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## A HPLC Analysis on Interpopulational Variations in the Flavonoid Composition of *Veronica chamaedrys*

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**Abstract:** Flavonoid profiles of aerial parts of two subspecies of *V. chamaedrys* L. growing at different altitudes were investigated. Five external flavones apigenin, luteolin, luteolin 3'-methyl ether (*chrysoeriol*), luteolin 7,4'-dimethyl ether and scutellarein 6,4'-dimethyl ether (*pectolarigenin*) were detected and quantified by HPLC. The study on the total and individual concentrations of flavonoid aglycones showed that their content was more abundant in the populations at alpine regions. *V. chamaedrys* ssp. *vindobonensis* M. Fish. accumulate greater amounts of flavonoid aglycones than *V. chamaedrys* ssp. *chamaedrys* and this correlate positively with more glandular hair in *V. chamaedrys* ssp. *vindobonensis*.

**Key words:** *Veronica chamaedrys*, flavonoid aglycones, altitude

### INTRODUCTION

Flavonoid metabolism is a sensitive biosynthetic system, reacting to changes in the environment (Cooper-Driver and Bhattacharya, 1998). It has been suggested that surface flavonoid aglycones have a number of properties for plant adaptation in harsh environmental conditions, especially against excessive solar radiation (Cuadra *et al.*, 1997; Markham *et al.*, 1998; Chaves *et al.*, 2001; Onyilagha and Grotewold, 2004). This supposes that flavonoid composition would be expected to vary between populations subjected to different environmental conditions. Across altitudinal gradient several abiotic and biotic factors are changed regularly. In the climatic zone in Bulgaria temperatures lowers by 0.7-1 °C at every 100 m above sea level (asl). Humidity also drops with increase of altitudes. Up to 3000 m asl intensity of direct solar radiation grows by 25%. With the increase of altitude ultraviolet radiation (UV) gets enriched in its biologically active B region (280-320 nm) (Sakali and Lingova, 1988; Lingova, 1995).

*V. chamaedrys* L. (Scrophulariaceae) is a strongly polymorphic species, that have a wide altitudinal distribution. Surface flavonoid aglycones: luteolin, apigenin and its methyl derivatives have been reported for *V. chamaedrys* population (Nikolova *et al.*, 2003). It has also been demonstrated that there is interpopulation flavonoid variability of *V. chamaedrys* s.l. in relation to altitude by TLC (Nikolova *et al.*, 2002). We therefore initiated a study to look at infraspecific flavonoid

variation of *V. chamaedrys* by more exact method as HPLC. The objective of the present research was to determine qualitative and quantitative changes of flavonoid composition in the acetone exudates of *V. chamaedrys* populations growing at different altitude and thereby contribute to understanding the functions of these compounds in plants.

### MATERIALS AND METHODS

**Chemicals and reagents:** Apigenin was purchased from Extrasynthese (Genay, France). The others flavonoid standards were kindly supplied by Prof. E. Wollenweber (Darmstadt, Germany). HPLC-grade methanol and analytical-grade chemicals (acetone, methanol, *t*-butanol, potassium dihydrogen phosphate and *ortho*-phosphoric acid) were provided by Merck (Germany).

**Plant material:** Plant material was carried out in the flowering period. Five samples of two subspecies of *V. chamaedrys* were collected from natural habitats in Bulgaria, details of which are given in Table 1. The sampling locations were chosen to cover regions at different altitude of the two subspecies. The samples were formed from individuals taken at random from different sides of the populations. The species were determined according to Walters and Webb (1972). Vouchers specimens are deposited at the Herbarium of the Institute of Botany (SOM).

Table 1: Voucher numbers (SOM) and collection sites of *Veronica* samples studied for externally accumulated flavonoid aglycones

Taxon	Sample No.	SOM	Habitat information <sup>a</sup>
<i>V. chamaedrys</i> ssp. <i>chamaedrys</i>	A	Co655	Rila Mountain, Mussala Hut, 2350 m asl, dry alpine pastures, Si
	B	Co1096	Stara Planina Mountain (Middle), Gabarevo, 800 m asl, Ca
<i>V. chamaedrys</i> ssp. <i>vindobonensis</i>	C	Co674	Vitosha Mountain, below peak of Cherni vrah, 2100 m asl, dry alpine pastures, Si
	D	Co454	Ljulin Mountain, Bankya, 750 m asl, Si
	E	Co493	Strandza Mountain, Brashlyan, 350 m asl, open places, Ca

<sup>a</sup>m asl-meters above sea level; Ca-calcareous bedrock; Si-siliceous bedrock

**Sample preparation:** Plant exudates were prepared from 2 g whole air-dried aerial parts by rinsing the material with 20 mL acetone for three minutes to dissolve the flavonoids stored in glandular hairs on the leaf and stem surfaces. The acetone extracts were evaporated and the residues dissolved in 200  $\mu$ L methanol for HPLC analysis. Variation of flavonoid compounds was estimated by analysis of three acetone exudates of each population.

**HPLC equipment and conditions:** The chromatographic analyses were performed on Varian (USA) chromatographic system, which includes tertiary pump Model 9012, Rheodyne injector with 20  $\mu$ L sample loop and UV-VIS detector Model 9050. Varian Star Chromatography workstation and computer software (version 4.5) were used for controlling the system and collecting the data. The separation was performed using Hypersil ODS RP18, 5  $\mu$ m, 250 $\times$ 4.6 mm I.D. column, (Chandon, UK) fitted with precolumn (30 $\times$ 4.6 mm I.D., Varian USA) dry packed with Perisorb RP-18, 30-40  $\mu$ m, (Merck Germany), the both maintained at room temperature. The mobile phase comprised *t*-butanol, methanol and 20 mmol L<sup>-1</sup> potassium dihydrogen phosphate buffer (adjusted to pH 3.22 by *ortho*-phosphoric acid) at a ratio 11:37:52 (v/v/v). The flow rate was 1 mL min. The chromatograms were recorded at 360 nm.

**Identification and quantification of flavonoids:** The identities of the HPLC peaks were definitely assessed by co-chromatography spiking the samples with reference compounds.

The analysis of the flavonoid aglycones was carried out using the external standard method. Because of poor availability, only apigenin was used as standard. The responses of luteolin, chrysoeriol, scutellarein 6,4'-dimethyl ether and luteolin 7,4'-dimethyl ether were related to apigenin, assuming the responses to be equal. Working solutions containing 0.15, 0.08, 0.04, 0.02, 0.01, 0.001, mg mL<sup>-1</sup> apigenin were prepared from stock solution 0.38 mg mL<sup>-1</sup> in methanol. The detection limit of apigenin was 0.1  $\mu$ g mL<sup>-1</sup> and quantification limit was 0.4  $\mu$ g mL<sup>-1</sup>.

Triplicate analyses were performed for each concentration and the peak area was detected at 360 nm. Calibration curve was constructed from peak areas

versus analytic concentrations. Slope, intercept and other statistics of calibration lines were calculated with linear regression program using Statistic program STL. For apigenin, good linearity of the response with a correlation coefficient of 0.9969, was obtained within the range 0.001–0.15 mg mL<sup>-1</sup> and the regression equation was  $y = 3.38 \times 10^7 x + 7.43 \times 10^4$ . For each sample the complete assay procedure was carried out in triplicate and the standard deviation was calculated.

The recovery of the method was checked by addition of the standard solutions of apigenin (0.01 mg mL<sup>-1</sup>) to untreated plant sample. Blank sample from the same plant material, without fortification, was treated and analyzed at the same time with spiked plant matrix under the conditions described in Experimental. The complete assay procedure was carried out in triplicate. The recovery of apigenin from the plant matrix was 102 $\pm$ 4%.

HPLC analysis was conducted in Faculty of Pharmacy, Medicinal University.

## RESULTS

The present study focused on the flavonoid profiles of the aerial parts of two subspecies of *V. chamaedrys* growing at different altitudes. The reversed phase liquid chromatography method applied by Nikolova *et al.* (2003) was used to achieve an optimum separation of the compounds. HPLC analysis demonstrated that the flavonoid aglycones present in *V. chamaedrys* were derivatives of the flavones apigenin and luteolin. Considering the substitution on the aglycone skeleton, the more methoxylated derivatives are eluted last. The detected flavones were (in sequence of their retention times) luteolin (1) 10.21 $\pm$ 0.08 min.; chrysoeriol (2) 14.78 $\pm$ 0.07 min.; apigenin (3) 16.48 $\pm$ 0.15 min.; scutellarein 6,4'-dimethyl ether (4) 26.24 $\pm$ 0.06 min. and luteolin 7,4'-dimethyl ether (5) 26.95 $\pm$ 0.07 min. (Fig. 1). The distribution of the flavones listed above in the different plant accessions of *V. chamaedrys* is shown in Fig. 3. The differences in flavone accumulation between the populations were quantitative than qualitative. Apigenin was the most abundant flavonoid in all samples then luteolin, chrysoeriol and scutellarein 6,4'-dimethyl ether. Luteolin 7,4'-dimethyl ether was detected in populations E and C only in trace amounts.

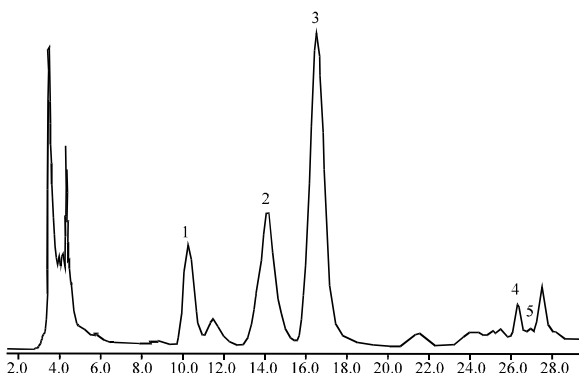


Fig. 1: HPLC chromatogram of the flavonoid aglycones from *Veronica chamaedrys* ssp. *vindobonensis* (sample E). 1. luteolin 2. luteolin 3'-methyl ether 3. apigenin 4. scutellarein 6,4'-dimethyl ether 5. luteolin 7,4'-dimethyl ether

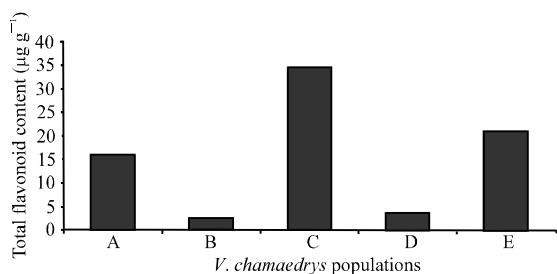


Fig. 2: Total flavonoid content in *V. chamaedrys* populations. Each value represents the sum of individual flavonoid aglycones

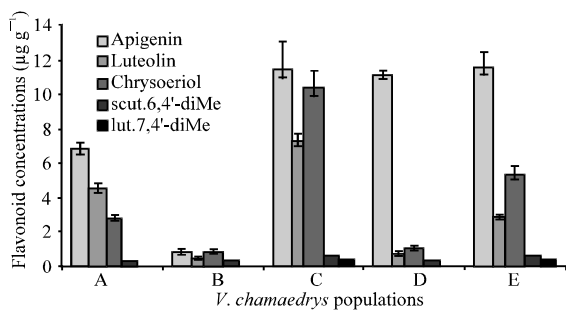


Fig. 3: Flavonoid aglycones in *V. chamaedrys* populations. Each value represents the mean of three independent determinations ±SD

The study on the total flavonoid content revealed increases in flavonoid level in the populations from alpine regions (A, C) and from Stranzha E (Fig. 2). The quantification of each of the five flavonoids present in the exudates, expressed in  $\mu\text{g g}^{-1}$  dry weight, showed that the flavonoid concentrations fluctuated according to localities (Fig. 3). The described trend to increased

flavonoid accumulation in the populations at alpine regions (A and C) as well as from Strandzha (E) was supported. On average the alpine populations contained ten-times more luteolin than the populations from lower altitude. The concentrations of chrysoeriol increased in the alpine populations too. The level of apigenin appeared was no effected by altitude.

Comparing the flavonoid composition of the populations, which are situated at the same altitude, of the both subspecies i.e., A with C and B with D we observed that the samples of *V. chamaedrys* ssp. *vindobonensis* exhibited higher flavonoid content. This is referring especially to individual concentrations of apigenin, luteolin and chrysoeriol. *V. chamaedrys* ssp. *vindobonensis* populations accumulated two time more apigenin than *V. chamaedrys* ssp. *chamaedrys* populations and this result correlate positively with more glandular hairs in the first subspecies. More that the populations of ssp. *vindobonensis* C and D contain additional flavonoid aglycone: luteolin 7,4-dimethyl ether.

## DISCUSSION

In this work the flavonoid profiles of five populations of *V. chamaedrys* were studied. The comparison of the total and individual flavonoid concentrations suggests that flavonoid content was higher in the alpine populations A and C. High irradiation, extreme climate and higher ozone concentrations are characteristic features of alpine regions. There is experimental evidence that flavonoid synthesis is increased by UV-B light radiation as well as by arid conditions and ozone (Cuadra *et al.*, 1997; Hofmann *et al.*, 2000; Saleem *et al.*, 2001; Onyilagha and Grotewold, 2004). Flavonoids absorbing strongly in the UV-B region of the solar spectrum may act as solar screens. Our results support the view that surface flavonoids play a role in plant defense against harsh environmental conditions.

The population E has relatively high flavonoid content. This result would be explained with its origin from Strandzha - region with strong Mediterranean influence. Wollenweber (1990) shows that these regions are suitable for accumulation on surface flavonoid aglycones.

Individual flavonoid quantification suggests that luteolin content fluctuated in the highest degree from populations to populations. The increased accumulation of luteolin in alpine populations is consistent with results of Markham *et al.* (1998), Ryan *et al.* (1998) and Hofmann *et al.* (2000). In their research they have been demonstrated that *orto*-dihydroxylated flavonoids like luteolin are more effective antioxidants than their monoxylated equivalents (apigenin) and this is account for their enhanced biosynthesis in plants under oxidative

stress like UV irradiation. The level of apigenin of studied samples appeared independent of altitude. Therefore luteolin have more importance role than apigenin for adaptation on plants to alpine conditions.

Comparing the populations of the both subspecies growing at the same altitude we observed that the populations of *V. chamaedrys* ssp. *vindobonensis* accumulate more flavonoid aglycones than populations of *V. chamaedrys* ssp. *chamaedrys*. Morphologically *V. chamaedrys* ssp. *vindobonensis* is differed from the *V. chamaedrys* ssp. *chamaedrys* mainly by presence of more glandular hair. Wollenweber and Dietz (1981) have noted correlations between surface flavonoids and certain plant structures such as glandular trichomes. The localization of flavonoid aglycones within glandular cells was frequently demonstrated for other plant species (Reiseberg *et al.*, 1987; Valkama *et al.*, 2003). Present results confirmed the correlation between surface flavonoids and glandular hair.

The results presented here demonstrate that total and individual concentrations of surface flavonoid aglycones of *V. chamaedrys* fluctuated in depending on the altitude and taxon. The alpine populations displayed the highest flavonoid level. Flavonoid content of *V. chamaedrys* ssp. *vindobonensis* are higher than those in *V. chamaedrys* ssp. *chamaedrys* and this correlate positively with more glandular hair in first subspecies.

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