Research on the biology, cultivation technology and valorification species of *Dracocephalum moldavica* L.

(SUMMARY OF THE DOCTORAL THESIS)

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INTRODUCTION

Dracocephalum moldavica L., traditionally known as Moldavian balm, bee balm, monastery balm, dragonhead, matica (from Old Slavic = queen of the bees), is a species belonging to the *Lamiaceae* family, widely recognized for its aromatic properties (Simea et al., 2018; Simea et al., 2023; Duda, 2023).

Edel (1835), cited by Muntean et al. (2007), considered that Moldavian balm is a "plant native to the Dacian territory, bordered, like others, by the Prut and Danube rivers". Later, it was found that this species is also widespread in other areas. However, in 1753, Linnaeus named it "moldavica," thereby recognizing its traditional area of distribution (Duda, 2023).

As part of the molecular analysis of the species *Dracocephalum moldavica L.*, the analysis of the genetic structure of some populations cultivated in our country and in the Republic of Moldavia. Genetic analysis can provide important data regarding intra- and inter-population genetic polymorphism, closely correlated with the adaptive capacity of the species to different soil and climatic conditions, as well as with the biosynthetic capacity of pharmacologically active compounds. This offers the possibility of correctly identifying and selecting new valuable genotypes. Molecular analysis can be performed using DNA molecular markers, among which SSR (simple sequence repeats) and ISSR (inter simple sequence repeats) markers are very frequently used due to their high reproducibility, relatively low cost, and the avoidance of using radioactivity (as is the case with other molecular markers).

The aim of the phytochemical analysis was to evaluate the chemical profiles and the potential cytotoxic and antimicrobial activities of three genotypes of *Dracocephalum moldavica L.*, in relation to their antioxidant capacities. The evaluation of their cytotoxic and antibacterial activities related to their antioxidant mechanisms is scarcely studied in the scientific literature. Considering all this, the present research introduces novel and original aspects by attempting to connect these biological activities with a less studied class of compounds in the species' composition: polyphenols. Additionally, this study aims to provide further arguments for establishing the appropriate cultivation conditions for this species and selecting the suitable variety for future studies on the medicinal potential of its plant product (Simea et al., 2023).

STRUCTURE OF THE THESIS

The doctoral thesis titled “Research on the Biology, Cultivation Technology, and Utilization of *Dracocephalum moldavica L.*” is structured into 8 chapters and comprises 120 pages, 42 tables, 37 figures, and 103 bibliographic references from recent national and international specialized literature. The thesis is divided into two main parts: the current state of knowledge and personal contribution.

The first part, **The current state of knowledge** consists of two chapters, which in turn include several subsections. This section spans 34 pages.

The second part, **Personal contribution,** contains 85 pages. It is structured into 7 chapters and includes the methods and materials, results and discussion, and conclusions and recomandations of the research conducted during the period 2018-2019.

PURPOSE AND OBJECTIVES OF THE RESEARCH

The aim of this research was to highlight the advantages of cultivating and utilizing *Dracocephalum moldavica L.* for phytotherapeutic purposes in the Transylvania region, considering that this species was named by Linnaeus with a term related to our country, namely "moldavica."

The research had several objectives:

- To establish the plant development across different phenological stages.
-To study the possibilities of establishing the crop through direct sowing and transplantation.
- To investigate the influence of planting time on growth and production outcomes.
- To perform genetic analysis of the genotypes A1, A2, B1, and B2.
-To assess the quality of the plant raw material obtained from cultivation by evaluating its chemical composition and biological properties.

RESEARCH MATERIAL AND METHOD

Field experiments were conducted at the experimental field of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca.

The experimental field has an extensive area, which allows the cultivation of a variety of plants, including field crops, medicinal plants and horticultural species. The soils in the area of the experimental field are predominantly chernozems, characterized by high fertility and good water retention capacity. They have a varied texture, from loam to clay, which allows for the cultivation of a wide range of plants.

The biological material used in the research was obtained from the Research and Development Station Buzău (B1, B2) and the Institute of Genetics, Physiology, and Plant Protection Chişinău (A1 and A2). Biometric measurements were carried out during the years 2018-2019 on different plants from each repetition, depending on their origin and density, for the following traits: height, emergence, and flowering of the plants.

To determine seed germination, the standardized method in STAS 1636/89 for a similar species, hyssop (*Hyssopus officinalis*), was applied. Germination energy and capacity were assessed. Germination energy is the rate of seed germination and is expressed as the percentage of seeds that germinate normally within a number of days equal to 1/3 to 1/2 of the time reserved for germination capacity. It reflects seed vigor and is directly related to the seedling's ability to emerge during germination (Duda et al., 2003).

In all cases, it was observed that plants grown in a greenhouse and then transplanted into the field exhibited significantly better development compared to plants sown directly in the field.

RESULTS AND DISCUSSION

Following the field experiments, a significant decrease in production was observed from year to year, with 2018 being more productive than 2019.

Genomic DNA Isolation

In our experiments, total genomic DNA was used. Due to its large molecular weight, it migrates near the start of the electrophoretic gel. The electrophoretic appearance of the DNA isolated from different individuals of *Dracocephalum moldavica* is shown in Figure 1. The electrophoretic profile of the genomic DNA bands indicates a large amount of DNA with very good quality, as it was not degraded.

To calculate the purity of the isolated DNA, absorbance readings at 260 and 280 nm were taken with a spectrophotometer. The ratio of these absorbances indicates DNA purity. In our case, this ratio ranged between 1.7 and 1.8, with an ideal value being 1.8. The concentrations obtained ranged between 30-60 µg DNA/ml. Given the concentrations were too high for subsequent PCR amplifications and to avoid contamination of the DNA stock, 3% solutions were prepared. These solutions were stored in a freezer at -20 °C.

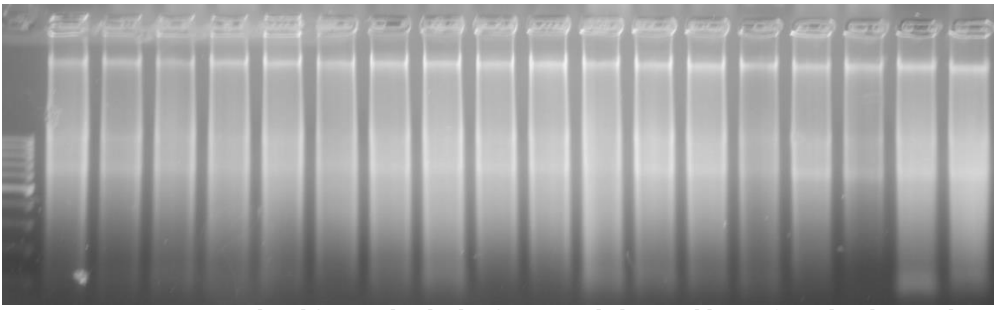


Fig. 1 **DNA isolated from individuals of Dracocephalum moldavica (1-molecular weight marker, 2-8-different genotypes A1, 9-12-different genotypes A2, 13-16- different genotypes B1, 17-19- different genotypes B2) . 1% agarose gel separation, ethidium bromide visualization.**

SSR Marker Analysis

Given that the SSR markers analyzed are not specific to the *Dracocephalum* genus, as mentioned previously, there are no specific markers developed for this genus, the preliminary results obtained were not as desired. The annealing temperature of the primers, as indicated in other studies conducted on lamiacee species, was 55°C, but in our case, clear bands were not obtained during amplification. We believe that further experiments are needed, with adjustments to the annealing

and elongation temperatures, considering that the size of the potential amplicons is not known.

ISSR Marker Analysis

Amplification of DNA with the 9 primers specific for ISSR markers revealed a varied polymorphism among individuals of *Dracocephalum moldavica*. An annealing temperature of 45°C was used for the primers, which was not optimal for some primers and also requires optimization. However, some primers produced well-defined fragments, allowing for a preliminary analysis of the plants. Primers UBC809, UBC811, and UBC856 did not generate any fragments during amplification; thus, reaction conditions will be modified specifically for these primers to determine whether this was due to the conditions or because these primers do not find complementarity with the genome of this plant species and therefore cannot be used for molecular analysis in this species.

Primers UBC808, UBC812, UBC818, and UBC855 generated polymorphic patterns, and it was checked whether these patterns are consistent after optimizing the amplification reactions. Primer UBC857 produced a single band, indicating that this marker is non-polymorphic. For illustration, only a few electrophoretic aspects are presented here, highlighting either the presence of genetic polymorphism (Fig. 2) or its absence (Fig. 3) with some of the primers used in the study. It can be observed that the highest number of bands was obtained with primers UBC812, UBC818, and UBC855, specifically 7 bands, which were present only in some plants, making the marker polymorphic. The lowest number of bands was obtained with primer UBC857, which produced a single band across all plants. For primer UBC808, polymorphism was evident in plants from groups A1 and B2. Primer UBC812 showed polymorphism only in plants from groups A1 and A2. Primer UBC818 generated polymorphic bands in all plants from all groups except for those in group A2, while primer UBC855 produced polymorphic patterns in plants from all groups except for those in group B2.

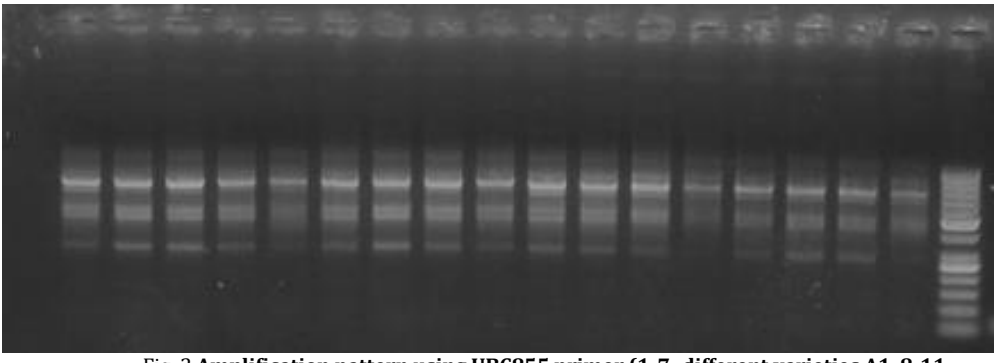


Fig. 2 **Amplification pattern using UBC855 primer (1-7- different varieties A1, 8-11- different varieties A2, 12-15- different varieties B1, 16-18- different varieties B2, 19- Fermentas molecular weight marker, SM1133). 1% agarose gel separation, ethidium bromide visualization.**

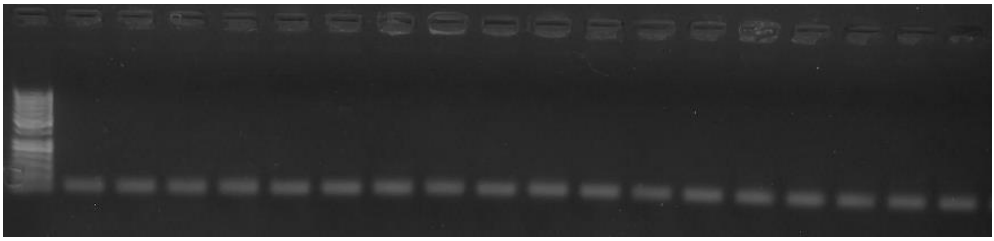


Fig. 3 **Amplification pattern using primer UBC857 (1-molecular weight marker Fermentas, SM1133, 2-8- different varieties A1, 9-12- different varieties A2, 13-16- different varieties B1, 17-19- different varieties B2). 1% agarose gel separation, ethidium bromide visualization.**

Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Phenolic Acids Content (TPAC)

Three different cultivations of *D. moldavica* (samples A1, A2, and B2) were subjected to phytochemical analysis and evaluation of their antioxidant, antimicrobial, and cytotoxic potential. The primary aim of this evaluation was to characterize the chemical compositions and selected biological activities, as well as to determine the cultivar-specific features and possible connections with their polyphenolic profiles.

Table 1 presents the results of the evaluation of total polyphenol content (TPC), total flavonoid content (TFC), and total phenolic acid content (TPAC), obtained through spectrophotometric methods.

Table 1. ***Total polyphenolic, flavonoid and phenolic acids contents of D. moldavica samples***

Sample	TPC (g GAE/100 g dry plant material)	TFC (g RE/100 g dry plant material)	TPA (g CAE/100 g dry plant material)
A1	4.862 ± 0.163	1.218 ± 0.096	4.267 ± 0.061*
A2	4.620 ± 0.151	1.100 ± 0.063	3.447 ± 0.161
B2	5.631 ± 0.175**	1.255 ± 0.167	5.806 ± 0.044**

Note: Each value represents the mean ± standard deviations of three independent measurements. GAE: Gallic Acid Equivalents; RE: Rutin Equivalents, CAE: Cafeic Acid Equivalents. * $p < 0.05$ B2 vs. A2 and A1 vs A2, ** $p < 0.001$ B2 vs A1 and A2

Differences were observed when comparing the total flavonoid and phenolic acid contents among the *D. moldavica* samples. The most significant variation ($p < 0.001$) was found for TPAC, with the highest level in the B2 cultivar. In fact, sample B2 showed significantly higher amounts of TPC compared to A1 ($p < 0.05$) and A2 ($p < 0.001$).

Antibacterial Tests

The results of the in vitro antimicrobial potential evaluation are presented in Table 2 (inhibition zones) and Table 3 (MIC index).

All three *D. moldavica* samples exhibited in vitro antimicrobial potential, with significant variations depending on the bacterial species. The greatest antibacterial activity was observed against the two Gram-positive bacteria (MSSA > MRSA). The B2 sample demonstrated the highest capacity to inhibit bacterial growth of *Staphylococcus*

aureus strains (MSSA and MRSA) with inhibition zones of 20.50 ± 0.55 mm and 23.50 ± 0.55 mm, respectively. Additionally, extracts from samples A1 and A2 produced inhibition zones similar to one of the positive controls, gentamicin ($p > 0.05$), but significantly smaller ($p < 0.05$) compared to extract B2. However, these diameters were significantly smaller ($p < 0.05$) compared to those of amoxicillin-clavulanic acid.

Regarding Gram-negative bacteria, no inhibitory effects were observed. Effects were recorded for the reference strain *Pseudomonas aeruginosa*, while *Escherichia coli* showed in vitro susceptibility, particularly to the B2 sample extract.

Table 2. ***In vitro antibacterial activity of the D. moldavica samples by well diffusion method***

Zone of Inhibition (mm)				
Sample	**MSSA**	**MRSA**	***Escherichia coli***	***Pseudomonas aeruginosa***
A1	19.67 ± 0.52	17.33 ± 0.52	15.50 ± 0.55	0
A2	20.17 ± 0.41	18.17 ± 0.41	15.83 ± 0.41	0
B2	23.50 ± 0.55 ^{a,b,d}	20.50 ± 0.55 ^{a,b,d}	17.33 ± 0.52	0
Amoxicillin-clavulanic acid	29 ± 0.00 ^{a,b,c}	28 ± 0.00 ^{a,b,c}	19 ± 0.00 ^{a,b,c}	0
Gentamicin	20 ± 0.00	17 ± 0.00	19 ± 0.00	18 ± 0.00

Note: MSSA -, MRSA - Methicillin-Resistant *Staphylococcus aureus*; Values represent the mean ± standard deviations of two independent measurements. $^{a-c}$ Means with different subscript letters within a row are significantly different at $p < 0.05$; A1 (4.862 mg GAE/mL), A2 (4.620 mg GAE/mL), B2 (5.631 mg GAE/mL), Antibiotic disks: Amoxicillin-clavulanic (20-10 µg), Gentamicin (10 µg).

Table 3. ***In vitro antibacterial activity of the D. moldavica samples by broth microdilution assay***

MIC index MBC (µmol GAE/100 µL) /MIC (µmol GAE/100 µL)			
Sample	**MSSA**	**MRSA**	***Escherichia coli***
A1	1 0.356/0.356	2 0.712/0.356	1 0.712/0.712
A2	2 0.343/0.171	4 0.687/0.171	4 2.750/0.687
B2	2 0.825/0.412	4 0.825/0.206	4 3.300/0.825

The MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values, collected using the broth microdilution method (Table 3), confirmed the superior efficacy of the extract obtained from sample B2. Additionally, these values indicated bactericidal effects manifested by all three extracts, as per the MIC index ($MBC/MIC \leq 4$).

Cytotoxicity Evaluation

The CCK-8 test was conducted to determine the cytotoxic potential of *D. moldavica* samples on BJ and DLD-1 cells. As shown in Figures 4a, 5a, and 6a, the evaluated samples (A1, A2, and B2) and Cisplatin did not have a significant effect ($p > 0.05$) on the survival of BJ cells. In DLD-1 cells, the tested samples led to a significant decrease ($p < 0.05$) in cell proliferation (Figures 4b, 5b, and 6b). Extract A1 resulted in cell viability percentages ranging from 71.80% ± 5.51 to 75.16% ± 3.44, at concentrations of 0.571 µmol GAE and 0.142 µmol GAE, respectively. Thus, the cytotoxic potential determined for all A1 extract concentrations was significant ($p < 0.05$ compared to the negative control) and still significantly lower ($p < 0.05$) than the positive control, represented by cells treated with Cisplatin (56.19% ± 4.02), with an IC50 of 0.466 µmol GAE (Figure 4b).

A similar cytotoxicity pattern was observed for extract A2, but only at concentrations ranging from 0.137 to 0.412 µmol GAE. Concentrations of 0.550 and 0.687 µmol GAE (IC50 = 0.40 µmol GAE) showed cytotoxic effects similar to those of the positive control, Cisplatin ($p > 0.05$) (Figure 5b). The most intense cytotoxicity was recorded for extract B2, where the average cell viability was 56.83% ± 3.58 ($p < 0.05$ compared to the negative control), with an IC50 of 0.54 µmol GAE. No significant differences were found between the viability percentages calculated for any of the tested concentrations and Cisplatin ($p > 0.05$) (Figure 6b). The decrease in cell viability is correlated with the concentration of TPC (mg/g GAE).

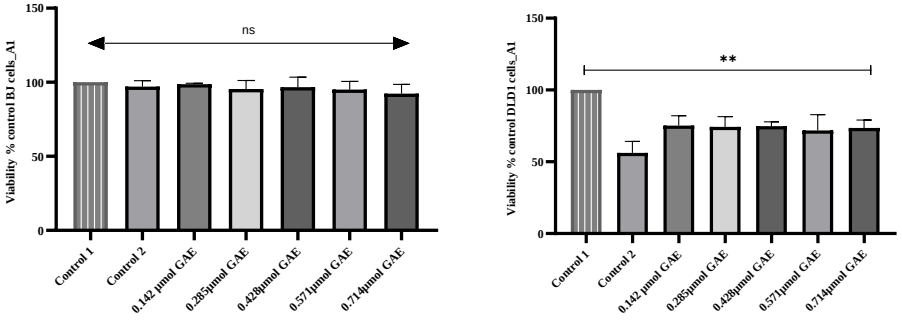
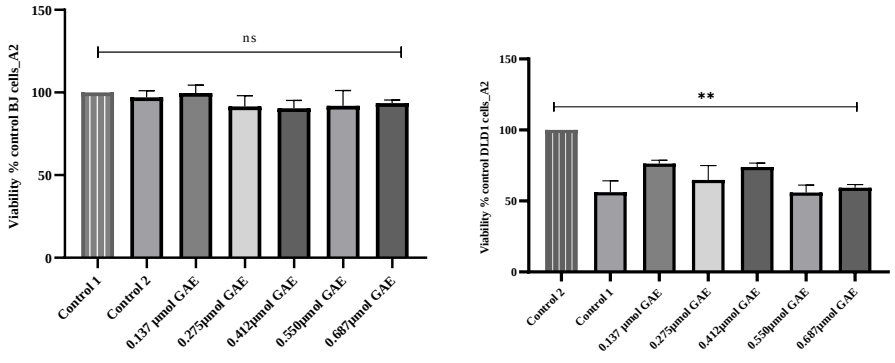
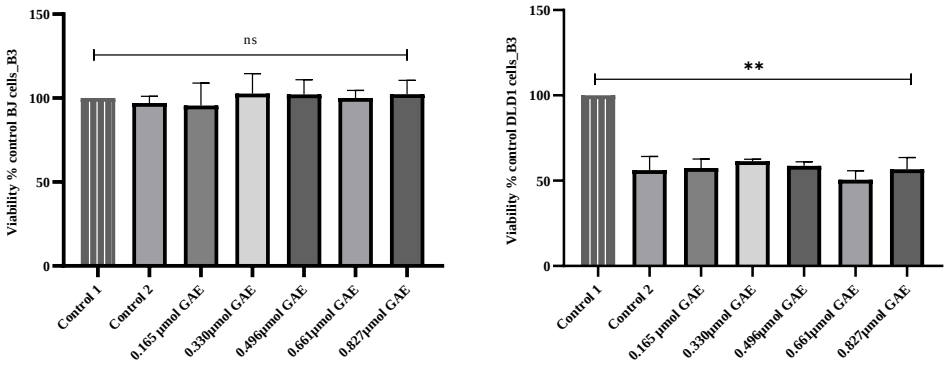


Fig. 4. **Viability percentages obtained for BJ (a) and DLD-1 (b) cells after 24 h incubation with A1 extract. The A1 concentrations were calculated according to the TPC µmol GAE (0.142 - 0.714 µmol GAE), Control 1 (Negative control) - untreated cells, Control 2 - cells treated with Cisplatin 25 µM). Data represent the mean ± SD of three independent experiments, ns – no significant differences, ** - *p* < 0.05**



(**a**) (**b**)

Fig. 5. **Viability percentages obtained for BJ (a) and DLD-1 (b) cells after 24 h incubation with A2 extract. The A2 concentrations were calculated according to the TPC µmol GAE (0.137 - 0.687 µmol GAE), Control 1 (Negative control) - untreated cells, Control 2 - cells treated with Cisplatin 25 µM). Data represent the mean ± SD of three independent experiments, ns – no significant differences, ** - *p* < 0.05**



(**a**) (**b**)

Fig. 6. Viability percentages obtained for BJ (a) and DLD-1 (b) cells after 24 h incubation with B2 extract. The B2 concentrations were calculated according to the TPC µmol GAE (0.165 - 0.827 µmol GAE), Control 1 (Negative control) - untreated cells, Control 2 - cells treated with Cisplatin 25 µM). Data represent the mean ± SD of three independent experiments, ns – no significant differences, ** - p < 0.05

Medicinal plants have a long history in treating a wide range of conditions, including infectious and cancerous disorders, with numerous species having been studied for their potential therapeutic properties (Nadeem et al., 2022). Antioxidant mechanisms play one of the most critical roles in addressing many of these conditions (Vrânceanu et al., 2022). Natural compounds, such as essential oils (Toma et al., 2010) or flavonoids (Panche et al., 2016), due to their natural ability to neutralize reactive oxygen species (ROS), are among the most studied compounds for their antimicrobial or cytotoxic activities, providing essential antioxidant mechanisms. In this context, the

present study introduces novelty and originality by aiming to provide scientific arguments for the cytotoxic and antibacterial activities of polyphenols from *D. moldavica*, demonstrating them through antioxidant mechanisms, as well as for the advantages of cultivating this species, specifically through changes in the chemical composition of the plant material obtained under cultivation conditions.

CONCLUSION AND RECOMMENDATIONS

Dracocephalum moldavica L. with its distinct citrus aroma due to the presence of neral and geranial in the essential oil, is widely used as a seasoning and for making teas. Dracocephalum moldavica has multiple uses, being employed not only for flavoring foods but also in perfumery, the alcoholic beverage industry, and in the manufacture of soaps and detergents. Additionally, the plant is cultivated as a honey plant and ornamental in gardens and parks.

The year 2018, compared to the year 2019, was much more productive from all points of view. In this sense, we recommend for sowing/planting seeds that are not more than 1 year old, if the highest productivity is desired.

Among the four genotypes, B2 Buzau proved to be the most productive, therefore we recommend that this genotype be cultivated.

ISSR markers analyzed revealed genetic polymorphism in the *Dracocephalum moldavica* plants. The highest polymorphism was detected with markers generated using primers UBC808, UBC812, UBC818, and UBC855. The markers generated with primers UBC818 and UBC855 demonstrated genetic polymorphism, including intergroup variation. The marker with primer UBC857 was non-polymorphic.

The superior quality of plant material obtained through cultivation is reflected in the higher content of polyphenolic compounds, associated with significant antioxidant and antimicrobial properties. Additionally, our results highlighted significant cytotoxic potential, particularly against colorectal adenocarcinoma cell lines. The antimicrobial potential was demonstrated, especially against Gram-positive bacteria, and a link was established between antioxidant capacity and both cytotoxic and antimicrobial tests, in relation to the polyphenolic compositions of the three tested cultivars. Among these cultivars, sample B2 was found to have the richest polyphenolic composition and the most significant biological potential. These findings provide further support for considering selected cultivars of *D. moldavica* (particularly B2 with blue flowers) as promising plant raw materials for the pharmaceutical industry producing phytotherapeutic remedies, which require additional investigation to elucidate the mechanisms of these biological activities and to prove the medicinal potential of this species as a plant-based medicinal product.

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

The research presented in this thesis is original due to its approach, the genotypes tested, and the research area. In Transylvania, according to available

literature, there have been no detailed studies on the cultivation of *Dracocephalum moldavica* via direct seeding.

The molecular analysis of the *Dracocephalum moldavica* species focused on the genetic structure of a cultivated plant population. Genetic analysis can provide crucial data on intra- and inter-populational genetic polymorphism, closely related to the species' adaptive capacity to various pedoclimatic conditions, as well as its biosynthetic capability for pharmacologically active compounds. This offers the potential for accurate identification and selection of valuable new genotypes.

The phytochemical analysis aimed to evaluate the chemical profiles and potential cytotoxic and antimicrobial activities of three *Dracocephalum moldavica* cultivars in relation to their antioxidant capacities. The evaluation of their cytotoxic and antibacterial activities linked to their antioxidant mechanisms is less studied in scientific literature. Given this, the current research introduces novelty and originality by attempting to connect these biological activities with a less-studied class of compounds in the species' composition, specifically polyphenols.

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