ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 22(2) 2023, 99–117
https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 https://doi.org/10.24326/asphc.2023.4913
ORIGINAL PAPER
Accepted: 26.10.2022
Issue published: 28.04.2023

COMPARATIVE ANALYSIS OF THE BIOTECHNOLOGICAL POTENTIAL OF *Knautia drymeia* Heuff. AND K. *macedonica* Griseb.

Małgorzata Chrząszcz[1], Katarzyna Dos Santos Szewczyk[1], Agnieszka Dąbrowska[2], Dorota Tchórzewska[3]

[1] Department of Pharmaceutical Botany, Medical University of Lublin, Poland
[2] Botanical Garden of Maria Curie-Skłodowska University, Lublin, Poland
[3] Department of Cell Biology, Institute of Biological Sciences, Maria Curie-Skłodowska University

ABSTRACT

The present study of *Knautia drymeia* and *K. macedonica* is in line with the current trend of searching for new plant species that can potentially be used as medicinal herb materials. A comparative analysis of the morphological and anatomical structure of both species was performed together with the distribution of polyphenolic compounds, which was correlated with the tissue structure of plant organs. Quantitative phytochemical analyses were performed to supplement the biophysical analyses. Both species had a similar morphological, anatomical, and histological structure. Polyphenolic compounds were accumulated in the parenchyma tissue in an organ-specific mode, mainly in the leaves. The phytochemical analyses revealed organ- and species-dependent variations in the polyphenol content. Thus, the highest polyphenol amount was observed in the leaves, with equal levels of total polyphenols and phenolic acids in the leaves of *K. macedonica* and *K. drymeia*, respectively. The present study integrates morphological/histological analyses with investigations of the biotechnological/pharmaceutical potential of the studied plants and constitutes an innovative and holistic approach to the current research problem.

Key words: *Knautia*, morphology, histology, phenolic compounds, flavonoids, phenolic acids

INTRODUCTION

Nowadays, many new threats are posed by e.g. multidrug-resistant pathogens (MDR) and infections with zoonotic viruses (e.g. SARS-CoV-2). Therefore, the search for new therapeutic substances is extremely important. Particularly valuable are substances of natural plant origin, which can not only be a source of new drugs but also enhance therapeutic effects through synergism and in many cases are safer for the patient and the environment [Chrząszcz et al. 2020, Magryś et al. 2021]. Many plant species contain biologically active substances (BAS), which have enormous potential as medicinal herb materials (MHM) but have been poorly understood and used in broad-sense medicine so far [Inoue and Hayashi 2021]. One of such valuable substances are phenolic compounds, which belong to secondary metabolites. They play an important role in growth, reproduction, and especially protection against various biotic and abiotic stresses. In addition

✉ dorota.tchorzewska@mail.umcs.pl

to the biological significance, phenolic compounds contribute to the sensory characteristics and colour of fruits and vegetables [Balasundram et al. 2006]. Importantly, considering the pharmaceutical issue, phenolics possess a wide range of biological activities considered for medicinal applications, such as anti-inflammatory, anti-allergic, anti-bacterial, antioxidant, anti-thrombotic, cardioprotective, and many more effects; thus, phenolic compounds present in plants represent one of the most valuable substances that can be used as a therapeutic agents; at the same time, plants are the biggest reservoir of such compounds, which have been poorly explored to date, bringing high capacity for the future development of new drugs [Zhang et al. 2022].

The research conducted so far on the determination of MHM in plants from the genus *Knautia* L. has indicated that some species exhibit pharmaceutical/biotechnological potential. For instance, *Knautia arvensis* has a high level of phenolic compounds [Tawaha et al. 2007, Moldoch 2011] with anti-proteolytic compounds [Selje et al. 2007, Hoffmann 2008]. It has also been found that various extracts from *K. arvensis* herb exhibit antibacterial, anti-inflammatory, and antioxidant, expectorant, diuretic, and analgesic properties [Launert 1981, Allen and Hatfield 2004, Kosch 2013]. The extract of this species is also a muscle relaxant and has a positive effect on blood parameters [Mattalia et al. 2013]. Furthermore, it has been reported that *K. sarajevensis* leaves, i.e. another representative of the genus *Knautia*, contain an high level of phenolic compounds with antioxidant and antimicrobial activity [Karalija et al. 2017, 2018].

Although there are a number of studies available, the current information about the therapeutic (pharmaceutical) potential of numerous *Knautia* species is still to be explored. Considering their biology, the natural habitats of *Knautia* species include meadows, roadside, bright forests, and forest margins in south-eastern Europe and the Mediterranean region [Ehrendorfer 1976, Rešetnik et al. 2016, Mabberley 2017]. One of interesting species are the perennial plants *Knautia drymeia* and *K. macedonica* which have not been analysed in terms of their morphology/anatomy and their suitability to be used as MHM. Therefore, to characterise the biological parameters together with the pharmaceutical potential of *K. macedonica* and *K. drymeia*, this study was focused on the analysis of the morphological, anatomical, and histological structure of the aboveground and underground shoots of both species. Concurrently, the distribution of polyphenolic compounds was correlated with the tissue structure of individual organs in the studied plants. The research was supplemented by quantitative spectrophotometric analyses of the total content of polyphenols and o-dihydroxyphenols in the plant organs (rhizome, stem, leaf, and flower). These correlative studies of the structure and distribution of biologically active substances of the *K. drymeia* and *K. macedonica* species constitute a holistic approach, combining the analysis of plant anatomy and cytology with biochemical research. The used approach allowed the identification, characterisation, and determination of the distribution of bioactive compounds in the tissues of the studied plant species, at the same time assessing the plants in terms of an effective use of their parts for acquisition of bioactive substances; the research meets the current requirements, to explore valuable natural biological resources for medicine as part of the sustainable development of society.

MATERIAL AND METHODS

Chemicals and reagents. Arnov's reagent, hydrochloric acid, sodium acetate, aluminum chloride were obtained from Chempur (Piekary Śląskie, Poland), while Folin-Ciocâlteu reagent was provided by Sigma-Aldrich (Steinheim, Germany). Methanol, sodium hydroxide, sodium chloride, and sodium carbonate were purchased from Avantor (Gliwice, Poland). Reference substances were provided by ChromaDex (Irvine, CA, USA).

Plant material. *Knautia drymeia* Heuff. and *K. macedonica* Griseb. were collected from the Botanical Garden of Maria Curie-Skłodowska University in Lublin. The plants were grown in brown soils prevailing in the Botanical Garden (Index Seminum 2014 Hortus Botanicus Universitatis Marie Curie-Skłodowska). The cultivation was carried out in a univariate randomised block design with 4 replications. The observations of the developmental phases and morphological traits and the collection of research material were carried out in 2020 and 2021 (from April to September). No herbicides, fungicides, or any chemi-

cals were used during cultivation, but manual weeding methods were used throughout the growing period of the plants. Macroscopic images of the plants were taken with a Nikon D300 photographic camera equipped with a 60 mm AF MICRO NIKKOR lens. Fragments of stems from nodes of the fourth order and internodes between the third and fourth nodes were imaged using a Nikon SMZ 74ST stereoscopic microscope, and photographic documentation was made with the use of the Delta Pix program.

Light microscopy (LM). Aboveground and underground shoots of *K. drymeia* and K. *macedonica* were collected for the histological and cytochemical studies. The material was sampled randomly from 20 plants. Hand-made cross-sections of fresh fragments of rhizomes (fragments near the aboveground shoot), stems (fragments from nodes of the fourth order and internodes between the third and fourth nodes), and leaves (fragments from the middle part of the leaf blade) were analysed. Peeled sections of the leaf epidermis were placed in a drop of distilled water for visualisation using a Nikon eclipse *Ni* light microscope. Photographic documentation was made with a digital camera and NIS-Elements BP software using the Extended Depth of Focus (EDF) module.

Fluorescence microscope (FM). To visualise phenolic compounds in the rhizome, stem, and leaf tissues of *K. drymeia* and *K. macedonica*, hand-made sections were prepared and placed in distilled water; next, blue autofluorescence of phenolic compounds was observed [Hutzler et al. 1998, Dmitruk et al. 2019, Ribeiro and Leitão 2020]. The slides were analysed under a fluorescence microscope Nikon eclipse *Ni*-U at an excitation wavelength of 330–380 nm and an emission wavelength over 480 nm (UV). Photographic documentation was made with a digital camera and NIS-Elements BP software with the use of the EDF module.

Scanning electron microscope (SEM). Fragments of *K. drymeia* and *K. macedonica* leaves were collected for the analysis of the top and bottom of the leaf lamina. The samples were fixed in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4) for 24 hours, washed in distilled water, and dehydrated in increasing concentrations of ethanol: 30%, 50%, 70%, 90%, 96%, and 100% [Hayat 1981]. The dehydrated samples were then dried in a Critical Point Dryer (Denton Vacuum, Moorestown, NJ, USA) using liquid CO_2. Next, the samples were mounted on aluminium stubs and sputtercoated with gold (Hummer 6.2 Sputter Coater, Anatech USA, Union City, CA, USA). The samples were analysed under a scanning microscope (LEO1430VP) with accelerating voltage of 15 kV equipped with a Bruker Ouantax 200XFlash EDX Spectrometer System attached to a Zeiss EVO 50 Variable Pressure SEM at 15 kV, using INCA-Mapping software (Billerica, MA, USA).

Preparation of extracts. The leaves, stems, flowers, and rhizomes of both species were washed and dried in the shade at room temperature (24°C ±0.5°C) to achieve constant weight [Polish Pharmacopoeia IX]. To obtain extracts, a methanol–acetone–water mixture (3 : 1 : 1, v/v/v; 3 × 20 mL) was added to 1 g of powdered plant materials, and the sample was sonicated for 15 min at a controlled temperature (45°C ±2°C). The combined extracts were filtered and concentrated using reduced pressure. After freezing, they were lyophilised in a vacuum concentrator (Free Zone 1 apparatus; Labconco, Kansas City, MO, USA) in order to obtain dried residues.

Determination of phenolic compounds. Total phenolic acid content (TPAC), total flavonoid content (TFC), and total phenolic content (TPC) were determined using the colorimetric assays described previously [Chrząszcz et al. 2021]. The absorbance was measured using a Pro 200F Elisa Reader (Tecan Group Ltd., Männedorf, Switzerland). The results of TPAC were expressed as mg of caffeic acid equivalent (CAE) per 1 g of dry extract (DE), TPC as mg of gallic acid equivalent (GAE) per 1 g of DE, and TFC as mg of quercetin equivalent (QE) per 1 g of DE.

RESULTS

Morphology and anatomy study of *Knautia drymeia*. *Knautia drymeia* analysed in this study is a perennial plant overwintering as underground rhizomes. Rosettes of lanceolate leaves and an erect stem reaching a height of approximately 100 cm appear from *K. drymeia* rhizomes at the beginning of the growing season (Fig. 1A). Each stem was characterised by limited growth and ended with a spherical capitulum inflorescence having 1.5–3 cm in diameter. The plant flowered from July to September. The inflorescence

Fig. 1. Morphology of the aboveground parts of *Knautia drymeia*. **A** – cultivation in the botanical garden, **B** – inflorescence with some flowers in the anthesis stage (arrow), **C** – inflorescence with all flowers in the anthesis stage, **D** – single flower in the anthesis stage, stamen (arrow), pistil (arrowhead)

was composed of numerous sessile simple flowers characterised by non-synchronous and irregular development, i.e. both marginal flowers and those located in the central part of the receptacle were in the anthesis phase (Fig. 1B). The perianth of the simple flowers consisted of small, serrated sepals and four pink corolla petals covered by numerous hairs (Fig. 1C, D). The flowers were hermaphrodite and had 4 stamens and 1 superior pistil. All the generative elements of the flower were light pink. The heads of the mature anthers opened along their entire length (Fig. 1D), and mature pollen grains were yellow.

The morphological analysis of a three-year-old *K. drymeia* rhizome showed its oval shape and no clear division into nodes and internodes. The rhizome produced adventitious roots and lanceolate leaves (Fig. 2A). The outer layer of this organ was covered by periderm, under which there was the cortex, phloem, cambium, and xylem as well as a relatively large medullary parenchyma visible in the centre (Fig. 2B). In this study, detailed analyses of the *K. drymeia* rhizome parts were performed (Fig. 2C, E, G). Biophysical methods with the use of a fluorescence microscope were employed to visualise the presence of phenolic compounds in the tissues of this organ (autofluorescence of phenolic compounds was employed). The analyses showed autofluorescence in the range of blue light in the cells of cortex parenchyma, medullary rays, and medulla, which indicated the presence of polyphenols in these tissues in significant amount (Fig. 2D, F, H).

The analysis of the aboveground part showed an erect, branched *K. drymeia* stem with leaves arranged oppositely (dichotomously) and growing from the nodes (Fig. 3A, B). The stem between the nodes formed the internodes (Fig. 3A, D). The analysis of the histological structure of the *K. drymeia* stem nodes showed that the entire circumference of this organ was covered by a single-layer of epidermis. Underneath, the assimilation parenchyma, phloem, xylem, and parenchyma-filled medulla layers were visible. A vascular bundle branch was present at the site where the leaf sheath surrounded the stem (Fig. 3C). The anatomical analysis of the internode part of the stem showed a single-layer of epidermis, assimilation parenchyma cells with numerous chloroplasts, a single-layer of endodermis, and vascular elements, i.e. phloem and xylem, which formed a layer around the entire circumference of the stem. At the boundary of the xy-

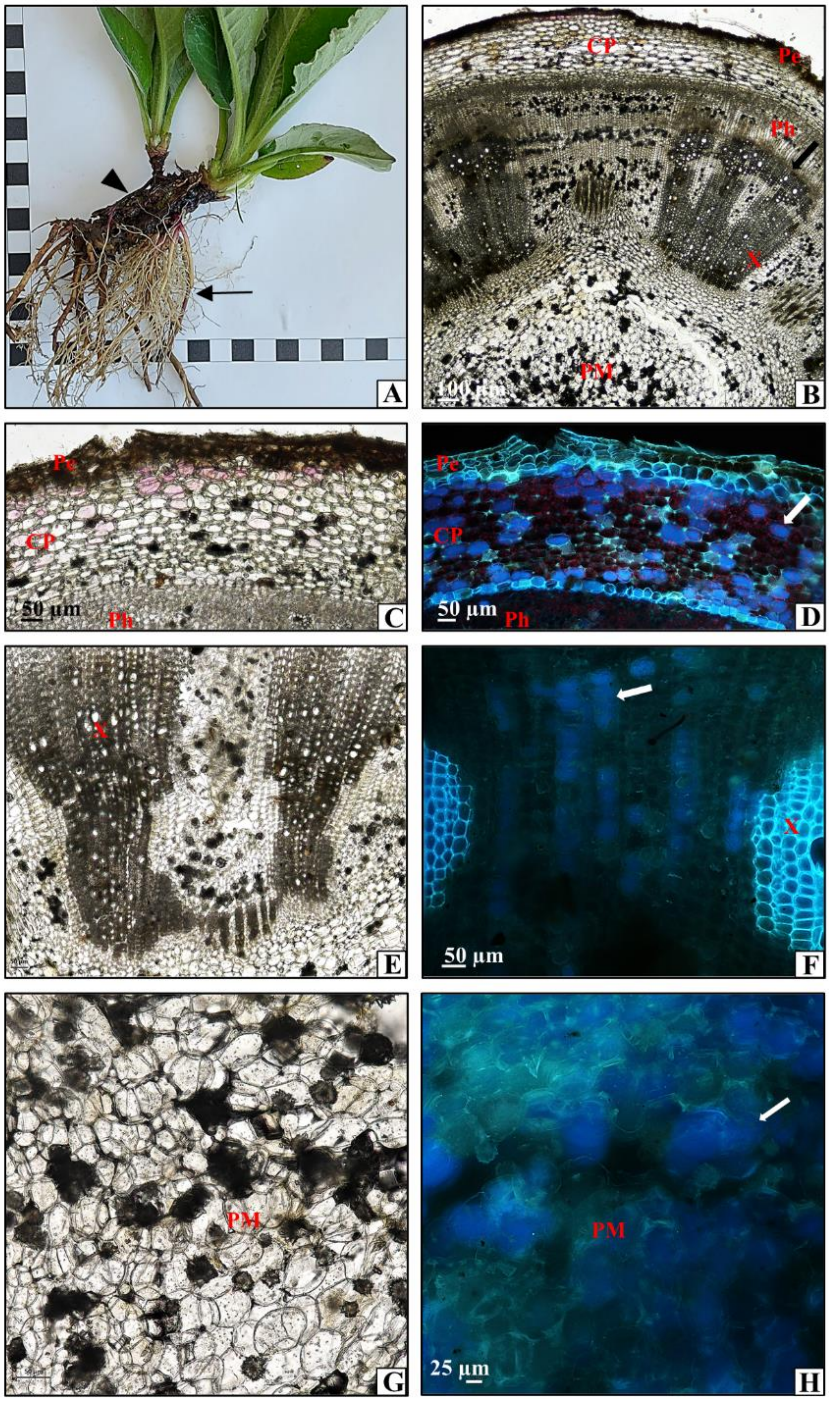

Fig. 2. Morphology, anatomy, and cytology of *Knautia drymeia* rhizome. **A** – rhizome (arrowhead) with roots (arrow) and leaves; **B–H** – cross through the rhizome: **B** – part of the rhizome: periderm (Pe), cortex parenchyma (CP), phloem (Ph), cambium (black arrow), xylem (X), parenchymal medulla (PM); **C** – fragment of cortex: periderm (Pe), cortex parenchyma (CP), phloem (Ph); **D** – phenolic compounds (arrow) in the cortex; **E** – part of xylem (X); **F** – phenolic compounds (arrow) in the xylem; **G** – fragment of parenchymal medulla (PM); **H** – phenolic compounds in the medulla (arrow). **B, C, E,** G light microscope; **D, F, H** fluorescence microscope

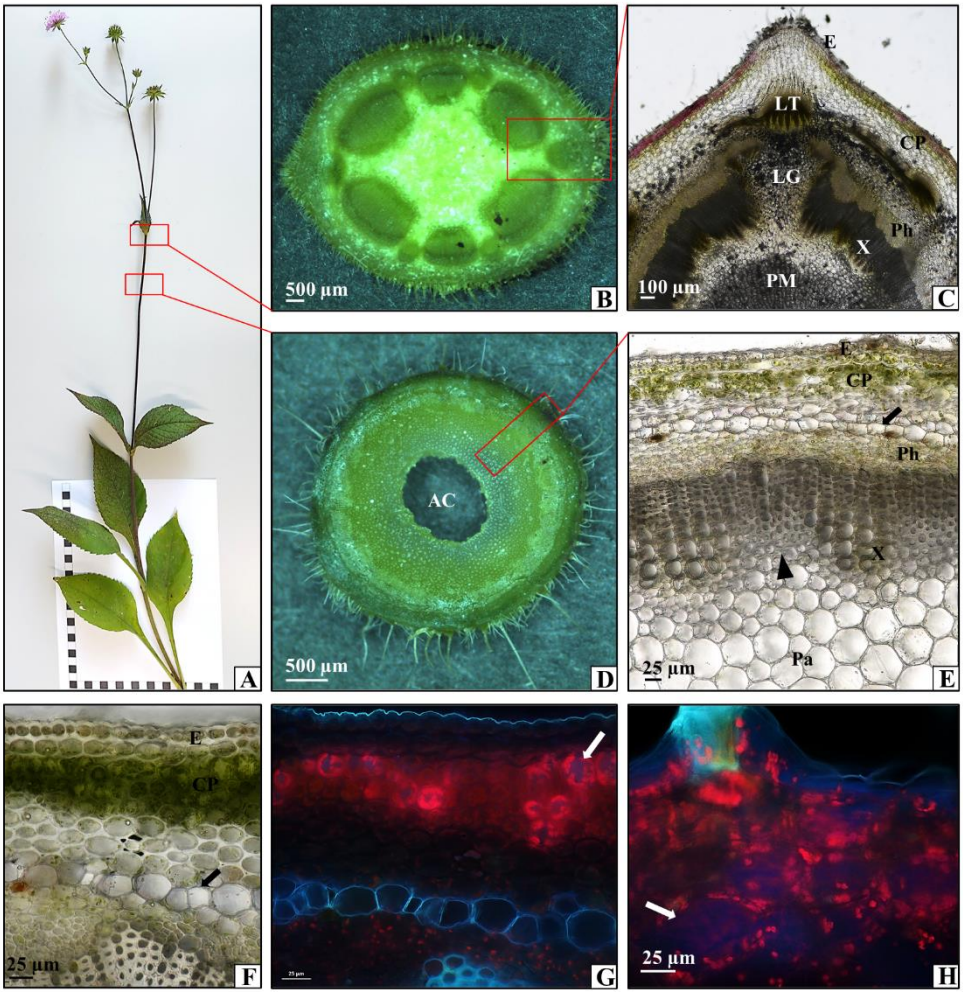

Fig. 3. Morphology, anatomy, and cytology of *Knautia drymeia* stem. **A** – fragment of a one-year-old stem; **B–C** – cross through the stem in the node part: **C** – parts of the stalk: epidermis (E), cortex parenchyma (CP), leaf trace (LT), phloem (Ph), xylem (X), leaf gap (LG), parenchymal medulla (PM); **D–H** – cross through the stem in the internode part: **D** – air channel (AC); **E** – parts of the stem: epidermis (E), cortex parenchyma (CP), endodermis (black arrow), phloem (Ph), xylem (X), sclerenchyma (arrowhead), parenchyma (Pa); **F–H** – cortex from an internode fragment, **G–H** – phenolic compounds (arrows) in the internode cortex. **B, D** stereoscopic microscope; **C, E, F** light microscope; **G, H** fluorescence microscope

lem and the subsequent parenchyma layer, there were layers of supporting sclerenchymatic fibres (Fig. 3E). A relatively small air channel (1000–2000 µm in diameter) was visible in the centre of the stem (Fig. 3D). The autofluorescence-based identification of phenolic compounds in the *K. drymeia* stem internode tissues (Fig. 3F) showed the presence of polyphenols in the parenchyma tissue only under the epidermis (Fig. 3G, H). In turn, in the node tissue (Fig. 4A, C, E), these substances were present in the parenchyma of the cortex, leaf gap, and medulla (Fig. 4B, D, F).

The species had simple leaves with an ovoid leaf blade with a pointed apex, a tapered base, and a serrated margin (Fig. 5A). The microscopic study showed that the epidermis cells on the upper side of the leaf blade (adaxial) had a slightly parentimatic polyhedral shape with wavy edges (Fig. 5B), while the cells of the lower (abaxial) epidermis were puzzle-shaped (Fig. 5C). Anomocytic stomata of the Amaryllis type were distributed on both sides of the leaf blade (Fig. 5B, C, F), but they were more numerous in the abaxial epidermis. The SEM imaging revealed characteristic linear

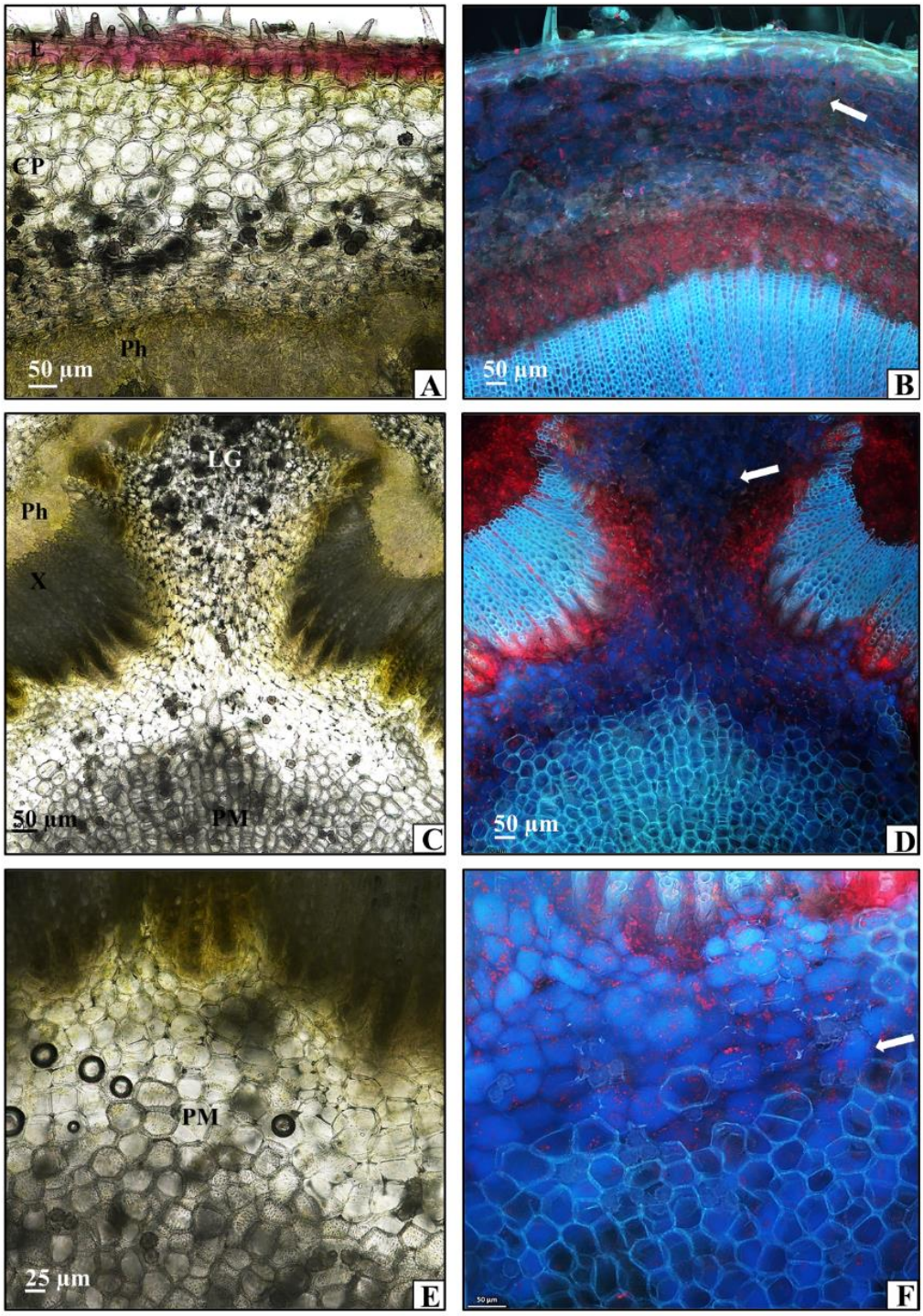

Fig. 4. Anatomy and cytology of *Knautia drymeia* stem in the node part. **A** – cortex: epidermis (E), cortex parenchyma (CP), phloem (Ph); **B** – phenolic compounds (arrow) in the cortex; **C** – leaf gap: phloem (Ph), xylem (X), leaf gap (LG), parenchymal medulla (PM); **D** – phenolic compounds (arrow) in the leaf gap; **E** – parenchymal medulla: parenchymal medulla (PM); **F** – phenolic compounds in the medulla (arrow). **A, C, E** light microscope; **B, D, F** fluorescence microscope

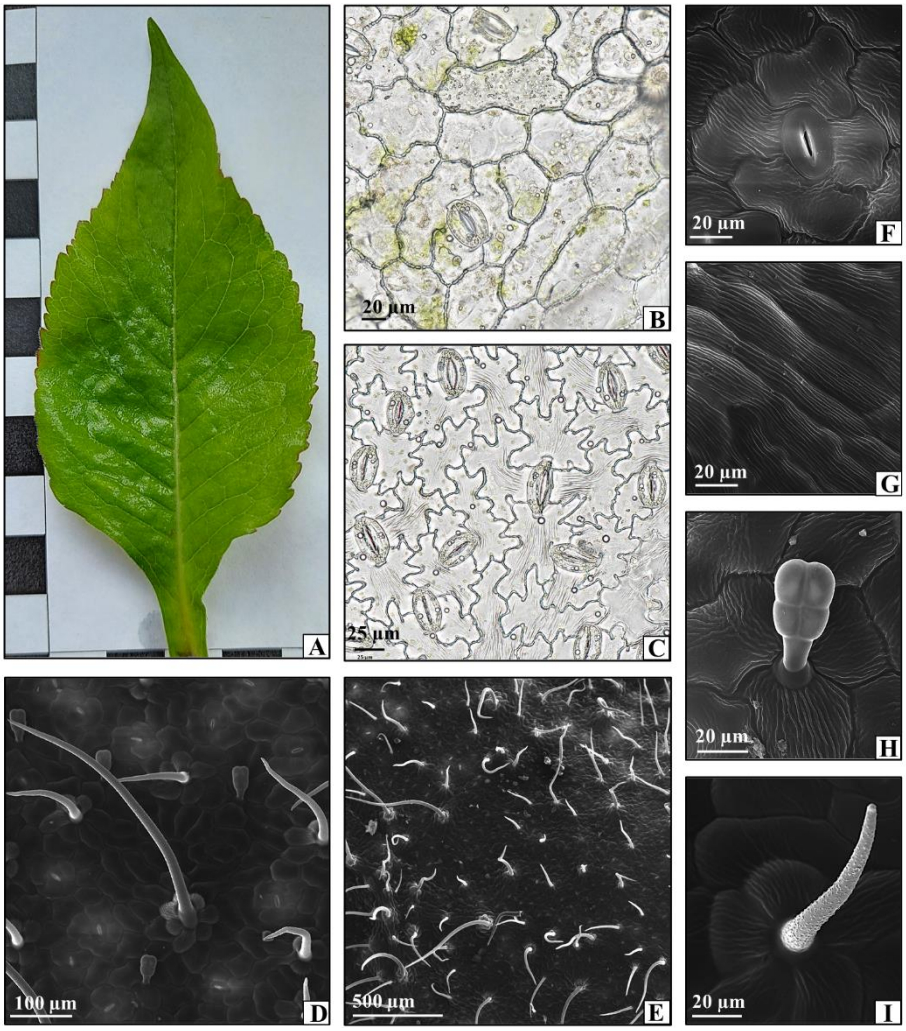

Fig. 5. Morphology and anatomy of *Knautia drymeia* leaf. **A** – macroscopic view; **B** – peeled sections of the upper leaf epidermis; **C** – peeled sections of the lower leaf epidermis; **D** – lower side of the leaf blade with visible trichomes; **E** – upper side of the leaf blade with visible trichomes; **F** – cuticle on the surface of the epidermis; **G** – leaf stoma; **H–I** – trichomes. **B, C** light microscope; **D–I** scanning electron microscope

sculpture of the epidermis cell cuticle (Fig. 5G). Two types of trichomes were observed on the upper (Fig. 5D) and lower (Fig. 5E) sides of the leaf blade: capitate trichomes consisting of a unicellular stalk and a four-celled head (Fig. 5H), and unicellular non-glandular trichomes with extensive sculpture (Fig. 5I). The cross sections through the leaf blade showed a single-layer upper and lower epidermis, a palisade mesophyll usually composed of one row of slightly elongated and closely adjacent cells, and a spongy mesophyll composed of loosely arranged oval cells (Fig. 6A). As in the previous analyses of the rhizome or the stem, the leaf was also analysed for the distribution of phenolic compounds. The autofluorescence-based analysis indicated a strong signal in the blue light range in the palisade and spongy assimilation parenchyma (Fig. 6B, C, D), in the parenchyma under the midrib epidermis (Fig. 6E), and in the bundle sheath cells (Fig. 6F).

Morphology and anatomy study of *Knautia macedonica*. The other analyzed species belonging to the genus *Knautia* was *K. macedonica*, which has not been thoroughly described. It is a perennial plant gro-

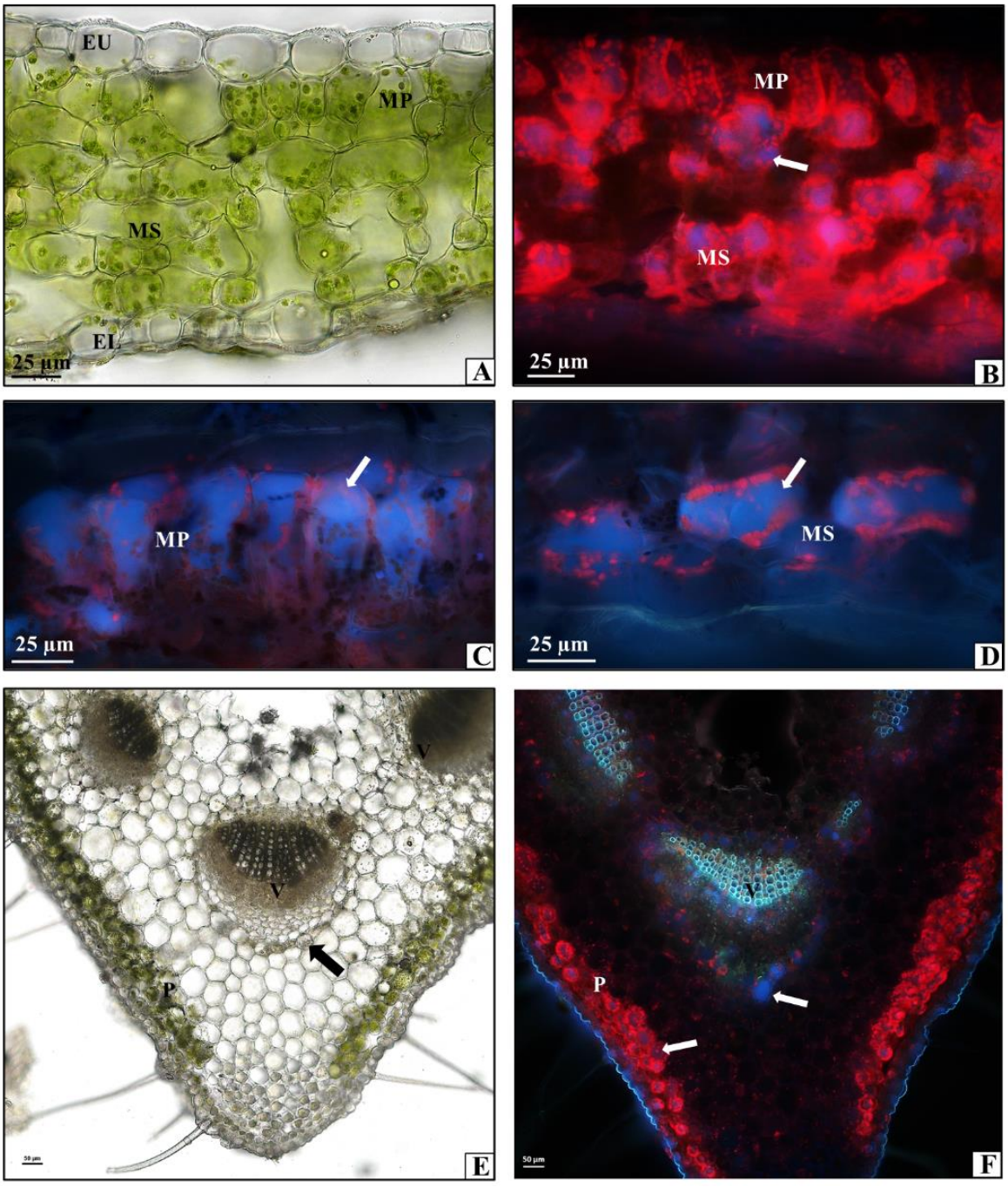

Fig. 6. Anatomy of *Knautia drymeia* leaf. **A–D** cross through the leaf blade: **A** – part of the leaf: upper epidermis (EU), palisade mesophyll (MP), spongy mesophyll (MS), lower epidermis (EL); **B** – phenolic compounds (arrow) in the leaf blade mesophyll: palisade mesophyll (MP), spongy mesophyll (MS); **C** – phenolic compounds (arrow) in the palisade mesophyll (MP); **D** – phenolic compounds (arrow) in the spongy mesophyll (MS); **E–F** – cross through the leaf midrib: vascular bundle (V), bundle sheath cells (black arrow), parenchyma (P); **F** – phenolic compounds (arrows), vascular bundle (V), parenchyma (P). **A, E** – light microscope; **B, C, D, F** – fluorescence microscope

Fig. 7. Morphology of the aboveground part of *Knautia macedonica*. **A** – cultivation in the botanical garden; **B** – inflorescence with some flowers in the anthesis stage (arrows); **C** – inflorescence with all flowers in the anthesis stage; **D** – single flower in the anthesis stage; stamen (arrow), pistil (arrowhead)

wing in compact clumps and reaching a height of approximately 100 cm (Fig. 7A). Each stem in this species ended with a capitulum inflorescence flowering from July to September. The inflorescence differed from that described above, i.e. the flowers were dark pink and they were characterised by a regular centripetal mode of opening, as the anthesis was initially observed only in marginal flowers (Fig. 7B). The simple flowers were composed of four petals fused to half their length, four stamens, and one superior pistil (Fig. 7C, D). The generative elements of the flower were light pink, and the heads of mature anthers were yellow (Fig. 7D).

The morphological analysis of the *K. macedonica* rhizome showed that, as in the case of *K. drymeia*, this organ was oval, did not exhibit a clear division into nodes and internodes, and produced adventitious roots and lanceolate leaves at the beginning of the growing season (Fig. 8A). The anatomical slides demonstrated that the rhizome was covered by a periderm, under which there was a cortex parenchyma, a phloem ring, a single layer of cambium cells, and a xylem ring. The centre was filled with a large medullary parenchyma (Fig. 8B). Detailed studies of the *K. macedonica* rhizome parts (Fig. 8C, E, G) showed blue light autofluorescence in the cells of the cortex parenchyma, medullary rays, and medulla, indicating the presence of polyphenols in these cells (Fig. 8D, F, H). Therefore, as in *K. drymeia*, the parenchyma of all layers of the rhizome was found to accumulate phenolic compounds.

The morphological studies of the aboveground parts showed an erect *K. macedonica* stem divided into nodes producing oppositely arranged leaves and lateral shoots (Fig. 9A, B). As demonstrated by the analysis of the tissues of this part of the plant, the stem was composed of an outermost single-layer of epider-

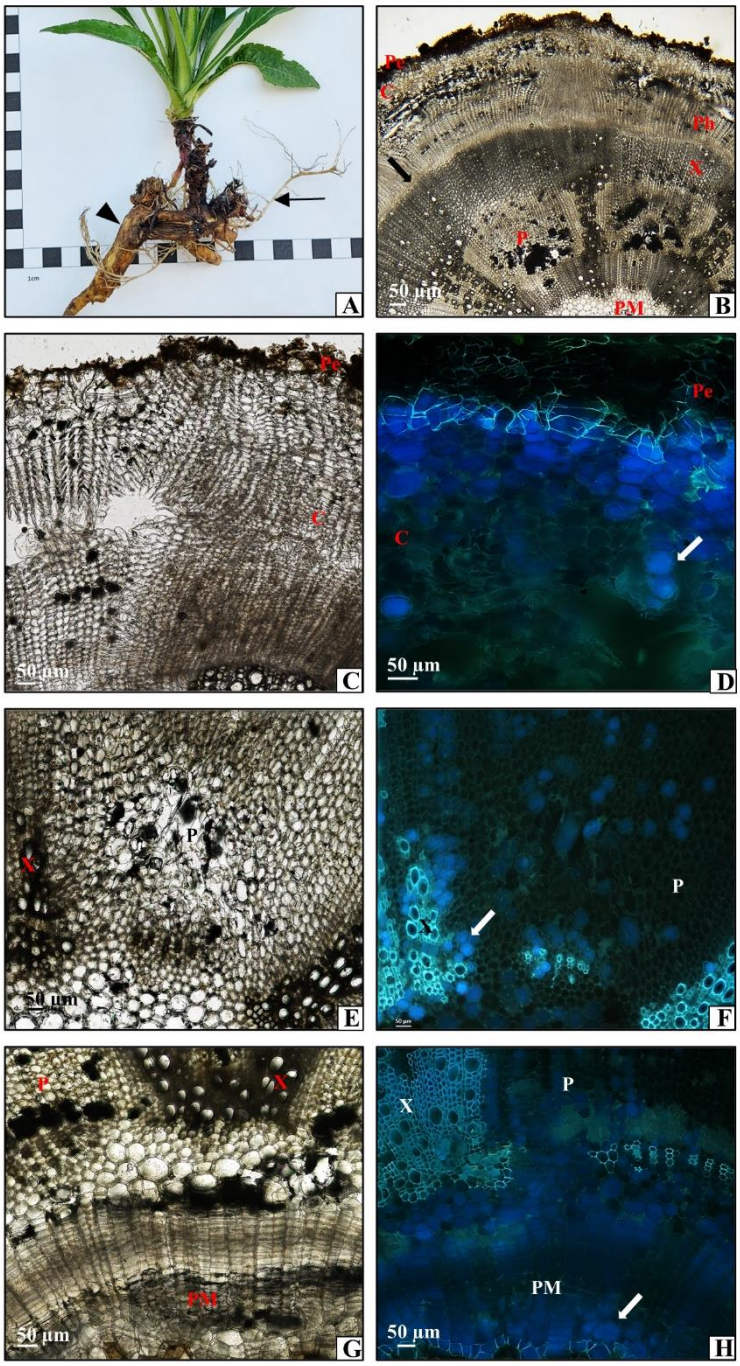

Fig. 8. Morphology, anatomy, and cytology of *Knautia macedonica* rhizome. **A** – rhizome (arrowhead) with roots (arrow) and leaves; **B–H** – cross through the rhizome: **B** – part of the rhizome: periderm (Pe), cortex (C), phloem (Ph), cambium (black arrow), xylem (X), parenchyma (P), parenchymal medulla (PM); **C** – fragment of cortex: periderm (Pe), cortex (C); **D** – phenolic compounds in the cortex (arrow), parenchyma (P), cortex (C); **E** – fragment of xylem (X), parenchyma (P); **F** – phenolic compounds (arrow) in the xylem (X), parenchyma (P); **G** – fragment of rhizome on the border of xylem and medulla: xylem (X), parenchyma (P), parenchymal medulla (PM); **H** – phenolic compounds (arrow) in the fragment of rhizome on the border of xylem and medulla: xylem (X), parenchyma (P), parenchymal medulla (PM). **B, C, E, G** light microscope; **D, F, H** fluorescence microscope

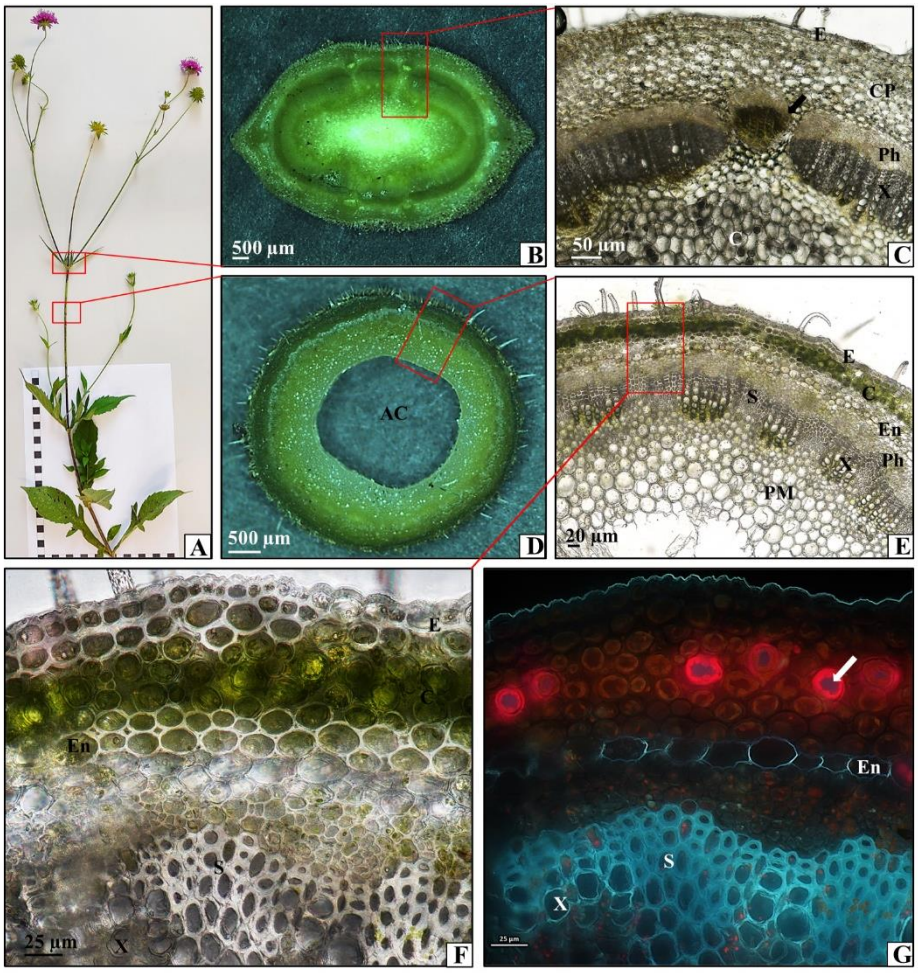

Fig. 9. Morphology of the aboveground part of Knautia macedonica. **A** – cultivation in the botanical garden; **B** – inflorescence with some flowers in the anthesis stage (arrows); **C** – inflorescence with all flowers in the anthesis stage; **D** – single flower in the anthesis stage; stamen (arrow), pistil (arrowhead)

mis, an assimilation parenchyma layer, a phloem layer, a xylem layer, and a parenchyma-containing medulla. A vascular bundle branch was visible at the site where the leaf sheath surrounded the stem (Fig. 9C). At the height of the internode, the stem in the centre had an air channel, with a diameter of approximately 2000 µm (Fig. 9D). Moreover, it was composed of the epidermis, an assimilation parenchyma layer with numerous chloroplasts, and a single-layer endodermis and phloem-xylem vascular bundles arranged in a single ring around the stem circumference. Cells of the supporting tissue, i.e. sclerenchyma, were visible between the bundles, and parenchyma surrounding the air channel (Fig. 9E, F). Phenolic compounds in the internode tissue in the *K. macedonica* stem were present only in the parenchyma tissue located under the epidermis (Fig. 9G). In contrast, in the node tissues (Fig. 10A, C, E), polyphenols were detected in the parenchyma of the cortex, leaf gap, and medulla (Fig. 10B, D, F).

The morphological analyses of the *K. macedonica* leaves showed dimorphism of the leaf blade: the basal leaves had a lanceolate blade with a serrated margin, whereas the shoot leaves were pinnatisect with an entire margin slightly serrated in the apical zone (Fig. 11A). Two types of trichomes were present on both the upper and lower sides of the leaf blade: capitate trichomes composed of a unicellular stalk and a four-

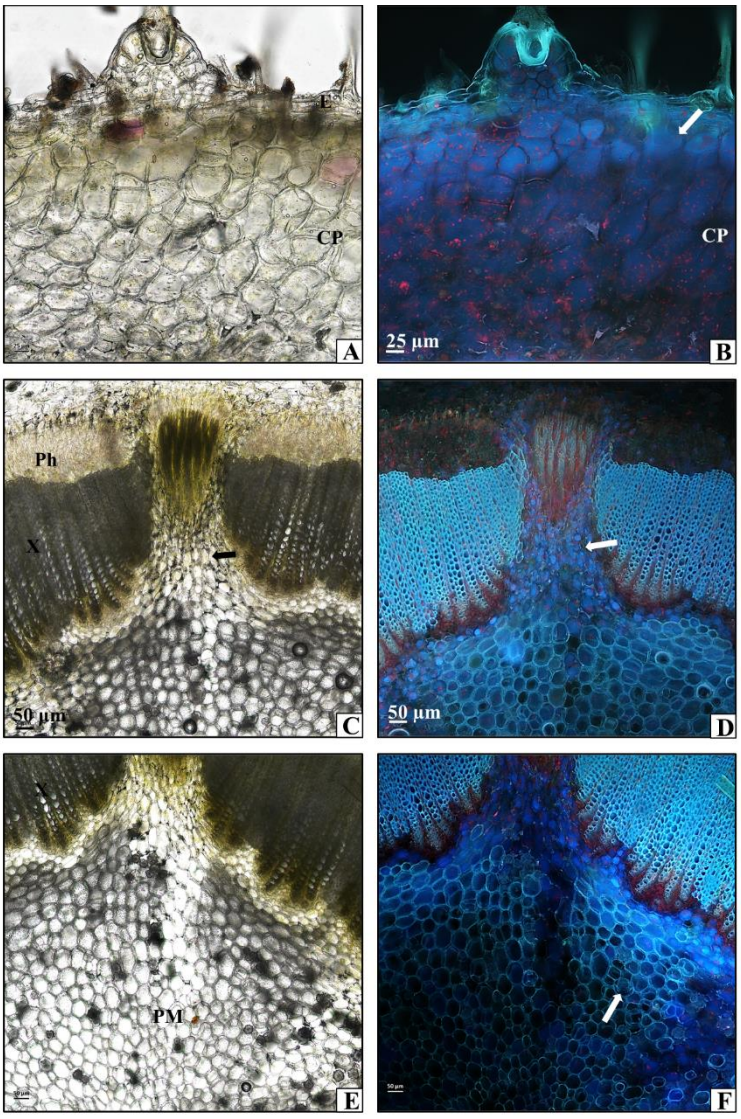

Fig. 10. Anatomy of *Knautia macedonica* stem in the node part. **A** – cortex: epidermis (E), cortex parenchyma (CP); **B** – phenolic compounds (arrow) in the cortex parenchyma (CP); **C** – leaf gap: phloem (Ph), xylem (X); **D** – phenolic compounds (arrow) in the leaf gap; **E** – parenchymal medulla: xylem (X), parenchymal medulla (PM); **F** – phenolic compounds in the medulla (arrow). **A, C**, **E** – light microscope; **B, D, F** – fluorescence microscope

-celled head and unicellular non-glandular trichomes with extensive sculpture (Figs 11B–D). Quite extensive linear striation of the epidermal cell cuticle was visible as well (Fig. 11E). The epidermis of the upper leaf blade was composed of polyhedron-shaped cells with straight edges, while the cells on the abaxial side had slightly wavy edges. Anomocytic stomata were visible on both sides of the leaf blade, but they were more numerous on the abaxial epidermis (Fig. 11F, G). The histological analysis of the leaf showed that the leaf blade was composed of a single-layer upper and lower epidermis and palisade and spongy mesophyll with phloem-xylem bundles typical of leaves (Fig. 12A). The autofluorescence-based identification of phenolic compounds in the *K. drymeia* leaf tissues revealed a high blue light signal in the assimilation (palisade

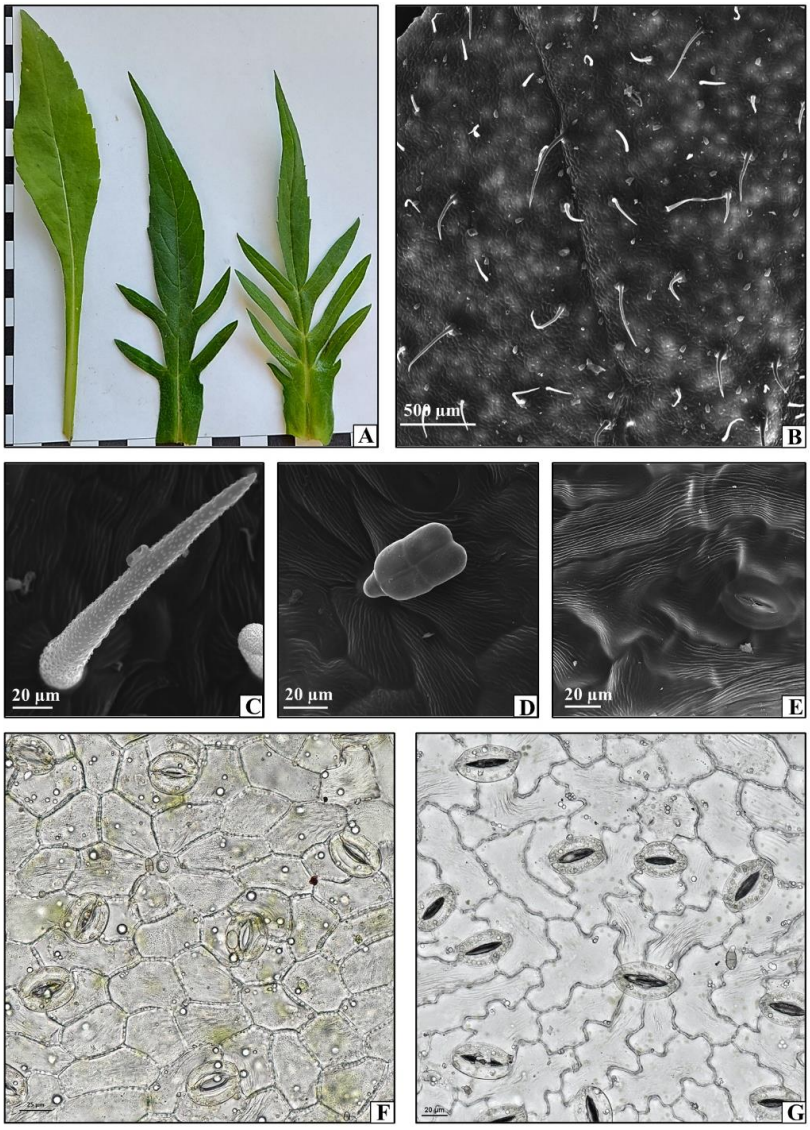

Fig. 11. Morphology and anatomy of *Knautia macedonica* leaf. **A** – macroscopic view; **B–D** trichomes on the lower side of the leaf blade; **E** – cuticle on the surface of the upper epidermis; **F** – peeled sections of the upper leaf blade epidermis; **G** – peeled sections of the lower leaf blade epidermis. **B–E** – scanning electron microscope; **F, G** – light microscope

and spongy) mesophyll, indicating high contents of polyphenols in the cells of these tissues (Fig. 12B, C, D). The midrib consisted of a centrally located phloem -xylem bundle surrounded by a parenchymal bundle sheath and a mesophyll composed of identical oval cells. No supporting tissue was observed in the midrib (Fig. 12E). The autofluorescence of polyphenols was visible in the parenchyma cells of the main midrib (Fig. 12F).

Phytochemical study of *Knautia drymeia* and *K. macedonica*. To characterise the phenolic content of the *K. drymeia* and *K. macedonica* in detail, leaves, stems, flowers and rhizomes were analysed using quantitative assays of their total polyphenols (TPC), phenolic acids (TPAC) and flavonoids (TFC). TPC in both studied species was determined using the Folin-Ciocâlteu assay, and the results were estimated as gallic acid equivalents (GAE). The total flavonoid

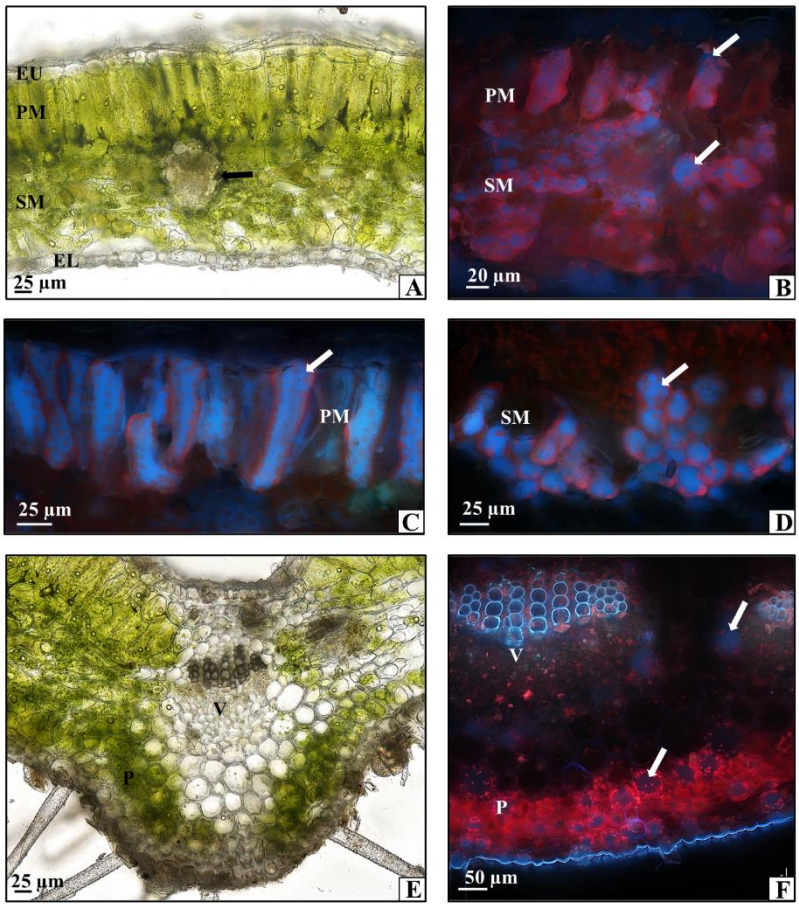

Fig. 12. Anatomy and cytology of Knautia macedonica leaf. **A–B** cross through the leaf blade: **A** – part of the leaf: upper epidermis (EU), palisade mesophyll (MP), spongy mesophyll (MS), vascular bundle (arrow), lower epidermis (EL); **B** – phenolic compounds (arrows) in the leaf blade mesophyll: palisade mesophyll (MP), spongy mesophyll (MS); **C** – phenolic compounds (arrow) in the palisade mesophyll (MP); **D** – phenolic compounds (arrow) in the spongy mesophyll (MS); **E–F** – cross through the leaf midrib: vascular bundle (V), parenchyma (P); **F** – phenolic compounds (arrows) in the leaf midrib: vascular bundle (V), parenchyma (P). **A, E** – light microscope; **B, C, D, F** – fluorescence microscope

content was determined using the previously described colorimetric method [Chrząszcz et al. 2021], and data were expressed as the quercetin equivalents (QE). As shown in Table 1 and Figure 13, the *K. drymeia* rhizome contained the smallest amounts of TPC and TFC (99.87 and 0.10 mg CAE/g DE respectively), compared with the other organs of this species. In turn, the content of TPAC was higher in the rhizome (81.93 mg CAE/g DE) than in the stems and flowers (51.92 and 51.50 CAE/g DE respectively). The quantitative analyses showed the TPC content in the stem of 101.12 mg CAE/g DE, which was higher than that of TPAC and TFC (51.92 and 0.31 mg CAE/g DE, respectively). However, these were moderate values in relation to those determined for the other organs (leaf, flower, and rhizome). Subsequent quantitative analyses showed that, in comparison with the stems, rhizomes, and flowers, the leaves contain the highest levels of TPC and TPAC (127.44 and 107.85 mg CAE/g DE, respectively), whereas slightly higher TFC values were determined in the *K. drymeia* flowers than in the leaves (0.53 and 0.49 mg CAE/g DE, respectively) (Tab. 1, Fig. 13).

In turn, the quantitative analyses of the *K. macedonica* organs carried out to determine the TPC, TPAC, and TFC levels showed low contents of these compounds in the rhizome extracts (95.21, 78.38, and 0.15 mg CAE/g DE, respectively), in comparison with the other

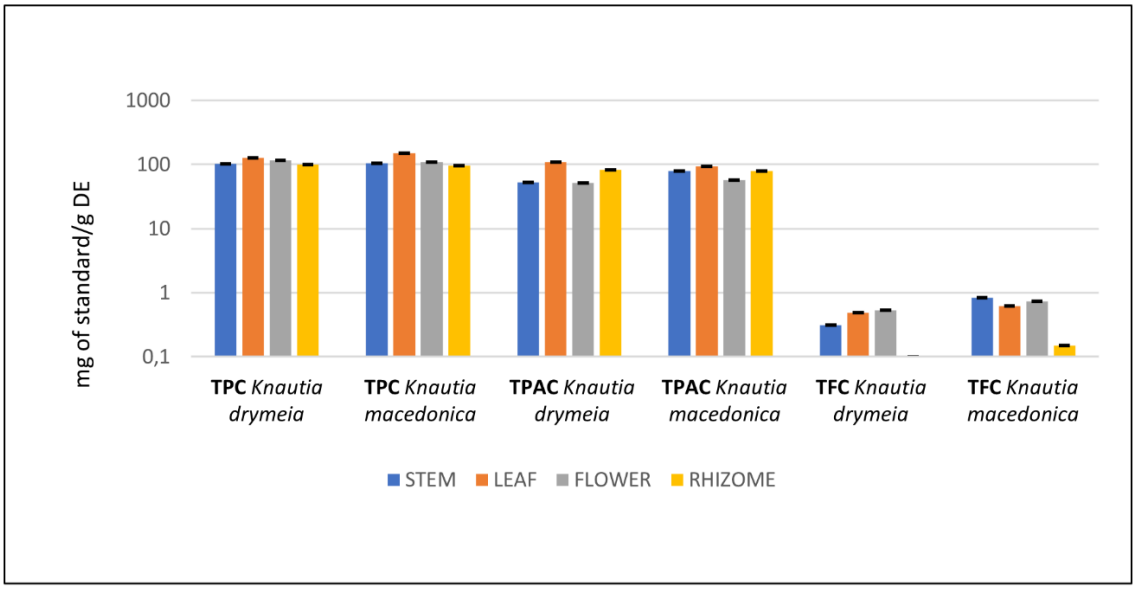

Fig. 13. Total content of phenolics (TPC), phenolic acids (TPAC), and flavonoids (TFC) in the organs of *Knautia drymeia* and *K. macedonica*

organs of this plant. In the *K. macedonica* stem extracts, the amounts of TPC (104.98 mg CAE/g DE), TPAC (79.27 mg CAE/g DE), and TFC (0.83 mg CAE/g DE) were higher, but the highest level of TPC (148.78 mg GAE/g DE) was recorded in the leaf extracts of this species (Tab. 1, Fig. 13).

DISCUSSION

The analyses presented in this manuscript aimed at determining the biological parameters in connection to the pharmaceutical potential of *K. macedonica* and *K. drymeia*, i.e. plant species that have never been characterized from the biotechnological point of view. *Knautia drymeia* and *K. macedonica* are perennial plants overwintering as underground rhizomes and growing in compact clumps [Ehrendorfer 1976, Jäger et al. 2017]. The observed morphological features of the aboveground organs of both studied species did not differ considerably from other plants of this genus growing in their natural habitats [Ehrendorfer 1976, Jäger et al. 2017]; therefore, the traits described in this study do not change in the conditions of temperate continental climate, indicating that *K. drymeia* and *K. macedonica* maintain their features irrespective of the growth conditions.

In the present report, we have characterized *K. drymeia* and *K. macedonica* taking into account their anatomical and biochemical traits. The initial analyses showed that both species have a similar anatomical and histological structure of the aboveground and underground shoots. Based on the analysis of the histological structure of the *K. drymeia* and *K. macedonica* stem, it can be concluded that the amount of the supporting tissue was inconsiderable in both species, which is associated with the similar low height of their aboveground shoots (about 100 cm high). A much larger area of the stem is filled with supporting tissue in species with long aboveground shoots, as described e.g. in *Cephalaria giganthea*, whose stem is 250 cm high [Chrząszcz et al. 2021]. In turn, the analysis of the morphological traits of the leaves of both species revealed extensive cuticle sculpture of the leaf epidermis. Such an extensive cuticle sculpture protects the leaf against excessive sunlight and transpiration, which is important in the dry steppe environment, which is a natural niche of the studied species [Solovchenko and Merzlyak 2003]. In addition to the anatomical and morphological analyses, the research was especially focused on showing the correlation of the distribution of polyphenolic compounds with the morphological/histological structure of individual organs in the

plants. Phenolic compounds, classified as secondary metabolites, usually occur as water-soluble substances accumulated in vacuoles, which are regarded as the main reservoir of pharmaceutically attractive bioactive compounds [Rice-Evans et al. 1997]. The correlative analyses revealed that polyphenols were accumulated in parenchyma tissue cells, and the amount of these substances was organ-specific. Therefore, the biophysical analysis performed with the aid of fluorescence microscopy imaging demonstrated that the rhizome of *K. drymeia* and *K. macedonica* emitted autofluorescence in parenchyma cells, but only in its small part, compared with the other organs of both species. As in the case of the *K. drymeia* stem, the biophysical analyses of the *K. macedonica* stem tissues showed that the phenolic compounds were accumulated only in parenchyma cells, mainly in the stem nodes with a relatively lower amount in the internodes. Subsequent analysis showed that the two analysed species did not exhibit significant differences in the histological and cytochemical structure of the leaves, and the leaves were the only organs where polyphenols were accumulated in all parenchymal cells. However, the capitate trichomes on the leaves did not contain phenolic compounds, in contrast to other plant species having this type of trichomes [Muravnik 2021]. Thus, based on the correlative analyses, it can be concluded that leaves may be the best material for acquisition of pharmaceutically important bioactive substances.

Next, the quantitative aspect of the polyphenols was considered, as the current literature showed that the quantitative data regarding polyphenols occurring in species of the genus *Knautia* are limited to only a few studies [Fraisse et al. 2007, Karalija 2020]. The quantitative spectrophotometric analyses of the total content of polyphenols and *o*-dihydroxyphenols in the individual organs provided detailed insight, showing differences in the content of polyphenolic substances not only between the plant organs but also between the studied species. The highest TPC and TPAC values were detected in the leaves, with a higher level of TPC in *K. macedonica* leaves and a higher amount of TPAC in *K. drymeia*, which was in line with the above mentioned correlative analyses. In turn, the highest content of TFC was found in the *K. macedonica* stem, indicating that this part of plant may be an attractive element from the active biocompound perspective. Moreover, the quantitative phytochemical analyses of *K. macedonica* indicated the highest level of TPC in the extracts from its leaves in comparison with not only the other organs but also *K. drymeia* leaves, underscoring the fact that this species is the most attractive plant from the pharmaceutical point of view.

Concluding, the integrated anatomical and biochemical analyses focused on the correlative studies have provided the first insight into the distribution of phenolic compounds in the tissues of the underground and aboveground organs of *K. drymeia* and *K. macedonica*. They have shown that the leaves of *K. macedonica* contain the highest amounts of biologically active substances and are the most attractive plant element in terms of their role as a bioactive compound resource. Additionally, this correlative study of *K. drymeia* and *K. macedonica* represents a new way of analyses carried out with a holistic approach integrating morphological/histological analyses with investigations of the biotechnological/pharmaceutical potential of these plants, opening the way to concentrate on the most relevant plant elements.

AUTHOR CONTRIBUTIONS

Conceptualization: K.D.S.S. and D.T., provided materials: A.D., methodology: M.C., K.D.S.S. and D.T., investigation: M.C., K.D.S.S. and D.T., data curation: K.D.S.S. and D.T., writing – original draft preparation: M.C., K.D.S.S. and D.T., editing: D.T., supervision, K.D.S.S. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SOURCE OF FUNDING

Research financed from the statutory funds of the Institute of Biological Sciences of the Maria Curie-Skłodowska University of Lublin and the Department of Pharmaceutical Botany of the Medical University of Lublin.

REFERENCES

Allen, D.E., Hatfield, G. (2004). Medicinal plants in folk tradition. Timber Press, Portland.

Balasundram, N., Sundram, K., Samman, S. (2002). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chem., 99(1), 191–203. https://doi.org/10.1016/j.foodchem.2005.07.042

Chrząszcz, M., Miazga-Karska, M., Klimek, K., Granica, S., Tchórzewska, D., Ginalska, G., Szewczyk, K. (2020). Extracts from Cephalaria uralensis (Murray) Roem. & Schult. and Cephalaria gigantea (Ledeb.) Bobrov as potential agents for treatment of seborrheic skin diseases: Chemical characterization and in vitro biological evaluation. Antioxidants, 9(9), 796. https://doi.org/10.3390/antiox9090796

Chrząszcz, M., Szewczyk, K., Tchórzewska, D. (2021). Biotechnological potential of Cephalaria uralensis (Murray) Roem. & Schult. and C. gigantea (Ledeb.) Bobrov – comparative analysis of plant anatomy and the content of biologically active substances. Plants, 10(5), 986. https://doi.org/10.3390/plants10050986

Dmitruk, M., Sulborska, A., Żuraw, B., Stawiarz, E., Weryszko Chmielewska, E. (2019). Sites of secretion of bioactive compounds in leaves of Dracocephalum moldavica L.: anatomical, histochemical, and essential oil study. Rev. Bras. Bot., 42, 701–715. https://doi.org/10.1007/s40415-019-00559-6

Ehrendorfer, F. (1976). Knautia L. In: Tutin, T.G. (ed.). Flora Europaea. Vol. 4. Plantaginaceae to Compositae (and Rubiaceae). Cambridge University Press, Cambridge.

Fraisse, D., Carnat, A., Viala, D., Pradel, P., Besle, J.M., Coulon, J.B., Felgines, C., Lamaison, J.L. (2007). Polyphenolic composition of a permanent pasture: variations related to the period of harvesting. J. Sci. Food Agric., 87, 2427–2435.

Hayat, M.A. (1981). Principles and techniques of electron microscopy. Biological applications. Edward Arnold, London.

Hoffmann, E.M., Selje-Assmann, N., Becker, K. (2008). Dose studies on anti-proteolytic effects of a methanol extract from Knautia arvensis on in vitro ruminal fermentation. Anim. Feed Sci. Technol., 145(1–4), 285–301. https://doi.org/10.1016/j.anifeedsci.2007.06.038

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T.P., Reuber, S., Schmitz, R., Veit, M., Weissenbök, G., Schnitzler J.P. (1998). Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J. Exp. Bot., 323, 953–965.

Inoue, M., Hayashi, S. (2021). Blessings of medicinal plants–history and prospects. Medicinal Plants. Domestication, biotechnology and regional importance. Ekiert, H.M., Romawat, K.G., Arora, J. (eds). Springer, Switzerland, pp. 771.

Jäger, E.J., Müller, F., Ritz, Ch.M., Wlek, E., Wesche, K. (2017). Rothmaler – Exkursionsflora von Deutschland [Rothmaler – Excursion flora of Germany]. Gefässpflanzen: Atlasband. 13th ed. Springer Spektrum, Berlin.

Karalija, E., Zeljković Ć.S., Tarkowski, P., Muratović, E., Parić, A. (2017). The effect of cytokinins on growth, phenolics, antioxidant and antimicrobial potential in liquid agitated shoot cultures of Knautia sarajevensis. Plant Cell Tiss. Organ Cult., 131(2), 347–357. https://doi.org/10.1007/s11240-017-1288-2

Karalija, E., Zeljković, S.Ć., Tarkowski, P., Muratović, E., Parić, A. (2018). Media composition affects seed dormancy, apical dominance and phenolic profile of Knautia sarajevensis (Dipsacaceae), Bosnian endemic. Acta Bot. Croat., 77(1), 70–79. https://doi.org/10.1515/botcro-2017-0011

Karalija, E., Zeljković, S.Ć., Parić, A. (2020). Harvest time -related changes in biomass, phenolics and antioxidant potential in Knautia sarajevensis shoot cultures after elicitation with salicylic acid and yeast. In Vitro Cell. Dev. Biol. Plant., 56, 177–183.

Kosch, A. (2013). Handbuch der Deutschen Arzneipflanzen [Handbook of German medicinal plants]. Softcover reprint of the original, Berlin and Heidelberg.

Launert, E. (1981). Edible and medicinal plants. Hamlyn, London.

Mabberley, D.J. (2017). Mabberley's plant-book. Cambridge University Press, pp. 491.

Magryś, A., Olender, A., Tchórzewska, D. (2021). Antibacterial properties of Allium sativum L. against the most emerging multidrug-resistant bacteria and its synergy with antibiotics. Arch. Microbiol., 203(5), 2257–2268. https://doi.org/10.1007/s00203-021-02248-z

Mattalia, G., Quave C.L., Pieroni A. (2013). Traditional uses of wild food and medicinal plants among Brigasc, Kyé, and Provençal communities on the Western Italian Alps. Genet. Resour. Crop Evol., 60(2), 587–603. https://doi.org/10.1007/s10722-012-9859-x

Moldoch, J., Szajwaj, B., Masullo, M., Pecio, L., Oleszek, W., Piacente, S., Stochmal, A. (2011). Phenolic constituents of Knautia arvensis aerial parts. Nat. Prod. Commun., 6(11), 1627–1630.

Muravnik, L.E. (2021). The structural peculiarities of the leaf glandular trichomes: a review. Plant cell and tissue differentiation and secondary metabolites. Fundamentals and applications. Ramawat, K.G., Ekiert, H.M., Goyal, S. (eds). Springer International Publishing.

Polish Pharmacopoeia IX (2011). PTFarm, Polish Pharmaceutical Society, Warsaw, Poland, pp. 150.

Rešetnik, I., Frajman, B., Schönswetter, P. (2016). Heteroploid Knautia drymeia includes K. gussonei and cannot be separated into diagnosable subspecies. Am. J. Bot., 103(7), 1300–1313. https://doi.org/10.3732/ajb.1500506

Ribeiro, V.C., Leitão C.A.E. (2020). Utilisation of Toluidine blue O pH 4.0 and histochemical inferences in plant sections obtained by free-hand. Protoplasma, 257, 993–1008. https://doi.org/10.1007/s00709-019-01473-0

Rice-Evans, C., Miller, N., Paganga, G. (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci., 2(4), 152–159. https://doi.org/10.1016/S1360-1385(97)01018-2

Selje, N., Hoffmann, E.M., Muetzel, S., Ningrat, R., Wallace, R.J., Becker, K. (2007). Results of a screening program to identify plants or plant extracts that inhibit ruminal protein degradation. Br. J. Nut., 98(1), 45–53. https://doi.org/10.1017/s0007114507472506

Solovchenko, A., Merzlyak, M. (2003). Optical properties and contribution of cuticle to UV protection in plants: experiments with apple fruit. Photochem. Photobiol. Sci. 2(8), 861–866. https://doi.org/10.1039/B302478D

Tawaha, K., Alali, F.Q., Gharaibeh, M., Mohammad, M., El-Elimat, T. (2007). Antioxidant activity and total phenolic content of selected Jordanian plant species. Food Chem., 104(4), 1372–1378. https://doi.org/10.1016/j.foodchem.2007.01.064

Zhang, Y., Cai, P., Cheng, G., Zhang, Y.A. (2022). Brief review of phenolic compounds identified from plants: their extraction, analysis, and biological activity. Nat. Prod. Commun. 17(1), 1–14. https://doi.org/10.1177/1934578X211069721