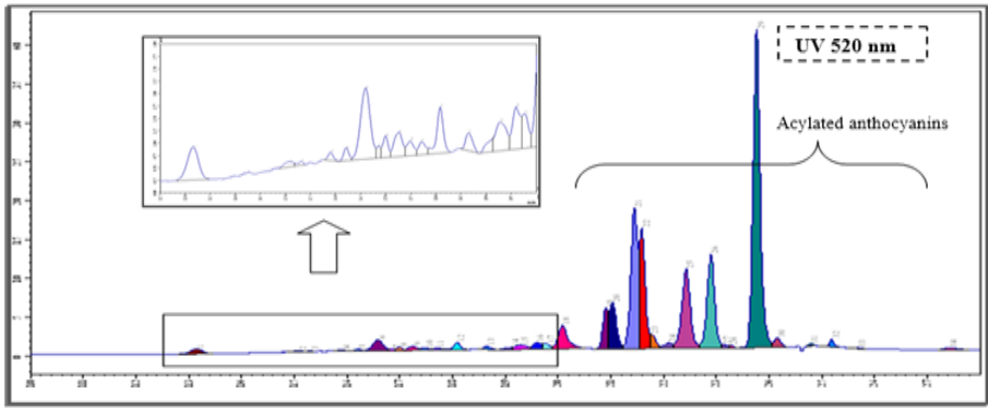
In samples of above-ground organs peonidin-3-glucoside was found in highest quantities.

The largest number of identified anthocyanins found in inflorescences: 2 delphinidin glycosides, 4 cyanidin glycosides, 3 peonidin glycosides, 2 malvidin glycosides and 1 glycoside of petunidin. There were also identified some unknown acylated anthocyanin derivatives.

Smaller number of anthocyanin derivatives was found in the stems of common self-heal, namely: 2 delphinidin glycosides, 3 cyanidin glycosides, 2 peonidin glycosides and acylated anthocyanins.

The lowest anthocyanin content was found in the self-heal roots: 3 delphinidin-galatosides and 2 cyanidin glycosides as well as anthocyanins acylated derivatives.

There were no found anthocyanins in prunella leaves.



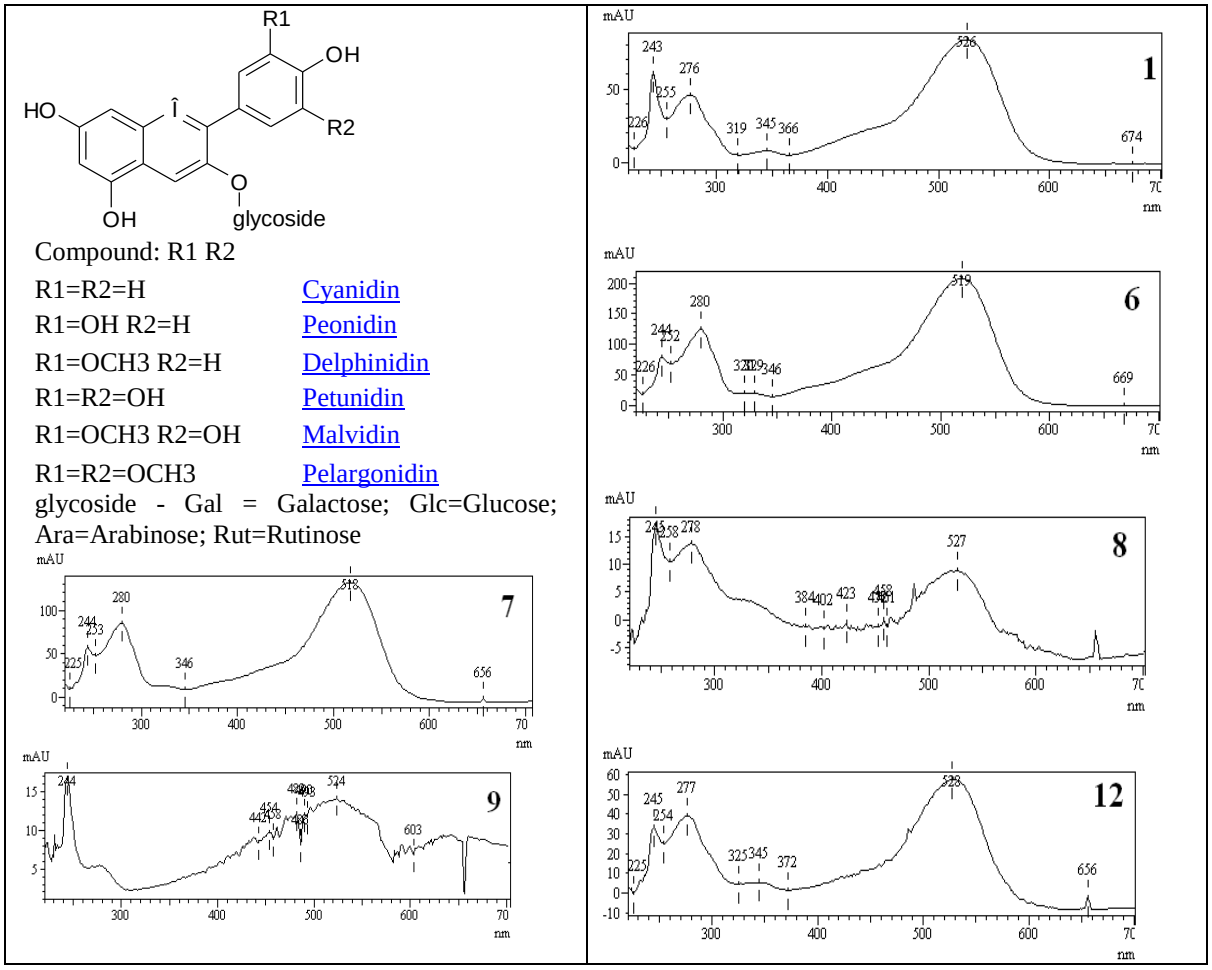


Fig 7: HPLC-DAD anthocyanin profile of self-heal spikes (spectra's numbers correspond to peak no. in table 3)

Table 3: Chromatographic, spectroscopic and spectrometric characteristics and content of the anthocyanins found in four parts of Prunella vulgaris

Peak No.	Rt (min)	λmax (nm)	Peak assignment	Spikes	Stems	Roots
1	13,19	526, 243, 278, 335	Dphd-3-gal	9,7	7,1	**62,2**
2	14,80	526, 280, 244, 335	Dphd-3-ara	-	3,3	-
3	15,03	527, 276, 244, 346	Dphd-3-rut	1,2	-	-
4	15,34	519, 279, 244, 335	Cyd-3-gal	0,6	-	-
5	17,21	519, 279, 244, 335	Cyd-3-arab	5,3	1,8	-
6	16,90	518, 244, 282, 376	Cyd-3-rut	18,2	17,1	25,4
7	16,43	517, 243, 280, 337	Cyd-3-glc	1,7	27,4	12,3
8	18,10	527, 278, 245, 334	Pnd-3-gal	8,1	5,6	-
9	19,90	524, 244, 274	Pnd-3-arab	2,2	-	-
10	18,90	534, 273, 245, 345	Pnd-3-glc	**37,0**	**49,6**	-
11	18,43	528, 278, 245, 336	Mvd-3-gal	3,2	-	-
12	19,40	528, 277, 245, 345	Mvd-3-glc	8,2	-	-
13	17,05	528, 242, 276, 346	Ptd-3-glc	3,1	-	-
Total content of identified anthocyanins				100	100	100

The highest content of anthocyanin component was established for an unknown anthocyanins acylated derivative with retention time 23.82 min. Aslo this is the first study regarding anthocyanin composition in *Prunella vulgaris* L.

4. Conclusions:

1. A comparative evaluation of the anthocyanin profile considering the phytochemical content of different parts of *Prunella vulgaris* was performed.
2. The above-ground and underground organs of common self-heal were analyzed by microscopy, TLC, UV/VIS spectrophotometry and HPLC for identification and the quantification of anthocyanins.
3. The total quantity of anthocyanins depends on herbal part.
4. The HPLC analysis showed that was the inflorescences were the richest one in anthocyanins.
5. Paeonidin and cyanidin glycosides were the major anthocyanin derivatives identified in spikes and stems of self-heal, especially the 3-O-glucosides and 3-O-rutinoside. The most representative anthocyanin in prunella roots were delphinidin-3-O-galactoside.
6. Our study demonstrates that the prunella spikes commonly consumed in folk and traditional

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