PAISII HILENDARSKI UNIVERSITY OF PLOVDIV
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BIOLOGICAL AND PHYTOCHEMICAL RESEARCH ON PLANTS IN THE FLORA OF BULGARIA WITH A POTENTIAL FOR BIOTECHNOLOGICAL APPLICATION

DOCTORAL DISSERTATION SUMMARY

for the acquisition of a PhD educational and scientific degree Field of Higher Education: 4. Natural sciences, mathematics and informatics; Professional Area: 4.3. Biological Sciences; Doctoral Programme: Botany

Academic Advisors:

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Plovdiv, 2024

This thesis consists of 128 pages. It illustrates its claims with 39 figures and 12 tables. The works cited include 257 titles (7 of which in Bulgarian).

The experimental studies were conducted in the scientific laboratories of: the department of *Botany and Biological Education* and the department of *Developmental Biology* at the Faculty of Biology, the department of *Chemical Technology* at the Faculty of Chemistry at Paisii Hilendarski University of Plovdiv; the department of *Medical Biochemistry* at the Faculty of Pharmacy of the Medical University of Plovdiv, and the *Centre of Plant Systems Biology and Biotechnology* - Plovdiv.

The dissertation has been discussed and proposed for defence at a meeting of the department of *Botany and Biological Education* at the Faculty of Biology of Paisii Hilendarski University of Plovdiv (Protocol № 173 /28.10.2024)

The public and conclusive meeting of the scientific jury will take place on 31.01.2025 г. at 11:00 in Lecture Hall 14 of the Faculty of Biology, at Paisii Hilendarski University of Plovdiv (Plovdiv, 2 Todor Samodumov Str.).

All materials pertaining to the defence have been made available at the University Library, Rectorate Building at 24 Tsar Asen Str., Plovdiv.

Scientific jury:

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Dissertation topic: Biological and Phytochemical Research on Plants in the Flora of Bulgaria with a Potential for Biotechnological Application

LIST OF ABBREVIATIONS

SEM	Scanning Electron Microscopy
LC-MS	Liquid Chromatography – Mass-Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
UV	Ultraviolet light
DPPH	2,2-diphenyl-1-picrylhydrazyl
ATP	Adenosine triphosphate
GC-MS	Gas Chromatography – Mass-Spectrometry
UPLC-MS	Ultra-Performance Liquid Chromatography - Mass-Spectrometry
UPLC-MS/MS	Ultra-Performance Liquid Chromatography – Synchronised Mass-Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MS	Mass Spectrometry
UPLC	Ultra-Performance Liquid Chromatography
QC	Quality Control

INTRODUCTION

Plants are among nature's many gifts (Oluwafemi *et al*., 2020). They have been used as a source of food and medicine since ancient times. According to the World Health Organization, approximately 80% of the world's population mainly uses medicinal plants for many diseases due to their healing properties, which are made possible by the biologically active substances they contain (Elansary *et al*., 2019).

It is because of the biologically active substances found in medicinal plants that the latter have antimicrobial, antiviral, antifungal, and antioxidant properties (Oluwafemi *et al*., 2020). Thus, they are widely studied and are essential for biotechnological and pharmaceutical research (Mumtaz *et al.,* 2017).

The core subjects of analysis in this dissertation are the species *Marrubium friwaldskyanum* Boiss., *Marrubium peregrinum* L., *Marrubium vulgare* L., and *Centaurea thracica* (Janka) Hayek, which are studied for their phytochemistry, biological activities, and potential for biotechnological application.

LITERATURE REVIEW

Botanical Characterization

Marrubium friwaldskyanum is a Bulgarian endemic, included in the Red Book of Bulgaria, Volume 1. Plants and Fungi (Peev, 2015), under the category "vulnerable" (VU) and listed in the Red List of Bulgarian vascular plants

(Petrova & Vladimirov, 2009) also under the "vulnerable" (VU) category. Its habitats in Bulgaria are located within the European ecological network of protected areas “Natura 2000” (Zheljazkov *et al.*, 2022). Its stem is single or branched, square and fibrous, and it can reach 20 to 60 cm. The leaves are simple and arranged on short 1.2-5.5 cm long and 0.7-3.4 cm wide petioles. The leaves are round or elliptical in shape and evenly serrated. The flowers are yellow to creamy yellow (Jordanov, 1989). It grows in shallow and dry soils, more often on limestone. *Marrubium friwaldskyanum* inhabits the middle and western parts of the Rhodope Mountains and the Thracian Plain (Meshinev, 2015).

Marrubium vulgare has similar morphological characteristics to those of *M. friwaldskyanum.* The stem is 20-80 cm tall, erect and branched. The leaves are ovate to rounded, up to 3 cm long, irregularly and bluntly serrated along the edge, and layered with white hairs. The flowers are arranged in beds of 20-50, with linear bracts. The calyx is bell-shaped. The corolla is white, bilabiate, and short-tubed. The upper lip is erect, bilobed to its middle, and the lobes are narrow and ribbon-shaped. The lower lip is curved and three-lobed, the middle lobe being rounded. The lateral lips are shorter and lanceolate, and the two lips above are densely covered with simple tiny hairs. There are four stamens; the two outer ones are longer but not coming out of the corolla tube. The dry fruit breaks into four elliptical, tubular, and bare nuts. (Kuzmanov et al., 2006). *Marrubium vulgare* was one of the best-selling herbal supplements to the general public in the USA in 2018 (Smith & Sawyer, 2019). The species is becoming increasingly rare in Bulgaria.

Marrubium peregrinum L. is a perennial plant of the Lamiaceae family with a square stem that is 25-105 cm tall and strongly branched. Its leaves are 0.2-1.4 cm long, single, lanceolate, absolutely entire at their base, then upwards unequal, sharp and rough to serrated. The flowers are white. The fruit is a nut (Jordanov, 1989). It inhabits dry grassy and rocky areas near roads and ruderal terrains up to 1000 m above sea level. It is found in Europe and Central Asia and belongs to the Pontic-Mediterranean floristic region (Diklić, 1974).

Most studies on the *Marrubium* genus have been conducted on species distributed in Turkey and Iran, as the plants of the genus are distributed in the Mediterranean region, Asia, and Africa (Akgül *et al*., 2008; Talebi *et al*., 2019).

Centaurea thracica is a perennial plant 50-90 cm tall and typically light green. The stem is erect and single or having 2 to 6 branches at the top. It thickens below the inflorescence and it is densely hairy with branched trichomes. The leaves are densely spaced and covered with branched trichomes. They are joint and lyre-like at the base of the plant, while the tips are oblong or triangular. The middle and upper leaves are simple, elongated, ovate-lanceolate, and pointed towards the tip. The petals that form the involucre are ovate or

dome-shaped, 23-35 x 20-28 mm in size, arranged in several rows, epidermal, yellow-green, and without hairs. Their veins extend from the tips as single spikes 4 to 8 mm long. The flowers are yellow and bisexual (Negaresh & Rahiminejad, 2015). It is common in Bulgaria, Greece, and Turkey. It inhabits mainly grassy areas and undergrowth 180 to 1300 m above sea level (Negaresh & Rahiminejad, 2015).

Phytotherapeutic Properties

Representatives of the *Marrubium* genus have a favourable impact on the human body. They are used in folk medicine worldwide to cure various diseases (Yabrir, 2018). Medicinal substances from plant species of the *Marrubium* genus manifest antinociceptive (De Jesus *et al*., 2000), antihypertensive (El Bardai *et al*., 2004), antispasmodic (Rigano *et al*., 2009), antiedematogenic (Stulzer *et al*., 2006), analgesic (Meyre-Silva *et al*., 2005), insecticidal (Pavela, 2004), anti-inflammatory (Sahpaz *et al*., 2002a), antimicrobial (Rigano *et al*., 2007), anti-helicobacterial (Ramadan & Safwat, 2009), cytoprotective (Martin-Nizard *et al*., 2003), and significant antileukemic activities (Alkhatib *et al*., 2010).

The *Centaurea* genus has attracted great interest in developing new pharmaceutical forms (Zengin *et al*., 2018). Representatives of this genus have a diuretic and slight astringent effect, and they are also used as bitter tonics for stomach issues (Al-Easa & Rizk, 1992). They are used to treat rheumatism, diabetes, diarrhoea, and hypertension (Sarker *et al*., 1997). The species of the genus have a variety of biological effects, including antimicrobial, antifungal, and antiplasmodial (Kaskoos, 2013). They display antiulcerogenic, antioxidant, antiviral, antiprotozoal, and anticancer properties (Pires *et al*., 2018).

Phytochemical Characterization

The *Marrubium* genus contains several biologically active substances, mostly phenolic compounds (Kozyra *et al*., 2020). Free phenolic acids have been identified in extracts of *M. vulgare* and *M. friwaldskyanum*: ferulic, p-coumaric, caffeic, and gentisic acids (Kozyra *et al*., 2020).

Various compound groups have been isolated in phytochemical studies on *M. peregrinum*: flavones (apigenin and luteolin) (Sahpaz *et al*., 2002b), flavonols (kaempferol) (Nagy *et al*., 1996), glycosylated flavonoids, derivatives of caffeic acid (Gruenwald & Brendler, 2000) and four diterpenoids (peregrinin, peregrinol, marrubiin, and premarrubiin) (Telek *et al*., 1997).

Zheljazkov *et al*. (2022) have discovered 45 compounds in *M. frivaldskyanum*, identified as flavonoids, phenolic acids, and (tri)terpenoids.

It has been established that *Marrubium vulgare* contains marrubin, a labdane diterpene characteristic of this genus, as well as a complex mixture of phenolic compounds (Aćimović *et al*., 2020).

For the *Centaurea* genus, the most common compounds are sesquiterpene lactones (cynaropicrin, grossgemin, repin). They are typical compounds in plants from the Asteraceae family (Rustaiyan & Faridchehr, 2021). Flavones (apigenin, luteolin, lutein, flavonols), quercetin, and cinnamic acids have also been identified (Fernandes *et al*., 2019).

The methanolic extract of fresh *Centaurea scabiosa* flowers contains coumaran (2,3-dihydrobenzofuran), 5-hydroxymethyl furfural, 3-hydroxy-4-methoxybenzoic acid, methyl ester and 3-methyl-coumarin. These components amount to 80% of all identified substances (Sharonova *et al*., 2021).

Biological Activity of Phenolic Compounds

Phenolic compounds are products of plant secondary metabolism. They are responsible for pigmentation and astringency, they act as protective agents against UV light, and protect plants from parasites and insects (Durazzo *et al*., 2019). They are natural bioactive molecules found mainly in plant tissues (Albuquerque *et al.,* 2021). The intake of antioxidants facilitates the decrease of reactive oxygen forms, so that particularly acute and chronic diseases may be prevented (Neha *et al*., 2019).

The flavonoids apigenin, chrysin, naringenin, kaempferol, quercetin, daidzein, and genistein prevent the formation of a biofilm, while quercetin, luteolin, myricetin, and baicalein inhibit the replication of bacterial DNA (Jucá *et al*., 2020).

Plant extracts obtained from herbs and applied as antitumour agents have a cytotoxic effect against tumour cells without affecting the cell viability of healthy cells (Mouhid *et al*., 2018). Little is known about the anticancer activity of the *Marrubium* genus (Kozyra *et al*., 2020).

Analyses have been made on the cytotoxic activity of phenolic acids featured in methanol extracts of a cultivated *M. friwaldskyanum* against cancer and normal cells. Cytotoxic activity of the non-hydrolyzed fraction of phenolic acids on a human melanoma cancer cell line has been identified, and no activity against normal cells has been detected (Kozyra *et al*., 2020).

PURPOSE AND OBJECTIVES OF THE DISSERTATION

The present dissertation aims to evaluate the significance of the species *Marrubium friwaldskyanum*, *Marrubium peregrinum, Marrubium vulgare,* and

Centaurea thracica for the development of biotechnological products and their application as medicinal plants through biological and phytochemical methods.

The following tasks were set as related to the aim:

1. Collection of samples from the species *Marrubium friwaldskyanum*, *Marrubium peregrinum, Marrubium vulgare*, and *Centaurea thracica* found in Bulgaria.
2. Anatomical analysis of *Marrubium friwaldskyanum* and *Marrubium peregrinum*.
3. Phytochemical study of the species *Marrubium friwaldskyanum*, *Marrubium peregrinum,* and *Centaurea thracica*.
4. Analysis of biological activities of *Marrubium friwaldskyanum and Marrubium peregrinum* extracts.
5. Determination of the protein content, oiliness, carbohydrate content, mineral substances, and crude fibers in *Centaurea thracica.*

MATERIALS AND METHODS

Materials

In the period 2019-2021, above-ground parts of the *Marrubium friwaldskyanum* Boiss. species were collected during the vegetation period on the territory of the medieval fortress Tsepina - in the Batashka mountain (figure 1).

In the period 2020-2021, above-ground parts of the *Marrubium peregrinum* L. species were collected during the vegetation period in the heights above the town of Krichim.

Marrubium vulgare above-ground parts have been collected during the vegetation period above the town of Kuklen.

In 2020 and 2021, above-ground parts of *Centaurea thracica* (Janka) Hayek during vegetation (blossoming and seed-formation) in the vicinity of the town ot Tsarevo. The blossoming inflorescences of *Centaurea thracica* were collected in June, and the mature inflorescences - in September.

Herbarium specimens have been deposited in the herbarium of the Agricultural University - Plovdiv (SOA) (figure 2).

Figure 1. Study and collection of *Marrubium friwaldskyanum.*

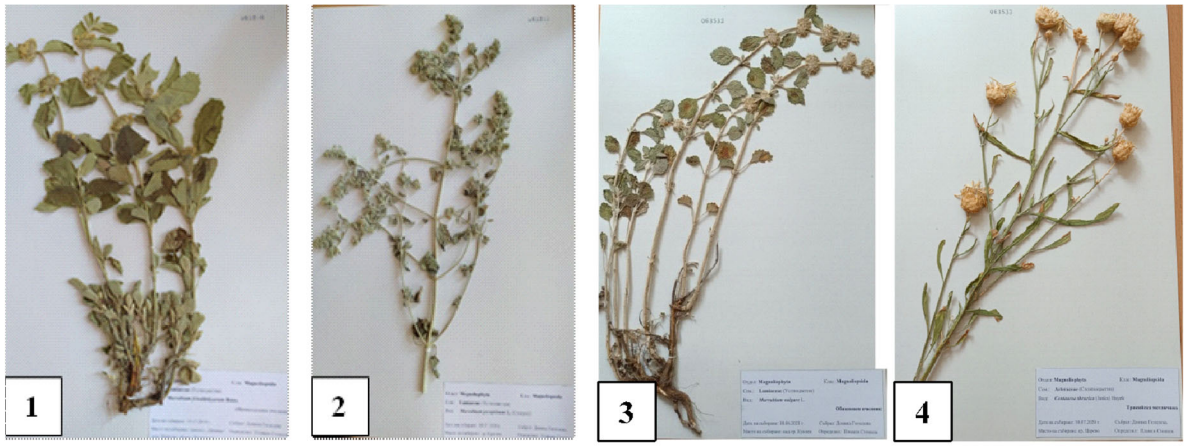

Figure 2. Herbarium specimens of the studied plants: 1- *Marrubium friwaldskyanum* (№ 063316); 2 - *Marrubium peregrinum* (№ 063315); 3 - *Marrubium vulgare* (№ 063532); 4 - *Centaurea thracica* (№ 063533).

Methods

Methods used in the study of the species of the *Marrubium* genus

The microscopic analysis of *M. friwaldskyanum, M. peregrinum, M. vulgare* was conducted with the Magnum T Trinocular light microscope that features the Si5000 photo documentation system. The leaves and stem analysis was performed following the classical methods of Metcalfe & Chalk (1950).

Dry powder of lyophilised samples of the entire *M. peregrinum* plant and of the leaves, stems, and flowers of *M. friwaldskyanum* was subjected to extraction and metabolite fractionation, the semipolar phase was used for GC-MS and UPLC-MS analyses, and the nonpolar one - for lipid study. The derivatization of the primary metabolites was performed according to Lisec *et al.* (2006).

The extraction was conducted as per Giavalisco *et al.* (2011) and Salem *et al.* (2020) with some small changes.

The identification and subsequent annotation of the metabolites were completed with the help of an in-house reference compound library, tandem MS (MS/MS) fragmentation, and metabolomics (Alseekh *et al.*, 2021).

For the annotation of metabolites measured by GC-MS, use was made of the Golm Metabolome Database (Kopka *et al.*, 2005). Lipid annotation was performed mainly by an in-house library search based on full scan MS^1 consisting of standalone standards as described in the method of Hummel *et al.* (2011).

For the identification of the mineral contents the samples were subjected to microwave digestion according to the protocol described by Miller (1998).

The GC and LC datasets were subjected to a multivariate analysis using MetaboAnalystR (Pang *et al.*, 2022) and R software, version 4.3.1.

The cytotoxicity and possible antitumor activity of *M. peregrinum* and *M. frivaldskyanum* extracts were analysed in vitro using four human cell lines: A549 (ATCC CCL-185™), isolated from lung adenocarcinoma; HeLa (ATCC CCL-2™) - cervical adenocarcinoma; HT-29 (ATCC HTB-38 HT29), derived from colorectal adenocarcinoma, and HFFC - fetal foreskin fibroblasts (CLS Cell Lines Service GmbH, Eppelheim, Germany).

Two *in vitro* cytotoxicity assays were performed: an MTT test based on reduction of 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide (MTT) of metabolically active cells (Edmondson *et al.*, 1988), and an NR test, whose principle of operation is connected to the uptake of neutral red (NR) in the lysosomes of live cells in the examined sample (Repetto *et al.*, 2008).

HT-29 cells were used to obtain tumour spheroids. Measurements were made of the obtained spheroids diameter after 24 and 96 hours of cultivation with the Inverso microscope (Medline Scientific, Chalgrove, Oxon, UK) and the help of a high-resolution Si-3000 camera, and specialised software (Medline Scientific, Chalgrove, Oxon, UK).

In order to examine the antibacterial activity of the *M. peregrinum* and *M. frivaldskyanum* samples, diffusion tests were conducted on two types of microorganisms – a Gram-negative /Escherichia coli (ATCC 25922□)/ and a Gram-positive /Bacillus cereus (ATCC 11778□)/ species.

The statistical analyses were made with the SPSS statistics software, version 17.0 (SPSS Inc., Chicago, IL, USA).

Methods used in the study of *Centaurea thracica*

- Total nitrogen and protein determination method (Association of Official Analytical Chemist, 2016).
- Oil content determination with a Soxhlet apparatus (ISO 659:2009).
- Total carbohydrate content determination (Food and Agriculture Organization of the United Nations, 2003).
- Crude fibre determination (BDS EN ISO 6865:2001).
- Ash content determination (BDS 13491:1976).
- Determination of moisture content and volatile substances (BDS ISO 711:1997).
- Energy value determination (Food and Agriculture Organization of the United Nations, 2003).
- Determination of individual fatty acid composition of glyceride oil (ISO 12966-1:2014, ISO 12966-2:2017).
- Iodine value determination (American Oil Chemists Society, 2022).

- Tocopherol composition determination (ISO 9936:2016).
- Isolation of phospholipids and individual phospholipids composition determination (Folch *et al.*, 1957, ISO 10540-1:2014).
- The index of Atherogenicity is calculated based on the oil's fatty acid composition (Ulbricht & Southgate, 1991).
- The index of Thrombogenicity is calculated as the ratio between prothrombogenic and antithrombogenic acids (Ulbricht & Southgate, 1991).
- The statistical analysis was performed using the statistical function of *Microsoft Office Excel*.

RESULTS AND DISCUSSION

Results obtained in the studies of the *Marrubium* species

Anatomical Analysis of *Marrubium friwaldskyanum* and *Marrubium peregrinum*

The leaf surface analysis established that the main cells in the two species possess cell walls curved in a zigzag pattern and that their size varies. This observation is consistent with the Aneli (1975) classification and the studies of Mladenova *et al.* (2019). The two taxons have mainly diacytic stomata, but some with anomocytic stomata are also present (figure 3).

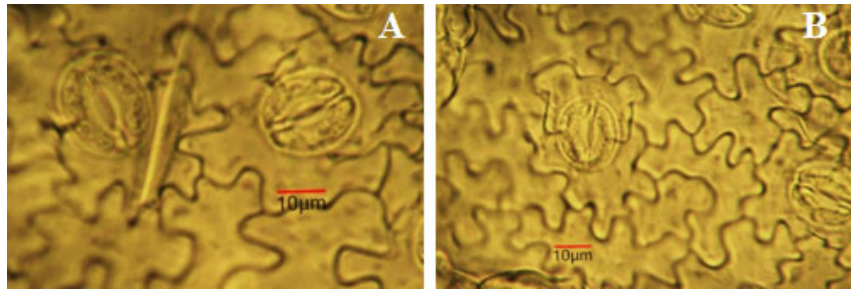

Figure 3. Diacytic and anomocytic stomatal apparatus in *Marrubium friwaldskyanum* (A) and *Marrubium peregrinum* (B).

The indumentum of *M. friwaldskyanum* is represented by two types of trichomes – non-glandular and glandular. The non-glandular trichomes can be unicellular linear and multicellular highly branched (figure 4A, B). According to their structure, the glandular trichomes are peltate stacked (figure 4C), with a short unicellular stalk (figure 4D, E), or with a bicellular structure (figure 4F).

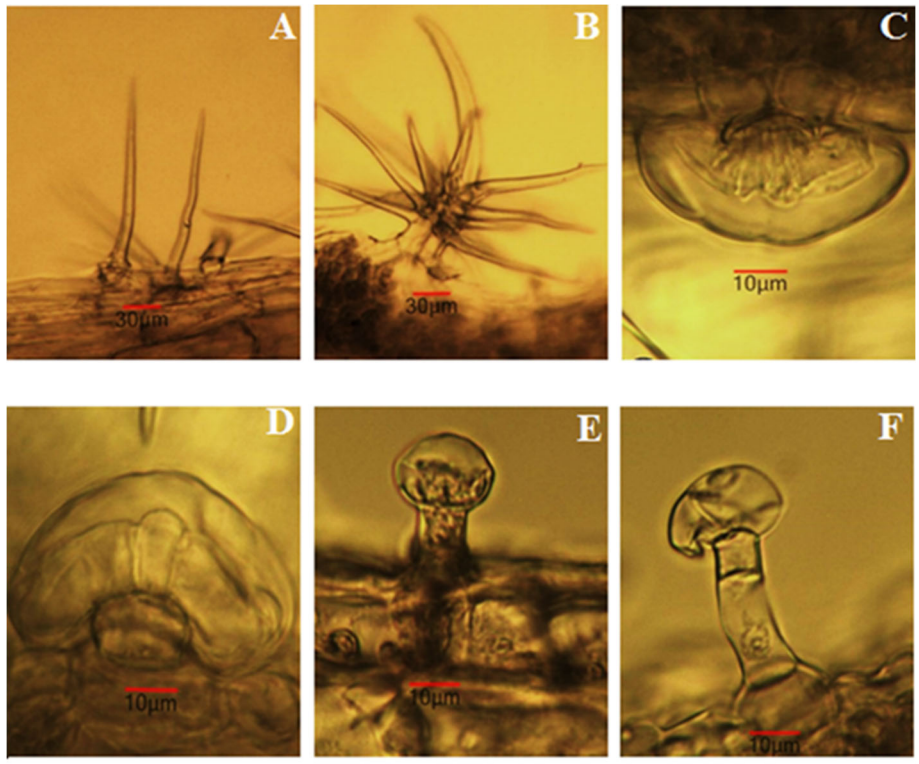

Figure 4. Types of trichomes in *M. friwaldskyanum*. A – unicellular linear non-glandular trichomes; B – multicellular branched non-glandular trichome; C – glandular peltate trichome; D, E – glandular trichome with unicellular stalk; F – glandular trichome with bicellular stalk.

The trichomes identified in *M. peregrinum* are displayed in figure 5.

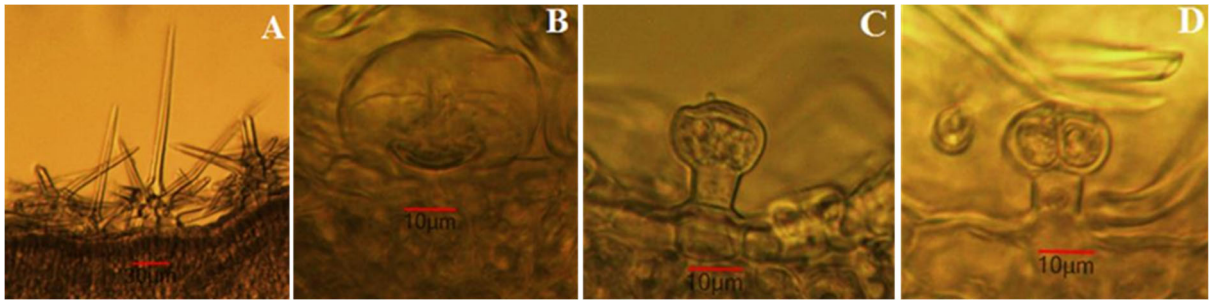

Figure 5. Types of trichomes in *M. peregrinum*. A – multicellular branched non-glandular trichomes; B – glandular peltate trichome; C – glandular trichome with a unicellular stalk; D – glandular trichome with a unicellular stalk and a two secretory cells.

Cross-sectional studies of the leaf lamina of the two species show a bifacial structure. In *M. friwaldskyanum*, adaxial and abaxial epidermises are easily distinguishable with a mesophyll consisting of one row of palisade tissue on the adaxial surface and spongy cells on the abaxial surface (figure 6A). In *M. peregrinum*, columnar cells are found on the two surfaces (figure 6B). The

central vein of the leaves in the two species is represented by closed collateral vascular bundles (figure 6C, D).

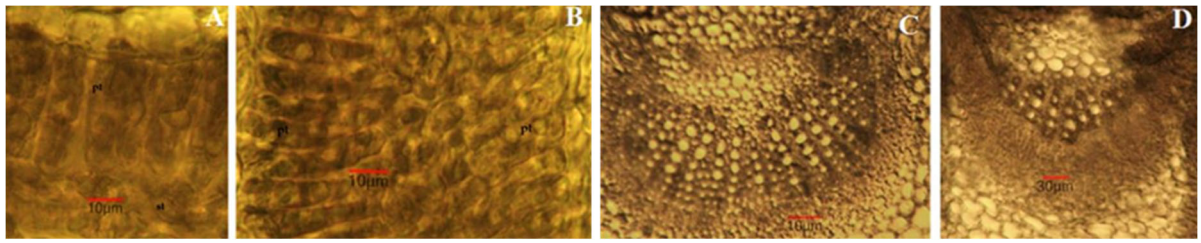

Figure 6. Cross-section of the leaf lamina. A – *Marrubium friwaldskyanum*; B – *Marrubium peregrinum*; C – collateral bundle in *Marrubium friwaldskyanum*; D – collateral bundle in *Marrubium peregrinum*. (pt – palisade tissue, st – spongy tissue).

A cross-section of the stem clearly reveals a cortex and a pith. Parenchymal cells are mainly present in the cortex, with several layers of collenchyma observed in the corners. The conductive tissues have no bundles in their structure (figure 7).

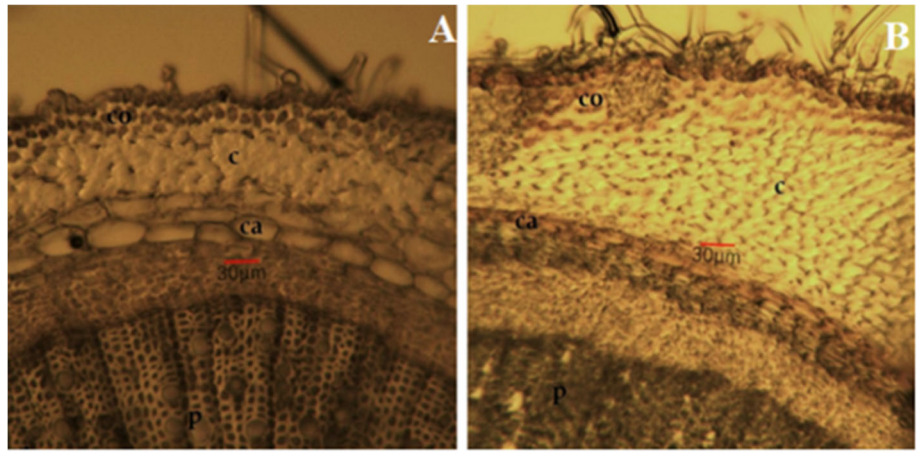

Figure 7. Cross-section of the stem. A – *Marrubium friwaldskyanum*; B – *Marrubium peregrinum*. (co – collenchyma, c – cortex, ca – cambium, p - pith).

On the stem surface of *M. friwaldskyanum,* there are both unicellular linear and multicellular branched non-glandular trichomes (figure 8 A, B). The glandular trichomes in this species are of two types: with a unicellular stalk and a unicellular secretory cell, and with a bicellular stalk and a two secretory cells (figure 8 C, D).

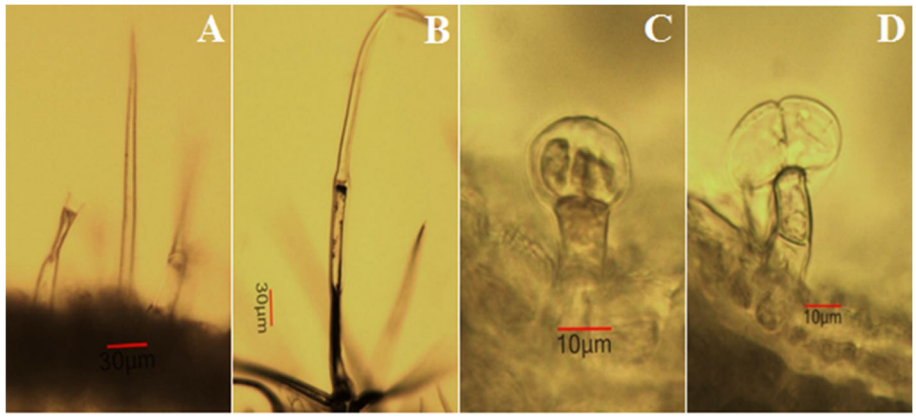

Figure 8. Types of trichomes in *Marrubium friwaldskyanum*. A – unicellular linear non-glandular trichomes; B – multicellular branched non-glandular trichomes; C – glandular trichome with a unicellular stalk and a secretory cell; D – glandular trichome with a bicellular stalk and a secretory cell.

In *M. peregrinum*, the non-glandular trichomes are multicellular branched, and the glandular trichomes are composed of a bicellular stalk and a two secretory cells (figure 9).

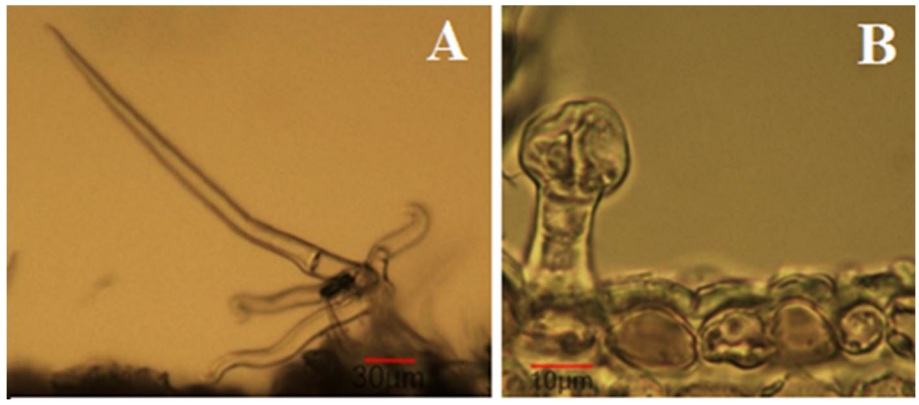

Figure 9. Types of trichomes in *Marrubium peregrinum*. A – a multicellular non-glandular trichome; B – a glandular trichome with a bicellular stalk and a bicellular secretory cell.

GC-MS analysis of primary metabolites

A total of 80 metabolomic markers (peaks) were identified and classified into amino acids, organic acids, sugars, and sugar alcohols.

The principal component analysis (figure 10) explains approximately 70.5% of the variation in the samples, with the first principal component (PC1) accounting for 53.5% of the variation, while the second principal component (PC2) – 17% of it.

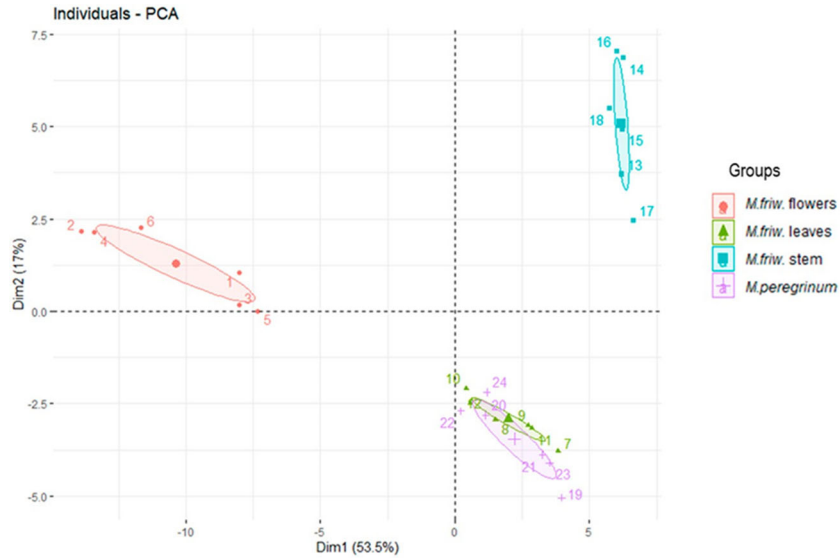

Figure 10. Score plots of PCA based on a GC-MS analysis, using Ward's clustering algorithm. The four samples are presented as follows: *Marrubium friwaldskyanum* flowers — red circles, *Marrubium friwaldskyanum* leaves — green triangles, *Marrubium friwaldskyanum* stem — cyan squares, and *Marrubium peregrinum* — purple crosses.

The four sample groups were clearly distinguished into three distinct clusters: *M. friwaldskyanum* flowers, *M. friwaldskyanum* stem, and *M. friwaldskyanum* leaves, and *M. peregrinum.* This indicates that the primary metabolite composition of *M. friwaldskyanum* leaves is not significantly different from that of *M. peregrinum.*

The hierarchical clustering analysis, HCA (figure 11), shows similar clustering as it recognizes the leaves of *M. peregrinum* and *M. friwaldskyanum* as having a higher degree of similarity than the leaves and stems of the respective species.

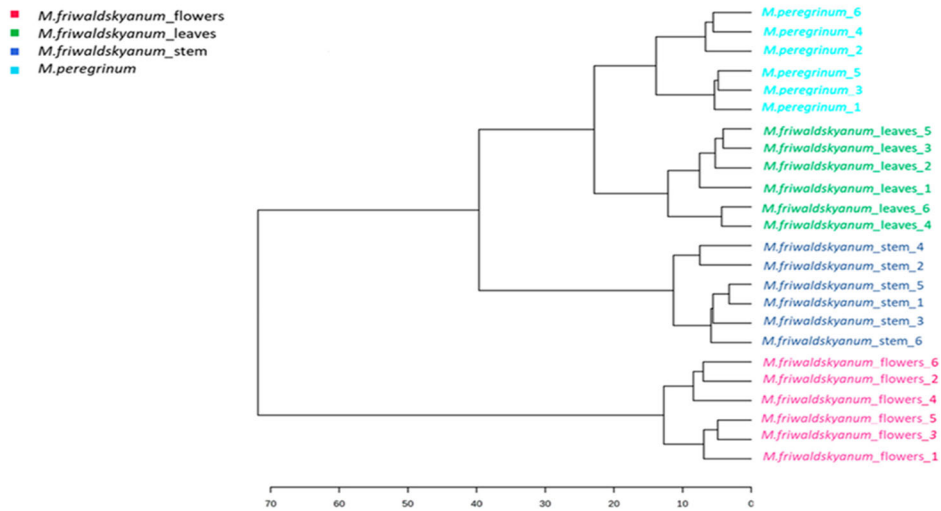

Figure 11. Hierarchical Clustering Analysis (HCA) based on a CG-MS analysis, employing Ward's clustering algorithm.

VIP (Variable Importance in Projection) scores were calculated for the top 20 metabolites in order to distinguish the most prominent markers among all sample groups (figure 12).

The unique compound from the selected top 20, distinguished in terms of its discrete levels, is the sugar alcohol galactinol, very often related to the protection of plant cells from oxidative damage and represented in the highest concentrations in the *M. peregrinum* samples (figure 12). The first 20 metabolites for each of the sample groups (figure 13A–D) have been specifically denoted.

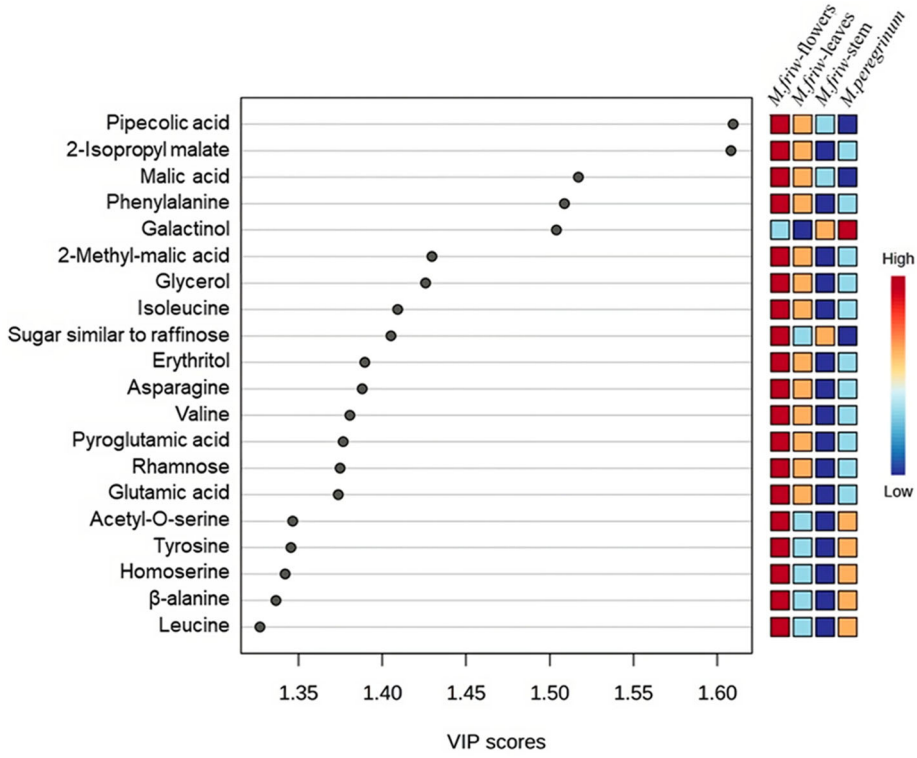

Figure 12. VIP scores indicate the potential markers among the four groups of samples.

The results show that the flowers of *M. friwaldskyanum* are clearly distinguishable from the rest of the samples mainly due to a high accumulation of amino acids. Leucine, β-alanine, homoserine, tyrosine, acetyl-O-serine, valine, asparagine, isoleucine, and phenylalanine are among the amino acids widely represented in *M. friwaldskyanum* flowers and have a contribution as important variables in comparison with the rest of the samples.

Malic acid and its derivatives, as members of the class of organic acids, were detected in addition to some sugars and sugar alcohols such as rhamnose, raffinose-like sugar, galactinol, and erythritol, but the main distinction was based on amino-acid composition.

In addition to the high levels of ornithine, homoserine, tyrosine, arginine, glutamine, pyroglutamic acid, glycine, glycerol, and isopropyl malate were detected in the flowers of *M. friwaldskyanum* (figure 13A).

In comparison with flowers, where the diversity of the group is mainly justified by the amino acid content (nearly 80% of the highly represented markers are amino acids), the distribution of the top 20 metabolites of leaves includes changes of approximately 45% in sugars and sugar alcohols, and nearly the same percentage of amino- and organic acid mixtures (figure 13B).

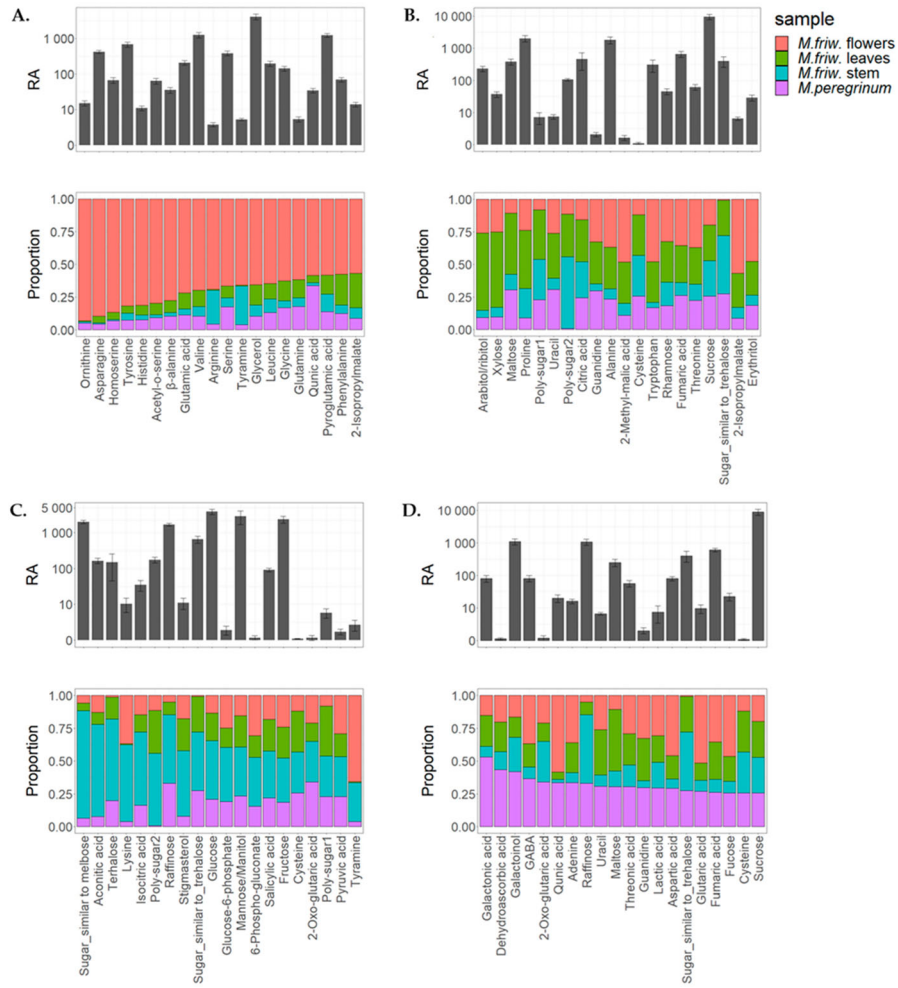

Figure 13. Tissue distribution of the top 20 primary metabolites in the flowers of *Marrubium friwaldskyanum* - A; B - leaves; C - stem; and D - *Marrubium peregrinum*. The four samples are presented as follows: *Marrubium friwaldskyanum* flowers – in red, *Marrubium friwaldskyanum* leaves – in green, *Marrubium friwaldskyanum* stems – in cyan, and *Marrubium peregrinum* – in purple. The upper part of the figure represents the relative abundance (RA) of the top 20 primary metabolites in the respective samples, and the lower part shows their proportion and distribution.

In *M. friwaldskyanum* stems, sugar variation shows a tendency of a slight increase when compared to leaves and it reaches 50%, but the amino acid

content is strongly decreased, which explains the prevalence of organic acids (figure 13C).

M. peregrinum forms a cluster with *M. friwaldskyanum* leaf samples, and based on the top 20 metabolites detected in *M. peregrinum*, we can confirm the presence of a similar proportion of organic acid variations and sugar content, as with that identified for *M. friwaldskyanum* leaves.

UPLC-MS/MS analysis of secondary metabolites

A total set consisting of nearly 400 metabolites was assessed, including different compound classes. Of the 400, we were able to tentatively annotate 320 compounds; 80 of them were labelled as not identified.

The PCA clearly identified four different classes of samples and this displayed their unique biochemical composition (figure 14).

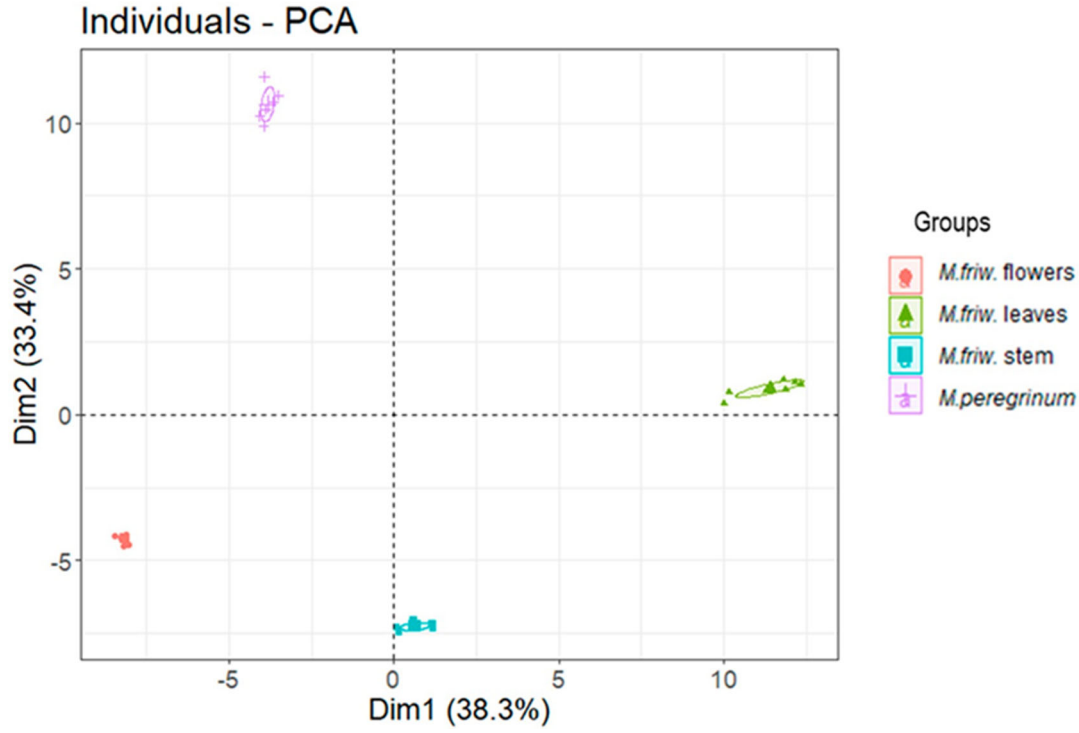

Figure 14. PCA score plots based on a UPLC-MS/MS analysis: *Marrubium friwaldskyanum* flowers - red circles, *Marrubium friwaldskyanum* leaves - green triangles, *Marrubium friwaldskyanum* stems - cyan squares, and *Marrubium peregrinum* - purple crosses.

Interestingly, while the primary metabolite composition of *M. friwaldskyanum* leaves and *M. peregrinum* are identical and clustered together, they differ markedly in terms of their secondary metabolome. This is clearly observed in figures 15 and 16 which display the top 20 metabolites with the highest contribution to PC1 and PC2. The difference between the samples is mainly due to flavonoid derivatives.

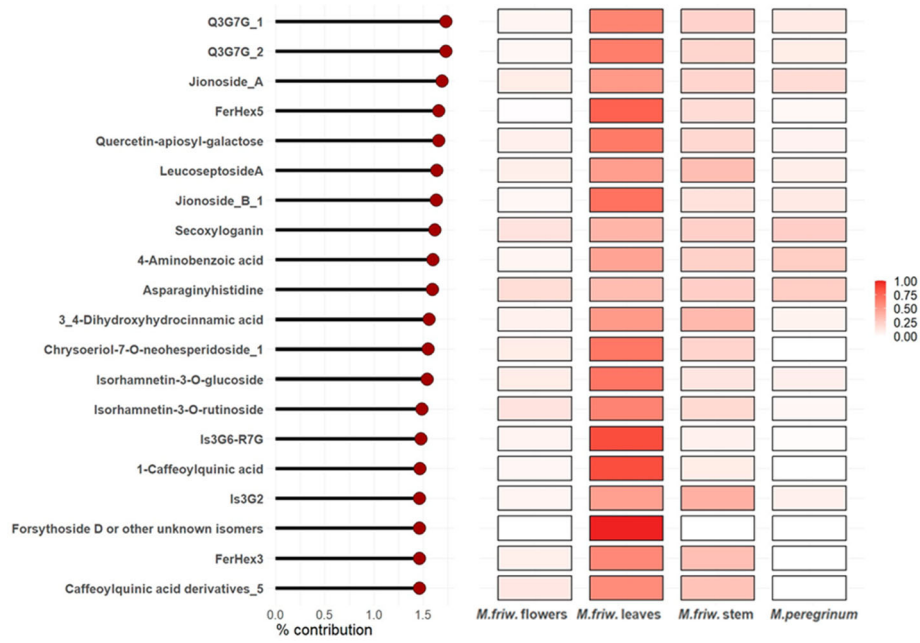

Figure 15. Top 20 metabolites with the highest contribution to PC1.

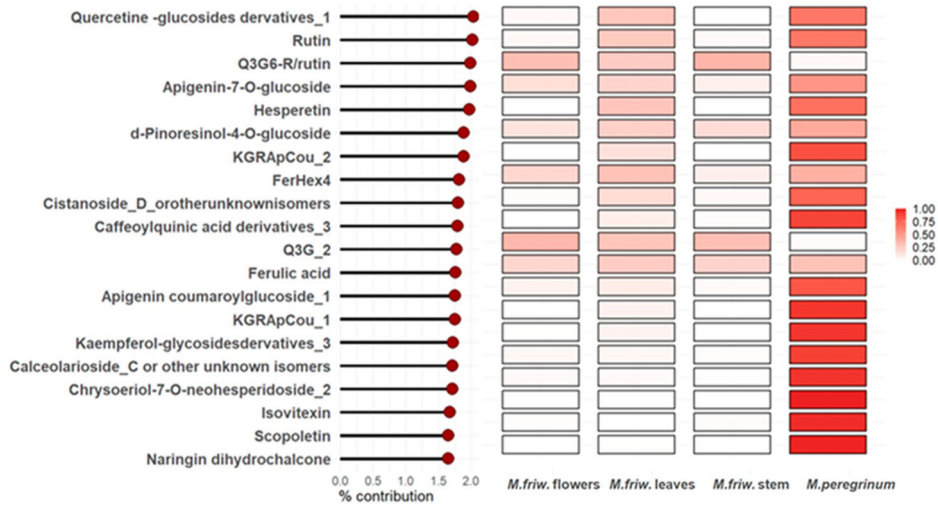

Figure 16. Top 20 metabolites with the highest contribution to PC2. Abbreviations used for the compounds: Q3G7G-quercetin-3-O-(2″-O-apiosyl-6-O″-rhamnosyl)glucosidase; Fer Hex-feruloyl-hexoside; Is3G6R7G-isorhamnetin-3-O-(6″-O-rhamnosyl)glucoside;Is3G2-isorhamnetin-3-O-(2″-O-apiosyl)glucoside; KGRApCou-kaempferol-3-O-Glc-2″-O-Api-6″-O-Rha pCou-7-O-G.

Along with the PCA, an analysis of the tissue-specific distribution of metabolites was performed (figure 17).

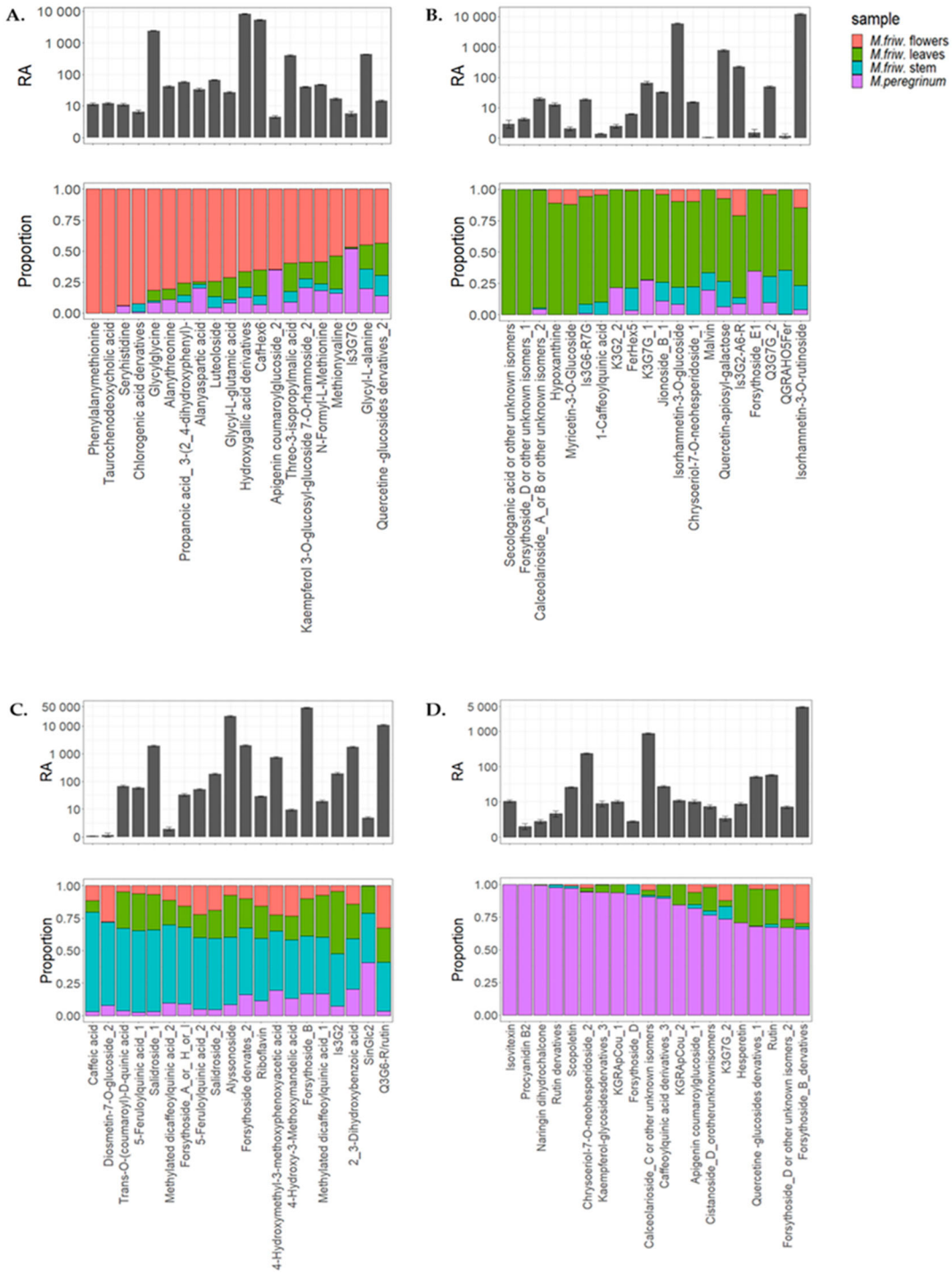

Figure 17. Tissue-specific distribution of secondary metabolites in *M. friwaldskyanum*: A-flowers; B-leaves; C-stem; D-*M peregrinum*. The four samples are presented as follows: *M. friwaldskyanum* flowers – in red, *M. friwaldskyanum* leaves – in green, *M. friwaldskyanum* stems – in cyan, and *M. peregrinum* – in purple. The upper part of the figure presents the relative abundance (RA) of the top 20 secondary metabolites in the respective samples, whereas the lower part displays their distribution across the four sample types, where 1 is the sum of signals from the four samples.

The results successfully underscore the distinctions between complex biochemical networks occurring in plant cells at a tissue level, which might be related to the tissue-specific synthesis of particular secondary metabolites.

Metabolic profiling can generate useful datasets that cover a wide range of various compound classes, and determine biochemical markers for a number of biological processes. In the case of *Marrubium* samples, attention should also be given to the compounds with greater prevalence.

The chemical profiling of the non-polar fraction revealed the lipid content of the two species. This study led to the identification of 175 lipid compounds, classified in 10 lipid classes. The results clearly show that the main lipid shares belong to only two lipid types: triacylglycerides (TAGs) and sphingolipids (SPs). The flowers of *M. friwaldskyanum* contain nearly 90% TAGs, followed by the leaves in which the proportion of TAGs:SPs is 74:22%. In the stem of *M. friwaldskyanum*, the two classes are represented almost equally, and there is an additional small share of lysophospholipids (LPL) that amounts to 15%. The remaining lipid types constitute a small share in the range between 1–7% (figure 18).

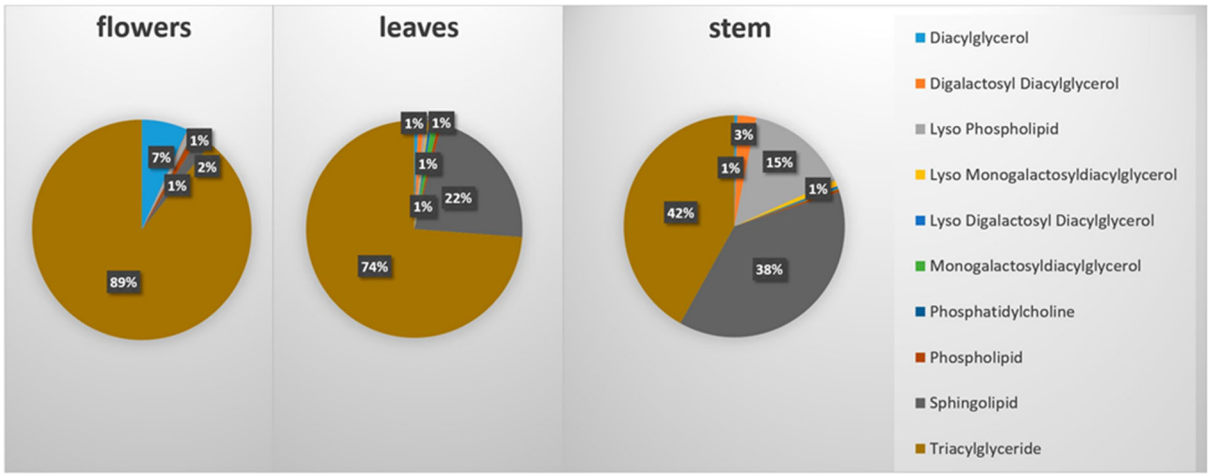

Figure 18. Lipid content in *Marrubium friwaldskyanum* tissue samples.

Remarkably similar results were observed for the *M. peregrinum* samples where the TAGs:SPs ratio was identical to that for *M. friwaldskyanum* leaves- 66:26% (figure 19).

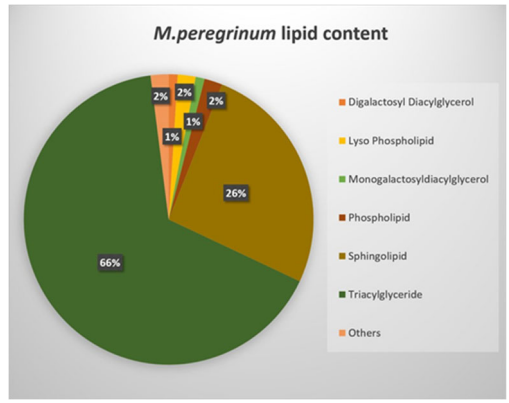

Figure 19. Lipid content in *Marrubium peregrinum*. Under *Others* fall the following lipid classes whose representation is below 1%: Diacylglycerols, Lyso Monogalactosyldiacylglycerols; Lyso Digalactosyldiacylglycerols; Phosphatidylcholine.

Mineral Contents

The dried plant samples were initially examined for the presence of 19 elements. Table 1 displays the mean values for three replicates of three independently prepared samples.

As seen in table 1, Ca, K, and Mg are the main mineral components of the analysed samples. Their concentrations increase in the following way Ca < Mg < K. The concentrations of the main microelements can be ordered as follows: Cu < Mn < Fe < Zn. The average Zn content in *M. friwaldskyanum* flowers reaches 581.3 ± 2.4 mg/kg, which is above the average Zn content for plant tissue (Kumar *et al.*, 2016).

Table 1. Concentrations (in mg/kg) of elements analysed in *Marrubium* samples. Mean values (n = 3) with relative standard deviation (RSD) in brackets.

	Ca	**K**	**Mg**	**Na**	**B**	**Al**
Marrubium friwaldskyanum flowers	187.7 (1.8)	9974.6 (1.9)	1726.3 (1.1)	12.4 (3.4)	3.9 (1.2)	12.6 (0.3)
Marrubium friwaldskyanum leaves	304.1 (1.2)	8212.9 (0.8)	2576.7 (0.8)	8.9 (1.1)	3.1 (0.6)	27.4 (0.1)
Marrubium friwaldskyanum stems	98.1 (1.0)	8945.9 (0.6)	824.3 (0.9)	6.9 (2.3)	4.1 (2.5)	4.7 (0.8)
Marrubium peregrinum	247.6 (1.5)	8787.8 (1.2)	1548.6 (2.1)	14.1 (2.4)	7.5 (3.7)	29.0 (1.0)
	Mn	**Fe**	**Cu**	**Zn**	**Sr**	**Ba**
Marrubium friwaldskyanum flowers	16.3 (0.8)	32.1 (0.7)	4.4 (2.2)	581.3 (2.4)	9.0 (1.8)	11.1 (0.7)
Marrubium friwaldskyanum leaves	26.3 (2.4)	73.9 (1.8)	2.7 (0.8)	559.8 (1.0)	9.4 (1.8)	10.4 (0.5)
Marrubium friwaldskyanum stems	9.1 (2.7)	9.4 (0.3)	2.3 (1.5)	472.9 (1.5)	7.8 (3.8)	14.3 (2.5)
Marrubium peregrinum	9.7 (1.5)	57.3 (1.8)	2.0 (1.7)	475.7 (3.7)	3.6 (3.4)	2.2 (1.1)

Another similar study (Rezgui *et al.*, 2021) demonstrates that the species of the *Marrubium* genus have the capability to accumulate Zn, Fe, Cu, Cd, Pb, and Bi. Heavy metal content in the analysed samples shows that Fe has the highest quantity (73.9 mg/kg) in *M. friwaldskyanum* leaf samples. Cu is accumulated mainly in the flower tissues of *M. friwaldskyanum* plants (4.4 mg/kg), and small quantities can be accumulated in leaves and stems. All metals were within the permissible limits of the regulatory standards of FAO/WHO [FAO/WHO], which means that the plants are safe for subsequent processing and use.

Cytotoxicity and Antitumour Potential

The extracts of *M. peregrinum* and *M. frivaldskyanum* were analysed by means of *in vitro* tests, which take into account an effect on cellular metabolism (chiefly mitochondrial activity) and vitality (MTT assay), as well as lysosome functionality and cellular vitality (NR assay). A panel of four cell lines was employed - three of carcinoma origin (HeLa, A549, HT29) and one normal fibroblast line (HFFC).

These studies afford an opportunity for the definition of both a common cytotoxic effect of the examined samples, and an antitumour potential value against various cancer cell types. The results displayed cytotoxic effects that depend on treatment time and the applied test-sample concentration. The highest sensitivity to all of the tested samples was demonstrated by the HeLa and HT29 cells, and it was fully distinct after a longer treatment period – 72 hours. For this test period, a calculation was made of IC_{50} values (displayed in table 2) using the data from the conducted NR assays.

As far as the HeLa cells are concerned, the strongest toxic effect was manifested by the *M. frivaldskyanum* flower sample, while the HT29 cells displayed higher sensitivity which can be proved by the lower IC_{50} values. Along with this, all four test samples of *Marrubium* caused a significant inhibitory effect on the HT29 line after 72 hours of treatment. These results (table 2) show that the antitumour effect of *M. peregrinum* and *M. frivaldskyanum* extracts is specific to cervical and colorectal carcinoma cells.

Table 2. Average IC_{50} values determined by an NR assay after a 72-hour treatment with *Marrubium* extracts.

Extract	IC_{50} (µg/mL)			
	A549	**HeLa**	**HT29**	**HFFC**
Marrubium peregrinum	-	-	221.1	-
Marrubium frivaldskyanum (flower)	-	390.1	272.7	-

Marrubium frivaldskyanum (stem)	-	-	303.4	-
Marrubium frivaldskyanum (leaf)	-	-	202.2	-
Mitomycin C	58.4	15.6	90.5	< 10

The results from the Neutral Red assay suggested that the examined *Marrubium* samples exhibit strong toxic effects which may potentially imply mechanisms of action specific to cellular lysosomes (figures 20-23).

The A549 cell line, derived from lung carcinoma, did not display high sensitivity to the four tested extracts, even after a longer treatment (figure 20). The highest level of inhibition was observed for the flower sample of *M. frivaldskyanum*, despite this inhibitory effect did not exceed 50%. A weak toxic effect (between 5 and 20 % of inhibition) was identified in the other samples, too, after 48- (in an NR assay) and 72-hour treatment.

The results obtained for the HeLa cell line indicate that the flower extract of *M. frivaldskyanum* has the highest cytotoxic effect because it causes more than 50% of inhibition of culture growth of HeLa cells with concentration of around 500 □g/mL already at the 48-hour treatment period. Meanwhile, with the HT29 line, a high level of inhibition was detected at a later stage – after 72 hours of treatment with extracts (figure 22). The HT29 cells reacted against all *Marrubium* test samples after a 48-hour treatment, and this effect grew significantly after 72 hours. The highest inhibition percentage was detected after testing the flower and leaf *M. frivaldskyanum* samples. A similar tendency was evident with the HeLa cells, as well as with HFFC fibroblasts (the percentage of inhibition being considerably lower when compared to cancer cell lines).

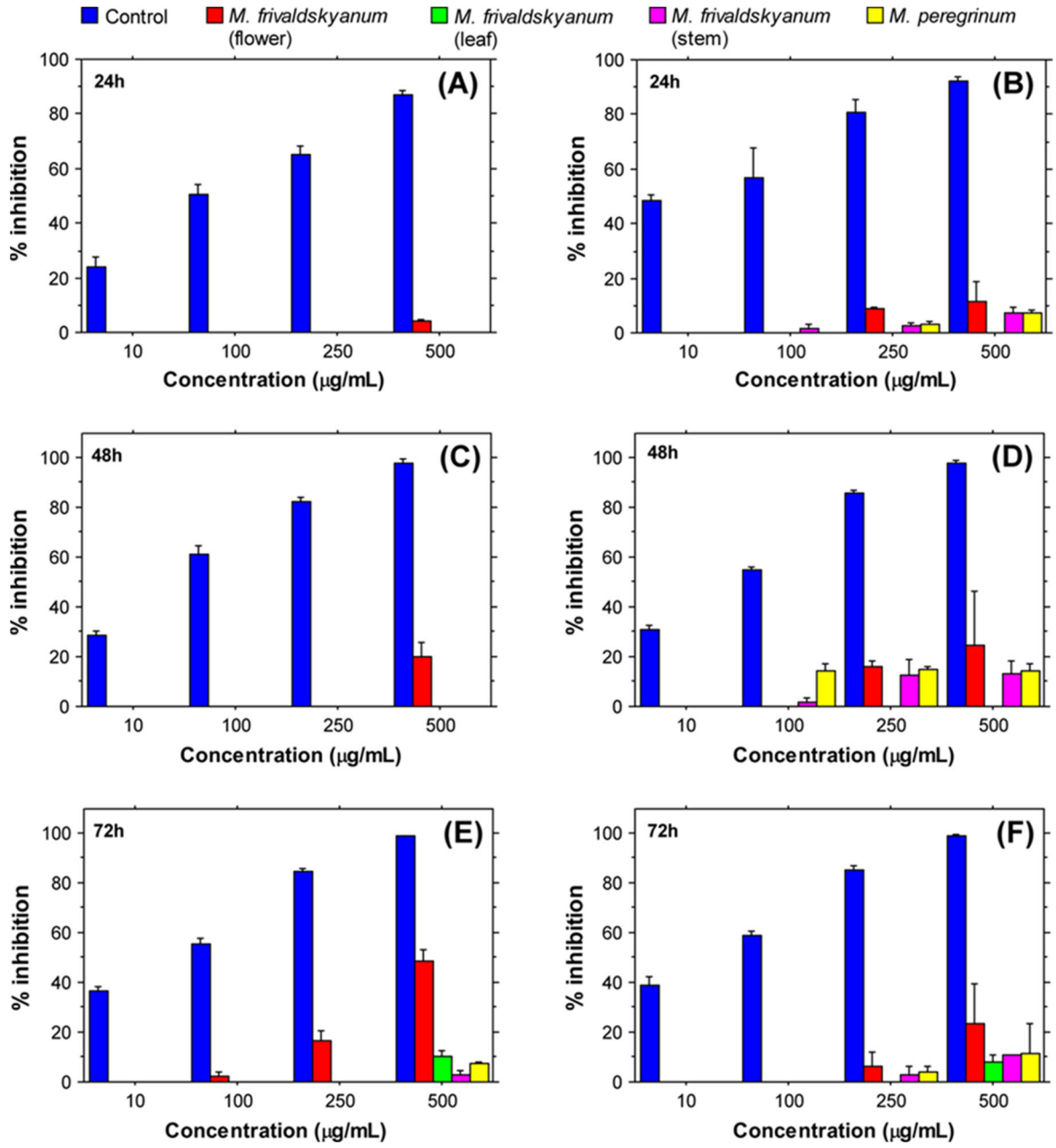

Figure 20. Cytotoxicity of *Marrubium* towards A549 cells. Graphs A, C, E display the percentage of inhibition of metabolic activity, detected in MTT assays. Graphs B, D, F – effects of test samples on cellular vitality and lysosomal activity determined in NR assays. The results are displayed as ± standard error of the mean (± SEM) and in comparison to the effect of the control sample for cytotoxic effect (mitomycin C).

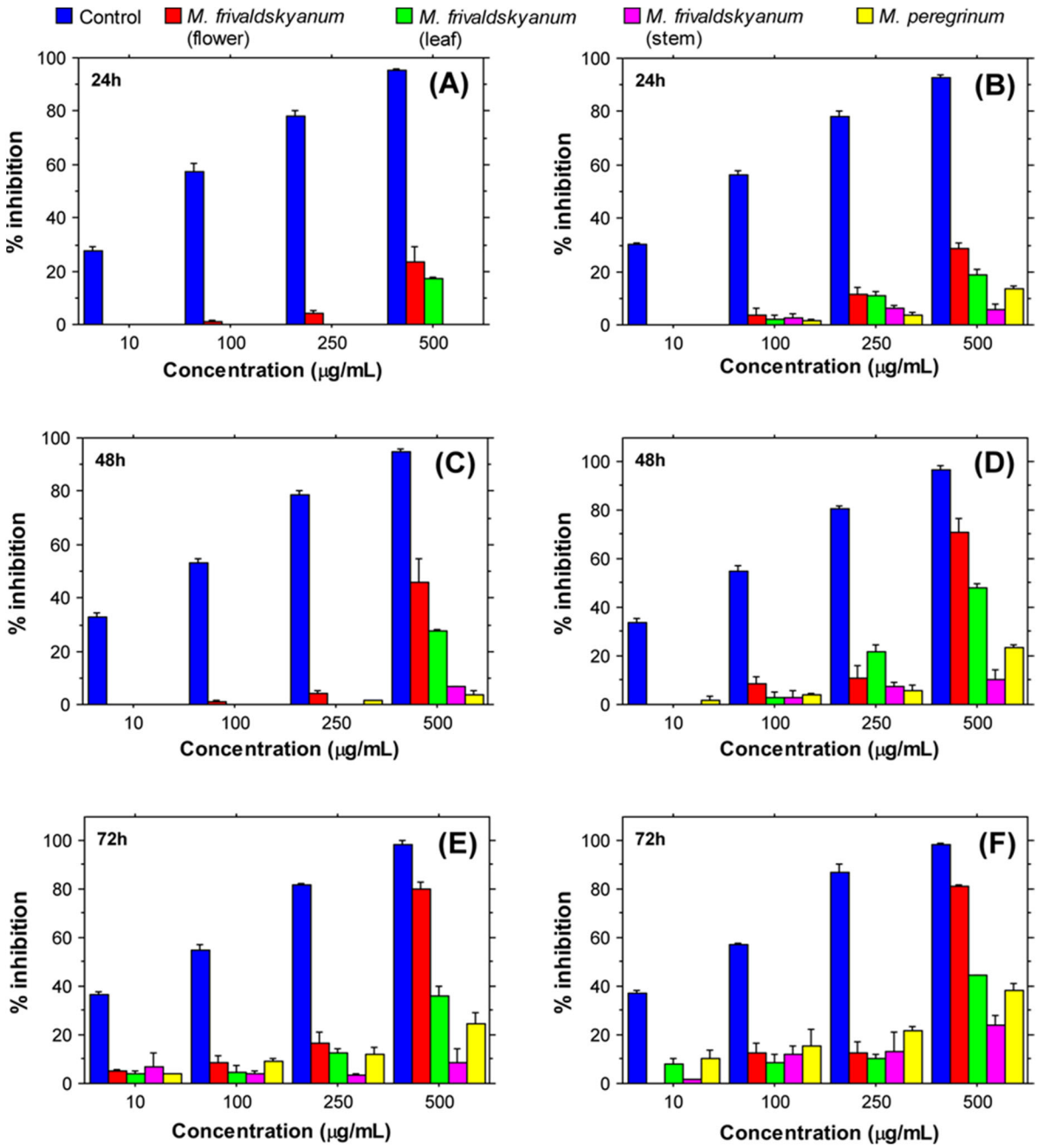

Figure 21. Cytotoxicity of *Marrubium* isolates analysed in HeLa cells. A, C, E display data of MTT assays. B, D, F – effects of test samples identified via an NR uptake assay. The results are displayed as ± SEM and in comparison to the effect of the control sample for cytotoxic effect (mitomycin C).

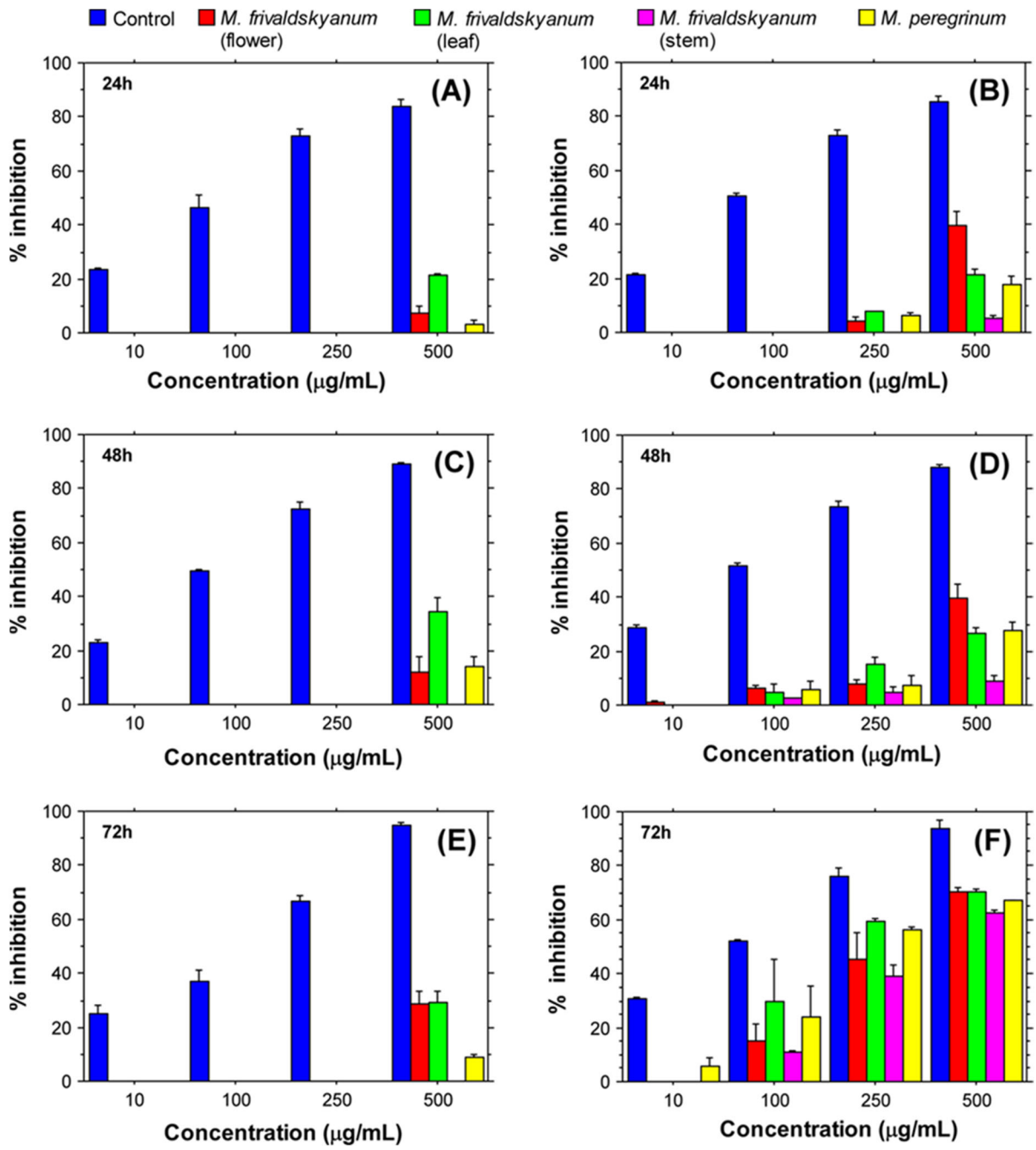

Figure 22. Cytotoxicity of *Marrubium* isolates analysed in NT29 cells. A, C, E represent the percentage of inhibition detected in MTT assays. B, D, F – results of NR assays. The results are presented as ± SEM and in comparison to the effect of the control sample for cytotoxic effect (mitomycin C).

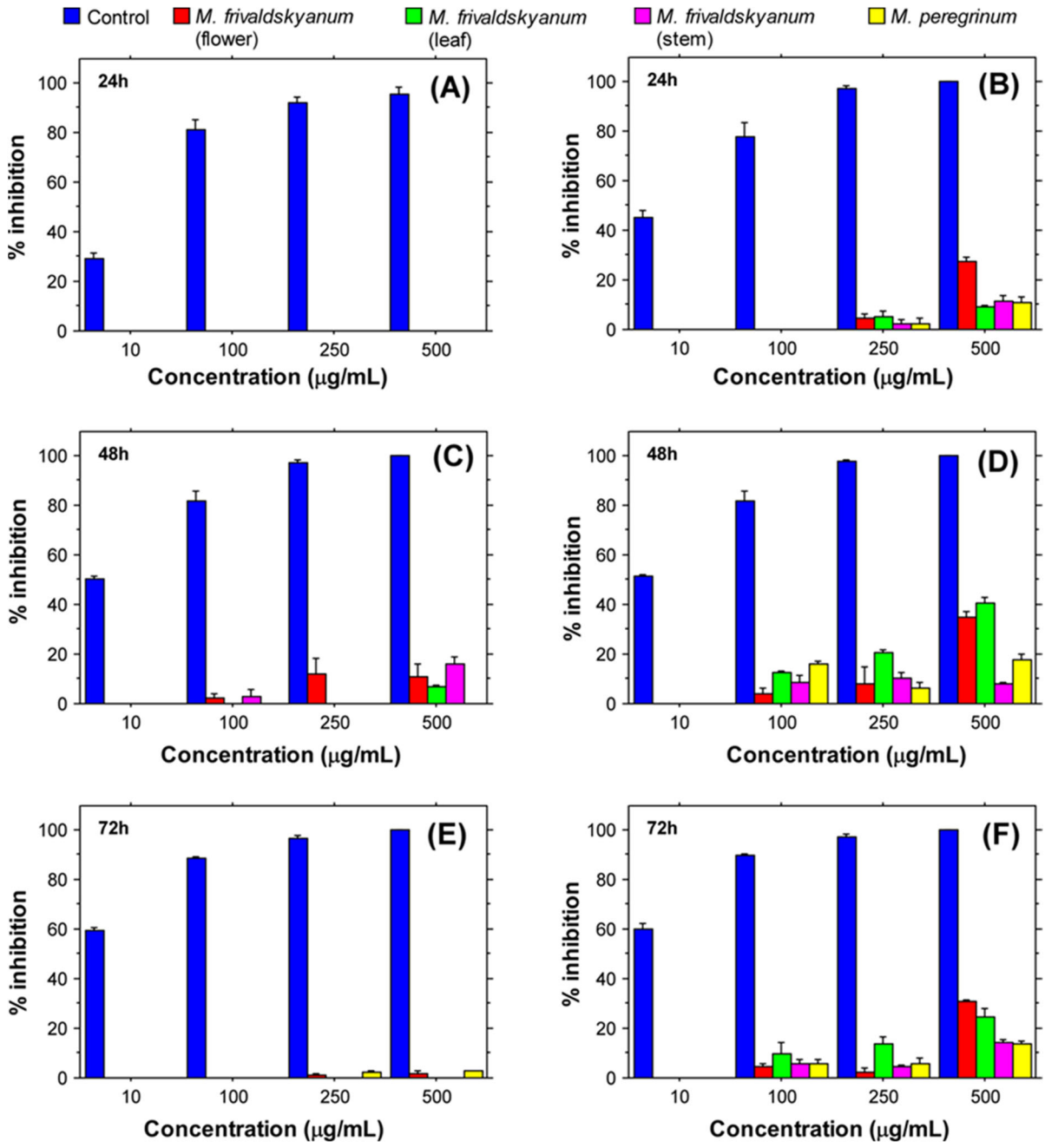

Figure 23. Effects of *Marrubium* extracts on the vitality and metabolic activity of HFFC fibroblasts. A, C, E represent the percentage of inhibition of metabolic activity detected in MTT assays. B, D, F – effects of the assays on cell viability and lysosomal activity as determined by NR assays. The results are presented as ± SEM and in comparison to the effect of the control sample for cytotoxic effect (mitomycin C).

The high cytotoxic effect of the *Marrubium* samples on the HeLa and HT29 cell lines is an indicator of antitumour activity, as in the case of normal cells (HFFC, figure 23) a significantly weaker cytotoxic effect of the tested extracts was reported.

In the HFFC cell line, a very prominent toxic effect was induced only by the flower extract of *M. frivaldskyanum.*

Along with this, however, the data for HFFC fibroblasts showed a maximum level of inhibitory effect 48 hours after treatment with the test samples, which was followed by a reduction at the 72nd hour, indicating the possibility of recovery/overcoming the toxic effect in non-cancerous cells. A similar response was not observed in the tumour cell lines for which the highest level of inhibition was recorded at the 72nd hour.

The potential antitumour effect of the *Marrubium* samples was also analysed with tumour spheroids formed from HT29 cells. They are an improved *in vitro* model for antitumour activity analysis as they offer the possibility to assess the antitumour effect at the level of a 3D multicellular structure of cancer cells. In these studies, a reduction in the average diameter of the spheroids treated with a flower or leaf sample of *M. frivaldskyanum* was detected both after 24 hours and after 96 hours of treatment. In the case of a more prolonged treatment (96 hours), these effects were more pronounced (figure 24).

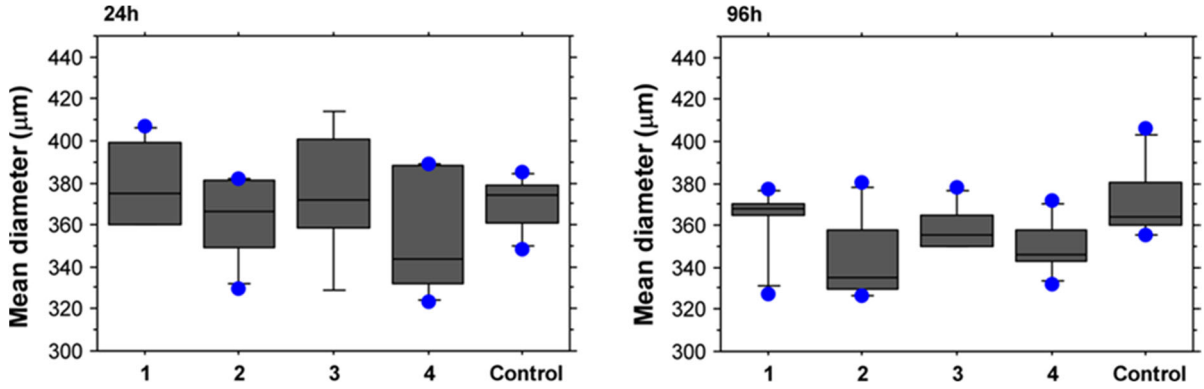

Figure 24. Comparison of spheroid sizes from HT29 cells treated with 200⌈g/mL isolates from *Marrubium* for 24 hours (left graph) and 96 hours (right graph). 1- *M. peregrinum*, 2- *M. frivaldskyanum* (flower), 3- *M. peregrinum* и *M. frivaldskyanum* (stem), 4- *M. frivaldskyanum* (leaf), Control – untreated spheroids cultivated under standard conditions.

The obtained results confirm the established antitumour activity of *M. frivaldskyanum* flower and leaf samples and indicate their potential to inhibit the development of multicellular aggregates of cancer cells.

Antibacterial Activity

The conducted tests for antibacterial activity distinguished the samples of *M. peregrinum* and *M. frivaldskyanum* flower (tables 3 and 4), which showed inhibitory effects on the development of both Gram-positive (*Bacillus cereus*)

and Gram-negative (*Escherichia coli*) bacteria. Concordantly, the leaf extract of *M. frivaldskyanum* displayed antibacterial activity but only against *B. cereus*.

The results represent the average size of the zone of inhibition (± standard error).

Table 3. Antibacterial activity of the *Marrubium* isolates.

Test-samples	Inhibition zone /diameter (mm)	
	Bacillus cereus	*Escherichia coli*
Marrubium peregrinum	7.67 (± 0.33)	7.9 (± 0.33)
Marrubium frivaldskyanum (flower)	8.25 (±0.45)	8.67 (±0.5)
Marrubium frivaldskyanum (stem)	-	-
Marrubium frivaldskyanum (leaf)	7.8 (± 0.45)	-
Control (penicillin+streptomycin)	23.8 (± 0.6)	25.5 (± 0.7)

Table 4. Minimum inhibitory and bactericidal concentrations of the *Marrubium* samples determined against *E. coli* and *B. cereus.*

Test-samples	Minimum inhibitory concentration (µg/mL)		Minimum bactericidal concentration (µg/mL)	
	B. cereus	*E. coli*	*B. cereus*	*E. coli*
Marrubium peregrinum	500	1000	1000	>1000
Marrubium frivaldskyanum (flower)	1000	1000	>1000	>1000
Marrubium frivaldskyanum (stem)	1000	>1000	>1000	>1000
Marrubium frivaldskyanum (leaf)	500	1000	>1000	>1000
Control (ceftriaxone)	7.5	7.5	30	15-30

A minimum inhibitory concentration was defined for *M. peregrinum*, *M. frivaldskyanum* flower and leaf against the two tested bacterial species, as well as *M. frivaldskyanum* stem against *B. cereus*. A minimum bactericidal concentration was determined only for the *M. peregrinum* sample against *B. cereus.* The MBC exceeded 1 mg/mL for the remaining extracts.

The obtained results show that the extracts of *M. peregrinum* and *M. frivaldskyanum* flower display an inhibitory effect on both Gram-negative and Gram-positive bacteria, while the *M. frivaldskyanum* leaf sample has selective activity against Gram-positive bacteria (*B. cereus*).

Results obtained from the studies of *Centaurea thracica*

Chemical composition of the fruit

The content of the main components in the composition of unripe and ripe fruit of *C. thracica* is presented in table 5.

The ripe fruit of *C. thracica* has a higher carbohydrate content and a lower protein and glyceride oil content which accounts for the increase in energy value compared to the unripe fruit (from 342 kcal/100g (1445 kJ/100g) to 354 kcal/100g (1503 kJ/100g).

Table 5. Changes in the total chemical composition of *Centaurea thracica* fruit.

Indicators	**Unripe fruit**	**Ripe fruit**
Proteins, %	8.7 ± 0.1	7.4± 0.2
Oil, %	2.0 ± 0.1	1.7± 0.1
Carbohydrates	72.3	77.2
» fibres, %	28.7 ± 0.4	35.8± 0.3
Ash, %	4.5± 0.1	4.2 ± 0.1
Moisture, %	12.5 ± 0.1	9.5 ± 0.1
Energy value, kJ/100g (kcal/100g)	1445 (342)	1503 (354)

Lipid composition of the oil isolated from fruit and seeds

The lipid composition of glyceride oil isolated from fruit and seeds of *C. thracica* has been tested, i.e. fatty acid composition of the glyceride oil, content of tocopherols, content and composition of the phospholipid fraction.

Figure 25 shows a chromatogram of methyl esters of the fatty acids of *C. thracica* glyceride seed oil.

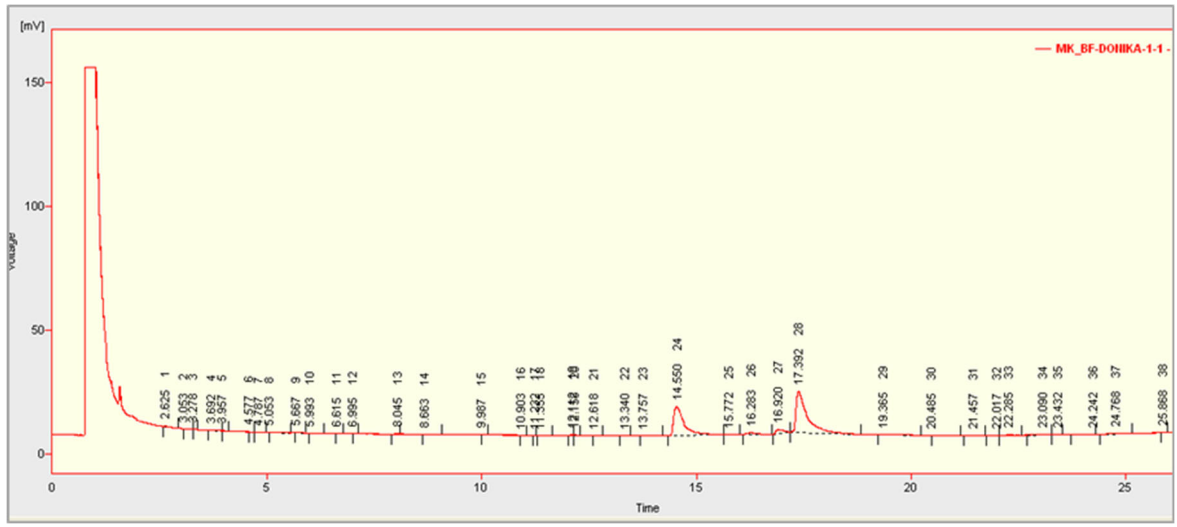

Figure 25. Chromatogram of methyl esters of fatty acids of *Centaurea thracica* seed oil.

Table 6 presents the fatty acid composition of glyceride oil isolated from the fruit and seeds of *C. thracica*.

Table 6. Fatty acid composition of oils isolated from *Centaurea thracica.*

Fatty acids, %		Unripe fruit	Ripe fruit	Seeds
C 4:0	butyric	3.4 ± 0.2	*	-
C 6:0	caproic	3.1 ± 0.1	0.7 ± 0.2	-
C 8:0	caprylic	-	0.1 ± 0.0	0.2 ± 0.02
C 10:0	capric	-	0.2 ± 0.05	0.1 ± 0.0
C 12:0	lauric	2.9 ± 0.2	1.1 ± 0.1	0.1 ± 0.0
C 14:0	myristic	2.5 ± 0.1	2.5 ± 0.15	0.2 ± 0.05
C 14:1	myristoleic	0.5 ± 0.1	0.8 ± 0.2	0.1 ± 0.0
C 15:0	pentadecanoic	1.0 ± 0.2	0.4 ± 0.1	0.2 ± 0.05
C 15:1	pentadecenoic	0.5 ± 0.1	0.1 ± 0.0	-
C 16:0	palmitic	32.6 ± 0.5	21.6 ± 0.6	36.2 ± 0.2
C 16:1	palmitoleic	3.8 ± 0.1	11.7 ± 0.2	-
C 17:0	heptadecanoic	0.9 ± 0.1	0.5 ± 0.06	0.3 ± 0.05
C 17:1	heptadecenoic	0.8 ± 0.1	0.5 ± 0.1	0.3 ± 0.0
C 18:0	stearic	4.9 ± 0.3	4.5 ± 0.2	3.7 ± 0.4
C 18:1	oleic	13.7 ± 0.5	30,9 ± 0.7	53.0 ± 0.5
C 18:2 (ω-6)	linoleic	13.8 ± 0.4	20.3 ± 0.3	1.4 ± 0.2
C 18:3 (ω-3)	linolenic	3.6 ± 0.3	0.7 ± 0.1	1.1 ± 0.1
C 20:0	arachidic	3.5 ± 0.5	0.2 ± 0.05	-
C 20:1	gadoleic	0.4 ± 0.1	0.1 ± 0.0	-
C 20:2 (ω-6)	eicosadienoic	0.5 ± 0.2	0.2 ± 0.05	0.4 ± 0.1
C 22:0	behenic	2.9 ± 0.3	1.5 ± 0.1	0.5 ± 0.1
C 22:1	erucic	0.3 ± 0.1	-	-
C 22:2 (ω-6)	docosadienoic	-	-	0.7 ± 0.1
C 20:5 (ω-3)	eicosapentaenoic	-	-	0.1 ± 0.0
C 23:0	tricosanoic	1.0 ± 0.2	0.4 ± 0.1	-
C 24:0	lignoceric	2.8 ± 0.4	1.0 ± 0.2	0.2 ± 0.1
C 24:1	nervonic	-	-	0.2 ± 0.05
C 22:6 (ω-3)	docosahexaenoic	0.6 ± 0.1	0,1 ± 0.0	1.0 ± 0.2
Saturated FA		61.5	34.6	41.7
Unsaturated FA		38.5	65,4	58.3
Monounsaturated FA		20.0	44.1	53.6
Polyunsaturated FA		18.5	21.3	4.7
Σ ω-6		14.3	20.5	2.5
Σ ω-3		4.2	0.8	2.2
Ratio ω-6/ω-3		3.4	26.6	1.14
Iodine value, $gI_2/100g$		47.2	66.5	53.2

* - not identified

In the oil composition of *C. thracica* fruit, there are 23 types of fatty acids, and in that of *C. thracica* seeds – 20 types of fatty acids.

It has been found that the ω-6/ω-3 ratio varies in quite a wide range from 1.14 to 26.6. The high ratio in the ripe fruit oil is due to the high content of linoleic acid (20.3%) and the small amount of linolenic acid (0.7%).

Figure 26 shows the ratio of saturated and unsaturated, including monounsaturated and polyunsaturated, fatty acids in the oil isolated from fruit at different stages of vegetation and seeds of *Centaurea thracica*.

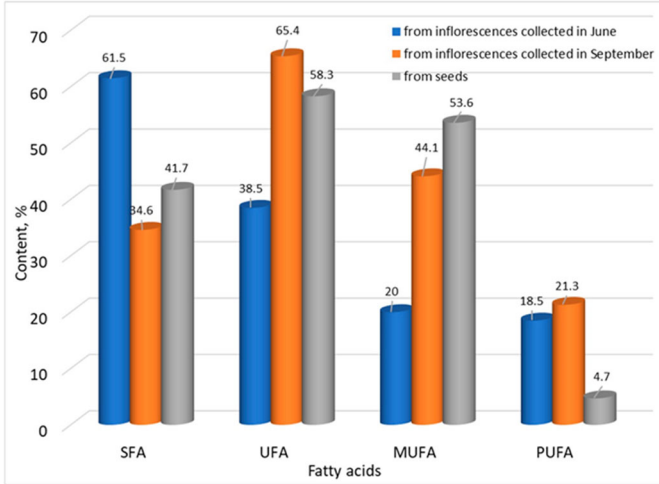

Figure 26. Content of saturated (SFA), unsaturated (UFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in oil isolated from unripe and ripe fruit and seeds of *C. thracica.*

The figure shows that the amount of saturated fatty acids predominates in the oil isolated from unripe fruit (61.5%), while in the oils isolated from ripe fruit and from seeds, the unsaturated fatty acids have the highest amount, respectively 65.4% and 58.3%. The main representative of saturated fatty acids is the palmitic acid (21.6 – 36.2%), followed by the stearic acid (3.7-4.9%). The quantities of monounsaturated fatty acids in the oils from ripe fruit (44.1%) and from seeds (53.6%) are significantly higher than those of the polyunsaturated fatty acids, respectively 21.3% and 4.7%, while in the oil from unripe fruit, the ratio of monounsaturated (20.0%) to polyunsaturated (18.5%) fatty acids is almost 1:1. The main representative of monounsaturated fatty acids is the oleic acid (13.7 – 53.0%), and of polyunsaturated acids - the linoleic (1.4 -20.3%) and the linolenic (0.7 – 3.6%) acid.

The iodine value, which is an indicator of the degree of unsaturation of fatty acids in oils, is relatively low (47.2 – 66.5 $gI_2/100g$), and this is a result of the high content of saturated fatty acids in the examined oils. The iodine value of the studied oils is similar to the one of palm olein oil (≥ 56 $gI_2/100g$) (CODEX STAN 210-1999). This gives one enough reason to classify the oils

under scrutiny as non-drying oils, which are characterised by an iodine value between 50-100 $gI_2/100g$.

Based on the data obtained as regards the fatty acid composition of the oils isolated from *C. thracica* fruit and seeds, indicators were determined for the first time related to the evaluation of the benefits of the oil for human health and which are criteria for their therapeutic effect: ratio between polyunsaturated and saturated fatty acids, atherogenic and thrombogenic index.

Table 7 presents data on the ratio between polyunsaturated and saturated fatty acids and atherogenic and thrombogenic indices.

Table 7. Ratio between polyunsaturated and saturated fatty acids (PUFA/SFA), atherogenic and thrombogenic indexes.

Oils from	PUFA/SFA	Atherogenic index	Thrombogenic index
unripe fruit	0.30±0.05	1.20±0.2	1.33±0.05
ripe fruit	0.62±0.02	0.50±0.1	0.82±0.02
seeds	0.11±0.01	0.64±0.1	1.13±0.03

The ratio of polyunsaturated to saturated fatty acids plays an important role in determining the different properties of cell membranes which help maintain normal metabolism in cells. The recommended minimum ratio of polyunsaturated fatty acids to saturated fatty acids is 0.45, and the optimal ratio that results in a reduced risk of cardiovascular disease – 1.0 – 1.5 (Kang *et al.*, 2005).

The PUFA/SFA ratio of the studied oils from *C. thracica* fruit and seeds is much lower than the same ratio of soybean oil (4.39), maize oil (4.10), sesame oil (2.94), and it is closer to that of palm oil (0.18) (Kang *et al.*, 2005).

The atherogenic index shows the connection between the sum of essential saturated fatty acids, considered as pro-atherogenic, and essential unsaturated fatty acids, which have an anti-atherogenic effect (Cottin *et al.*, 2011). The oil from unripe fruit has two-times higher atherogenic index (1.20) when compared to the oils isolated from ripe fruit and seeds (0.50 and 0.64). The anti-atherogenic lipids inhibit plaque build-up and reduce the levels of esterified fatty acids and cholesterol, thus preventing the onset of micro- and macro-coronary diseases (Hooper *et al.*, 2006).

The thrombogenic index determines the tendency to thrombogenesis in blood vessels. The value of this index for the studied oils ranges from 0.82 to 1.33.

The atherogenic and thrombogenic indexes of the oils from fruit and seeds of *C. thracica* are significantly higher than those of olive oil (0.1250 and 0.3230,

respectively), argan oil (0.1577 and 0.4498), and sesame oil (0.1235 and 0.3623), (Alvites Misajel, 2017).

It is considered that atherogenic and thrombogenic index values below 1.0 are an indication of better anti-atherogenic and anti-thrombogenic properties of lipids (Ulbricht & Southgate, 1991). The values of these indexes for the oil isolated from ripe fruit are lower than 1, while for the other two studied oils, they are above 1 but below 1.5.

Tocopherol Content

Tocopherols are of utmost importance to the protection of polyunsaturated fatty acids (PUFA) in plants and animals against oxidation. They manifest their antioxidant effect by means of many biochemical and biophysical mechanisms, including the absorption of active oxidants and free radicals (Kamal-Eldin & Appelqvist, 1996). The tocopherol content and their individual composition were determined directly in the oil, and the results are displayed in table 8.

The presence of only one tocopherol - α- tocopherol - was established in the examined oils of *C. thracica* fruit and seeds.

Table 8. Tocopherol content and individual tocopherol composition in *Centaurea thracica* fruit and seeds.

Indicators	**Unripe fruit**	**Ripe fruit**	**Seeds**
Tocopherols, mg/kg	58 ± 5	110 ± 5	260 ± 10
Individual tocopherol composition			
α – tocopherol, %	100 ± 0.0	100 ± 0.0	100 ± 0.0

The tocopherol composition coincides with that of oils isolated from *Centaurea albonitens* and *Centaurea balsamita,* in which the α-tocopherol is also prevalent. The individual tocopherol composition of *C. thracica* fruit and seeds oil is close to the composition of sunflower and safflower oils which contain primarily α-tocopherol (CODEX STAN 210-1999). Chemically, α-tocopherol is more effective than γ-tocopherol. It removes free radicals but has the disadvantage of acting as "pro-oxidant" under certain conditions (Kamal&Eldin & Andersson, 1997). In plants, α-tocopherol is biosynthesised by means of β- or γ-tocopherol (Furuya *et al.*, 1987).

Fernandes *et al*. (2019) have found that during the flower development of plants from the *Centaurea* genus, the tocopherol content in isolated oil is reduced from 3.0 to 2.4 mg/100g dw, and the individual tocopherol composition differs from the one in the oils we tested, and their oils contain all four main

tocopherol representatives (α-, β-, γ-, and δ-tocopherol), the prevalent one being α- tocopherol (Fernandes *et al.*, 2019).

Phospholipid Content and Individual Phospholipid Composition

The results regarding the phospholipid content of lipids isolated from *C. thracica* fruit and seeds are displayed in table 9. The phospholipid content in the oil of unripe fruit is similar to that of sunflower, linseed, maize, soybean, and rapeseed oils (0.7-1.0%) (Popov and Ilinov, 1986).

The individual phospholipid composition was determined spectrophotometrically after separating the components by means of two-dimensional thin-layer chromatography (figure 27).

Table 9. Individual phospholipid composition of lipids, isolated from *Centaurea thracica*.

Phospholipids, %	**Unripe fruit**	**Ripe fruit**	**Seeds**
Phosphatidylcholine	6.6 ± 0.2	23.9 ± 0.5	9.1 ± 0.5
Phosphatidylinositol	14.6 ± 0.3	10.4 ± 0.4	25.0 ± 1.5
Phosphatidylethanolamine	6.9 ± 0.5	13.9 ± 0.4	31.8 ± 1.1
Sphingomyelin	7.3 ± 0.1	7.6 ± 0.5	11.3 ± 1.1
Phosphatidylserine	12.5 ± 0.4	4.7 ± 0.1	7.0 ± 1.0
Lysophosphatidylcholine	6.1 ± 0.1	9.4 ± 0.4	2.8 ± 0.4
Lysophosphatidylethanolamine	2.5 ± 0.3	2.0 ± 0.2	2.5 ± 0.6
Monophosphatidylglycerol	*	1.0 ± 0.1	5.6 ± 0.5
Diphosphatidylglycerol	21.4 ± 0.4	7.3 ± 0.3	4.9 ± 0.2
Phosphatidic acids	22.1 ± 0.1	19.8 ± 0.4	-
Total phospholipid content, %	0.70 ± 0.10	0.35 ± 0.05	0.30 ± 0.04

* - not identified

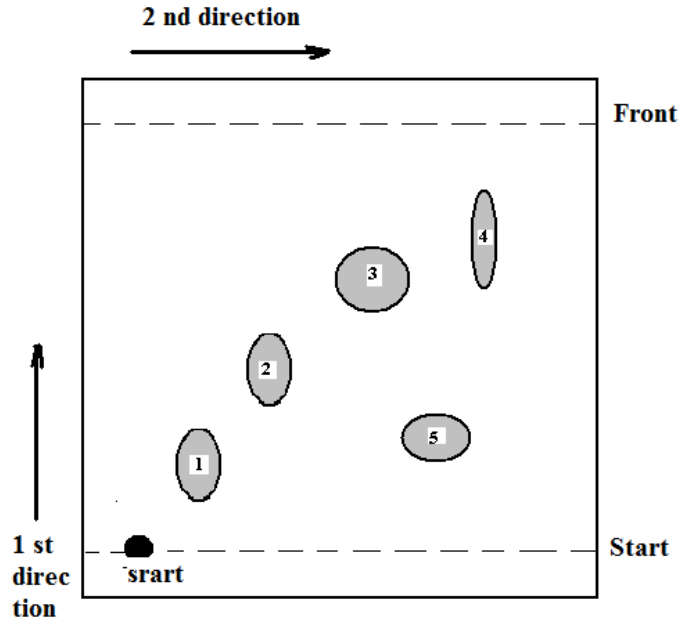

Figure 27. Thin-layer chromatogram of individual phospholipids: 1–Phosphatidylinositol, 2–Phosphatidylcholine, 3–Phosphatidylethanolamine, 4–Diphosphatidylglycerol, 5–Phosphatidic acids.

Almost all major classes of phospholipids were identified in the phospholipid fraction of lipids from *C. thracica* fruit and seeds.

It was established that the main representatives of phospholipids isolated from seeds are phosphatidylethanolamine (31.8%) and phosphatidylinositol (25.0%). No phosphatidic acids were identified in the phospholipids of seeds.

CONCLUSIONS

Conclusions drawn from the study of the *Marrubium friwaldskyanum and Marrubium peregrinum* species

As a result of the studies conducted on the *Marrubium friwaldskyanum* and *Marrubium peregrinum* species, the following conclusions can be drawn:

1. The anatomical study of the *M. friwaldskyanum* and *M. peregrinum* species found in Bulgaria enriches the knowledge of the biology of the genus.

2. The comparative anatomical analysis of the characteristic features of *M. friwaldskyanum* and *M. peregrinum* establishes:

- amphistomatic leaf structure with diacytic and anomocytic type of stomata;

- chlorenchyma differentiation into palisade and spongy types;

- unicellular and multicellular non-glandular trichomes with linear and branching structure;

- peltate stacked, unicellular and bicellular glandular trichomes, covering the leaf and stem surfaces.

3. The flavonoids apigenin, quercetin, rutin, and their derivatives were identified in large quantities.

4. Significant quantities of phenylethanoid and phenylpropanoid glycosides such as forsythoside, calceolarioside, and caffeoylquinic acids were registered.

5. Successful identification of 175 lipid compounds, classified in 10 lipid classes.

6. Identification and quantity determination of the elemental composition of plants was performed, the highest concentration being that of the important for human health microelements K, Mg, Ca, and Zn.

7. The extracts derived from *M. peregrinum* and the flower of *M. frivaldskyanum* display an inhibitory effect against both Gram-negative and Gram-positive bacteria.

8. *M. frivaldskyanum* leaf extract has a selective activity against the Gram-positive *Bacillus cereus* bacteria.

9. The extracts derived from *M. peregrinum* and *M. frivaldskyanum* have a specific antitumour effect against cervical and colorectal carcinoma cells. The highest sensitivity to all tested samples was demonstrated by HeLa and HT29 cells.

10. The antitumour activity of *M. frivaldskyanum* flower and leaf samples has the potential to inhibit the growth of multicellular aggregates of cancer cells.

Conclusions from the study of *Centaurea thracica*:

1. The total chemical composition of the unripe and ripe *C. thracica* fruit was determined. The results obtained as regards the total chemical composition of fruit show a higher content of carbohydrates in the ripe fruit and a lower content of protein and glyceride oil, which conditions the increase in the energy value of the ripe fruit (from 342 kcal/100g (1445 kJ/100g) to 354 kcal/100g (1503 kJ/100g)).

2. The fatty acid composition of glyceride oils isolated from *C. thracica* fruit and seeds was determined. In the composition of oil isolated from unripe fruit, saturated fatty acids prevail (61.5%), while in the oils isolated from ripe fruit and from seeds, unsaturated fatty acids have the prevalence - 65.4% and

58.3%, respectively. The main representatives of saturated fatty acids are palmitic acid (21.6 – 36.2%) and stearic acid (3.7 – 4.9%). The only representative of monounsaturated fatty acids is the oleic acid (13.7 – 53.0%), and of polyunsaturated fatty acids are the linoleic acid (1.4 – 20.3%) and linolenic acid (0.7 – 3.6%).

3. The values of atherogenicity and thrombogenicity indexes indicate good antiatherogenic and antithrombogenic properties of the studied oils, and the values of the PUFA/SFA ratio confirm the nutritional value of the oil.

4. The individual composition of the tocopherol fraction and, for the first time, of the phospholipid fraction of *C. thracica* fruit and seed lipids was determined.

- In the tocopherol composition study, it was established that the main component is α- tocopherol.

- In the phospholipids, isolated from unripe fruit, phosphatidic acids (22.1%) and diphosphatidylglycerol (21.4%) are prevalent, whereas in ripe fruit - phosphatidylcholine (23.9%) and phosphatidic acids (19.8%). The main representatives in the phospholipid fraction of the studied *C. thracica* seeds are phosphatidylethanolamine (31.8%) and phosphatidylinositol (25.0%).

5. The high content of monounsaturated fatty acids and other biologically active substances, such as tocopherols (58 – 260 mg/kg) and phospholipids (0.3 – 0.7%) determines the nutritional and biological activity of oils isolated from *C. thracica* fruit and seeds, and this makes them suitable raw materials for the development of nutritional supplements and pharmaceutical products for the prevention of the onset of a number of chronic diseases.

CONTRIBUTIONS

Original scientific contributions

1. These detailed studies on the chemical composition, including lipid composition, of *Centaurea thracica* fruit and seeds are the first in Bulgaria.

Scientific contributions of applicable nature

1. The differences obtained as a result of the statistical processing of data on the characteristic features of *M. friwaldskyanum* and *M. peregrinum* in terms of: the width of the stem epidermal cells, the thickness of the cortex, and xylem and phloem thickness, can be truly useful for future taxonomic studies within the *Marrubium* genus.

2. *M. friwaldskyanum* and *M. peregrinum* are rich in their content of secondary metabolite and trace elements, important for human health. This phytochemical composition has proven antioxidant, antibacterial, anticancer,

and other bioactive effects, making the two species valuable source materials for the biotechnology industry.

3. The unique tissue-specific compounds with important bioactivities make *M. friwaldskyanum* and *M. peregrinum* suitable for extraction and subsequent application in the pharmaceutical sector.

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PARTICIPATION IN SCIENTIFIC FORUMS ON THE TOPIC OF THE DISSERTATION

5TH BALKAN SCIENTIFIC CONFERENCE ON BIOLOGY. April, 15-16, 2021. Plovdiv, Bulgaria. Poster: Leaf and stem anatomy of Bulgarian endemic *Marrubium frivaldskyanum* Boiss. (Lamiaceae). Authors: **Donika Gyuzeleva**, Plamen Stoyanov, Tsvetelina Mladenova, Anelia Bivolarska, Rumen Mladenov, Krasimir Todorov.

2ND INTERNATIONAL CONFERENCE ON PLANT SYSTEMS BIOLOGY AND BIOTECHNOLOGY (ICPSBB). September, 25-27, 2023. Plovdiv, Bulgaria. Poster: Metabolome profiling of *Marrubium peregrinum* L. and *Marrubium friwaldskyanum* Boiss. reveals their potential as sources of plant-based pharmaceuticals. Authors: **Donika Gyuzeleva**, Maria Benina,

Valentina Ivanova, Saleh Alseekh, Tsvetelina Mladenova, Rumen Mladenov, Krasimir Todorov, Anelia Bivolarska, Plamen Stoyanov.

ANNIVERSARY CONFERENCE *PHARMACY - A SCIENCE WITH A FUTURE*, 17-19 November 2023, Plovdiv. Poster: Some antioxidant properties of water extracts from *Marrubium peregrinum* and *Marrubium friwaldskyanum*, and their influence over the contractile activity of smooth muscles in rats. Authors: **Donika Gyuzeleva,** Tsvetelina Mladenova, Krasimir Todorov, Plamen Stoyanov, Rumen Mladenov, Ivitsa Dimov, Anelia Bivolarska, Viktor Yotov, Raina Ardasheva, Natalia Prisadova, Ekaterina Zaitseva, Valentin Turiiski.

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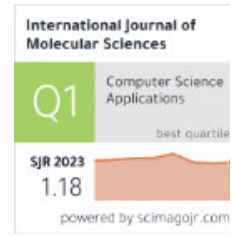
Gyuzeleva, D., Stoyanov, P., Bivolarska, A., Mladenov, R., Mladenova, Ts., Petkov, V., Todorov, K. (2022). Anatomical Investigation of *Marrubium friwaldskyanum* Boiss. and *Marrubium peregrinum* L. (Lamiaceae) from Bulgaria. *Ecologia Balkanica*, 14(1): 87-101. WoS, Scopus Q4 SJR 0.202



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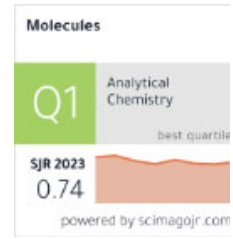

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1. Gyuzeleva, D., Stoyanov, P., Bivolarska, A., Mladenov, R., Mladenova, Ts., Petkov, V., Todorov, K. (2022). Anatomical Investigation of *Marrubium friwaldskyanum* Boiss. And *Marrubium peregrinum* L. (Lamiaceae) from Bulgaria. *Ecologia Balkanica*, 14(1): 87-101.

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