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Research on the biology of some plant species with medicinal potential from the wild flora

DOCTORAL THESIS SUMMARY

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*** The numbering of the subsections, figures, graphics, and tables included in this summary follows the original structure of the thesis as outlined in the table of contents above.

KEYWORDS

Plant morpho-anatomy, plant extracts, hydroalcoholic extract, aqueous extract, phytochemistry, polyphenols, bioactive compounds, *Tussilago farfara* L., *Geranium robertianum* L., *Leonurus cardiaca* L., *Oenothera biennis* L., medicinal plants, wild flora, pedoclimatic factors, influence of environmental factors, HPLC, Q-GIS, optical microscopy, electron microscopy, TEM, SEM, micromorphology, cellular ultrastructure.

LIST OF ABBREVIATIONS

ECN - 7β-(3-ethyl-ciscrotonoyloxy)-1α-(2-methyl butyryloxy)-3(14)-dehydro-Z-notonipetranone
3-CQA - 3-caffeoylquinic acid
4-CQA - 4-caffeoylquinic acid
5-CQA - 4-caffeoylquinic acid
3,4-diCQA - 3,4-dicaffeoylquinic acid
3,5-diCQA - 3,5-dicaffeoylquinic acid
4,5-diCQA – 4,5-dicaffeoylquinic acid
EMDT - 2-ethyl-5-methoxy-N,N-dimethyltryptamine
Ppm - parts per million
HO-1 - heme oxygenase-1
NF-kappaB - nuclear factor kappa-light-chain-enhancer of activated B cells
MAP - mitogen-activated protein kinase
Nrf2 - nuclear factor erythroid 2-related factor 2
iNOS - inducible nitric oxide synthase
COX-2 - cyclooxygenase 2
LPS - lipopolysaccharide
IFN-gamma - gamma interferon
NO - nitric oxide
TNF-alfa - tumor necrosis factor alpha
MIC - minimum inhibitory concentration
C% - concentration percentage
IC_{50} - 50% maximal inhibitory concentration
GI_{50} - 50% maximal inhibitory concentration for cell proliferation
EC_{50} - effective half-maximal concentration
RM – Rhesus monkey renal cell line
MPP+ - 1-methyl-4-phenylpyridinium
DPPH - 2,2-diphenyl-1-picrylhydrazyl antioxidant scavenging assay
ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) antioxidant assay
FRAP - iron ion-reducing antioxidant potential
PQ - atrial depolarization interval on an electrocardiogram
QT - ventricular action interval on an electrocardiogram
QRS - ventricular depolarization complex on an electrocardiogram
ECG - electrocardiogram
GTPCH1 - guanosine triphosphate cyclooxygenase 1
DHFR - dihydrofolate reductase
GABA - gamma-aminobutyric acid
DMSO - dimethyl sulfoxide
KIM-1 - kidney molecule 1
Alfa-SMA - alpha-smooth muscle actin
TGF-beta - transforming growth factor beta
IL-6 - interleukin 6
IL-1beta - interleukin 1-beta
PAF - platelet-activating factor
TLR4 - Toll-like receptor 4
BuOH - butanol
GAE - gallic acid equivalent
LDL-C - low-density lipoprotein cholesterol

INTRODUCTION

Current global initiatives are focused on identifying and developing new compounds with therapeutic value. Medicinal plants have been used since ancient times and continue to be a primary source of bioactive compounds with structural diversity and inestimable therapeutic value (Dehelean and Pinzaru, 2024; Latif and Nawaz, 2025). Therefore, global efforts are concentrated on research of medicinal plants—particularly those used in traditional medicine—with the aim of discovering new bioactive compounds and exploring therapeutic applications beyond those already known. This urgency is highlighted by the limitations of conventional medicine in addressing complex medical problems such as metabolic syndrome, neurodegenerative disorders, cardiovascular disease, cancer, and antibiotic resistance. Conventional treatments are often associated with numerous side effects, limited accessibility, and high costs. Furthermore, there are recognized current financial and technological limitations to the synthesis of bioactive compounds, most of which are obtained from plants harvested from wild and cultivated flora (WHO, 2000; Rates, 2001; Süntar, 2020).

In this context, medicinal plants and the vast body of knowledge accumulated empirically from antiquity to the present day in traditional medicine, as well as knowledge regarding the evolution and adaptations of plants to their environment, significantly reduce the time required for identification, the list of species that could provide important bioactive compounds, and the associated costs (WHO, 2000; Dehelean *et al.*, 2024). Natural herbal remedies would be preferable in this regard, as they have limited side effects and are more affordable (Rates, 2001). Although their therapeutic value is still viewed with skepticism in Western countries, scientific studies are demonstrating an increasing number of beneficial effects. Access to this data is now facilitated by technology and the implementation of new standards in education (e.g., Open Access sources) (Gwynn and Hylands, 2000; Dehelean *et al.*, 2024; Latif and Nawaz, 2025).

Many of the endogenous receptors identified in humans, which play important roles in the body, are activated by phytochemicals (e.g., opioid and cannabinoid receptors). An increasing number of researchers are suggesting the possibility of a much larger number of structure-activity relationships—of physiological and pharmacological importance—involving phytochemicals that have not yet been characterized (Gwynn and Hylands, 2000). Natural plant compounds have greater biocompatibility than synthetic ones and act

synergistically, often through similar metabolic pathways (Rout *et al.*, 2009). Moreover, when combined with conventional medication, they can enhance the effectiveness of treatment (Chaudhary *et al.*, 2015; Pezzani *et al.*, 2019).

Research conducted to date shows that natural plant-derived compounds act as therapeutic agents in inflammatory conditions; in particular, polyphenols have been identified as significant metabolic modulators due to their ability to influence several key cellular and molecular pathways (Sivapragasam *et al.*, 2016; Liu *et al.*, 2023). Along with inflammation, oxidative stress has also become a strategic target in the development of new therapies and medications, as studies have continued to demonstrate its role in the pathogenesis of various conditions (Hussain *et al.*, 2016). According to the specialized literature, the species that are the subject of this doctoral thesis - *Tussilago farfara* L., *Geranium robertianum* L., *Leonurus cardiaca* L., and *Oenothera biennis* L., have common mechanisms of antioxidant and anti-inflammatory action, including Nrf2 activation, inhibition of certain inflammatory cytokines, TNF-α, and NO production (Schmidt *et al.*, 2003; Armatu *et al.*, 2010; Zhi *et al.*, 2012; Li *et al.*, 2012; Granica *et al.*, 2013; Avila *et al.*, 2013; Song *et al.*, 2014; Ratz-Lyco *et al.*, 2015; Bartlam *et al.*, 2017; Kyung *et al.*, 2017; Catarino *et al.*, 2017). These increasingly studied mechanisms are becoming strategic targets in the treatment of complex conditions such as those mentioned above (Hussain *et al.*, 2016; Roh and Sohn, 2018; Tonelli *et al.*, 2018; Yahfoufi *et al.*, 2018).

Before the 19th century, plants were the main source of medicines, and currently they continue to account for 20–25% to 40% of pharmaceutical products (Rates, 2001; Süntar, 2020; Abbott, 2024), serving as direct therapeutic agents and sources of model molecules for the synthesis and semisynthesis of medications (Dehelean *et al.*, 2024). Moreover, according to the World Health Organization (WHO), approximately 75–80% of the global population relies on plant-based medicines for their primary health care needs (Dehelean *et al.*, 2024; Latif and Nawaz, 2025).

Despite intensified efforts in recent years to study medicinal plants, their potential remains largely unexplored. Of the estimated 400,000 to 500,000 plant species in the global flora, only 15% have been studied phytochemically, and even fewer (6%) have been investigated for their therapeutic properties (Seidel, 2020). In most cases, only screenings and preliminary studies were conducted (Rates, 2001; Rout *et al.*, 2009); however, species such as *Taxus baccata*, *Camptotheca accuminata*, and *Artemisia annua* are among the best-known and most successful studies, which have led to the development of new drugs that are still used today to treat cancer and malaria (Cseke *et al.*, 2006). Galantamine from

Galanthus woronowiis was approved in 2001 for the treatment of mild to moderate Alzheimer's disease, and remains a first-line treatment option (Seidel, 2020). Plants of the genus *Salix* and *Populus* are sources of salicylic acid and form the basis of one of the world's best-known and most widely used medications—aspirin—while *Papaver somniferum* is the source of morphine, codeine, and papaverine, which are used for their analgesic, antitussive, and antispasmodic effects, respectively (Dehelean *et al.*, 2024). Such examples contribute to interest in the use of plants for the development of new medicines.

The concept of personalized therapy was anticipated as early as the 1990s by some scientists and visionaries, following advances in genomics. In Traditional Chinese Medicine (TCM) and Ayurvedic medicine, treatments customized for each patient—using personalized blends of medicinal herbs—have been practiced for centuries (Gwynn and Hylands, 2000). Phytotherapy is a branch of medicine that uses plants and the active ingredients extracted from them to prevent and treat various conditions; it differs from other natural therapies in its emphasis on scientific evidence, standardization, and clinical studies (Petkova *et al.*, 2019; Allegra *et al.*, 2023). The relevance of phytotherapy has grown alongside the trend toward individualized therapy, as its holistic, patient-centered approach aligns with the principles of personalized care, focusing on a person's unique constitution rather than on a single symptom or disease. Furthermore, the trend in recent decades toward the use of natural plant-based products and the integration of complementary and alternative medicine has intensified since the COVID-19 pandemic (Latif and Nawaz, 2025). Phytotherapeutic extracts, like conventional medicines, act on the body through the biological/pharmacological activity of the biochemical compounds they contain, with their effects depending on the nature and concentration of these compounds (Allegra *et al.*, 2023). Unlike most conventional medications, which are based on a single active ingredient, plant extracts contain multiple bioactive compounds that act synergistically. The therapeutic potential and biological activity of complex plant extracts yield new effects that go beyond the properties observed in isolated compounds. This complexity allows for a nuanced approach to treatment that can be adapted to the specific and multifaceted needs of each individual. As early as the year 2000, Gwynn and Hylands foresaw the emergence of a new class of prescription remedies—featuring complex blends of plant extracts and compounds with synergistic effects—for the treatment of various conditions and their associated symptoms, as well as for prophylactic purposes.

Along the same lines, the WHO provides guidelines for research on traditional medicine (TM), focusing on quality, safety, and efficacy, with an emphasis on botanical

verification, chemical analysis, and rigorous clinical studies to generate consistent evidence, and integrating TM into health systems through standards, research, and the promotion of regulatory frameworks at the national level, all in accordance with the new Global Strategy for Traditional Medicine 2025–2034. Key methodologies include harmonization of terminology, botanical identification, biological and pharmacognostic studies, clinical evaluation, analysis, and documentation of evidence (WHO, 2025). The first essential step involves the precise identification of plants (botanical verification); the subsequent steps involve isolating and identifying the active constituents (such as polyphenols, alkaloids, and tannins), understanding the chemical profiles of medicinal plants (including variations among populations, among plant organs, the influence of genetic and environmental factors), and standardizing the ingredients to ensure consistent quality, safety, and efficacy (WHO, 2025).

The approach to discovering new plant-based therapeutic agents depends on the desired outcome. The strategies employed and the requirements differ between the development of phytotherapeutic products marketed as dietary supplements and the development of isolated active compounds used in the pharmaceutical industry or marketed as medicine. The initial steps are common and include: the selection of relevant plants, typically based on studies of their use in traditional medicine; botanical identification and characterization; analysis of the phytochemical composition; and isolation of bioactive compounds using chromatographic and spectrophotometric methods. Given that plant extracts contain a large number of bioactive compounds but in low concentrations, the techniques must be sensitive to a variety of compounds in small quantities, simple, reproducible, and cost-effective (Rates, 2001). Thereafter, the strategy aimed at developing a medicine involves the characterization and large-scale partial or total synthesis of the isolated compound, which is necessary for costly preclinical, clinical, and toxicological studies. The strategy aimed at developing phytotherapeutic products is less costly both financially and in terms of time, even though it also involves studies on the product's efficacy and safety (Rates, 2001). Although the research in this doctoral thesis follows the common stages, we focused on the end objective in the form of phytotherapeutic products.

Traditional medical practices vary by country and region, influenced by local flora, culture, history, and other factors. Their safety and efficacy are supported by knowledge and experience accumulated over centuries, supplemented by scientific studies conducted in recent decades (WHO, 2000; Süntar, 2020; Latif and Nawaz, 2025). In Romania, the traditional use of medicinal and aromatic plants dates back to the first century CE, to the

time of the Thracian settlements (Kupke *et al.*, 2000). Favoured by its biogeographic location and diverse landscape, Romania has a high level of biodiversity. It is a meeting point for various ecosystems and bioregions, which is also reflected in the richness of its vegetation cover (Kathe *et al.*, 2003). Regarding medicinal and aromatic plants, Romania is an important resource for Europe, as significant quantities are cultivated—but especially harvested from wild flora—for economic purposes. It has been estimated that Romania's flora comprises over 3,700 taxa, representing nearly 30% of Europe's vascular flora, of which over 20% are medicinal plants (Murariu, 2002; Segneanu *et al.*, 2018).

The active compounds in plants are secondary metabolites, which, although they do not play a role in plant growth and development, are essential for survival and mediate interactions with the environment. Therefore, their presence and concentration are strongly influenced by genetic and environmental factors (Borges *et al.*, 2017; Zhan *et al.*, 2022). In the case of medicinal plants, it is important to examine the influence of specific environmental conditions from the harvesting sites on the composition of active principles, through local and comparative studies.

The research presented in this doctoral thesis is anchored in the global effort to identify new compounds with medicinal value and new solutions for the prevention and treatment of complex conditions such as those mentioned above, which are rooted in oxidative stress and inflammation. Furthermore, this research aligns with the current trend toward personalized medicine and the growing interest in phytotherapy.

Our research highlights the therapeutic potential of four species of medicinal plants that have been known and used in traditional medicine in various regions of the world since ancient times: *Tussilago farfara* L., *Geranium robertianum* L., *Leonurus cardiaca* L., and *Oenothera biennis* L. Their therapeutic properties and their content of important bioactive compounds are supported by scientific studies, with notable antioxidant and anti-inflammatory properties, as well as a number of common biological mechanisms of action, as mentioned above and summarized in Chapter 1, which is dedicated to the scientific literature. Some of these properties are recognized in conventional medicine, with all four species being included in numerous pharmacopoeias around the world. All the aspects discussed above, along with the lack of necessary biological studies in the scientific literature, formed the basis for the selection of these four species.

The results obtained fill in gaps in the scientific literature with data on the morpho-anatomy of the species studied, which are essential for characterizing and distinguishing between species, hold botanical interest, and are necessary for quality control of plant

material used in phytotherapeutic preparations. In order to conduct comprehensive analyses of the structure and ultrastructure of the vegetative organs—which, in addition to morpho-anatomical characterization, aimed to identify the organs and structures responsible for the production and storage of bioactive compounds in these four species—we employed optical, scanning electron (SEM), and transmission electron microscopy (TEM). We emphasize that numerous morphological, anatomical, and phytochemical aspects are entirely new to the scientific literature and of fundamental scientific value (Bota *et al.*, 2020; Bota *et al.*, 2022a; Bota *et al.*, 2022b; Bota *et al.*, 2022c; Bota *et al.*, 2025). The results often include the first description of the morpho-anatomical structure of the vegetative organs in the species under study; the subsections on the ultrastructure of cells in various tissues present entirely new data for these four species and make a substantial contribution to the knowledge and understanding of certain cellular structures and processes that are currently poorly understood. Furthermore, the results supplement the scientific literature with data on the composition of active compounds, analyzed using modern chromatographic and spectrophotometric methods (LC/MS and GC/MS), with some compounds identified for the first time in the species studied. At the same time, this research addresses the need for comparative studies and analyses of Romania's plant populations. The morpho-anatomical descriptions of the species are followed by subsections that present a comparative analysis of the bioactive compound composition of two types of plant extracts (aqueous and hydroalcoholic), obtained from plants belonging to two different populations, each from the lowland and mountain regions, respectively, of Romania's wild flora. The comparative analysis of active principles content is placed in the context of the different pedoclimatic factors that characterize the two areas where the plant material was collected, factors which, according to the scientific literature, influence the plants' composition of bioactive compounds (Liu *et al.*, 2016; Suyal *et al.*, 2020; Zhang *et al.*, 2020; Chelghoum et al, 2021). For each sampling site, we analyzed the altitude, climatic conditions over three years—encompassing the entire ontogenetic development of the species—and a series of basic edaphic parameters. The spatial distribution of climatic parameters (annual average temperature, cumulative annual precipitation) was modeled using QGIS technology and the IDW (Inverse Distance Weighting) interpolation method, with the results converted into thematic maps—an approach that follows developments in the field of GIS (Geographic Information Systems) and applied studies, highlighting the usefulness of spatial analysis for understanding landscape dynamics and their impact on plants.

RESEARCH PURPOSE AND OBJECTIVES

The research presented in this doctoral thesis aimed, on the one hand, to characterize the morpho-anatomical and biochemical properties of the species *Tussilago farfara* L., *Geranium robertianum* L., *Leonurus cardiaca* L. and *Oenothera biennis* L. found in Romania's wild flora, including the identification of the organs responsible for the production and storage of bioactive compounds; and, on the other hand, to analyze the influence of environmental factors on their content of secondary metabolites of therapeutic interest.

Research objectives:

O1 – morpho-anatomical investigations of vegetative organs: analysis of the structure, micromorphology, and ultrastructure of the leaf, stem and/or rhizome, and root, using optical microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM), respectively; observation and description of anatomical features important for the identification and characterization of species, as well as of structures involved in the production and storage of bioactive compounds of pharmacological interest.

O2 – Obtaining hydroalcoholic and aqueous extracts from the vegetative organs of taxa collected from two geographical regions in Romania with different ecological conditions, and their biochemical characterization.

O3 – assessment of environmental factors at the harvesting sites that may influence phytochemical composition: analysis of soil fertility (pH, total nitrogen, phosphorus, and potassium—in assimilable forms); analysis of climatic conditions throughout the life cycle of the species under study (annual average temperature, average temperatures in January and July, annual maximum and minimum temperatures, cumulative annual precipitation, frost-free days), altitude.

O4 – a comparative analysis of the polyphenolic profile of specimens belonging to the taxa of interest collected from two geographical regions of the country with different ecological conditions (plains and mountains).

PART I: CURRENT STATE OF KNOWLEDGE

CHAPTER 1
RESEARCH HISTORY ON THE BIOLOGY OF THE SPECIES UNDER STUDY

1.1. Biology of Tussilago farfara L. species – research history

1.1.1. Uses

Tussilago farfara L. is a species with a long-standing reputation for medicinal use (Săvulescu, 1964). Following the etymology of its name, which derives from the Latin „*tussio*" - *cough* and „*agere*" – *to heal, to drive away*, the primary use of plants belonging to this species is for treating coughs (Săvulescu, 1964; Barkley, 2006; Zhi *et al.*, 2012). In Romania, the species is commonly known as "coltsfoot" or "donkey's hoof," a name that reflects the shape of its leaves, which are used in traditional European medicine (Drăgulescu and Drăgulescu, 2000; Sârbu *et al.*, 2013).

In Traditional Chinese Medicine (TCM), flower buds (*Farfarae flos*/Kuandonghua) are used; they have been mentioned in Chinese medical texts as far back as the Qin and Han dynasties (approximately 2,000 years ago) for the treatment of coughs, bronchitis, and asthma (Zhi *et al.*, 2012; Wu *et al.*, 2016; Liu *et al.*, 2020), and *Collective Notes to Canon of Materia Medica* (Tao Hongjing, A.D. 456-536 cited by Chen *et al.*, 2021) includes an early mention of them in the treatment of diabetes, along with cough, sore throat, shortness of breath, terror-induced epilepsy, chills, fever, and nausea.

In the westernmost part of Asia, in the region of Anatolia, in addition to the primary uses of the species discussed so far, the leaves and flowers are also used to treat eye inflammation, varicose veins, wounds, burns, menstrual disorders, and dyspepsia (Uzun *et al.*, 2004). In the Russian Pharmacopoeia, the leaves are recommended as an expectorant and sedative for the treatment of colds and other respiratory conditions (asthma, bronchitis), and according to Vereschagin et al. (1959), cited by several authors, they are used in Siberia – administered internally to improve digestion, treat diarrhea, and as an analgesic, and externally for skin conditions (Shikov *et al.*, 2014; Jarić *et al.*, 2018). For example, in 2018, Jarić *et al*., along with other authors, mention their external use for skin conditions (wounds, burns, ulcers) in Turkey, Ukraine, Poland, and several countries in the Balkan Peninsula, while Rigat *et al*., in 2015, note their use in the Iberian Peninsula.

Unlike in Asia, where countries with medicinal traditions similar to China's use the species in the same way (Liu *et al*., 2020), in Europe and the Americas other plant' organs

(flowers, leaves, roots) are used for both medicinal and culinary purposes, with a preference for the leaves (Tobyn *et al.*, 2011; Zhi *et al.*, 2012; Liu *et al.*, 2020).

According to Chen *et al.* (2021), the traditional use of its leaves in Europe extends to the treatment of gastrointestinal and urinary disorders, wounds, burns, and eye inflammation. In the United States, coltsfoot roots are used as an infusion for coughs and asthma, as a stimulant, relaxant, demulcent, expectorant, and tonic, as well as for treating chronic inflammation (catarrh), liver disorders (as a depurative and tonic), and tuberculosis (Tobyn *et al.*, 2011; Herrick, 1977, Hopper and Chandler, 1984 cited by Liu *et al.*, 2020).

In Europe, including Romania, this species is also used for food (Săvulescu, 1964; Łuczaj and Szymański, 2007; Łuczaj, 2012; Kalle *et al.*, 2012; Dénes *et al.*, 2012; Adamczak *et al.*, 2013; Dogan *et al.*, 2015; Ghosh *et al.*, 2017; Uysal *et al.*, 2018). Given its dual role as both a food and a medicinal remedy, there is potential for its use as a functional food, which has not yet been sufficiently explored.

1.1.2. Pharmacological and pharmacokinetic studies

Pharmacological and pharmacokinetic studies conducted to date, both *in vitro*, *in vivo*, and *in silico*, largely on flower buds and leaves, but also on certain isolated phytochemicals, have revealed biological activities consistent with their use in traditional medicine. Furthermore, a number of studies have been reported that support the species' medicinal potential beyond the aforementioned uses, particularly in the field of oncology.

In support of the use of this species for treating respiratory system disorders, references can be made to the studies conducted by Li *et al.* (2012), Wu *et al.* (2016), Li Jing *et al.* (2018), Yang *et al.* (2020), Xu *et al.* (2022), and Lin *et al.* (2022) on flower buds. The uses for respiratory and skin conditions mentioned in traditional medicine are further supported by the antiviral and antimicrobial effects reported for all parts of the plant. These properties are supported by studies conducted by Kokoska *et al*. in the year 2002 on the aerial organs and rhizome; Turker and Usta (2008), Danilets *et al.* (2010), and Uysal *et al.*, (2018) on the leaves; Zhao *et al.* (2014) on the aerial organs (including the case of tuberculosis); Hleba *et al.* (2014) on the flowers and stem; by Boucher *et al.* (2020) on the flowers; by Ding *et al.* (2018), Lee *et al.* (2019), and Sun *et al.* (2019) on the flower buds. Both antimicrobial and antipsoriatic/antidermatitic effects have been reported for isolated tussilagone (Kim *et al.*, 2017; Lee *et al.*, 2020). Promising results, which support the species' effectiveness in treating diabetes, have been obtained for the flower buds by Gao *et al.* (2008), Park *et al.* (2008), and Song *et al.* (2020), and for the leaves by Kuroda *et al.* (2016) and Kikuchi *et al.* (2020).

Furthermore, positive results were also reported for the treatment of gastrointestinal disorders by Szentmihályi *et al.* in 2013 for an infusion made from leaves and flower buds, by Safonova *et al*. in 2018 for isolated polysaccharides, and by Cheon *et al*. in 2018.

Most studies conducted in this regard explore the species' potential beyond its uses in traditional medicine. For example, flower buds have been reported to have beneficial effects in treating cardiovascular diseases (Hwang *et al.*, 1987; Li and Wang, 1988), neurodegenerative diseases (Cho *et al.*, 2005; Lim *et al.*, 2008; Lim *et al.*, 2015; Lee *et al.*, 2018), cerebral ischemia (Hwang *et al.*, 2018) and neuropathy (Khan *et al.*, 2021), as well as colon, lung, and breast cancer (Ryu *et al.*, 2014; Qin *et al.*, 2014; Qu *et al.*, 2018; Jang Hyeri *et al.*, 2019; Lee *et al.*, 2019; Song *et al.*, 2021). Other studies demonstrate the cytoprotective capacity of flower buds (Kang *et al.*, 2016; Sun *et al.*, 2019), as well as efficacy against enterovirus 71 (Chiang *et al.*, 2017), allergies (Jin *et al.*, 2020), allergic rhinitis (Wu *et al.*, 2021), and in the prevention of osteoporosis (Ryoo *et al.*, 2020).

Similar activities have been reported for tussilagone, sesquiterpenes, and isolated polysaccharides. The results obtained by Safonova and colleagues regarding the isolated polysaccharides show not only antitumor activity but also an increase in the efficacy of conventional treatment, genoprotective and regenerative activity, promoting the repair of tissues damaged by polychemotherapy and cytostatics, a reduction in fluoracil toxicity, and an increase in the number of hematopoietic stem cells in the bone marrow (Safonova *et al.*, 2016, 2018a, 2018b, 2019, 2020a, 2020b).

1.1.3. Chemical composition

Given the predominant use of coltsfoot flower buds and leaves, these are the most extensively studied plant organs in terms of their chemical composition, followed by the flowers and other parts of the plant. Existing assessments in the scientific literature show a similar chemical profile for these plant organs, which nevertheless exhibit significant variability in content among flower buds, flowers, and leaves, and across plant populations. (Zhi *et al.*, 2012; Xue *et al.*, 2012; Li *et al.*, 2018). Since 1980, several studies have been conducted on the chemical composition of flower buds, revealing the presence of approximately 175 compounds, including terpenes, organic acids, flavonoids, alkaloids, chromones, essential oils, bioactive polysaccharides, as well as tannins, amino acids, steroids, ketones, lipids, aldehydes, iaraxanthin, and minerals. Research often focuses on the content of sesquiterpenes and caffeoylquinic acids for their pharmacological activities, and on pyrrolizidine alkaloids for their toxic potential (Liu *et al.*, 2020).

1.1.4. Pyrrolizidine alkaloid content and species toxicity

Although *T. farfara* is a species with a long history in traditional medicine, the safety of its therapeutic use has been called into question due to the presence of unsaturated pyrrolizidine alkaloids (PA) (Evans *et al.*, 2019). The controversy surrounding the safety of consuming plants containing PA led to the formation of a team of experts, which conducted a comprehensive analysis of the evidence gathered to date. According to the team, the evidence regarding adverse effects from the consumption of *T. farfara* is scarce, of poor quality, and does not allow for the establishment of a direct causal relationship. Reports of toxicity resulted from misidentification of the species, adulteration of plant-based medicinal products, or substitution of the species. (Evans *et al.*, 2019). An analysis of all case studies conducted by Avila *et al.* in 2020 shows some level of association between an ingested material and an adverse effect, but the lack of unequivocal identification of the species consumed casts significant doubt on the conclusions. None of the existing case studies provides solid evidence on which to draw conclusions regarding the plant's toxicity. However, it has been observed that PA levels can be significantly reduced under controlled growing conditions (Wawrosch, 2000).

1.1.5. Antioxidant and anti-inflammatory potential

The plant's therapeutic potential is primarily attributed to its antioxidant and anti-inflammatory effects. The antioxidant activity of the flower buds has been supported by several studies conducted both *in vitro* and *in vivo*, on extracts and isolated compounds (Ryu *et al.*, 1999; Cho *et al.*, 2005; Kim *et al.*, 2006; Li *et al.*, 2012; Qin *et al.*, 2014; Jang *et al.*, 2016; Song *et al.*, 2020). The antioxidant activity of the leaves is supported by studies conducted by Georgescu and Antemir (2002), Arnold *et al.* (2015), Uysal *et al.* (2018), and Tajner-Czopek *et al.* (2020) on aqueous and alcoholic extracts.

With regard to anti-inflammatory activity, the flower buds are once again the most studied, whether in the form of buds fried with honey (Yang *et al.*, 2020), as an extract (Hwang *et al.*, 1987; Hwang *et al.*, 2018), or as sesquiterpenes isolated from them (Lim *et al.*, 2008; Hwangbo *et al.*, 2009; Jin *et al.*, 2020; Khan *et al.*, 2021). The anti-inflammatory activity of the leaves is supported by Arnold *et al.* (2015) and Conea *et al.* (2016), through *in vitro* and *in vivo* studies, respectively, and the aqueous and ethanolic extracts analyzed by Ravipati *et al*. in 2012 on macrophages activated by LPS and IFN-gamma demonstrated inhibitory effects on NO and TNF-alpha.

1.2. Biology of Geranium robertianum L. species – research history

1.2.1. Uses

Geranium robertianum L. is a species that has been used medicinally since ancient times, as mentioned by Dioscorides in his work ”De Materia Medica” (50 B.C., p. 362). The origin of the species' name is less clear, with various versions presented in the literature (Larkin, 2011). In Romanian, the species has a variety of common names, many of which allude to the shape of the fruit, its scent, its use, or the species' ecology; the best known of these being „năpraznic” (Săvulescu, 1958; Drăgulescu, 2013; Sârbu *et al.*, 2013; Drăgulescu, 2018).

Regarding the medicinal uses of this species in the Middle Ages, in the 12th century, in Chapter CXLIV of the work *Physica*, written by the Benedictine nun Hildegard of Bingen, it is mentioned that *G. robetianum* powder was snorted for congestion, baked into cakes and eaten for coughs or lung constriction, dissolved in warm wine and drunk for chest and throat pain or loss of voice, and, when mixed with feverfew (*Tanacetum parthenium*) or nutmeg (*Myristica fragrans*), sprinkled on bread or licked from the hand to relieve heartache (Larkin, 2011). In the 15th-century work *Ein Gard der Gesundheit*, the plant is recommended as a diuretic, for the treatment of kidney stones, and as a cardiac tonic, while the 16th-century writings of John Gerard follow Dioscorides' recommendations for its use in treating bleeding wounds (Larkin, 2011).

The use of this species in traditional medicine is based on the principles of the *Theory of Signatures*, which was developed as far back as antiquity and reached its peak during the Middle Ages. The Theory of Signatures seeks to explain the surrounding world by highlighting connections between appearance and properties, particularly in the case of medicinal plants. According to this theory, a plant with red pigments, such as *G. robertianum*, should regenerate blood (Machon and Motard, 2018) or treat conditions related to blood circulation, explaining the species' use as a hemostatic, a wound-healing agent, and for cardiac problems, some of these properties having subsequently been scientifically confirmed (Lacoste, 2015).

The scientific literature on the traditional medicinal uses of *G. robertianum* mentions its astringent, hemostatic, wound-healing (Pedro *et al.*, 1992; Radulović *et al.*, 2011), antiseptic, antispasmodic, and diuretic properties (Fodorea *et al.*, 2005), as well as hypoglycemic, antitumor, tonic (Radulović *et al.*, 2011), anti-inflammatory, antibacterial, antihepatotoxic (Graça *et al.*, 2016a), antidiarrheal (Kartig and Bucar-Stachel, 1991; Papović *et al.*, 2021), and antihypertensive properties (Tiță *et al.*, 2009). Infusions made from the

leaves, stems, and flowers have hypoglycemic, antispasmodic, and antitumor effects (Kahouaji, 1995, cited by Bnouham *et al.*, 2002; Cunha *et al.*, 2009; Bautista *et al.*, 2015).

According to several authors cited by Fodorea *et al.* in the year 2005, the species is used in traditional medicine to treat wounds, bleeding, and heart conditions, as well as diarrhea (Leclerc, 1973; Hoppe, 1975; Reader's Digest, 1985; Ody, 1993; Chevalier, 1996) and cancer (Leclerc, 1973). Other sources also mention its use in treating digestive disorders, diabetes, and allergies (Graça *et al.*, 2016a).

Concerning internal use, infusions, tinctures, decoctions, and essential oils are mentioned, while external use includes poultices for treating eye and mouth conditions, as well as fresh leaves applied directly to skin rashes (herpes, eczema, infections) (Lanzara, 1997; Della Beffa, 1999, cited by Farcas, 2018).

Regarding the uses of this species in traditional Romanian medicine, in 2016, Tudor Ilie noted its popularity in mountainous regions as a tonic and for fertility-related issues.

G. robertianum is also an edible species, even in its raw state (Botineau, 2013). According to Couplan and Duke (2013), the fresh leaves can be used in salads or in tea and have an earthy, bitter, and astringent taste.

1.2.2. Pharmacological and pharmacokinetic studies

The first studies on the antimicrobial activity of this species were conducted by Hersch-Martinez *et al.* (2005), Tosun *et al.* (2005), Schelz *et al.* (2006), and Lima (2009). Radulović *et al.* (2012) observed stronger antimicrobial activity in the essential oils extracted from the underground organs (MIC 0.312-10 mg/mL), compared to those from the aerial ones. According to a 2014 study conducted on 70 patients with acute external otitis (AEO), the essential oil of *G. roberianum*, in combination with those of *Syzygium aromaticum* and *Lavandula angustifolia*, was just as effective as the antibiotic ciprofloxacin, which is often used to treat this condition (Panahi *et al.*, 2014). Subsequently, antimicrobial effects were reported for volatile oils extracted from the herba (Renda *et al.*, 2016; Gębarowska *et al.*, 2017), as well as for extracts from the flowers, leaves, stems, and roots, against a range of Gram-positive and Gram-negative bacteria and the fungus *Candida albicans* (Shawarb *et al.*, 2017; Alhage and Elbitar, 2019; Suručić *et al.*, 2021).

Although several sources mention the species G. robertianum as a taxon used in traditional medicine to treat diabetes, with hypoglycemic effects, we have identified only one study in the scientific literature that provides scientific evidence to support this claim (Ferreira *et al.* 2010a).

The enzymatic inhibitory activity of aqueous extracts from the herba, purified and concentrated by microfiltration and ultrafiltration, respectively, was demonstrated for the enzymes urease and alpha-chymotrypsin. The results obtained support the use of this species in the treatment of gastric disorders such as *Helicobacter pylori*-induced ulcers; a possible explanation in this case is the synergistic action of the polyphenolic compounds identified in the tested extracts (Păun *et al.*, 2014).

The species' cytotoxic activity against tumor cells was studied using a taxon from Romanian flora (Păun *et al.*, 2012). Subsequently, a correlation was established between the antioxidant and cytostatic effects and the polyphenol content of aqueous leaves extracts (Neagu *et al.*, 2017). The cytotoxic activity of the aqueous and organic extracts was also observed in the MCF-7, NCI-H460, HeLa, and HepG2 tumor cell lines, without affecting healthy liver cells (Graça *et al.*, 2016; Graça *et al.*, 2017). Similar results were also observed for the leaves decoction (Catarino *et al.*, 2017).

The tannin-rich infusion exhibits chelation capacity for heavy metals (Cu, Cd, Ni, Pb, Hg), reducing their bioavailability in the body and promoting the detoxification process (Apostu *et al.*, 2017).

A neuroprotective effect of the aqueous extract was recently observed, protecting cells in differentiated SHSY-5Y cell cultures from toxicity induced by 1-methyl-4-phenylpyridinium (MPP+), an effect that suggests a potential benefit of the extract in the treatment of Parkinson's disease (Aslan and Yilmaz, 2023). Recent research also provides evidence regarding the gastroprotective capacity of the aqueous leaves extract (Bawish *et al.*, 2023).

1.2.3. Chemical composition

Phytochemical studies on the species *G. robertianum* are based on solid-liquid extracts and focus on phenolic compounds and essential oils (Graça *et al.*, 2016a). Tannins were the first class of compounds reported in *G. robertianum* (Hegnauer, 1966, cited by Graça *et al.*, 2016a). Together with flavonoids and phenolic acids, they are the most extensively studied classes of compounds found in *Geranium* species, including *G. robertianum* (Graça *et al.*, 2016a). It is well known that *Geranium* species contain both hydrolyzable and condensed tannins, with the latter concentrated in the underground organs and the ellagitannins in the leaves (Graça *et al.*, 2016a).

Among phenolic acids, ellagic acid has been identified in significant amounts in various extracts (Bate-Smith, 1962; Kobakhidze and Alaniya, 2004; Fodorea *et al.*, 2005;

Santos *et al.*, 2013; Păun *et al.*, 2014; Catarino *et al.*, 2017; Graça *et al.*, 2017; Neagu *et al.*, 2017). Other phenolic acids present in this species were identified by Amaral *et al*. (2009) and by Păun and collaborators during 2011–2012.

Flavonoids were first described in *G. robertianum* by Bate-Smith in the year 1962. They represent the main class of secondary metabolites in this species, with flavonols predominating (Graça et al., 2016a; 2017), and variations in their relative abundance are sometimes correlated with the geography of the genus (Bate-Smith, 1973).

Other types of compounds identified in *G. robertianum* include lectins, which were detected by Rybak and Rudik in 2013 in the rhizome. The presence of alkaloids was detected by Hultin and Torssell in 1965 in the dried plant, and by Igwenyi and Elekwa in 2014 in the fresh leaves. Saponins have been reported in similar quantities (Păun et al., 2011; Igwenyi and Eleka, 2014), small amounts of glycosides, oxalates (Igwenyi and Elekwa, 2014), and lutein in aqueous extracts from the herba (Loranty et al., 2010), as well as acetovanilone and 3',4'-dimethoxyflavone (Amaral *et al.*, 2009).

An early study on the composition of essential oils extracted from the aerial organs of plants belonging to this species, published in 1992 by Pedro *et al*., detected over 100 compounds, of which 72 were identified (86% of the total oil). Later, the study conducted by Radulović *et al*. (2012) included both the aerial and underground organs of the plants, identifying 152 compounds in the former (95.8% of the oil) and 53 compounds in the latter (98% of the total compounds). The differences in composition could be explained by the species' genetic variability or by the influence of environmental factors (Radulović *et al*., 2012). Overall, the amount of volatile oils in *G. robertianum* is low, a fact that, according to Radulović *et al*. (2012), is due to low production of volatile compounds rather than the extraction methods used, a view supported by the predominance of fatty acids and their derivatives, along with carotenoid derivatives, in the composition of the analyzed volatile oil samples.

1.2.4. Antioxidant and anti-inflammatory potential

Antioxidant activity is the most studied biological activity of this species, the scientific literature comprising a number of 17 studies (published through June 2023) conducted on various plant organs, of diverse origins, primarily using aqueous or alcoholic extracts and the DPPH assay (2,2'-diphenyl -1-picrylhydrazyl) test, and to a lesser extent other *in vitro* methods (ABTS—2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) antioxidant test, beta-carotene, metal chelating activity, FRAP—ferric reducing antioxidant power, etc.)

(Katalinic *et al.*, 2006; Amaral *et al.*, 2009; Lima, 2009; Nikolova *et al.*, 2010; Neagu *et al.*, 2010b, 2017; Păun *et al.*, 2011, 2012; Ben Jemia *et al.*, 2013; Santos *et al.*, 2013; Graça *et al.*, 2016b, 2017; Shawarb, 2017; Ilic *et al.*, 2021; Bawish *et al.*, 2023). Although there are differences in the results obtained in these studies, the unanimous conclusion is that *G. robertianum* extracts possess significant antioxidant capacity.

A review of the scientific literature revealed only four studies that refer to the anti-inflammatory activity of this species (Amaral *et al.*, 2009; Piwowarski *et al.*, 2011, 2014; Catarino *et al.*, 2017).

1.3. Biology of Leonurus cardiaca L. species – research history

1.3.1. Uses

Leonurus cardiaca L. is a medicinal plant whose benefits for the cardiovascular, nervous, and female reproductive systems have been known since ancient times in the traditional medicine of both Asia and Europe (Sermukhamedova *et al.*, 2017; Fierascu *et al.*, 2019). Later, the species' medicinal potential was also recognized by modern medicine, being included in most of the world's pharmacopoeias, and used in the preparation of numerous dietary supplements (Fierascu *et al.*, 2019; Sadowska *et al.*, 2019).

In conventional medicine, it is recommended to use the first 40 cm from the top of the *Leonurus cardiaca* L. herba, harvested at the beginning of flowering, either whole or dried and chopped. Some pharmacopoeias, such as those of the Commonwealth and the Russian Pharmacopoeia, also consider as suitable the use of raw material harvested from *Leonurus quinquelobatus* Gilib. ex Usteri, or *Leonurus villosus* Desf. ex Spreng. This clarification is due to the fact that, prior to the end of the 20th century, *L. villosus* was considered a subspecies of *L. cardiaca*, whereas *L. quinquelobatus* was considered a synonym of *L. cardiaca* L. (Shikov *et al.*, 2014, and several authors cited by Sermukhamedova *et al.*, 2017).

Since the 1930s, numerous studies have been conducted on the phytochemical composition and biological activities of several *Leonurus* species, primarily *L. japonicus* (from Asia), *L. cardiaca* (from Europe), *L. persicus* (Iran and Turkey), and *L. sibiricus* (from Mongolia and Siberia), and occasionally other minor species (*L. rurkestanicus*, *L. glaucescens*, and *L. macranthus*) (Zhang, 2018). In the scientific literature, there is sometimes confusion between species, on the one hand—of a taxonomic nature—as differences have been demonstrated between plants that were later recognized as distinct species, for example, *L. japonicus* and *L. sibiricus* (Pitschmann et al., 2017)— and, on the other hand, confusion regarding chemical composition, as information is cited regarding the

presence of certain compounds or biological activities in a particular species, while the original sources refer to other species.

In Traditional Chinese Medicine (TCM), the dried aerial parts (Leonuri herba or Yimucao) and, sometimes, the dried fruits (Chong-Wei-Zi) of *L. japonicus* are used. (Zhang *et al.* 2018; Lam *et al.*, 2022). Known as the "plant that benefits mothers", it has been used for centuries to treat gynecological and obstetric conditions (Zhang *et al.*, 2018; Lam *et al.*, 2022). Similar references are found in the literature for *L. cardiaca*, but it is listed as *Leonuri cardiacae herba* (EMA, 2010), which can easily be confused with *Leonuri herba* (L. japonicus), especially when the Latin name is not specified, thus leading to a possible confusion in the literature between the two species. Zhang *et al.* (2018) are among the few authors who point out that the plant commonly known as "motherwort"—used in China for gynecological conditions and in Europe for cardiac and nervous disorders—actually refers to two different species: *L. japonicus* and *L. cardiaca*, respectively.

The specialized literature mentions the culinary use of this species as a seasoning in vegetable soups (lentil, pea, etc.) and for flavoring beer and teas (Shikov *et al.*, 2017, cited by Fierascu *et al.*, 2019). Either fresh or dried flowers are used for this purpose (Facciola, 1990).

1.3.2. Pharmacological and pharmacokinetic studies

In the scientific literature, the biological activities of the species are described first from the perspective of original research and clinical studies conducted on *L. cardiaca* preparations, and second from the perspective of the effects observed in studies on isolated compounds.

Cardiovascular activity underlies the main applications of *L. cardiaca* L. species (Fierascu *et al.*, 2019).

To date, there have been reports of several effects of *L. cardiaca* preparations and isolated compounds on the nervous system, including analgesic, sedative, anxiolytic, and antidepressant effects (Rezaee-Asl *et al.*, 2014; Rauwald *et al.*, 2015; Liu *et al.*, 2016; Zhang *et al.*, 2018; Koshovyi *et al.*, 2021).

Moreover, studies were carried out on the antimicrobial and antiviral activity of the species (Agnihotri *et al.*, 2008; Tahmouzi and Ghodsi, 2014; Samoilova *et al.*, 2014; Todorov *et al.*, 2014; Micota *et al.*, 2016).

Additional medicinal uses of this species are based on its nephroprotective, hepatoprotective, genoprotective, and antigenotoxic effects (Xu *et al.*, 2014; Cheng *et al.*, 2015; Pereira *et al.*, 2019; Angeloni *et al.*, 2021; Oalde *et al.*, 2021).

1.3.3. Chemical composition

Considering the use in traditional and conventional medicine of *Leonurus cardiacae herba*, which consists of the aerial parts of the plant harvested during the flowering period, research on the chemical composition of this species has focused primarily on the aerial parts as a whole rather than on individual components. To date, several types of bioactive compounds have been identified, including terpenes, nitrogen-containing compounds, phenylpropanoids, flavonoids, phenols, and, in small quantities, volatile oils, sterols, and tannins (Wojtyniak *et al*., 2012), and recent studies continue to identify compounds whose presence in *L. cardiaca* was previously uncertain or even denied (EMA, 2010; Angeloni *et al.*, 2021).

1.3.4. Antioxidant and anti-inflammatory potential

According to Ali *et al*. (2007), ursolic acid isolated from the ethanolic extract of *L. cardiaca* has anti-inflammatory potential comparable to that of aspirin and indomethacin, as well as antioxidant activity. The anti-inflammatory activity of various extracts obtained from *L. cardiaca* is supported by studies conducted by Flemming *et al.*, 2015; Sadowska *et al.*, 2017 and Wu *et al.*, 2018.

Antioxidant activity has been studied more extensively than anti-inflammatory activity, and there are significant differences between the results obtained. Overall, the antioxidant activity of this species is considered moderate or weak (Matkowski *et al.*, 2006; Armatu *et al.*, 2010; Jafari *et al.*, 2010; Ebrahimzadeh *et al.*, 2010b; Yi and Wetzstein, 2011; Tahmouzi and Ghodsi, 2014; Zhogova *et al.*, 2014; Sadowska *et al.*, 2017; Ji *et al.*, 2017; Ziyatdinova *et al.*, 2017, 2018; Pereira *et al.*, 2019; Oalde *et al.*, 2021; Angeloni *et al.*, 2021).

1.4. Biology of Oenothera biennis L. species – research history

1.4.1. Uses

The modern name of the genus *Oenothera* was given by Carl Linnaeus in his 1735 work *Systema Naturae*. The species name "*biennis*" refers to its biennial life cycle (Steckel *et al.*, 2019). The English name "Evening-primrose" and the Romanian name "Luminița nopții" refer to the flowers that open at sunset (Britton and Brown, 1898, cited by Steckel *et*

al., 2019). Just as evocative are the English names “King’s cure-all” and “Fever plant” (Calapai *et al*., 2015), which recall the time when the plant was considered a universal remedy and its use in treating fever, respectively.

Given the species’ origin on the American continent, its earliest uses are rooted in the medicinal and dietary practices of Native Americans. Although seeds are currently the most widely used part of the plant, from which an oil rich in essential fatty acids is extracted and marketed as a dietary supplement or ingredient in various products (cosmetics, pharmaceuticals, etc.), and the scientific literature abounds with studies on their composition and biological activity, these seeds had no application in traditional medicine (Balch, 2000, Kuhns and Winston, 2008, Stonemetz, 2008). Instead, the entire plant was used to treat inflammations in the body and various associated diseases (EMA, 2018a), as well as to make an ointment applied topically to skin lesions; the leaves, flowers, and outer root layer were used to treat spastic cough and irritable bowel syndrome, and, together with *Hypericum perforatum* and *Veronicastrum virginicum*, for depression associated with gastrointestinal disorders (Kuhns and Winston, 2008; EMA, 2018a).

It is important to note that in numerous studies on the phytochemical composition and biological activity of this species, the plants were not collected from areas corresponding to the settlements of the Native Americans who used the species. Often, the plants are collected from other regions where they grow natively or have been introduced and naturalized, which can significantly influence their chemical composition and biological activity due to different climatic and geographical conditions (Vanhaelen *et al.*, 1991; Setzer, 2018).

Several species of Oenothera were introduced to Europe in the early 1700s, including *O. biennis*, which for a long time was studied intensively not from a medicinal perspective, but as a model in genetic and cytogenetic studies, playing an important role in the development of evolutionary biology and ecology (Balch, 2000; Johnson, 2011). According to the EMA (2018a), the species was initially introduced to Europe for medicinal purposes, becoming a popular remedy, particularly in the United Kingdom.

In 2018, the oils of *O. biennis* L. and *O. lamarckiana* L. were included in the European Union Herbal Monograph. The EMA has approved the use of oil obtained from the seeds of these two species through solvent extraction or pressing to relieve symptoms of itching and irritation—whether short-term or long-term—associated with dry skin in individuals over 12 years of age, through oral administration of doses (2–3 g, or 4–6 g daily dose) (EMA 2018a). Although *O. lamarckiana* is an invalid name, it was decided to include it in the European Monograph to acknowledge its occasional use in reference to the species *O. glazioviana*

Micheli (synonym *O. erythrosepala*) (Calapai, 2015, WFO, 2024). Oil from the seeds of *O. biennis* is also recognized as an ingredient in cosmetic products, along with extracts from the flowers, leaves, roots, and shoots, each of which is registered with its own CAS number (Calapai *et al.*, 2015).

In China, the first study on the chemical composition of the oil from the seeds of the plant growing in the wild, and its possible uses, was conducted in 1962. Independent studies on the oil and gamma-linolenic acid began in 1980, in parallel with those conducted in Western countries, though they were less well-known than the latter due to being published in Chinese. The results led to the approval in 1986 of two medications, for which licensed equivalents were subsequently developed in the West to treat eczema, atopic dermatitis, and mastalgia. (Deng *et al.*, 2001; Calapai *et al.*, 2015, EMA, 2018a). After 1989, emulsions and other medicinal products were developed for a variety of conditions, ranging from diabetes to premenstrual syndrome, and research continued into the use of GLA in the treatment of inflammatory diseases, nephrosis, obesity, and hyperlipidemia (Deng *et al.*, 2001).

According to sources cited by Hather in 1988, the roots of *O. biennis* are edible, have nutritional value (Medsger, 1939), and can be eaten from late autumn until early spring (Morton, 1963). Native Americans incorporated the seeds, roots, and young leaves into their diet (Kuhn and Winston, 2008).

1.4.2. Pharmacological and pharmacokinetic studies

The potential use of this species in the treatment of diabetes and related conditions has been explored mainly in terms of the biological activity of the seed oil (EPO) and the isolated gamma-linolenic acid (GLA) (Doormaal *et al.*, 1988; Arisaka *et al.*, 1991; Cameron *et al.*, 1991; Oon *et al.*, 1992; Stevens *et al.*, 1993a; Fang *et al.*, 1997; Söğütlü *et al.*, 2019; Mert *et al.*, 2022). The activity of *O. biennis* seed extracts has been less studied (Aitani *et al.*, 2003; Wang *et al.*, 2019, 2021).

The scientific literature contains several studies demonstrating the effects of *O. biennis* seed oil (EPO) on the circulatory system, often in the context of diabetes mellitus (Uccella *et al.*, 1989; Singer *et al.*, 1990; Stevensen, 1993b; Sadi *et al.*, 1996; Fan *et al.*, 1996, 2001; De la Cruz *et al.*, 1997; Villalobos *et al.*, 1998; Jack *et al.*, 2002; Riaz *et al.*, 2009; Zaitone *et al.*, 2011; Zaitone *et al.*, 2011; Zaitone *et al.*, 2011; Mosaad *et al.*, 2017; Andjic *et al.*, 2021).

Several studies show that EPO increases HDL cholesterol levels and reduces serum triglyceride levels (Singer *et al.*, 1986; Sugano *et al.*, 1986; Uccella *et al.*, 1989; Singer *et*

al., 1990; Fragoso and Skinner, 1992; De la Cruz *et al.,* 1997; Vilallobos *et al.,* 1998; Khorshidi *et al.,* 2020; Mert *et al.,* 2022). The majority of studies also show a reduction of total cholesterol (Singer *et al.,* 1990; Sadi *et al.,* 1990; De la Cruz *et al.,* 1997; Fukushima *et al.,* 1997; Vilallobos *et al.,* 1998).

EPO is effective in treating diabetes-related neuropathies (Tomlinson *et al.,* 1989; Cameron *et al.,* 1991; Lockett and Tomlinson, 1992; Dines *et al.,* 1995; Karasu *et al.,* 1995; Julu, 1996; Kuruvilla *et al.,* 1998; Head *et al.,* 2000; Ford *et al.,* 2001; Omran, 2012).

When combined with hemp oil, EPO proved effective in treating autoimmune encephalomyelitis in mice by modulating certain immune-related transcription factors and stimulating remyelination of the spinal cord (Rezapour-Firouzi *et al.,* 2020).

The anti-tumour activity of EPO, of compounds isolated from the roots, and of certain extracts from the seeds and aerial parts is supported by several studies (Yoshida *et al.,* 1991; Arimura *et al.,* 2003a,b, 2004, 2005; Pellegrina *et al.,* 2005; Singh *et al.,* 2017; Fecker *et al.,* 2020; Lee *et al.,* 2022; Abd-Alhaseeb *et al.* 2022; Zeppa *et al.,* 2022; Ciszewski *et al.,* 2022; Chmielewska-Kassassir *et al.,* 2023; Lee *et al*., 2024).

Extracts obtained from the seeds and leaves, Evening Primrose oil, and compounds isolated from this species exhibit antimicrobial activity (Borchardt *et al.,* 2009; Matsumoto-Nakano *et al.,* 2011; Cybulska *et al.,* 2011; Singh *et al.,* 2012; Kim *et al.,* 2015) as well as antifungal activity (Shukla *et al.,* 1999).

The meta-analysis carried out by Morse and collaborators in 1989, based on nine clinical trials, supports the efficacy of EPO in the treatment of atopic dermatitis.

1.4.3. Chemical composition

According to the scientific literature, plants belonging to the genus *Oenothera* are sources of a variety of bioactive compounds such as phenolic acids, flavonoids, biflavonols, triterpenes, saponins, alkaloids, tocopherols, and sterols (Munir *et al.,* 2017), as well as sources of lipids and carbohydrates that are important for the proper functioning of the body (Singh *et al.,* 2012). In the case of the O. biennis L. species, most research has been carried out on the composition of the seeds and products derived from them (meal, cake), particularly the seed oil (EPO), which is of particular economic and medicinal interest. The appeal of EPO stems from its composition, which includes the essential omega-6 fatty acids linoleic acid and gamma-linolenic acid, along with other bioactive compounds such as triterpenes, phenolic acids, tocopherols, and phytosterols (Farag *et al.,* 2023). A lower number of studies examine the composition of the leaves, roots, aerial parts, and the whole

plant (Zinsmeister and Bartl, 1971; Miyazawa *et al.*, 1978; Krzaczek and Bogucka-Kocka, 1994; Krzaczek *et al.*, 1995; Shuckla *et al.*, 1999; Lakshman *et al.*, 2014; Noge and Tamogami, 2018; Sultana *et al.*, 2018).

1.4.4. Antioxidant and anti-inflammatory potential

The antioxidant effect of extracts from the herba, seeds, meal, derived compounds and seed oil was assessed by Budinčević *et al.*, 1995; Shahidi *et al.*, 1997; Muñoz *et al.*, 1998; De La Cruz *et al.*, 1999; Birch *et al.*, 2001; Hamburger *et al.*, 2002; Šardzíková *et al.*, 2002; Peschel *et al.*, 2007; Borchardt *et al.*, 2008; Ratz-Łyko *et al.*, 2013; Granica *et al.*, 2013; Selek *et al.*, 2018; Fecker *et al.*, 2020; Wang *et al.*, 2021. Oenotheralanosterol A and B, oenotheraphenoxyacetone, and dihydroxyphenyl xanthone, isolated from the roots, and the hydroethanolic extract from the sprouts, respectively, exhibit antioxidant and anti-inflammatory activity (Singh *et al.*, 2012; Ahmad *et al.*, 2014; Kwak *et al.*, 2021).

The anti-inflammatory activity of this species has been demonstrated in a series of *in vitro*, *in vivo*, and clinical studies (Hamburger *et al*., 2002; Singh *et al.*, 2012; Montserrat de la Paz *et al.*, 2012, 2014a; Ratz-Łyko *et al.*, 2015; Ma *et al.*, 2020; Fecker *et al.*, 2020; Mert *et al.*, 2022; Kim *et al.*, 2021a,b; Kwak *et al.*, 2021). According to clinical studies analysed by Sharifi *et al*. in 2024, there is evidence to support the benefits of using seed oil in inflammatory conditions such as diabetes mellitus, eczema, mastalgia, rheumatoid arthritis, and hot flushes associated with the menopause.

CHAPTER 2
GENERAL CHARACTERIZATION OF THE STUDIED SPECIES

2.1. Botanical characterization of the Tussilago farfara L. species

2.1.1. Taxonomic classification

Kingdom - *Plantae*
Division - *Tracheophyta*
Subdivision - *Spermatophytina*
Class - *Magnoliopsida*
Superorder - *Asteranae*
Order - *Asterales*
Family - *Asteraceae* Giseke
Tribe - *Senecioneae* Cass.
Genus - *Tussilago* L.

The family Asteraceae Berchtold and J. Presl (Compositae Giseke) comprises 16 subfamilies with 2,675 genera, consisting largely of annual, biennial, or perennial herbaceous species, and occasionally shrubs, subshrubs, climbing plants, and trees.

The genus *Tussilago* L. belongs to the family Asteraceae Giseke (WFO, 2023), subfamily Asterioideae, tribe *Senecioneae* Cass (Greuter, 2006+). According to The Plant List (Greuter, 2006+) and WFO (2023) databases, the genus *Tussilago* includes several species, of which the only accepted species name is *Tussilago farfara* L., the remaining names being considered synonyms or belonging to unevaluated species.

2.1.2. Morphological characteristics of *Tussilago farfara* L. species

The most comprehensive morphological description of the species was provided by Săvulescu in 1964, in „Flora României". Other morphological aspects are mentioned in the specialized literature by Akçin (2007), Świeboda *et al.* (1975), Myerscough *et al.* (1966), Gaffal (2007), Upton *et al.* (2011), Nedelcheva *et al.* (2015), and WFO (2023). Morphological aspects of the species are presented in Figure 2.1.

Fig. 2.1. *Tussilago farfara* L. – morphological features. A – inflorescence, B – flowering stems, rhizome, and adventitious roots, C – leaves and rhizome, D – rhizome with buds. (Original photos)

2.1.3. Anatomical characteristics of *Tussilago farfara* L. species

2.1.3.1. Structural aspects

Anatomically, the species has been described by several authors (Świeboda *et al.*, 1975; Toma and Rugină, 1998; Akçin, 2007; Upton *et al.*, 2011; Nedelcheva *et al.*, 2015; Rodideal *et al.*, 2021; Akhtar *et al.*, 2022).

2.1.3.2. Ultrastructural aspects

At the ultrastructural level, the glandular trichomes of the foliar limb were described by Muravnik and Kostina during 2014–2016.

2.1.4. Distribution and habitat

Tussilago farfara L. is a species with a Eurasian distribution, ranging eastward from the British Isles to Siberia and from northwestern Africa to the Arctic Circle. Native to most of Europe except Portugal, it has been introduced and naturalized in other parts of the world (Iceland, the United States, Canada) (Myerscough *et al.*, 1966; Greuter, 2006+).

According to several authors cited by Myerscough *et al*. (1966), *T. farfara* L. grows under a variety of substrate and climatic conditions and in various plant communities. In Romania, it is widely distributed from the lowlands to the mountains (Săvulescu, 1964; Sârbu *et al.*, 2013).

The species' ecological preferences are summarized in Table 2.1, along with the Ellenberg index values.

Table 2.1.

Ecological preferences of *T. farfara* L. species (d. Chytrý M. *et al.*, 2018; Păcurar, 2020)

Species	Evaluated aspect						References
	Bioform	Light index	Temperature index	Humidity index	pH index	Trophic index (Nitrogen)	
		*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	
***Tussilago farfara* L.**	G geophyte	8 - heliophile	5 - meso-thermophile	6 - mesophile	7 - neutrophile	6x - mesotrophic (moderate nitrophile), euritrophic	Chytrý M. *et al*., 2018; Păcurar, 2020

*VIE – Ellenberg index value (1991, 1992), interpreted according to Chytrý M. *et al*., 2018, and Păcurar, 2020

2.2. Botanical characterization of the Geranium robertianum L. species

2.2.1. Taxonomic classification

Kingdom - *Plantae*
Division - *Tracheophyta*
Subdivision - *Spermatophytina*
Class - *Magnoliopsida*
Superorder - *Rosanae*
Order – *Geraniales Juss. ex Bercht. & J. Presl*
Family – *Geraniaceae Juss.*
Genus - *Geranium* L.

The family Geraniaceae Juss. comprises 14 genera, consisting of herbaceous plants—annuals or perennials—and, rarely, shrubs or subshrubs (WFO, 2023).

The genus *Geranium* L. belongs to the family Geraniaceae Juss. and comprises 436 taxa. This genus includes herbaceous, annual, biennial, and perennial species, as well as, rarely, shrubs and subshrubs. (WFO, 2023). In Romania, Săvulescu (1958) mentions only the presence of herbaceous species. According to the review conducted by Tofts (2004), within the genus *Geranium*, subgenus *Robertium* Picard, section *Ruberta* Dumontier, some specialists recognize three subspecies: *robertianum*, *maritimum* (Bab.) H. G. Baker, and *celticum* Ostenf., but others (Stace, 1997) consider that the reported differences are not of significant taxonomic value.

The species *Geranium roberianum* L. belongs to the genus *Geranium* L. and has two subspecies*: G. roberianum* subsp. *celticum* and *G. roberianum* var. *genuinum* Gren. & Godr. It was first described by Carl Linnaeus in 1753 in *Species Plantarum*.

2.2.2. Morphological characteristics of *Geranium roberianum* L. species

The species G. robertianum exhibits an extraordinary capacity for adaptation, as well as morphological, anatomical, and genetic variability among populations, suggesting the

existence of ecotypes (Tofts, 2004; Bozchaloyi *et al.*, 2017). Consequently, the morphological descriptions in the literature vary to a greater or lesser extent. They mention variations in the degree of pubescence, differences in the floral parts, erect or prostrate stems (Tofts, 2004), and a variable number of vascular bundles in the petiole (Rybak *et al.*, 2014). In addition to pubescence, Săvulescu noted in 1958 variations in petiole length, flower diameter, and the ratio of petals to sepals, and distinguished between 7 subspecies present in the flora of Romania. The populations themselves are often uniform, but there are notable differences between populations (Tofts, 2004). Variability may be induced by environmental conditions, although some traits of taxonomic importance are genetically determined, such as the degree of pubescence (Baker, 1956). Plants growing in close proximity to one another tend to remain distinct as a result of self-pollination (Yeo, 1985). The species' morpho-anatomical variability has, over time, led to the definition of forms, subspecies, and various classifications (Săvulescu, 1958; Tofts, 2004; Salimpour *et al.*, 2009; Bozchaloyi and Zaman, 2018). The WFO database (2023) contains descriptions of the species that are more or less similar, based on sources from the Flora of China (FOC), the Flora of Pakistan (FOP), of the United States (Gleason and Cronquist, 1991), and of Brazil (Reflora).

The morphological characteristics of *G. robertianum* L. plants belonging to the populations analyzed in this doctoral thesis are presented in Figure 2.2.

Fig. 2.2. *Geranium robertianum* L. – morphological features. A, C – leaves rosette; B, E – appearance of leaves, flowers, and fruits; D – section of the root and stem branches with leaves and fruits. (Original photos)

2.2.3. Anatomical characteristics of *Geranium robertianum* L. species

2.2.3.1. Structural aspects

The subgenus *Geranium* L. comprises over 370 species grouped into sections based on morpho-anatomical characteristics such as tuber- or rhizome-like underground organs, palmate leaf structure, or the absence of these features, nodal organization, and fruit characteristics (Salimpour *et al*., 2009). In 2009, Salimpour and collaborators classified the species *G. robertianum* L. among the species with rhizomes.

In 2006, Paolillo analyzed the root anatomy of *G. robertianum*, focusing on the structural relationships between the taproot and its branches during secondary growth. In 2014, Rybak and collaborators described anatomical features of the foliar limb and petiole.

2.2.3.2 Ultrastructural aspects

During 1990–1991, Pedro *et al*. identified three types of uniseriate glandular trichomes, which develop through periclinal divisions from a single cell in the protoderm.

Ultrastructural studies of *G. robertianum*, based on TEM and/or SEM images, focus primarily on floral elements (Weber, 1996a, b; Sexton *et al.*, 1983; Yeo, 2004; Endress, 2010; Bozchaloyi and Zaman, 2018).

2.2.4. Distribution and habitat

A comprehensive overview of the distribution and habitat of the *G. robertinanum* L. species was provided by Tofts in the year 2004. The species has a wide distribution in Europe, being native to most countries, including Romania, and widely naturalized outside its native range (Tofts, 2004; Euro+Med Plantbase).

A ruderal plant with a wide ecological range, which grows in biomes ranging from Mediterranean to boreal, under conditions varying from oceanic to continental (Tofts, 2004). Ellenberg and collaborators classified it in 1991 as an intermediate species between oceanic and suboceanic, while Preston and Hill classified it in 1997 as a temperate European species. In Romania, it is common throughout the country in moist and shady forests, particularly beech and spruce forests, and rarely in oak forests (Săvulescu, 1958), within the *Stipion calamagrostis*, *Galio-Urticetea*, *Fagetalia,* and *Tilio-Acerion* plant communities (Sârbu *et al.*, 2013).

G. robertianum L. is a hemicryptophyte or therophyte species with a high capacity to adapt to environmental conditions; it is described in the scientific literature as a mesoheliophobic to mesosciaphilous, mesothermic or eurythermic, mesophilic or euryphilic,

weakly acidophilic or euryacidophilic, eutrophic or nitrophilic plant (Tofts, 2004; Chytrý M. *et al.*, 2018; Păcurar, 2020). Classified as an intermediate type between ruderal and competitive-stress-tolerant-ruderal (Grime *et al.*, 1988; Tofts, 2004). The species' ecological preferences are summarized in Table 2.3, along with the Ellenberg index values.

Table 2.3.

Ecological preferences of *G. robertianum* L. species (d. Chytrý M. *et al.*, 2018; Păcurar, 2020)

Species	Evaluated aspect						References
	Bioform	Light index	Temperature index	Humidity index	pH index	Trophic index (Nitrogen)	
		*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	
***G. robertianum* L.**	H, TT hemicryptophyte, terophyte	4, 5x - meso-heliophobe, meso-sciaphile	X, 5 - meso-therm, euritherm	X, 6x - mesophile, euriphile	X, 6x - weakly-acidophile, euriacidophile	7 - eutrophic, nitrophile	Tofts, 2004; Chytrý M. *et al.*, 2018; Păcurar, 2020

* VIE – Ellenberg index value (1991, 1992), interpreted according to Chytrý M. *et al*., 2018, and Păcurar, 2020

2.3. Botanical characterization of the Leonurus cardiaca L. species

2.3.1. Taxonomic classification

Kingdom - *Plantae*
Division - *Tracheophyta*
Subdivision - *Spermatophytina*
Class - *Magnoliopsida*
Superorder - *Asteranae*
Order – *Lamiales Bromhead*
Family – *Lamiaceae Martinov*
Genus - *Leonurus* L.

The Lamiaceae family comprises 316 taxa, typically herbaceous plants, sometimes shrubs or subshrubs, annuals or perennials, and often aromatics. A characteristic feature is the shape of the stem or branches, which are usually quadrangular (WFO, 2023).

According to the World Flora Online database (2023), the genus Leonurus L. comprises 33 taxa. Annual, biennial, or perennial herbaceous plants with an erect growth habit, featuring 3–7-lobed leaves—those at the base are palmately lobed, while those on the stem are whole, incised, or trilobed—and whorled inflorescences with numerous flowers, forming long spikes (WFO,2023).

Leonurus cardiaca L. is the species' current official name, which has 24–26 synonyms (Euro+med; WFO, 2023).

2.3.2. Morphological characteristics of *Leonurus cardiaca* L. species

Leonurus cardiaca L. was described by Săvulescu in 1961 in „Flora României". In the international scientific literature, it was described by Gleason and Croquist in 1991, in the Flora of Pakistan, and in Flora Helvetica, which are included in the WFO database (2023), as well as by Sermukhamedova *et al*. in 2017. Figure 2.3 shows morphological aspects of *L. cardiaca* L. plants analyzed in the original research.

Fig. 2.3. *Leonurus cardiaca* L. – morphological features. A – whole plant, B – stems with leaves, flowers, and fruits, showing leaf polymorphism, D – stem tip with inflorescences and fruits. (Original photos)

The taxonomy of this species has been the subject of controversy over time regarding its division into subspecies; however, in databases such as The Plant List, these are often considered synonyms of *L. cardiaca*, and no comprehensive studies exist, with the exception of some descriptions in local or regional floras (Soorni *et al*., 2014). The shape of the leaves and pubescence are considered key characteristics in distinguishing the species (Soorni *et al.*, 2014).

2.3.3. Anatomical characteristics of *Leonurus cardiaca* L. species

2.3.3.1. Structural and ultrastructural aspects

The anatomy of *Leonurus cardiaca* L. is poorly documented in the scientific literature. The available data are limited to the stem, leaves, and flowers. Due to the structural characteristics of its stem, this species serves as a model for biomimetic designs. (Kaminski *et al.*, 2019).

No information regarding the structure of underground organs was found in the specialized literature.

2.3.4. Distribution and habitat

Leonurus cardiaca L. is a species found in the Mediterranean and Atlantic regions, as well as in Central and Eastern Europe, Anatolia, Mongolia, and China; it is an invasive species in North America and is gradually spreading throughout southern and western Siberia (Chikov, 1983, cited by Sermukhamedova *et al.,* 2017). According to Vermeil (2002), the species is native to Asia and has become naturalized in Europe over the past millennium. It is widespread, except in the northern regions, in semi-desert, and in desert areas. (Sermukhamedova *et al.,* 2017). It prefers the eco-phytocenotic conditions of the Baltic region and Belarus, being considered a characteristic species (Muravyova *et al.,* 2002). It grows along roadsides, in disturbed areas such as wastelands, sites with rubble and trash, and ruderal sites (Săvulescu, 1961), as well as in clear-cut areas and plantations, ranging from the forest-steppe to the oak zone, within the *Artemisietea* and *Stellarietea mediae* plant communities (Sârbu *et al.,* 2013).

A hemicryptophytic, heliophilous, mesothermic species with moderate requirements for moisture levels, that is neutrophilic and nitrophilic or eutrophic (Table 2.4).

Table 2.4.

Ecological preferences of *L. cardiaca* L. species (d. Chytrý M. *et al.*, 2018; Păcurar, 2020)

Species	**Evaluated aspect**						**References**
	Bioform	Light index	Temperature index	Humidity index	pH index	Trophic index (Nitrogen)	
		*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	
***Leonurus cardiaca* L.**	H hemicryptophyte	7 - meso-heliophile	6 - mesotherm	5 - mesophile	7 - neutrophile	8 - eutrophic (nitrophile)	Chytrý M. *et al.,* 2018; Păcurar, 2020

* VIE – Ellenberg index value (1991, 1992), interpreted according to Chytrý M. *et al*., 2018, and Păcurar, 2020

2.4. Botanical characterization of the Oenothera biennis L. species

2.4.1. Taxonomic classification

Kingdom - *Plantae*
Division - *Tracheophyta*
Subdivision - *Spermatophytina*
Class - *Magnoliopsida*
Superorder - *Rosanae*
Order – *Myrtales Juss. ex Bercht. & J. Presl*
Family – *Onagraceae Juss.*
Genus - *Oenothera* L.

The family Onagraceae Juss. comprises 23 genera and includes annuals or perennials, sometimes shrubs, and rarely trees up to 30 m tall. Onagraceae species often contain oil-

bearing epidermal cells and internal phloem, and, with the exception of the genus *Ludwigia*, a distinct, nectar-producing floral tube (WFO, 2023).

According to Săvulescu (1957), the genus *Oenothera* L. comprises 100–140 species distributed throughout North America. Other sources mention approximately 200 (Flora of Panama, 2013) or 168 taxa (WFO, 2023). The genus comprises herbaceous plants or subshrubs (Săvulescu, 1957). Most species are polyploid, with occasional mutations, frequent recombinations, and hybridizations, which over time have given rise to new phenotypes (Renner, 1942).

Oenothera biennis L. is the current official name (WFO, 2023).

2.4.2. Morphological characteristics of *Oenothera biennis* L. species

The morphology of the species is described by Săvulescu in Volume V of „Flora României" (1957). Outside the country, Hall and collaborators presented the morphology of the species from Canada in 1988, supplementing earlier works from the period 1950–1970. Descriptions compiled by Gleason and Croquist (1991), Zomlefer (1994), Miller and Miller (1999), Bryson and DeFelice (2010), and Wagner (2017) were summarized by Steckel and collaborators in the year 2019. The morphology and reproductive biology of the species cultivated in India are described by Thakur *et al*. in the year 2019. World Flora Online (2023) also provides a series of morphological descriptions from various other databases. Figure 2.4 illustrates morphological aspects of plants belonging to populations of the Romanian flora analyzed in this doctoral thesis.

Fig. 2.4. *Oenothera biennis* L. – morphological features. A – flowering stem, B – appearance of the stem and cauline leaves, C – leaves rosette. (Original photos)

2.4.3. Anatomical characteristics of *Oenothera biennis* L. species

2.4.3.1. Structural aspects

The scientific literature provides little information on the anatomy of *Oenothera biennis* L. species. Images of the collet and stem are available in a plant anatomy atlas (Schweingruber *et al.,* 2011), a description of the root is found in a thesis on plant archaeology (Hather, 1988) and there are several studies on the reproductive organs (De Vos, 1981; Takahashi and Skwarla, 1990; Noher de Halac & Harte, 1995; Sodmergen *et al.*, 1997; Chapman & Mulcahy, 1997; Abd-El Maksoud, 2012; Enache *et al.*, 2021), as well as on callus formation and embryogenesis (Ghasemnezhad *et al.,* 2011). Micromorphological aspects of the foliar limb epidermis were studied in a plant cultivated in Egypt (Abd-El Maksoud, 2012) and in four varieties of *O. biennis* (Tverskoj, Genoteros, Svetlyachok, Fonarik) by Savchenko and collaborators in the year 2021. The study confirms findings described by Popov *et al*. (2009) and Cheryatova (2014) for the Russian species. In his 1988 thesis on plant archaeology, Hather provides a description of the secondary root. A brief overview of the anatomy of the foliar limb is provided by Cheryatova in 2014 (cited by Savchenko *et al*., 2021). Micromorphological aspects of the foliar limb were also studied by Savchenko and collaborators in 2021 in four cultivated varieties of *O. biennis*, in the context of its ecological plasticity.

2.4.3.2. Ultrastructural aspects

Regarding the ultrastructure of this species, the scientific literature contains only studies on pollen grains (Takahashi and Skvarla, 1990; Noher de Halac and Harte, 1995; Sodmerger *et al.*, 1997).

2.4.4. Distribution and habitat

The species is endemic to North America; its range, according to Steckel *et al.* (2019), extends from Mexico to Florida, northward to central Ontario and Quebec (Canada), westward to North Dakota and Oklahoma, and along the Pacific coast. It was subsequently introduced by humans to all other continents except Antarctica (Steckel *et al*., 2019) and is the most common and widespread species of the *Oenothera* subsection (Dietrich *et al*., 1997). It is presumed that its spread in Europe is based on two distinct groups of *Oenothera* spp.: one native to North America, which is biennial and adapted to continental conditions with lower average temperatures and moderate monthly precipitation; and one from South

America, which is annual or perennial and prefers an oceanic climat (Dietrich *et al.*, 1997; Hall *et al.*, 1988; Steckel *et al.*, 2019).

In Romania, it is an introduced species with unknown status, classified as a pioneer species (Săvulescu, 1957; Sârbu *et al.,* 2013). The species' ecological preferences are summarized in Table 2.5, along with the Ellenberg index values.

Table 2.5.

Ecological preferences of *O. biennis* L. species (d. Chytrý M. *et al.*, 2018; Păcurar, 2020)

Species	**Evaluated aspect**						**References**
	Bioform	Light index	Temperature index	Humidity index	pH index	Trophic index (Nitrogen)	
		*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	*VIE - Interpretation	
***Oenothera biennis* L.**	H hemicryptophyte	9 - extreme-heliophile	7 - thermophile	4 - meso-xerophile	6x - weakly-acidophile-euriacidophile	4 - Oligomesotrophic (moderately nitrophile)	Chytrý M. *et al.*, 2018; Păcurar, 2020

* VIE – Ellenberg index value (1991, 1992), interpreted according to Chytrý M. *et al*., 2018, and Păcurar, 2020

PART II: PERSONAL CONTRIBUTIONS

CHAPTER 3
MATERIALS AND METHODS

3.1. Collecting and processing of plant material

The plant material consists of whole plants of the species *Tussilago farfara* L., *Geranium robertianum* L., *Oenothera biennis* L., and *Leonurus cardiaca* L., collected from wild flora in two different regions of Romania: West—Macea, a lowland area; and Northeast—Vatra Dornei, a mountain area. Several specimens belonging to the same population were collected from each location; one specimen from each population was preserved as a voucher (Figure 3.1), while the rest were used for extraction. The specimens were identified by Assoc. Prof. Violeta Turcuș, Ph.D. The number of specimens collected from each location varied depending on availability and plant biomass. The species were collected in accordance with applicable legislation, research standards in traditional medicine (Law No. 491/2003; Directive 92/43/CEE; OUG57/2007) as well as the recommendations in the specialized literature regarding weather conditions at the time of collection—warm weather, no precipitation—and the ontogenetic stage—at maturity, specifically during the flowering period (Pirnă *et al.*, 2012). In accordance with the study's objectives and the biology of the species, *T. farfara* was collected during vegetative growth stage. The collection sites and periods are shown in Table 3.1. Species identification in the field was performed using techniques described in the scientific literature, with reference to botanical identification guides (Sârbu *et al*., 2013) and herbaria (Ardelean and Mohan, 2008 - Flora medicinală a României).

The working methodology is shown schematically in Figure 3.2.

Fig. 3.1. Herbarium sheets with voucher specimens of the species studied. A – *Tussilago farfara* L. collected from Macea, B – *Tussilago farfara* L. collected from Vatra Dornei, C – *Geranium robertianum* L. collected from Macea, D – *Geranium robertianum* L. collected from Vatra Dornei, E – *Leonurus cardiaca* L. collected from Macea, F – *Leonurus cardiaca* L. collected from Vatra Dornei, G – *Oenothera biennis* L. collected from Macea, H – *Oenothera biennis* L. collected from Vatra Dornei.

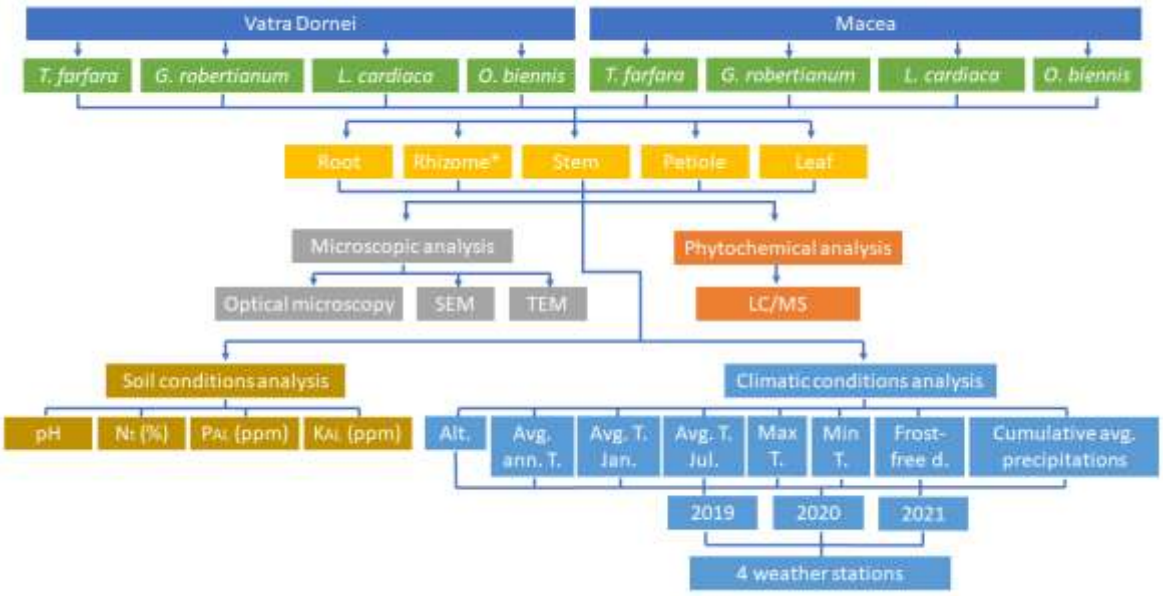


Fig. 3.2. Diagram of materials and methods. SEM – scanning electron microscopy, TEM – transmission electron microscopy, LC/MS – liquid chromatography–mass spectrometry, Nt(%) – total nitrogen percentage, PAL (ppm) – assimilable phosphorus content calculated in parts per million, KAL (ppm) – assimilable potassium content calculated in parts per million.

3.2. Microscopic examination of plant material

3.2.1. Optical microscopy

The analysis of the internal structure of the main vegetative organs of the species taken into study was conducted according to the techniques described by Șerbănescu-Jitariu and collaborators in 1983. The specimens were observed and photographed using an Olympus BX43 microscope with an Olympus XC30 camera, operated by CellSens Life Science Imaging Software (Olympus), and a Novex Holland microscope with a built-in camera, operated by BEL Capture Software, provided by the "Aurel Ardelean" Institute of Life Sciences, "Vasile Goldiș" Western University of Arad.

3.2.2. Scanning electron microscopy (SEM)

In order to analyze the micromorphological features using a scanning electron microscope, fragments were sectioned from the vegetative organs—root, rhizome, aerial stem, petiole, and foliar limb—of whole plants from each species under study, which had been preserved in 70% ethanol. The specimens were examined using a Quanta 250 scanning electron microscope (FEI Company), located in the Electron Microscopy Laboratory of the "Aurel Ardelean" Institute of Life Sciences, at the "Vasile Goldiș" Western University of Arad, under vacuum conditions, using a secondary electron detector (Bray, 2000; Talbot and White, 2013).

3.2.3. Transmission electron microscopy (TEM)

In order to analyze the ultrastructural elements of the vegetative organs of *T. farfara* L., *G. robertianum* L., *L. cardiaca* L., and *O. biennis* L. species, the plant material was processed using the transmission electron microscopy techniques described in the scientific literature (Crăciun, 2010; Hayat, 2000).

Semifine sections with a thickness ranging from 250 to 500 nm were prepared using the LEICA UC7 ultramicrotome and glass knives. These sections were mounted on degreased glass slides, stained with Epoxy Tissue Stain solution, and then observed and photographed under an Olympus BX 51 microscope.

Finally, the samples were examined using the Jeol JEM 1010 transmission electron microscope (Japan), located in the Electron Microscopy Laboratory of the "C. Crăciun" Electron Microscopy Center at Babeș-Bolyai University, at an electron acceleration of 80 kV, and images were captured at various magnifications.

3.3. Phytochemical composition analysis of plant material

3.3.1. Preparation of plant extracts

3.3.1.1. Preparation of hydroalcoholic extracts

The hydroalcoholic extracts were prepared from whole plants of *G. robertianum* L., *L. cardiaca* L., and *O. biennis* L. species, collected during the flowering period, and *T. farfara* L., collected during the vegetative growth period. The dried and ground plant material was extracted in 70% ethanol at a mass-to-volume ratio of 1:5, in accordance with the European Pharmacopoeia, 9th Edition (2021), and the Homöopathische Arzneibuch (HAB) (2021). The 70% ethanol required for extraction was prepared from 96% ethanol (Coman Prod, Ilfov, Romania) and purified water (Millipore). The final concentration of the extract prepared in this manner was 0.2 g of dried plant material per mL of extract.

3.3.1.2. Preparation of aqueous extracts (infusion)

The aqueous extracts were prepared by drying the plant material—consisting of whole plants from the four studied species—for 7 days in the shade at room temperature. Extraction was performed at a ratio of 1:10 dried and ground plant material to hot water (brought to a boil), in accordance with the European Pharmacopoeia, 10th Edition (1993). The final concentration of the extract prepared in this manner was 0.1 g dried plant material per mL of extract.

3.3.2. Liquid chromatography-mass spectrometry method (LC/MS)

The LC/MS analysis of plant extracts was performed using a Shimadzu Nexera I LC/MS-8045 UHPLC system (Kyoto, Japan), equipped with a quaternary pump and an autosampler, as well as an ESI probe and a quadrupole mass spectrometer.

The separation was performed on a Luna C18 reverse-phase column (150 mm × 4.6 mm × 3 µm, 100 Å) from Phenomenex (Torrance, CA, USA). The mobile phase (Annex 1, Table 1, and Fig. 1) consisted of a gradient of methanol (Merck, Darmstadt, Germany) and ultrapure water prepared using the Simplicity Ultra Pure Water Purification System (Merck Millipore, Billerica, MA, USA). Formic acid (Merck, Darmstadt, Germany) was used as the organic modifier. The methanol and formic acid were of a quality suitable for the LC/MS method. Detection was performed using a quadrupole mass spectrometer operated with electrospray ionization (ESI) in both negative and positive multiple reaction monitoring (MRM) modes (Annex 1, Table 2).

The standards are represented by the substances listed in Annex 1, Table 3. From each standard, at each concentration, 1 µl was injected. Identification was performed by comparing the MS spectra and transitions between the separated compounds and the standards. Identification was based on the major and minor transitions in the MS spectra of the substances, and quantification was based on the major transitions. For the purpose of quantification, calibration curves were also determined (Annex 1, Figures 1–2). Table 3 in Annex 1 also shows the equations of the calibration curves, their correlation coefficients, and the determined limits of detection and quantification. Annex 1, Table 2 also presents the retention times and specific MS spectrum data for the standards used, and Annex 1, Figures 3–37 present the chromatograms and MS spectra. Tables 4–7 in Appendix 1 list the retention times and major transitions for each compound identified in the hydroalcoholic and aqueous extracts from each species studied. For the LC/MS analyses, "ND" is defined as "not detected" or "below the limit of detection," and "<QL" is defined as a component that can be identified (at a concentration higher than the limit of detection) but cannot be quantified within a reasonable margin of error.

3.4. Pedoclimatic conditions analysis in collecting areas

The analyzed factors were selected according to results presented in the scientific literature, which, based on the observed correlations, indicate that they are among the main environmental factors affecting the content of bioactive compounds in various other species (Liu *et al.*, 2016; Neagu *et al.*, 2019; Chelgoum *et al.*, 2021).

3.4.1. Soil conditions analysis

Soil samples were collected from each collecting site to determine the soil's fertility status by analyzing several basic parameters: pH, total nitrogen (N_t), and the amounts of phosphorus and potassium in their assimilable forms (P_{AL}, K_{AL}).

Soil samples were collected in accordance with the recommendations in the literature (Liebig *et al.*, 2010).

The soil pH was determined using the potentiometric method (Kalra, 1995),

The total nitrogen index (IN) of the soil samples was calculated based on the soil humus content (H%) and the base saturation (V%) using the following formula:

$$IN = H\%*V\%/100$$

The amount of assimilable phosphorus and assimilable potassium in the soil samples was determined using the Egner-Rhiem-Domingo method (Egner *et al*., 1960) and expressed in parts per million (ppm).

3.4.2. Climatic conditions analysis

3.4.2.1. Altitude

GPS data from the sampling sites was collected using the GPS Finder app for Android, available at https://play.google.com/store/apps/details?id=com.developers.gps&pcampaignid=web_share. The corresponding altitude was obtained by entering the GPS coordinates into the system provided by GPS-Coordinates, available at https://www.gps-coordinates.net/.

3.4.2.2. Temperature and precipitations

In order to assess the climatic conditions in the sampling regions, meteorological data were collected and analyzed from the Meteomanz database (http://www.meteomanz.com/), from four weather stations near the sampling locations, for the years 2019–2021, in order to cover the entire ontogenetic cycle of the plants.

The following data were collected: average annual temperature, average temperatures for January and July, maximum and minimum temperatures, and cumulative annual precipitation. The frost-free period was determined by subtracting the number of frost days (available in the database) from the total number of days in the year.

Maps were created for the two sampling regions by interpolating data (average annual temperature, cumulative annual precipitation) from four weather stations located near the sampling sites. The spatial distribution of climate parameters was modeled using QGIS 3.40 "Bratislava" software, employing the Inverse Distance Weighting (IDW) interpolation method (Philip and Watson, 1982; Watson and Philip, 1985).

3.5. Statistical data analysis

The quantitative values of the compounds identified in the hydroalcoholic and aqueous extracts represent the arithmetic mean ± standard deviations of three independent measurements of replicate injections from the same extract, for each plant/extract type. Statistical analysis was performed using Excel software from the Microsoft Office suite.

The morphological parameters of the scanning electron microscopy (SEM) images were analyzed using IS Scandium Image Analysis software. The values represent the arithmetic mean ± standard deviation of at least 3 measurements.

CHAPTER 4
EVALUATION OF ENVIRONMENTAL FACTORS

4.1. Altitude and habitat

An increasingly discussed topic in the scientific literature is the influence of geographic location and habitat conditions on the morpho-anatomy, biochemical composition, and biological activity of plants (Liu *et al.*, 2016; Chelghoum et al, 2021). One of the environmental factors considered important in inducing variability is altitude, which is closely related to the other factors (Wulf *et al.*, 1999; Dong *et al.*, 2011; Zlatev, 2012; Suyal *et al.*, 2020).

In the present study, the four species were collected from two different locations in Romania's wild flora, each characterized by a specific set of ecological conditions (as described in Chapter 3) (Table 4.1).

Table 4.1.

Altitude, climate, and vegetation zones corresponding to the locations where the analyzed plant material was collected

Species	GPS Coordinates	Location	Altitude	Climatic zone*	Vegetation Zone/Level **
T. farfara L.	Lat. 46.390821 Long. 21.308288	Macea	99 m	Temperate continental with oceanic influences	The forest-steppe zone (50–150 m) – northern forest-steppe: *Quercus robur* L.
G. robertianum L.	Lat. 46.381613 Long. 21.310076				
L. cardiaca L.	Lat. 46.391422 Long. 21.309233				
O. biennis L.	Lat. 46.388241 Long. 21.302165				
T. farfara L.	Lat. 47.373752 Long. 25.46664	Vatra Dornei	1027 m	Cool continental climate with Scandinavian-Baltic influences	The nemoral zone – the subzone of beech and mixed beech-coniferous forests (600–1,350 m): *Fagus sylvatica* L., *Abies alba* Mill.
G. robertianum L.	Lat. 47.347652 Long. 25.340239		863 m		
L. cardiaca L.	Lat. 47.346267 Long. 25.384017		791 m		
O. biennis L.	Lat. 47.341726 Long. 25.331948		926 m		

According to bibliography: *Administrația Națională de Meteorologie, Clima României, Ed. Academiei Române, București, 2008; **Pacurar, F. Specii Indicator Pentru Evaluarea și Elaborarea Managementului Sistemelor de Pajiști cu Înalta Valoare Naturală—HNV; Casa Cărții de Știință, Cluj-Napoca, Romania, 2020; pp. 16–18.

4.2. Climatic conditions

4.2.1. Average annual temperature

According to data recorded by nearby weather stations, the average annual temperature in the Macea area ranged from 11.7 to 12.2°C in 2021, from 12.2 to 12.7°C in 2020, and from 13 to 13.3°C in 2019 (Plate 4.1. A–C).

In the Vatra Dornei area, the average annual temperature in 2021 ranged from 0.7 to 9.8°C; in 2020, it ranged from 1.9 to 10.5°C; and in 2019, it ranged from 2.1 to 11.4°C (Plate 4.1. D–F).

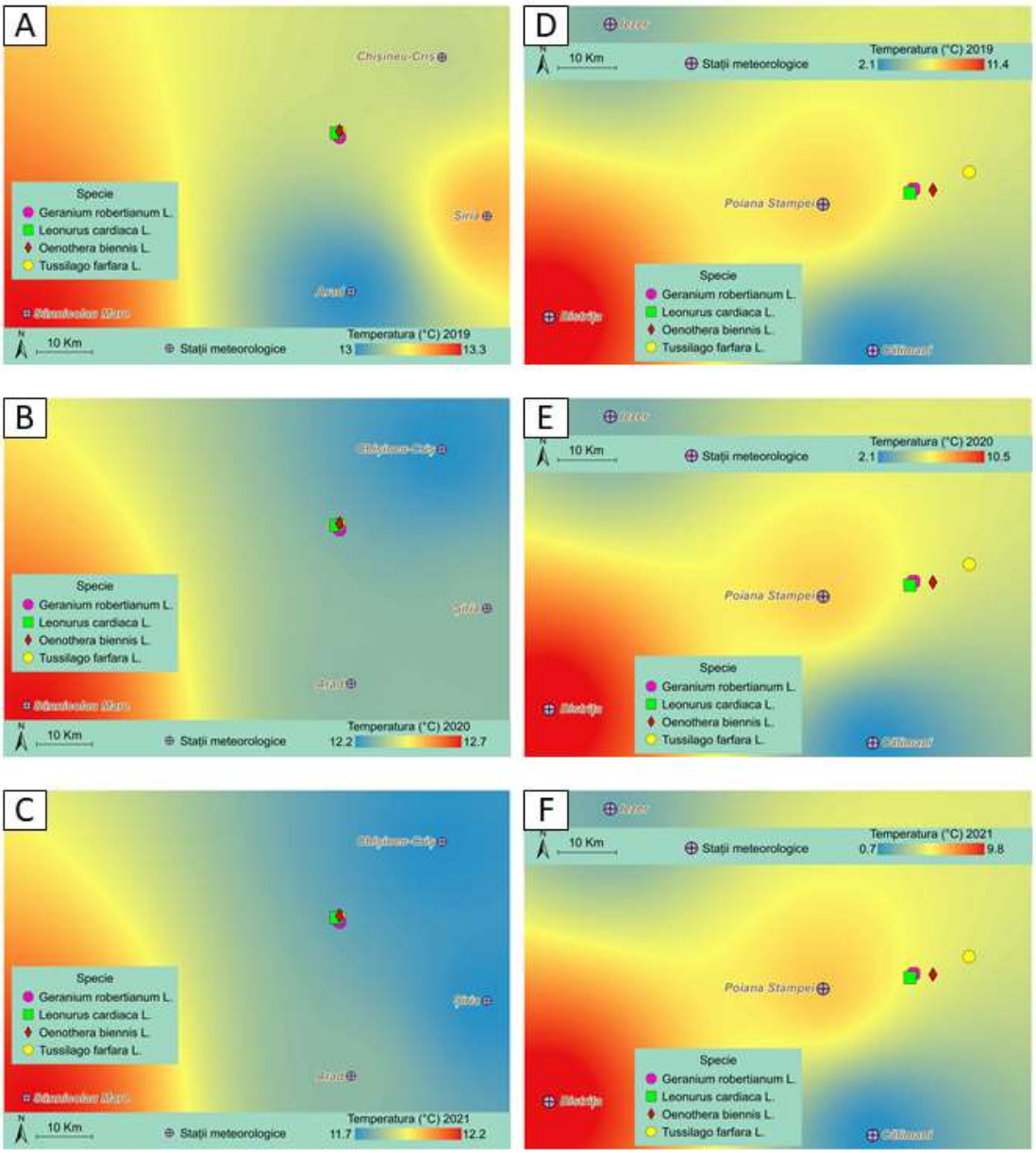


Plate 4.1. Maps of average annual temperatures based on data interpolated from four weather stations near the sampling sites for the years 2019–2021. A – Macea 2019, B – Macea 2020, C – Macea 2021, D – Vatra Dornei 2019, E – Vatra Dornei 2020, F – Vatra Dornei 2021.

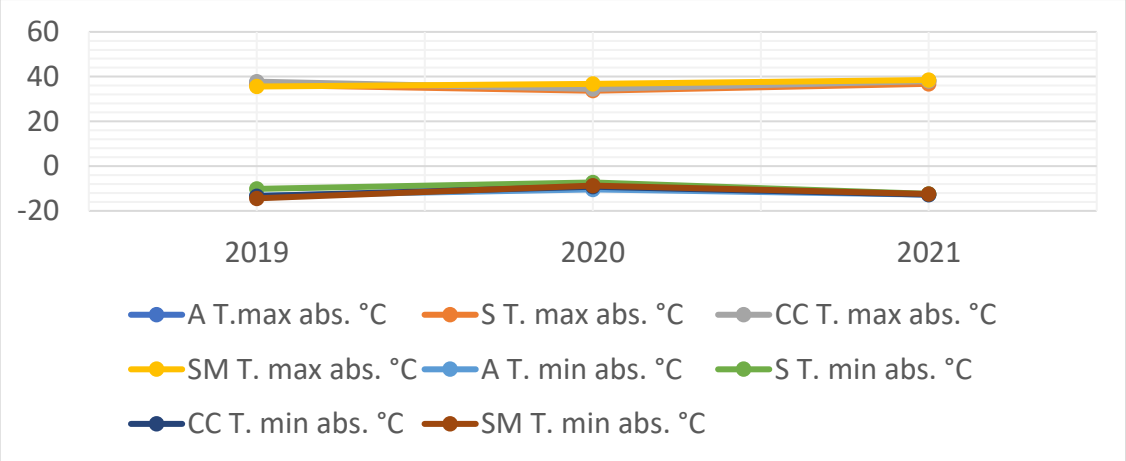
4.2.2. January and July mean temperatures

In the Macea area, during the plants' ontogenetic cycle, the trend was toward warmer winters, with the average January temperature rising from one year to the next, reaching positive values in the year the plants were collected (2021 and 2020, respectively, for *T. farfara* L.). In January 2021, the average temperature ranged from 1.1 to 2.2°C. In 2020, the values ranged from -0.9 to -0.4°C, and in 2019, from -1.8 to -0.6°C. The average temperature in July 2021 ranged from 24.9 to 25.6°C, in 2020 from 21.4 to 22.3°C, and in 2019 from 21.8 to 22.2°C.

In January 2021, the average temperature in the Vatra Dornei area ranged from -7.7 to 0.2°C; in 2020, it ranged from -4.4 to -2.8°C; and in 2019, it ranged from -9.8 to -1.7°C.

4.2.3. Maximum and minimum temperatures

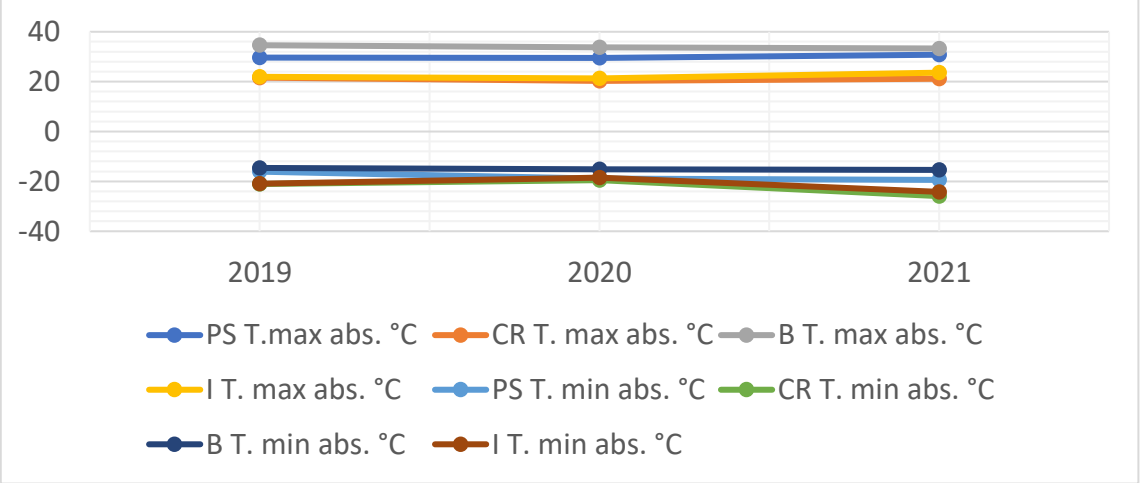
Maximum temperatures in the Macea area reached their highest levels in the year the plants were collected (2021), ranging from 36.8 to 38.5°C. In 2020, temperatures were the lowest during the analysis period, ranging from 33.7 to 36.7°C, while in 2019, they ranged from 35.7 to 37.7°C. In the same area, minimum temperatures in 2021 reached -12.8 and -12.4°C, in 2020 they were -10.4 and -7.3°C, and in 2019 they were -14.4 and -10.2°C (Graph 4.1).



Graph. 4.1. Absolute maximum and minimum temperatures in the Macea area for the period 2019–2021, according to the four nearby weather stations. A – Arad, S – Șiria, CC – Chișineu-Criș, SM – Sânicolaul Mare.

Both maximum and minimum temperatures were lower in the year the plants were collected (2021 and 2020, respectively, for *T. farfara* L.), compared to their ontogenetic development period. In 2021, maximum temperatures in the Vatra Dornei area ranged from 21.2 to 33.3°C; in 2020, they ranged from 20.3 to 33.7°C; and in 2019, they ranged from

21.6 to 34.6°C. The minimum temperatures recorded in the Vatra Dornei area were between -25.9 and -15.4°C in 2021, between -19.5 and -15.1°C in 2020, and between -21.1 and -14.6°C in 2019 (Graph 4.2).



Graf. 4.2. Absolute maximum and minimum temperatures in the Vatra Dornei area for the period 2019–2021, according to the four nearby weather stations. PS – Poiana Stampei, CR – Călimani Reținș, B – Bistrița, I – Iezer, grC – degrees Celsius.

4.2.4. Cumulative annual precipitation

In the Macea area, cumulative precipitation in 2021 ranged from 395.3 to 578 mm, with the highest values recorded at the stations closest to the Macea locality (Plate 4.2. A-C). In 2020, annual precipitation ranged from 412.9 to 603.8 mm, with the highest value recorded at the Șiria station, and in 2019, the values ranged from 480.7 to 575.2 mm.

In the Vatra Dornei area, cumulative precipitation in 2021 ranged from 757.4 to 1,528.1 mm, in 2020 from 691.4 to 1419.5 mm, and in 2019 between 660.6 and 1340.1 (Plate 4.2. D-F).

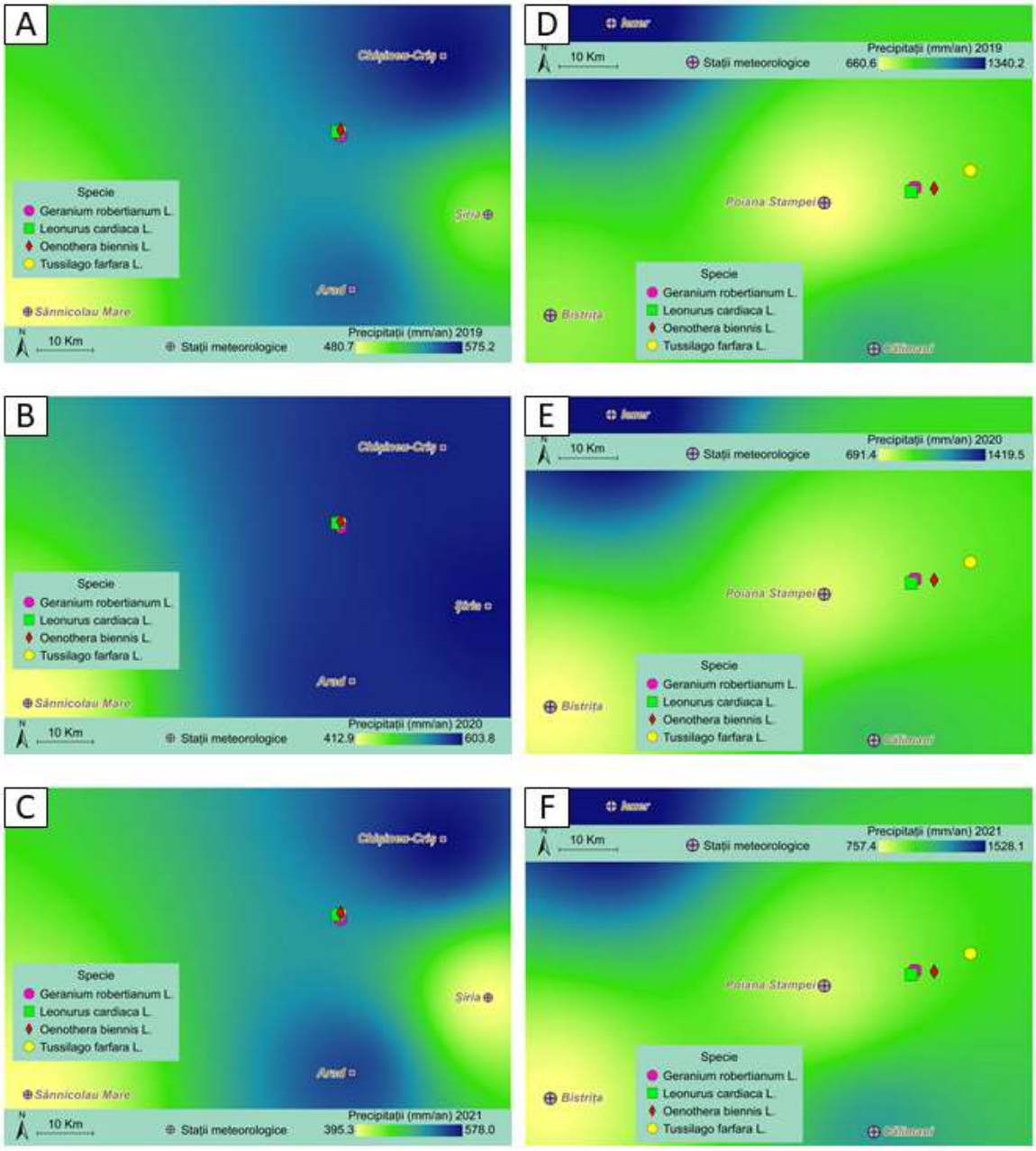
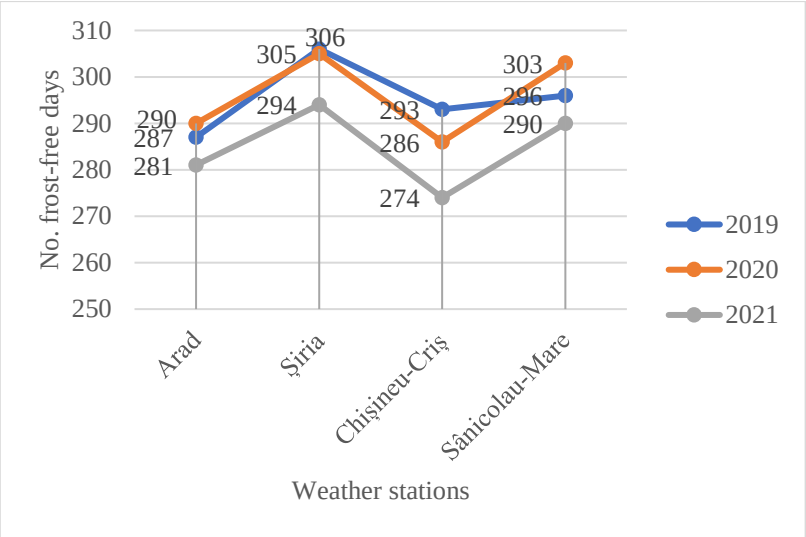


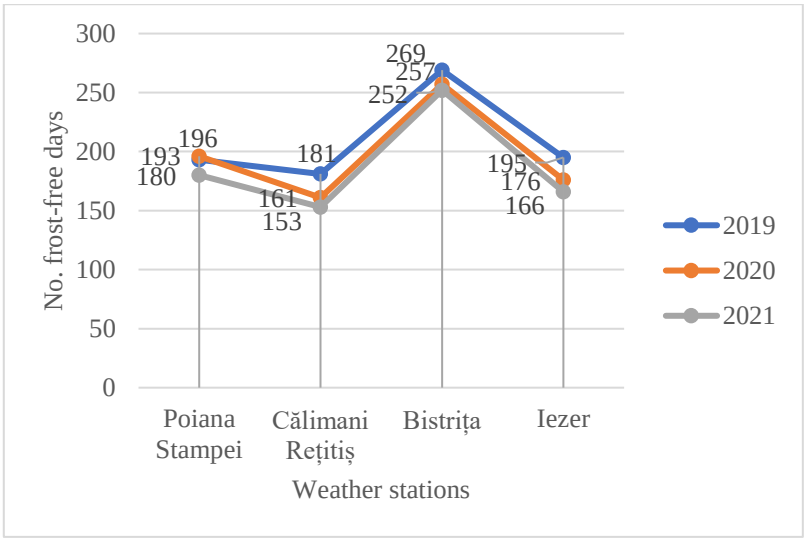
Plate 4.2. Maps of cumulative annual precipitation based on data interpolated from four weather stations near the sampling sites for the years 2019–2021. A – Macea 2019, B – Macea 2020, C – Macea 2021, D – Vatra Dornei 2019, E – Vatra Dornei 2020, F – Vatra Dornei 2021.

4.2.5. Frost-free days

The number of days without ground frost decreased over the years analyzed at both locations, reaching its lowest value in the year the plant material under study was collected. The fluctuations recorded in the lowland area were greater than those in the mountainous area, particularly for the data from the Arad station (Graphs 4.3, 4.4).



Graph. 4.3. Number of frost-free days in the Macea area from 2019 to 2021, according to the four nearby weather stations. A – Arad, S – Șiria, CC – Chișineu-Criș, SM – Sânicolaul Mare.



Graph. 4.4. Number of frost-free days in the Vatra Dornei area from 2019 to 2021, according to the four nearby weather stations. PS – Poiana Stampei, CR – Călimani Reținș, B – Bistrița, I – Iezer.

4.3. Edaphic conditions

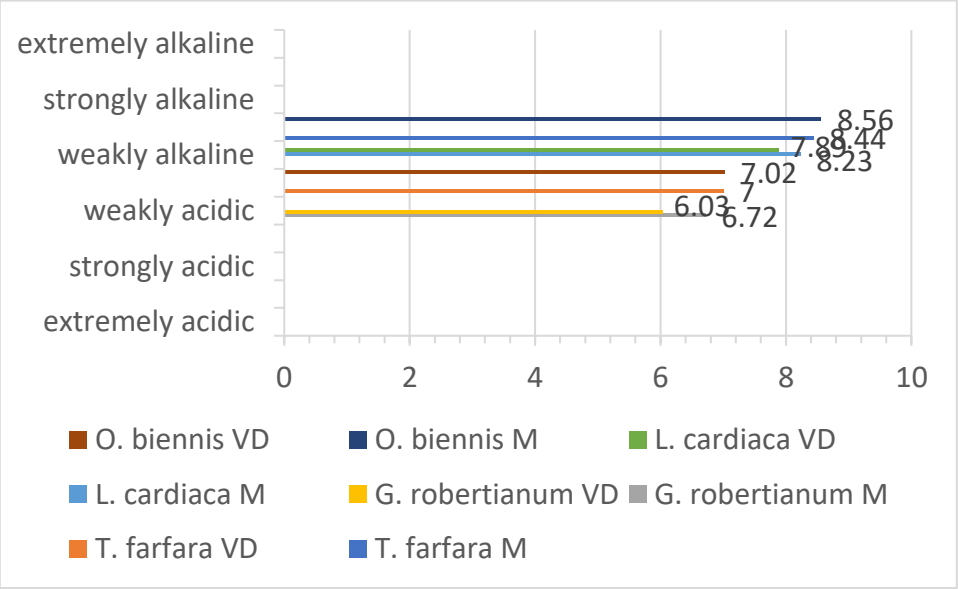
According to the scientific literature (Liu *et al.*, 2016; Zhang *et al.*, 2020), among the main edaphic factors influencing the composition of bioactive compounds in plants growing on a given soil are pH, the amounts of phosphorus and potassium in forms readily assimilable by plants, and the soil's nitrogen supply. In the present study, these parameters were analyzed for each sampling site of the four species under study, and the results are summarized in Table 4.2 and Graphs 4.5–4.8.

Table 4.2.

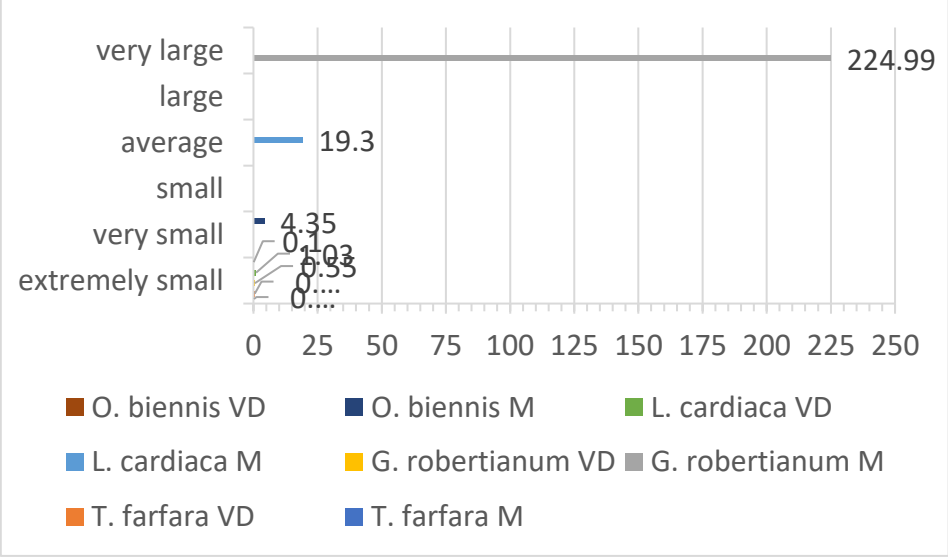
Edaphic conditions corresponding to the collection sites for the analyzed plant material

Species	Location	pH (unit. pH)	P_{AL} (ppm)	K_{AL} (ppm)	N_t (%)
T. farfara L.	Macea	8,44	0,21	663	1,11
G. robertianum L.		6,72	224,99	626	2,56
L. cardiaca L.		8,23	19,30	625	1,86
O. biennis L.		8,56	4,35	280	1,89
T. farfara L.	Vatra Dornei	7,00	0,49	149	0,08
G. robertianum L.		6,03	0,55	148	0,25
L. cardiaca L.		7,89	1,03	109	4,62
O. biennis L.		7,02	0,10	67	4,29

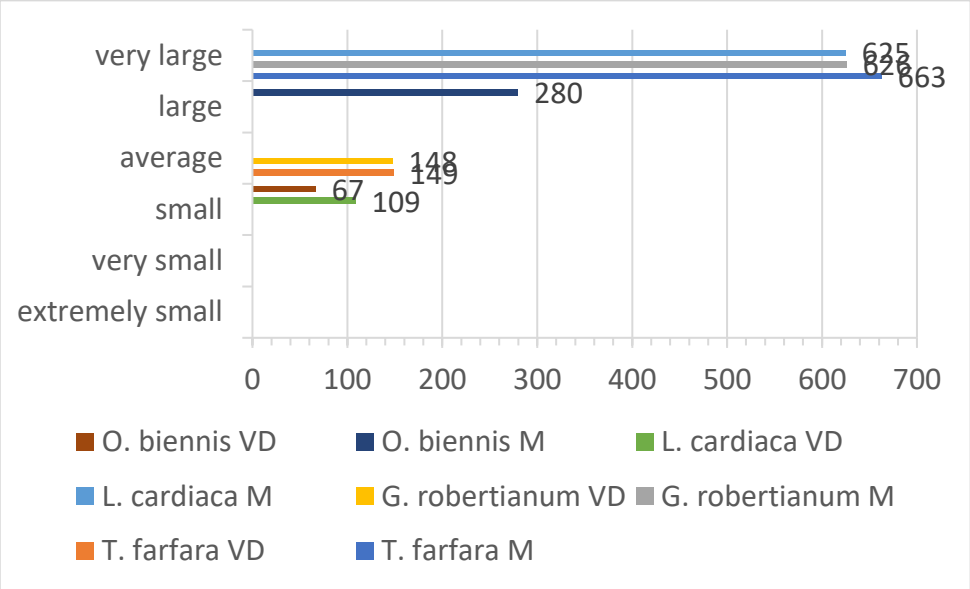
Legend: unit. pH – pH units, PAL (ppm) – amount of available phosphorus calculated in parts per million, KAL (ppm) – amount of available potassium calculated in parts per million, Nt – total nitrogen index; its classification into different classes represents the soil's nitrogen supply.



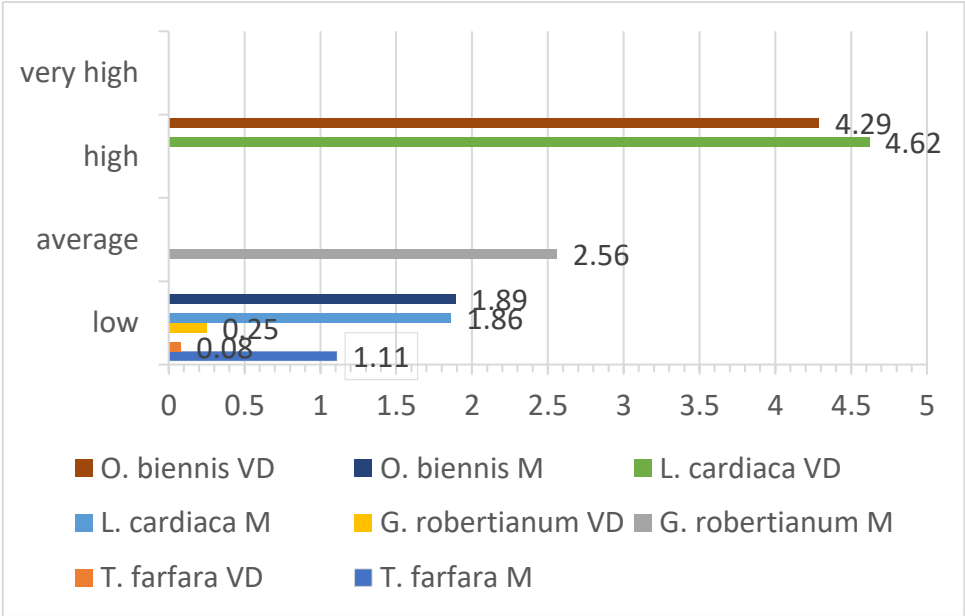
Graph. 4.5. pH index in water of the soil corresponding to the collection sites for the analyzed plant material. M – Macea, VD – Vatra Dornei.



Graph. 4.6. Amount of assimilable phosphorus (ppm) in the soil corresponding to the collection sites for the analyzed plant material. M – Macea, VD – Vatra Dornei.



Graph. 4.7. Amount of assimilable potassium (ppm) in the soil corresponding to the collection sites for the analyzed plant material. M – Macea, VD – Vatra Dornei.



Graph. 4.8. Total nitrogen content in the soil corresponding to the collection sites for the analyzed plant material. M – Macea, VD – Vatra Dornei.

CHAPTER 5
MORPHO-ANATOMICAL AND PHYTOCHEMICAL RESEARCH OF *TUSSILAGO FARFARA* L. SPECIES

5.6. Considerations regarding the morpho-anatomical characteristics and phytochemical composition of the vegetative organs of Tussilago farfara L. species

Morpho-anatomical results obtained for *Tussilago farfara* L. species:

– Structural analysis performed using optical microscopy provided the first anatomical description of the adventitious root, which includes features for species identification (Plate 5.1.); Certain structural aspects are confirmed, and the scientific literature is supplemented with a detailed description of the rhizome's anatomy; presented elements can be correlated with the climatic conditions affecting plant growth at the sampling site, such as the presence of numerous small aerenchyma cells in the cortical parenchyma under low-humidity conditions* (Plate 5.2.); A detailed description of the anatomy of the petiole is provided, including the identification of glandular trichomes with short, elongated stalks supporting a globulose glandular cell, as well as aspects of variability concerning trichomes, the hypodermis, and vascular bundles that contrast with those mentioned in the literature (Plate 5.3.); A detailed description is provided of the cross-sections through the foliar limb, including elements for species identification and aspects regarding the variability of the air chambers and mesophyll cells in the context of environmental factors (high humidity, hypoxia, light intensity); it confirms the appearance of the epidermis and the presence of two types of trichomes – non-glandular, multicellular, uniseriate, with a long, flagellated terminal cell, and glandular with a bicellular stalk and a globulose gland (Plate 5.4.);

* Bota, V. B., Neamtu, A.-A., Olah, N.-K., Chișe, E., Burtescu, R. F., Pripon Furtuna, F. R., Nicula, A.-S., Neamtu, C., Maghiar, A.-M., Ivănescu, L.-C., Zamfirache, M.-M., Mathe, E., Turcuș, V., 2022 - A Comparative Analysis of the Anatomy, Phenolic Profile, and Antioxidant Capacity of *Tussilago farfara* L. Vegetative Organs. Plants, 11(13), 1663.

Plate 5.1. A–D Cross-section through the adventitious root of *Tussilago farfara* L. R – rhizodermis, Co – cortex, Ph – phloem, X – xylem, Ex – exodermis, Pa – cortical parenchyma, En – endodermis, P – pericycle, Ca – air chamber, black arrow – root hairs.

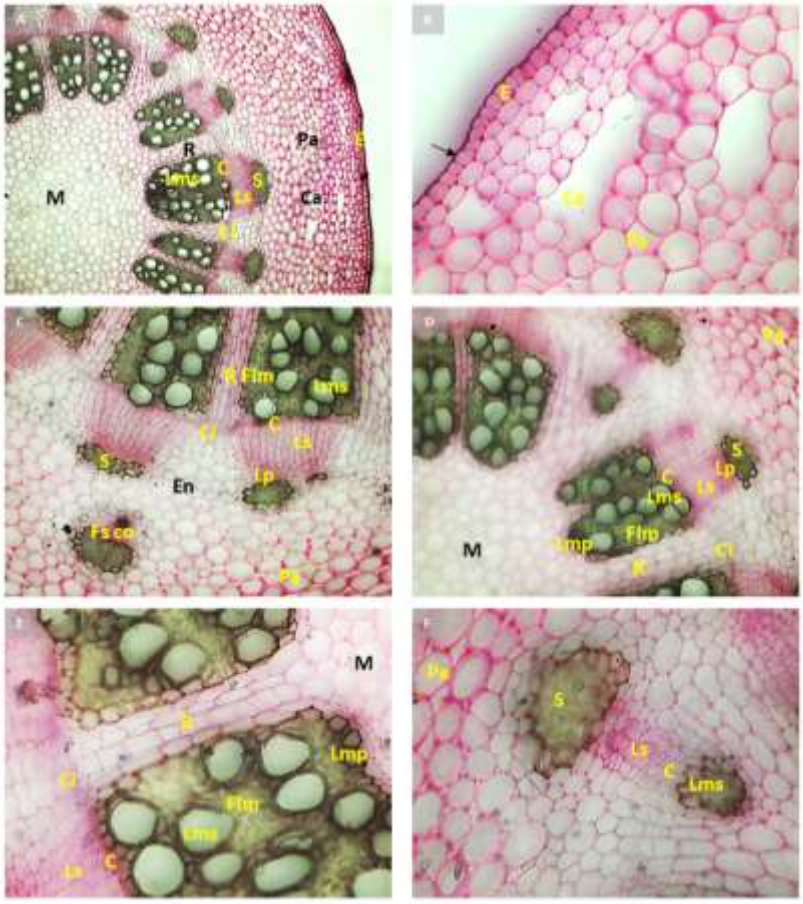


Plate 5.2. A–F Cross-section through the rhizome of *Tussilago farfara* L.; B – detail of the epidermis, cortical parenchyma, and air chambers; C–E – details of the central cylinder; F – detail of a cortical vascular bundle. E – epidermis, Pa – cortical parenchyma, Ca – air chamber, S – sclerenchyma, Ls – secondary phloem, C – intrafascicular cambium, Ci – interfascicular cambium, Lms – secondary xylem, R – medullary ray, M – pith, Lp – primary phloem, Lmp – primary xylem, Flm – libriform fibers, black arrow – cuticle.

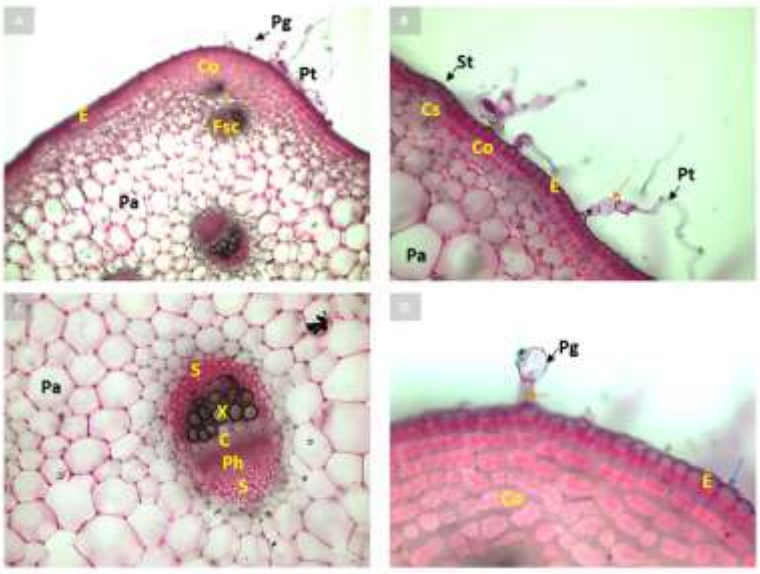


Plate 5.3. A-D Cross-section through the petiole of *Tussilago farfara* L. E – epidermis, Co – collenchyma, Fsc – vascular bundle, Pa – fundamental parenchyma, Pg – glandular trichome, Pt – non-glandular trichome, S – sclerenchyma, X – xylem, C – cambium, Ph – phloem, orange arrow – stalk cells, blue arrow – cuticle.

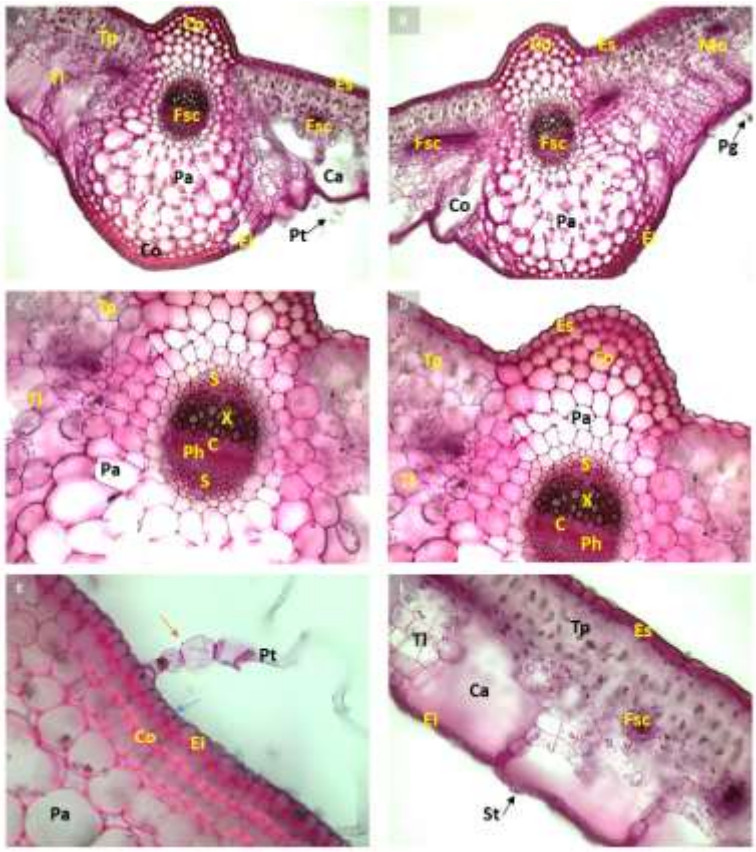


Plate 5.4. A–F Cross-section through the foliar limb of *Tussilago farfara* L.; A–B – general views of the midrib; C–D – details of the midrib and the vascular bundle; E – trichome detail; F – general view of the mesophyll. Es – upper epidermis, Ei – lower epidermis, Co – collenchyma, Fsc – vascular bundle, Pa – fundamental parenchyma, Ca – air chamber, Tp – palisade tissue, Tl – spongy tissue, Pt – non-glandular trichome, Pg – glandular trichome, St – stomata, orange arrow – stalk cells, blue arrow – cuticle.

– Based on scanning electron microscopy (SEM) images, an initial description of the micromorphology of the rhizome, petiole, and foliar in this species was obtained. These images reveal fine longitudinal striations and crystalline particles in small depressions on the surface of the rhizome (Plate 5.5.); The results confirm the presence of two types of non-glandular and glandular trichomes, and indicate stomata dimensions ranging from 48.92 ± 6.25 μm in length to 27.44 ± 4.65 μm in width on the petiole (Plate 5.6.); They also confirm the amphistomatic nature of the foliar limb, with anomocytic stomata; those on the abaxial surface being more numerous, shorter in length (L = 40.94 ± 3.54 μm), and wider in width (l = 31.34 ± 2.80 μm), compared to those on the adaxial surface (L = 43.08 ± 4.23 μm, l = 28.87 ± 2.88 μm), with 6 subsidiary cells, one of which is smaller than the others, with wavy lateral walls similar to those of epidermal cells (Plates 5.7.-5.8.);

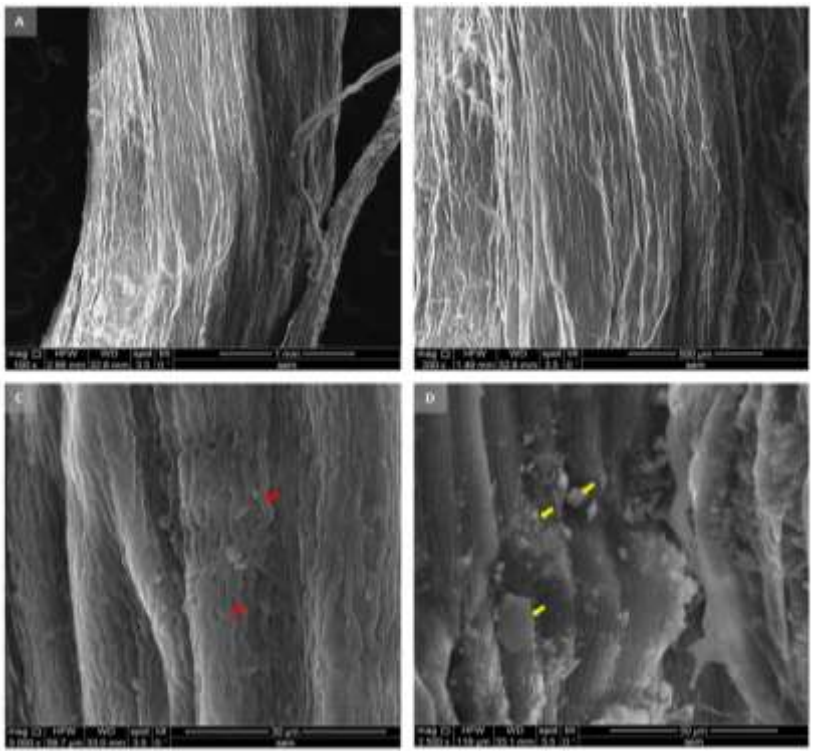


Plate 5.5. A-B – Surface microtopography of the rhizome of *Tussilago farfara* L.; C – crenate appearance of the cuticle; D – presence of crystals and fine particles on the surface of the epidermis. Red arrow – crenate cuticle, yellow arrow – crystals.

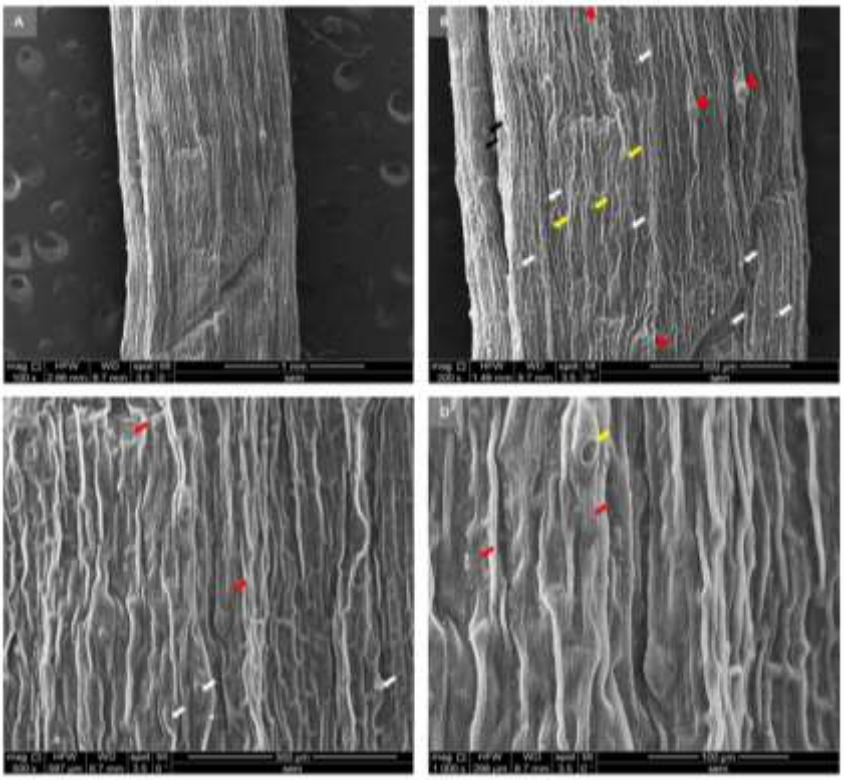


Plate 5.6. A-D – Surface microtopography of the petiole of *Tussilago farfara* L. Red arrowhead – stomata; black arrow – glandular trichome; yellow arrow – base of a broken hair; white arrow – excrescence; red arrow – cuticular striations.

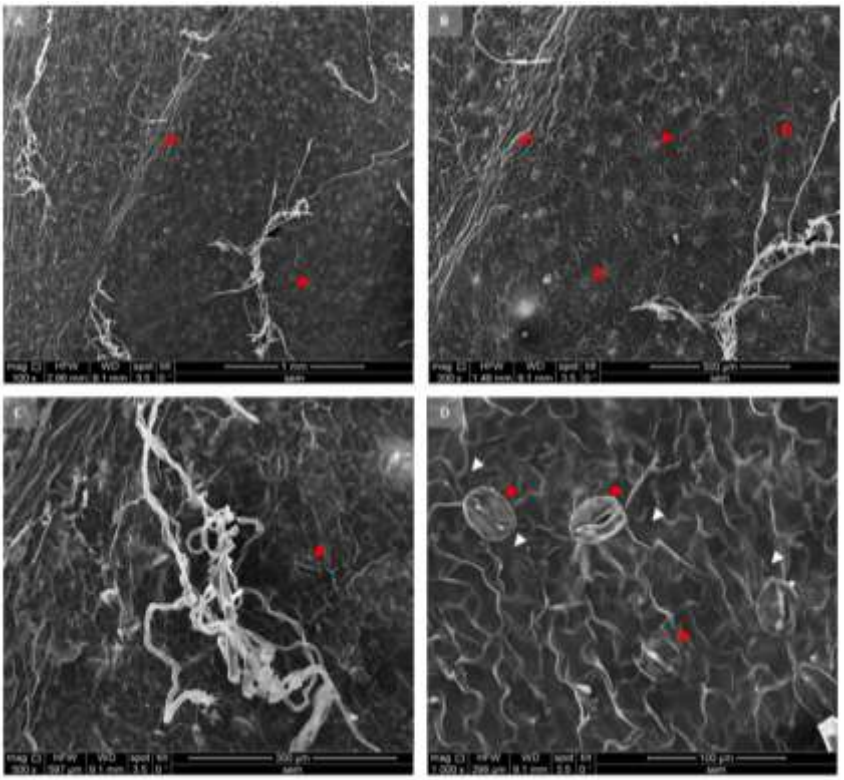


Plate 5.7. A-D – Micromorphological aspects of the lower epidermis of the foliar limb in *Tussilago farfara* L. Red arrowhead – stomata; red arrow – midrib; white arrowhead – subsidiary cells; black arrow – mycelial hyphae; white arrow – non-glandular trichome.

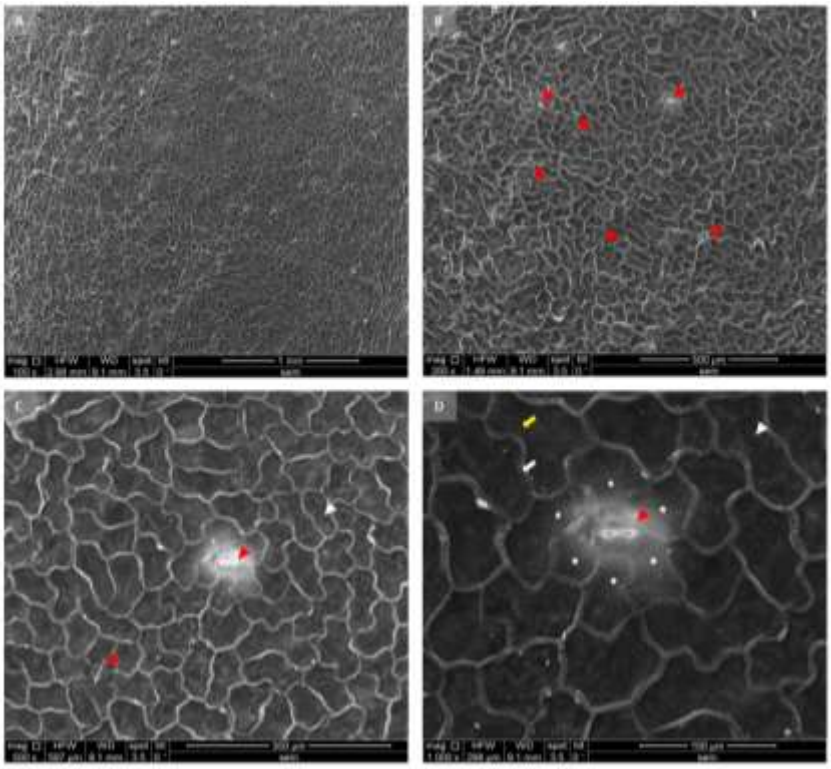


Plate 5.8. A-D – Micromorphological aspects of the upper epidermis of the foliar limb in *Tussilago farfara* L. Red arrowhead – stomata, white arrowhead – epidermal cell, yellow arrow – cuticular striations, white arrow – outline of the outer cell wall, white star – subsidiary cell.

– Analysis of transmission electron microscopy (TEM) images provided the first description in the scientific literature regarding the ultrastructure of the rhizome, petiole, and foliar limb of this species. Ultrastructural analysis of the rhizome indicates an accumulation of primary and secondary metabolites in the intercellular space of the cortical parenchyma near the vascular bundles (Plates 5.9., 5.11., 5.13.); Ultrastructural analysis of the petiole reveals features of the vascular bundle and indicates the presence of secondary metabolites in the phloem parenchyma and companion cells (Plates 5.14.-5.16.); Ultrastructural analysis of the foliar limb mesophyll allowed for the characterization of palisade and spongy cells and air chambers, the first observation in this species of chloroplast stromule in palisade cells, and the presence of secondary metabolites in the form of electron-dense deposits and acicular structures in palisade cells (Plates 5.17., 5.19., 5.20.).

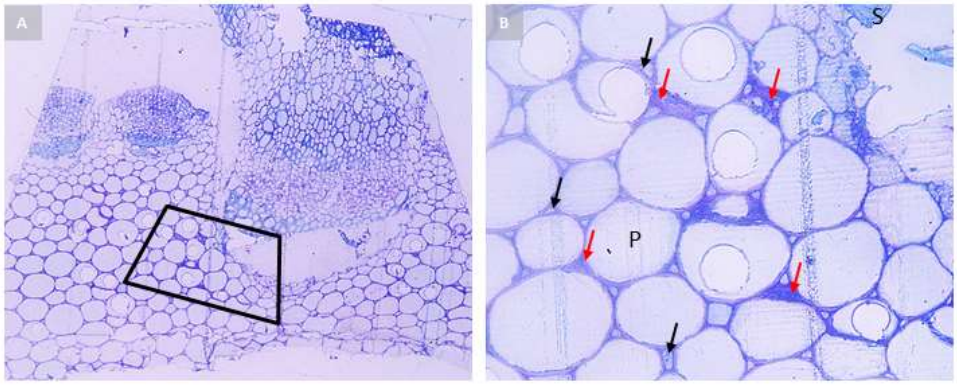


Plate 5.9. A – Semi-thin cross-section through the rhizome of *Tussilago farfara* L. showing the area of interest for TEM (trapezoidal area), 10X; B – detail of the cortical parenchyma with extracellular deposits, 40X. P – cortical parenchyma, black arrow – intercellular space, red arrow – deposit.

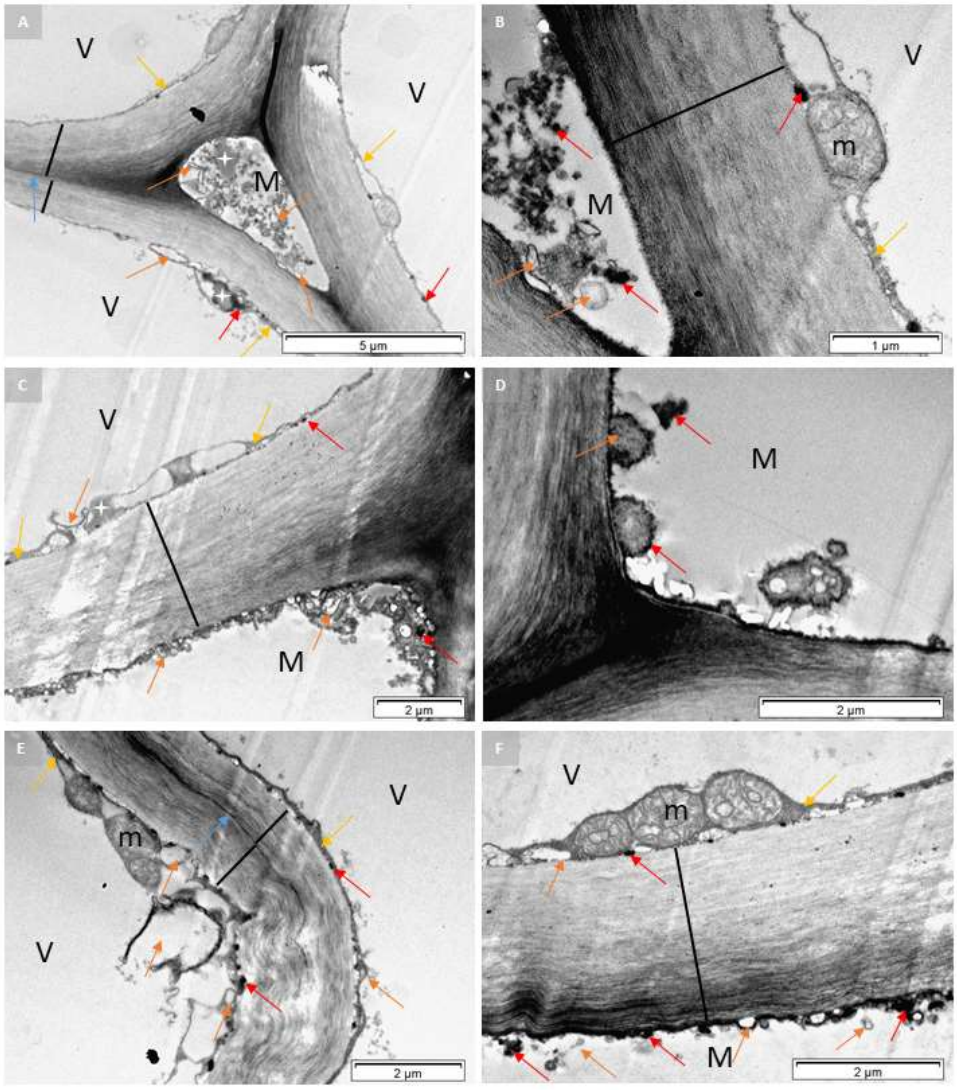


Plate 5.11. A-F – Ultrastructural aspects of the cortical parenchyma of the *Tussilago farfara* L. rhizome, showing deposits in the intercellular space and within cortical cells; B – detail from Figure A. V – vacuole, M – intercellular space, m – mitochondria, black bar – cell wall, blue arrow – middle lamella, yellow arrow – cytoplasmic filament, orange arrow – vesicles, red arrow – granular electron-dense deposits, white star – lipid droplet.

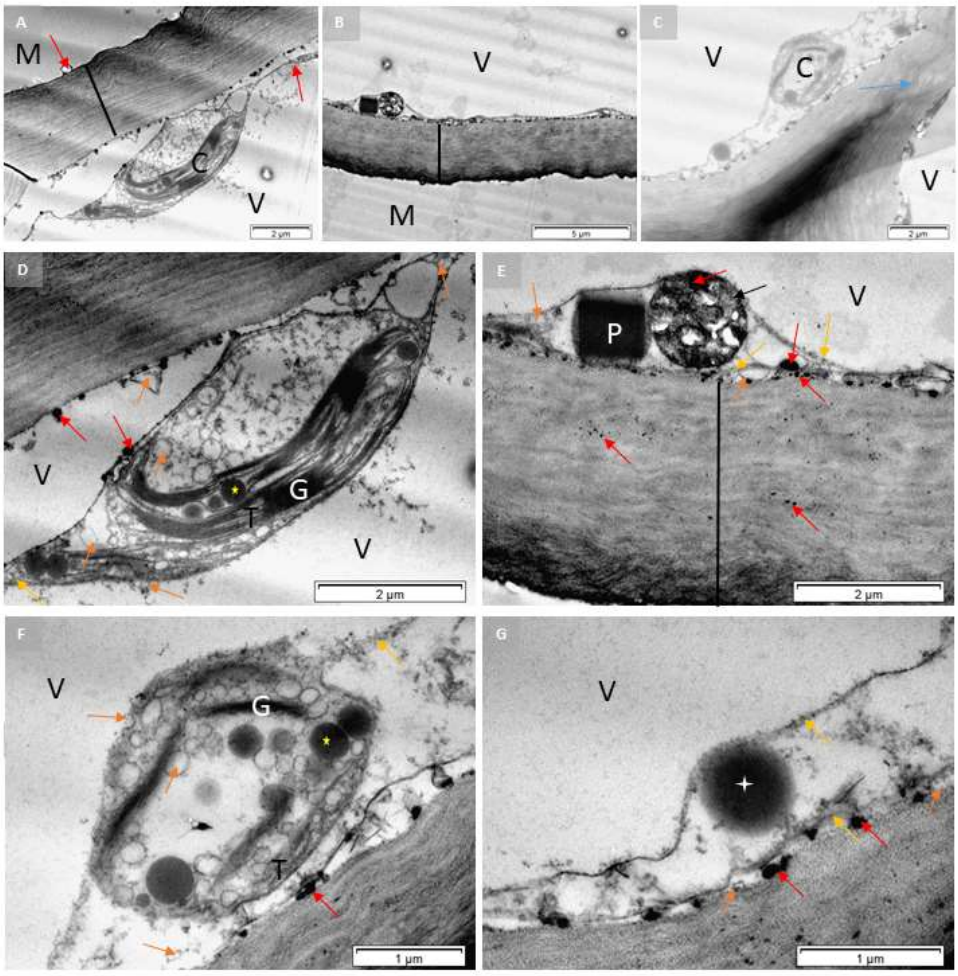


Plate 5.13. A-C – Ultrastructural features of the cortical parenchyma in *Tussilago farfara* L.; D – detail from Figure A; E – detail from Figure B; F–G – details from Figure C. M – intercellular space, V – vacuole, C – chloroplast, T – thylakoid, G – grana, P – peroxisome, black bar – cell wall, blue arrow – middle lamella, yellow arrow – cytoplasmic filament, orange arrow – vesicles, red arrow – granular electron-dense deposits, black arrow – multivesicular body, white star – lipid droplet, yellow star – plastoglobule.

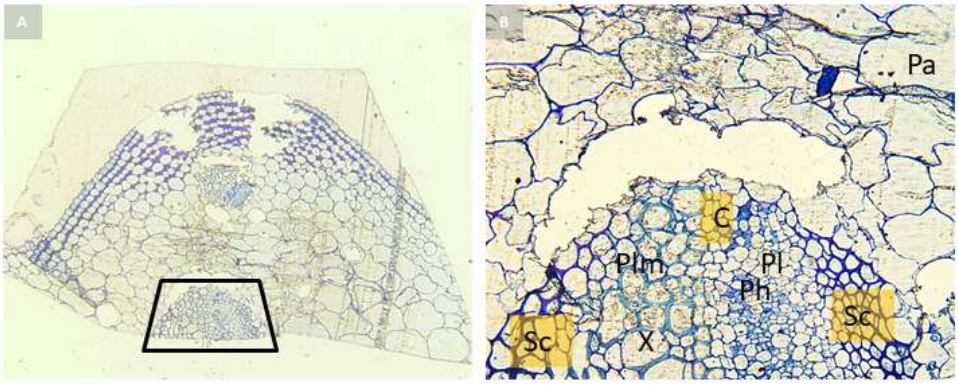


Plate 5.14. A – Semi-thin cross-section through the petiole of *Tussilago farfara* L., with the area of interest for TEM (trapezoidal area), 10X; B – detail of a section showing fundamental parenchyma and a vascular bundle, 40X. Pa – fundamental parenchyma, Sc – sclerenchyma, Pl – phloem parenchyma, Ph – phloem, C – cambium, Plm – wood parenchyma, X – xylem.

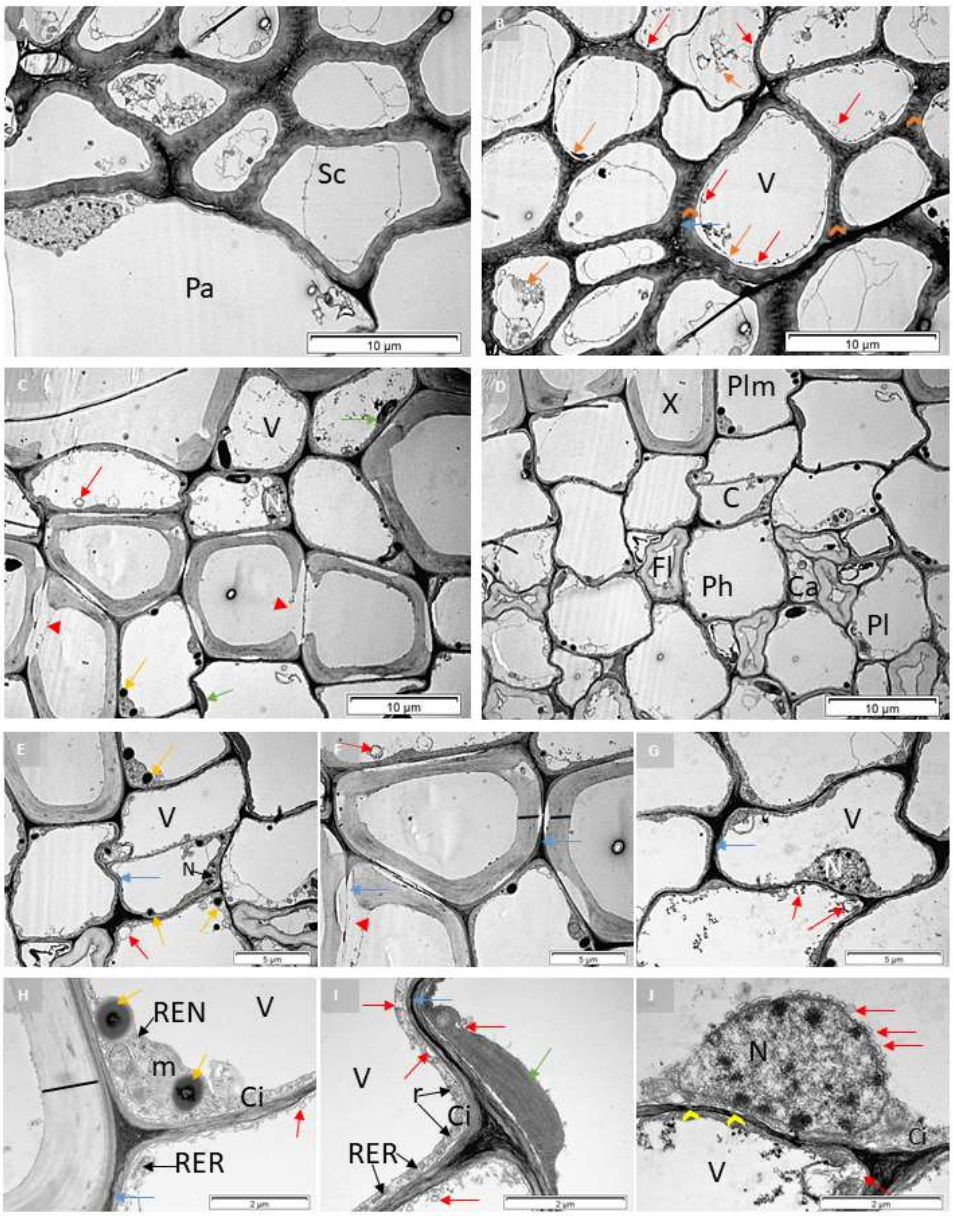


Plate 5.15. A – Section showing fundamental parenchyma and perixylary sclerenchyma in the petiole of *Tussilago farfara* L.; B – section showing sclerenchyma; C – section showing wood tissue; D – section showing cambium, wood, and secondary phloem; E, H, I – ultrastructural details of the cambium and wood parenchyma (Figure D); F – detail of the xylem ultrastructure (Figure C), G, J – detail of the cambium ultrastructure. Pa – fundamental parenchyma, Sc – sclerenchyma, Plm – wood parenchyma, X – xylem, C – cambium, Fl – phloem fiber, Ph – phloem, Ca – companion cell, Pl – phloem parenchyma, V – vacuole, N – nucleus, Ci – cytoplasm, m – mitochondria, REN – smooth endoplasmic reticulum, RER – rough endoplasmic reticulum, r – ribosomes, blue arrow – middle lamella, red arrow – vesicles, yellow arrow – lipid droplets, orange arrow – cytoplasmic filaments, green arrow – chloroplast, orange chevron arrow – Z-band-like junctions of the cell walls, yellow chevron arrow – cytoplasmic extensions into the apoplastic space, red arrowhead – bordered pit, black bar – cell wall.

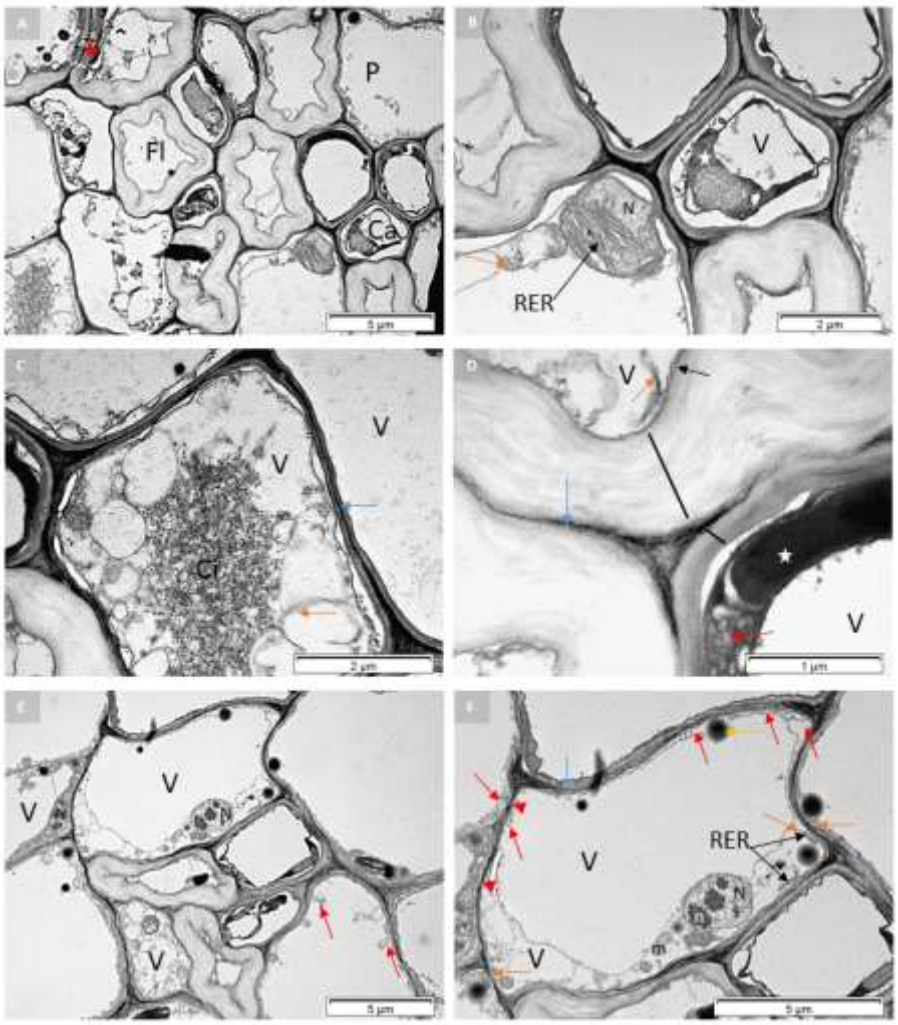


Plate 5.16. A–D – Ultrastructural aspects of the secondary phloem in the petiole of *Tussilago farfara* L.; E–F – ultrastructural aspects of the cambium; B–D show magnified details from Figure A. P – phloem parenchyma, Fl – phloem fiber, Ca – companion cell, V – vacuole, N – nucleus, n – nucleolus, RER – rough endoplasmic reticulum, Ci – cytoplasm, m – mitochondria, blue arrow – middle lamella, red arrow – vesicles, orange arrow – cytoplasmic filaments, black arrow – plasmalemma, red arrowhead – plasmodesmata, black bar – cell wall, white star – electron-dense deposit.

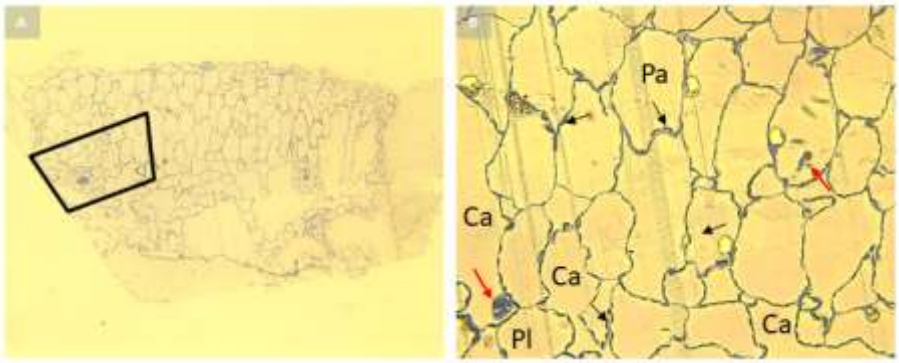


Plate 5.17. A – Semifine cross-section through the foliar limb of *Tussilago farfara* L., showing the area of interest for TEM (trapezoidal area), 10X; B – Detail of the transition zone from spongy tissue to palisade tissue, 40X. Pa – palisade tissue, Pl – spongy tissue, Ca – air chamber, black arrow – chloroplasts, red arrow – storage tissue.

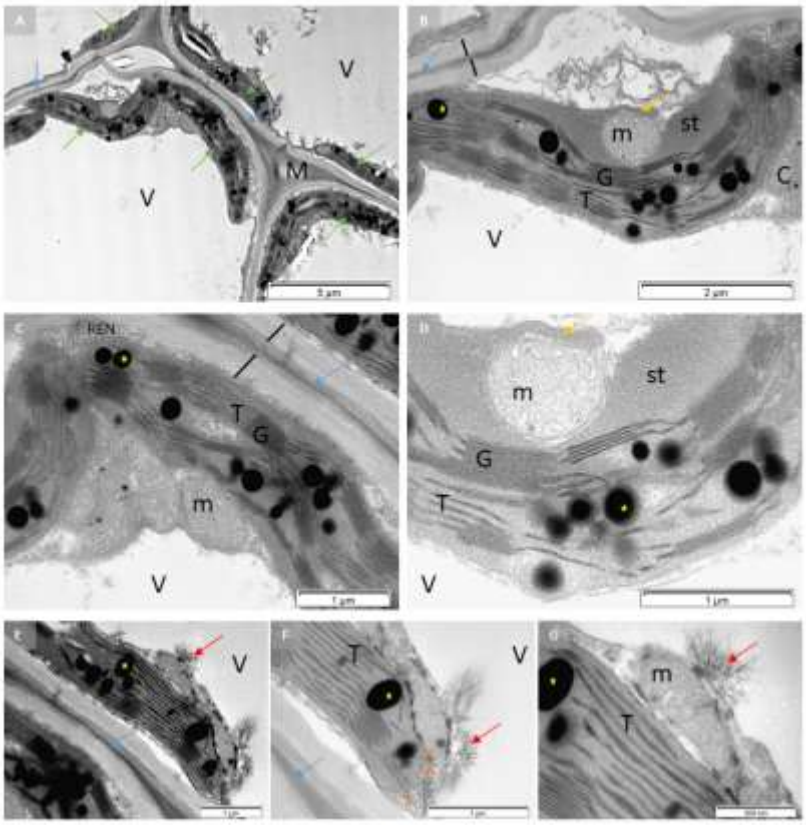


Plate 5.19. A – Ultrastructural aspects of cells in the palisade tissue of the foliar limb of *Tussilago farfara* L.; B–C, E–G – details from Figure A; D – detail from Figure B. V – vacuole, M – intercellular space, m – mitochondria, st – stroma, G – grana, T – thylakoids, C – cytoplasm, REN – smooth endoplasmic reticulum, green arrow – chloroplast, blue arrow – middle lamella, yellow arrow – stromule, red arrow – acicular structures, black bar – cell wall, yellow star – plastoglobules, orange chevron arrow – direct contact between the chloroplast membrane and the acicular structures.

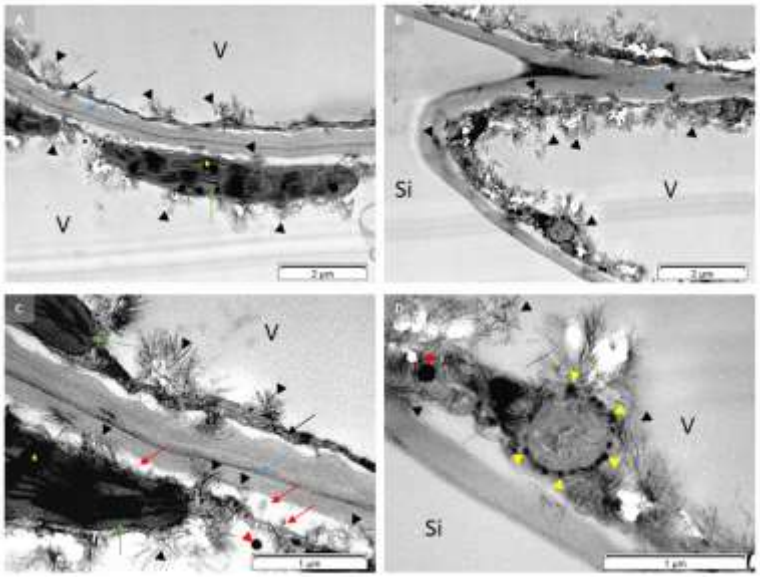


Plate 5.20. A–D – Ultrastructural details of mesophyll cells in *Tussilago farfara* L. with acicular deposits. V – vacuole, Si – intercellular space, blue arrow – middle lamella, black arrow – cytoplasmic filament, green arrow – chloroplast, red arrow – transport vesicles, black arrowhead – acicular structures, red arrowhead – intracellular electron-dense structure, yellow arrowhead – electron-dense structures outside the mitochondrial membrane.

Results obtained following phytochemical analysis of extracts derived from *Tussilago farfara* L. plants belonging to the two populations collected from the wild flora of the two geographic regions in Romania (lowland region—Macea; mountain region—Vatra Dornei):

The LC/MS analysis of extracts from *T. farfara* L. herba revealed, in the context of the pedoclimatic conditions from Macea, 5 polyphenols in the hydroalcoholic extract (Table 5.1) and 8 in the infusion (Table 5.2), and under the pedoclimatic conditions of Vatra Dornei, 10 polyphenols in the hydroalcoholic extract and 8 in the infusion. According to the specialized literature consulted, salicylic acid and carnosol were identified for the first time in this species; the presence of myricetin, naringenin, and carnosic acid was detected for the first time in the herba, and luteolin was found for the first time in the aqueous extract, while trans-p-coumaric acid was quantified for the first time, at levels of 43.5 ± 0.3 μg/g dry plant weight (THVD) and 8700 ± 130 μg/g dry plant weight (TAVD) in the extracts obtained from specimens collected fromVatra Dornei*. Fewer polyphenols, but in higher amounts, were observed in the hydroalcoholic extract from plants growing under pedoclimatic conditions found in the lowland sampling site, while more polyphenols, but in smaller amounts, were observed in the same type of extract from plants collected in the mountain sampling site. In the infusions, there was a higher amount of caffeic acid, apigenin, chrysin, naringenin, rutoside, and hyperoside in the extract from *T. farfara* herba collected from the lowland area, but higher amounts of trans-p-coumaric acid, chlorogenic acid, salicylic acid, luteolin, and gallic acid in the extract from *T. farfara* herba collected from the mountains area (Graphs 5.1-5.2).

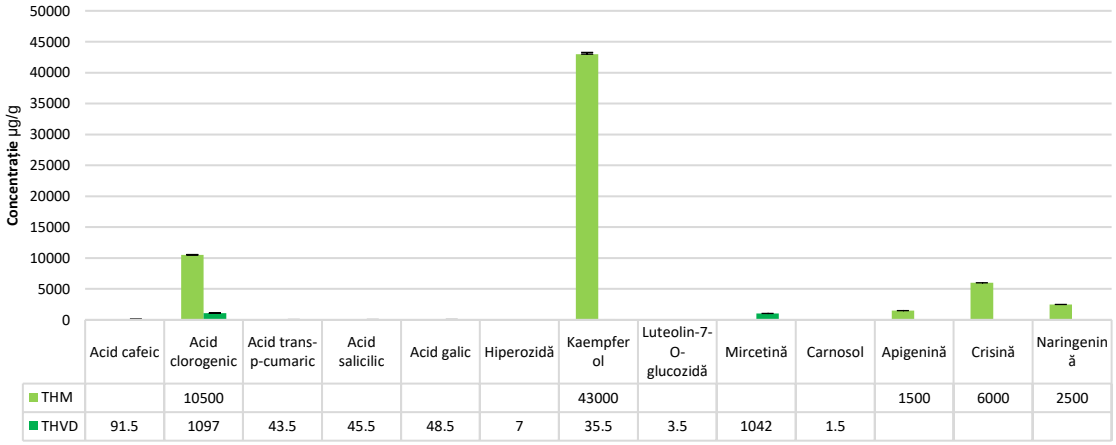
Table 5.1.

Qualitative and quantitative analysis of polyphenols in hydroalcoholic extracts of *Tussilago farfara* L. by LC/MS

No.	Compounds	Sample THM [μg/g dry plant weight]	Sample THVD [μg/g dry plant weight]
1	Caffeic acid	<QL	91.5 ± 0.8
2	Chlorogenic acid	10500 ± 70	1097.0 ± 7.9
3	Trans-p-coumaric acid	<QL	43.5 ± 0.3
4	Gallic acid	<QL	48.5 ± 0.4
5	Salicylic acid	<QL	45.5 ± 0.4
6	Luteolin	<QL	<QL
7	Luteolin-7-O-glucoside	<QL	3.5 ± 0.1
8	Hyperoside	<QL	7.0 ± 0.1
9	Rutoside	<QL	<QL
10	Kaempferol	43000 ± 270	35.5 ± 0.2
11	Myricetin	<QL	1042.0 ± 8.8
12	Apigenin	1500 ± 10	<QL
13	Chrysin	6000 ± 40	<QL
14	Naringenin	2500 ± 10	<QL

No.	Compounds	Sample THM [µg/g dry plant weight]	Sample THVD [µg/g dry plant weight]
15	Carnosol	<QL	1.5 ± 0.1

Abbreviations: Sample THM = hydroalcoholic extract, sample collection site in Macea; Sample THVD = hydroalcoholic extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.



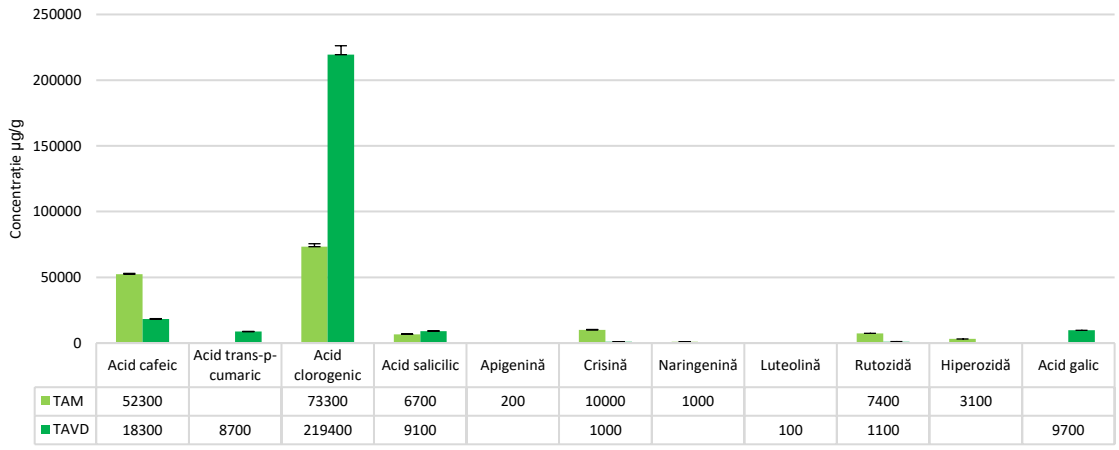
Graph. 5.1. LC/MS analysis of the hydroalcoholic extract of *Tussilago farfara* L. (herba) collected from Macea (THM) and Vatra Dornei (THVD).

Table 5.2.

Qualitative and quantitative analysis of polyphenols in aqueous extracts of *Tussilago farfara* L. by LC/MS

No.	Compounds	Sample TAM [µg/g dry plant weight]	Sample TAVD [µg/g dry plant weight]
1	Caffeic acid	52300 ± 700	18300 ± 270
2	Chlorogenic acid	73300 ± 2270	219400 ± 6800
3	Trans-p-coumaric acid	<QL	8700 ± 130
4	Gallic acid	<QL	9700 ± 300
5	Salicylic acid	6700 ± 100	9100 ± 130
6	Luteolin	<QL	100 ± 10
7	Luteolin-7-O-glucoside	<QL	<QL
8	Hyperoside	3100 ± 100	<QL
9	Rutoside	7400 ± 230	1100 ± 30
10	Kaempferol	<QL	<QL
11	Myricetin	<QL	<QL
12	Apigenin	200 ± 10	<QL
13	Chrysin	10000 ± 310	1000 ± 30
14	Naringenin	1000 ± 30	<QL
15	Carnosol	<QL	<QL

Abbreviations: Sample TAM = aqueous extract, sample collection site in Macea; Sample TAVD = aqueous extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.



	Acid cafeic	Acid trans-p-cumaric	Acid clorogenic	Acid salicilic	Apigenină	Crisină	Naringenină	Luteolină	Rutozidă	Hiperozidă	Acid galic
TAM	52300		73300	6700	200	10000	1000		7400	3100	
TAVD	18300	8700	219400	9100		1000		100	1100		9700

Graph. 5.2. LC/MS analysis of the aqueous extract of *Tussilago farfara* L. (herba) collected from Macea (TAM) and Vatra Dornei (TAVD).

5.5. Comparative analysis of the polyphenol content in extracts of Tussilago farfara L. species in the context of environmental factors

The quantitative and qualitative differences between the two types of extracts—hydroalcoholic and aqueous—can find their explanation in the varying degrees of solubility in the solvent of the polyphenolic compounds analyzed in the previous section. The differences observed between the two populations of *T. farfara* L. from lowland and mountain regions—which are characterized by completely different soil and climate conditions—can be explained, according to the scientific literature, by genotype and/or environmental factors (Liu *et al.*, 2016; Tsusaka, 2019; Chelghoum *et al.*, 2021). Among the environmental factors that significantly influence the content of bioactive compounds in plants are the soil and climate factors analyzed in Chapter 4. In the case of the species *T. farfara* L., environmental factors are known to have a greater influence than genotype on the content of pyrrolizidine alkaloids, which are maintained at an acceptable level in commercial products by ensuring controlled growing conditions (Wawrosch, 2000).

The lowland area where *T. farfara* L. plants were collected for phytochemical analysis is characterized by a low elevation (99 m), a temperate-continental climate with oceanic influences, where in the year of plant sampling (2020) the average annual temperature ranged between 12.2–12.7°C (according to data from nearby weather stations), approximately one degree lower than in the previous year. Also in the sampling year, the winter was warmer than the previous year, with January averages ranging from -0.9 to -0.4°C, and the summer was warmer, with July averages ranging from 21.4 to 22.3°C. Maximum temperatures ranged from 33.7 to 36.7°C, while minimum temperatures were below freezing, ranging

from -10.4 to -7.3°C. Precipitation decreased in the sampling year (2020) compared to the previous year, ranging from 412.9 to 603.8 mm. In the lowland sampling area, there were between 286 and 305 frost-free days in 2020, with smaller fluctuations compared to the previous year than in the mountain area. The soil at the collection site exhibited a moderately alkaline pH (8.44), an extremely low amount of assimilable phosphorus (0.21 ppm), a very high amount of assimilable potassium (663 ppm), and a low total nitrogen content (1.11%). Given the species' ecological preferences, described in section 2.1.4, we can conclude that the pedoclimatic parameters recorded at the lowland collection site generally meet the requirements of *T. farfara* plants, with the exception of the total nitrogen index, which is slightly below the optimum for this species (nitrophilic), but still within the tolerance range.

Under the conditions mentioned above, a higher number of polyphenols was identified in the aqueous extract (8) than in the hydroalcoholic extract (5) obtained from plants collected in the lowland area. Notably, the hydroalcoholic extract contained a high level of kaempferol (43000 μg/g dry plant weight), and was completely absent from the aqueous extract, while it also contained higher levels of apigenin and naringenin (1500 and 2500 μg/g dry plant weight, respectively) compared to the aqueous extract. Under lowland growing conditions, the infusion of *T. farfara* L. herba showed a higher chlorogenic acid content (73300 μg/g dry plant weight) compared to the hydroalcoholic extract (10500 μg/g dry plant weight), with chlorogenic acid being the main compound. The infusion contained caffeic acid, salicylic acid, rutoside, and hyperoside, as well as a higher amount of chrysin but lower amounts of apigenin and naringenin.

The mountain area where *T. farfara* L. plants were collected for phytochemical analysis is characterized by a higher elevation (1027 m), with a cold continental climate and Scandinavian-Baltic influences. In contrast to the lowland area, based on data from nearby weather stations, average annual temperatures are approximately 2–10°C lower (1.9–10.5°C in the sampling year, 2020). Although the warming trend compared to the year prior to the sampling persists in the mountain region as well, winters and summers are cooler, with average temperatures for January and July 2020 ranging from -4.4 to -2.8°C and 10 to 19.9°C, respectively; with low maximum and minimum temperatures in the sampling year (2020), ranging from 20.3 to 33.7°C and 19.5 to 15.1°C, respectively. In contrast to the plains, the cumulative annual precipitation in the mountain region is higher, ranging from 691.4 to 1,419.5 mm in 2020, and the number of frost-free days is lower and marked by greater fluctuations compared to the previous year (176–257 days in 2020, 181–269 days in 2019). The soil at the sample collection site, located in a mountain area, was characterized

by a neutral pH (7), an extremely low amount of assimilable phosphorus, though higher than in the lowlands (0.49 ppm), an average amount of assimilable potassium (149 ppm), and a lower nitrogen supply than in the lowlands (0.08%). Consequently, the pedoclimatic conditions in the mountain area fall within the tolerance range but are slightly below the optimum preferred by *T. farfara* L. species in terms of temperature, moisture, and nitrogen indices.

Under these conditions, the following differences in the polyphenol content of the extracts obtained were observed in the population analysed from Vatra Dornei: a higher number of polyphenols in the hydroalcoholic extract (10) compared to the aqueous extract (8), the absence of apigenin and naringenin in both types of extract and of hyperoside in the infusion, as well as a higher concentration of the analyzed compounds in the aqueous extract. Of particular note is the concentration of chlorogenic acid in the infusion (219400 μg/g dry plant weight) and of caffeic acid (18300 μg/g dry plant weight). In contrast, the hydroalcoholic extract additionally contained carnosol, myricetin, kaempferol, and luteolin-7-O-glucoside, while the aqueous extract contained rutoside, luteolin, and chrysin.

CHAPTER 6
MORPHO-ANATOMICAL AND PHYTOCHEMICAL RESEARCH OF *GERANIUM ROBERTIANUM* L. SPECIES

6.6. Considerations regarding the morpho-anatomical characteristics and phytochemical composition of the vegetative organs of Geranium robertianum L. species

Morpho-anatomical results obtained for *Geranium robertianum* L. species:

– A detailed characterization, unprecedented in the scientific literature, of the adventitious root, rhizome, aerial stem, petiole, and foliar limb of this species, based on optical microscopy*, SEM, and TEM images;

– Among the species' characteristic features are the arrangement of the secondary xylem in the central cylinder in the form of two opposing triangles of xylem vessels and two triangles of homogeneous parenchymatous medullary rays, respectively, which rest their bases on the cambium, both in the structure of the adventitious root and in that of the rhizome*. A distinctive feature observed in cross-sections through the rhizome is the presence of an aquiferous parenchyma located in two opposing clusters at the boundary between the woody parenchyma and libriform fibers, which can be explained by the plant's role in the phytoremediation of the substrate (Plates 6.1–6.3). SEM images confirm the irregularly oval shape of the rhizome, the presence of cork and the outer mucilage layer, along with amorphous, semispherical deposits, fine particles, and crystals (Plate 6.8). The mucilages are also confirmed by TEM images (Plates 6.13-6.14);

– The analysis of cross-sections through the aerial stem adds new details about the anatomical structure to the scientific literature and highlights aspects of variability in cross-sectional contour and cortical differentiation, which are important for classifying the species within the tuberous *Geranium* group (Plate 6.4). Micromorphologically, the stem is characterized by a surface with parallel striations spaced at equal intervals of 23.59 ± 3.15 μm, and by the presence of three types of hairs: (1) unicellular, non-glandular trichomes, 853.12 ± 79.12 μm in length, with a broad base and a pointed tip, smooth cell walls, translucent under optical microscopy, containing lipid droplets inside; (2) unicellular, non-glandular trichomes, 296.18 ± 33.85 μm in length, with a sharp tip bent in the same direction, rarely erect, smooth cell walls, arranged in clusters, and less numerous than the previous type, (3) short, conical trichomes, with a constriction before the short, conical tip, possibly glandular. The morphological differences from the trichomes mentioned in the literature led

to the designation of these trichomes as type IV, Type V, and short trichomes, respectively (Plate 6.9).

– Analysis of the petiole's anatomical structure revealed the presence of large, unicellular trichomes on this organ as well, a finding that contradicts the data in the literature (Plate 6.5). According to SEM images, these correspond to type IV and V, the latter being rarer and arranged in a column along the striations (Plate 6.10);

– Analysis of cross-sections through the foliar limb provides a complete description of the anatomical structure of the midrib and mesophyll; together with the SEM images, this confirms the presence of type IV trichomes along the veins, of type I and II glandular trichomes mentioned in the literature, and the presence of crystals in the cortical cells, which support the inclusion of the species in the tuberous *Geranium* group; according to the results obtained, stomata and non-glandular trichomes are present on both surfaces of the foliar limb, being noticeably more numerous on the abaxial surface (Plates 6.6-6.7, 6.11-6.12);

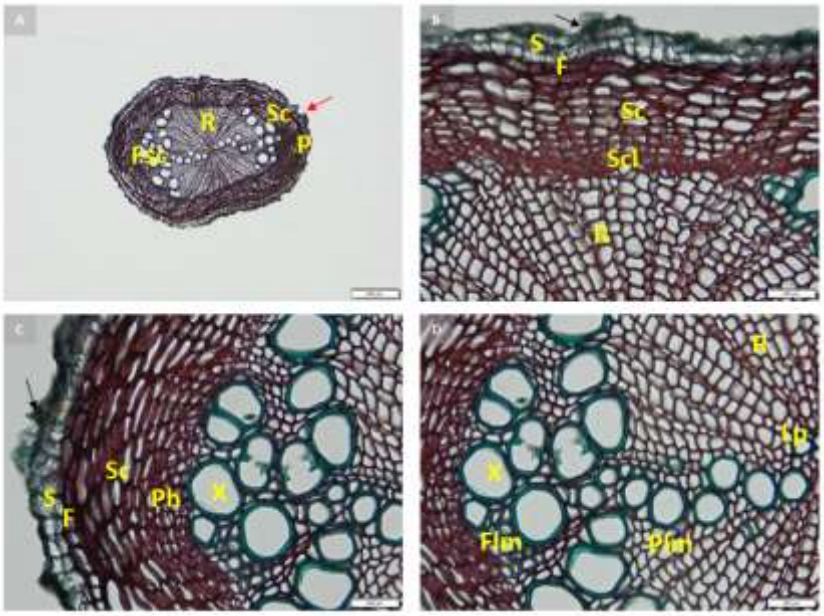


Plate 6.1. A–D Cross-section through the adventitious root of *Geranium robertianum* L. P – periderm, Fsc – vascular bundle, S – cork, F – phellogen, Sc – cortex, Scl – sclerenchyma, Ph – phloem, X – xylem, R – medullary ray, Flm – wood fibers, Plm – wood parenchyma, Lp – primary wood, red arrow – exfoliating periderm, black arrow – gelatinous substances.

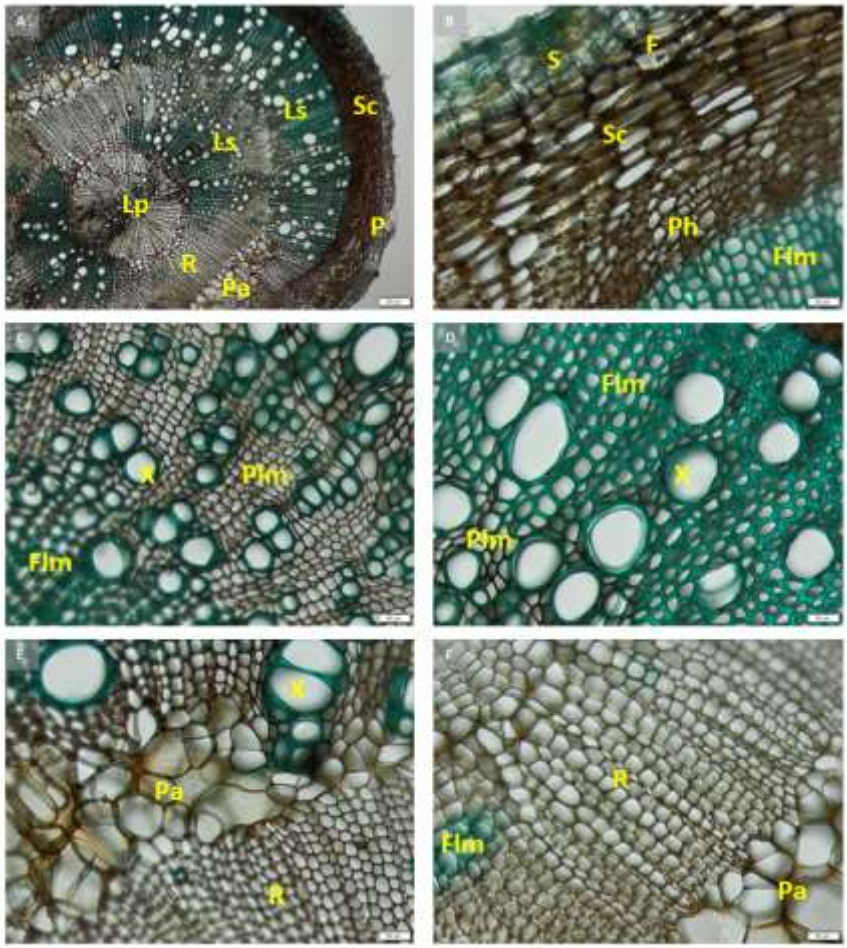


Plate 6.2. A-F Cross-section through the rhizome of *Geranium robertianum* L. P – periderm, Sc – cortex, Ls – secondary wood, Lp – primary wood, R – medullary rays, Pa – aquiferous parenchyma, S – cork, F – phellogen, Ph – phloem, X – xylem, Flm – wood fibers, Plm – wood parenchyma.

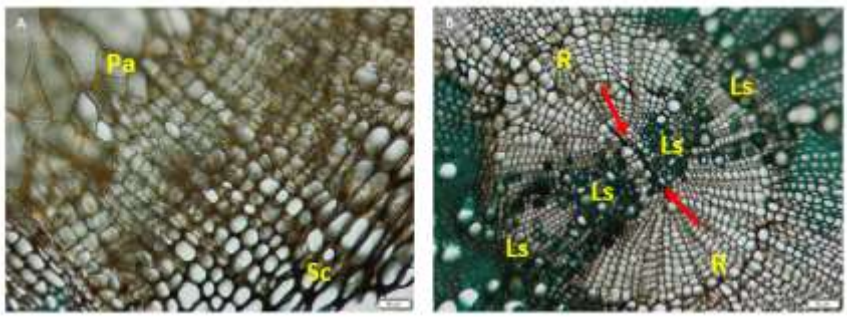


Plate 6.3. A-B Cross-section through the rhizome of *Geranium robertianum* L.; A – Detail of the transition zone from the aquiferous parenchyma to the cortex; B – Detail of the central sector showing primary wood and the tips of the two secondary wood triangles. Pa – aquiferous parenchyma, Sc – cortex, R – medullary rays, Ls – secondary wood, red arrow – primary wood.

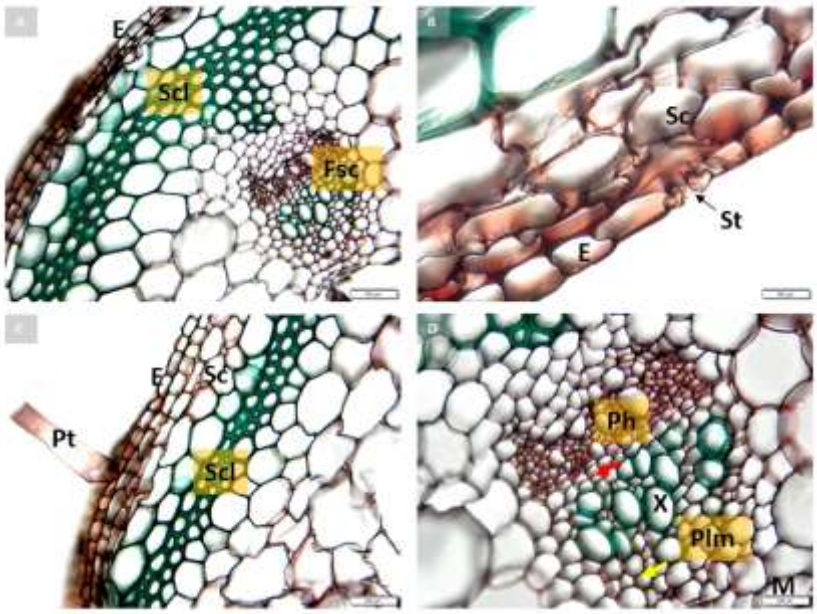


Plate 6.4. A-D Cross-section through the aerial stem of *Geranium robertianum* L. E – epidermis, St – stomata, Pt – trichome, Sc – cortex, Scl – sclerenchyma, Fsc – vascular bundle, Ph – phloem, X – xylem, Plm – wood parenchyma, M – pith, red arrow – procambium, yellow arrow – protoxylem.

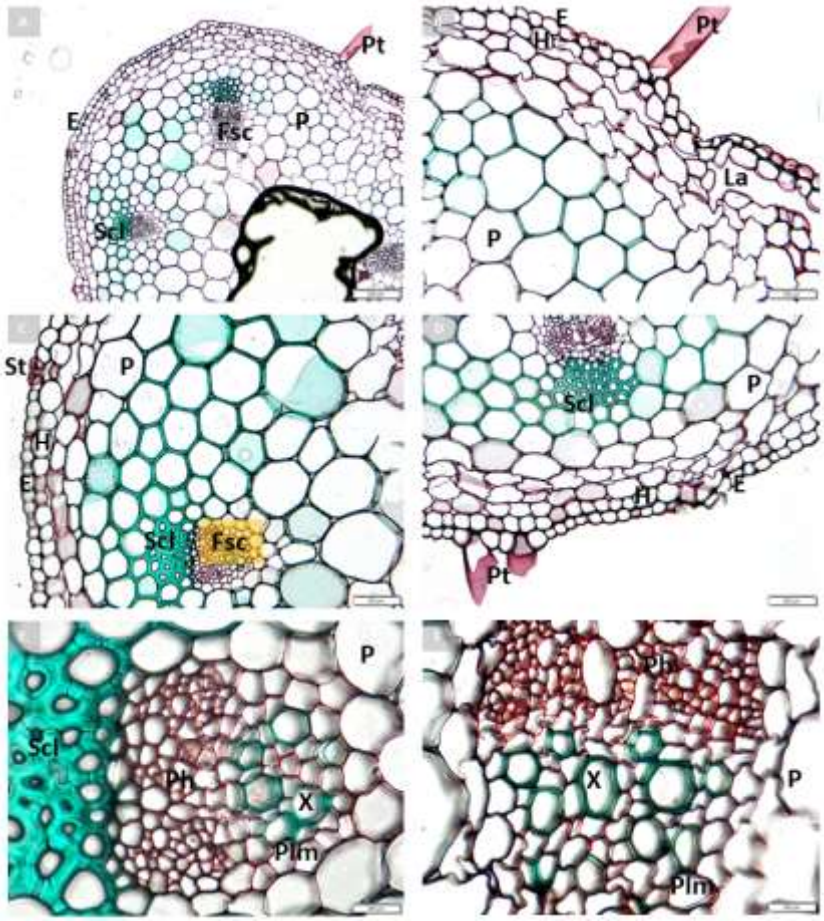


Plate 6.5. Cross-section through the petiole of *Geranium robertianum* L. E – epidermis, H – hypodermis, P – parenchyma, Pt – trichome, St – stomata, Scl – sclerenchyma, Fsc – vascular bundle, Ph – phloem, X – xylem, Plm – woody parenchyma.

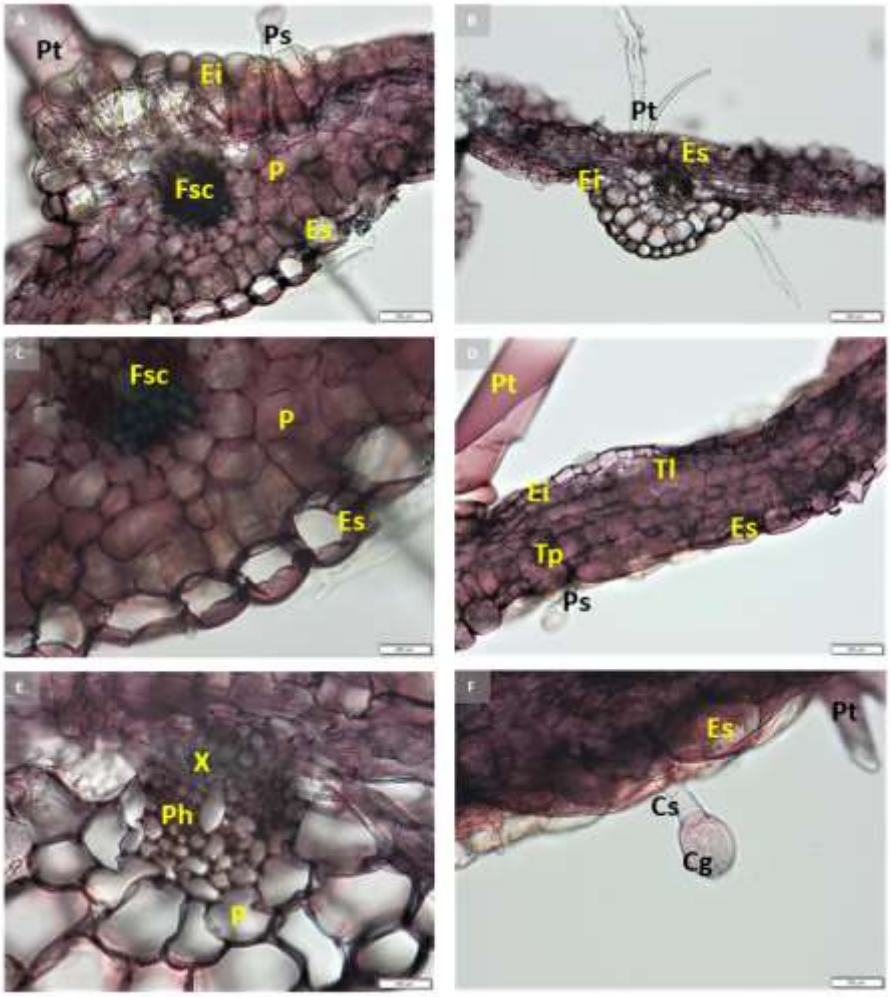


Plate 6.6. A-F Cross-section through the foliar limb of *Geranium robertianum* L. A, C, E – Details of the midrib; B – General view; D, F – Details of the mesophyll. Ei – lower epidermis, Es – upper epidermis, Fsc – vascular bundle, P – parenchyma, Pt – non-glandular trichome, Ps – glandular trichome, Tp – palisade tissue, Tl – spongy tissue, X – xylem, Ph – phloem, Cs – stalk cells, Cg – glandular head.

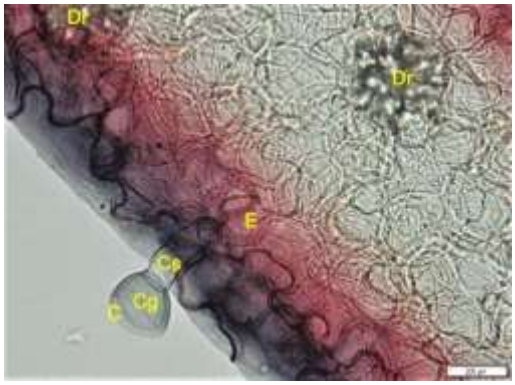


Fig. 6.7. Transverse-superficial section through the internervural region of a *Geranium robertianum* L. foliar limb; rosette crystals (druses) are visible through transparency, and a glandular trichome is visible on the epidermis. E – epidermis, Dr – druses, Cs – stalk cells, Cg – glandular head, C – cuticle.

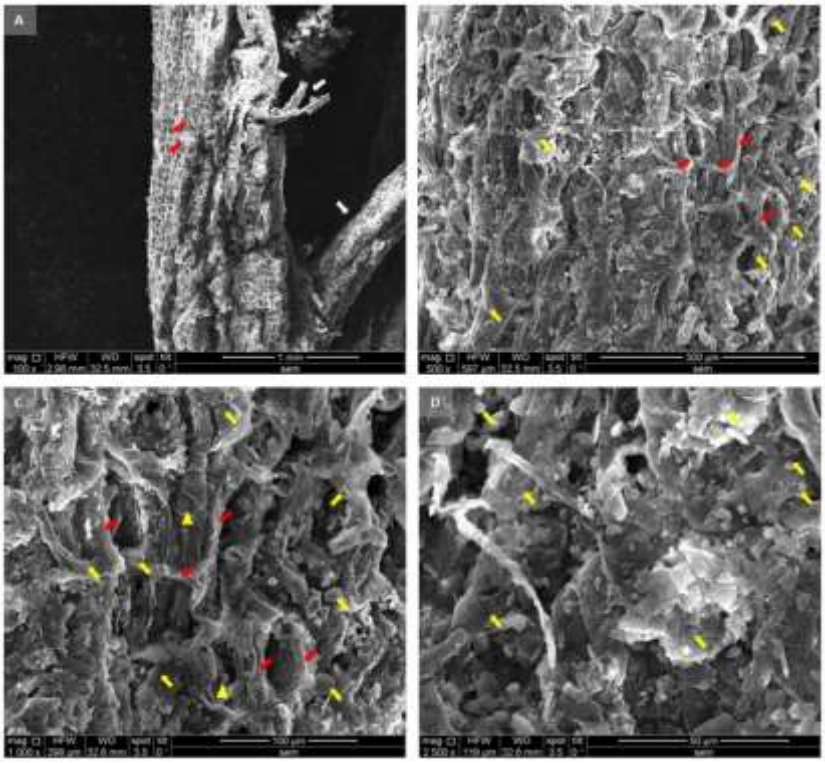


Plate 6.8. A-D Surface microtopography of the rhizome of *Geranium robertianum* L. White arrow – lateral ramification; red arrow – cell wall; yellow arrow – mucilage; yellow arrowhead – crystal.

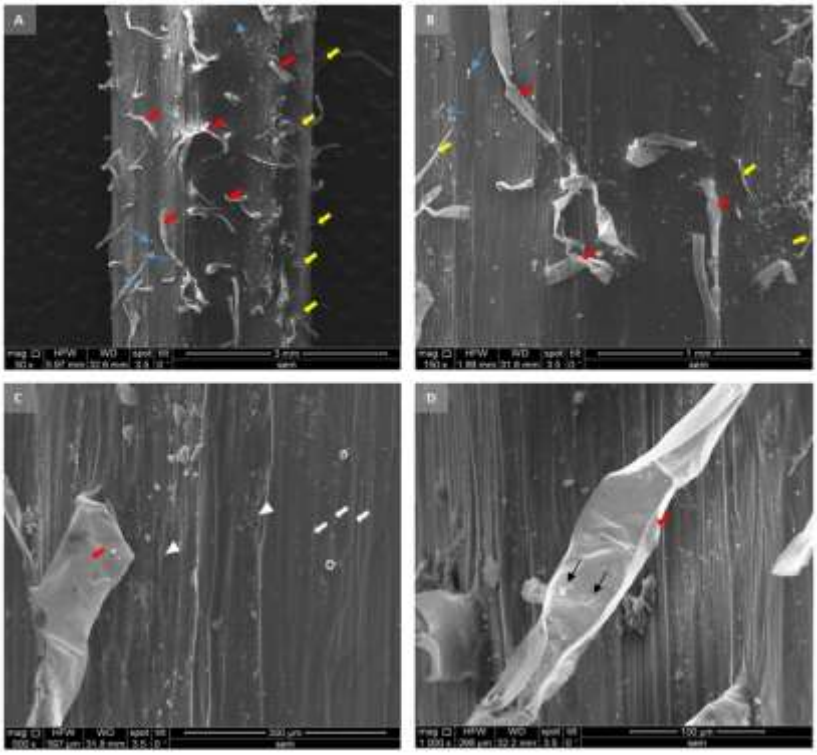


Plate 6.9. A-D Microtopography of the aerial stem of *Geranium robertianum* L.; C – details of the epidermis showing trichomes and stomata; D – details of a Type IV trichome. Red arrow – Type IV trichome, yellow arrow – Type V trichome, blue arrow – short trichome, white arrow – epidermal striations, white arrowhead – stomata, black arrow – fine particles on the surface of Type IV trichome walls.

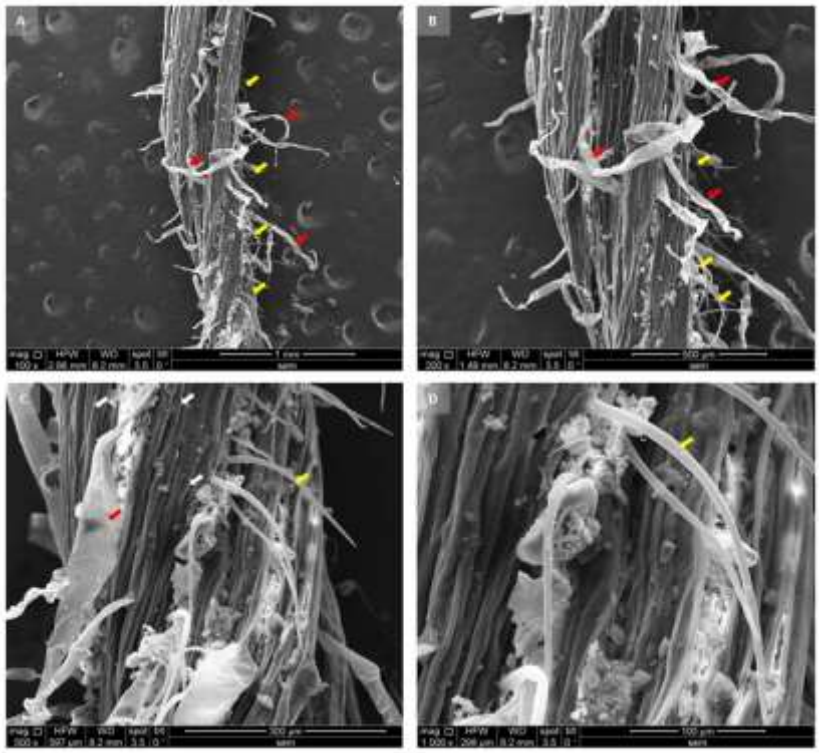


Plate 6.10. A-D Microtopography of the petiole in *Geranium robertianum* L. Red arrow—Type IV trichome; yellow arrow—Type V trichome; white arrow—striations; black arrow—smooth cuticle appearance.

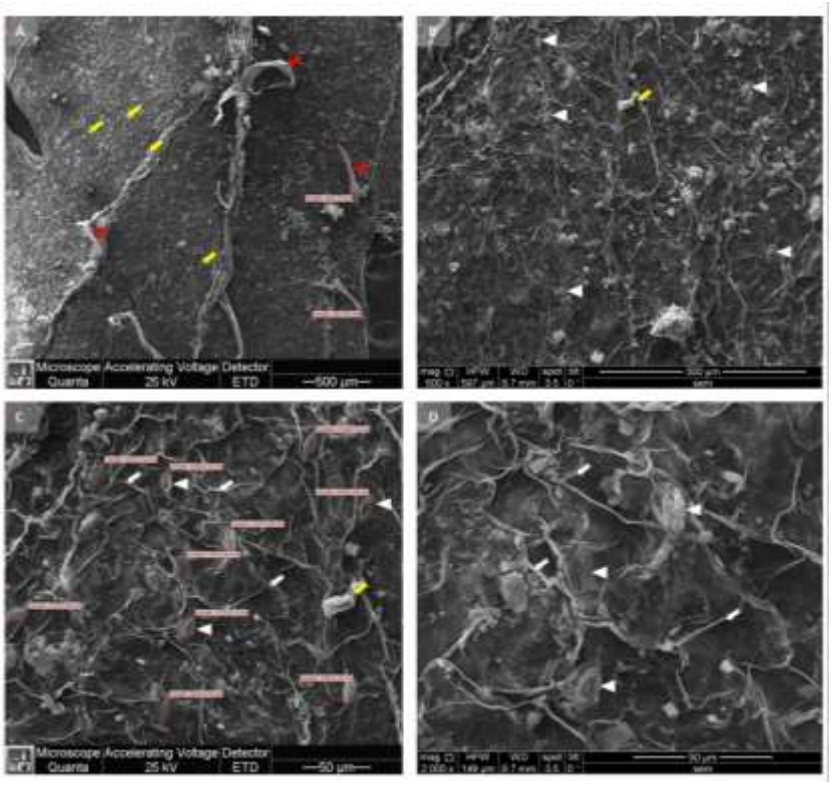


Plate 6.11. A-D Microtopography of the lower epidermis of the foliar limb in *Geranium robertianum* L. Red arrow—Type IV trichome; yellow arrow—glandular trichome; white arrow—cuticle; white arrowhead—stomata.

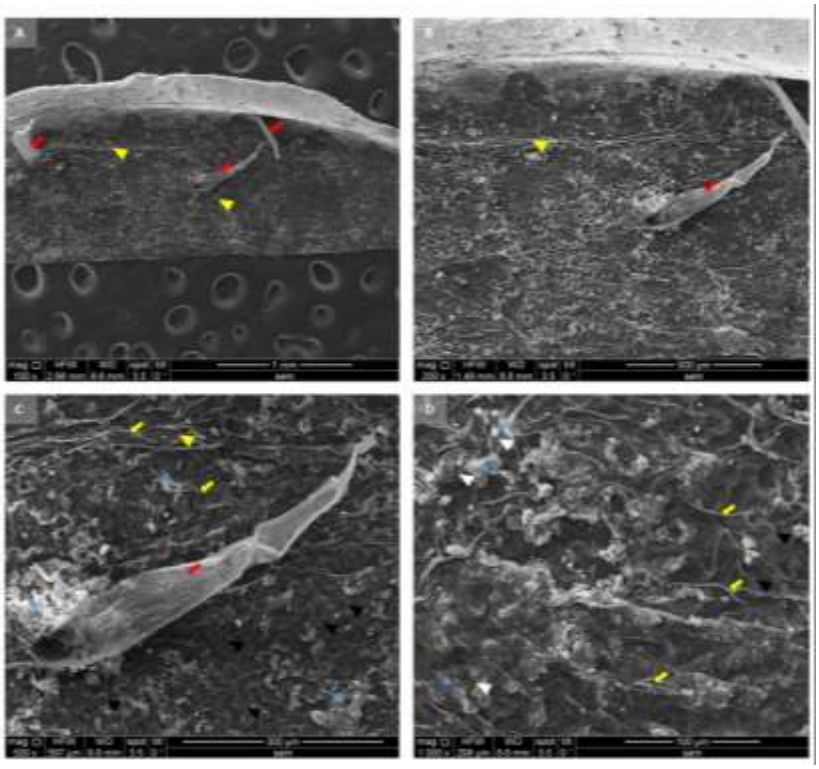


Plate 6.12. A-D Microtopography of the upper epidermis of the foliar limb in *Geranium robertianum* L. Red Arrow – Type IV trichome, yellow arrowhead – vein, yellow arrow – wrinkled cuticle, black arrowhead – undulating lateral walls of epidermal cells, white arrowhead – stomata, blue arrow – salt deposits and amorphous particles.

TEM images of the adventitious root show the presence of tannin deposits in the periderm cells, the abundance of which gradually decreases toward the cortex, as well as secondary metabolites found in the cells of the periderm, phloem, and secondary xylem (Plates 6.13–6.14). In the rhizome, numerous intra- and intercellular deposits were observed between the endodermis and the cambium; their appearance suggests a phenolic or lipidic nature, and crystalline formations were observed in the cells of the woody parenchyma (Plates 6.17-6.18, 6.20). Ultrastructural analysis of the aerial stem reveals features of hypodermal cells, such as intravacuolar acicular deposits and electron-dense structures in chloroplasts associated with the rupture of their membranes, for which no explanation has been found in the scientific literature; electron-dense structures of unknown nature, the presence of chloroplasts, and multivesicular bodies in the phloem parenchyma (Plates 6.21-6.22). Acicular deposits were also observed in the epidermal cells of the petiole, toward the interior of the cell wall, and on the exterior of the hypodermal cells, toward the air chamber (Plates 6.24–6.26). Within the foliar limb, the cells of the upper adaxial epidermis are larger than the other cells in the organ; most of the intravacuolar deposits observed are accumulated at this level and, less frequently, in palisade, spongy, or abaxial epidermal cells; Deposits of

tannins were observed outside the vacuole, and large lipid droplets in chloroplasts, which may be associated with abiotic environmental stress caused by low nitrogen supply, as confirmed by soil analysis at the sampling site (Plates 6.27-6.28).

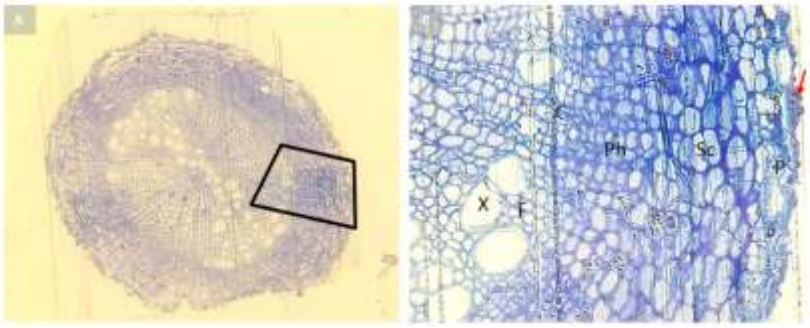


Plate 6.13. A – Semi-thin cross-section through the adventitious root of *Geranium robertianum* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of the analyzed marginal sector, 40X. P – periderm, Sc – cortex, Ph – phloem, C – cambium, X – xylem, F – wood fibers, red arrow – mucilage.

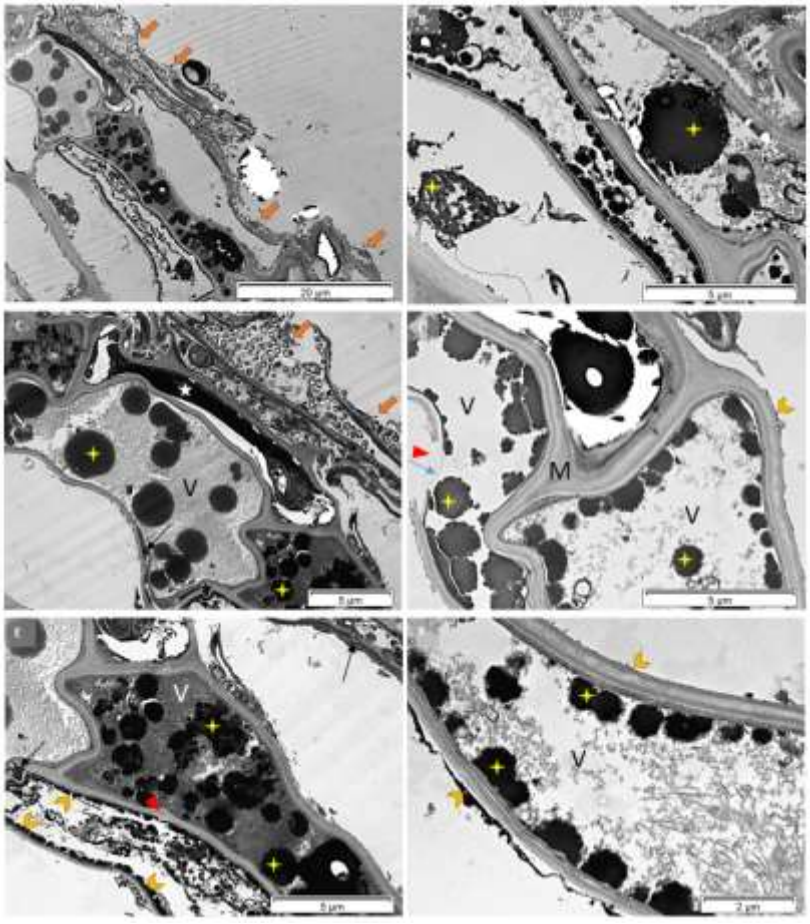


Plate 6.14. A-F Ultrastructural features of cells in the periderm of the adventitious root of *Geranium robertianum* L. that play a role in the synthesis and storage of bioactive compounds; C-E show details from Figure A. V – vacuoles with granular deposits, M – parenchyma, blue arrow – middle lamella, orange arrow – mucilages on the outside of the periderm, red arrowhead – pit, yellow star – tannin deposits, white star – compact tannin deposit, black arrow – intercellular deposit, yellow chevron arrow – early-stage electron-dense deposits along the tonoplast.

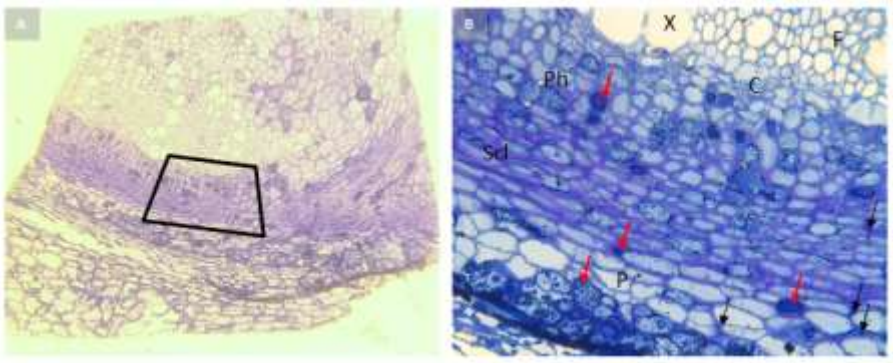


Plate 6.17. A – Semi-thin cross-section through the rhizome of *Geranium robertianum* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of a sector from the central cylinder, 40X. P – pericycle, Scl – sclerenchyma, Ph – phloem, X – xylem, C – cambium, F – wood fibers, red arrow – intracellular deposits, black arrow – intercellular deposits.

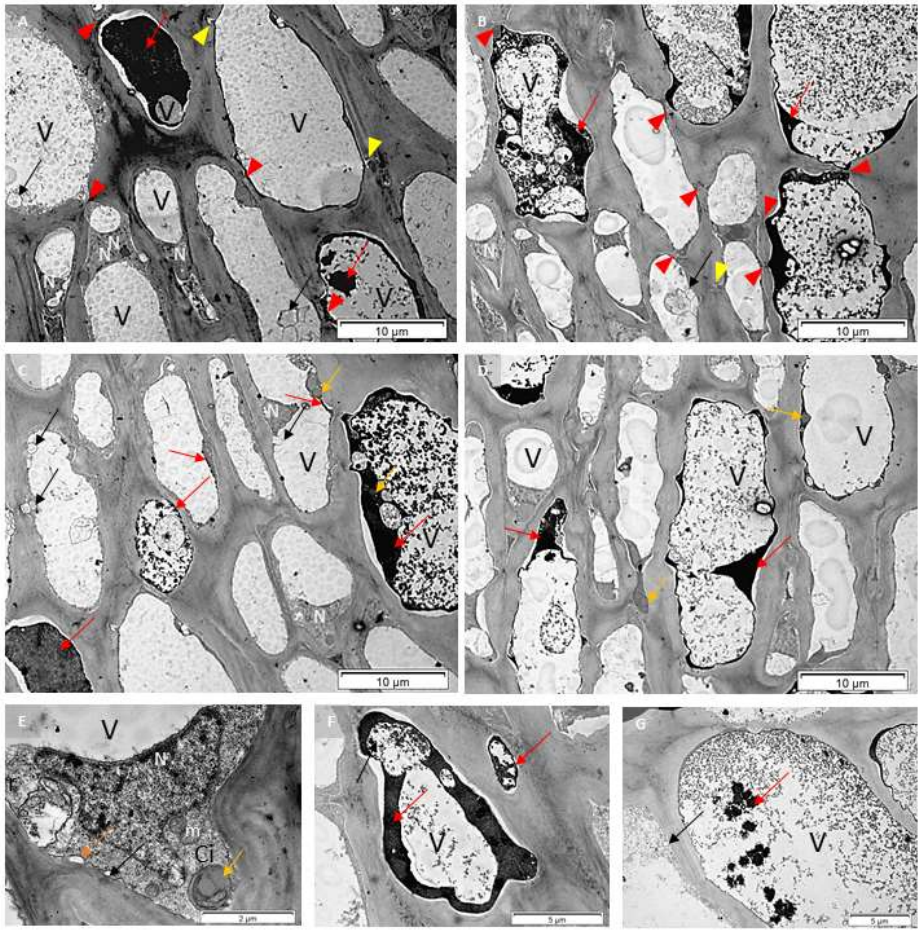


Plate 6.18. A-D – Periphloemic sclerenchyma sector in the rhizome of *Geranium robertianum* L.; E-F – ultrastructural details of sclerenchymatic cells. V – vacuole, N – nucleus, m – mitochondria, Ci – cytoplasm, r – ribosomes, orange arrow – rough endoplasmic reticulum, red arrow – electron-dense deposit, black arrow – vesicles, yellow arrow – lipid droplets, red arrowhead – plasmodesmata, yellow arrowhead – pit communicating with the apoplast.

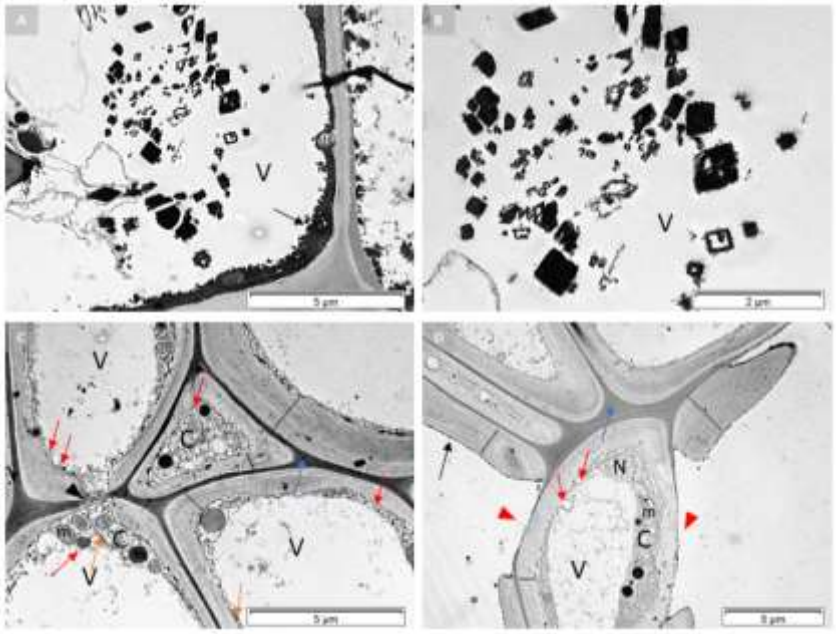


Plate 6.20. A-B – A section of the woody parenchyma of the rhizome of *Geranium robertianum* L. showing intracellular electron-dense deposits and intravacuolar crystals; C–D – a section of xylem and woody fibers. V – vacuole, C – cytoplasm, N – nucleus, m – mitochondria, black bar – cell wall, blue arrow – middle lamella, orange arrow – rough endoplasmic reticulum, red arrow – vesicles, black arrow – electron-dense deposits, red arrowhead – bordered pit on the xylem side, black arrowhead – plasmodesmata.

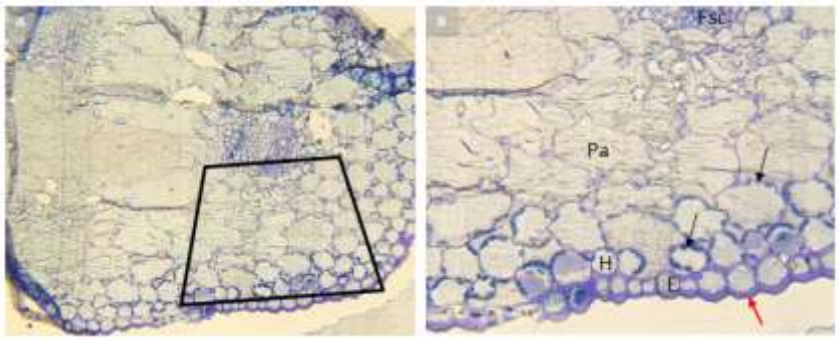


Plate 6.21. A – Semi-thin cross-section through the aerial stem of *Geranium robertianum* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of the analyzed marginal sector, 40X. E – epidermis, H – hypodermis, Pa – cortical parenchyma, Fsc – vascular bundle, red arrow – cuticle, black arrow – chloroplasts.

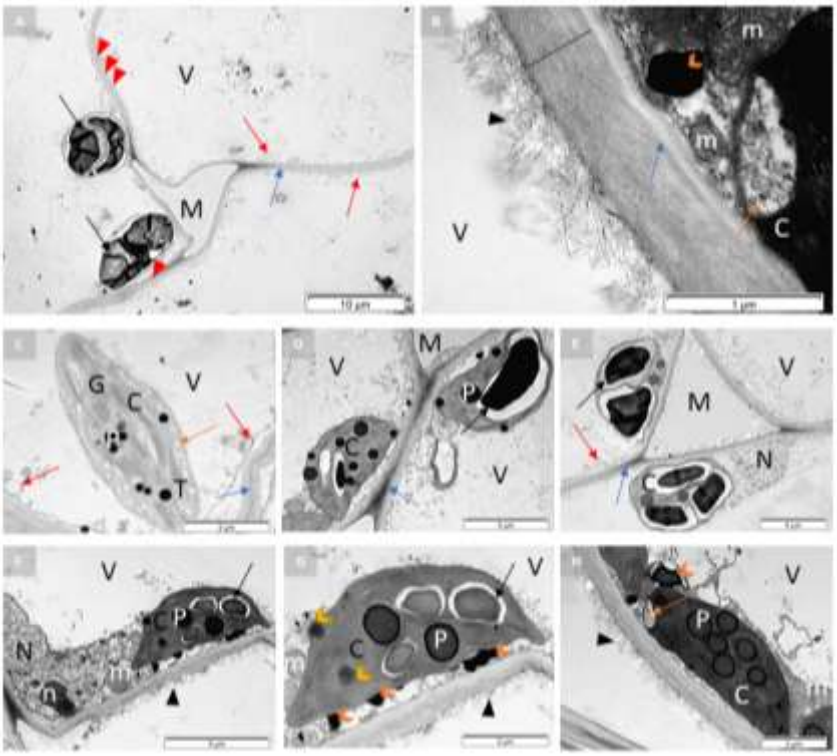


Plate 6.22. A-H Ultrastructural features of the cortical parenchyma cells of the aerial stem in *Geranium robertianum* L.; A – section of parenchyma cells with starch deposits; B – detail from Figure H showing acicular deposits; C–H – details of the ultrastructure of plastids and the nucleus. V – vacuole, M – intercellular space, C – chloroplast, m – mitochondria, G – grana, T – thylakoid, P – plastoglobule, N – nucleus, n – nucleolus, black arrow – starch granules, blue arrow – middle lamella, red arrow – transport vesicles, orange arrow – vesicles in the chloroplast stroma, black arrowhead – acicular deposits, black bar – cell wall, yellow chevron arrow – electron-dense structures in the chloroplast associated with membrane rupture, orange chevron arrow – intracellular electron-dense structures, red arrowhead – plasmodesmata.

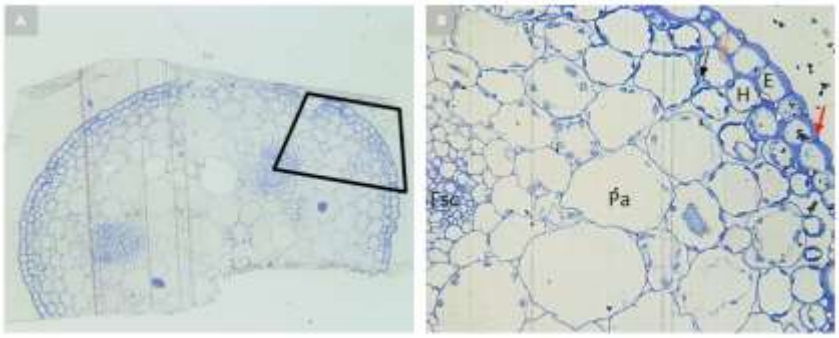


Plate 6.24. A – Semi-thin cross-section through the petiole of *Geranium robertianum* L. showing the area selected for TEM analysis (trapezoidal area), 10X. B – detail of the analyzed marginal sector, 40X. E – epidermis, H – hypodermis, Pa – fundamental parenchyma, Fsc – vascular bundle, red arrow – cuticle, black arrow – chloroplasts, orange arrow – nucleus.

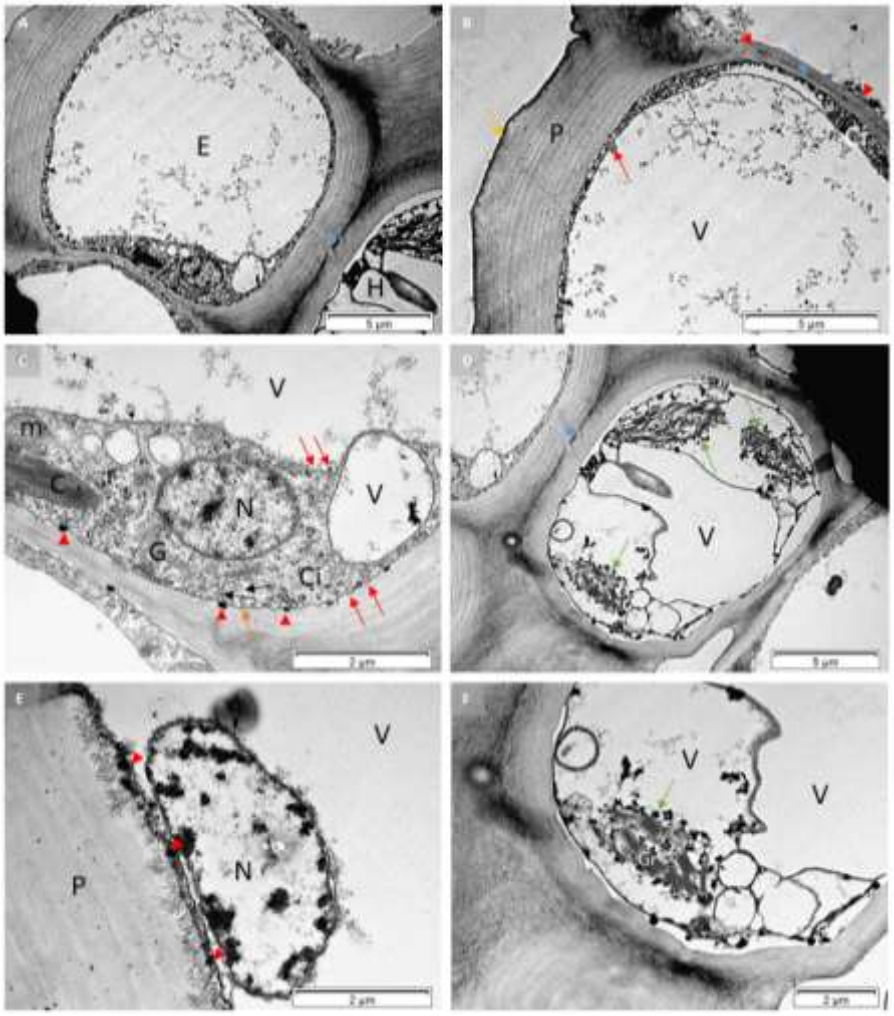


Plate 6.25. A-C, E – Ultrastructural features of the epidermal cells of the petiole in *Geranium robertianum* L.; D – hypodermal cell, with a detail shown in Figure F; B and C represent details from Figure A. E – epidermis, P – outer cell wall, V – vacuole, N – nucleus, Ci – cytoplasm, G – Golgi apparatus, r – ribosomes, m – mitochondria, C – chloroplast, Gr – grana, yellow arrow – cuticle, blue arrow – middle lamella, red arrow – transport vesicles, black arrow – Golgi vesicles, green arrow – partially degraded chloroplast, red arrowhead – electron-dense deposits.

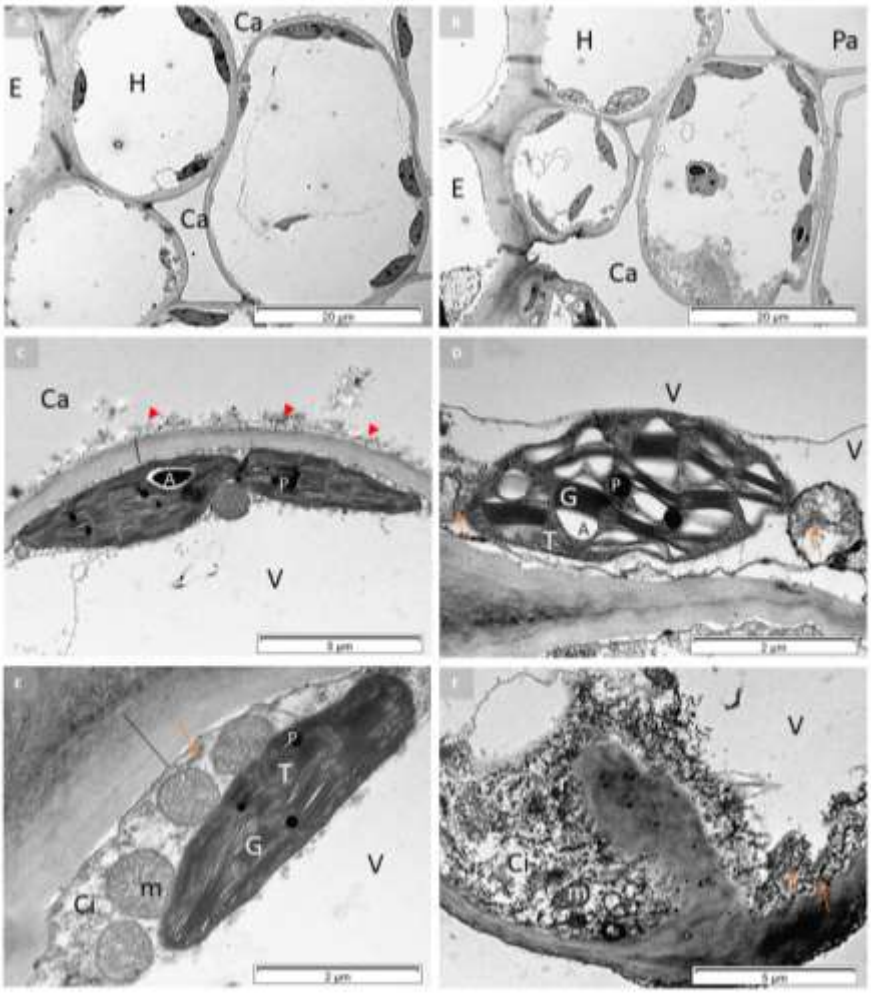


Plate 6.26. A-F – Ultrastructural features of cells in the hypodermis and fundamental parenchyma of the petiole in *Geranium robertianum* L.; C and E show details from Figure A (hypodermis); D and F show details from Figure B (outer layer of the fundamental parenchyma). E – epidermis, H – hypodermis, Ca – air chamber, Pa – parenchyma, V – vacuole, A – starch, P – plastoglobule, T – thylakoid, G – grana, m – mitochondria, Ci – cytoplasm, black bar – cell wall, orange arrow – rough endoplasmic reticulum, red arrowhead – acicular electron-dense deposits.

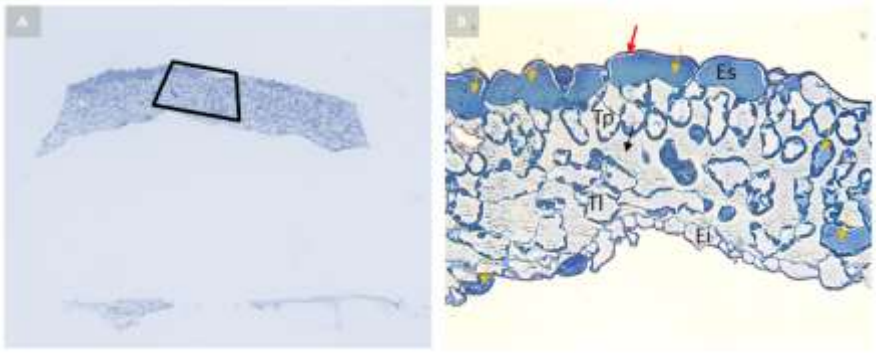


Plate 6.27. A – Semi-thin cross-section through the foliar limb of *Geranium robertianum* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of the analyzed mesophyll sector, 40X. Es – upper epidermis, Ei – lower epidermis, Tp – palisade tissue, Tl – spongy tissue, red arrow – cuticle, black arrow – air chamber, yellow arrow – intravacuolar deposits.

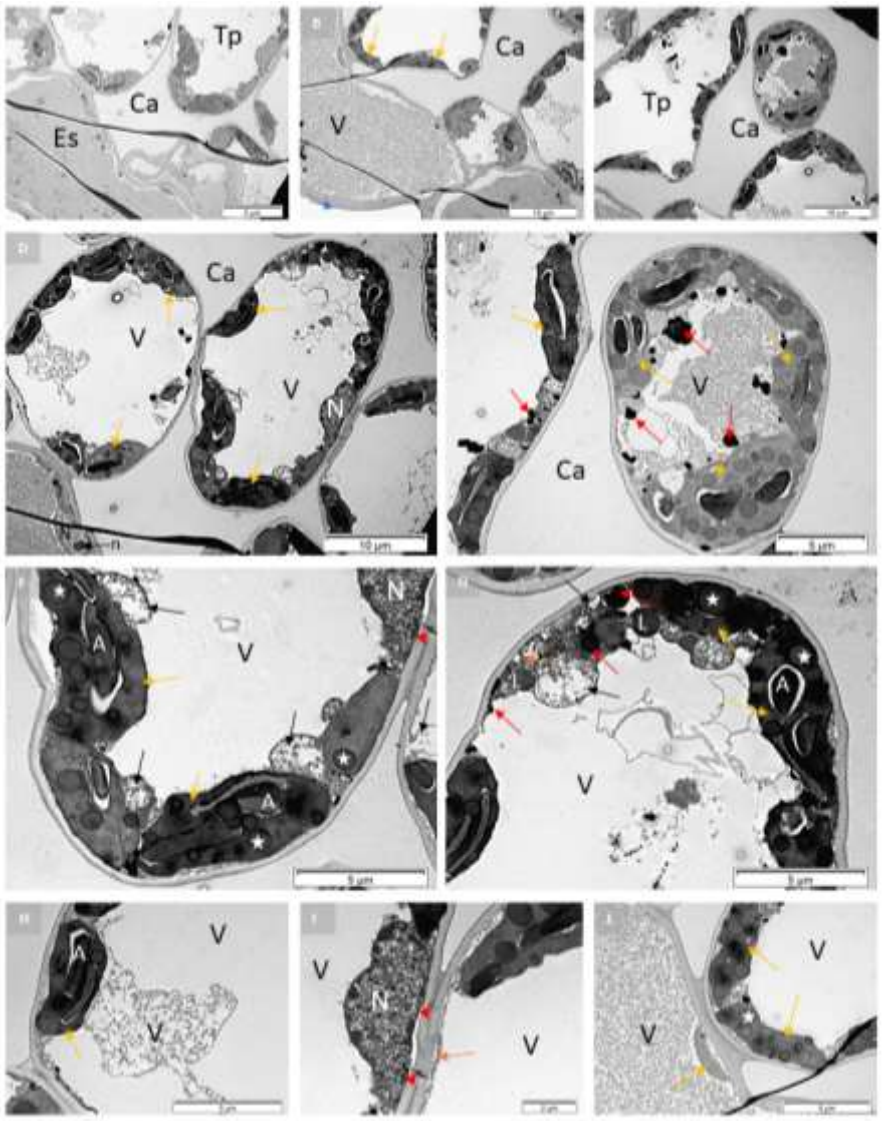


Plate 6.28. A-J – Ultrastructural features of the upper epidermis and palisade tissue of the foliar limb in *Geranium robertianum* L.; D – detail from Figure A; F–I – details from Figure D; E – detail from Figure C; J – detail from Figure B. Es – upper epidermis, Ca – air chamber, Tp – palisade tissue, V – vacuole, N – nucleus, n – nucleolus, L – lysosome, A – starch, yellow arrow – chloroplast, red arrow – electron-dense deposit, black arrow – vesicle, blue arrow – cuticle, orange arrow – rough endoplasmic reticulum, white star – lipid droplet, red arrowhead – plasmodesma.

Results obtained following phytochemical analysis of extracts obtained from *Geranium robertianum* L. plants belonging to the two populations collected from the wild flora of the two geographic regions in Romania (lowland region—Macea; mountain region—Vatra Dornei):

The LC/MS analysis of whole-plant extracts of *Geranium robertianum* L. allowed the identification of 14 polyphenols in the hydroalcoholic extracts obtained from plants collected from Macea and Vatra Dornei (Table 6.1), 12 polyphenols in the infusion from plants collected from Macea, and 7 in the infusion from plants collected from Vatra Dornei (Table

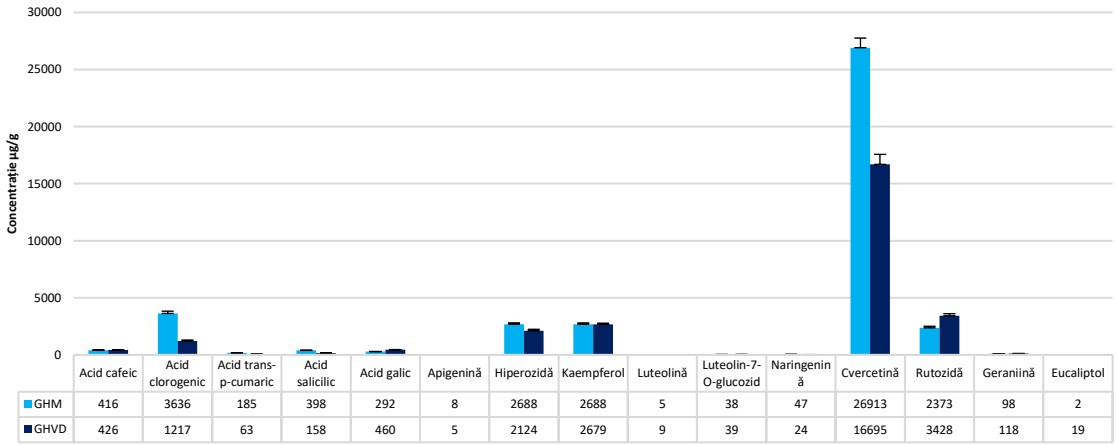
6.2). The GC/MS analysis revealed the presence of eucalyptol in all the *G. robertianum* L. extracts obtained, with higher concentrations found in plant material from the mountain region. For this species, the presence of eucalyptol, salicylic acid, apigenin, luteolin-7-O-glucoside, and trans-p-coumaric acid was identified for the first time. Significantly higher amounts of chlorogenic acid, quercetin, trans-p-coumaric acid, salicylic acid, and naringenin were observed in the hydroalcoholic extract from plants collected from Macea, while higher amounts of gallic acid, rutoside, and geraniin were found in the extract from plants collected from Vatra Dornei. With the exception of eucalyptol, higher levels of polyphenols were observed in the plant material collected from Macea compared to that from Vatra Dornei. However, all identified polyphenols are present in much higher quantities in the hydroalcoholic extracts than in the aqueous ones. The main compound in the analyzed extracts was quercetin, present in higher quantities in the plant material collected from the plains region (Graf. 6.1.-6.2.).

Table 6.1.

Qualitative and quantitative analysis of polyphenols in hydroalcoholic extracts of *Geranium robertianum* L. by LC/MS and GC/MS

No.	Compounds	Sample GHM [µg/g dry plant weight]	Sample GHVD [µg/g dry plant weight]
1	Caffeic acid	416 ± 10.4	426 ± 10.2
2	Chlorogenic acid	3636 ± 197.5	1217 ± 87.2
3	Trans-p-coumaric acid	185 ± 3.8	63 ± 2.4
4	Salicylic acid	398 ± 10.1	158 ± 3.5
5	Gallic acid	292 ± 8.6	460 ± 10.6
6	Apigenin	8 ± 0.1	5 ± 0.1
7	Hyperoside	2728 ± 117.2	2124 ± 121.2
8	Kaempferol	2688 ± 108.9	2679 ± 101.9
9	Luteolin	5 ± 0.1	9 ± 0.1
10	Luteolin-7-O-glucoside	38 ± 0.5	39 ± 0.5
11	Naringenin	47 ± 0.7	24 ± 0.5
12	Quercetin	26913 ± 847.2	16695 ± 875.2
13	Rutoside	2373 ± 134.5	3428 ± 179.4
14	Geraniin	98 ± 3.1	118 ± 3.7
15	Eucalyptol (GC/MS)	2 ± 0.1	19 ± 0.5

Abbreviations: Sample GHM = hydroalcoholic extract, sample collection site in Macea; Sample GHVD = hydroalcoholic extract, sample collection site in Vatra Dornei.



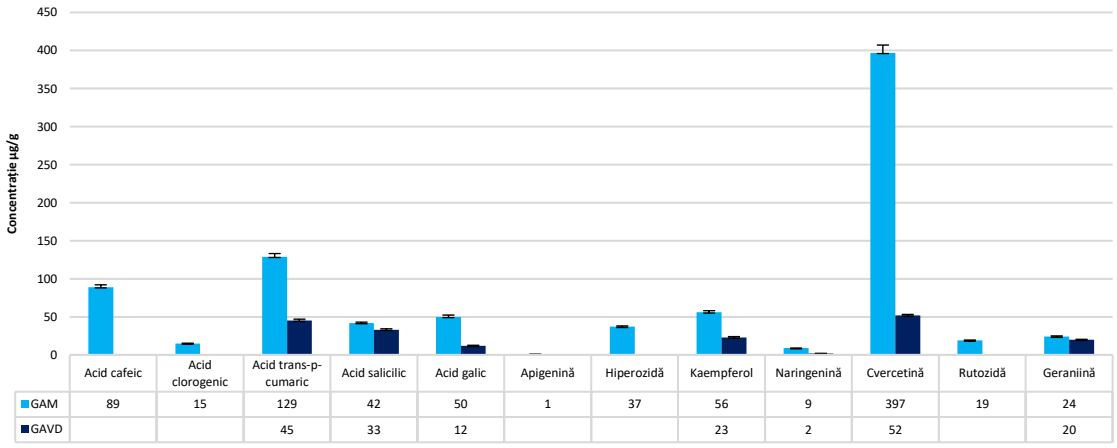
Graf. 6.1. LC/MS analysis of the hydroalcoholic extract obtained from whole *Geranium robertianum* L. plants belonging to the two populations studied—from Macea (GHM) and from Vatra Dornei (GHVD).

Table 6.2.

Qualitative and quantitative analysis of polyphenols in aqueous extracts of *Geranium robertianum* L. by LC/MS and GC/MS

No.	Compounds	Sample GAM [µg/g dry plant weight]	Sample GAVD [µg/g dry plant weight]
1	Caffeic acid	89 ± 3.1	<QL
2	Chlorogenic acid	15 ± 0.4	<QL
3	Trans-p-coumaric acid	129 ± 4.2	45 ± 2.0
4	Salicylic acid	42 ± 1.1	33 ± 1.3
5	Gallic acid	50 ± 2.4	12 ± 0.7
6	Apigenin	1 ± 0.1	<QL
7	Hyperoside	37 ± 1.0	<QL
8	Kaempferol	56 ± 2.1	23 ± 1.1
9	Luteolin	<QL	<QL
10	Luteolin-7-O-glucoside	<QL	<QL
11	Naringenin	9 ± 0.1	2 ± 0.1
12	Quercetin	397 ± 10.2	52 ± 1.2
13	Rutoside	19 ± 0.6	<QL
14	Geraniin	24 ± 0.9	20 ± 0.5
15	Eucalyptol (GC/MS)	3 ± 0.1	14 ± 0.3

Abbreviations: Sample GAM = aqueous extract, sample collection site in Macea; Sample GAVD = aqueous extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.



	Acid cafeic	Acid clorogenic	Acid trans-p-cumaric	Acid salicilic	Acid galic	Apigenină	Hiperozidă	Kaempferol	Naringenină	Cvercetină	Rutozidă	Geraniină
GAM	89	15	129	42	50	1	37	56	9	397	19	24
GAVD			45	33	12			23	2	52		20

Graf. 6.2. LC/MS analysis of the aqueous extract obtained from whole *Geranium robertianum* L. plants belonging to the two populations studied—from Macea (GAM) and from Vatra Dornei (GAVD).

6.5. Comparative analysis of the polyphenol content in extracts of Geranium robertianum L. species in the context of environmental factors

In the case of the *Geranium robertianum* L. species, as cited in subsection 2.2, the plants exhibit a remarkable ability to adapt to a wide range of environmental conditions; their morpho-anatomical and biochemical variability, induced by environmental factors, is well known, extending to the possibility of the existence of ecotypes. The reviewed literature presents aspects related to the genetic variability and conservation of certain morphological traits, along with limited data on biochemical variability.

Taking into account the species' wide ecological range, particularly with regard to climatic factors, the plants' requirements were met at both the lowland and mountain collection sites. As discussed in section 2.2.4, the species is somewhat sensitive to light conditions, but the plants at both collection sites were located in permanent shade provided by the tree canopy and surrounding vegetation, exhibiting well-developed biomass. The pH level corresponds exactly to the species' requirements at both collection sites; however, the nitrogen level can be considered suboptimal in the mountain area.

The collection site for *G. robertianum* L. plants in the lowland area is located at a low elevation (99 m), within a temperate-continental climate with oceanic influences, where, in the year the plants were collected for phytochemical analysis (2021), the average annual temperature ranged between 11.7 and 12.2°C, according to data from nearby weather stations. The average January temperature was above freezing in 2021, ranging from 1.1 to 2.2°C, in contrast to 2019–2020, when it was below freezing point. The average temperature for July, ranging from 24.9 to 25.6°C in 2021, confirms the general warming trend throughout the ontogenetic development of plants. The maximum temperatures recorded

during the sampling year ranged from 36.8 to 38.5°C, while the minimum temperatures were -12.8 and -12.4°C. At the Macea sampling site, in 2021, cumulative precipitation ranged from 395.3 to 578 mm, and the number of frost-free days ranged from 274 to 294. The soil at the collection site was characterized by a slightly acidic to neutral pH (6.72), a very high amount of assimilable phosphorus (224.99 ppm), a high amount of assimilable potassium (626 ppm), and a moderate total nitrogen content (2.56%).

Under these conditions, 15 bioactive compounds were identified in the hydroalcoholic extract (14 polyphenols and the monoterpene eucalyptol), and 13 in the aqueous extract (12 polyphenols and eucalyptol); in the latter, luteolin and luteolin-7-O-glucoside were below the quantification limit. In both types of extracts, quercetin stands out as the quantitatively predominant compound compared to other compounds in the same extract. All identified polyphenols are present in much higher quantities in the hydroalcoholic extract compared to the aqueous extract, the difference in eucalyptol content being only 1 μg/g dry plant.

The collection site for *G. robertianum* L. plants in the mountain region is located at an altitude of 863 m, characterized by a cold continental climate with Scandinavian-Baltic influences, where, in the year the plants were collected for phytochemical analysis (2021), the average annual temperature ranged from 0.7 to 9.8°C. In the sampling year (2021), the average temperature in January was above freezing, ranging from 7.7 to 0.2°C, while the average temperature in July ranged from 12.2 to 22.3°C. At the collection site, the maximum temperature in 2021 did not exceed 33.3°C, and the minimum temperatures did not fall below -25.9°C, reaching as high as 15.4°C, depending on the nearby weather station. The collection site for the analyzed plants in the mountain area is characterized by heavy precipitation, with cumulative values in 2021 ranging from 757.4 to 1,528.1 mm. The number of days without ground frost in 2021 ranged from 166 to 252. Soil conditions at the plant collection site included a slightly acidic pH (6.03), extremely low levels of assimilable phosphorus (0.55 ppm), moderate levels of assimilable potassium (148 ppm), and low levels of total nitrogen (0.25%).

In the context of the environmental factors analyzed in the mountain region, it was observed that, similar to the extracts from the lowland region, the hydroalcoholic extract contains a higher number of polyphenols (14) than the aqueous extract (7). The monoterpene eucalyptol is also present in the extracts obtained from plants in the mountain region, in greater quantities than in those obtained from plants in the lowland region (14–19 μg/g dry plant weight, depending on the type of extract, compared to 2–3 μg/g dry plant weight). The polyphenols present in the hydroalcoholic extract but below the quantification limit in the

infusion prepared from plants from the mountain region are caffeic acid, chlorogenic acid, apigenin, hyperoside, rutoside, luteolin, and luteolin-7-O-glucoside. The main compound continues to be quercetin, but in much smaller quantities than in plants from the lowland area (10218 μg/g of dry plant weight less in the hydroalcoholic extracts, and 345 μg/g of dry plant weight less in the aqueous extracts). With regard to kaempferol content, the differences are small compared to lowland plants in hydroalcoholic extracts; a similar pattern is observed for the content in caffeic acid, apigenin, luteolin, and luteolin-7-O-glucoside (differences ranging from 1 to 10 μg/g dry plant weight). These differences are more accentuated in aqueous extracts, where kaempferol is present in less than half the amount observed in infusion prepared from the lowland plants. Naringenin, another reference compound for environmental-condition-induced variability (according to the scientific literature), is present in lower amounts in the extracts obtained from *G. robertianum* plants growing in the mountain region. Geraniin, a characteristic ellagitannin, was found in higher quantities in the hydroalcoholic extract from plants growing in the mountain region and in amounts up to 6 times lower in infusions, regardless of origin.

CHAPTER 7
MORPHO-ANATOMICAL AND PHYTOCHEMICAL RESEARCH OF *LEONURUS CARDIACA* L. SPECIES

7.6. Considerations regarding the morpho-anatomical characteristics and phytochemical composition of the vegetative organs of Leonurus cardiaca L. species

Morpho-anatomical results obtained for *Leonurus cardiaca* L. species:

– Following the analysis of optical microscopy and SEM images, we have obtained a first description of the anatomical structure of the adventitious root, rhizome, and petiole in this species, as well as the first detailed characterization of the aerial stem and foliar limb; following the analysis of TEM images, a first description of the ultrastructure of its vegetative organs was obtained;

– The surface of both underground organs is covered with mucilage and small crystals; the rhizome features cork, lenticels, and a more developed central cylinder, with secondary phloem and xylem organized into distinct layers; rare intracellular deposits in the cortical parenchyma and in the outer layers of the secondary phloem indicate deposits of secondary metabolites whose significance is currently uncertain* (Plates 7.1-7.3, 7.11);

– The stem retains its general structure along its entire length and is characterized by: a square-rhombic cross-section, 4 large ribs with monticules filled with angular collenchyma and bearing non-glandular trichomes at their tips, some of which are inserted into a cup formed by two cells and have protuberances on the surface of the cell wall, 4 attenuated intermediate ribs, a crenellated cuticle, several types of glandular trichomes (7)—sessile, short- and long-stalked, with uni-, bi-, and pluricellular glands, more frequent in the upper part, and a medullary lacunae that gradually disappears toward the stem apex (Plates 7.4-7.6, 7.12);

– The surface of the petiole bears non-glandular trichomes with bent tips, as well as sessile and short-stalked glandular trichomes, with 1–2 to 4 secretory cells; According to SEM images, the non-glandular trichomes are bicellular, uniseriate, with a slightly conical base and a long, sharp tip, with the boundary between the two cells marked by a bulging node (Plates 7.7-7.8, 7.13);

– The foliar limb is hypostomatic, with unicellular non-glandular trichomes that have either verrucose or smooth walls, and glandular trichomes that are more frequent on the lower surface; according to SEM images, on the abaxial surface, the non-glandular trichomes

are arranged along the veins, and their number and size decrease with each order of branching, while the glandular trichomes are present between the meshes of the veins network and on the lower-order veins; on the adaxial surface, the distribution of glandular and non-glandular trichomes is reversed (glandular ones along the veins and non-glandular ones in the meshes of the veins and on the lower-order veins) (Plates 7.9-7.10, 7.14-7.15);

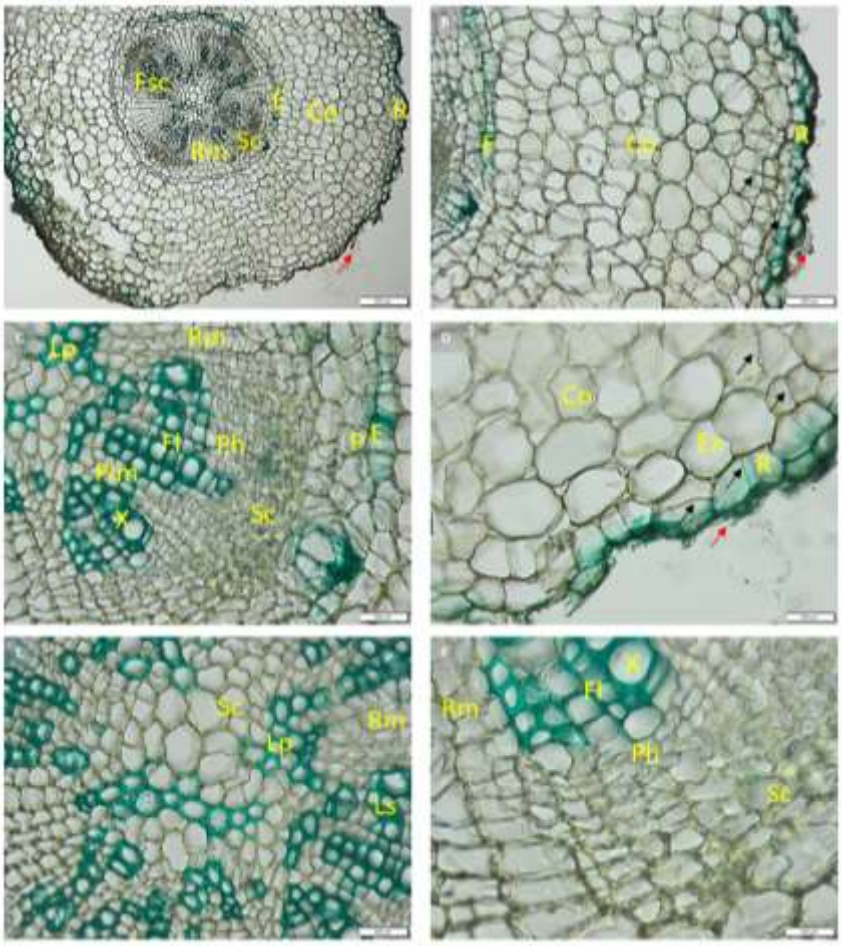


Plate 7.1. A-F Cross-section through the adventitious root of *Leonurus cardiaca* L. R – rhizodermis, Co – cortex, E – endodermis, Sc – sclerenchyma, Rm – medullary rays, Fsc – vascular bundle, Ex – exodermis, P – pericycle, Ph – phloem, X – xylem, Fl – wood fibers, Plm – wood parenchyma, Lp – primary wood, Ls – secondary wood, black arrow – division walls, red arrow – mucilages.

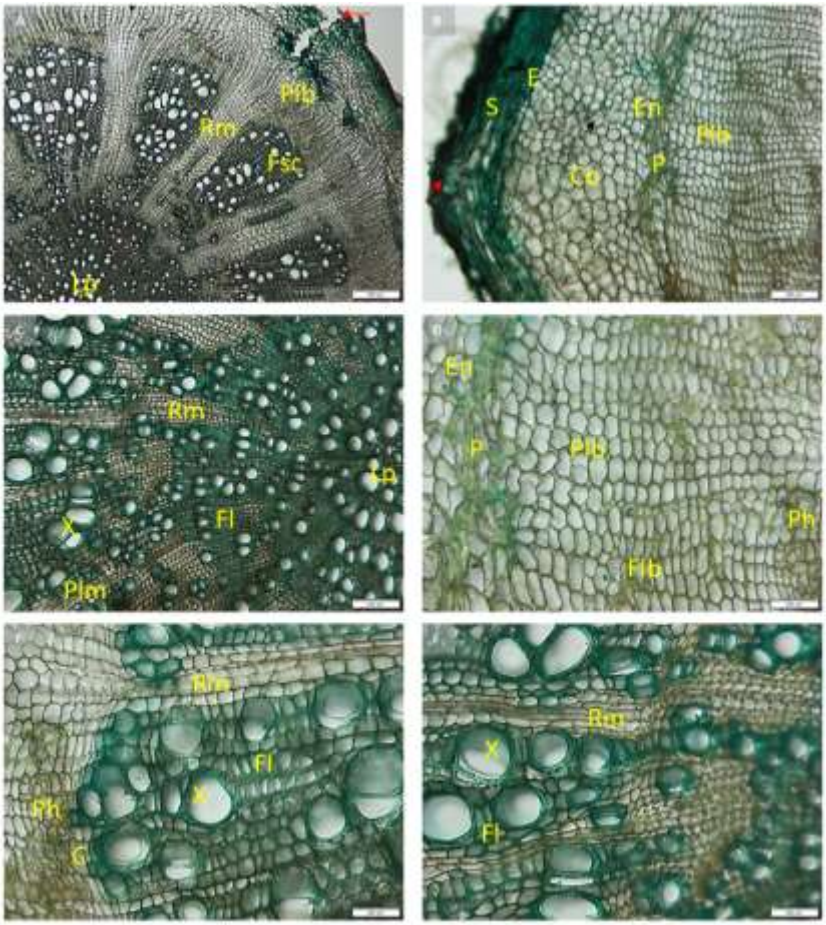


Plate 7.2. A-F Cross-section through the rhizome of *Leonurus cardiaca* L. E – epidermis, S – cork, Co – cortex, En – endodermis, P – sclerenchymatic pericycle, Plb – phloem parenchyma, Flb – phloem fibers, Rm – medullary rays, Fsc – vascular bundle, Lp – primary wood, X – xylem, Fl – wood fibers, Plm – wood parenchyma, Ph – phloem, C – cambium, black arrow – calcium oxalate crystals, red arrow – lenticel.

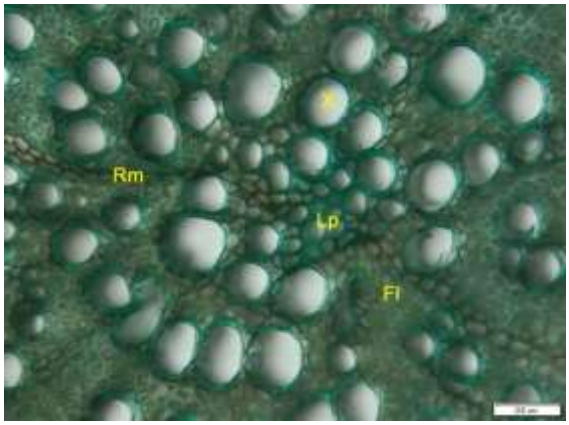


Fig.7.3. Cross-section through the rhizome of *Leonurus cardiaca* L., central region. Lp – primary wood, Fl – wood fibers, Rm – medullary ray.

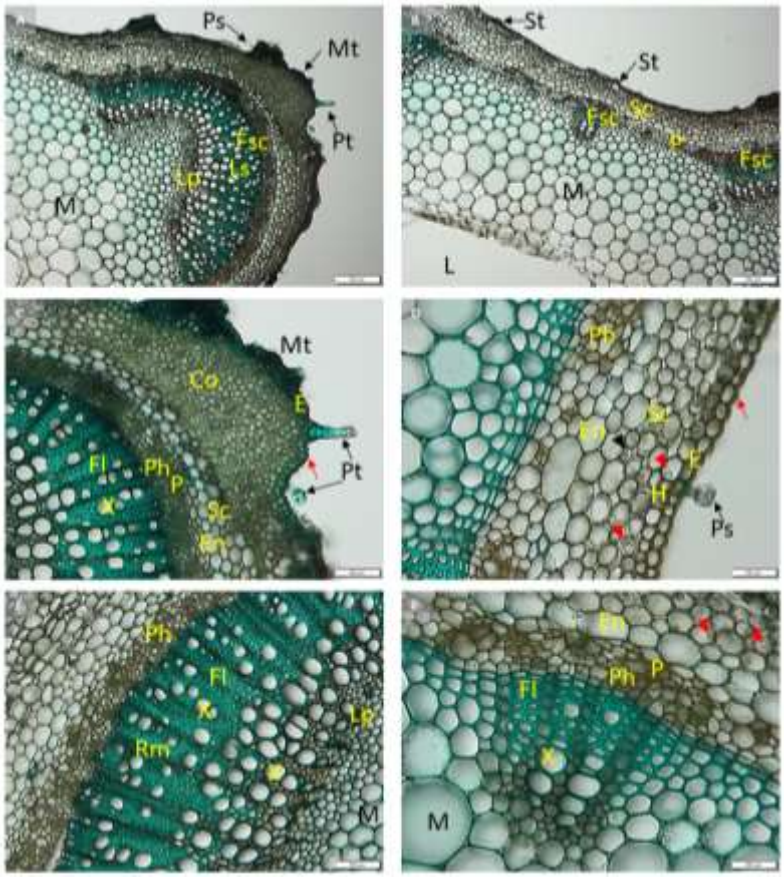


Plate 7.4. A-F Cross-section through the lower part of the stem of *Leonurus cardiaca* L. Mt – monticle, Ps – glandular trichome, Pt – non-glandular trichome, Fsc – vascular bundle, Ls – secondary wood, Lp – primary wood, M – pith, St – stomata, Sc – cortex, P – sclerenchymatic pericycle, L – air chamber, E – epidermis, Co – angular collenchyma, En – endodermis, Ph – phloem, X – xylem, Fl – wood fibers, Rm – medullary ray, H – hypodermis, red arrow – crenate cuticle, black arrowhead – raphides, red arrowhead – inclusions.

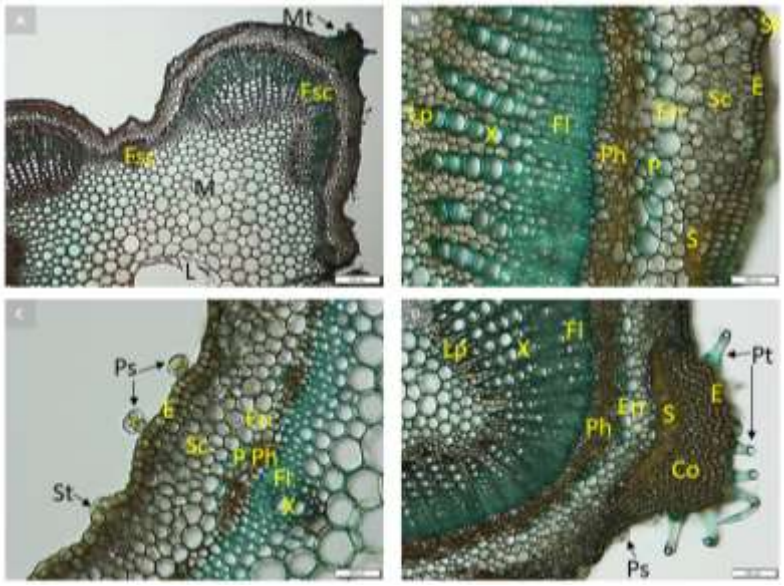


Plate 7.5. A-D Cross-section through the upper part of the stem of *Leonurus cardiaca* L. Mt – monticle, Fsc – vascular bundle, M – pith, L – air chamber, St – stomata, E – epidermis, Sc – cortex, En – endodermis, P – pericycle, Ph – phloem, Fl – wood fibers, X – xylem, Lp – primary wood, Co – angular collenchyma, S – sclerenchyma, Pt – non-glandular trichome, Ps – glandular trichome.

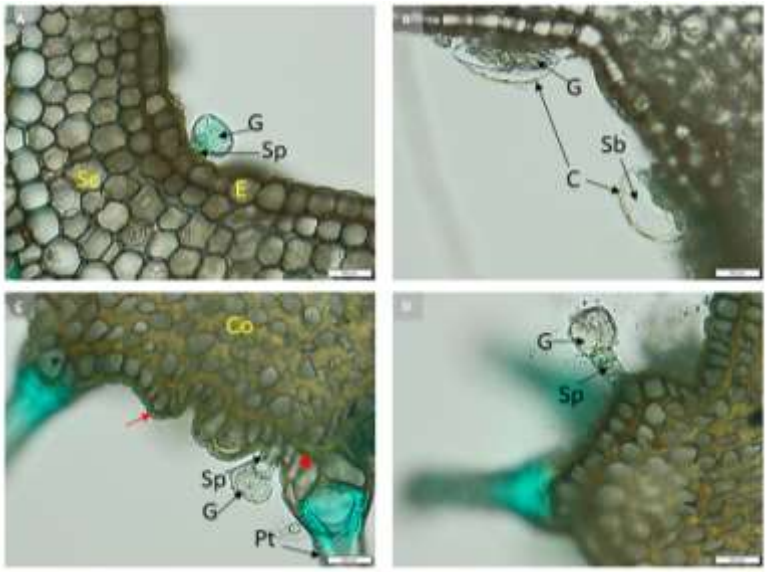


Plate 7.6. A–D Cross-section through the upper part of the stem of *Leonurus cardiaca* L. – details of glandular trichomes (A–D) and non-glandular trichomes (C). G – secretory glands, Sp – stalk cells, C – cuticle, Sb – subcuticular storage space, Pt – non-glandular trichome, E – epidermis, Sc – cortex, Co – angular collenchyma, red arrow – crenate cuticle, red arrowhead – cup-shaped basal cells at the base of non-glandular trichome.

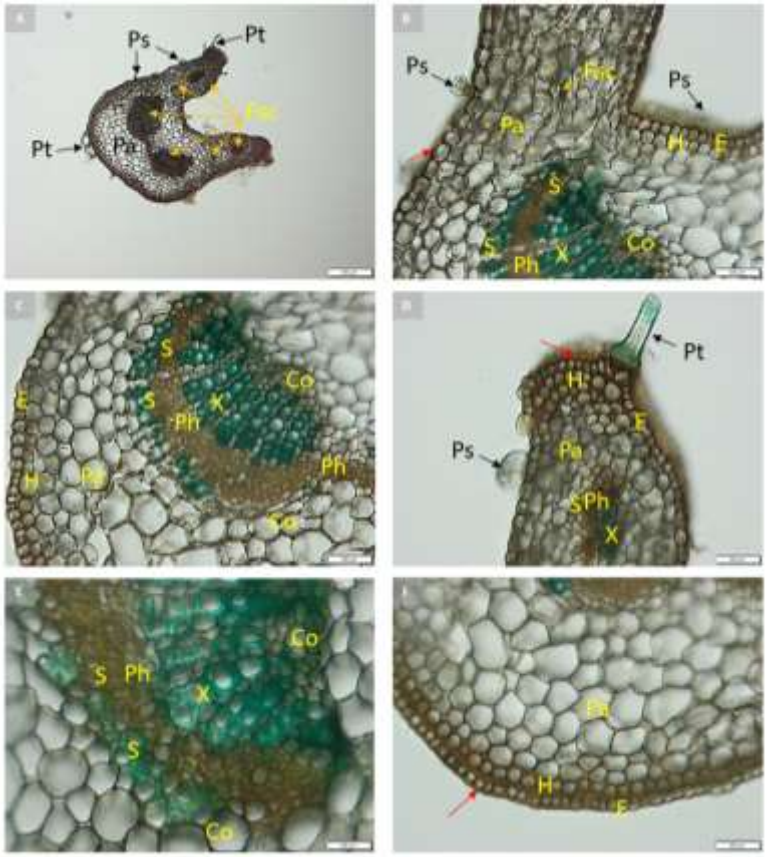


Plate 7.7. A-F Cross-section through the petiole of *Leonurus cardiaca* L.; A – general view; B – section from the base of the lateral cret and the adaxial sulcus; C – detail from the central part; D – detail from the apex of the lateral crest; E – detail of the central vascular bundle; F – section from the abaxial side. Pa – fundamental parenchyma, Fsc – vascular bundle, E – epidermis, H – colenchyma-rich hypodermis, S – sclerenchyma, Co – collenchyma, Ph – phloem, X – xylem, Pt – non-glandular trichome, Ps – glandular trichome, red arrow – cuticle.

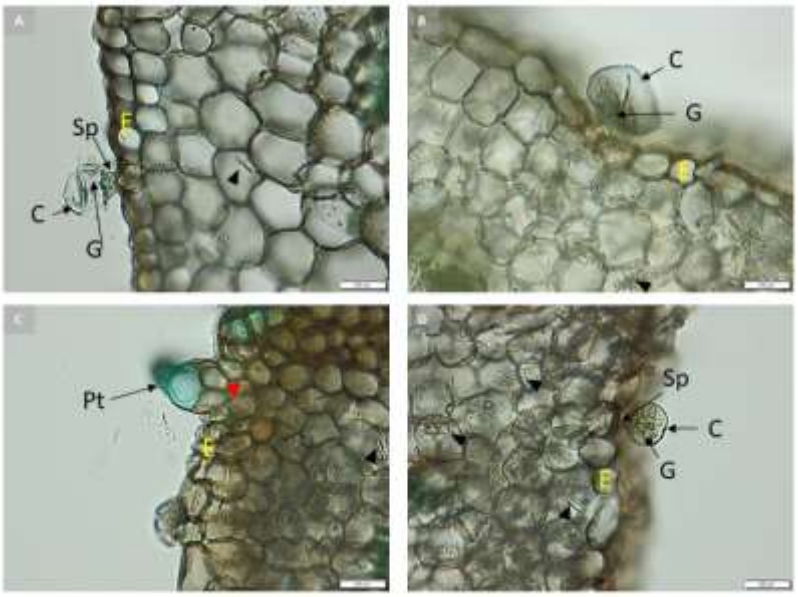


Plate 7.8. A-D Cross-section through the petiole of *Leonurus cardiaca* L., showing details of the trichomes. E – epidermis, Pt – non-glandular trichome, Sp – stalk cells, G – secretory glands, C – cuticle, black arrowhead – crystals, red arrowhead – cup-shaped basal cells at the base of non-glandular trichome.

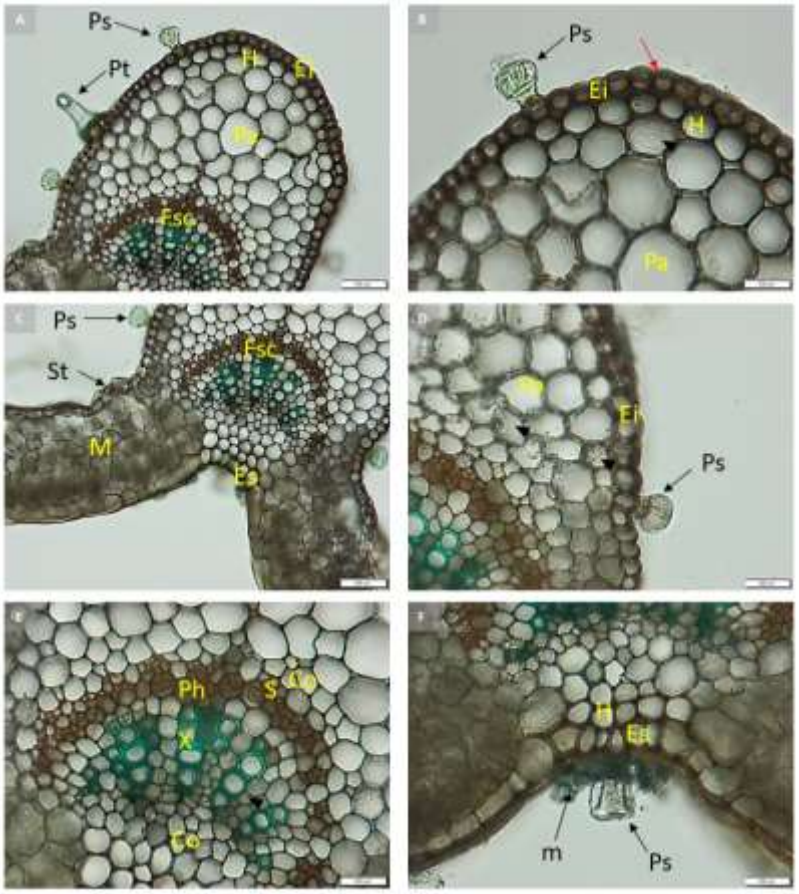


Plate 7.9. A-F Cross-section through the foliar limb of *Leonurus cardiaca* L., showing aspects of the midrib. Ps – glandular trichome, Pt – non-glandular trichome, Fsc – vascular bundle, Ei – lower epidermis, Es – upper epidermis, H – colenchyma-rich hypodermis, Pa – ground parenchyma, S – sclerenchyma, Ph – phloem, X – xylem, Co – collenchyma, m – mucilage, red arrow – crenate cuticle, black arrowhead – crystals.

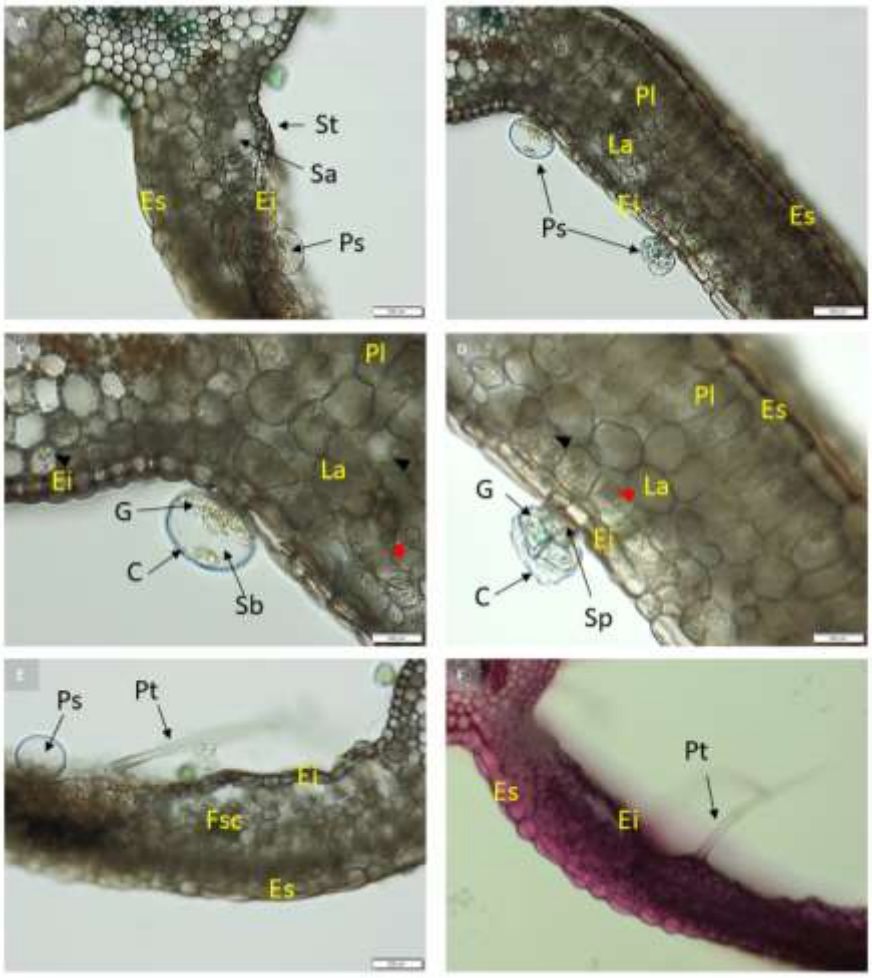


Plate 7.10. A-F Cross-section through the foliar limb of *Leonurus cardiaca* L., showing aspects of the mesophyll and details of the trichomes. Ei – lower epidermis, Es – upper epidermis, Ps – glandular trichome, Pt – non-glandular trichome, Fsc – vascular bundle, G – secretory glands, c – cuticle, Sb – subcuticular space, Sp – stalk cells, St – stomata, Sa – substomatal chamber, black arrowhead – crystals, red arrowhead – spherical structures.

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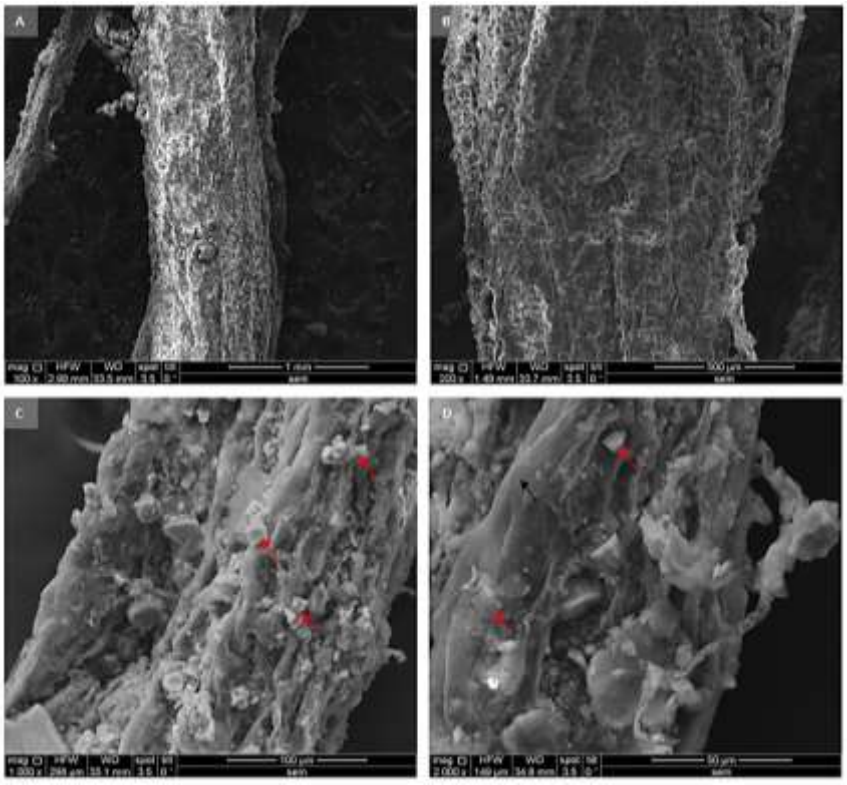


Plate 7.11. A- B Surface microtopography of the rhizome of *Leonurus cardiaca* L.; C–D – surface details. Black arrow – smooth appearance of the epidermal surface; red arrow – crystals.

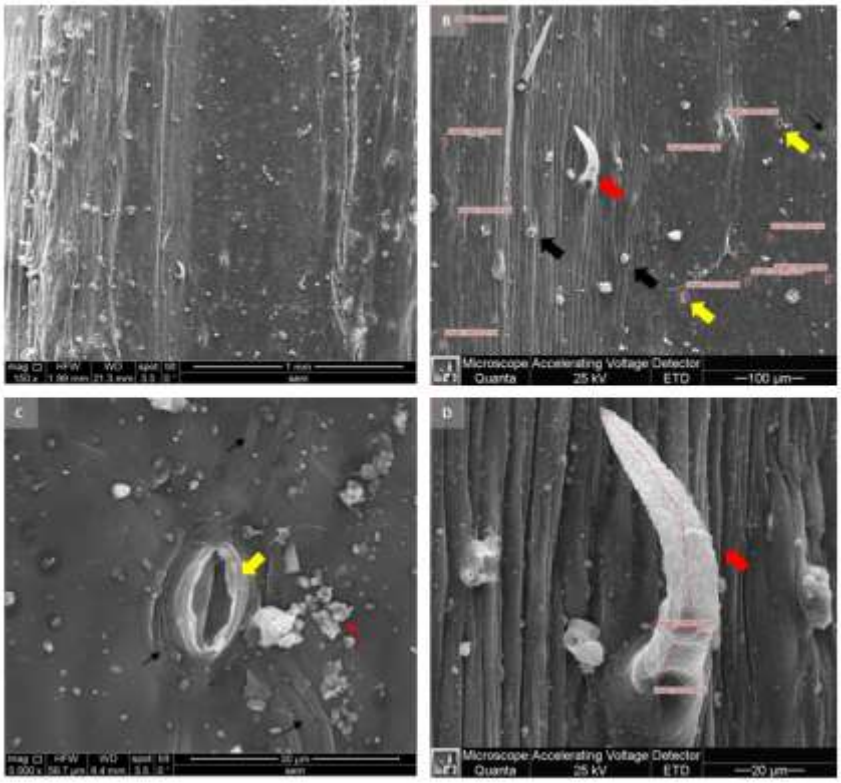


Plate 7.12. A-B Surface microtopography of the stem of *Leonurus cardiaca* L.; C – close-up of a stomata; D – detail of a non-glandular trichome. Yellow arrow – stomata, red (thick) arrow – non-glandular trichome, black (thick) arrow – glandular trichome, black arrow – cuticular folds/striations around the stomata.

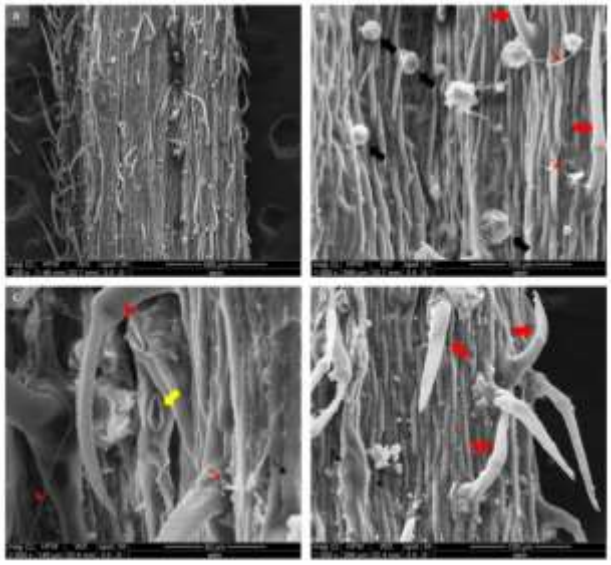


Plate 7.13. A – General view of the surface microtopography of the petiole of *Leonurus cardiaca* L.; B–D – details of the epidermis showing glandular and non-glandular trichomes. Yellow arrow – stomata, red (thick) arrow – non-glandular trichome, black (thick) arrow – glandular trichome, red arrow – mycelial hyphae, black arrow – cuticular striations, red arrowhead – protuberances on the surface of non-glandular trichome.

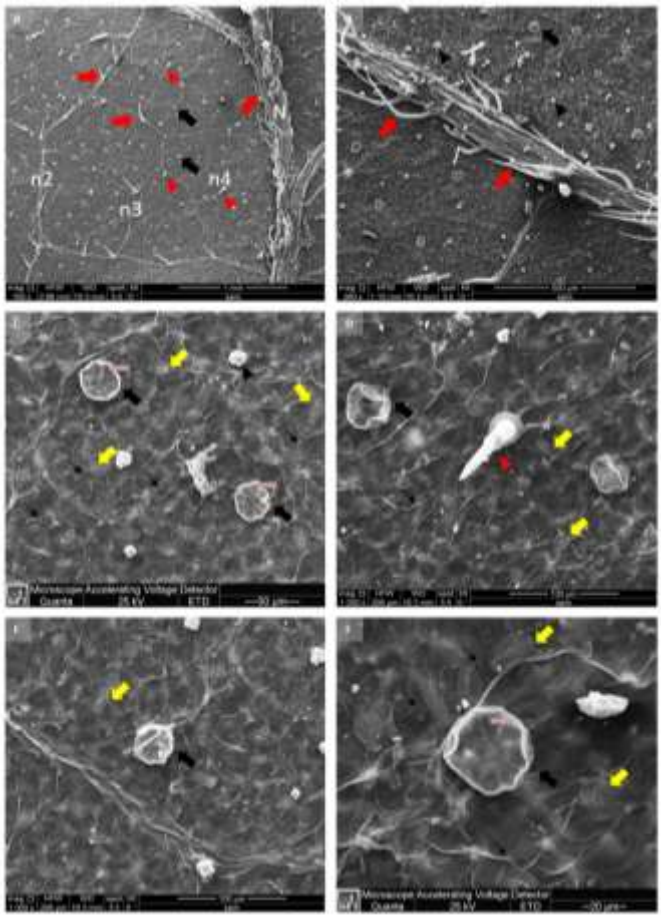


Plate 7.14. A-B Microtopography of the lower epidermis of the foliar limb in *Leonurus cardiaca* L.; C-F – details of the epidermis, glandular and non-glandular trichomes, and stomata. N – midrib, n2 – second-order vein, n3 – third-order vein, n4 – fourth-order vein, red arrowhead – small non-glandular trichome, red arrow – large non-glandular trichome, black (thick) arrow – multiglandular trichome, black arrowhead – uniglandular trichome, yellow arrow – stomata, black arrow – cuticular striations.

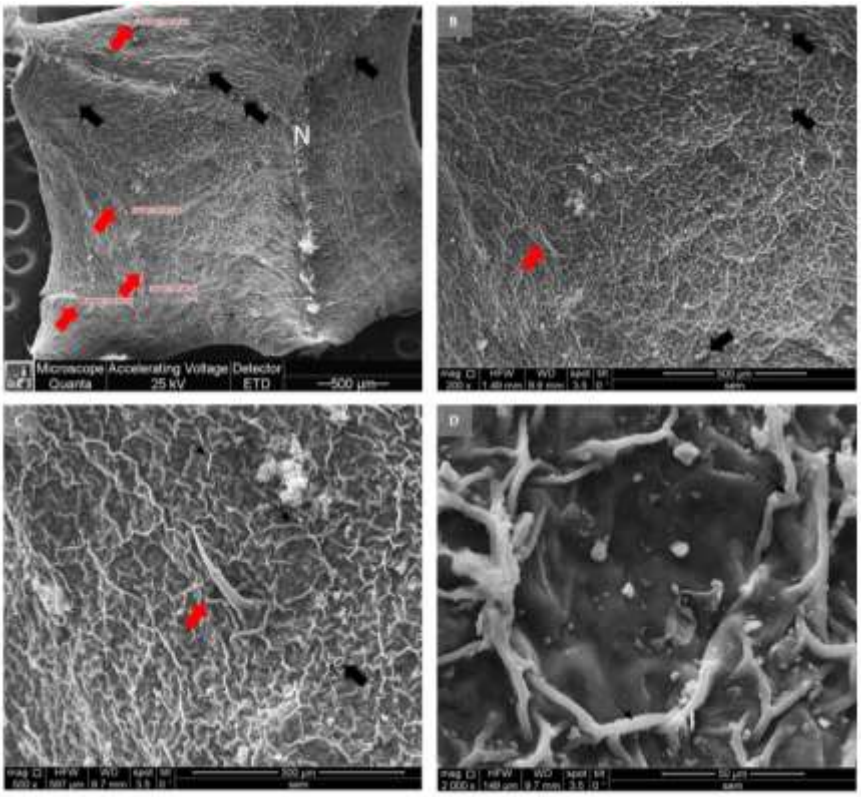


Plate 7.15. A-C Microtopography of the upper epidermis of the foliar limb of *Leonurus cardiaca* L.; D – details of cuticular folds and striations. N – midrib, red arrow – non-glandular trichome, black (thick) arrow – glandular trichome, black arrow – folds.

– At the rhizome level, the TEM images reveal ultrastructural aspects of the cambium as it differentiates into phloem and secondary xylem, electron-dense deposits in the symplast and apoplast, various types of pits and thickenings of the cell walls, and indicate the presence of polyphenolic compounds in the wood fibers (Plates 7.16–7.18). At the aerial stem level, lipid deposits were observed in the cortical cells and acicular deposits in the secondary phloem cells; the spaces in the wood parenchyma, between the primary wall and the middle lamella, suggest a transport process via the apoplast (Plates 7.20–7.23); notable features identified in the cortical cells include multivesicular and multilamellar bodies involved in secretory pathways and autophagy processes (Plate 7.27). Ultrastructural analysis of the petiole reveals the cuticle and the processes involved in its formation, various intravacuolar deposits with crystalline, granular, and fluid appearances in the vascular bundles and the periphloemic sclerenchymatic sheath, as well as vesicular structures in the intercellular space between the phloem and periphloemic tissues (Plates 7.28–7.29, 7.33–7.34). Ultrastructural analysis of the foliar limb indicates processes of synthesis and storage of certain metabolites, particularly lipids and polyphenols, in the spongy parenchyma cells surrounding the vascular bundles (Plates 7.35-7.36, 7.38).

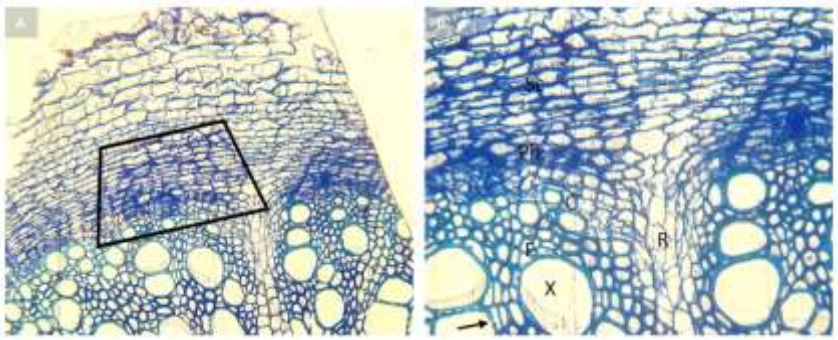


Plate 7.16. A – Semi-thin cross-section through the rhizome of *Leonurus cardiaca* L. showing the TEM analysis area (trapezoidal area) at 20X; B – detail of the central cylinder with medullary rays and a sector of the vascular bundle at 40X. Sc – sclerenchyma (lignified fibers), Ph – phloem, C – cambium, F – wood fibers, X – xylem, R – medullary ray.

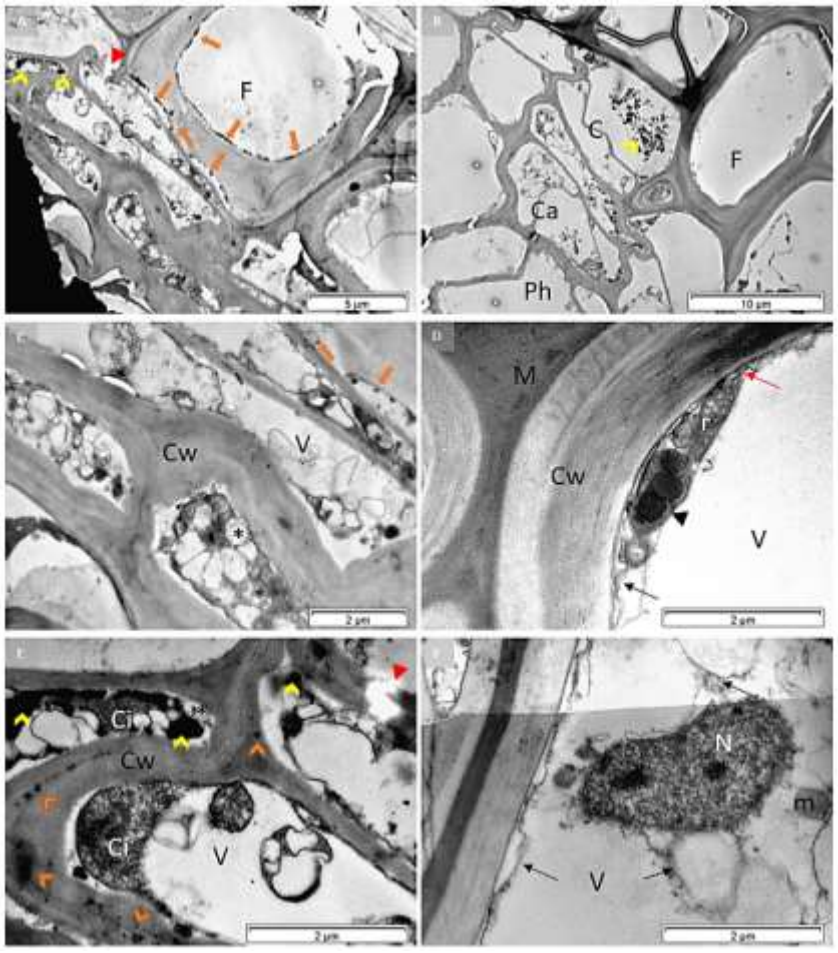


Plate 7.17. A-F Ultrastructural aspects of the secondary phloem, cambium, and wood fibers of the rhizome in *Leonurus cardiaca* L. F – wood fiber, C – cambium, Ca – companion cells, Ph – phloem, Cw – cell wall, V – vacuole, M – intercellular space, Ci – cytoplasm, N – nucleus, m – mitochondria, r – smooth endoplasmic reticulum, red arrowhead – pits, yellow chevron arrow – intracellular electron-dense deposits, orange chevron arrow – electron-dense deposits in the apoplast, orange arrow – electron-dense deposits at the cell wall edge, red arrow – rough endoplasmic reticulum, black arrow – cytoplasmic filament, asterisk – mucilage-containing vesicle, black arrowhead – body with osmophilic deposits.

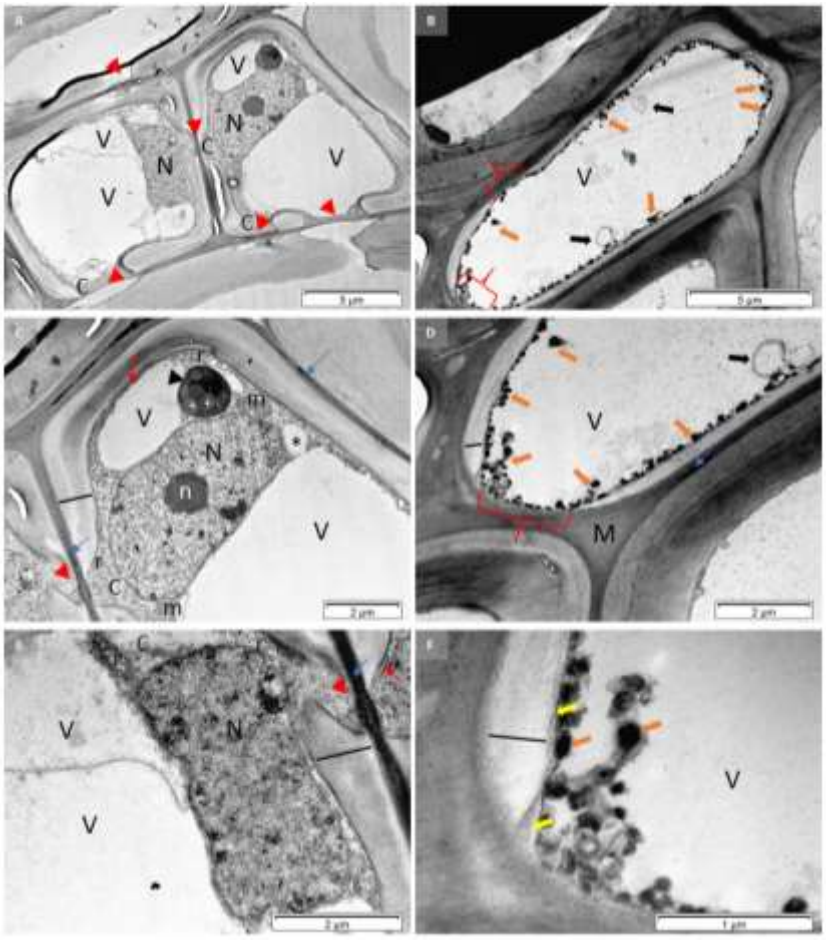


Plate 7.18. A, C, E Ultrastructural aspects of young wood fibers in the rhizome of *Leonurus cardiaca* L. at various stages of differentiation; B, D, F – mature wood fibers. N – nucleus, n – nucleolus, C – cytoplasm, V – vacuole, m – mitochondria, r – smooth endoplasmic reticulum, M – parenchyma, red arrowhead – pits, curly bracket – non-thickened portions of the cell wall, black arrow – intravacuolar vesicles, orange arrow – electron-dense deposits in the symplast, yellow arrow – cell membrane, red arrow – rough endoplasmic reticulum, blue arrow – middle lamella, black arrowhead – structure containing osmophilic deposits and lipid droplets (white star), asterisk – mucilage-containing vesicle, black bar – secondary wall.

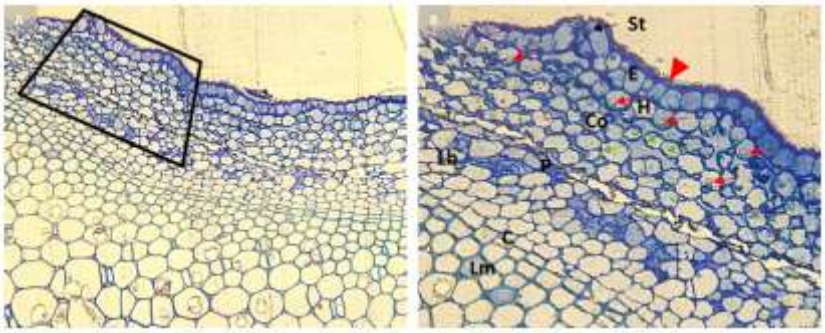


Plate 7.20. A – Semi-thin cross-section through the stem of *Leonurus cardiaca* L. showing the area of interest for TEM (trapezoidal area), 10X; B – detail from the marginal zone, between the ridges, 40X. St – stomata, E – epidermis, H – hypodermis, Co – cortex, P – pericycle, Lb – phloem, C – cambium, Lm – wood, green arrow – chloroplasts, red arrow – translucent deposits, red arrowhead – crenate cuticle.

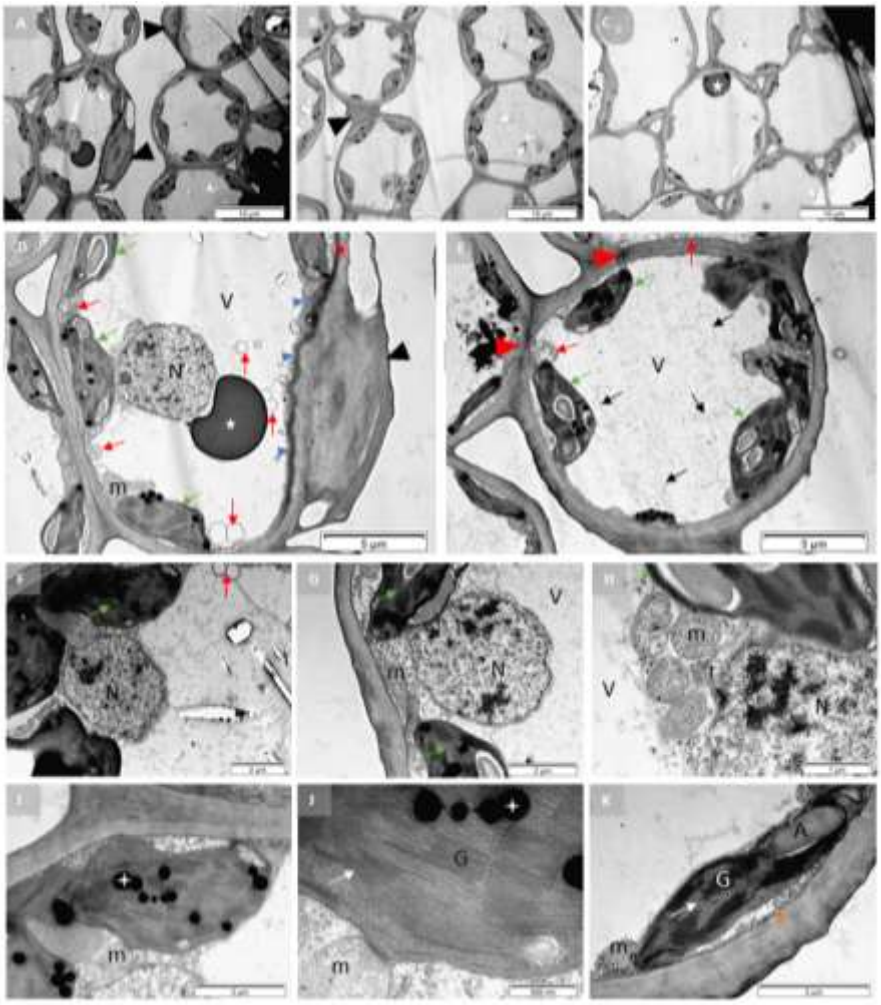


Plate 7.21. A-C – Overview of the cortex ultrastructure in the stem of *Leonurus cardiaca* L.; D–H – ultrastructural details of cortical cells; I–K – details of chloroplasts in cortical cells. N – nucleus, m – mitochondria, V – vacuole, G – grana, A – starch granule, green arrow – chloroplast, white arrow – thylakoids, red arrow – vesicles, red arrowhead – pit, black arrowhead – cell wall protrusions, blue arrowhead – diffuse region of the cytoplasm, orange arrow – endoplasmic reticulum, black arrow – intravacuolar filamentous structures, white star – lipid droplet, white star (4 points) – plastoglobule.

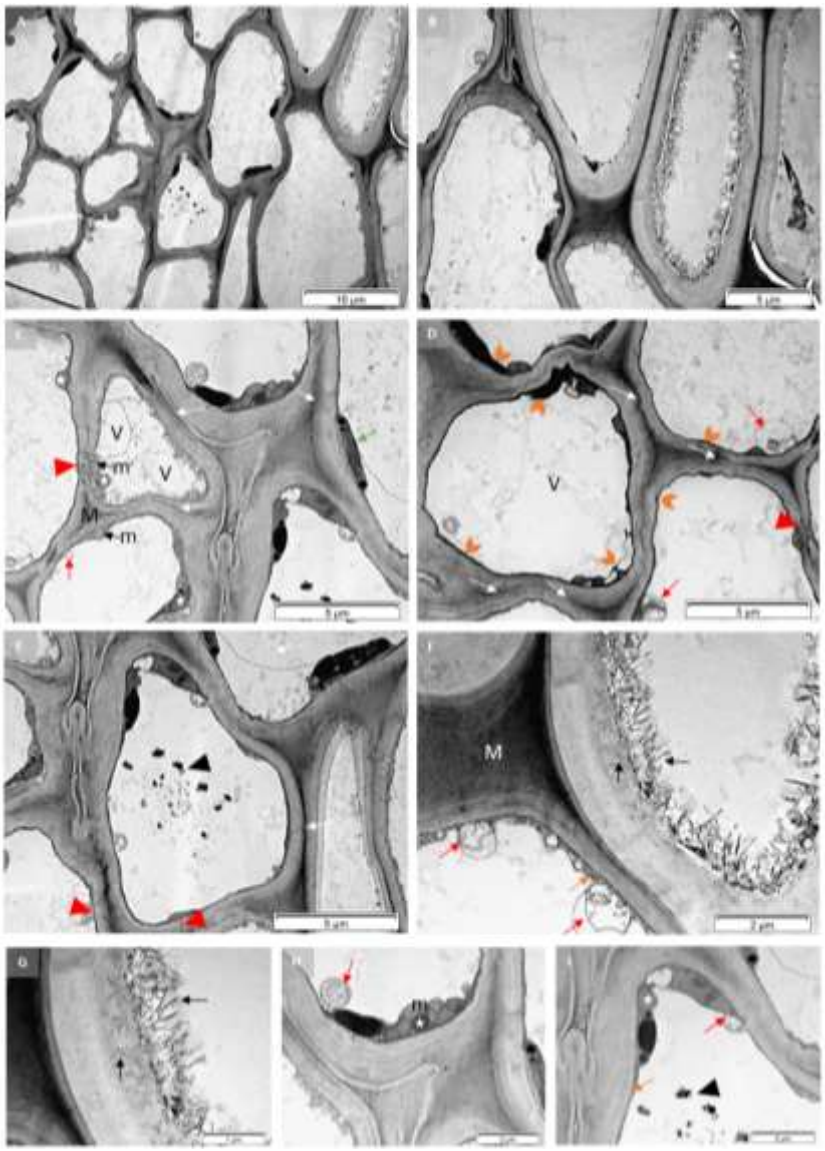


Plate 7.22. A-F – Ultrastructural aspects of the secondary phloem ring in the stem of *Leonurus cardiaca* L.; C, D, E – details from Figure A; F – detail from Figure B; G–I – details of higher magnifications from Figures B/F and A/E, respectively. V – vacuole, M – intercellular space, m – mitochondria, red arrowhead – pit, black arrowhead – intravacuolar electron-dense deposits, white arrow – middle lamella, red arrow – vesicles, black arrow – acicular deposits, orange arrow – endoplasmic reticulum, green arrow – chloroplast, chevron arrow – electron-dense cytoplasm, white star – lipid droplet.

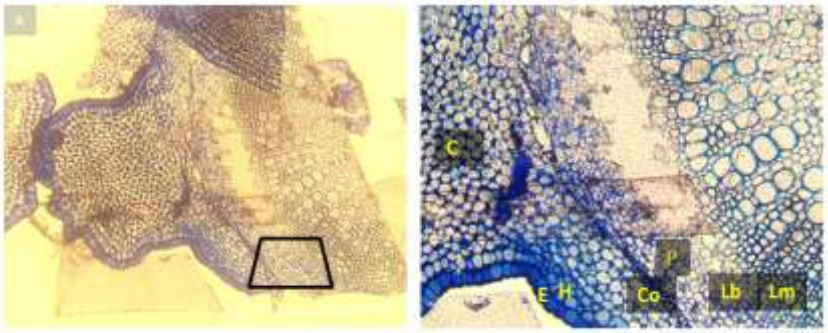


Plate 7.23. A – Semi-thin cross-section through the stem of *Leonurus cardiaca* L., showing the area of interest for TEM (trapezoidal area), 10X; B – detail from the area adjacent to a monticle, 20X. C – angular collenchyma, E – epidermis, H – hypodermis, Co – cortex, P – pericycle, Lb – phloem, Lm – wood.

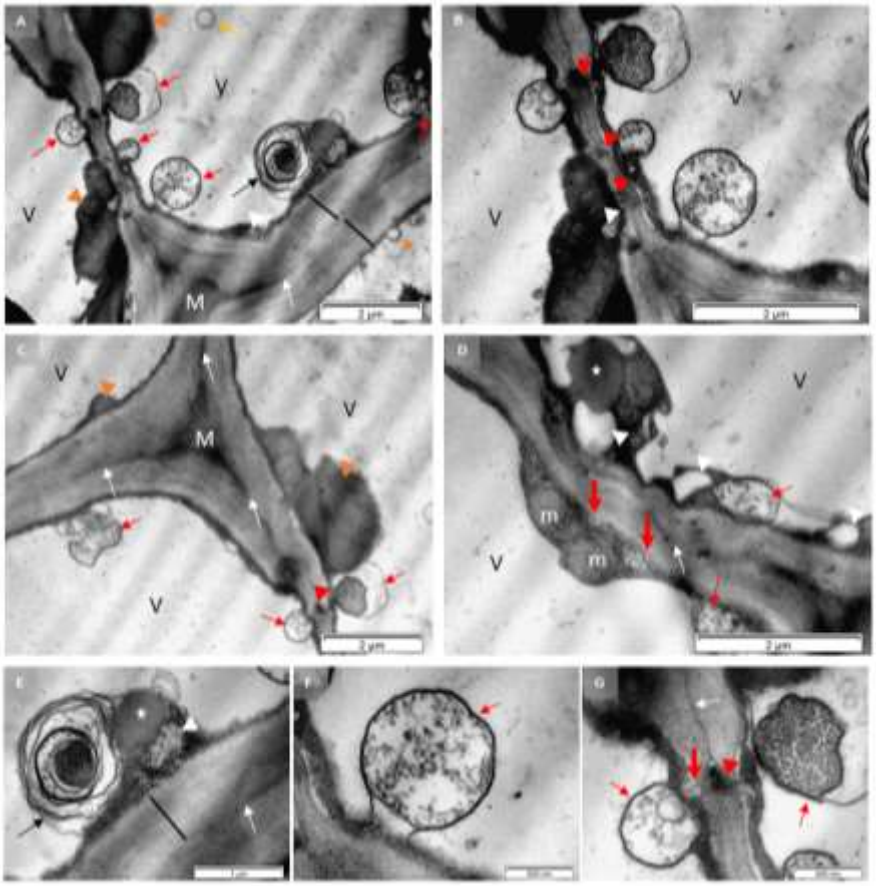


Plate 7.27. A, D – Multivesicular bodies and a paramural body in cortical cells of the stem of *Leonurus cardiaca* L.; B, E–G – magnified details of the image in A; C – continuation of the upper region in A, shown in detail in G. V – vacuole, M – intercellular space, m – mitochondria, red arrowhead – plasmodesma, white arrowhead – intracellular vesicle, orange arrowhead – intracellular electron-dense structure, black bar – cell wall, white arrow – middle lamella, white star – lipid droplet, black arrow – multilamellar body, red arrow – multivesicular body, thick red arrow – vesicles in contact with the cell wall, yellow arrow – intravacuolar vesicle, orange arrow – vesicle in formation.

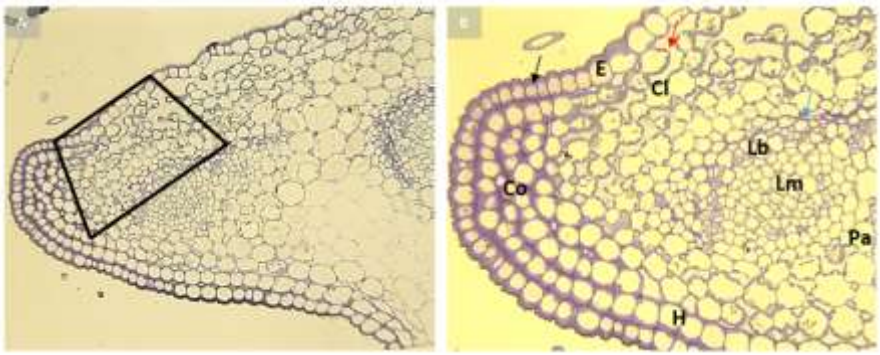


Plate 7.28. A – Semi-thin cross-section through the petiole of *Leonurus cardiaca* L. showing the area of interest for TEM (trapezoidal area), 20X; B – detail of the marginal zone, 40X. E – epidermis, H – hypodermis, Co – collenchyma, Cl – chlorenchyma, Lb – phloem, Lm – wood, Pa – parenchyma, black arrow – crenate cuticle.

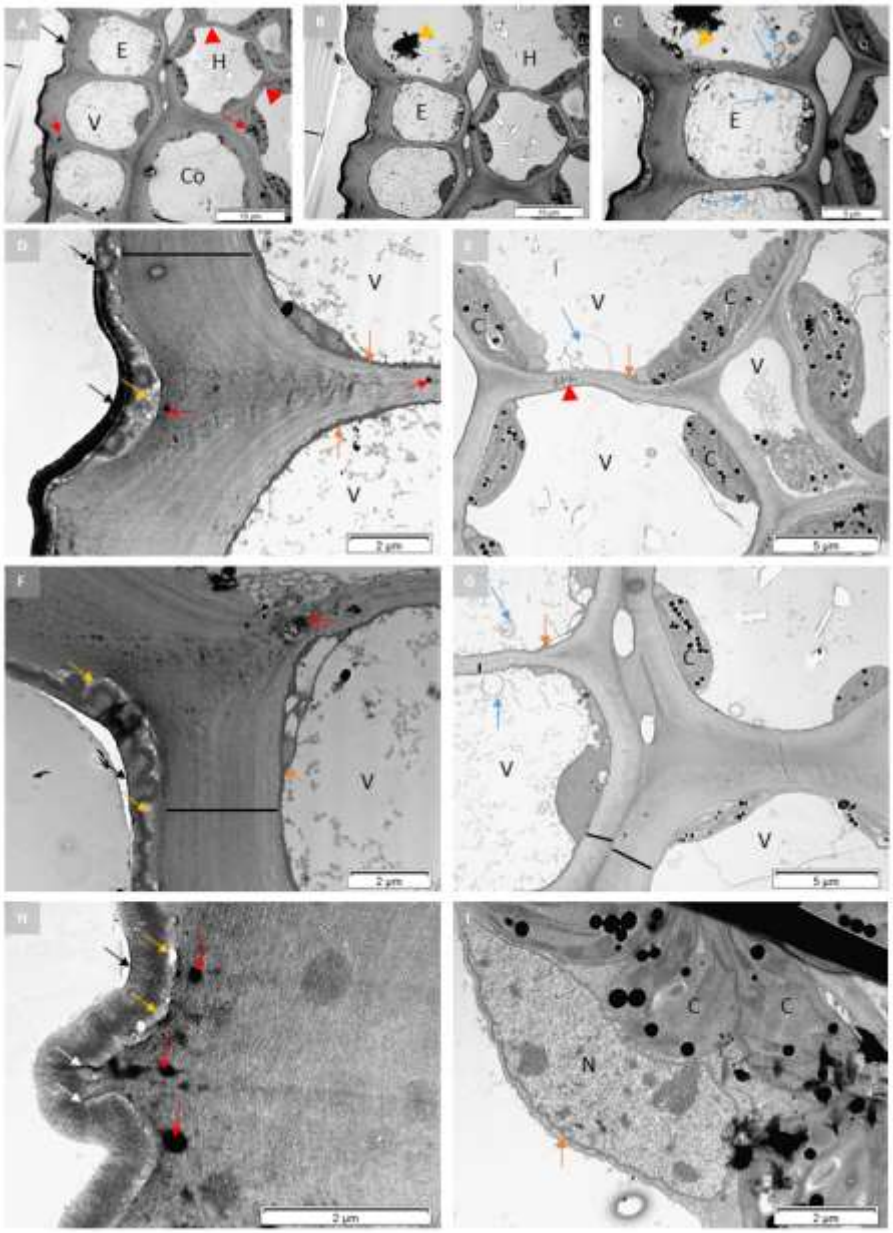


Plate 7.29. A-I – Ultrastructural aspects of the epidermis and hypodermis in the region adjacent to the collenchyma ridge of the petiole in *Leonurus cardiaca* L.; D, F, H – details of the cuticle's ultrastructure. E – epidermis, H – hypodermis, V – vacuole, Co – collenchyma, C – chloroplast, N – nucleus, red arrowhead – plasmodesmata, black bar – cell wall, orange arrow – cytoplasm, yellow arrowhead – electron-dense intravacuolar deposits, blue arrow – vesicles, red arrow – electron-dense deposits at the cell wall, yellow arrow – electron-light vesicles accumulated in the space between the cuticle and the cell wall, black arrow – epicuticle, white arrow – fibrils.

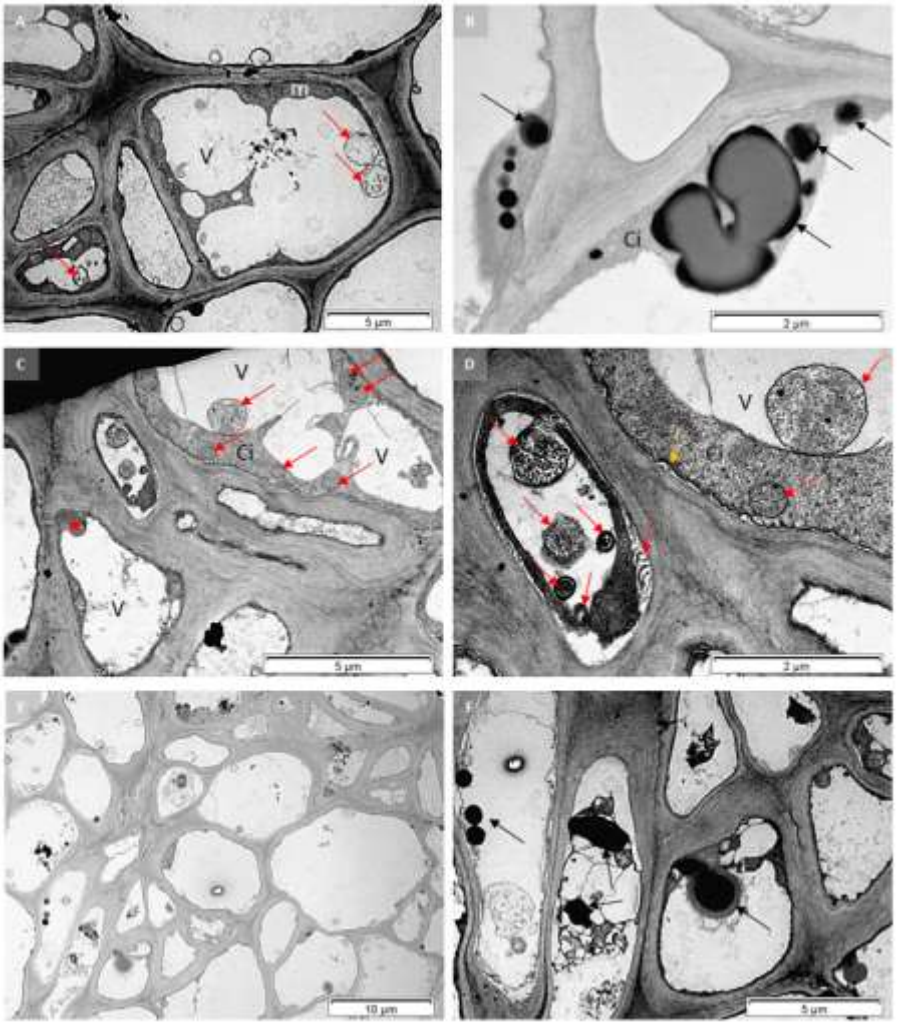


Plate 7.33. A-F Ultrastructural features of the periphloemic sclerenchymatic arch of the petiole in *Leonurus cardiac*a L. V – vacuole, Ci – cytoplasm, m – mitochondria, yellow arrow – rough endoplasmic reticulum, red arrow – multivesicular body, black arrow – electron-dense deposits.

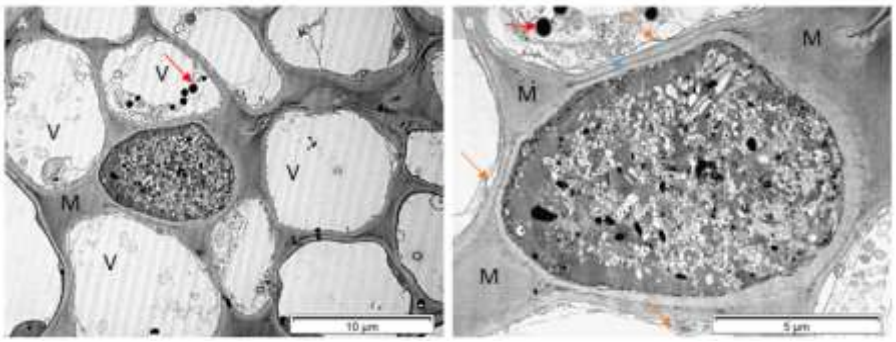


Plate 7.34. A-B Storage cells in the periphloemic sclerenchymatic arch of the petiole of *Leonurus cardiaca* L. V – vacuole, M – intercellular space, blue arrow – middle lamella, orange arrow – endoplasmic reticulum, red arrow – spherical electron-dense deposits.

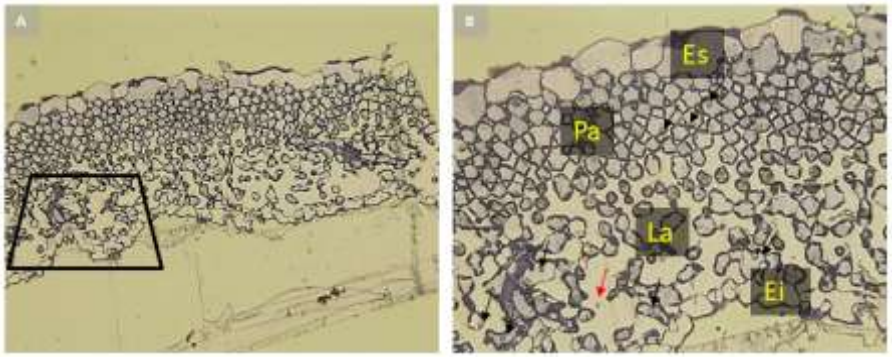


Plate 7.35. A – Semi-thin cross-section through the foliar limb of *Leonurus cardiaca* L. showing the TEM analysis area (trapezoidal area), 20X; B – detail of the epidermis and mesophyll, 40X. Es – upper epidermis, Ei – lower epidermis, Pa – palisade tissue, La – spongy tissue, red arrow – aeriferous lacunae, black arrow – lipid droplets.

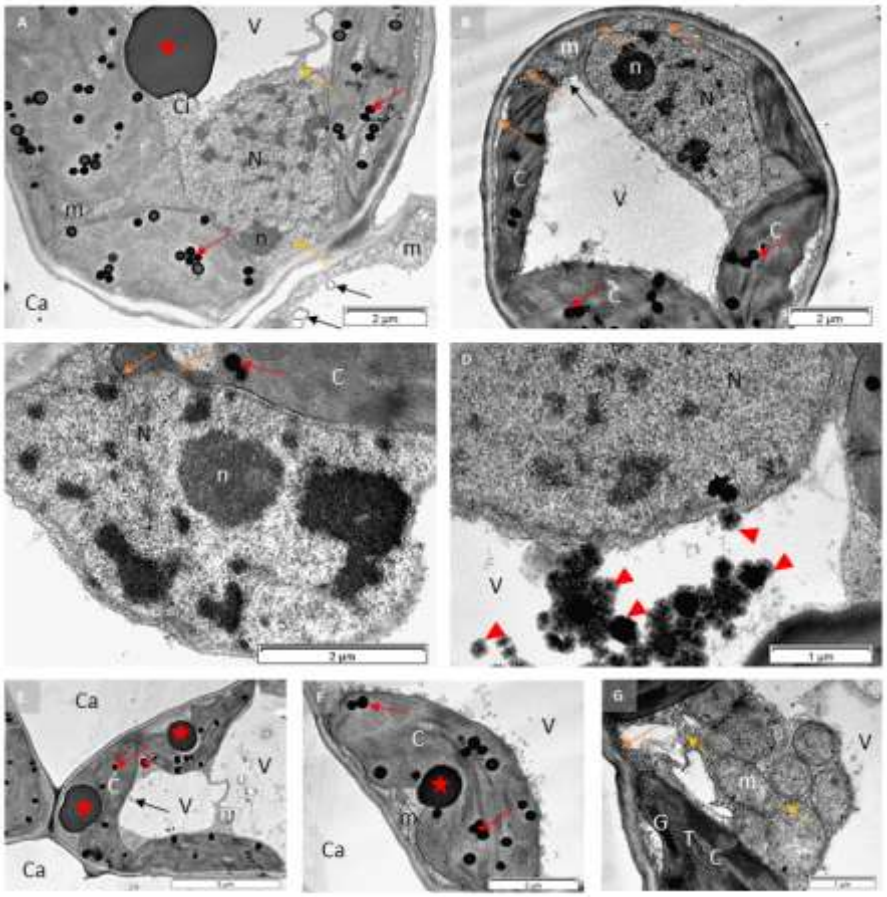


Plate 7.36. A-G – Cross-section through the mesophyll of the foliar limb of *Leonurus cardiaca* L.; A–B – Ultrastructure of cells in the spongy tissue; C–D – Details of the nucleus ultrastructure in cells of the spongy tissue and intravacuolar osmophilic deposits; E–G – Details of the plastids ultrastructure. V – vacuole, N – nucleus, n – nucleolus, Ci – cytoplasm, m – mitochondria, C – chloroplast, T – thylakoid, G – grana, red star – lipid droplet, red arrow – plastoglobules, orange arrow – rough endoplasmic reticulum, yellow arrow – smooth endoplasmic reticulum, black arrow – vesicles, red arrowhead – intravacuolar osmophilic deposits.

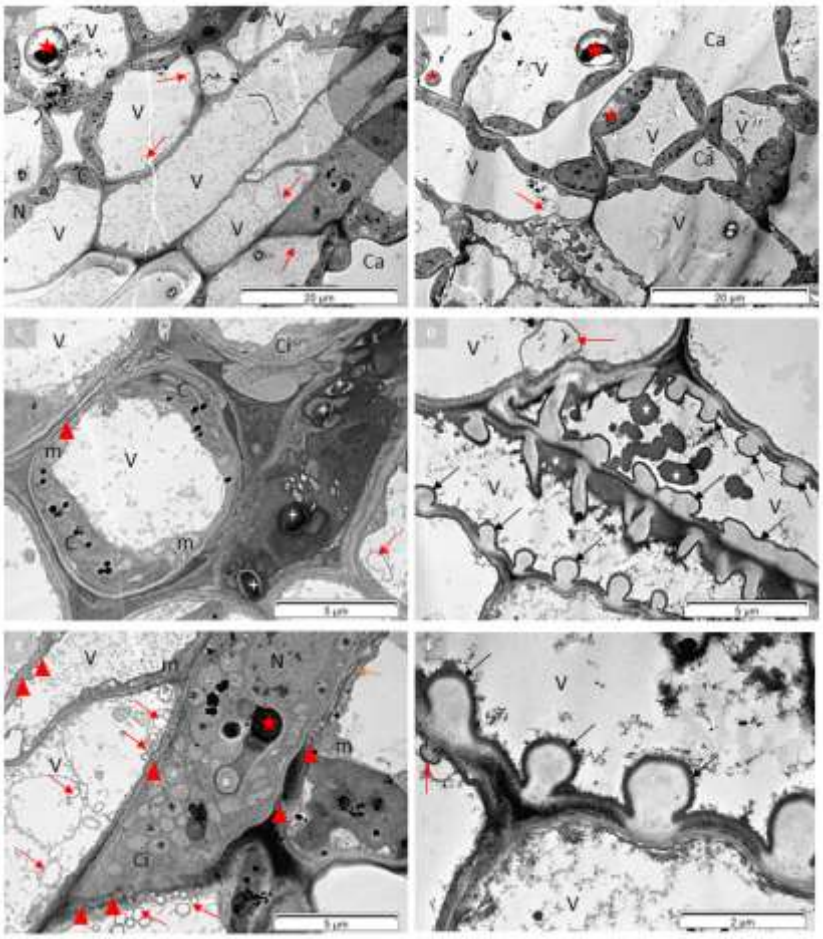


Plate 7.38. A-F – Cross-section through the mesophyll of the foliar limb of *Leonurus cardiaca* L. Ultrastructure of parenchyma cells involved in the synthesis and storage of secondary compounds; C, E – details from Figure A; D, F – details from Figure B. V – vacuole, Ca – air chamber, C – chloroplast, m – mitochondria, N – nucleus, Ci – cytoplasm, red star – lipid droplet, red arrow – vesicles, red arrowhead – plasmodesmata, orange arrow – endoplasmic reticulum, asterisk – membrane-bound storage bodies, white star – osmophilic storage sites, black arrow – vesicular invaginations of the plasmalemma.

Results obtained following the phytochemical analysis of extracts prepared from *Leonurus cardiaca* L. plants belonging to the two populations collected from the wild flora of the two geographical areas in Romania (lowland area—Macea; mountain area—Vatra Dornei):

In the context of pedoclimatic conditions of the two collection sites, LC/MS analysis revealed the presence of 10 polyphenols in the hydroalcoholic extracts from whole *Leonurus cardiaca* L. plants collected from Macea and Vatra Dornei (Table 7.1), 6 in the infusion prepared from plant material collected from Macea, and 7 in the infusion prepared from plant material collected from Vatra Dornei (Table 7.2). Chrysin and esculetin were identified for the first time in this species. In terms of quantity, esculetin was the major compound in the hydroalcoholic extracts and in the infusion obtained from *L. cardiaca* from Vatra Dornei, where the highest concentration was also observed. With the exception of salicylic acid, the

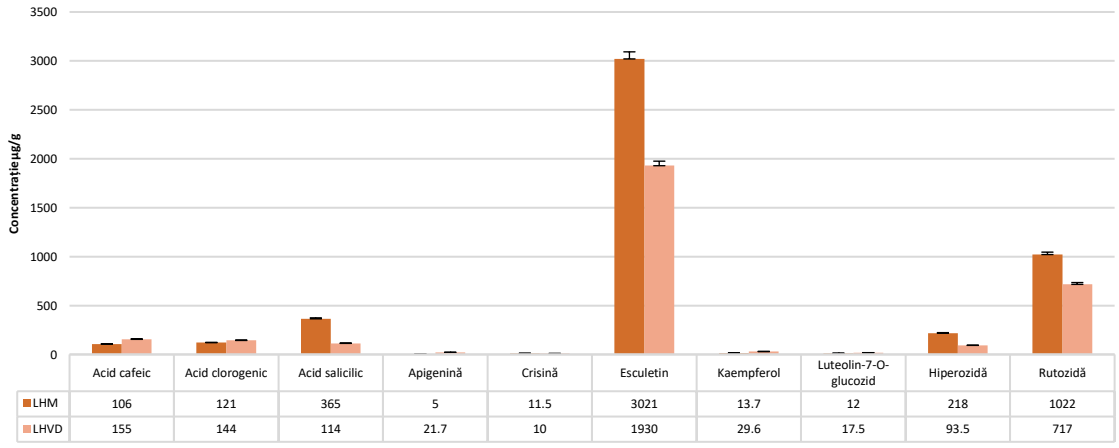
infusion made from plant material collected from the lowland contains higher concentrations of polyphenols compared to the hydroalcoholic extract from the same site, as well as to the infusion prepared from plant material collected from the mountain region (Graphs 7.1-7.2).

Table 7.1.

Qualitative and quantitative analysis of polyphenols in hydroalcoholic extracts of *Leonurus cardiaca* L. by LC/MS

No.	Compounds	Sample LHM [µg/g dry plant weight]	Sample LHVD [µg/g dry plant weight]
1	Caffeic acid	106 ± 2.7	155 ± 3.8
2	Chlorogenic acid	121 ± 3.0	144 ± 3.6
3	Salicylic acid	365 ± 9.1	114 ± 2.8
4	Apigenin	5 ± 0.1	21.7 ± 0.8
5	Chrysin	11.5± 0.72	10.1± 0.6
6	Esculetin	3021± 071.4	1930 ± 45.6
7	Hyperoside	218 ± 5.4	93.5 ± 2.51
8	Kaempferol	13.7 ± 0.84	29.6 ± 1.1
9	Luteolin-7-O-glucoside	12 ± 0.7	17.5 ± 1.04
10	Rutoside	1022 ± 24.1	717 ± 17.9

Abbreviations: Sample LHM = hydroalcoholic extract, sample collection site in Macea; Sample LHVD = hydroalcoholic extract, sample collection site in Vatra Dornei.



Graph. 7.1. LC/MS analysis of the hydroalcoholic extract obtained from whole plants of *Leonurus cardiaca* L. belonging to the two populations studied—from Macea (LHM) and from Vatra Dornei (LHVD).

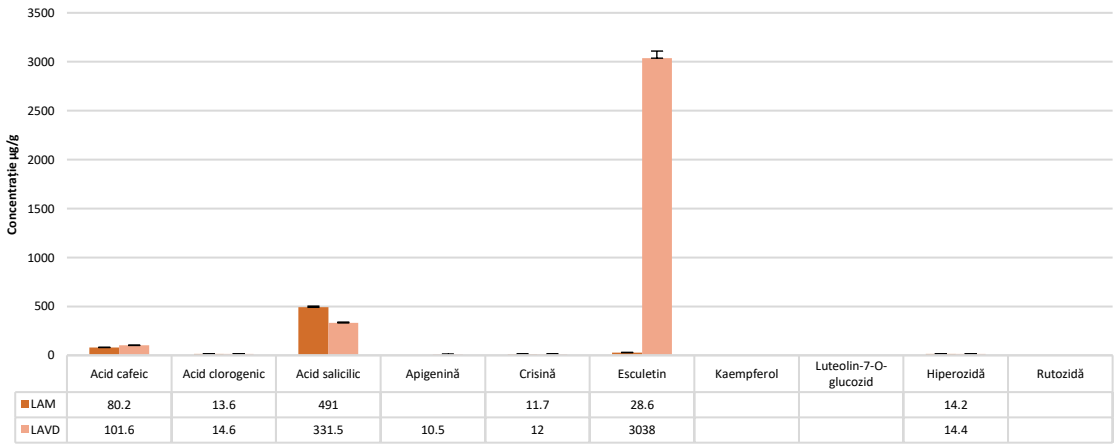
Table 7.2.

Qualitative and quantitative analysis of polyphenols in aqueous extracts of *Leonurus cardiaca* L. by LC/MS

No.	Compounds	Sample LAM [µg/g dry plant weight]	Sample LAVD [µg/g dry plant weight]
1	Caffeic acid	80.2 ± 2.1	101.6 ± 2.54
2	Chlorogenic acid	13.6 ± 0.8	14.6 ± 0.80
3	Salicylic acid	491 ± 12.2	331.5 ± 8.34
4	Apigenin	<QL	10.5 ± 0.69
5	Chrysin	11.7± 0.73	12.1± 0.71
6	Esculetin	28.6± 1.17	3038 ± 71.8

No.	Compounds	Sample LAM [µg/g dry plant weight]	Sample LAVD [µg/g dry plant weight]
7	Hyperoside	14.2 ± 0.82	14.4 ± 0.82
8	Kaempferol	<QL	<QL
9	Luteolin-7-O-glucoside	<QL	<QL
10	Rutoside	<QL	<QL

Abbreviations: LAM sample = aqueous extract, sample collection site in Macea; LAVD sample = aqueous extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.



Graph. 7.2. LC/MS analysis of the aqueous extract obtained from whole plants of *Leonurus cardiaca* L. belonging to the two populations studied—from Macea (LAM) and Vatra Dornei (LAVD), respectively.

7.5. Comparative analysis of the polyphenol content in extracts of Leonurus cardiaca L. species in the context of environmental factors

The collection sites for the *Leonurus cardiaca* L. plants analyzed phytochemically are located in the lowland area, at an elevation of 99 m, and in the mountain area, at an elevation of 791 m. The climatic conditions during the plant's ontogenetic development correspond to those analyzed for the year 2021 (the year the plant material was collected) in Chapter 4 and mentioned in Section 6.5. Taking into account the species' wide ecological range and its average temperature and humidity requirements, as described in section 2.3.4, the climatic conditions in the lowland area tend to better meet the preferences of *L. cardiaca* L. plants, a fact also supported by the visibly more developed biomass at this location. With regard to edaphic conditions, the soil pH and total nitrogen index in the mountain area are closer to the species' requirements than those in the lowland area. At the sampling site in Macea, the soil had an extremely low content of assimilable phosphorus but a very high content of assimilable potassium, whereas this ratio was reversed in the mountain area, with average levels of assimilable phosphorus and very low levels of assimilable potassium.

Under these conditions, 10 polyphenols were identified in the hydroalcoholic extract and 6 in the aqueous extract of the plant material analyzed from the lowland population of *L. cardiaca*. The situation was similar for the plant material from the mountain population, with the exception that apigenin was also present in the aqueous extract, at a concentration of 10.5 μg/g dry plant. In contrast, kaempferol, luteolin-7-O-glucoside, and rutoside were absent from the infusion in both populations studied. In hydroalcoholic extracts, regardless of the origin of the plant material, esculetin stands out as the main compound, present in nearly double the amount in the extract obtained from lowland plants (3021 μg/g dry plant), a value that is very close to the amount found in the aqueous extract of plants from the mountains (3038 μg/g dry plant). In the hydroalcoholic extracts, the next most abundant compound—exceeding the levels of the other identified polyphenols—is rutoside, with a quantified value that is once again higher in the plant material collected from the lowlands (1022 vs. 717 μg/g dry plant). In terms of quantity, the following significant compounds are caffeic acid, chlorogenic acid, and salicylic acid, at levels exceeding 100 μg/g of dry plant weight, with the exception of chlorogenic acid in infusions and caffeic acid in the LAM extract (lowland area). Caffeic acid shows similar values between the two populations in hydroalcoholic extracts (106 μg/g dry plant weight – LHM, 155 μg/g dry plant weight – LHVD) and in the aqueous extract from plants collected from the mountains (101.6 μg/g dry plant weight – LAVD). Chlorogenic acid and salicylic acid, present in equal amounts in the hydroalcoholic extract from plant material collected from the mountain region, vary in the aqueous extract (the amount of chlorogenic acid decreases to 14.6 μg/g dry plant weight, and the amount of salicylic acid increases to 331.5 μg/g dry plant weight) compared to plant material collected from the lowland area. Lower but similar values were observed for chlorogenic acid— 121 μg/g of dry plant weight in the hydroalcoholic extract and 13.6 μg/g of dry plant weight in the infusion, respectively, and higher levels of salicylic acid—365 μg/g of dry plant weight in the hydroalcoholic extract and 491 μg/g of dry plant weight in the infusion. Therefore, the highest concentration of salicylic acid, considered a marker for distinguishing populations of *L. cardiaca* L. (Lam *et al*., 2022), was observed in the aqueous extract of plant material collected from the lowland area. The remaining identified polyphenols were quantified at levels below 100 μg/g dry plant, with the lowest concentration (5 μg/g dry plant weight) recorded for apigenin in the hydroalcoholic extract from *L. cardiaca* L. plants collected from the lowland area.

CHAPTER 8
MORPHO-ANATOMICAL AND PHYTOCHEMICAL RESEARCH OF *OENOTHERA BIENNIS* L. SPECIES

8.6. Considerations regarding the morpho-anatomical characteristics and phytochemical composition of the vegetative organs of Oenothera biennis L. species

Morpho-anatomical results obtained for *Oenothera biennis* L. species:

– A detailed initial characterization of the root, stem, petiole, and foliar limb of this species found in Romania's wild flora using optical microscopy, SEM, and TEM techniques;

– Anatomical analysis of the root revealed structural differences between the fleshy base and the slender tip, with the base exhibiting a thinner bark, a thicker secondary phloem ring, a predominance of phloem parenchyma, storage parenchyma cells between the periderm and the external phloem, a higher frequency of raphides, and a more developed secondary xylem. Optical microscopy images confirm the presence of interxylary phloem in this species, as well as libriform cells with gelatinous deposits—which appear dark—and SEM images confirm the presence of numerous crystal deposits on the surface of the periderm* (Plates 8.1-8.3, 8.10);

– Morphological and anatomical analysis of the stem reveals the presence of interxylary phloem, as well as four types of trichomes: in the lower part—(Pt-1) inserted perpendicular to the epidermis, unicellular, long and straight, with a rounded tip, smooth and transparent walls; (Pt-2) located at the tips of small ridges, unicellular, with pointed tips, short, straight, or slightly curved, with heavily thickened walls and surface protuberances, semi-transparent; and in the upper part (Pt-3), unicellular, long, with the terminal end bent, parallel to the epidermis, with thick walls covered by small protuberances; (Pt-4), unicellular, short, clavate, and curved* (Plates 8.4-8.5, 8.11). The petiole structure contains Pt-3 and Pt-4 trichomes, which are more numerous on the upper surface (Plates 8.6–8.7, 8.12). In the structure of the foliar limb, we find Pt-1 and Pt-4-type trichomes and very short glandular trichomes with a strongly swollen gland; a characteristic feature of the mesophyll is the presence of spherical structures with a granular, rarely translucent content* (Plates 8.8-8.9, 8.13-8.14);

* Bota, V.B., Turcuș, V., Mihali, C.V., Arsene, G.-G., Ivănescu, L.C., Zamfirache, M.-M., 2020 - Anatomical investigations on *Oenothera biennis* L. using optical microscopy and scanning electron microscopy (SEM), Research Journal of Agricultural Science 52(1): 61-71.

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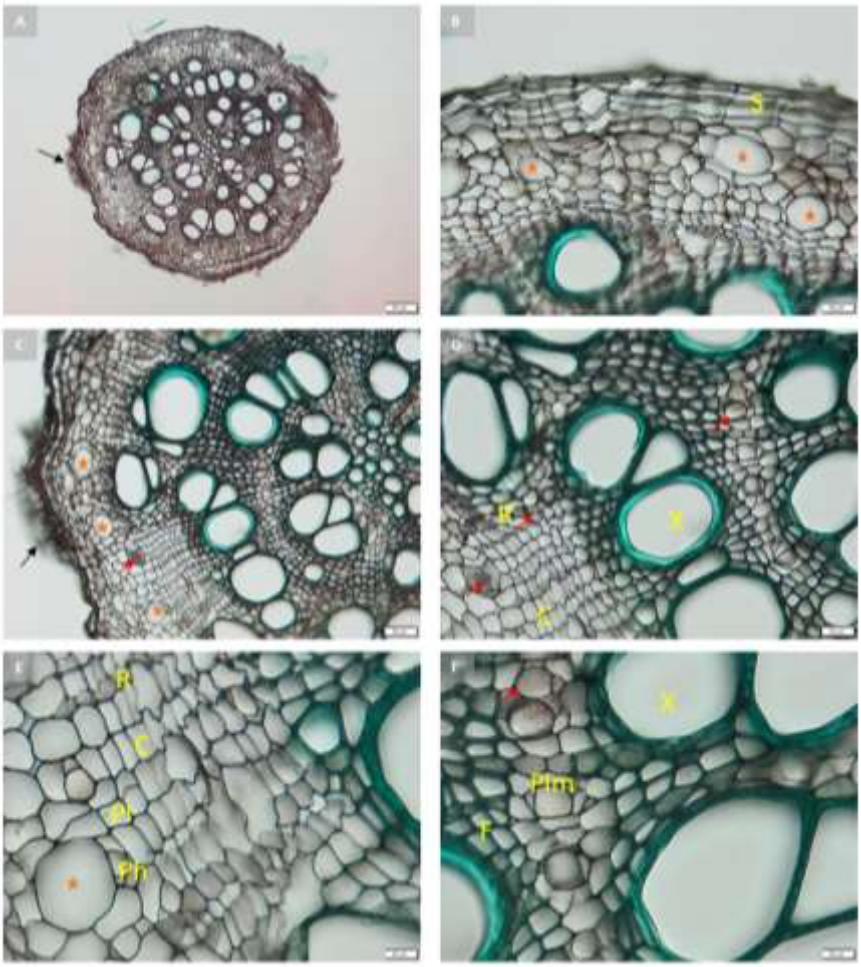


Plate 8.1. A-F Cross-section through the lower part of the root of *Oenothera biennis* L. S – bark, R – medullary ray, C – cambium, Ph – phloem, Pl – phloem parenchyma, X – xylem, Plm – wood parenchyma, orange star – cavities, black arrow – exfoliating periderm, red arrow – cells with granular deposits.

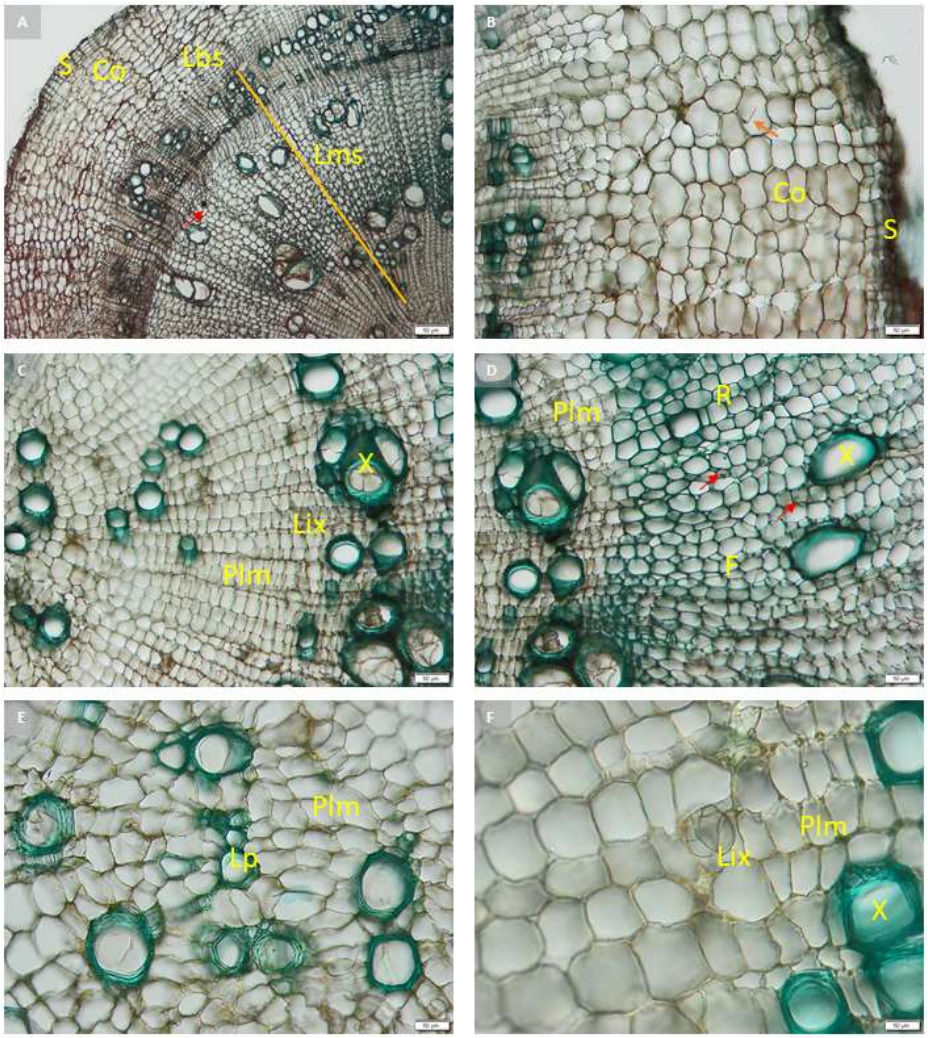


Plate 8.2. A-F Cross-section through the upper part of the root of *Oenothera biennis* L. S – bark, Co – cortex, Lbs – secondary phloem, Lms – secondary xylem, X – xylem, Lix – interxylary phloem, Plm – woody parenchyma, R – medullary ray, F – wood fibers, Lp – primary wood, orange arrow – raphides, red arrow – storage cells.

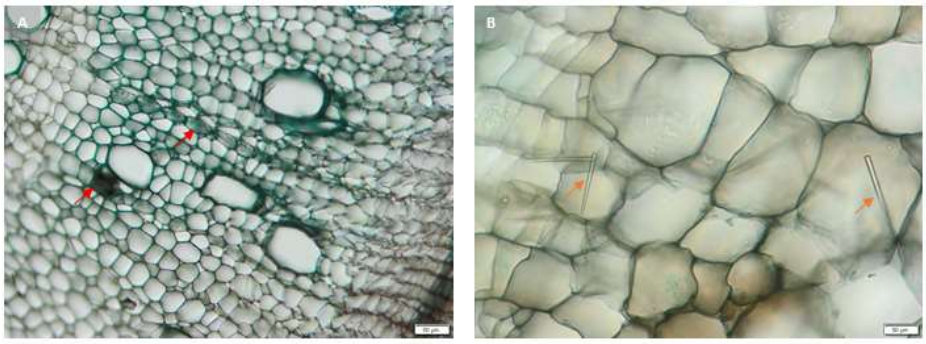


Plate 8.3. A-B – Cross-section through the upper part of the root of *Oenothera biennis* L. A – Cells containing dark or mucilaginous deposits in the medullary and libriform rays; B – raphides in the phloem parenchyma; orange arrow – raphides, red arrow – storage cells.

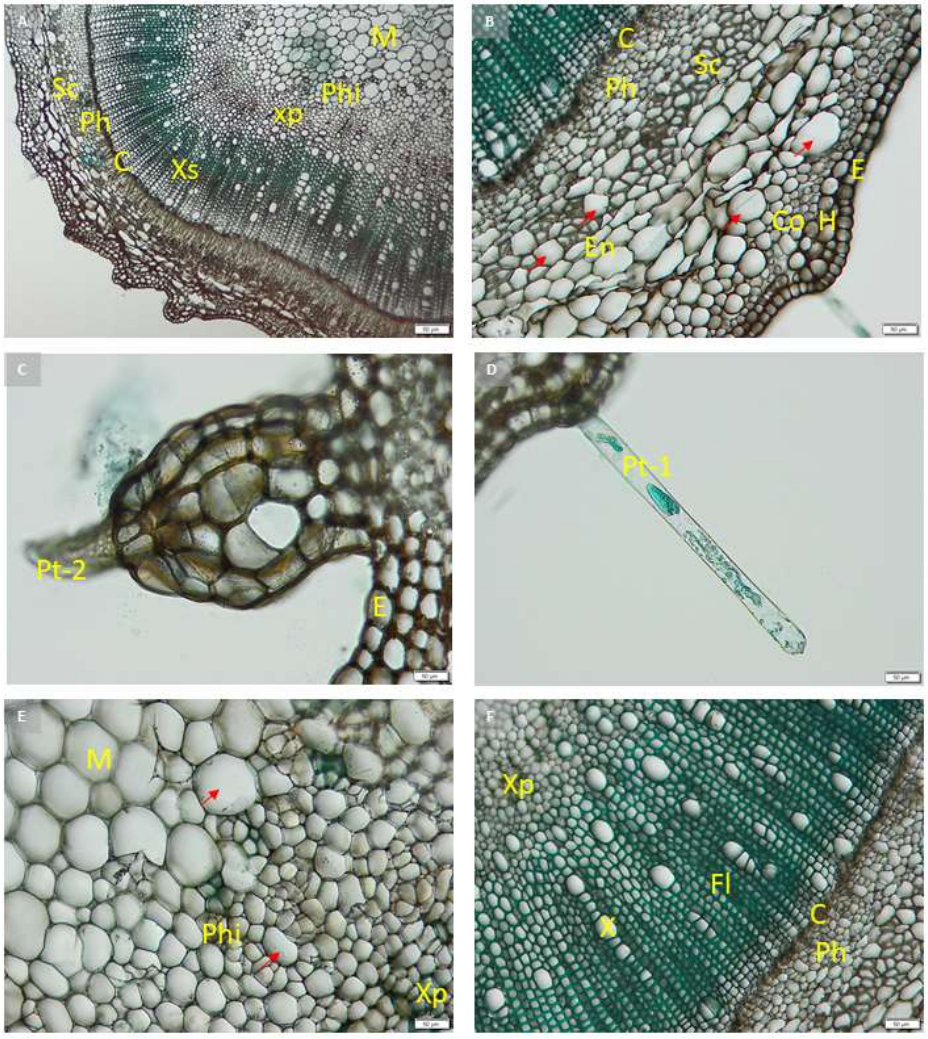


Plate 8.4. A–F Cross-section through the lower part of the stem of *Oenothera biennis* L. Sc – sclerenchymatic pericycle, Ph – phloem, C – cambium, Xs – secondary xylem, Xp – primary xylem, Phi – internal phloem, M – pith, E – epidermis, H – collenchyma hypodermis, Co – cortex, En – endodermis, Pt-1 – type 1 trichome, Pt-2 – type 2 trichome, Fl – libriform, X – xylem, red arrows – large, characteristic cells.

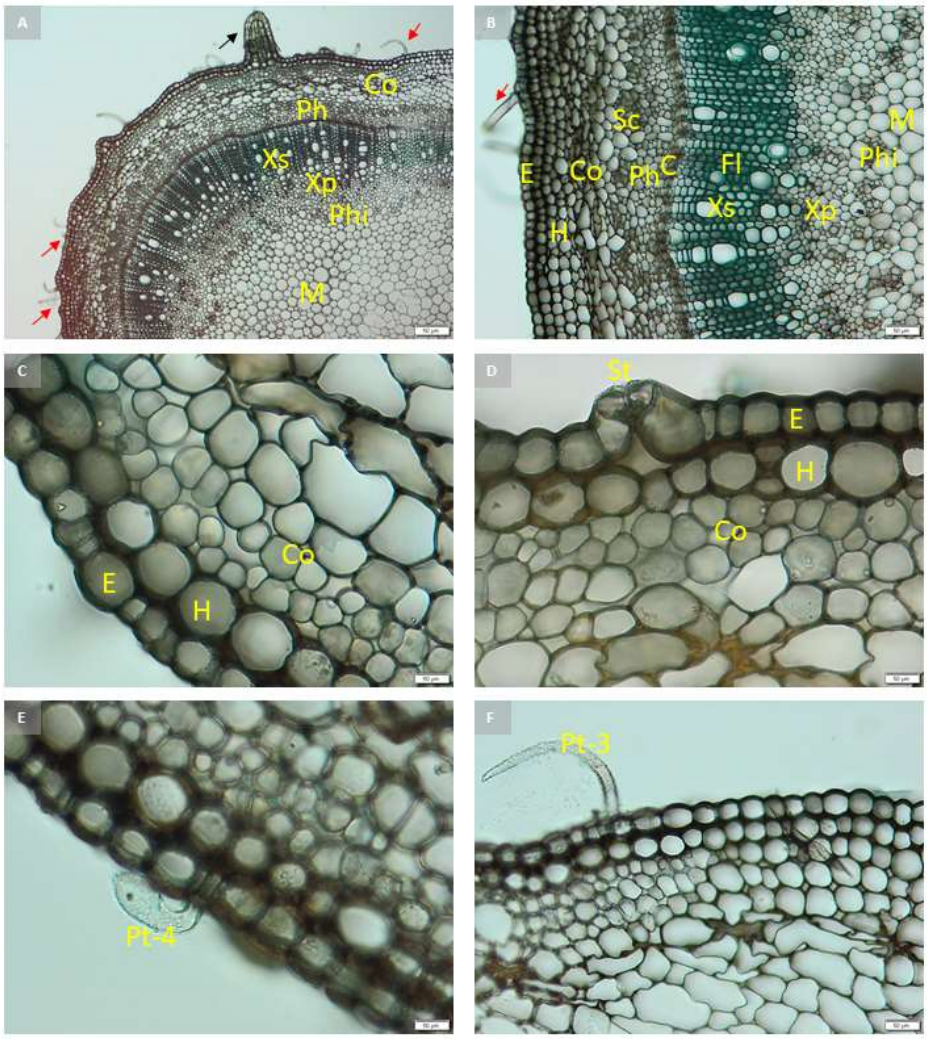


Plate 8.5. A-F Cross-section through the upper part of the stem of *Oenothera biennis* L. Co – cortex, Ph – phloem, Xs – secondary xylem, Xp – primary xylem, Phi – internal phloem, M – pith, E – epidermis, H – collenchyma hypodermis, Sc – sclerenchyma pericycle, C – cambium, Fl – libriform, St – stomata, Pt-3 – type 3 trichome, Pt-4 – type 4 trichome, black arrow – crest with type 2 trichome, red arrow – type 3 and 4 trichomes.

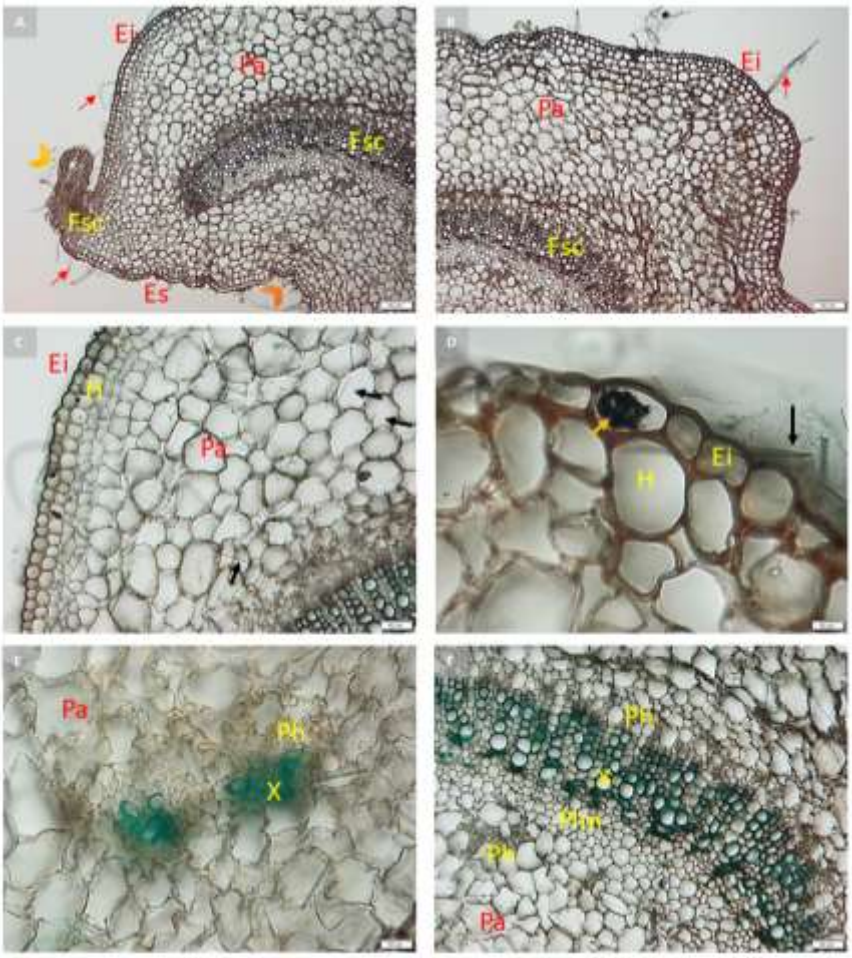


Plate 8.6. A-F Cross-sections through the petiole of *Oenothera biennis* L. Ei – lower epidermis, Es – upper epidermis, Fsc – vascular bundle, Pa – parenchyma, H – collenchyma hypodermis, Ph – phloem, X – xylem, Plm – woody parenchyma, red arrow – trichome, yellow chevron arrow – lateral ridge, orange chevron arrow – adaxial middle depression, black arrow – raphides, orange arrow – dark crystalline deposit.

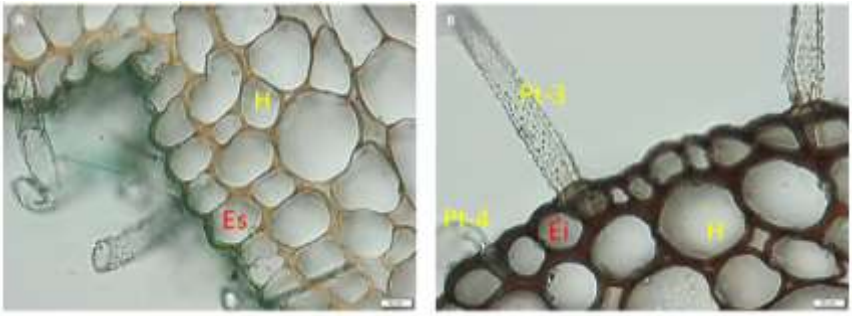


Plate 8.7. Cross-section through the petiole of *Oenothera biennis* L. A – Section at the level of the adaxial depression showing the cuticle-bearing epidermis (green), the bases of type 3 and 4 trichomes, and the multilayered, collenchyma-rich hypodermis; B – View of the lower epidermis showing the bases of type 3 and 4 trichomes; Es – upper epidermis, Ei – lower epidermis, Pt-3 – type 3 trichome, Pt-4 – type 4 trichome.

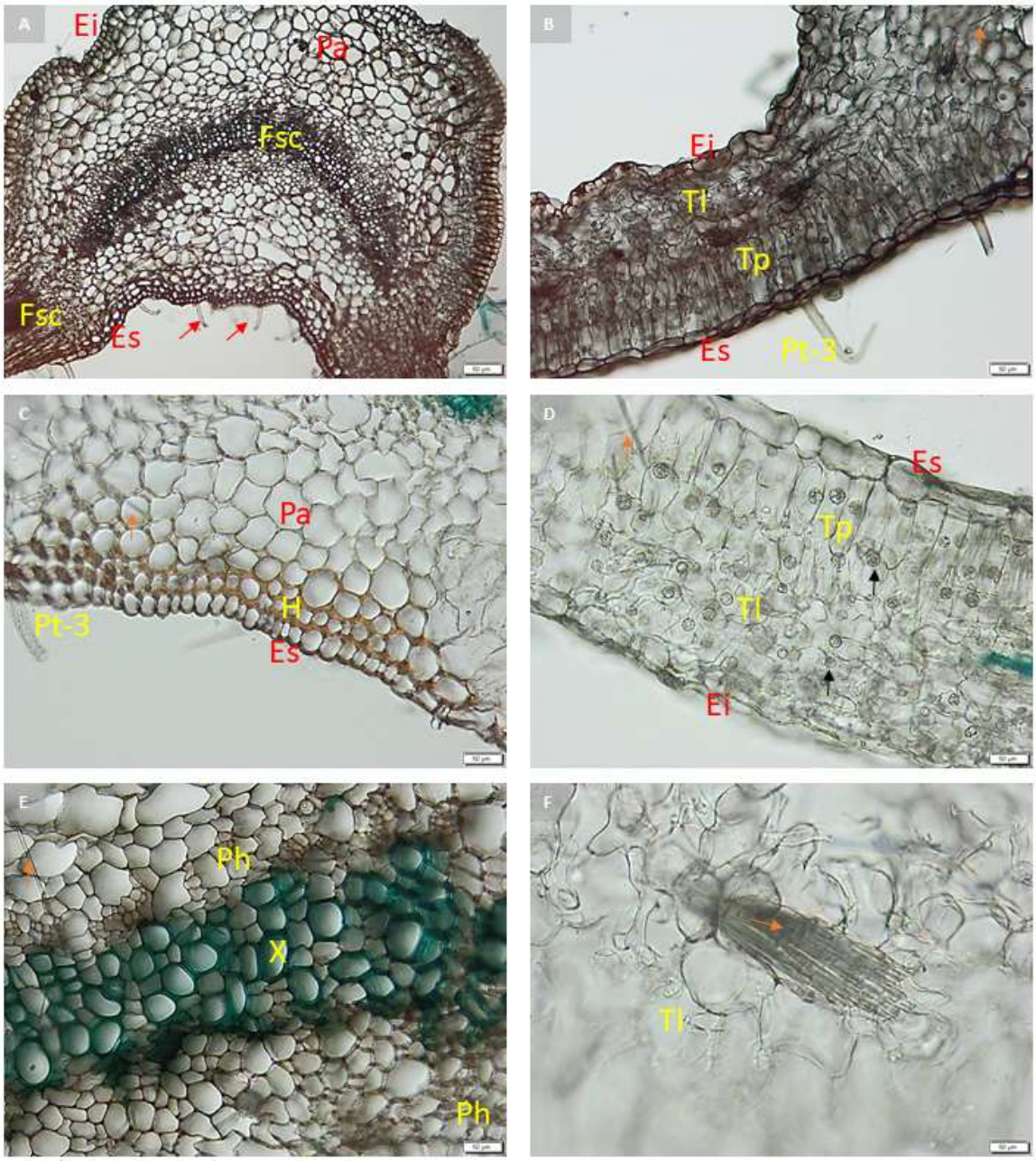


Plate 8.8. A-F – Cross-section through the foliar limb of *Oenothera biennis* L.; A-C-E – views of the midrib; B-D – views of the foliar limb between the veins; F – section of mesophyll with a bundle of raphides. Ei – lower epidermis, Es – upper epidermis, Pa – parenchyma, Fsc – vascular bundle, Tp – palisade tissue, Tl – spongy tissue, Pt-3 – type 3 trichome, H – hypodermis, Ph – phloem, X – xylem, red arrow – trichomes, orange arrow – acicular raphides, black arrow – spherical deposits.

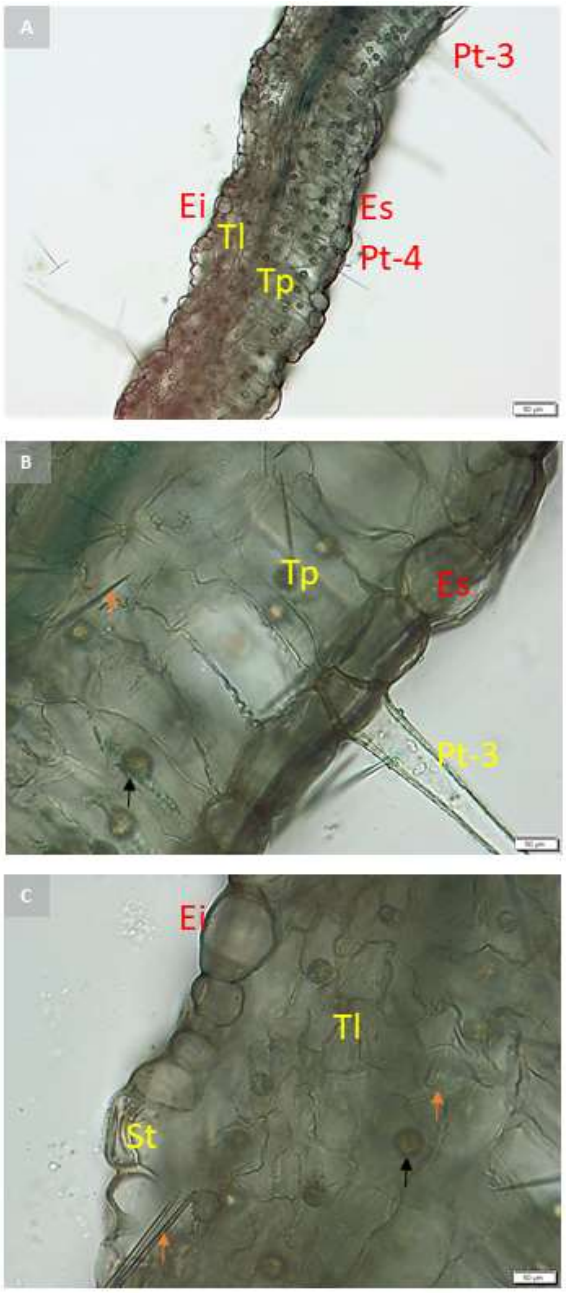


Plate 8.9. A-C Cross-section through the foliar limb of *Oenothera biennis* L. Ei – lower epidermis, Es – upper epidermis, Tp – palisade tissue, Tl – spongy tissue, Pt-3 – type 3 trichome, Pt-4 – type 4 trichome, St – stomata, orange arrow – raphides, black arrow – spherical deposits.

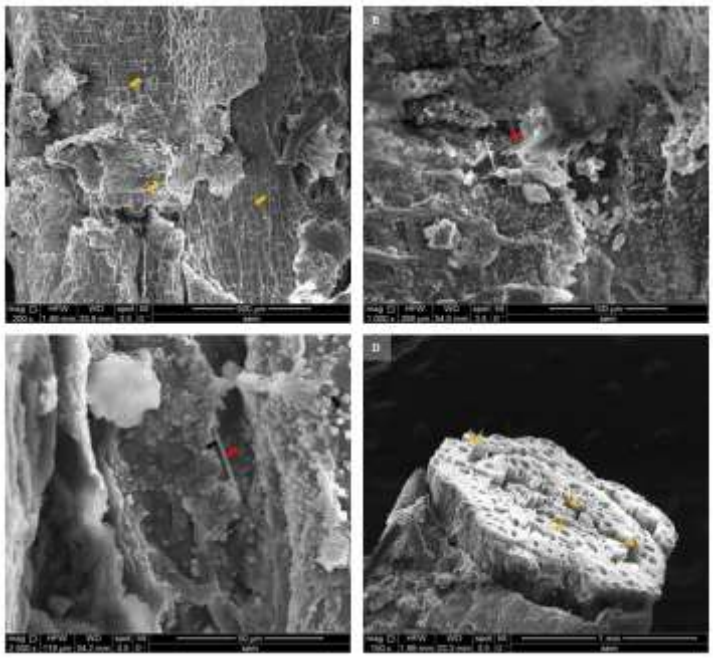


Plate 8.10. A – surface microtopography of the root of *Oenothera biennis* L.; B–C – types of crystals on the root surface; D – appearance of the conducting vessels in a longitudinal-transverse section. Orange arrows – rectangular cells, red arrows – raphides, black arrows – prismatic crystals, yellow arrows – appearance of the perforations in the walls of xylem vessels.

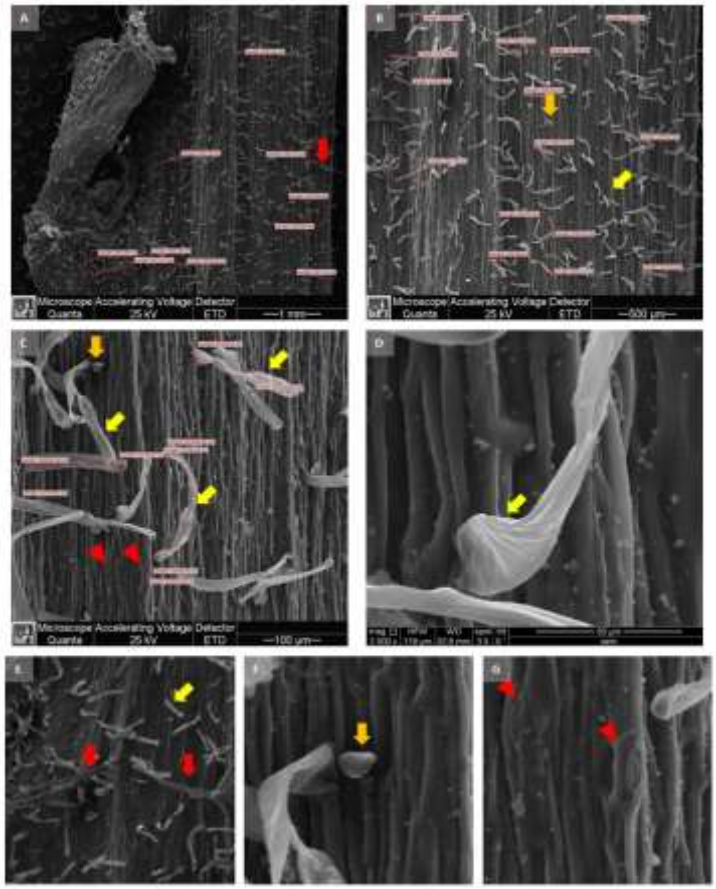


Plate 8.11. A-D Micromorphological aspects of the stem of *Oenothera biennis* L.; E–G—details of trichomes and stomata. Red arrows—pointed trichomes; yellow arrows—Pt-1-type trichomes; orange arrows—Pt-4-type trichomes; red arrowheads—stomata.

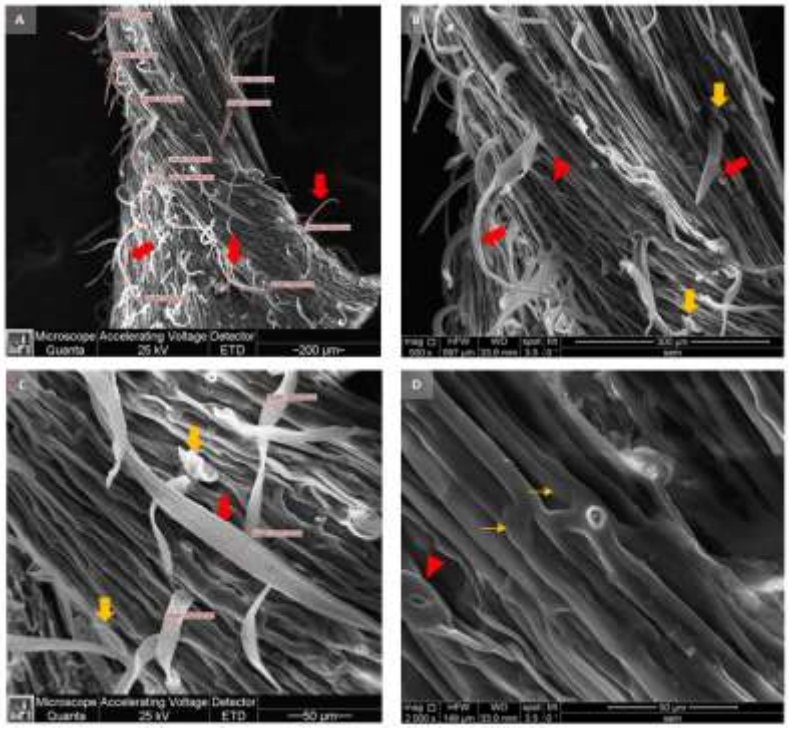


Plate 8.12. A-B Micromorphological aspects of the petiole in *Oenothera biennis* L.; C – close-up of Pt-3 trichome with a papillate surface and Pt-4-type trichome; D – close-up of the surface showing cuticle striations and a stomata. Red arrows – Pt-3-type trichome; orange arrows – Pt-4-type trichome; thin arrows – cuticle striations; red arrowheads – stomata.

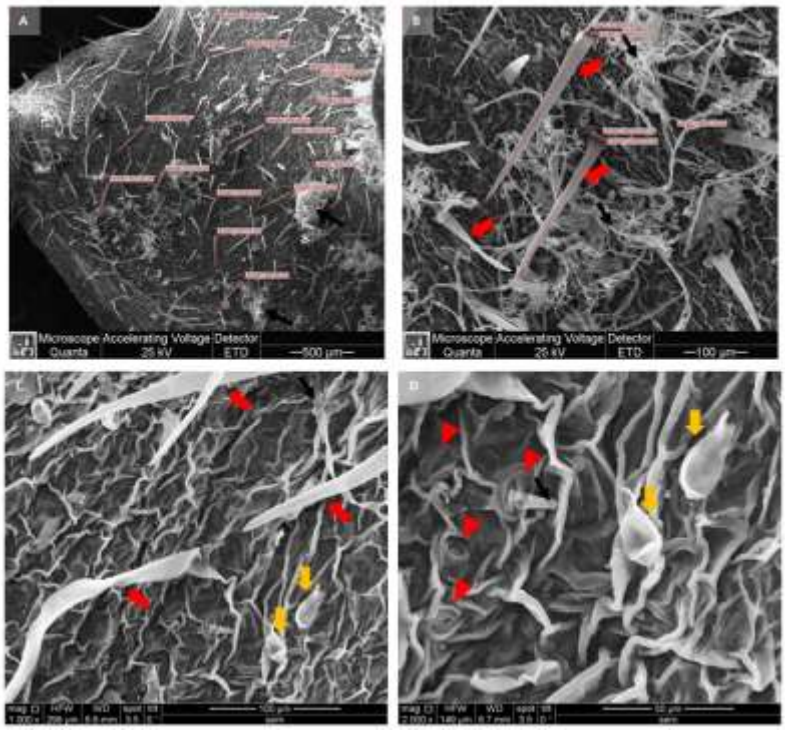


Plate 8.13. A - General view of the lower epidermis of *Oenothera biennis* L.; B – detail of trichomes and mycelial hyphae; C – detail of Pt-3 and Pt-4 trichomes; D – appearance of the cuticle, stomata, mycelial hyphae, and Pt-4 trichomes. Red arrows – Pt-3-type trichomes, orange arrows – Pt-4-type trichomes, black arrows – mycelial hyphae, red arrowheads – stomata.

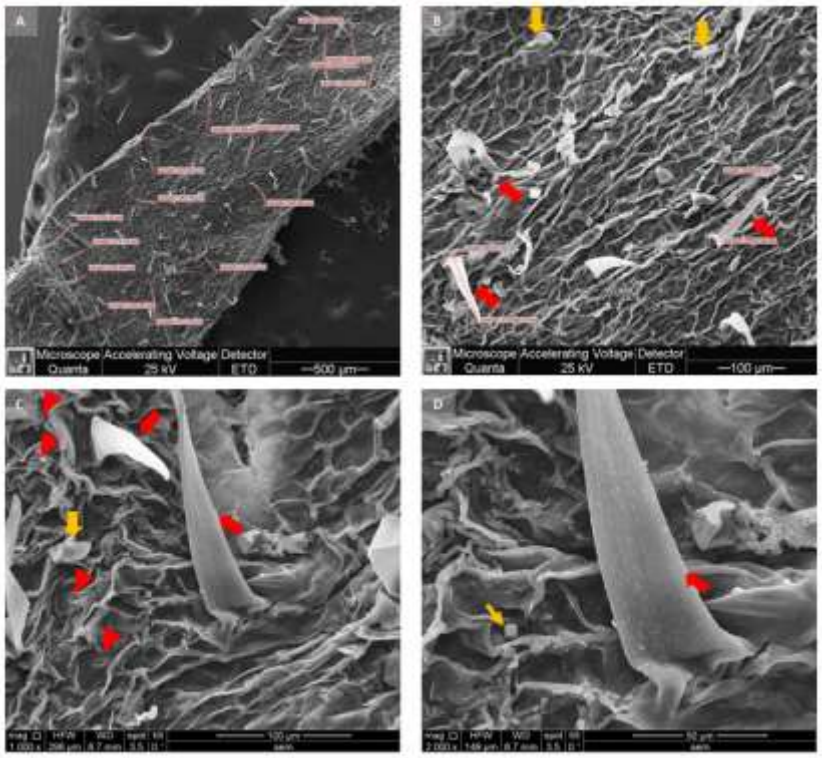


Plate 8.14. A – General view of the upper epidermis of *Oenothera biennis* L.; B–C – details of the cuticle, Pt-3 and Pt-4 trichomes, and stomata; D – detail of protuberances on the surface of the Pt-3 trichome and the presence of crystals.

– The TEM images reveal processes of secondary wall thickening, of transport and storage of certain metabolites (polyphenols, lipids, mucilages) within the ultrastructure of the root's vascular bundles (Plates 8.15 -8.17), as well as intracellular and intravacuolar deposits, along with the less common presence of chloroplasts and multilamellar bodies in the medullary rays and wood fibers of the stem (Plates 8.19, 8.22-8.23, 8.25). Ultrastructural analysis of the petiole revealed the presence of secondary metabolite deposits in the extracellular spaces, in the cell walls, and in the vacuoles of parenchyma cells that were irregular in shape and size in cross-section (Plates 8.26–8.28). In the ultrastructure of the spongy tissue of the foliar limb, three types of cells were observed: cells in which the vacuole and chloroplasts containing starch granules predominate; cells with abundant, granular cytoplasm, chloroplasts, lipid, and/or crystalline deposits; and nucleated cells with abundant, homogeneous cytoplasm and no chloroplasts (Plates 8.30-8.33).

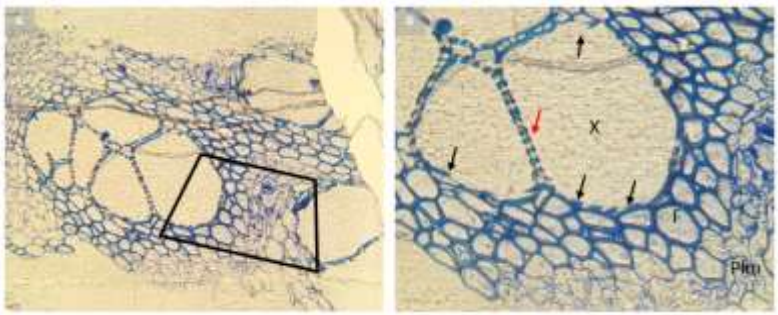


Plate 8.15. A – Semi-thin cross-section through the root of *Oenothera biennis* L. showing the TEM analysis area (trapezoidal area), 20X; B – detail of secondary wood, 40X. X – xylem, F – wood fibers, Plm – wood parenchyma, black arrow – uneven thickening of cell walls, red arrow – bordered pits.

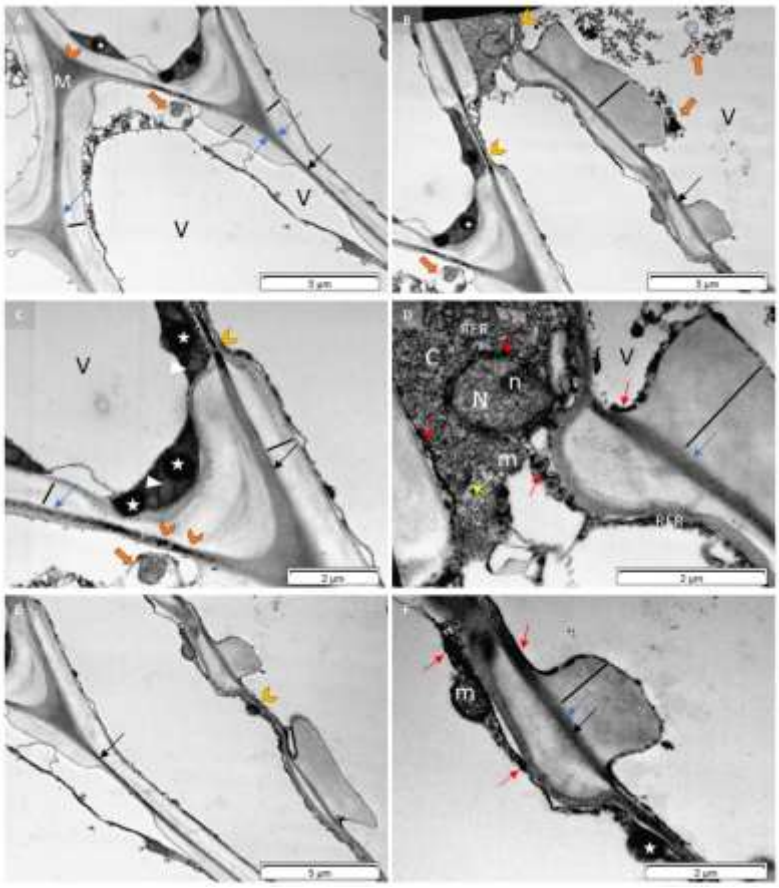


Plate 8.16. A-F – Ultrastructural features of the wood fibers in the root of *Oenothera biennis* L. V – vacuole, N – nucleus, n – nucleolus, C – cytoplasm, m – mitochondria, RER – rough endoplasmic reticulum, yellow arrow – Golgi apparatus, red arrow – electron-dense structures in the cytoplasm, white arrowhead – lipid droplets, orange arrows – transport vesicles, yellow chevron arrow – non-thickened portions of the cell wall, orange chevron arrow – electron-dense deposits in the middle lamella and intercellular space, white star – spherical electron-dense bodies, blue arrow – primary wall, black arrow – middle lamella, black bar – secondary wall.

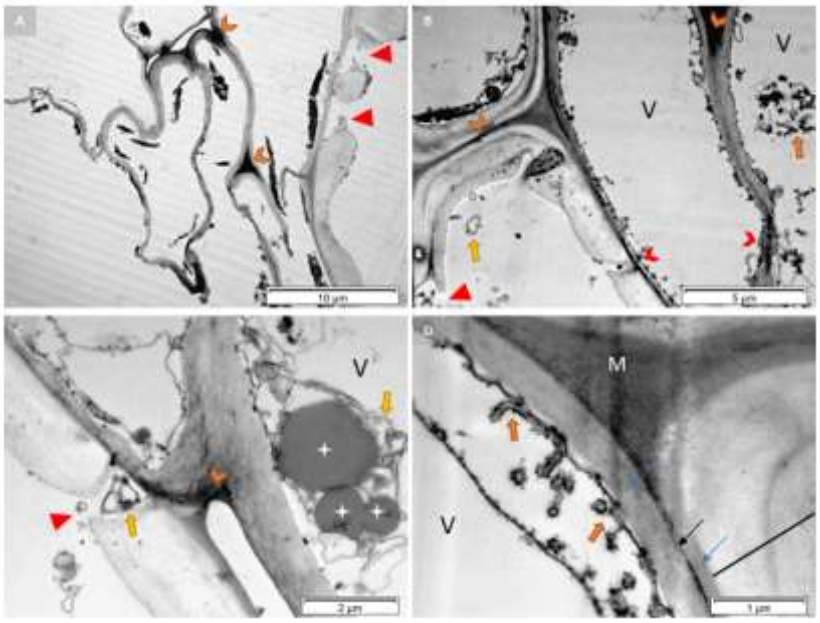


Plate 8.17. A – Wood parenchyma cells in contact with a wood vessel in the root of *Oenothera biennis* L.; B – wood parenchyma in contact with wood fibers; C–D – details of a wood fiber section. V – vacuole, M – intercellular space, blue arrow – primary wall, black arrow – middle lamella, black bar – secondary wall, orange chevron arrow – electron-dense deposits, red chevron arrow – plasmodesmata, yellow arrow – intravacuolar vesicular formations, orange arrows – transport vesicles, white star – lipid droplets, red arrowhead – pits.

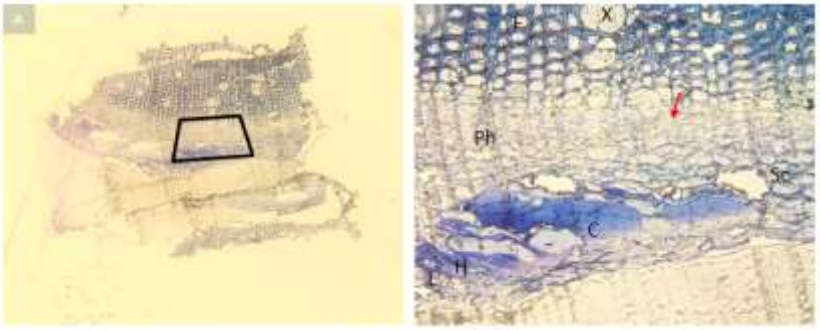


Plate 8.19. A – Semi-thin cross-section through the stem of *Oenothera biennis* L. showing the TEM analysis area (trapezoidal area), 20X; B – detail of the bark and central cylinder, 40X. E – epidermis, H – hypodermis, Sc – sclerenchymatic pericycle, C – cortex, Ph – phloem, X – xylem vessel, F – wood fibers, red arrow – cambium.

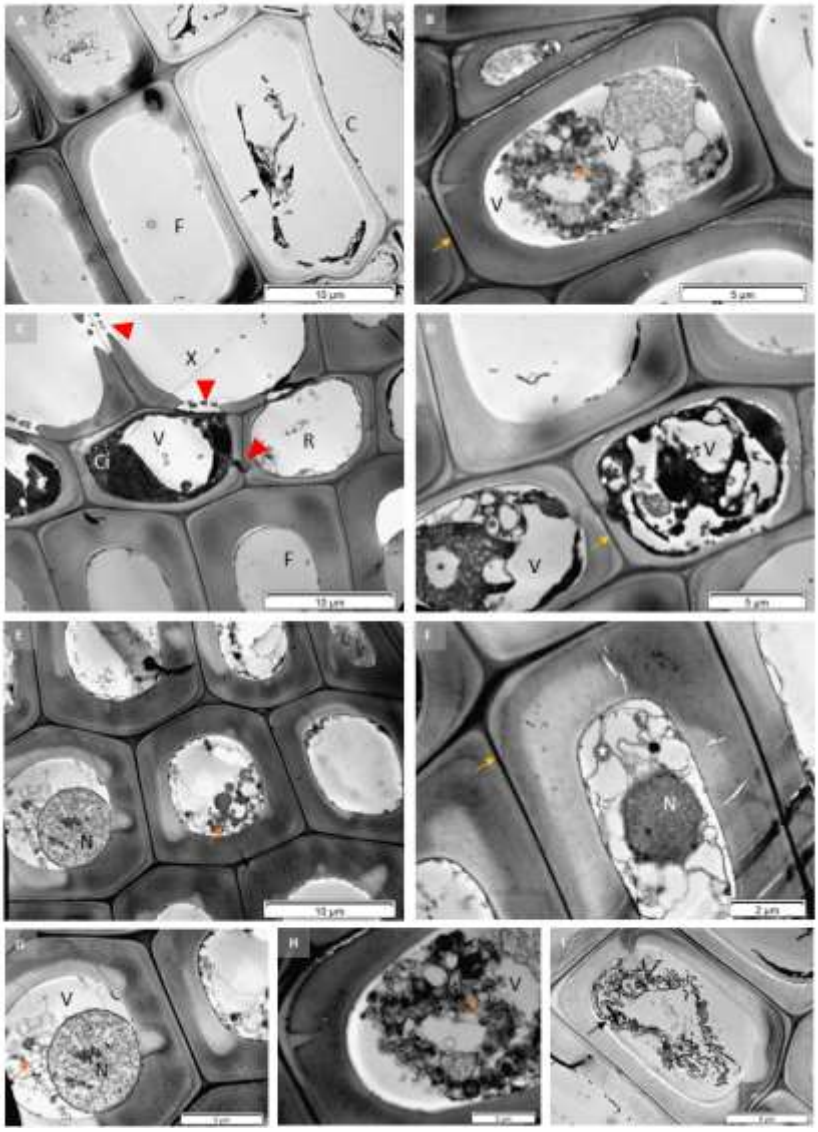


Plate 8.22. A-I – Ultrastructural features of cells in the secondary xylem of the stem of *Oenothera biennis* L; G, H – details of Figures E and B, respectively. C – cambium, F – wood fiber, X – xylem, R – medullary ray, V – vacuole, Ci – cytoplasm, N – nucleus, red arrowhead – bordered pits, yellow arrow – middle lamella, orange arrow – vesicles, black arrow – cellular debris remaining after cambium differentiation, asterisk – mucilage-containing vesicles.

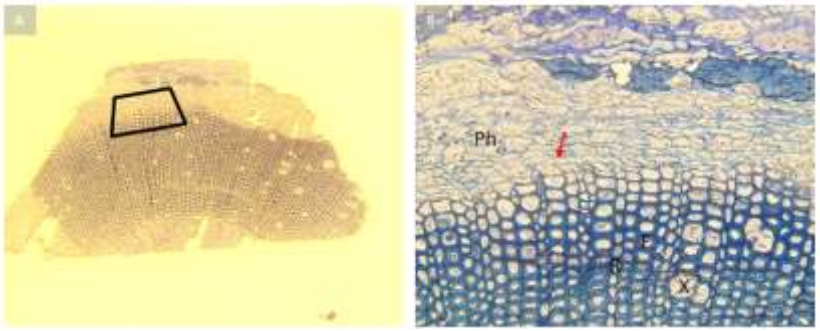


Plate 8.23. A – Semi-thin cross-section through the stem of *Oenothera biennis* L. showing the TEM analysis area (trapezoidal area), 20X; B – detail of a section of the central cylinder, 40X. Ph – phloem, R – medullary ray, F – wood fibers, X – xylem, red arrow – cambium.

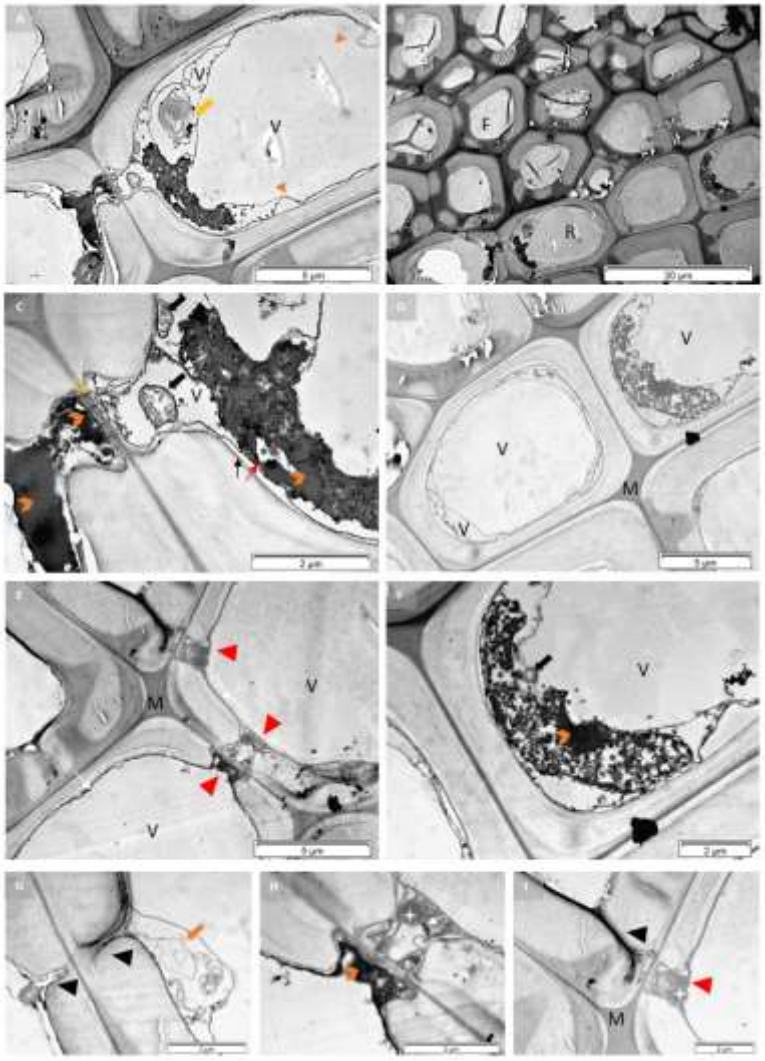


Plate 8.25. A – Ultrastructural features of medullary ray cells and wood fibers in the secondary xylem of the stem of *Oenothera biennis* L. F – wood fiber, R – medullary ray, V – vacuole, M – parenchyma, red arrowhead – bordered pits, black arrowhead – canalicular pits, orange arrowhead – vesicles partially fused with the tonoplast or the cell membrane, thick black arrow – transport vesicles, yellow arrow – plasmodesma, black arrow – plasma membrane, white arrow – cytoplasmic filament, red arrow – rough endoplasmic reticulum, white star – lipid droplet, thick yellow arrow – multilamellar body, orange arrow – multilamellar vesicles, asterisk – mucilage-containing vesicle, orange chevron arrow – osmophilic deposit.

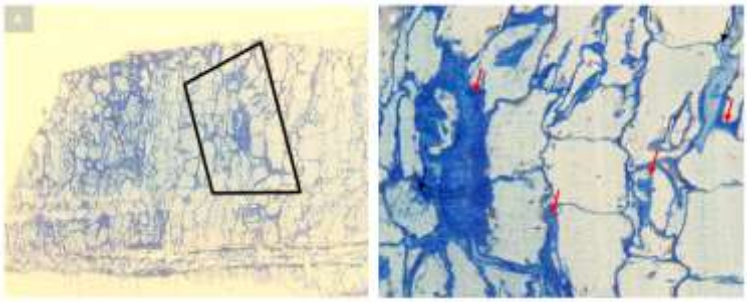


Plate 8.26. A – Semi-thin cross-section through the petiole of *Oenothera biennis* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of the analyzed area showing storage parenchyma, 40X. Red arrow – deposits intensely stained with Epoxy Tissue Stain; black arrow – lightly stained deposits indicating different substances or concentrations.

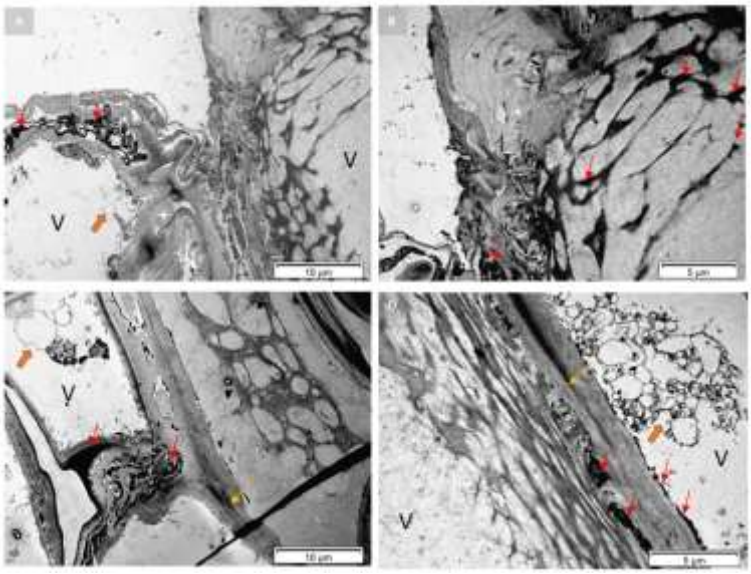


Plate 8.27. A-D – A section of parenchyma cells from the petiole of *Oenothera biennis* L., showing numerous electron-dense deposits and transport vesicles. V – vacuole, red arrow – electron-dense deposits, orange arrow – transport vesicles, yellow arrow – middle lamella, white star – lipid droplet.

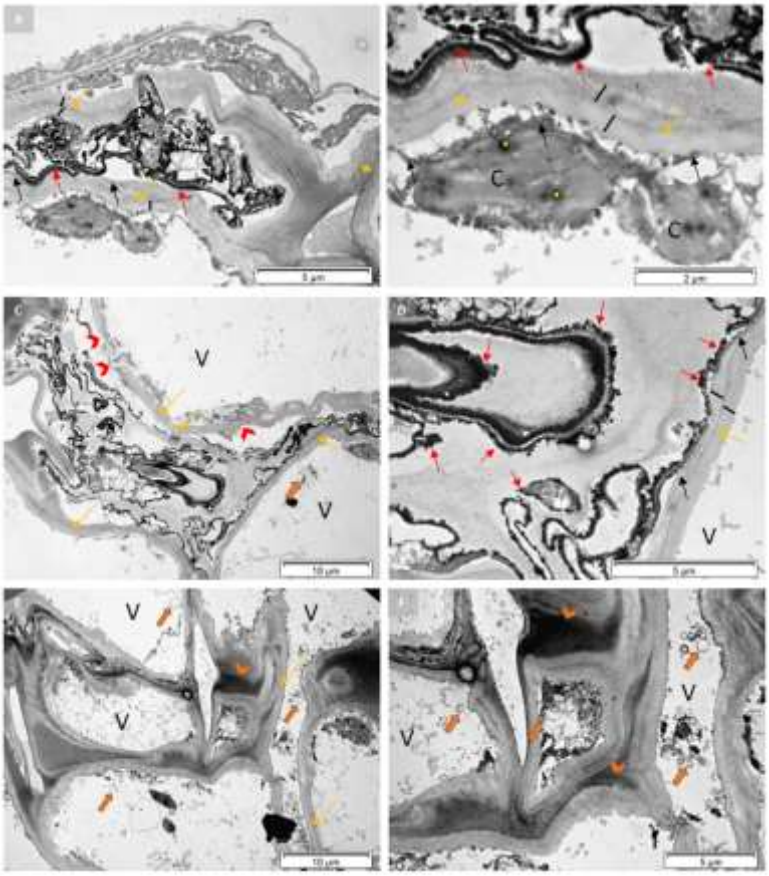


Plate 8.28. A-D – Parenchyma cells section of the petiole of *Oenothera biennis* L. with intracellular deposits of secondary metabolites; E–F – parenchyma cells section with extracellular deposits on the cell walls and multiple intravacuolar vesicles. V – vacuole, C – chloroplast, black bar – cell wall, black arrow – cytoplasmic filaments, yellow arrow – middle lamella, red arrow – osmophilic droplets, orange arrow – transport vesicles, red chevron arrow – pits, orange chevron arrow – extracellular electron-dense deposits, yellow star – plastoglobule.

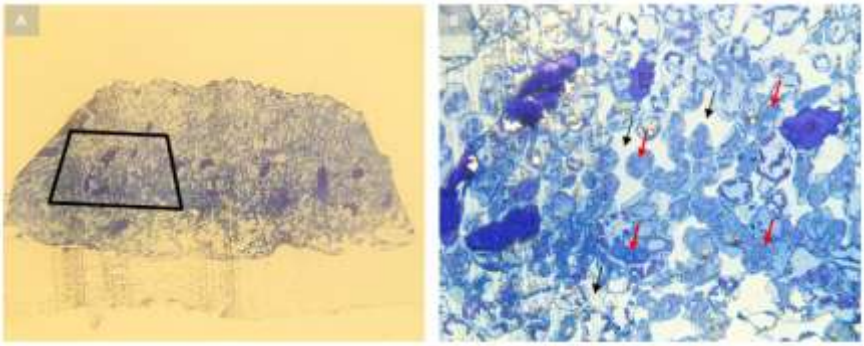


Plate 8.30. A – Semi-thin cross-section through the foliar limb of *Oenothera biennis* L. showing the area selected for TEM analysis (trapezoidal area), 10X; B – detail of the analyzed area with spongy parenchyma, 40X. Red arrow – storage cells, black arrow – air chambers.

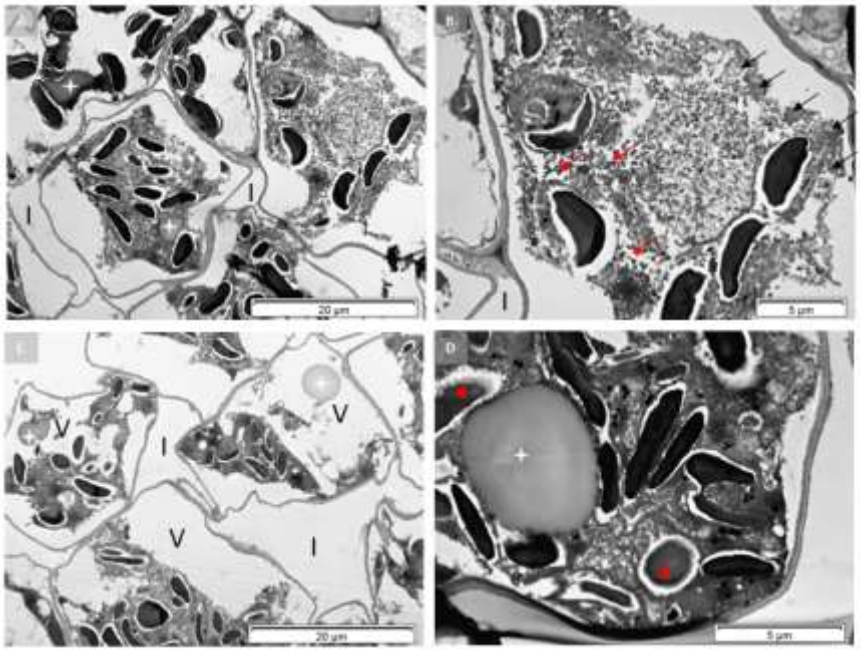


Plate 8.31. A-D – Ultrastructure of spongy tissue cells of the foliar limb of *Oenothera biennis* L. V – vacuole, l – air-chamber, white star – lipid droplet, black arrow – mitochondria, red arrow – ribosomes, red arrowhead – mucilaginous deposits.

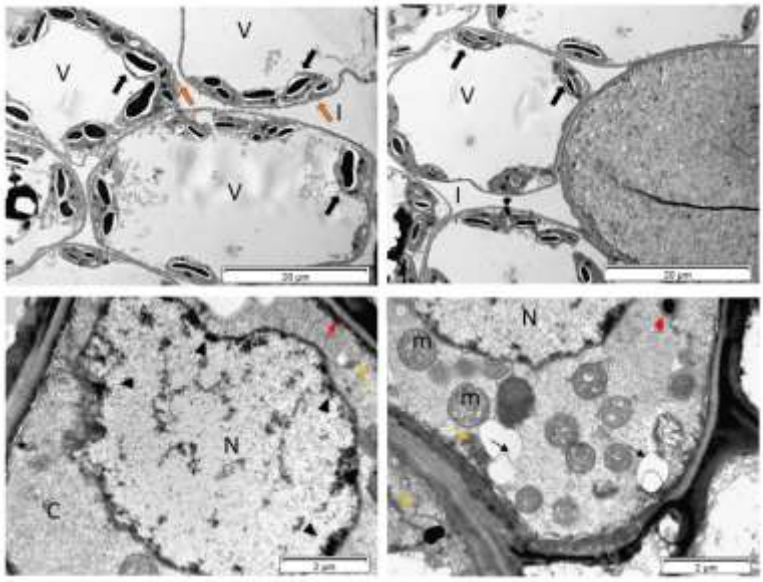


Plate 8.32. A-D – Ultrastructure of spongy tissue cells of the foliar limb of *Oenothera biennis* L. V – vacuole, l – air-chamber, N – nucleus, C – cytoplasm, m – mitochondria, black arrow (A, B) – chloroplast with large starch granules and plastoglobules, red arrow – rough endoplasmic reticulum, yellow arrow – smooth endoplasmic reticulum, black arrow (D) – vesicles, black arrowhead – inactive chromatin, red arrowhead – osmophilic structure in a single-membrane body.

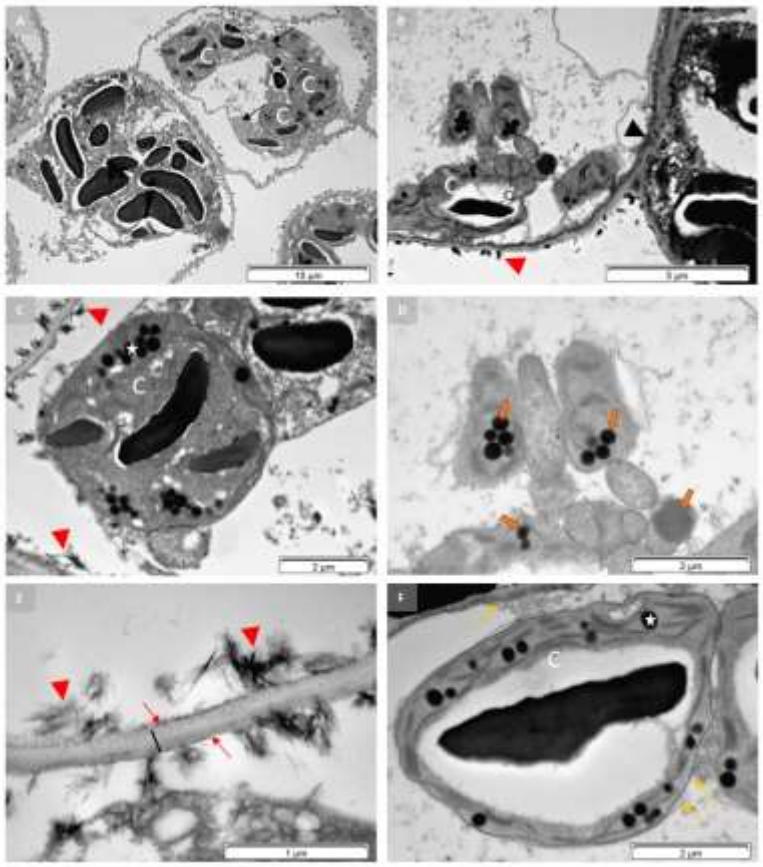


Plate 8.33. A-F – Ultrastructural features of spongy tissue storage cells of the foliar limb of *Oenothera biennis* L. C – chloroplast; white star – plastoglobule; black arrow – mitochondria; red arrow – cell membrane, black bar – cell wall, red arrowhead – osmophilic acicular structures, black arrowhead – vesicles at the level of cell wall, orange arrow – vesicles containing osmophilic droplets.

Results obtained from the phytochemical analysis of extracts prepared from *Oenothera biennis* L. plants belonging to the two populations collected from the wild flora of the two geographical areas in Romania (lowland area—Macea; mountain area—Vatra Dornei):

Qualitative and quantitative analysis of extracts from whole *Oenothera biennis* L. plants, grown under the pedoclimatic conditions of Macea, revealed 13 polyphenols in the hydroalcoholic extract and 10 in the infusion, and under the pedoclimatic conditions of Vatra Dornei, 14 polyphenols in the hydroalcoholic extract and 5 in the infusion (Tables 8.1-8.2). In accordance with the scientific literature reviewed, seven flavonoids were identified for the first time in this species: apigenin, chrysin, esculetin, hesperetin, hyperoside, luteolin, and naringenin. The quantified compounds are well known in the specialized literature for their medicinal properties and for the role they play in plant interactions with environmental factors, indicating the species' therapeutic potential. Overall, the polyphenol content of the hydroalcoholic extract is higher than that of the aqueous extract, both quantitatively and qualitatively. The polyphenol content of the extract obtained from samples collected in the lowland area is higher than that of the extract obtained from samples collected in the mountain area. Notable differences were observed in the content of chlorogenic acid, esculetin, kaempferol, luteolin, and naringenin in the hydroalcoholic extract of O. *biennis* collected from the mountain area. The amount of gallic acid was similar in both types of extract* (Graphs 8.1-8.2).

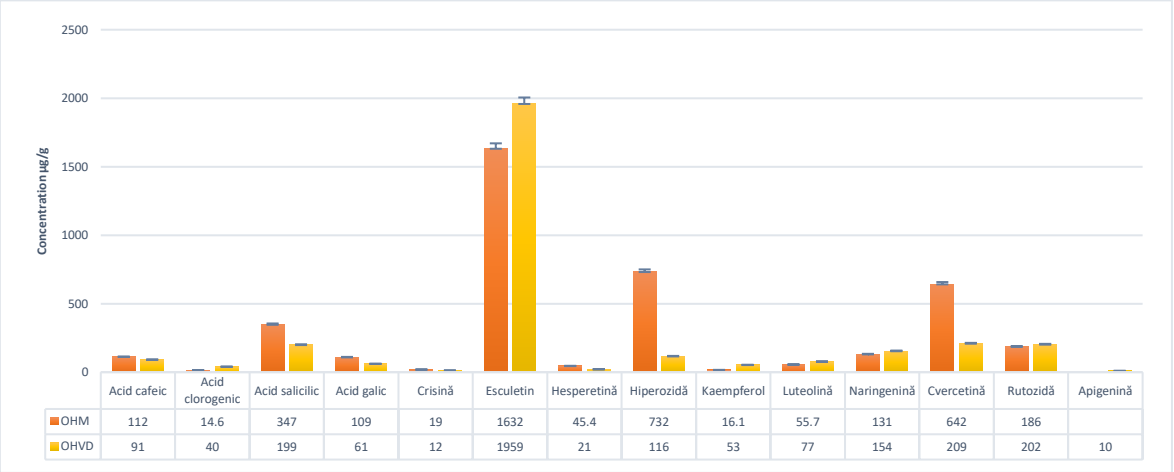
Table 8.1.

Qualitative and quantitative analysis of polyphenols in hydroalcoholic extracts of *Oenothera biennis* L. by LC/MS

No.	Compounds	Sample OHM [µg/g dry plant weight]	Sample OHVD [µg/g dry plant weight]
1	Caffeic acid	112 ± 2.8	91 ± 2.4
2	Chlorogenic acid	14.6 ± 0.81	40 ± 1.0
3	Salicylic acid	347 ± 8.7	199 ± 4.9
4	Gallic acid	109 ± 2.7	61 ± 1.6
5	Apigenin	<QL	10 ± 0.6
6	Chrysin	19 ± 1.1	12 ± 0.7
7	Esculetin	1632 ± 38.5	1959 ± 46.3
8	Hesperetin	45.4± 1.25	21± 1.2
9	Hyperoside	732 ± 18.3	116 ± 2.9
10	Kaempferol	16.1 ± 0.91	53± 1.4
11	Luteolin	55.7 ± 1.54	77± 2.0
12	Naringenin	131 ± 3.2	154 ± 3.8
13	Quercetin	642 ± 16.0	209 ± 5.2
14	Rutoside	186 ± 4.6	202 ± 5.0

Abbreviations: Sample OHM = hydroalcoholic extract, sample collection site in Macea; Sample OHVD = hydroalcoholic extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; the values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.

* Bota, V.B., Oláh, N.-K., Chișe, E., Burtescu, R.-F., Furtună, F.-R. P., Ivănescu, L.-C., Zamfirache, M.-M., Máthé, E., Turcuș, V., 2025 - A Comparative Analysis of the Polyphenolic Content and Identification of New Compounds from *Oenothera biennis* L. Species from the Wild Flora. Molecules, 30(20), 4059.



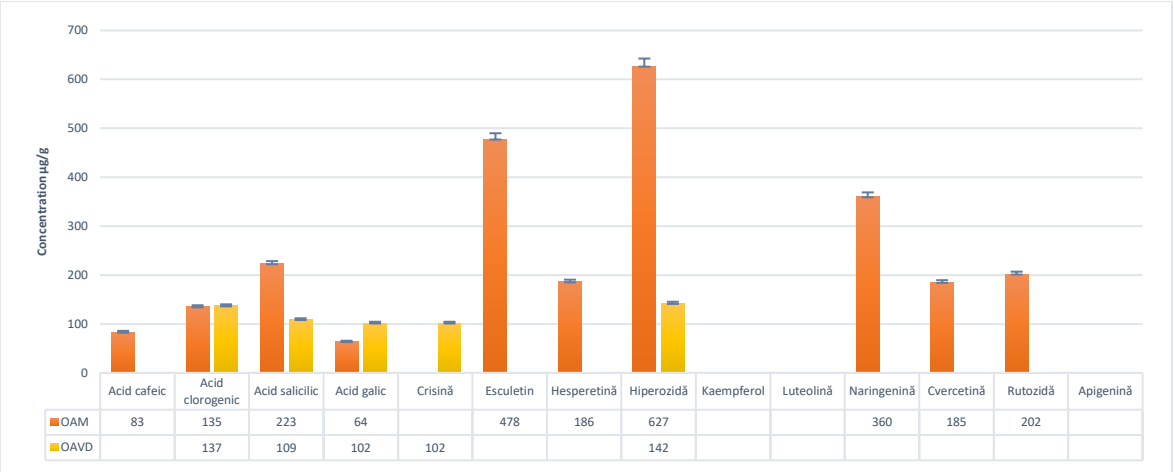
Graph. 8.1. LC/MS analysis of the hydroalcoholic extract obtained from whole *Oenothera biennis* L. plants belonging to the two populations studied—from Macea (OHM), and Vatra Dornei (OHVD).

Table 8.2.

Qualitative and quantitative analysis of polyphenols in aqueous extracts of *Oenothera biennis* L. by LC/MS

No.	Compounds	Proba OAM [µg/g dry plant weight]	Proba OAVD [µg/g dry plant weight]
1	Caffeic acid	83 ± 3.0	<QL
2	Chlorogenic acid	135 ± 3.3	137 ± 3.2
3	Salicylic acid	223 ± 5.5	109 ± 2.7
4	Gallic acid	64 ± 1.7	102 ± 2.6
5	Apigenin	<QL	<QL
6	Chrysin	<QL	102 ± 2.6
7	Esculetin	478 ± 11.9	<QL
8	Hesperetin	186± 4.6	<QL
9	Hyperoside	627 ± 15.6	142 ± 3.5
10	Kaempferol	<QL	<QL
11	Luteolin	<QL	<QL
12	Naringenin	360 ± 9.0	<QL
13	Quercetin	185 ± 4.6	<QL
14	Rutoside	202 ± 5.0	<QL

Abbreviations: Sample OAM = aqueous extract, sample collection site in Macea; Sample OAVD = aqueous extract, sample collection site in Vatra Dornei; <QL = compound below the limit of quantification; values represent the arithmetic mean ± standard deviation of three independent measurements of replicate injections from the same extract.



Graph. 8.2. LC/MS analysis of the aqueous extract obtained from whole *Oenothera biennis* L. plants belonging to the two populations studied—from Macea (OAM), and Vatra Dornei (OAVD).

8.5. Comparative analysis of the polyphenol content in extracts of Oenothera biennis L species in the context of environmental factors

The plant material subjected to phytochemical analysis in the case of *Oenothera biennis* L. species was collected in the year 2021 from the lowland area, at an elevation of 99 m, and from the mountain area at 926 m; the corresponding climatic conditions are presented in Chapter 4 and summarized in Section 6.5. The temperate-continental and cold-continental climate conditions, respectively, along with the biennial nature observed in the individuals studied, indicate that the analyzed populations belong to the group of *Oenothera* species native to North America. Therefore, the *O. biennis* L. plants analyzed are adapted to continental conditions, with lower average temperatures and moderate amounts of precipitation. Although the species' climatic requirements are better met in the lowland area, the general warming trends in Vatra Dornei—with milder winters and warmer summers—have allowed the plants to thrive even in the highland area. With regard to the edaphic factors considered, as mentioned in Chapter 4, attention must be paid to the influence that the tendency toward alkalinity of the soil at the sampling sites—particularly in the lowland area—may have on the polyphenolic composition observed in the present study, taking into account the species' preference for more acidic soils. The soil at the lowland sampling site was characterized by low levels of assimilable phosphorus, assimilable potassium, and total nitrogen, while the soil at the mountain sampling site exhibited high levels of assimilable potassium and total nitrogen. These factors may constitute an additional stress, given the species' average requirements regarding soil trophic indices.

Considering the pedoclimatic conditions of the lowland sampling site, 13 polyphenols were identified in the hydroalcoholic extract and 10 in the aqueous extract. The difference observed is marked by the absence of the compounds chrysin, kaempferol, and luteolin. Quantitatively, in the first type of extract, the main compound was esculetin and the secondary compound was hyperoside, while in the latter, the main compound was hyperoside and the secondary compound was esculetin. The hydroalcoholic extract contains higher amounts of quercetin, salicylic acid, caffeic acid, and gallic acid, while the aqueous extract contains higher amounts of naringenin, rutoside, chlorogenic acid, and hesperetin.

By comparison, in extracts obtained from *O. biennis* L. specimens collected from the mountain region, 14 polyphenols were identified in the hydroalcoholic extract and 5 in the infusion. Esculetin was the major compound in the hydroalcoholic extract, with the highest concentration among all extracts analyzed for this species (1959 μg/g dry plant). In the aqueous extract, the main compound by weight was hyperoside (142 μg/g dry plant), but in much smaller quantities than in the extracts obtained from specimens collected from the lowland. Among all four extracts analyzed, salicylic acid was present in the lowest amount recorded in the OAVD aqueous extract, which lacked most of the analyzed polyphenols; in contrast, chrysin and chlorogenic acid were present in the highest amounts (102 μg/g dry plant weight and 137 μg/g dry plant weight, respectively). Chlorogenic acid, salicylic acid, gallic acid, and hyperoside were consistently detected across all analyzed extracts. Apigenin was quantified only in the hydroalcoholic extract obtained from plants collected in the mountains, suggesting a considerable influence from both environmental factors and the extraction method.

CONCLUSIONS

Building on national and international research trends in medicinal plants over the past few decades, with the aim of identifying new compounds with therapeutic value and developing new medicinal remedies, the research conducted sought to characterize the morpho-anatomical and phytochemical properties of specimens belonging to four plant species with medicinal potential found in Romania's wild flora: *Tussilago farfara* L., *Geranium robertianum* L., *Leonurus cardiaca* L., and *Oenothera biennis* L, collected from two distinct geographic areas (lowland area—Macea, and mountain area—Vatra Dornei).

In this context, particular attention was paid to identifying the plant organs and structures involved in the production and storage of bioactive compounds in the analyzed specimens, as well as to the influence of environmental factors on their content of secondary metabolites of therapeutic interest. The results thus obtained support the therapeutic potential of the taxa studied.

The morpho-anatomical research conducted has yielded comprehensive descriptions of the structure, micromorphology, and ultrastructure of the vegetative organs of the taxa under study, representing a novelty within the scientific literature. Moreover, the TEM results obtained for the species *G. robertianum* L., *L. cardiaca* L., and *O. biennis* L. provide new data on structures such as multivesicular and multilamellar bodies, which are important for understanding cellular processes of secretion, transport, and autophagy.

Regarding *T. farfara* L. plants, the first anatomical description of the adventitious root, as well as the micromorphology and ultrastructure of the rhizome, petiole, and foliar limb, was obtained. Among the secretory and storage structures of particular interest, primarily located on/in the petiole and foliar limb, we mention the glandular trichomes. Ultrastructural analysis of the vegetative organs—the first of its kind in the scientific literature for this species—revealed accumulations of secondary metabolites in the cortical parenchyma of the rhizome, in the phloem tissues of the petiole, and in the palisade tissue of the foliar limb. Furthermore, the results contribute new data to the scientific literature regarding stromules—structures that are currently poorly understood—which were observed for the first time in this species within the palisade cells of the foliar limb.

The results obtained for taxa belonging to the species *G. robertianum* L. provide important data for its identification, characterization, and taxonomic classification, highlighting the variability of the species within Romania's wild flora. The optical and

electron microscopy analyses represent a novelty in the scientific literature. Among the most notable features observed in this species are the aquiferous parenchyma in the rhizome structure, the three types of trichomes on the stem, petiole, and foliar limb, the accumulation of tannins in the periderm and secondary vascular tissues of the adventitious root, and the accumulation of lipids and phenols in the phloemic, periphloemic and secondary woody tissues of the rhizome, as well as secondary metabolites in the hypodermis of the aerial stem, the epidermis and hypodermis of the petiole, and the epidermis and palisade tissue of the foliar limb.

The morpho-anatomical analysis conducted on individuals of *L. cardiaca* L. species provides an initial description of the structure of the underground organs and the petiole, the ultrastructure of the vegetative organs, and an initial comprehensive characterization of the structure and micromorphology of the aerial stem and foliar limb. The results highlight secretory and storage structures in the cortical parenchyma and in the secondary phloem and xylem of the rhizome, in the cortical parenchyma and secondary phloem of the aerial stem, in the vascular tissues and the periphloemic sclerenchymatic sheath of the petiole, in the spongy parenchyma of the foliar limb, as well as seven types of glandular trichomes on the stem, petiole, and foliar limb. Of particular importance to the scientific literature and a first for this species are the observations made on the multivesicular and multilamellar bodies associated with secretory pathways and autophagy processes in plants.

The morpho-anatomical studies conducted on *O. biennis* L. plants contribute to the scientific literature with the first comprehensive description of the root, stem, petiole, and foliar limb, using both optical and electron microscopy techniques. Structures involved in the secretion and storage of secondary metabolites were observed in the secondary xylem and phloem tissues of the root and stem, in the petiole parenchyma, and in the spongy tissue of the foliar limb, as well as glandular trichomes arranged on the epidermis of the aerial organs. Of particular importance are the TEM results, which reveal processes of secondary wall thickening and the unusual presence of chloroplasts and multilamellar bodies in the central cylinder of the stem.

The phytochemical analysis of extracts obtained from plants belonging to two populations of each species studied, collected from the wild flora of two distinct regions in Romania (the lowland area—Macea; the mountain area—Vatra Dornei), led to the first-ever identification of certain polyphenols in these species, as reported in the scientific literature. Furthermore, the results confirm the presence of important bioactive compounds, known in the specialized literature for their therapeutic effects, thereby supporting the medicinal

potential of the taxa under study. Qualitative and quantitative differences in polyphenol content were observed both between aqueous and hydroalcoholic extracts, as well as between extracts obtained from individuals belonging to lowland populations and those obtained from individuals belonging to mountain populations; the results are presented in the context of the pedoclimatic factors analyzed at the collection sites.

The inclusion of only two populations of each species in the study severely limited the possibility of statistical analysis and the establishment of correlations between the content of the identified polyphenols and the analyzed pedoclimatic parameters. To better understand the possible correlations between ecological factors and the variability of active compounds in the taxa under study, further research is needed, involving multiple collection sites for plant material, larger sample sizes collected over a longer period of time, and supplemented by genetic studies. In addition, a comparative qualitative and quantitative analysis of polyphenol content in different plant organs would provide valuable information regarding their sites of biosynthesis and accumulation in the plant material used in the preparation of phytotherapeutic and pharmaceutical products.

TEM images of the plant material under study often reveal distinctive subcellular structures that are not yet well understood; these will be examined in greater detail in future research that goes beyond the scope and limitations of this doctoral thesis. Throughout the analysis and discussion of the data obtained, their presence is noted, and, whenever possible, current specialized references are cited, that may offer pertinent explanations for questions that still await answers. This information gives the present scientific endeavor the potential to contribute to the description of cellular structures and processes of real importance to the field of applied plant biology.

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ANNEX 1

The contents of Annex 1 supplementing Chapter 3. Materials and Methods—3.3.2. Liquid Chromatography-Mass Spectrometry Method (LC/MS)—and the List of Scientific Activities can be found at the following link:
https://drive.google.com/file/d/1Wuq4iYDDHumj9acoVFiabx2bwt4HX1Zv/view?usp=sharing