

Comparative Leaf Epidermis Analyses of Micromeria frivaldszkyana (Degen) Velen. and Clinopodium vulgare L. (Lamiaceae) from Bulgarka Nature Park, Bulgaria

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Abstract. The present work presents a comparative anatomical study of the leaf epidermis of the Bulgarian endemic *Micromeria frivaldszkyana* (Degen) Velen. and *Clinopodium vulgare* L., belonging to the family Lamiaceae. In both species they occur diacytic and anomocytic stomatal type, while the indumentum is presented by multicellular, linear covering trichomes and multicellular, stacked glandular trichomes. The results obtained from the statistical data processing contribute to the distinction between the two species with respect to the peculiarities of the leaf epidermis. Taxonomically significant anatomical features for their determination are: number, width and length of stomata on the abaxial epidermis; width of on the adaxial epidermis; number, width and length of basic epidermal cells along both epidermis and thickness of upper and lower cuticle.

Key words: *Micromeria frivaldszkyana*, *Clinopodium vulgare*, leaf epidermis, Bulgarka Natural Park.

Introduction

Globally, the Lamiaceae family includes about 258 genera and 3,500 species (DUARTE & LOPES, 2007). According to HARLEY *et al.* (2004) and SINGH (2010) it comprises about 236 genera with 7173 species. Many of them have medicinal properties due to the essential oils contained in their various parts and are widely used as a raw material in the cosmetic industry.

Others are used in the official medicine of Anatolia, Europe, China and others (BAYTOP 1999; CUI *et al.*, 2003).

Genus *Micromeria* Benth. belongs to the Nepetoideae subfamily, the Menthae tribe. According to MORALES (1993) and BRÄUCHLER *et al.* (2005), it includes about 70 species distributed from the Himalayas to the Macaronesian Archipelago, and from the

Mediterranean to South Africa and Madagascar. ARABACI *et al.* (2010) describe the genus as a complicated complex comprising about 54 species, 32 subspecies and 13 varieties. In Europe, the genus is represented by 21 species (TUTIN *et al.*, 1972), of which *M. frivaldszkyana* and *M. juliana* are also found in our country.

There are 4 species of Bulgarian flora, (STOJANOV *et al.*, 1967; KOZHUHAROV, 1992; DELIPAVLOV & CHESHMEDZHIEV, 2003), of which 1 Balkan endemic - *Micromeria dalmatica* Benth. ssp. *bulgarica* (Velen) Guinea and 1 Bulgarian endemic - *Micromeria frivaldszkyana* (Degen) Velen.). The species *M. frivaldszkyana* is included in Biological Diversity Act (2002), Red list of Bulgarian vascular plants (PETROVA & VLADIMIROV, 2009) with a category "endangered", Red Data Book of the Republic of Bulgaria. Vol. 1. Plants and Fungi (PEEV *et al.*, 2011).

The genus *Clinopodium* L. is cosmopolitan (HARLEY *et al.*, 2004). Recently, based on biosystematic studies, a number of taxa have been added to the genus *Clinopodium*, thus making the species composition in the genus approximately 100 (FIRAT *et al.*, 2015). *Clinopodium vulgare* L. is the only one represented in Bulgaria (DELIPAVLOV & CHESHMEDZHIEV, 2003). The species is ubiquitous in the Bulgarian flora, known for its medicinal properties and included in the Bulgarian Medicinal Plants Act (MPA, 2000).

A number of molecular and morphological studies have shown a close relationship between the genera *Micromeria* (section *Pseudomelissa*) and genus *Clinopodium* (BRÄUCHLER *et al.*, 2005; 2008).

Although highly influenced by environmental factors, anatomical features, including epidermal structures, are an important marker for determining the boundaries of taxa from different plant families (METCALFE & CHALK, 1950; 1979; STENGLEIN *et al.*, 2003; SATIL & KAYA, 2007; SALMAKI *et al.*, 2011; CELEP *et al.*, 2014).

The highest taxonomic value for the representatives of the Lamiaceae family are the structure and distribution of the trichomes covering their vegetative organs, as well as the

type of stomatal apparatus (METCALFE & CHALK, 1972; KAHRAMAN *et al.*, 2010; VENKATESHAPPA & SREENATH, 2013).

According to METCALFE & CHALK (1985) at labiate plants have covering and glandular trichomes. According to the authors, the glandular trichomes can be in the form of unbranched papillae, stellate and others.

A number of studies show that there are mainly two types of stomatal apparatus in the Lamiaceae family - anomocytic and anisocytic (HARUNA & ASHIR, 2017).

The data on anatomical studies of both genera are relatively small. One of the recent studies is that of KAYA (2016), in which the stem and root anatomy of 6 species of the genus is affected *Clinopodium*. A year earlier AL-ZUBAIDY *et al.* (2015) gives a full description of the stem and leaf anatomy of the genus, also describing the type of trichomes covering the vegetative organs.

There are a number of studies for representatives of the genus *Micromeria* providing information on their morphological characteristics (ARABACI *et al.*, 2010; KREMER *et al.*, 2012; MARIN *et al.*, 2013). SLAVKOVSKA *et al.* (2017), analyzing the leaf and stem anatomy of 9 species of the genus *Micromeria*, they find that the anatomical features reflect deeper genetic differences and have a taxonomic value. In addition, some anatomical features of the genus are also affected in the studies of KOCA (1996; 2002) и MOON *et al.* (2009).

The main objective of the present work is a comparative anatomical study of the leaf epidermis of the taxonomically controversial species *M. frivaldszkyana* and *C. vulgare*, with the aim of separating the features serving to distinguish the two taxa.

Material and Methods

The plant material (leaves) of the two studied species was collected during the 2019 growing season from the territory of Bulgarka Nature park - Stara planina floristic region (Central). The species were identified at the Department of Botany and Methods of Teaching Biology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski" according to DELIPAVLOV &

CHESHMEDZHIEV (2003). The voucher materials were deposited in the Herbarium of Agricultural University - Plovdiv (SOA).

The leaf material is fixed in 70% ethanol, then histological preparations of leaf epidermis are made. The following qualitative and quantitative features were followed: hair type; type, number (1 mm²), width (µm) and length (µm) the stomata on the adaxial and abaxial epidermis; number (1 mm²), width (µm) and length of base cells on both epidermis; cuticle thickness (µm) on both leaf surfaces.

The results for quantitative traits are based on a review of 50 fields of view. Light-microscopic images of each of the scars examined were taken using a microscope *Magnum T*, equipped with photo documentation system *Si5000* at magnification x100 до x400 in Department of Botany and Methods of Teaching Biology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski". The processing of the quantitative data received for each sign is done using a statistical processing program IBM SPSS, ver. 20 (IBM, 2018). The critical level of significance used is p=0.05.

Results and Discussion

In the analysis of the adaxial and abaxial leaf epidermis of the two species studied, a single-layered structure was found, with main cells having different sizes and degrees of folding of anticlinal walls (Fig. 1). According to the classification of ANELI (1975) they refer to zig-zag folded cell walls, but according to the classification of SVESHNIKOVA (1970) are

defined as curved to highly curved. In addition to these cells, the epidermis of *M. frivaldszkyana* and *C. vulgare* also include stomata cells, subsidiary cells and trichomes cells.

The leaf surface is covered with a well-defined cuticle of varying thickness, with different anatomically distinct trichomes.

Stomata

In the epidermis of the two analyzed taxa predominates diacytic stomatal type, but in places they are also found anomocytic stomatal type. In the first type, there are two around the stomatal cells perpendicular to the ostiolum, while for anomocytic stomata are characteristic subsidiary cells, which do not differ in shape from the underlying epidermal cells (Fig. 2). The location of the stomata on both epidermis defines the leaves as amphistomata. These results are in line with the statements of METCALFE & CHALK (1950) and HARUNA & ASHIR (2017) for leaf type and stomatal apparatus with members of family Lamiaceae.

Trichomes

Two types of trichomes were found on the surface of the adaxial and abaxial epidermis of the two species studied - covering and glandular (Fig. 3). The glandular trichomes are multicellular, stacked. These results contradict the data obtained from YA'NI *et al.* (2018), for other species of family Lamiaceae, in which the authors establish multicellular glandular trichomes with unicellular head. Established by AL-ZUBAIDY *et al.* (2015) two cell structure of the glandular trichomes of genus *Clinopodium*, was not found in this study.

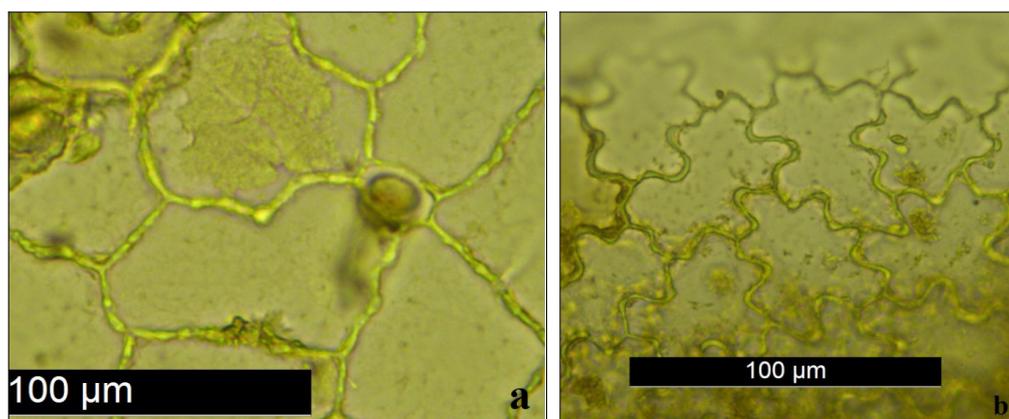


Fig. 1. Basal epidermal cells of *Micromeria frivaldszkyana* (a) and *Clinopodium vulgare* (b).

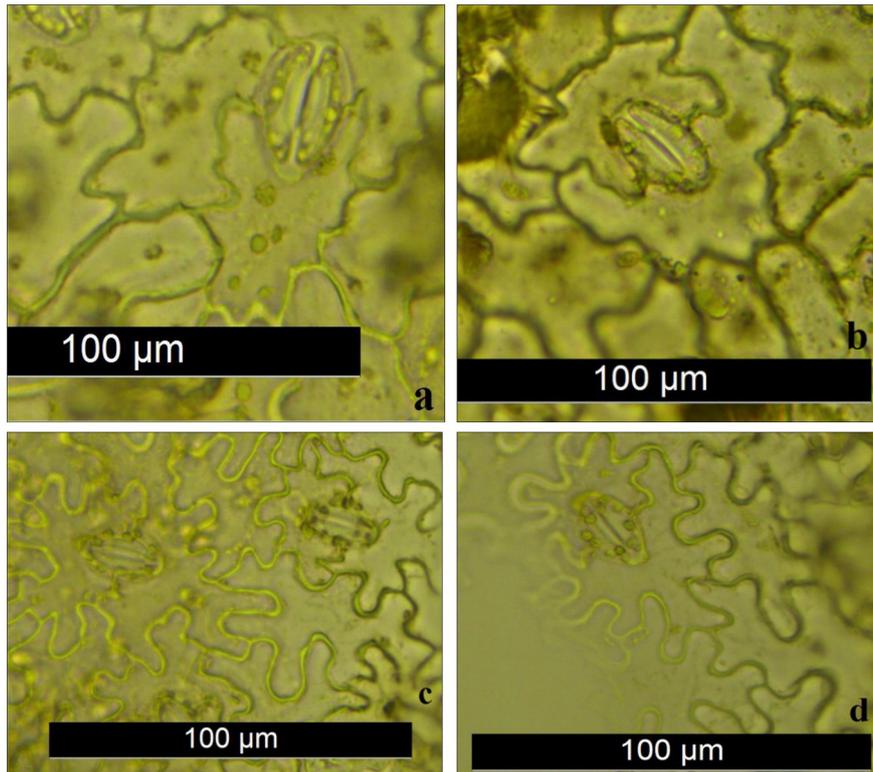


Fig. 2. Anomocytic and diacytic stomatal type of *Micromeria frivaldszkyana* (a, b) and *Clinopodium vulgare* (c, d).

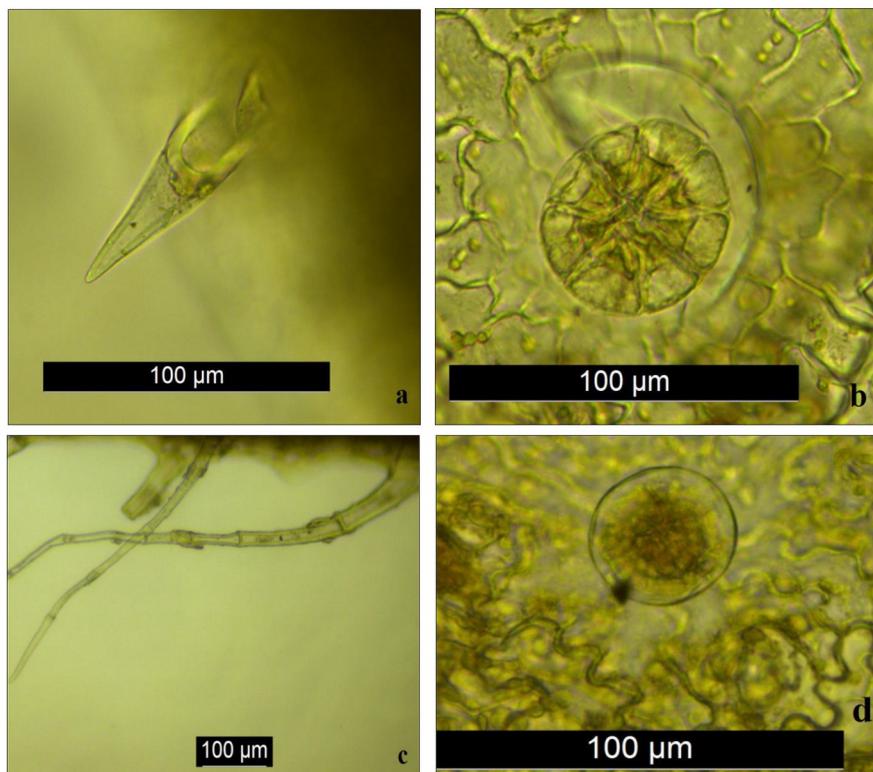


Fig. 3. Covering and glandular trichomes of *Micromeria frivaldszkyana* (a, b) and *Clinopodium vulgare* (c, d).

Abaxial epidermis

The results of the statistical processing of quantitative data by using Students' T-Test, for the abaxial epidermis in both species are presented on Table 1.

The table shows that a statistically significant difference ($p \leq 0.001$) between the two types exists with respect to all seven quantitative features. The number of stomata at *M. frivaldszkyana* (mean value of 201.95 number/mm²) is significantly larger than that of *C. vulgare* (mean value of 100.49 number/mm²). In proportion to this trend is the reduction in the size of the stomata at *C. vulgare*. *M. frivaldszkyana* shows higher mean values for width and length of the stomatal cells. The statistical differences with the highest significant difference ($p \leq 0.001$) regarding these three quantitative dimensions of the stomata apparatus between the two species necessitate further ecological studies of the two

taxa. The need for these studies is based on the claims made by ZARINKAMAR (2007), who reports that regulation of the size, density and placement of the stomata is a major way of adapting plants to environmental conditions. A smaller number is found in the major epidermal cells of *C. vulgare* (696.10 number/mm²) at the expense of larger sizes (width and length) than at *M. frivaldszkyana* ($P \leq 0.001$). One of the major contributing factors of small cells according to NINOVA & DUSHKOVA (1981) are the lower temperatures in the habitat. Mean cuticle thickness at *M. frivaldszkyana* (1.81 μm) are twice as large as those of *C. vulgare* (0.91 μm). The statistically significant difference between the two species for this sign is again of the highest significance ($p \leq 0.001$).

Adaxial epidermis

Table 2 shows the results obtained from the treatment of adaxial epidermis data for both taxa.

Table 1. Comparison of the mean values of anatomical characteristics in *Micromeria frivaldszkyana* and *Clinopodium vulgare* for abaxial epidermis. Legend: X_1 – Mean value of *M. frivaldszkyana*; X_2 – Mean value of *C. vulgare*; SE – Standart Error of Mean value; T – Students' T-test; p – Accuracy indicator; $p \leq 0.001$ - ***.

Feature	<i>M. frivaldszkyana</i> $X_1 \pm \text{SE}$	<i>C. vulgare</i> $X_2 \pm \text{SE}$	T	p
Stomata (Number)	201.95±3.34	100.49±3.53	20.87	0.000***
Stomata (Width)	4.02±0.06	0.95±0.03	44.15	0.000***
Stomata (Length)	5.87±0.07	5.05±0.05	9.92	0.000***
Epidermal cells (Number)	865.37±8.46	696.10±6.04	16.29	0.000***
Epidermal cells (Width)	5.41±0.07	5.86±0.06	-4.73	0.000***
Epidermal cells (Length)	14.81±0.27	18.07±0.26	-8.71	0.000***
Cuticle (Thickness)	1.81±0.03	0.91±0.03	22.43	0.000***

Table 2. Comparison of the mean values of anatomical characteristics in *Micromeria frivaldszkyana* and *Clinopodium vulgare* for adaxial epidermis. Legend: X_1 – Mean value of *M. frivaldszkyana*; X_2 – Mean value of *C. vulgare*; SE – Standart Error of Mean value; T – Students' T-test; p – Accuracy indicator; $p \leq 0.05$ - *; $p \leq 0.001$ - ***.

Feature	<i>M. frivaldszkyana</i> $X_1 \pm \text{SE}$	<i>C. vulgare</i> $X_2 \pm \text{SE}$	T	p
Stomata (Number)	42.93±2.57	37.56±2.33	-1.55	0.125
Stomata (Width)	3.03±0.04	2.87±0.05	-2.51	0.014*
Stomata (Length)	6.10±0.04	4.83±0.05	-21.08	0.000***
Epidermal cells (Number)	714.15±6.43	465.37±7.97	-24.30	0.000***
Epidermal cells (Width)	6.34±0.07	7.96±0.03	20.04	0.000***
Epidermal cells (Length)	17.09±0.18	13.01±0.07	-21.23	0.000***
Cuticle (Thickness)	1.91±0.03	1.12±0.03	-19.72	0.000***

Analyzing the upper leaf surface, it was found that there is no statistically significant difference between the number of stomata in the two species, which defines the sign as unstable. The least significant difference ($p \leq 0.05$) is the difference between the width of the stomata, where *M. frivaldszkyana* shows higher mean values (3.03 number/mm²). The trend in the mean values for the other features is similar to that of the abaxial epidermis. The only difference is in the length of the epidermal cells, which in the adaxial epidermis is larger in *M. frivaldszkyana*. The statistically significant difference ($p \leq 0.001$) for the sign length, number, width, and length of the major epidermal cells and thickness of the upper cuticle defines them as stable and suitable for the correct determination of the two taxa.

Conclusions

A comparative anatomical study of the leaf epidermis in the species *M. frivaldszkyana* and *C. vulgare* was made for the first time in Bulgaria. The two species are indistinguishable in terms of the quality characteristics tracked - the type of stomata apparatus (diacytic and anomocytic) and type of trichomes (multicellular, linear covering trichomes and multicellular, stacked glandular trichomes). The differences between the values of the quantitative marks (number, width and length of the stomata on the lower epidermis; length of the stomata on the upper epidermis; number, width and length of the main epidermal cells on the two epidermis; thickness of the upper and lower cuticles) are significant, which defines them as suitable for distinguishing the two taxa.

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