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# Phytochemical and Biological Traits of Endemic *Betonica bulgarica* (Lamiaceae)

**Tsvetelina Mladenova [1], Plamen Stoyanov [1,2], Krasimir Todorov [1], Delyana Davcheva [3,4], Gergana Kirova [4,5], Tanya Deneva [3,4], Donika Gyuzeleva [1], Rumen Mladenov [1,2] and Anelia Bivolarska [6,*]**

1 Department of Botany and Methods of Biology Teaching, Faculty of Biology, University of Plovdiv "Paisii Hilendarski", 24 Tzar Assen Street, 4000 Plovdiv, Bulgaria; cmladenova@uni-plovdiv.bg (T.M.); rummlad@uni-plovdiv.bg (R.M.); pstoyanov@uni-plovdiv.bg (P.S.); ktodorov@uni-plovdiv.bg (K.T.); donikag@uni-plovdiv.bg (D.G.)
2 Department of Bioorganic Chemistry, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vasil Aprilov Boulevard, 4000 Plovdiv, Bulgaria
3 Department of Clinical Laboratory, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vasil Aprilov Boulevard, 4000 Plovdiv, Bulgaria; Delyana.Davcheva@mu-plovdiv.bg (D.D.); Tanya.Deneva@mu-plovdiv.bg (T.D.)
4 Division of Pharmaceutical Innovations for Personalized Medicine, Research Institute at Medical University of Plovdiv, 15A Vasil Aprilov Boulevard, 4000 Plovdiv, Bulgaria
5 Department of Chemical Sciences, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vasil Aprilov Boulevard, 4000 Plovdiv, Bulgaria; Gergana.Kirova@mu-plovdiv.bg
6 Department of Medical Biochemistry, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vasil Aprilov Boulevard, 4000 Plovdiv, Bulgaria
* Correspondence: anelia.bivolarska@mu-plovdiv.bg; Tel.: +359-887611786

**Abstract:** *Betonica bulgarica* is an endemic species distributed in Bulgaria. The chemical composition of the essential oil analysed by GC–MS (Gas chromatography–mass spectrometry) and the content of trace elements analysed by ICP–MS (Inductively coupled plasma mass spectrometry) were determined. Additionally, a study on the types and distribution of trichomes was done using a microscope with a camera. The essential oil was characterized using a high concentration of sesquiterpene hydrocarbons, whose major compounds are β-caryophyllene (17.4%), germacrene D (9.9%), and β-bourbonene (6.7%). The contents of manganese (177.2 μg/g) and strontium (156.8 μg/g) were highest among the investigated micronutrients. Two types of trichomes were identified on the adaxial and abaxial epidermises of the leaves of *B. bulgarica*—covering and glandular. Peltate stacked glandular trichomes with a four-celled head of type B were observed on the leaf surface.

**Keywords:** *Betonica bulgarica*; ICP–MS (Inductively coupled plasma mass spectrometry); GC–MS (Gas chromatography–mass spectrometry); multielement analysis; trace elements; trichomes; endemic; essential oil; lamiaceae



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## 1. Introduction

Endemic plants make up 12.8% of the representatives of Bulgarian flora and have specificity and certain genetic peculiarities [1]. They are some of the most sensitive and vulnerable natural ecosystems in the country [2].

The genus *Betonica* L. is separate from the genus *Stachys* L., with four species [3]. The taxonomic independence of two of them—*Betonica officinalis* and *Betonica bulgarica*—is currently controversial.

In Bulgaria, *B. officinalis* can be found throughout the country at an altitude of up to 1400 m in grassy and shrubby areas [4]. *B. officinalis* is used in folk medicine for the treatment of nervous exhaustion, gallstones, stomach acids, high blood pressure, migraine, and neuralgia, as an ointment for cuts and wounds as well as against sweating [5,6].

The Bulgarian endemic *B. bulgarica* Degen et Neič. (*Stachys bulgarica* Hayek), described by the Hungarian botanist A. V. Degen and the Bulgarian botanist I. Neichev in 1906 on the basis of materials collected in the Central Stara Planina Mountains, grows outdoors in stony and sandy soils in oak and beech forests of the Stara Planina Mountains (Central, East) and the Thracian Lowlands [7–9]. *B. bulgarica* is an endemic plant protected by the Biological Diversity Act [10] and included in the *Red Data Book of Bulgaria* under the category "endangered" [9]. Three flavonoids have been found in significant amounts in *B. bulgarica*—rutin, quercetin, and hispidulin—associated with the antioxidant activity of the plant [11].

In recent years, the Bulgarian populations of *B. bulgarica* have been studied by Grozeva et al. [12–14]. Along with morphological, palinomorphological, and ecological studies, the authors also draw attention to the epidermis covering the leaves, stems, and flower parts of the plants as the main structure responsible for the accumulation of essential oils.

Although the healing properties of the species of the Lamiaceae family are likely due to their essential oils and flavonoids, in recent years, there has also been increasing interest in certain trace elements [15], whose presence could have a synergistic effect with these components [16]. Most trace elements are essential for higher plants, mainly in the composition of metalloenzymes and metalloproteins. They are also involved in a number of redox processes, processes of photosynthesis, breathing, expression and regulation of genes, protein synthesis, and protective mechanisms of plants [17]. So far, there have been no data on the presence of trace elements in *B. bulgarica* species.

The purpose of this study was to complement the available information about the endemic species *B. bulgarica* with regard to the anatomic morphological structures of the epidermis, the essential oil composition, and the microelement composition of the species from the plant habitat, which we discovered in the Bulgarka Nature Park.

## 2. Results and Discussion

### *2.1. Biological Traits*

Analysing the leafy and stem surfaces of *B. bulgarica*, two types of trichomes were identified: covering trichomes and glandular trichomes. The results obtained are in accordance with the claims of Metcalfe and Chalk [18], Maleci Bini and Giuliani [19], and Mladenova et al. [20] regarding the types of trichomes in the representatives of the Lamiaceae family.

In this study, two types of trichomes were identified on the upper and lower epidermises of the leaves of *B. bulgarica*-covering and glandular. Covering trichomes are multicellular linear unbranched, whereas glandular trichomes are peltate stacked with a four-celled head (Figure 1).

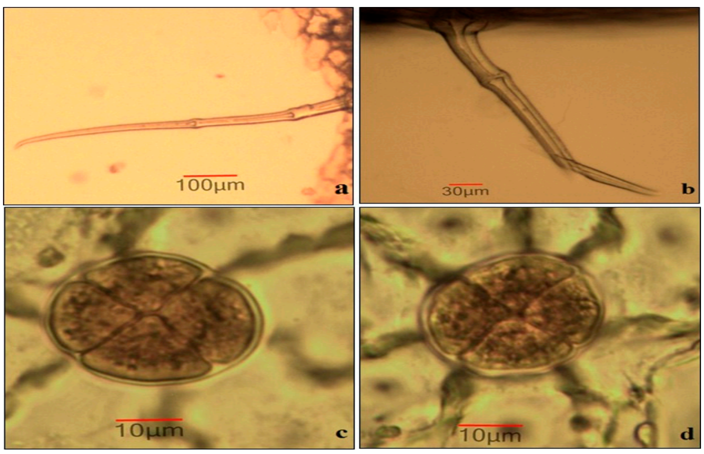


**Figure 1.** Trichomes on the leaf surface: (**a**) multicellular linear unbranched covering trichome on the adaxial epidermis; (**b**)multicellular linear unbranched covering trichome on the abaxial epidermis; (**c**) peltate stacked glandular trichome with a four-celled head on the adaxial epidermis; (**d**) peltate stacked glandular trichome with a four-celled head on the abaxial epidermis.

According to the descriptions of Giuliani and Maleci Bini [21], this type of glandular trichomes belongs to type B, where the secreting cell has a bicellular or quadricellular structure. Giuliani et al. [22] reported that this type of trichomes is mainly characterized by polysaccharide content.

Upon examining the stem surface, apart from multicellular linear unbranched trichomes, the presence of capitate stacked trichomes was also identified, which, however, end with a unicellular head (Figure 2). The presence of such multicellular glandular trichomes with a unicellular head was reported by Ya'ni et al. [23] regarding other species of the Lamiaceae family as well.

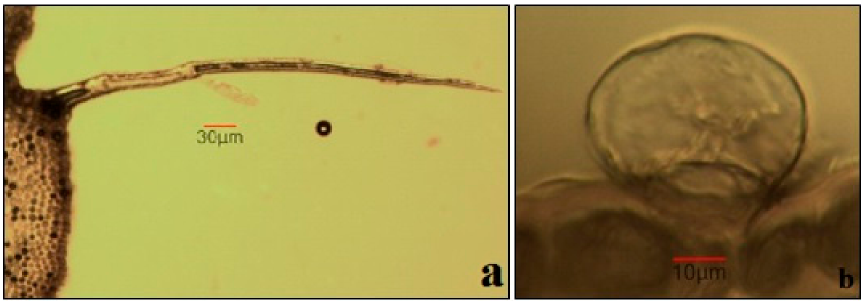


**Figure 2.** Trichomes on the stem surface: (**a**) multicellular linear unbranched covering trichome; (**b**) capitate stacked glandular trichome with a unicellular head.

### 2.2. Analysis of Essential Oils (GC–MS)

The total number of chemical constituents identified in essential oils was 64 for *B. bulgarica*. The highest yield of essential oil was extracted from the leaves, then from the flower, and then from the stem. The specific identified compounds and their percentages are compiled in Tables 1 and 2. The main constituents of the essential oil were sesquiterpene hydrocarbons (β-caryophyllene, 17.4%, and germacrene D, 9.9%).

**Table 1.** Essential oil content of the samples being examined.

| Sample | Quantity of Essential Oil | |
|---|---|---|
| | **mg** | **% *w/w* (Dry Matter)** |
| *Betonica bulgarica*—leaves | 5.04 | 0.10 |
| *Betonica bulgarica*—stem | 1.85 | 0.04 |
| *Betonica bulgarica*—flower | 3.73 | 0.07 |

**Table 2.** Phytochemical composition [%] of essential oils.

| No. | KI | Compound | Leaf | Stem | Flower |
|---|---|---|---|---|---|
| 1 | 855 | trans-Hexenal * | 0.6 | tr | 0.1 |
| 2 | 939 | α-Pinene | 0.9 | tr | 2.0 |
| 3 | 960 | Benzaldehyde | tr | tr | 0.3 |
| 4 | 979 | 1-Octen-3-ol | 3.6 | 0.5 | 0.7 |
| 5 | 985 | 6-Methyl-5-hepten-2-one | tr | n.d. | 0.1 |
| 6 | 988 | 2-Pentylfuran | tr | n.d. | 0.1 |
| 7 | 990 | Myrcene | 0.1 | n.d. | n.d. |
| 8 | 1007 | trans, trans-2,4-Heptadienal | 0.2 | n.d. | 0.1 |
| 9 | 1031 | Benzyl alcohol | tr | n.d. | 0.2 |
| 10 | 1042 | Benzene acetaldehyde | 0.5 | 0.1 | 1.0 |
| 11 | 1081 | 4-Methylbenzaldehyde | 0.1 | n.d. | 0.4 |
| 12 | 1096 | Linalool | 1.7 | 1.2 | 0.7 |
| 13 | 1100 | Undecane | 0.1 | 0.7 | tr |
| 14 | 1108 | Phenyl ethyl alcohol | 0.6 | 0.8 | 0.9 |
| 15 | 1126 | α-Campholenal | tr | tr | 0.2 |

**Table 2.** *Cont.*

| No. | KI | Compound | Leaf | Stem | Flower |
|---|---|---|---|---|---|
| 16 | 1144 | trans-Verbenol | 0.4 | 0.2 | 0.3 |
| 17 | 1188 | α-Terpineol | n.d. | 0.4 | 0.1 |
| 18 | 1195 | Myrtenol | n.d. | n.d. | 0.1 |
| 19 | 1167 | Octanoic acid | 1.1 | 1.1 | 0.6 |
| 20 | 1205 | Verbenone | 0.2 | tr | 0.1 |
| 21 | 1284 | trans-Anethole | 0.2 | 0.7 | 0.4 |
| 22 | 1293 | 2,4-Decadienal | tr | n.d. | 0.2 |
| 23 | 1351 | α-Cubebene | 1.0 | 0.9 | 0.4 |
| 24 | 1359 | Eugenol | 0.6 | 0.7 | 0.3 |
| 25 | 1375 | α-Ylangene | 0.5 | 0.4 | 0.3 |
| 26 | 1376 | α-Copaene | 1.6 | 1.1 | 1.1 |
| 27 | 1388 | β-Bourbonene | 6.7 | 5.4 | 2.0 |
| 28 | 1420 | β-Ylangene | 0.8 | 0.4 | tr |
| 29 | 1390 | β-Elemene | 0.2 | 0.5 | 1.6 |
| 30 | 1408 | Iso-caryophyllene | tr | 0.1 | 0.2 |
| 31 | 1419 | β-Caryophyllene | 8.6 | 5.9 | 17.4 |
| 32 | 1432 | β-Copaene | 2.8 | 2.1 | 1.0 |
| 33 | 1434 | trans-α-Bergamotene | tr | tr | 0.3 |
| 34 | 1433<br>1441 | Gurjunene + Aromadendrene | 0.3 | 0.3 | 0.4 |
| 35 | 1454 | α-Humulene | 3.9 | 2.8 | 6.4 |
| 36 | 1456 | trans-β-Farnesene | 1.3 | 1.7 | 1.8 |
| 37 | 1479 | γ-Muurolene | 5.7 | 4.8 | 2.6 |
| 38 | 1481 | Germacrene D | 9.9 | 2.4 | 3.6 |
| 39 | 1488 | trans-β-Ionone + Unknown | 1.1 | 1.1 | n.d. |
| 40 | 1500 | α-Muurolene | 0.5 | 2.4 | tr |
| 41 | 1512 | Amorphene | 0.3 | 0.3 | 0.3 |
| 42 | 1513 | γ-Cadinene | 4.8 | 4.3 | 0 |
| 43 | 1523 | δ-Cadinene | 6.0 | 4.6 | 2.9 |
| 44 | 1534 | Cadina-1,4-diene | 0.3 | 0.5 | 0.1 |
| 45 | 1538 | α-Cadinene | 0.5 | 0.6 | 0.1 |
| 46 | 1545 | α-Calacorene | 0.4 | 0.6 | 0.2 |
| 47 | 1563 | 1-nor-Bourbonanone | 0.2 | 0.6 | 0.1 |
| 48 | 1565 | β-Calacorene | 0.2 | 0.6 | 0.2 |
| 49 | 1563 | Nerolidol | 0.2 | tr | 0.2 |
| 50 | 1578 | Spatulenol | 0.8 | 0.5 | 0.3 |
| 51 | 1582 | Caryophyllene oxide | 3.4 | 4.7 | 5.2 |
| 52 | 1584 | β-Copaen-4-α-ol | 1.0 | 1.2 | 0.3 |
| 53 | 1594 | Salvial-4(14)-en-1-one | 0.6 | 0.8 | 0.2 |
| 54 | 1608 | Humulene 1,2-epoxide | 1.3 | 1.9 | 1.5 |
| 55 | 1640 | τ-Cadinol | 1.0 | 1.5 | 0.6 |
| 56 | 1650 | β-Eudesmol | 0.7 | 2.1 | 1.3 |
| 57 | 1654 | α-Cadinol | 1.1 | 1.8 | 1.3 |
| 58 | 1913 | Farnesyl acetone | 0.2 | 0.3 | 0.1 |
| 59 | | Hexahydrofarnesyl acetone | 0.5 | 0.6 | 1.3 |
| 60 | 1943 | Phytol | 1.1 | 1.4 | 0.2 |
| 61 | 1960 | Palmitic acid | tr | 0.1 | 0.1 |
| 62 | 2500 | Pentacosane | tr | 0.2 | 0.6 |
| | | Monoterpene | 4.1 | 3.2 | 4.4 |
| | | Sesquiterpene | 56.6 | 54.7 | 49.9 |
| | | Others | 39.3 | 42.1 | 45.7 |

* The specific isomer is not identified; tr—traces, <0.1%; n.d.—not detected.

Our results confirm data from the literature that the essential oils of plants of the genus *Stachys* are poor in mono- but rich in sesquiterpene hydrocarbons [24–29]. Sesquiterpene hydrocarbons are defined as main components of the oils of six plant species of the genus *Stachys* (including *B. officinalis* and *Stachys sylvatica*) from Serbia [26]. Iso-caryophyllene and β-caryophyllene (22.9%) are predominant in the essential oils of *B. officinalis* plants from

Montenegro [24]. Sesquiterpene hydrocarbons are the most widespread class of isolated volatile compounds in both *B. officinalis* and *S. sylvatica* that grow in Bulgaria, but unlike in our study on *B. bulgarica*, γ-muurolene prevails [30].

### *2.3. Analysis of Trace Elements*

In recent years, there has been increasing interest in certain trace elements in the environment that are considered to be a factor necessary for the proper functioning of living organisms. The contents of microelements in *B. bulgarica* are compiled in Table 3. The minerals are distributed in the following order: Mn > Sr > Fe > Ba > Si > Ti > Zn > Cu > Ba > Cu > Al > Rb > Ni > Pb > Mo > Cr > Cs. Except for Ba, all other elements are found to be present in greater amounts in the leaves and inflorescences compared with the stem. The content of manganese is highest in the plant. Its main function is to participate in the biosynthesis of lipids, lignins, and carbohydrates. It is involved in photosystem II (PSII), and it plays a structural role as a cofactor in many enzymes [31]. Despite the reports of several transporter families in Mn uptake in some plants, we still have limited knowledge about many other plant species [31]. The element strontium comes second in terms of amount. Due to limited data related to the toxicity and intake of strontium, the World Health Organization has not determined ranges for the safe and adequate nutritional level of strontium. The distribution of strontium in the human body is similar to that of calcium, which means that more than 99% is deposited in bones, connective tissues, and teeth [32]. Compared with studies on other plants [15,33,34], we found high concentrations of manganese (177.2 μg/g) and strontium (156.8 μg/g) in the dry mass of the plant being examined by us, which seems to be promising for the purposes of commercial exploitation.

**Table 3.** ICP–MS (Inductively coupled plasma mass spectrometry) analysis of plant samples (μg/g).

| Element | Cu | Zn | Fe | Cr | Rb | Si | Ti | Ni | Sr | Mn | Ba | Al | Mo | Cs | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. bulgarica* (leaves) | 16.9 ± 1.7 | 28.2 ± 0.4 | 119.9 ± 1.5 | 0.29 | 8.71 | 75.6 | 71.9 | 1.02 | 123.2 | 177.2 | 62.1 | 19.4 | 0.31 | 0.16 | 0.79 |
| *B. bulgarica* (flower) | 24.7 ± 1.5 | 29.05 ± 0.3 | 95.3 ± 4.43 | 0.16 | 13.95 | 67.7 | 60.2 | 0.96 | 156.8 | 67.5 | 76.2 | 12.3 | 0.16 | 0.20 | 0.90 |
| *B. bulgarica* (stem) | 19.3 ± 2 | 20.3 ± 0.5 | 85.9 ± 4.8 | 0.24 | 10.42 | 63.7 | 63.6 | 0.73 | 134.3 | 104.0 | 90.3 | 16.1 | 0.17 | 0.20 | 0.91 |

Results are presented as mean ± SD, where $n = 6$.

## 3. Materials and Methods

### *3.1. Plant Material*

Aerial parts from *Betonica bulgarica* were collected during flowering in June–July 2019 from the Bulgarka Nature Park, the areas of Uzana (42°45′02″ N, 25°14′18″ E). Plant materials were authenticated by Assoc. Prof. Plamen Stoyanov. Collected raw materials were dried at 25 °C and powdered. Voucher specimens for *Betonica bulgarica* (n. 062646) were deposited at the Herbarium of the University of Agriculture, Plovdiv, Bulgaria.

Applying the classic methods of Metcalfe and Chalk [35], histology samples of fresh plant materials (leaf and stem epidermises) were prepared, after which the morphology of the identified trichomes was examined. With the help of a Magnum T microscope equipped with a photo documentation system Si5000, light microscope pictures were taken at 50× and 400× magnifications.

### *3.2. Determination of Essential Oil Content*

Essential oil content was determined by means of a Likens-Nickerson apparatus. Ground plant material (5 g) was placed in a 100 mL round-bottom flask, and distilled water (50 mL) was added to it. Diethyl ether (3 mL) was placed in a 5 ml round-bottom flask. Distillation lasted for 2 hours. The diethyl ether was dried with water-free $Na_2SO_4$ and distilled under vacuum to constant weight.

The determination of the qualitative and quantitative compositions of the essential oil obtained was carried out through chromatographic analysis (GC-MS) using a gas chromatograph Agilent 7890B with a mass selective detector Agilent 5977A; carrier gas—helium, DB-5 MS column (5% phenylmethylpolysiloxane, 30 m, 0.25 mm I.D.).

Temperature mode: Injector and detector temperature: 260 °C. Column temperature: 60 °C. The temperature was held at 60 °C for 4 min, followed by raising it to 220 °C at a rate of 4 °C per min. Then the temperature was increased to 300 °C at a rate of 10 °C per min and was held at this level for 3 min.

### 3.3. Quantitative and Semiquantitative Multielement Analysis

In the current study, an inductively coupled plasma mass spectrometry (ICP–MS) method was used for the quantitative multielement analysis of Cu, Zn, and Fe and the semiquantitative analysis of Cr, Rb, Si, Ti, Sr, Mn, Ba, Al, Mo, Cs, and Pb in nitric acid solution of *B. bulgarica* obtained after microwave (MW)-assisted acid mineralization. All element standards used for the preparation of the calibration standard solutions were NIST traceable.

Microwave-assisted acid mineralization was performed by a Multiwave GO microwave digestion system with closed vessels provided by Anton Paar (Graz, Austria). Multielement determination was carried out by iCAP Qc ICP–MS (Thermo Scientific, Erlangen, Germany).

## 4. Conclusions

This is the first time that the structure of trichomes that cover the leaves and stems of populations of *B. bulgarica* in the Bulgarka Nature Park has been studied. For the first time, peltate stacked glandular trichomes with a four-celled head of type B have been identified. These trichomes could be used as a characteristic for the taxonomic discussion of *B. bulgarica* and *B. officinalis* species. The levels of manganese (177.2 μg/g) and strontium (156.8 μg/g) are predominant in the microelement composition of the endemic *B. bulgarica* in the researched habitat. It is the first time that we report on the essential oil composition of *B. bulgarica*, with the predominance of sesquiterpene hydrocarbons and major components: β-caryophyllene (17.4%), germacrene D (9.9%), and β-bourbonene (6.7%).

Since the growth and development of plants, as well as the synthesis and accumulation of essential oils in them, depends on the micronutrients absorbed by the roots [36], in the future, we plan to continue our research on the relationship between micronutrients and essential oils.

**Author Contributions:** Conceptualization, T.M. and D.G.; collection of the plants, P.S. and K.T.; methodology, D.D. and G.K.; software, D.G.; validation, T.D. and D.D.; formal analysis, P.S.; investigation, T.M.; data curation, A.B.; writing—original draft preparation, R.M.; writing—review and editing, A.B.; visualization, K.T.; supervision, A.B.; project administration, R.M. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that they have no competing interests.

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