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Anatomical Investigation of Marrubium friwaldskyanum Boiss. and Marrubium peregrinum L. (Lamiaceae) from Bulgaria

Donika P. Gyuzeleva[1], Plamen S. Stoyanov[1,2], Anelia V. Bivolarska[3], Rumen D. Mladenov[1,2], Tsvetelina R. Mladenova[1], Venelin H. Petkov[4], Krasimir T. Todorov[1*]

[1]University of Plovdiv "Paisii Hilendarski", Faculty of Biology, Department of Botany and Biological education, 24 TzarAssen Str., Plovdiv 4000, BULGARIA

[2]Medical University of Plovdiv, Faculty of Pharmacy, Department of Bioorganic Chemistry, 120 Bratya Bukston Blvd., Plovdiv 4000, BULGARIA

[3]Medical University of Plovdiv, Faculty of Pharmacy, Department of Medical Biochemistry, 15 A Vasil Aprilov Blvd., Plovdiv 4000, BULGARIA

[4]Department "Global Quality", LEO Pharma A/S, 285 Cashel Rd, Crumlin, D12 E923, Dublin, IRELAND

[*]Corresponding author: ktodorov@uni-plovdiv.bg

Abstract. The present study presents data on the anatomical characteristics of the leaf and stem in *Marrubium friwaldskyanum* and *Marrubium peregrinum*. The leaves in both species are amphistomatic with diacytic and anomocytic stomata. The leaf lamina shows differentiation of palisade and spongy tissue, and collateral vascular bundles. Non-glandular and glandular trichomes have been found on the epidermis of the leaves and stem. The non-glandular trichomes are unicellular linear and multicellular branched. Glandular trichomes are peltate, with a single-celled or two-celled structure. There is a difference between the two species in terms of the width of the epidermal cells of the stem, the thickness of the cortex, the thickness of the xylem and the phloem in the stem. The established differences in the anatomical features can be useful in future taxonomic studies within the genus *Marrubium*.

Key words: Plant anatomy, Marrubium, Lamiaceae.

Introduction

The genus *Marrubium* L. belongs to the family Lamiaceae and is represented worldwide by about 40 species (Akgül et al., 2008). Representatives of the genus are herbaceous, annual and perennial plants, distributed mainly in the Iranian-Turanian and Mediterranean phytogeographical region (Hedge, 1992). Some of them have medicinal properties and are used as spices, and others are grown as ornamental plants (Meyre-Silva & Cechinel-Filho, 2010; Estilai & Hatemi, 1990; Büyükkartal et al., 2016). The genus *Marrubium* is represented with the greatest diversity in Turkey (Akgül & Ketenoğlu, 2014), where 21 taxa are found, 12 of which are endemic (Cullen, 1982; Ekim et al., 2000; Aytaç et al., 2012). In the flora of Europe, the genus is represented by 12 species (Tutin et al., 1972). There are 4 species in the Bulgarian flora – *M. friwaldskyanum* Boiss., *M. vulgare* L., *M.*

peregrinum L., *M. parviflorum* Fisch et Mey (Andreev et al., 1989).

M. friwaldskyanum Boiss. is a rare species and Bulgarian endemic (Kozhuharov, 1992; Delipavlov & Cheshmedzhiev, 2011), included in the Red Book of Bulgaria (Meshinev, 2015), as well as in the Red list of Bulgarian vascular plants (Petrova & Vladimirov, 2009) with category "vulnerable". The plant is perennial, herbaceous with yellow to creamy yellow flowers, gathered in short verticillaster and elliptical to oval leaves.

M. peregrinum is also a perennial herbaceous plant with lanceolate to elliptical leaves, narrowed at the base and to the top. The species is included in the list of medicinal plants of the Medicinal Plants Act (2000).

So far, cytotoxic activity of methanolic extraction from *M. friwaldskyanum* herbs (Kozyra et al., 2020), antioxidant (Kaurinović et al., 2010, 2011; Stanković, 2011) and antimicrobial activity of *M. peregrinum* extracts (Radojević et al., 2013), composition of diterpenoids and phenolic compounds in *M. friwaldskyanum* and *M. peregrinum* (Piozzi et al., 2006; Hennebelle et al., 2007; Kozyra et al., 2020; Zheljazkov et al., 2022), have been studied. Studies on the reproductive biology and reproductive capacity, leaves, stem, calyx, corolla, seeds and pollen surfaces analysis by Scanning Electron Microscopy of *M. friwaldskyanum* have been held by Zheljazkov et al. (2022). Data concerning anatomical structure of vegetative organs of these species are missing in the literature.

Since the main characteristics of taxonomic value in members of the Lamiaceae family are the type of trichomes (Marin et al., 1994; Navorro & El-Qualidi, 2000), the type of epidermal structure is covered in the Cantino studies (1990), which described two main types - non-glandular and glandular trichomes, as non-glandular are more common. In species of the genus *Marrubium*, stellate non-glandular and branched non-glandular trichomes are found on all parts of plants (Bosabilidis, 1990; Kaya et al., 2003; Upson & Andrew, 2004; Ahvazi et al., 2016).

The structure of glandular trichomes of *M. cuneatum* and *M. vulgare* has been studied by Baher et al. (2004), Belhattab & Larous (2006), Dmitruk & Haratym (2014).

Haratym & Weryszko-Chmielewska (2017) conduct a detailed study of the structure of glandular trichomes in *M. vulgare*. The authors describe peltate trichomes with a single-celled stalk and an 8-cell head and capitate trichomes of 3 types: with long stalk and unicellular head, with a short stalk and a two-celled head, and with a short stalk and a four-celled head. Two types of non-glandular trichomes are also mentioned: multicellular linear (found on the stem and the lower surface of the leaves and calyx) and multicellular branched (located mostly on the upper leaf surface).

There are descriptive anatomical studies of *M. vulgare* concerning bioaccumulative properties (Moreno–Jimenez et al., 2006) and M. anisodon (Talebi et al., 2019a) concerning leaf structure. In another study, Talebi et al. (2019b) examine the anatomical structure of the stem of 6 species of the genus *Marrubium* found in Iran. Ahvazi et al. (2017) describe the taxonomic, morphological and pharmacological features of *M. vulgare* from Iran.

It is obvious that at present research on the genus mainly concerns the features of pollen, anatomomorphological and chemical characteristics (Akgül et al., 2008; Ahvazi et al., 2016; Kharazian & Hashemi, 2017).

Cross-sectional studies of the leaf lamina of *M. trachyticum* show a bifacial structure with collateral vascular bundles (Akçin & Camili, 2018). The stomata are of the anomocytic type, located on both leaf surfaces, which defines the structure as amphistomatic.

The analysis of the literature data and the identified lack of information on comprehensive anatomical studies on *M. friwaldskyanum* and *M. peregrinum*

determined the purpose of the present study, namely the anatomical study of *M. friwaldskyanum* and *M. peregrinum*, in order to enrich the information on the genus and Bulgarian endemic plants.

Material and Methods

Plant material

Plant material (leaves and stems) from *M. friwaldskyanum* and *M. peregrinum* (Fig. 1) was collected from natural habitats during the 2020 growing season (Table 1). In order to prepare histological preparations, the vegetative organs were initially fixed in 70% ethanol.

Fig. 1. General view of *M. friwaldskyanum* (A) and *M. peregrinum* (B).

Table 1. List of studied taxa and localities of their collection.

Species	Locality(latitude/longitude and altitude)	Floristic region and year of sampling
M. friwaldskyanum Boiss.	42°09' N; 24°12'E/1136 m	Fortress Cepina, Rhodopes Mts. (western), 2020
M. peregrinum L.	42°04' N; 24°47'E/389 m	Krichim, Rhodopes Mts. (western), 2020

Anatomical methods

The analysis of leaves and stems was done following the classical methods of Metcalfe & Chalk (1950). For easier description of the epidermal structures, as well as the underlying tissues, a large amount of trichomes on the leaf and stem surface has been removed. The following qualitative and quantitative features were observed: type of trichomes on the leaf and stem surface; length and width of leaf and stem epidermal cells (μm); type, number (mm^2), length and width (μm) of the stomata on the adaxial and abaxial epidermis; mesophyll thickness (palisade and spongy) in the leaves (μm); stem cortex thickness (μm); thickness of xylem and phloem in the stem (μm). In order to ensure the reliability of the results for each quantitative trait, 30 prominent fields were examined and 30 measurements were made, respectively. The light microscope images were taken with a Magnum T Trinocular microscope, equipped with a Si5000 photo documentation system in the Department of Botany and Biological Education at the Faculty of Biology, Plovdiv University "Paisii Hilendarski".

Statistical methods

Statistical analyzes were performed with statistical program SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The main descriptive statistical values of the variation variables: mean value (mean), median (median), standard deviation (SD) and standard error (SE) were calculated according to standard methods. Quantitative (variational) variables were tested for normality of distribution with the Kolmogorov-Smirnov D-Test. The difference between the mean values of the normally distributed quantitative variables of two independent groups was estimated using the non-pair t-test for independent groups (Independent-samples t-test).

The possibilities of graphic analysis are used to illustrate the processes and phenomena.

In all analyzes for a level of statistical significance at which the null hypothesis is rejected (i.e. there is a difference between the compared values) p <0.05, respectively confidence probability greater than 0.95 (95%).

Results and Discussion

Leaf anatomy

Analyzing the leaf surface of *M. friwaldskyanum* and *M. peregrinum*, the epidermis was found, including ordinary epidermal cells, stomata and trichomes of different structures. The basic cells in both species have undulated cell walls and different sizes, which is in accordance with the classification of Aneli (1975) and the studies of Mladenova et al. (2019) in other members of the Lamiaceae family. In both taxa, the predominant stomata are of the diacytic type, and in some places an anomocytic type of stomata is found (Fig. 2).

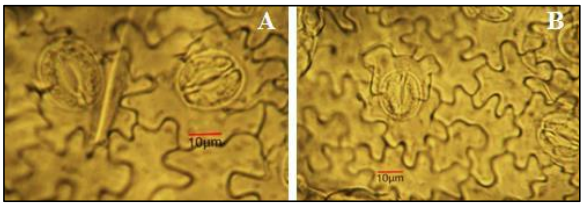

Fig. 2. Stomatal apparatus in *M. friwaldskyanum* (A) and *M. peregrinum* (B).

Their location on the two leaf surfaces defines the leaves as amphistomatic, which is in line with the statement of Büyükkartal et al. (2016) and Tüylü et al. (2017), for other species of the genus *Marrubium*. The indumentum in *M. friwaldskyanum* is represented by two types of trichomes - non-glandular and glandular. Non-glandular trichomes are unicellular linear and multicellular strongly branched (Fig. 3A, B). Glandular trichomes are peltate, stacked (Fig. 3C), with a short unicellular stalk (Fig. 3D, E) or bicellular structure (Fig. 3F).

These results are similar to those reported by Zheljazkov et al. (2022) for the same species collected from another locality. In *M. peregrinum*, only a large number of multicellular branched non-glandular trichomes (Fig. 4A) were found, while in structure glandular trichomes are peltate, stacked (Fig. 4B), with a unicellular stalk and a secretory cell (Fig. 4C) and with a unicellular stalk and a bicellular secretory cell (Fig. 4D).

The cross-section of the leaf lamina in the two studied species showed epidermal cells who are arranged in one row, oval in shape. In *M. friwaldskyanum*, the adaxial and abaxial epidermises are distinguished with a mesophyll consisting of one row of palisade tissue to the adaxial surface and spongy cells to the abaxial surface (Fig. 5A). In *M. peregrinum*, palisade tissue is found on both surfaces (Fig. 5B). The central vein of the leaves in the two studied species is represented by closed collateral vascular bundle (Fig. 5C, D).

The data from the statistical processing of the quantitative indicators reflecting the leaf structure of *M. friwaldskyanum* are presented in Table 2.

Based on the performed statistical correlation analyzes between leaf traits within the species, no statistical reliability was established (p> 0.05). Analyzing the mark length of the stomata on both epidermis (p = 0.250), the obtained average values on the abaxial epidermis are close to those on the adaxial epidermis. As for the width of the stomata on the two leaf surfaces, similar mean values are again established (p = 0.648). Regarding the number of stomata (per mm^2), despite the significant difference in the average values of the scar on the two epidermis, no statistically significant difference was found (p = 0.890). This fact determines the amphistomatic nature of the leaves, as noted by Büyükkartal et al. (2016) and Akcin & Camili (2018) for other species of the genus *Marrubium*. The values of p when comparing the length and width of epidermal cells are 0.322 and 0.147, respectively. Palisade and spongy tissue occupy almost the same space in the leaf structure, which is evident from the close values of the two scars (p = 0.147).

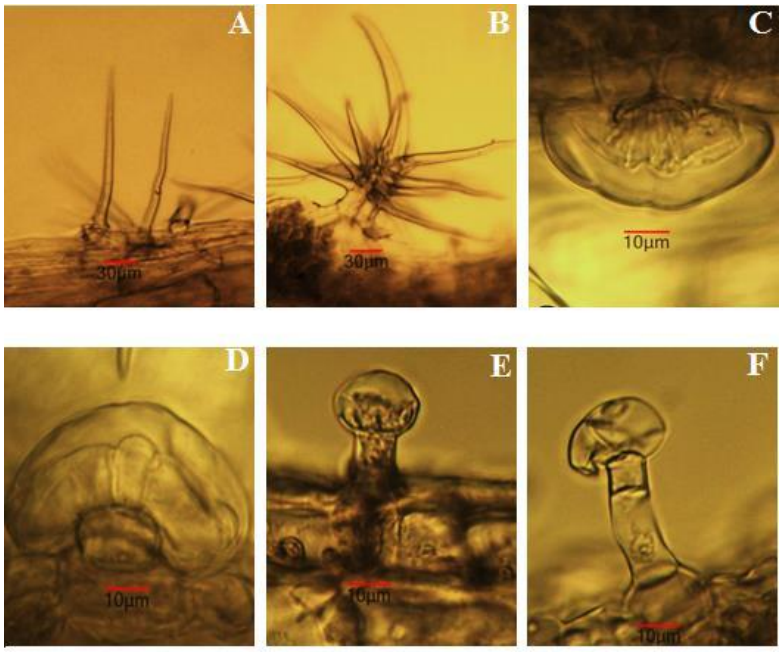

Fig. 3. Non-glandular and glandular trichomes in *M. friwaldskyanum*. A - unicellular linear non-glandular trichomes; B - multicellular branched non-glandular trichome; C – glandular peltate trichome; D, E - glandular trichomes with unicellular stalk; F - glandular trichomes with bicellular stalk.

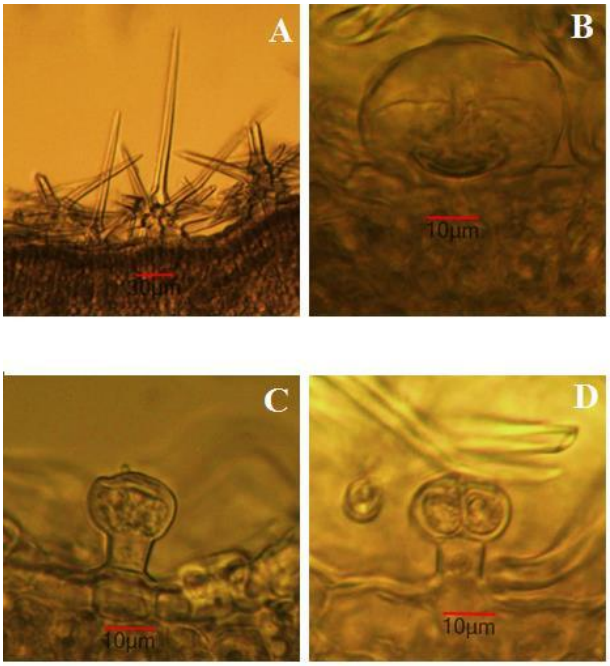

Fig. 4. Non-glandular and glandular trichomes in *M. peregrinum*. A - multicellular branched non-glandular trichomes; B - glandular peltate trichome; C – glandular trichome with a unicellular stalk and secretory cell; D – glandular trichome with a unicellular stalk and a bicellular secretory cell.

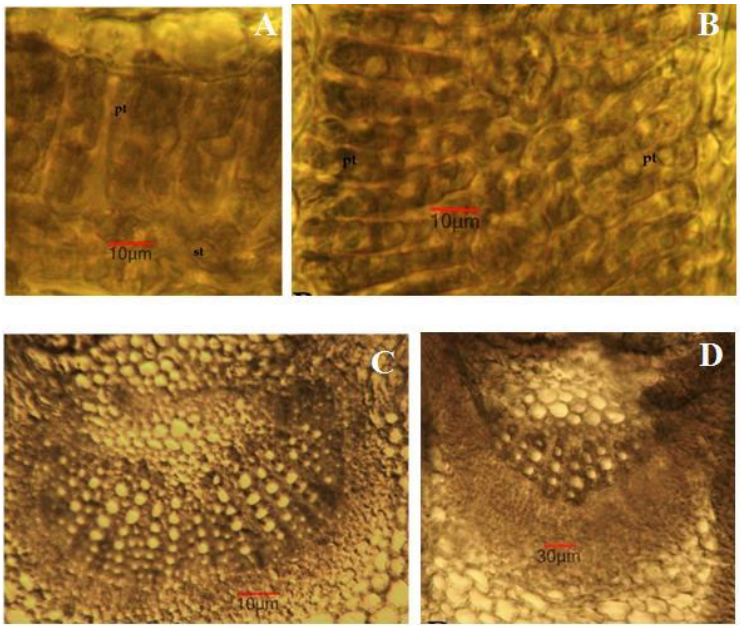

Fig. 5. Cross section of the leaf lamina. A – *M. friwaldskyanum*; B – *M. peregrinum*; C – Vascular bundle by *M. friwaldskyanum*; Vascular bundle by *M. peregrinum*; pt – palisade tissue; st – spongy tissue.

Table 2. Descriptive statistics *of M. friwaldskyanum*. Legend: SD – Standard deviation; SEM – Standard Error of Mean.

Feature	Mean±SD	95% Confidence Interval	Median	SEM	Minimum	Maximum	One-Sample Kolmogorov-Smirnov Test
Abaxial epidermis stomata (Length, µm)	5.12±0.28	5.02-5.22	5.06	0.05	4.14	5.52	p=0.054
Abaxial epidermis stomata (Width, µm)	3.95±0.34	3.82-4.07	3.91	0.06	3.22	4.37	p=0.470
Abaxial epidermis stomata (Number, per mm^2)	140.57±18.85	133.53-147.61	146.00	3.44	98	171	p=0.052
Adaxial epidermis stomata (Length, µm)	4.81±0.31	4.70-4.93	4.83	0.55	4.37	5.29	p=0.247
Adaxial	3.90±0.17	3.84-3.97	3.91	0.03	3.68	4.14	p=0.117

epidermis stomata (Width, µm)							
Adaxial epidermis stomata (Number, per mm^2)	45.47±20.15	37.94-52.99	49.00	3.68	24.00	98.00	p=0.083
Epidermal cells abaxial epidermis (Length, µm)	3.57±0.24	3.48-3.66	3.68	0.04	3.22	3.91	p=0.156
Epidermal cells abaxial epidermis (Width, µm)	2.51±0.33	2.38-2.63	2.30	0.06	2.07	3.22	p=0.008
Epidermal cells adaxial epidermis (Length, µm)	4.38±0.86	4.06-4.70	4.37	0.16	2.53	5.75	p=0.035
Epidermal cells adaxial epidermis (Width, µm)	2.97±0.47	2.79-3.14	3.10	0.08	1.84	3.45	p=0.043
Palisade tissue (Thickness, µm)	9.03±0.60	8.81-9.25	9.20	0.11	7.59	10.12	p=0.280
Spongy tissue (Thickness, µm)	9.12±1.39	8.60-9.64	8.28	0.25	7.82	11.96	p=0.034

The results obtained from the analysis of leaf characteristics in *M. peregrinum* are presented in Table 3.

The same structure in the internal structure of the two epidermis of *M. peregrinum* determines the smaller number of features described in the table. The stomata frequency on both surfaces is close in average to that of the lower epidermis in *M. friwaldskyanum*.

Stem anatomy

The cross-section of the stem of the two studied species shows the typical for the representatives of the family Lamiaceae four-edged shape. Cortex and pith are clearly distinguishable. Parenchymal cells are mainly present in the cortex, with several layers of collenchyma being observed in the corners. The vascular tissues have a non-bundled structure (Fig. 6).

The epidermis consists of round or rectangular cells covered with a thick cuticle. These features of the covering tissue are also mentioned by Akçin & Camili (2018) in another species of the genus *Marrubium*. On the stem surface of *M. friwaldskyanum* and *M. peregrinum* a large number of trichomes are observed, which in structure and type highly correspond to the trichomes covering the leaves. In *M. friwaldskyanum*, both unicellular linear and multicellular branched non-glandular trichomes are found (Fig. 7A, B). Glandular trichomes in this species are of two types: with a unicellular stalk and a secretory cell, and with a bicellular stalk and a secretory cell (Fig. 7C, D).

In *M. peregrinum*, the non-glandular trichomes are multicellular branched (Fig. 8A), and the glandular trichomes are composed of a bicellular stalk and a bicellular secretory cell (Fig. 8B).

The analyzed values of the quantitative indicators affecting the stem of the studied taxa are presented in Table 4.

The comparative analysis (Independent Samples T-test) between the stem indicators of the two taxa showed that there was no statistically significant difference in epidermal cell length alone. For the other studied traits, the values for the two species show significant differences ($p < 0.0001$). The identified significant differences are presented graphically in Fig. 9.

Table 3. Descriptive statistics *of M. peregrinum*. Legend: SD – Standard deviation; SEM – Standard Error of Mean.

Feature	Mean±SD	95% Confidence Interval	Median	SEM	Minimum	Maximum	One-Sample Kolmogorov-Smirnov Test
Stomata (Length, µm)	4.41±0.24	4.31-4.50	4.37	0.04	3.91	4.83	p=0.089
Stomata (Width, µm)	3.15±0.37	3.01-3.29	3.22	0.07	2.53	3.68	p=0.111
(Number, per mm^2)	120.47±24.63	111.27-129.66	122.00	4.50	73.00	171.00	p=0.060
Leaf epidermal cells (Length, µm)	4.73±0.54	4.53-4.93	4.83	0.10	3.45	5.98	p=0.713
Leaf epidermal cells (Width, µm)	2.94±0.68	2.68-3.19	2.99	0.12	1.84	4.60	p=0.679
Palisade tissue (Thickness, µm)	5.95±0.91	5.61-5.29	5.75	0.16	4.60	7.59	p=0.479

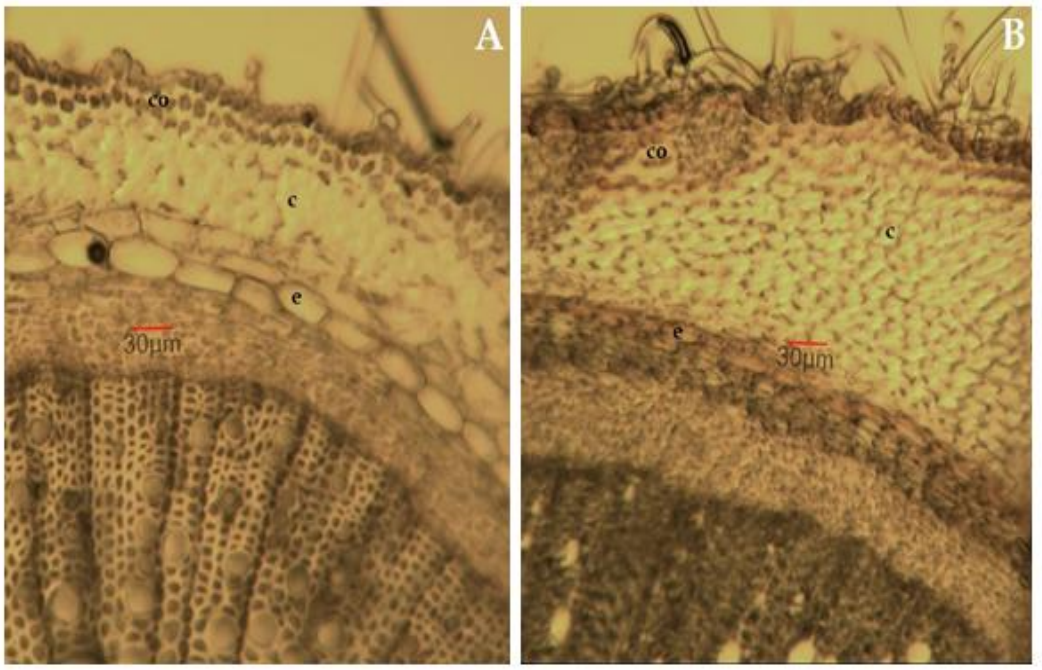

Fig. 6. Cross section of the stem. A - *M. friwaldskyanum*; B – *M. peregrinum*; co – collenchyma; c – cortex; e – endodermis.

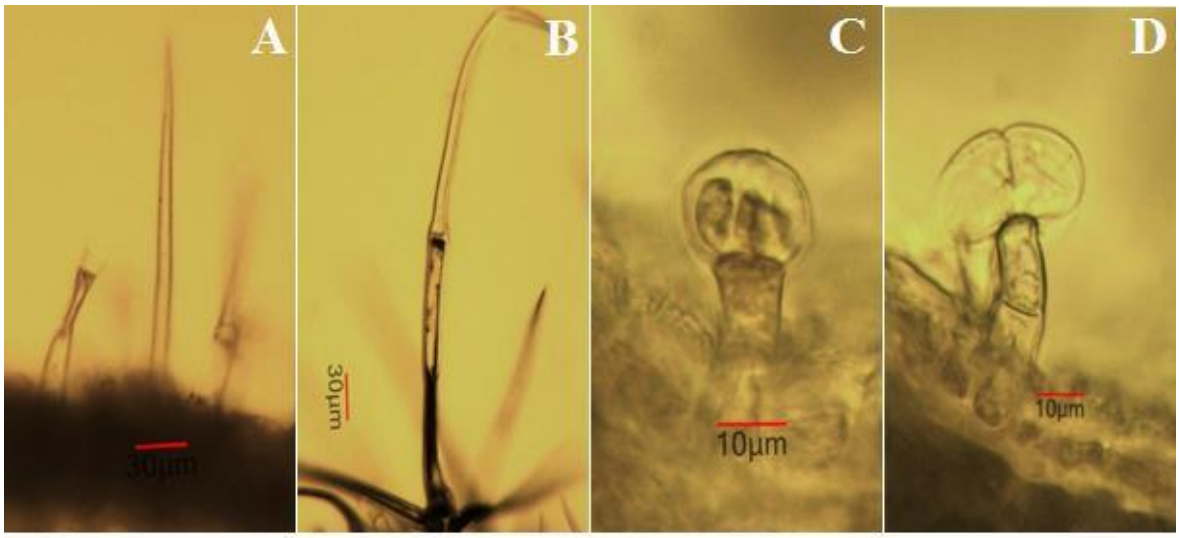

Fig. 7. Type of trichomes on the stem surface of *M. friwaldskyanum*. A - unicellular linear non-glandular trichome; B - multicellular branched non-glandular trichome; C – glandular trichome with a unicellular stalk and a secretory cell; D – glandular trichome with a bicellular stalk and a secretory cell.

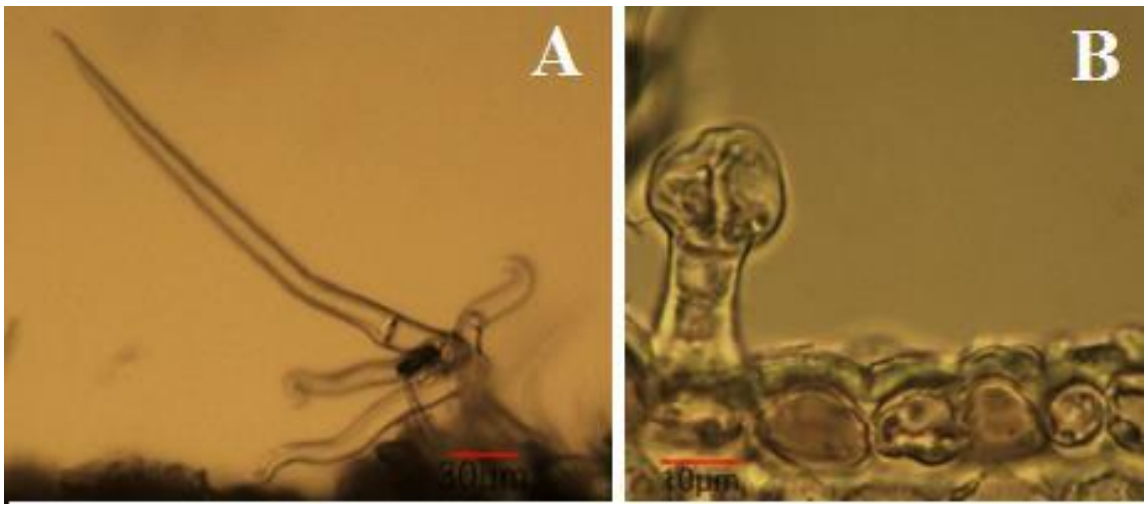

Fig. 8. Type of trichomes on the stem surface of *M. peregrinum*. A - multicellular branched non-glandular trichome; B – glandular trichome with bicellular stalk and a secretory cell.

Table 4. Descriptive statistics of the studied stem features of *M. friwaldskyanum* and *M. peregrinum*. Legend: SD – Standard deviation; SEM – Standard Error of Mean.

Feature	Mean±SD	95% Confidence Interval	Median	SEM	Minimum	Maximum	One-Sample Kolmogorov-Smirnov Test
			M. friwaldskyanum				
Stem epidermal cells (Length, µm)	4.00±0.58	3.78-4.22	4.02	0.11	2.99	4.83	p=0.305
Stem epidermal cells (Width, µm)	2.81±0.24	2.72-2.90	2.76	0.04	2.53	3.22	p=0.070
Stem cortex (Thickness, µm)	36.57±5.88	34.38-38.77	36.57	1.07	23.92	45.08	p=0.142
Xylem (Thickness, µm)	81.16±2.53	80.21-82.10	81.65	0.46	74.98	84.18	p=0.316
Phloem (Thickness, µm)	14.60±1.56	14.02-15.19	15.64	0.28	11.96	16.10	p=0.006

	M. peregrinum						
Stem epidermal cells (Length, µm)	4.05±0.44	3.89-4.22	4.14	0.08	3.22	4.60	p=0.059
Stem epidermal cells (Width, µm)	1.93±0.23	1.84-2.02	1.95	0.04	1.61	2.30	p=0.096
Stem cortex (Thickness, µm)	26.56±3.15	25.38-27.73	24.95	0.57	23.00	34.04	p=0.080
Xylem (Thickness, µm)	97.14±7.13	94.47-99.80	95.45	1.30	86.94	113.16	p=0.349
Phloem (Thickness, µm)	13.62±1.87	12.92-14.31	13.80	0.34	9.66	16.33	p=0.606

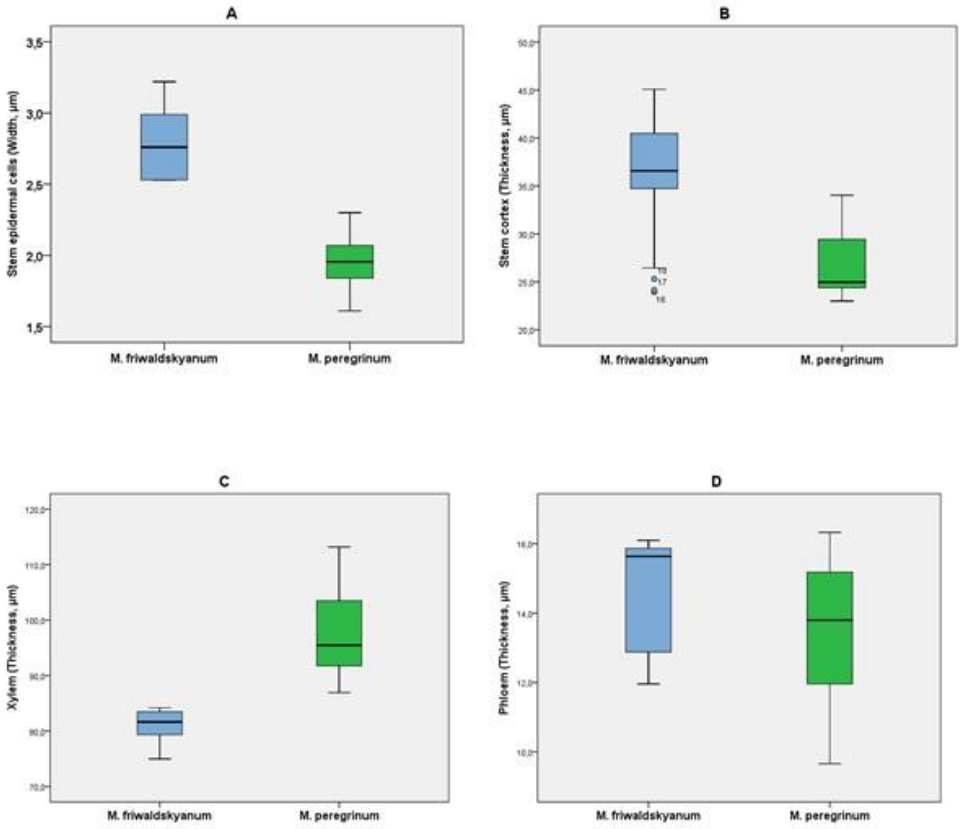

Fig. 9. Significant differences between the mean values of the stem indicators of *M. friwaldskyanum* and *M. peregrinum*.

M. friwaldskyanum is characterized by wider epidermal cells than *M. peregrinum* (Fig. 9A). The cortex of the stem in *M. friwaldskyanum* is statistically significantly thicker than that of *M. peregrinum* (Fig. 9B). Comparing the thickness of the two vascular tissues in both species, it was found that *M. friwaldskyanum* had a thinner layer of xylem (Fig. 9C) and a thicker layer of phloem (Fig. 9D).

Conclusions

The anatomical study of the species *M. friwaldskyanum* and *M. peregrinum* enriches the information about the genus and the endemic plants of Bulgaria. Described are: amphistomatic leaf structure with diacytic and anomocytic stomata, palisade and spongy chlorenchyma, unicellular and multicellular non-glandular trichomes with linear and branched structure, peltate, stacked, unicellular and bicellular glandular trichomes covering the leaf and the stem surface. The structure of the stem in both species is typical of the Lamiaceae family. Differences resulting from statistical processing of trait data: stem epidermal cell width, cortex thickness, and xylem and phloem thickness can be useful in future taxonomic studies within the genus *Marrubium*.

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