UNIVERSITY OF MEDICINE AND PHARMACY
"CAROL DAVILA", BUCHAREST
DOCTORAL SCHOOL
DOCTORAL FIELD OF PHARMACY

Ph.D. THESIS

PhD Supervisor:
PROF. UNIV. DR. GÎRD CERASELA ELENA

PhD student:
RUSTEM (CĂS. IONUS) R. ELIS

2024

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PRELIMINARY RESEARCH ON THE PHYTOCHEMICAL AND BIOLOGICAL PROFILE OF THE *AJUGA CHAMAEPITYS* SPECIES, SPONTANEOUS IN ROMANIA

PHD THESIS SUMMARY

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Table of Contents

List of published scientific papers

Articles published in ISI rated journals:

1. **Elis Ionus**, Laura Adriana Bucur, Carmen Elena Lupu, Cerasela Elena Gîrd. Evaluation of the chemical composition of *Ajuga chamaepitys* (L.) Screb. from the spontaneous flora of Romania (2021). *Farmacia*, 69(3), 461-466, ISI indexed journal, with impact factor **1.433**, ISSN: 2065-0019 (*for the Online Edition*) and 0014-8237 (*for the Printed Edition*), link: https://doi.org/10.31925/farmacia.2021.3.8
2. **Elis Ionus**, Verginica Schröder, Carmen Lidia Chiţescu, Laura Adriana Bucur, Carmen Elena Lupu, Denisa-Elena Dumitrescu, Liliana Popescu, Dragos Paul Mihai, Octavian Tudorel Olaru, George Mihai Nițulescu, Boscencu Rica, Gîrd Cerasela Elena. Phytochemical, *In Vitro*, *In Vivo*, and *In Silico* Research on the Extract of *Ajuga chamaepitys* (L.) Schreb. (2024). *Plants*, 13, 1192, ISI indexed journal, with impact factor **4.0**, ISSN: 2223-7747 (*for the Online Edition*), link: https://doi.org/10.3390/plants13091192

Participation in poster sessions in national or international symposia or congresses with the publication of abstracts:

1. **Elis Ionus**, Cerasela Elena Gîrd, Laura Bucur, *Ajuga chamaepitys* (L.) Schreb., potential resource of active principles (2021). National Congress of Pharmacy XVIIIth Edition, Oradea, September 15-17, summary published in the Congress Brochure: Pharmacy. From innovation to good pharmaceutical practice, ISBN: 978-606-10-2144-4.
2. **Elis Ionus**, Verginica Schröder, Sorin Grigore, Cerasela Elena Gîrd, Biological effects of the selective dry extract *Ajuga chamaepitys* (L.) Schreb. (2023). Symposium with international participation : Alternative and complementary therapies (Homeopathy/Phytotherapy), 7th edition, Constanța, 27-28 october, summary published in the *Book of abstracts*, ISSN: 2601-1476 (certified with 1st prize).

Scientific works communicated at national events

- **Elis Ionus**, Cerasela Elena Gîrd, Laura Bucur, Evaluation of antioxidant capacity and phytochemical screening of dry extract of *Ajuga chamaepitys* (L.) Schreb. (2021). Symposium with international participation : Alternative and complementary therapies (Homeopathy/Phytotherapy), 4th edition, Constanța, 26-27 march, summary published in the *Book of abstracts*, ISSN: 2601-1476 .

LIST OF ABBREVIATIONS

- FC – flavones content;
- PAC – phenolcarboxylic acids content;
- TPC – total polyohenols content;
- BSLA – Brine shrimp letality assay;
- UHPLC-HRMS/MS - Identification and quantification of polyphenolic compounds in ACHE by ultra-performance liquid chromatography and high-resolution mass spectrometry;

INTRODUCTION

Medicinal plants have been used since ancient times and are recognized as having an important interest in the pharmaceutical industry because they can alleviate and treat various pathologies. The plants used traditionally have had an important impact in research, many of which are currently used on a large scale, because they have proven through experimental *in vivo* and *in vitro* studies their pharmacotherapeutic utility, optimizing the methods of processing and incorporating the principles active ingredients of these phytopreparations.

Along with the modernization of research technologies, phytotherapy has gained momentum as an alternative to allopathic treatments that frequently exhibit pharmacotoxicological effects. Recognition of plants in the medicinal field is not surprising because they have demonstrated effectiveness and benefits for the human body.

The chemical constituents of plants, called phytonutrients, are not indispensable to life; however, by adding them to the diet (or by taking food supplements), they can have important properties in the protection or prevention of certain diseases. For example, they can have an antioxidant role to protect the body's cells against harmful free radicals, cardioprotective role to regulate blood pressure and support optimal cardiac functions, and immunoprotective role to prevent common colds and fight infections, among others.

In modern times, people tend to place great importance on lifestyle and the prevention of certain diseases; therefore, greater attention is paid to traditional phytopreparations in medicinal areas, and the benefits of medicinal plants as therapeutic resources are recognized. Moreover, in recent decades, scientific research on herbal medicines has shown an upward trend, indicating an increased interest by the population in this field. However, as in any field, many elements remain unexplored and misunderstood, prompting researchers to continue researching, exploring, and exploiting these medicinal plants.

In this research thesis, we have proposed conducting preliminary research regarding the pharmacobotanical, phytochemical, and pharmacological profile of the species *Ajuga chamaepitys* (L.) Schreb, spontaneous in the area of Central Dobrogea.

To justify for selecting this theme was the fact that *Ajuga chamaepitys* (L.) Schreb. is a traditional plant. To provide evidence that they represent a potential resource of active compounds, we aimed to establish a correlation between phytoconstituents identified and quantified using

methods specific to the field of phytochemistry and potential therapeutic effects through *in vitro*, *in vivo*, and *in silico* research.

The originality of the research was the phytochemical and phytotherapeutic evaluation of the species that inhabit this area of the country, the area of Central Dobrogea.

Research objectives: Phytochemical evaluation of various types of plant products (roots, aerial parts, flowers) harvested from the species *Ajuga chamaepitys* (L.) Schreb. with the aim of establishing the type of plant organ most ennobled in active principles; establishing the optimal harvesting interval of the vegetable product/products (harvesting of vegetable raw materials in the May-August period); establishing the type of solvent (water, ethanol of different concentrations) that solubilizes the largest amount of active principles, with the aim of obtaining a dry extract rich in active principles; establishing the methodology for obtaining the dry extract; establishing the quality of the dry extract through the quantitative determination (spectrophotometric and HPLC methods) of the active chemical constituents; evaluation of the antioxidant action *in vitro* by three methods (DPPH, ABTS, FRAP method); assessment of *in vivo* cytotoxicity on *Artemia* and *Daphnia* species; *in silico* studies in order to establish the potential enzymatic interactions of polyphenolic derivatives (with possible utility in the treatment of neurodegenerative pathologies and neuropathic pain); dissemination of the results obtained in specialized journals and within scientific events.

1. CURRENT STATE OF KNOWLEDGE

Pharmacobotanical data for the species *Ajuga chamaepitys* (L.) Schreb

Chapter 1 systematizes information from specialized literature on *Ajuga* species and discusses the most widespread species in the Dobrogea area, *Ajuga reptans* (L.), *Ajuga genevensis* (L.), *Ajuga laxmanni* (L.) Benth., *Ajuga salicifolia* (L.) Schreb., *Ajuga chamaepitys* (L.) Schreb.

2. PREVIOUS RESEARCH ON THE *AJUGA CHAMAEPITYS* (L.) SCHREB. SPECIES

Chapter 2 systematizes information on phytochemical and phytopharmacological research on *Ajuga chamaepitys* (L.) Schreb. and other intermediates of this species (subspecies).

PERSONAL RESEARCH

3. DETERMINATION OF MORPHO-ANATOMIC PARTICULARS IN VEGETABLE PRODUCTS HARVESTED FROM *AJUGA CHAMAEPITYS* (L.) SCHREB. SPREAD IN DOBROGEA

Chapter 3 presents the research conducted to highlight the anatomical-morphological peculiarities based on the macroscopic and microscopic analyses (cross-sections) conducted on different types of plant organs harvested from the species *Ajuga chamaepitys* (L.) Schreb.

Ajuga chamaepitys (L.) blooms in the summer from May to August, and plant products (*radix*, *folium*, *flores*, *caulis*, *herba*) were collected in each month of flowering, resulting in four batches of analysis. In this way, we can establish the main groups of active principles and determine which of these batches contains the most abundant active compound composition for comparison. The species *Ajuga chamaepitys* (L.) Schreb. was found and collected in the Dobrogea Gorges, 44°29'16.5"N - 28°27'09.5"E.

The data obtained from the evaluation of the different types of plant products and species were in accordance with the data from the specialized literature. The entire plant has a strong, pine-like smell.

The anatomical-morphological observations indicated that the species morphologically fell within the characteristics of the genus. The study of *Ajuga*'s organs had several objectives, including improving the methods of microscopic observation of the structures. For this purpose, as a novelty, this study presents the application of fluorescence microscopy techniques, which ensure the completion of information by visualizing additional details related to the topochemistry of cell walls and the synthesis of specific compounds. The use of these UV visualization methods and the realization of vital stainings provides a new perspective regarding the understanding and interpretation of morphological details because the physiological phenomena of the analyzed cellular structures, which are correlated with the synthesis of specific compounds (lignin, cellulose, cutin, chlorophyll pigments, etc.) or pH changes (autolysis, secretory products).

4. ESTABLISHING THE QUALITY STANDARDS OF VEGETABLE RAW MATERIALS

4.1. Obtaining vegetable raw materials

Harvesting was carried out in 2019, after the 15th of each flowering month (May, June, July, August), in the morning, in dry and sunny weather, followed by the process of sorting and drying the plant material under the conditions from the Pharmacognosy laboratory, resulting in four

batches of analysis (May - sample 1, June - sample 2, July - sample 3, August - sample 4). Also, six plant organs were sorted from each batch, which were subsequently subjected to phytochemical study (flowers, leaves, stems, roots, fruits and aerial parts). Harvesting was carried out as follows: for the aerial parts (H – aerial parts), resulting in sample H1 (collected in May), sample H2 (collected in June), sample H3 (collected in July) and sample H4 (collected in August); for leaves (F - leafs), resulting in sample F1 (May), sample F2 (June), sample F3 (July) and sample F4 (August); for flowers (FL - flowers), resulting in sample FL1 (May), sample FL2 (June), sample FL3 (July) and sample FL4 (August); for stems (C - stems), resulting in sample C1 (May), sample C2 (June), sample C3 (July) and sample C4 (August); for roots (R – roots), resulting in sample R1 (May), sample R2 (June), sample R3 (July) and sample R4 (August). We decided to exclude the fruits, because they were in a very small amount, not allowing us to carry out the necessary studies. The plant products were dried in the shade, at room temperature, under the conditions of the Pharmacognosy laboratory of the Faculty of Pharmacy, "Ovidius" University of Constanța, plant material being stored in the collection of the Pharmacognosy laboratory.

4.2. Phytochemical analyzes - identification reactions of the main chemical compounds

Due to the fact that *Ajuga chamepitys* (L.) Schreb. is a species that has not been studied from a phytochemical point of view and is an unknown species, we used qualitative phytochemical analysis to identify the main active compounds. Qualitative phytochemical analysis requires that plant products be subjected to successive extraction with different solvents and subsequently carry out specific identification reactions for each group of phytochemical compounds or certain chemical constituents. The identification reactions were carried out for each type of plant organ in each batch, precisely to highlight the qualitative differences between the types of plant products.

Conclusions. The analyses performed on the ether extract revealed the presence mainly of carotenoids. The residue was buttery and had a pleasant smell, indicating the presence of volatile oils and fatty acids. According to the obtained results, phenylcarboxylic acids, catechin tannins, reduced compounds, coumarins, and triterpene heterosides was mainly found in the methanolic extract. Finally, the following chemical compounds were highlighted in the aqueous extract: mucilages and saponosides with detergent properties.

4.3. Determination of humidity

The results obtained fall within the quality standards of vegetable products.

4.4. Determination of soluble substances

Through this determination, we highlight the amount (expressed in g%) of substances that can be extracted depending on the solvent.

Table IV.1. Determination of soluble substances in different solvents for each type of vegetable product.

Type of vegetal product	Sample	Type of solvent	Soluble substances g% ± standard deviation (*n*=3)	Type of vegetal product	Sample	Type of solvent	Soluble substances g% ± standard deviation (*n*=3)
Herba (aerial parts)	H1	ethanol 70%	30.4721 ± 0.2164	*Folium* (leafs)	F1	ethanol 70%	30.9230 ± 0.0530
		ethanol 50%	34.3678 ± 0.0030			ethanol 50%	37.3263 ± 0.0533
		etanol 20%	36.4618 ± 0.2068			ethanol 20%	39.9193 ± 0.0379
		water	34.3406 ± 0.0023			water	39.8207 ± 0.0006
	H2	ethanol 70%	27.7466 ± 0.5178		F2	ethanol 70%	30.8745 ± 0.2317
		ethanol 50%	29.5662 ± 0.0371			ethanol 50%	36.5696 ± 0.0284
		ethanol 20%	34.1802 ± 0.0842			ethanol 20%	39.4866 ± 0.0685
		water	31.9468 ± 0.0012			water	33.7807 ± 0.1063
	H3	etanol 70%	28.3446 ± 0.0224		F3	ethanol 70%	38.6295 ± 0.1959
		ethanol 50%	32.2954 ± 0.0377			ethanol 50%	44.8601 ± 0.0901
		ethanol 20%	34.1353 ± 0.0074			ethanol 20%	47.3890 ± 0.1955
		water	30.0335 ± 0.2001			water	40.4680 ± 0.3408
	H4	ethanol 70%	25.9839 ± 1.0013		F4	ethanol 70%	29.5840 ± 0.1151
		ethanol 50%	31.2247 ± 0.0825			ethanol 50%	36.2109 ± 0.1165
		ethanol 20%	35.5771 ± 0.1530			ethanol 20%	42.1847 ± 0.1023
		water	25.6663 ± 0.1086			water	37.5457 ± 0.1438
Flores (flowers)	FL1	ethanol 70%	36.4342 ± 0.3314	*Caulis* (stems)	C1	ethanol 70%	26.1248 ± 0.6893
		ethanol 50%	36.7727 ± 0.2728			ethanol 50%	33.4137 ± 0.0530
		ethanol 20%	37.3017 ± 0.3611			ethanol 20%	35.6965 ± 0.0991
		water	36.8094 ± 0.0005			water	36.9994 ± 0.0011
	FL2	ethanol 70%	30.4120 ± 0.8151		C2	ethanol 70%	19.1318 ± 0.1293
		ethanol 50%	32.0383 ± 0.3561			ethanol 50%	24.0898 ± 0.2056
		ethanol 20%	33.0591 ± 0.2718			ethanol 20%	25.2178 ± 0.1202
		water	28.1583 ± 0.5214			water	23.8367 ± 0.0226
	FL3	ethanol 70%	31.9521 ± 0.4447		C3	ethanol 70%	24.6953 ± 0.0528
		ethanol 50%	33.9347 ± 0.0693			ethanol 50%	29.4511 ± 0.1333
		ethanol 20%	34.8945 ± 0.0076			ethanol 20%	29.7833 ± 0.0489
		water	30.2317 ± 0.0722			water	29.0932 ± 0.5202
	FL4	ethanol 70%	35.3686 ± 0.3942		C4	ethanol 70%	22.3889 ± 0.5361
		ethanol 50%	38.3826 ± 0.2928			ethanol 50%	28.4214 ± 0.1513
		ethanol 20%	41.0420 ± 0.0922			ethanol 20%	29.8708 ± 0.1901
		water	38.9171 ± 0,0801			water	28.2125 ± 0.1271
Radix (roots)	R1	ethanol 70%	16.5349 ± 0.2114				
		ethanol 50%	19.1022 ± 0.1783				
		ethanol 20%	21.3447 ± 0.2716				
		water	17.1352 ± 0.0025				
	R2	ethanol 70%	20.2281 ± 0.6557				
		ethanol 50%	25.4451 ± 0.1281				
		ethanol 20%	24.7377 ± 0.0444				
		water	22.4734 ± 0.0015				

	R3	ethanol 70%	27.8101 ± 0.0073
		ethanol 50%	32.5118 ± 0.1795
		ethanol 20%	34.4623 ± 0.0962
		water	28.8712 ± 0.0454
	R4	ethanol 70%	23.8863 ± 0.1588
		ethanol 50%	33.2257 ± 0.0507
		ethanol 20%	29.7707 ± 0.0513
		water	24.5510 ± 0.1187

Notes: H1, H2, H3, H4 (aerial parts collected in May, June, July, August), F1, F2, F3, F4 (leaf samples collected in May, June, July, August), FL1, FL2, FL3, FL4 (flowers samples collected in May, June, July, August), C1, C2, C3, C4 (stems samples collected in May, June, July, August), R1, R2, R3 , R4 (roots samples collected in May, June, July, August). The highest values for the respective vegetable products are highlighted (in green).

Conclusions. According to the results obtained, we noticed that the compounds tended to be extracted at a higher percentage with ethanol 20% (v/v), this can also be favored by the fact that the plant material contained a large amount of mucilage.

4.5. Quantitative determination of bioactive constituents

4.5.1. Determination of flavone content

The principle of this method consists of determining the intensity of the yellow color through a method based on the complexation reaction with aluminum chloride ($AlCl_3$), where the flavones are extracted using hydrophilic solvents to form complex combinations. The reading of the absorbances is performed at λ = 427 nm compared with the control, which rutin is used as a reference substance.

Conclusions. We note that the results of the spectrophotometric determination regarding the flavone content, in the case of the radix plant product, were not detected in any sample, and the highest flavone content was recorded in the FL1-70 sample, 7.1501 ± 2.4560 g rutin equivalents/100 g dry vegetable product (Figure 4.1).

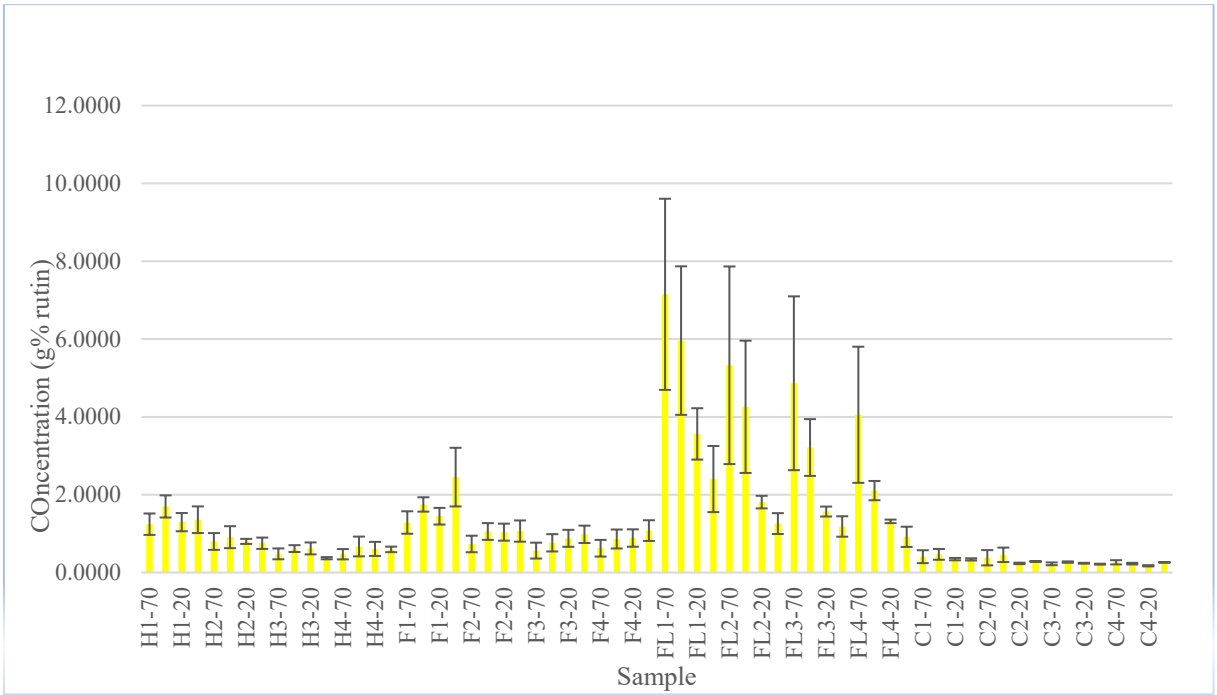

Figure 4.1. The results of the spectrophotometric determination of flavonoid content expressed in g flavones (rutin equivalents)/100 g dry vegetable product.

4.5.2. Determination of phenolcarboxylic acids content

The principle of this method is based on the ability of the phenolcarboxylic acids to form with nitrous acid (HNO_2) nitrosoderivatives, which, through spontaneous tautomerization, pass into isonitroderivatives (oximes), which, due to their weak acid character, dissolve in alkaline solutions, resulting in red color [63]. The absorbances are read at λ = 525 nm compared with the control, and chlorogenic acid was used as the reference substance.

Conclusions. According to the results of the spectrophotometric determinations, the highest phenolcarboxylic acids content was recorded in the FL1-50 sample, 2.1480 ± 0.4541 g chlorogenic acid equivalents/100 g dry vegetable product, while in the radix vegetable product had the lowest amount (Figure 4.2.).

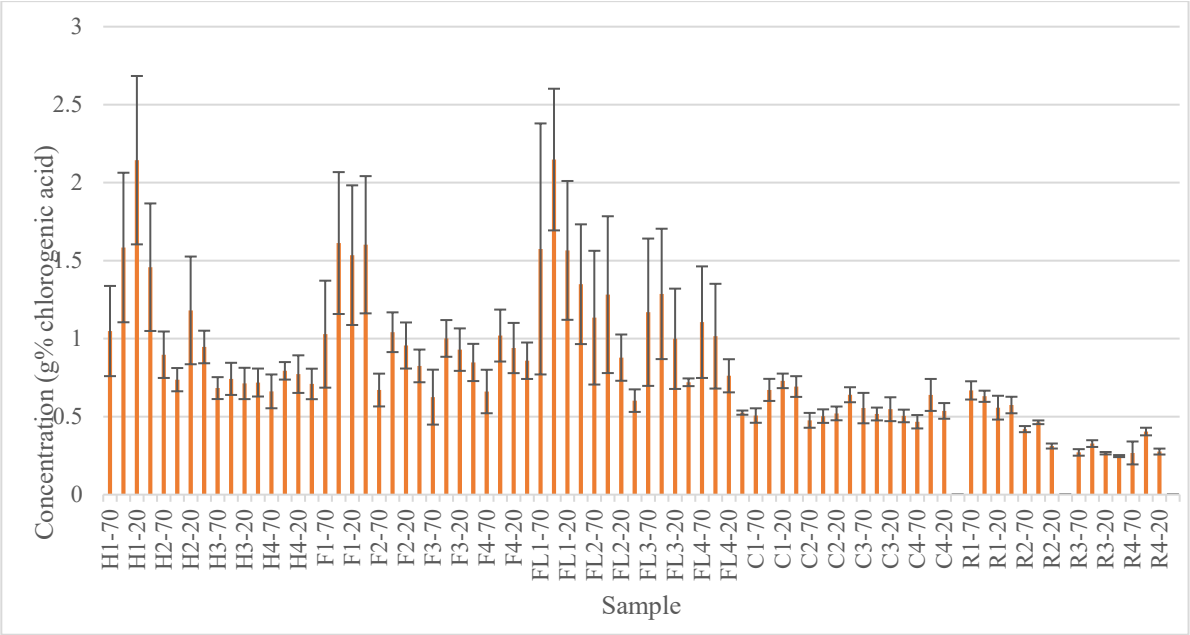

Figure 4.2. The results of the spectrophotometric determination of the phenolcarboxylic acids content expressed in g (chlorogenic acid equivalents)/100 g of dry vegetable product.

4.5.3. Determination of total polyphenol content

The principle of this method is based on the ability of polyphenols to form a blue color in the presence of the Folin-Ciocâlteu reagent in the dark [63, 64]. The absorbances are read at λ = 763 nm compared with the control, and tannic acid was used as a reference substance.

According to the results, for the total polyphenol content, the highest value was recorded for the FL1-50 sample, obtaining 5.9601 ± 1.1547 g of tannic acid equivalents/100 g dry vegetable product, whereas in the type vegetable product radix, sample R4-A, the lowest amount was recorded, 0.3785 ± 0.0141 g of tannic acid equivalents/100 g dry vegetable product (Figure 4.3.).

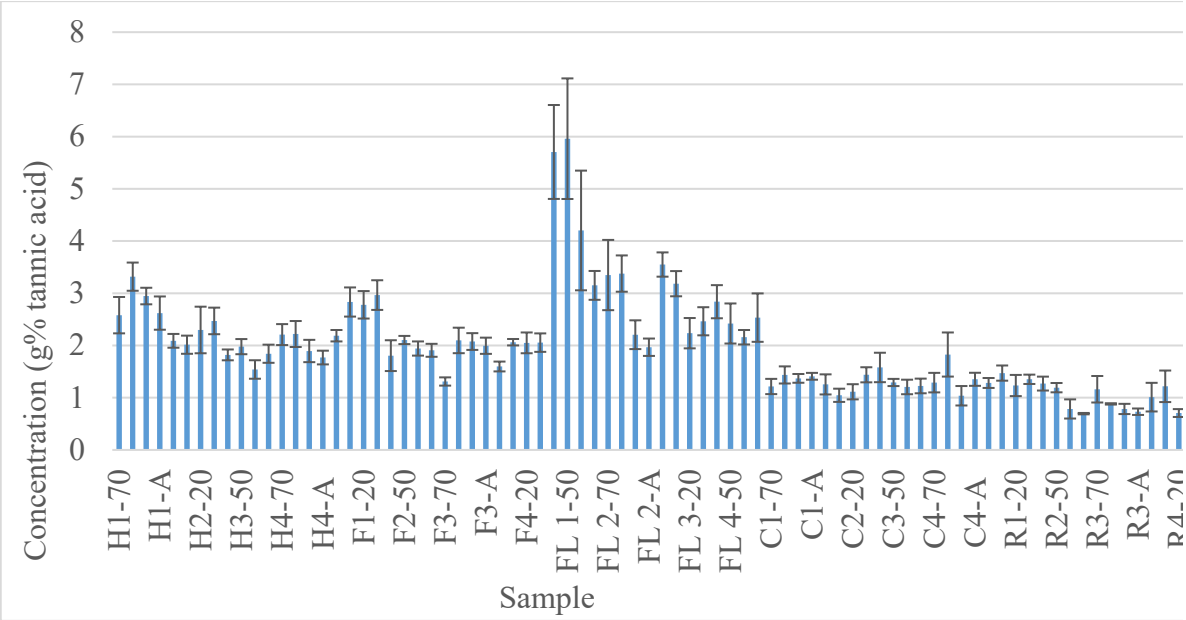

Figure 4.3. The results of the spectrophotometric determinations, regarding the content of total polyphenols expressed in g total polyphenols (tannic acid equivalents)/100 g dry vegetable product.

4.5.4. Conclusions of the three spectrophotometric determinations

According to the results obtained, the most abundant amount of chemical compounds was mainly found in the collection batch from May, sample FL1-70 contained the highest content of flavones, 7.1501 ± 2.4560 g rutin equivalents/100 g dry plant product, sample FL1-50 contained the highest content in phenolcarboxylic acids, registering 2.1480 ± 0.4541 g chlorogenic acid equivalents/100 g dry plant product, and at the same time sample FL1-50 the highest content of total polyphenols, 5.9601 ± 1.1547 g tannic acid equivalents/100 g dry vegetable product.

4.5.5. Statistical analysis of the obtained data

Analysis of variance (ANOVA) was performed to assess the optimal harvest time. Post hoc comparisons of mean amounts were made using the least significant difference (LSD) post hoc test. Differences were considered significant when p values were less than 0.05 ($p < 0.05$). The time of harvest (month) was considered a fixed factor.

The chemical composition depends on the meteorological (pedo-climatic) conditions for each month during which the harvesting was performed. However, this observation overlaps with the dynamics of compound accumulation, which varies according to the month in which

the plant is collected. ANOVA indicated significant differences ($p < 0.05$) between the plant products of the analyzed plants and the collection time for flavones in terms of aerial parts ($F = 16.539$, $p < 0.001$) and leaves ($F = 8.451$, $p = 0.002$), phenolic acids in roots ($F = 62.545$, $p < 0.000$), and total polyphenols in leaves ($F = 14.308$, $p = 0.001$) and flowers ($F = 10.943$, $p = 0.003$). These observations refer to the results obtained in 70% (v/v) ethanol.

For ethanol 50% (v/v), ANOVA indicated significant differences ($p < 0.05$) between the plant products of the analyzed plants and collection time for flavones in terms of aerial parts ($F = 11.230$, $p < 0.001$) and leaves ($F = 10.590$, $p = 0.001$), phenolic acids in stems ($F = 8.230$, $p = 0.003$), total polyphenols in flowers ($F = 13.540$, $p = 0.001$), and roots ($F = 9.470$, $p = 0.002$). Post-hoc analysis with the LSD test revealed statistically significant differences in the content of flavones in aerial parts and leaves ($p < 0.05$), between phenolic acids in stems and other parts ($p < 0.05$), and between total polyphenols in flowers and roots ($p < 0.05$).

For 20% (v/v) ethanol, ANOVA results indicated significant differences ($p < 0.05$) between plant products of the analyzed plants and collection time for flavones in terms of aerial parts ($F = 16.539$, $p < 0.001$) and leaves ($F = 8.451$, $p = 0.002$), phenolic acids in roots ($F = 62.545$, $p < 0.001$), and total polyphenols in leaves ($F = 14.308$, $p = 0.001$) and flowers ($F = 10.943$, $p = 0.003$). Post hoc analysis with the LSD test revealed statistically significant differences between the content of flavones in aerial parts and leaves ($p < 0.05$), between phenolic acids in roots and other parts ($p < 0.05$), and between total polyphenols in leaves and flowers ($p < 0.05$).

For the distilled water (v/v) used as the extraction solvent, the ANOVA results indicated significant differences ($p < 0.05$) between the plant products of the analyzed plants and the collection time for flavones in terms of aerial parts ($F = 9.345$, $p < 0.001$) and leaves ($F = 7.543$, $p = 0.003$), phenolic acids in stems ($F = 12.234$, $p < 0.001$), total polyphenols in flowers ($F = 10.789$, $p = 0.002$), and roots ($F = 8.456$, $p = 0.004$). Post hoc analysis with the LSD test revealed statistically significant differences in the content of flavones in aerial parts and leaves ($p < 0.05$), phenolic acids in stems and other parts ($p < 0.05$), and total polyphenols in flowers and roots ($p < 0.05$).

4.6. Quantitative determination of lipophilic constituents: dosing volatile oil

According to a specialized literature review, the presence of volatile components [20, 22, 24] in the composition of the studied species was highlighted. We set out to obtain volatile oil from each type of fresh plant organ and subsequently identify the constituents of each batch.

Conclusions. The volatile oil appeared as a viscous, yellowish liquid adhering to the walls of the apparatus. Organoleptic characteristics could not be determined due to the addition of xylene. Because the amount of volatile oil was very small, for these reasons, we decided to abandon this analysis.

5. ESTABLISHMENT OF A TECHNOLOGICAL PROCEDURE TO OBTAIN A DRY EXTRACT (AJUGAE CHAMAEPITYSIS HERBA EXTRACTUM - LABORATORY PHASE)

In order to continue our research, we decided to obtain a lyophilized extract from sample H1-50 (aerial parts from from May, using 50% ethanol (v/v) as the extraction solvent) because the flowers, after drying, are presented in a very small amount to continue our analyses, and the 50% (v/v) ethanolic extract represents the optimal solvent for extracting the phytochemical compounds. The solvents and experimental conditions used in each step are described. In addition, we note that this species is distributed in the area of Dobrogea does not indicate any problems related to endangering the plant raw material. This species has been adapted to the geoclimatic conditions in this region, it grows spontaneously and is very abundant. Additionally, Ajuga species have been documented as insect repellants that are pest-resistant and well adapted to the environment.

Results. After lyophilization, 52 g of the lyophilized extract was obtained and stored in a desiccator. The obtained extract is presented as a green hygroscopic powder with a persistent characteristic smell. The yield of the lyophilized extract was 28.4463% dry plant product.

5.1. Determination of the stability of the dry extract – Ajugae chamaepitysis herba extractum (ACHE)

In order to assess whether the lyophilized extract was stable from a qualitative and quantitative point of view, the content of active principles, namely the content of flavones, phenolcarboxylic acids, and total polyphenols, was determined according to a previously described methodology. These determinations will be made approximately one month after obtaining the extract and at 3, 6, 9, and 12 months.

Results. The obtained results are shown in Figure 5.1.

Conclusions. According to the results obtained, the lyophilized extract stabilized over time, and its organoleptic characteristics did not change because the extract was maintained under optimal conditions.

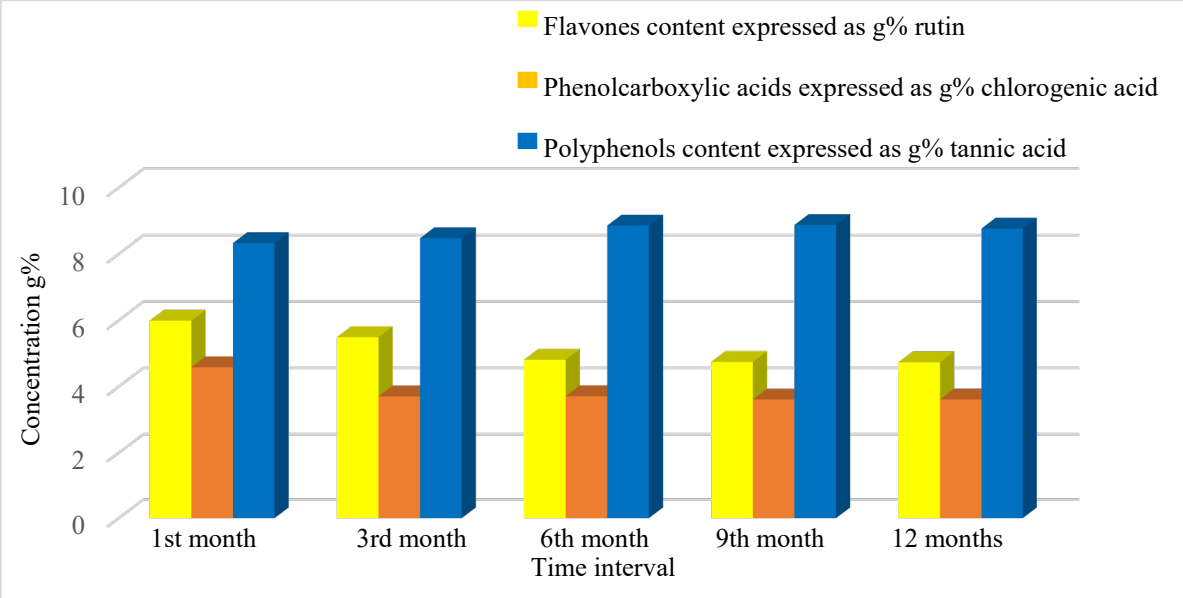

Figure 5.1. Determination of the content of phytochemical constituents on the ACHE dry extract at different time intervals.

5.2. Identification and quantification of polyphenolic compounds in ACHE by ultra-performance liquid chromatography and high-resolution mass spectrometry (UHPLC-HRMS/MS)

Results. A total of 19 polyphenolic compounds were identified: apigenin, kaempferol, naringenin, chrysin, genistin, 2′,6-dihydroxyflavone, caffeic acid, biochanin A, pratensein, kaempferol-3-O-rutinoside, kaempferol (or luteolin)-O- glucoside/isomers, vitexin (apigenin 8-C-glucoside)/isovitexin, apigetrin (apigenin-7-glucoside), cinnaroside (luteolin-7-O-glucoside), apigenin 7-O-glucosylglucoside, hispidulin, luteolin, apigenin-7 -O-glucuronide, and chlorogenic acid. These compounds are in the group of flavonoids, isoflavones, and phenolic acids. 13 phytochemical compounds were quantitatively quantified.

6. DETERMINATION OF CYTOTOXICITY OF THE EXTRACT

Artemia and Daphnia are crustaceans widely used for the pharmacotoxicity and environmental toxicity evaluation of chemical compounds and plant extracts. Both models provide preliminary data on biologically active concentration ranges and more accurately predict lethal concentrations than the in silico test. The Daphnia embryo test can provide additional information regarding the influence of the extract on embryo development and the potential risks of teratogenicity. These preliminary data are useful for non-clinical tests on more evolved species (e.g. mice and rats).

6.1. Testing for in vivo cytotoxic activity in the Artemia salina L. model

The cytotoxicity evaluation of larvae of Artemia salina L. was developed and adapted as one of the fastest and most effective tests due to the sensitivity of larvae to a variety of chemical substances [81, 82, 83]. The test is considered a useful tool for evaluating the preliminary toxicity of vegetable or animal extracts. The test is an efficient, cheap, and relatively rapid method for detecting toxic compounds and requires only a small amount of sample (less than 20 mg). Probit analysis is a method for analyzing the relationship between stimulus (dose) and binomial response.

Conclusions. The LC_{50}, which expresses mortality in 50% of the subjects, is higher than 1000 μg/mL, indicating low cytotoxic activity.

6.2. Testing for in vivo cytotoxic activity in the Daphnia species

Conclusions. Our research found the use of both species to be advantageous, as each species exhibited unique sensitivity responses, thus serving as a valuable tool for identifying potential toxic effects induced by plant extracts. However, toxicity was evident at concentrations significantly higher than those commonly used in phytotherapy.

6.3. Daphnia magna Embryo test

Toxicity tests on Daphnia species showed that the extract exhibited low to moderate toxicity, with effects being concentration-dependent. Although higher concentrations significantly affected Daphnia pulex and delayed Daphnia magna embryo development, no teratogenic effects were observed at lower concentrations, indicating relative safety. The embryo test on D. magna can be used to identify potential teratogenic effects. In our research, embryonic development testing on D. magna further elucidated the impact of the extract. Thus, between 50 and 500 μg/mL, developmental stages and viability were slightly lower or

comparable to those of the untreated control, whereas at 1000 µg/mL, a very high concentration, viability was reduced, larval development was delayed, and abnormal changes were observed.

7. IN VITRO STUDIES OF THE ANTIOXIDANT ACTIVITY

The antioxidant activities of the extracts were determined based on their free radical scavenger capacity, DPPH (2,2-diphenyl-2-picryl-hydrazyl), $ABTS^{\bullet+}$ (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), and ferric reduction capacity.

Our results showed that ACHE has significant antioxidant activity, confirming the results of other studies. Moreover, even if the free radical scavenging effect of ACHE is several times lower than the reference antioxidant activity (IC_{50}DPPH extract = 529.4 µg/mL higher than IC_{50} ascorbic acid = 16.5 µg/mL), we can observe that the Pearson coefficients regarding DPPH values and concentrations of chemical compounds (values from the first month after extraction: 59.932 ± 21.167 mg rutin equivalents/g dry extract, 45.864 ± 4.434 mg chlorogenic acid equivalents/g dry extract and 83.307 ± 3.989 mg equivalents, respectively acid tannic acid/g dry extract) that generate the effect reached the highest significant level (DPPH vs. FC (FC = content in flavones): $r = 0.9959$, $p < 0.05$; DPPH vs PAC (PAC = content in phenolic acids) $r = 0.9991$, $p < 0.001$; DPPH vs TPC (TPC = content in total polyphenols): $r = 0.9704$, $p < 0.05$). As a result, a strong positive correlation was observed between DPPH and FC ($r = 0.9959$, $p = 0.009$), PAC ($r = 0.9991$, $p < 0.001$), and TPC ($r = 0.9704$, $p = 0.0367$). A significant negative correlation was found between $ABTS^{\bullet+}$ and FC ($r = -0.9639$, $p < 0.0386$) and PAC ($r = -0.9719$, $p = 0.0249$). No significant correlation was observed between FRAP levels and polyphenolic compound content. These results highlighted the precision of the correlations between variables and the high degree of accuracy of the DPPH method compared with other antioxidant techniques.

7.5. Conclusions

The results obtained were conclusive, indicating that the phytochemical compound content plays a major role in improving oxidation by capturing free radicals, chelating metal ions, and removing oxygen from biological systems.

8. DETERMINATION OF ANTIMICROBIAL ACTIVITY

The determination of the antimicrobial action of the extract was carried out using the diffusimetric method and the dilution method on culture medium with the reference strains of gram (-) negative bacilli: Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922) and gram (+) cocci positive: Enterococcus faecalis ATCC 29212 and Staphylococcus aureus ATCC 23235. ACHE exhibits an inhibitory effect on bacterial strains and is more effective against Escherichia coli and Staphylococcus aureus. Bactericidal values are recorded at concentrations exceeding 18 and even 40 mg/mL, revealing their antimicrobial potential.

9. MOLECULAR DOCKING SIMULATIONS FOR IN SILICO PREDICTION OF BIOLOGICAL TARGETS

9.1. Prediction of molecular targets and molecular docking

Potential molecular targets were predicted for several phytochemicals (apigenin, galangin, genistein, kaempferol, naringenin, caffeic acid, ferulic acid and p-coumaric acid), which were found in higher amounts based on UHPLC-HRMS/MS analysis. Following an analysis of the prediction results, we selected CDK5 and GSK-3β as potential target candidates for the investigated polyphenols, both of which are involved in the pathophysiology of neurodegenerative diseases and neuropathic pain. As shown, apigenin was predicted as a CDK5 inhibitor with 100% probability using SwissTargetPrediction and 7.8% probability using PASS. Furthermore, apigenin had a maximum TC value of 61.54% when paired with known CDK5 inhibitors. Additionally, apigenin had a predicted probability of 100% to show GSK-3β inhibitory activity according to the SwissTargetPrediction algorithm and a maximum TC value of 100% when paired with known GSK-3β inhibitors. After searching the ChEMBL database [114], we found that apigenin inhibited both CDK5/p25 and GSK-3β activities with similar potencies (IC_{50} values of 1.6 μM and 1.9 μM, respectively). Another interesting compound was kaempferol, for which the SwissTargetPrediction algorithm predicted a 51.79% probability of inhibiting CDK5 and a 65.80% probability of inhibiting GSK-3β activity, whereas the PASS algorithm gave a probability of 6.9% to act as a GSK-3β inhibitor. A previous study showed that kaempferol inhibits CDK5/p25 with an IC_{50} value of 51 μM, but the activity on GSK-3β was not investigated. Additionally, galangin was predicted using

SwissTargetPrediction to have a 14.97% probability of inhibiting CDK5 and 16.61% of inhibiting GSK-3β, while PASS predicted a 9.80% probability of being active on CDK5. Interestingly, SEA calculated a similarity of 45.65% between galangin and CDK5 inhibitors. Although naringenin was predicted as a potential CDK5 and GSK-3β inhibitor only by SwissTargetPrediction, previous studies have shown that its glycoside, naringin, inhibits GSK-3β with an IC_{50} of 100 μM.

Molecular docking studies were further performed to estimate the binding potential between the evaluated polyphenols and the protein targets that were selected based on PASS, SwissTargetPrediction, and SEA predictions. Thus, docking simulations were performed against CDK5 and GSK-3β, both of which are in active conformations (αCin architecture) because αC-helix glutamate residues (Glu51 in CDK5 and Glu97 in GSK-3β) formed salt bridges with β3 - lysine chain residues (Lys33 in CDK5 and Lys85 in GSK-3p). The docking protocol was validated by superimposing the predicted positions of the co-crystallized ligands onto the experimental conformations. The calculated RMSD values were 0.7023 Å for the CDK5 inhibitor and 1.2459 Å for the GSK-3β inhibitor, both values being less than the 2.0 Å threshold.

Conclusions. In silico studies have revealed the possibility of inhibiting the activities of CDK5 and GSK-3b protein kinases in the presence of apigenin, galangin, and kaempferol, with potential utility for treating neurodegenerative pathologies and neuropathic pain. In conclusion, according to our analysis, Ajuga chamaepitys (L.) Schreb., which is widespread in the spontaneous flora of Dobrogea, Romania, can be an important source of polyphenols with potential inhibitory activity against specific targets in neurodegenerative diseases and pain.

9.2. Molecular dynamics simulations

Molecular docking studies were further performed to estimate the binding potential between the evaluated polyphenols and the protein targets that were selected based on PASS, SwissTargetPrediction, and SEA predictions. Thus, docking simulations were performed against CDK5 and GSK-3β, both of which are in active conformations (αCin architecture) because αC-helix glutamate residues (Glu51 in CDK5 and Glu97 in GSK-3β) formed salt bridges with β3 - lysine chain residues (Lys33 in CDK5 and Lys85 in GSK-3p). The docking protocol was validated by superimposing the predicted positions of the co-crystallized ligands onto the experimental conformations. Molecular dynamics (MD) simulations were performed

to further evaluate the results of the molecular docking study and to analyze the stability of the predicted protein–ligand complexes. Thus, the following structures were subjected to simulations: CDK5/p25 without ligand (apo) – negative control structure; GSK-3β without ligand (apo) – negative control structure; CDK5/p25 in complex with apigenin – positive control structure (complex with reference inhibitor); GSK-3β in complex with apigenin – positive control structure (complex with reference inhibitor); CDK5/p25 in complex with galangin – investigated complex with putative inhibitor; GSK-3β in complex with kaempferol – investigated complex with putative inhibitor.

Conclusions. Molecular dynamics simulations performed for CDK5 and GSK-3β in the free and bound states provide comprehensive insights into the stability, conformational changes, and dynamics of the interactions of these protein kinases with phytocompounds. The simulations lasted 100 ns and included analyses of RMSD, RMSF, the radius of gyration, and the number of intramolecular hydrogen bonds. For both proteins, apigenin was used as a reference ligand, which has demonstrated inhibitory activity on the 2 kinases. Regarding the CDK5 complex with p25-activating proteins, it appears that apigenin and galangin induce conformational changes in it, possibly altering the stability of the activated complex in addition to the putative competitive mechanism. Conversely, apigenin and kaempferol increased the conformational stability of GSK-3β over time.

In conclusion, the results of the computational study suggest that the interactions between kaempferol and GSK-3β are more stable than those between galangin and CDK5/p25, and the possible inhibitory activity of kaempferol is supported by MD simulations to a greater extent.

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

General conclusions

The research carried out within this thesis was carried out in order to collect preliminary data regarding the chemical composition of the species *Ajuga chamaepitys* (L.), collected from the spontaneous flora of the Dobrogea Gorges, Romania. (44°29′16.5″ N–28°27′09.5″ E), which involved determining the content of flavones, polyphenols, and phenolic acids in hydroalcoholic (ethanol 70%, 50%, 20%) and aqueous extracts collected in different months of flowering. The results obtained from all four batches were higher for the flower samples from May. Thus, the optimal collection interval, solvent, and plant product with the highest

number of phytoconstituents were established (herba plant product was used, because the flowers were present in a small amount to perform the analyses). According to the obtained results, it was demonstrated that *Ajuga chamaepitys* (L.) Schreib., from the spontaneous flora, possesses notable amounts of chemical constituents, which makes us consider the importance of this plant from a therapeutic perspective. Later, based on these considerations, a selective dry extract was obtained and evaluated from a qualitative and quantitative perspective. Research on *Ajugae chamaepitys herba extractum* takes place in several stages: establishing the quality of the plant raw material; obtaining and chemical characterization of the dry extract by spectrophotometric (flavones, phenolcarboxylic acids and total polyphenols content) and chromatographic (UHPLC-HRMS/MS) methods; assessment of antioxidant capacity by 3 methods (DPPH, $ABTS^{\bullet+}$, FRAP); determination of cytotoxicity *in vivo* on larvae of *Artemia sp.* (BSLA) and *Daphnia*; determination of the teratogenic potential by the embryo test on *Daphnia magna* embryos; *in silico* determinations (molecular docking studies for the potential to inhibit the activity of protein kinases CDK5 and GSK-3b for apigenin, galangin and kaempferol, with possible utility for treating neurodegenerative and neuropathic pathologies); determination of antimicrobial activity on pathogens of clinical interest. All results are processed statistically.

According to the obtained results, it was demonstrated that *Ajuga chamaepitys* (L.) Schreb., from the spontaneous flora, possesses notable amounts of chemical constituents, which makes us consider the importance of this plant from a therapeutic perspective. According to the studies presented, from the literature, and from the analyses conducted by us, in terms of chemical composition, this plant includes several groups of active principles such as: volatile oil, iridoid, triterpenes, neoclerodane diterpene, steroidal heterosides (ecdysterones), flavonoids, anthocyanins, derivatives of polyphenolic carboxylic acids, total polyphenols, and resins, which explains the use of this species in traditional medicine.

The spontaneous flora of Romania offers several possibilities for exploration and valorization through research; these species represent a scientifically-based source of bioactive compounds.

Degree of originality

In our country, this species, which has important medicinal potential, has not been explored, and no studies have been conducted regarding its therapeutic effects and chemical

composition. At the same time, in other countries, studies have been conducted using *in vitro* and less *in vivo* models, giving the opportunity to continue research from this perspective. We mentioned that this is the first study on these species in our country.

Research Perspectives

This species can allow the inclusion of phytoconstituents in the form of a food supplements with benefits for the human body. Due to the content of iridoids, the anti-inflammatory and analgesic effects can be explored *in vivo* models, allowing to establish a pharmaceutical form with topical application (gel or patch) for the suppression of pain in arthralgias (e.g. in gout arthritis).

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