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

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

PhD student:
LUȚĂ EMANUELA ALICE

THE YEAR

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THE UNIVERSITY OF MEDICINE AND PHARMACY
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PHYTOSOCIOLOGICAL STUDY AND ITS IMPLICATIONS ON THE BIOSYNTHESIS OF BIOLOGICALLY ACTIVE COMPOUNDS FROM DIFFERENT MEDICINAL SPECIES

PHD THESIS SUMMARY

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THE YEAR

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CONTENT

List of published scientific papers

Articles published in ISI rated journals

1. **Emanuela Alice Luță** , Manuela Ghica , Teodora Costea, Cerasela Elena Gîrd. Phytosociological study and its influence on the biosynthesis of active compounds of two medicinal plants *Mentha piperita* L. and *Melissa officinalis* L. (2020) . *Farmacia*, 68(5) 919-924, ISI indexed journal, with impact factor **1.433** , ISSN: **2065-0019** (*for the On-Line Edition*) and **0014-8237** (*for the Printed Edition*)
link: https://doi.org/10.31925/farmacia.2020.5.20

2 . **Emanuela Alice Luță** , Manuela Ghica , Cerasela Elena Gîrd . The Initiation of a Phytosociological Study on Certain Types of Medicinal Plants (2022). *Agriculture* , 12(2), 283, ISI indexed journal, with impact factor **3.408 ,** ISSN: **2077-0472**
link: https://doi.org/10.3390/agriculture12020283

Works communicated at national events with volume publication of summaries

1. **Emanuela Alice Luţă** *, Teodora Costea, Ligia Elena Du ţu, Cerasela Elena Gîrd. Phytosociological Study Influence on the Content of Active Principles (2021). ***Anniversary Symposium Romanian Ethnopharmacology at 20 years*** *Șirnea* , June 17-20, summary published in Book of Abstracts – Romanian Ethnopharmacology On Its 20th Anniversary, Volume 20, 2021, ISSN 1844-6604, ISSN-L 1844-6604 (Romanian Society of ethnopharmacology)

2. **Emanuela Alice Luță** , Teodora Deculescu-Ioniță, Cerasela Elena Gîrd. Phytosociology. Case studies (2021). *Congress of the "Carol Davila" University of Medicine and Pharmacy, from Bucharest , the 9th edition, held online between November 25-27,* with participation in the Young Researcher Session

3. **Emanuel Alice Luţă** , Cerasela Elena Gîrd. Phytosociological observations regarding some crops of *Thymus vulgaris* L. and *Calendula officinalis* (2021). *Symposium with International Participation "Alternative And Complementary Therapies (Homeopathy / Phytotherapy)" 4th Edition,* March 26-27, abstract published in Book of abstracts, Volume 4, 2021, ISSN 2601-1476, SSNL 2601-1476

4. **Emanuel Alice Luţă** , Cerasela Elena Gîrd . Introduction In a Phytosociology - Study Note I. Comparative analysis of *Rosmarinus officinalis* L. and *Matricaria Chamomilla* L. growth in common crops (2021). *National Pharmacy Congress XVIII Edition, Pharmacy: From Innovation to Good Pharmaceutical Practice, Bucharest , September 15-17* , abstract published in the Book of Abstracts, Volume 18, 2021, ISBN 978-606-10-2144-4 (University of Oradea - Faculty of Medicine and Pharmacy Oradea, Publishing House of the University of Oradea)

LIST OF ABBREVIATIONS

ABTS = benzothiazoline sulfonic acid
AFC = phenolcarboxylic acids
BA = betulinic acid
CAF = caffeic acid
CF = phytosociological lot thyme
CHL = hydrochloric acid
CM = control batch thyme
CODF = phytosociological batch mouse tail
CODM = control lot mouse tail
COU = p-coumaric acid
DPPH = 2,2-diphenyl-1-picrylhydrazyl
ESI +/- = positive/negative ionization
F = found, unquantifiable
FER = ferulic acid
FRAP = iron reduction method
GF = marigold phytosociological batch
GM = control batch marigolds
HUM = humus
ISO = isoquercitrin
KAE = kaempferol
LUT = luteolin
M = missing
M Bio – mint batch enriched with Bio fertilizer
M NPK – batch mint enriched with NPK fertilizer
MF = phytosociological batch mint
ML Bio – lemon balm batch enriched with Bio fertilizer
ML NPK – lemon balm batch enriched with NPK fertilizer
MLF = lemon balm phytosociological lot
MLM = lemon balm control lot
MM = control batch mint
MS = mass spectrometer
MUF = chamomile control lot
MUM = control lot chamomile
OA = oleanolic acid
pa = active principle
PRO = protocatechuic acid
QUE = quercetin
Res. Cond. = determination of electrical conductivity
RF = phytosociological batch rosemary
RM = control batch rosemary
ROS = rosmarinic acid
RUT = rutoside
SF = St. John's wort phytosociological batch
SI = similarity index
SIRT = sirtuin
SM = bellflower control lot
TFL = total flavone content
TPAC = total phenolcarboxylic acid content
TPC = total polyphenol content
UA = ursolic acid

INTRODUCTION

Phytosociology is a branch of plant science that deals with actual plant communities studied at a scale included in the field of investigation. Its main objectives are the delimitation and characterization of vegetation types based on complete floristic composition (species). By analogy with plant taxonomy, phytosociological classification (syntaxonomy) places vegetation units in a hierarchical system based on varying degrees of floristic similarity.

Particularly in the anglo-american scientific field, the value of a typology of plant communities was fundamentally questioned at one time, based on an individualistic community concept.

In retrospect, it is evident that only phytosociology succeeded in creating comprehensive and widely applied classifications of vegetation, types for larger spatial areas, while all other approaches remained regional or ceased to exist.

The first researchers, aware of the existence of plant groups, which did not appear by chance, but with a certain constancy of characters and physiognomy, tried to delimit them as an area, to describe them based on the environmental characteristics that are repeated when the groups reproduce in itself, dependent on the pedoclimatic conditions but also on the endogenous factors involved in the perpetuation of the species.

Nowadays, phytosociology is the main method used throughout Europe, it is also applied in northern Asia and various regions of Africa and Latin America. While its application in North America has remained limited, relatively recently the US National Vegetation Classification (USNVC) has been launched, which recognizes the importance of consistent hierarchical classification systems and adopts ideas from the Braun-Blanquet approach in a modified terminology.

While traditional phytosociologists believed that an entire vegetation assemblage (portion) could be represented with a single "typical" relevance, current phytosociology is seen as a statistical approach that seeks to characterize vegetation types through combined information from several different crops.

Therefore, it is generally better to sample more smaller crops than fewer larger crops. Sampling strategies normally aim to cover the full variability of vegetation within the defined geographical and ecological boundaries of a study while minimizing within-crop heterogeneity.

In the past, phytosociologists believed that a "minimum area" could be determined for each type of vegetation beyond which the number of species would not increase further. Vegetation sampling in crops equal to or larger than the specific minimum area would produce comparable results regardless of the different sizes of the study area.

According to modern empirical and theoretical knowledge, however, average species richness increases monotonically with area, and a minimum area is a delusion caused by the nonlinear nature of species–area relationships. Since most vegetation classification approaches are sensitive to different crop sizes, the recommendation is to apply uniform crop sizes in structurally bounded formations [1].

The major applications of phytosociology are:

- ecological assessment (for example, the values of the Ellenberg indicators , a useful tool to delimit the relationship between plants and the environment, recognizing each species' functional role as a biological indicator);
- vegetation mapping;
- monitoring environmental changes.

The utility of molecular phylogeny and phylogenomics in chemodiversity prediction and bioprospecting is highlighted in the context of natural drug discovery and development based on plant product or extract.

The justification for choosing the research topic was based on the potential ennobling in biologically active compounds of some species of medicinal plants grown in common crops, versus single crops, as well as the generation of a much higher plant mass production. Potentially, the biosynthesis of certain classes of secondary metabolites leads to new types of therapeutic effects not yet demonstrated.

the impact expected on the development of knowledge in the field :

- a larger mass of plant raw material can be generated for the industry producing supplements or phytomedicines;
- plant raw materials ennobled in active principles can be generated .

The working hypothesis of this research started from the following foundation:

- two medicinal plants that coexist in the same soil, having approximately the same active principles, but belonging to different species or families, can influence each other;
- they can positively modify their content in the active principles, with direct influences on the therapeutic values.

Research objectives were :

- ✓ selection of medicinal plants that can be cultivated in common crops, species that can belong to the same type of family or can come from different botanical families;

- ✓ establishing the agro-technical parameters for establishing the respective crops;
- ✓ crop monitoring over a period of 4 years;
- ✓ establishing the morphological characters for certain types of plant organs from the species included in this extensive phytosociological study;
- ✓ determination of the content in active principles of the plant products recognized for association in phytotherapy with the scoring of the differences between the phytosociological batches and the reference ones;
- ✓ evaluation of volatile oil content for some aromatic species;
- ✓ determining the antioxidant action and highlighting the antiradical effect, correlated with the content in secondary metabolites and especially in polyphenols;
- ✓ following the ascending-descending course of the concentration of active principles in a species of medicinal plants in the sense: basal leaves > middle leaves > tip leaves and vice versa, during 28 hours;
- ✓ determining the mass production and following the horizontal and vertical development of the medicinal plants in the studied crops;
- ✓ comparative study of the parallel development of a batch of plants located in a soil enriched with organic fertilizer versus synthetic fertilizer;
- ✓ dissemination of results in journals from the international scientific flow, as well as communication at different profile sessions.

I mention that all the medicinal plants in the phytosociological groups were compared with control plants, which were subjected to the same monitoring (humidity, sun, temperature, soil pH, etc.).

CURRENT STATE OF KNOWLEDGE

1. THEORETICAL DATA REGARDING PHYTOSOCIOLOGY

Phytosociology or phytocenology is a subgroup of vegetation science that deals with the study of existing plant communities and places special emphasis on the classification of their structure and their geographical distribution [2].

If we were to summarize the literature data up to this point, from the point of view of chronology, phytosociology has experienced three distinct periods in its evolution [3], namely:

Period **1400-1921**	Period **1921-2000**	Period **2000….**

❁Linné distinguishes most plant taxa of species rank, creates binary nomenclature and classifies the respective species in higher taxa: family, class, order. ❁Floristic studies become the main interest of the research activity. ❁Species are revised, names are changed, and new species described by different taxonomists are discovered.	❁Braun-Blanquet founds the French school and provides the foundations of the sociology of plants (phytosociology). The Russian school is founded by Bîkov and Soceava. ❁Borza and Boşcaiu make a critical analysis of the principles of the two Romanian schools, adopting to a large extent the principles of the French school. ❁Floristic research continues on a global and national scale. ❁An attempt is made to find a relationship between the structure of the vegetation and the plant biomass.	❁Point studies are starting to regain the interest of researchers, they examine the relationship between individuals of a plant species and pollinators, for example. ❁The competition between individuals of two plant species is examined. ❁The number of studies related to the plant community as a whole in inter-relationship with the control factors is reduced. ❁Revisions to species begin again. ❁The habitat-specific syntaxonomic unit is used to define different types of habitats.

2. PHYTOSOCIOLOGY . RESEARCH AND PREVIOUS STUDIES

Phytosociological studies on leguminous species . In 2016, a study was conducted that evaluated the effect on weed dynamics and bean yield following the application of herbicides using the Heightson sprayer through a field experiment. A 3x4+1 factorial design was used, comprising a combination of three herbicides (fomesafen, fluazifop-p-butyl and a mixture of both) with four different spray heights (0.20; 0.30; 0.40 ; 0.50 m above the target) and one control (without weed control) [4].

Phytosociological studies on tree species . A paper published in 2017 presents a phytosociological study of the tree community in a lower montane forest located on Mount Batulanteh, Sumbawa, Indonesia [5]. Although Indonesia's forests are known worldwide for their high species diversity, many regions remain poorly known [6,7,8]. Crops of 1,800 m^2 each were chosen , two from secondary forests and one from a deforested primary forest. Each crop was further divided into 18 squares of 100 m^2 (10m × 10m) each. Division 1 was studied on a clayey soil, and Division 3 on a friable black soil, being located in secondary forests. Division 2 also located on friable black soil was located in an affected primary forest. Each crop was placed on a more or less flat, gently sloping area. Through this study, quantitative measures of floristic composition and structure were made and carbon storage was also estimated [9].

Phytosociological studies on weed species . In 2018, a scientific paper was published that carried out a phytosociological survey of the weed community in two types of vegetable systems: ecological (management with natural products) and conventional (management with synthetic products) in the state of Alagoas, Brazil [10].

Phytosociological studies on a plant community. A 2019 paper published by Ilie Silvestru Nuță and Mariana Niculescu presents new phytosociological and ecological findings regarding the distribution of the plant community formed by *Myricaria germanica* L. (false tamarisk) and willow species - *Salix purpurea* L . in the Lotru Valley (Carpathian Mountains) [11].

Phytosociological studies on parasitic plant species . Another study published in 2019 presents the phytosociology and some ecological attributes of the cereal infesting flora in Tehsil Charsadda, Khyber Pakhtunkhwa Province, Pakistan. In order to carry out this work, Fawad Ali together with the rest of the team carried out a survey in the field using the method of Squares (a method by which the frequency of a population is studied) in the months of March and April 2013 [12].

3. GENERAL ASPECTS ABOUT THE MEDICINAL SPECIES USED IN THE STUDY

The scientific research started with the choice of a plant material of phytotherapeutic interest. Species belonging to the same family (*Mentha x piperita* L. and *Melissa officinalis* L.) but also different ones (*Calenduls officinalis* L. and *Thymus vulgaris* L.; *Hypericum perforatum L. and Melissa officinalis* L., etc.). For each plant species, we have summarized some aspects related to the therapeutic importance. We note that all photos of the medicinal species presented in this chapter are original.

EXPERIMENTAL PART

4. EXPERIMENTAL PHYTOSOCIOLOGICAL STUDY

Research stages. In this phytosociological study, plant raw materials with active silimary principles, but belonging to different species or families, were selected. In order to carry out the scientific research, it was necessary to draw up a plan structured in stages over the course of several years, including both practical and theoretical parts.

Study design. The research began with the arrangement of a space intended for the planting of different medicinal species. Before this first step was taken, wooden signs were made, painted white, with the scientific and popular names of all plant raw materials. The scientific experiment took place in the suburban area of Turnu Măgurele, Teleorman County, Romania (43 44044,1600 N, 24 52053,4000 E), starting in 2018. The average annual temperature in Turnu Măgurele is 11.5°C, the average in the warm months it is 23°C, and the average in the cold months drops below 2°C. It is characterized by a high calorific potential , large amplitudes of air temperature, low amounts of precipitation, often torrential in summer, and frequent periods of drought [13] . This phytosociological investigation started from the selection of plant raw materials with approximately identical active ingredients, but from distinct species or families. Based on the therapeutic benefits, the following categories of medicinal plants have been associated in cultures: *Rosmarinus officinalis* L. and *Matricaria chamomilla* L. - source of volatile oil ; *Hypericum perforatum* L. and *Chelidonium majus* L. - associated in hepatobiliary diseases; *Mentha* x *piperita* L. and *Melissa officinalis* L. - aromatic medicinal plants from the Lamiaceae family; *Thymus vulgaris* L. and *Calendula officinalis* L. - associated in gastrointestinal disorders. Each phytosociological culture was accompanied by the appropriate controls [14] .

Monitoring the evolution of culture during the period of scientific research – 2018-2021

In 2018, only the preparation of the space for the study was carried out and the evolution of medicinal plants was followed.

Results and discussion. We only exemplify with a single experimental year, all extended results can be found in the thesis. Following the macroscopic analysis and the vertical and horizontal development of all the phytosociological crops, it can be observed: interesting expansion of the mint - lemon balm crop but also of the marigolds - thyme crop at the time before the harvest in June, 3 months after the cultivation of the seedling. In comparison with the controls, all the plants in the study developed more and grew taller together : MF - mint from the phytosociological group reached a height of 39 cm, compared to MM - mint from the control group with a height of only 23 cm, growing together with MLF - lemon balm from the phytosociological group with a height of 42 cm, compared to MLM - lemon balm from the control group with a height of only 33 cm; the rosemary – chamomile batch presented some particularities as well: RF - the rosemary in the phytosociological batch developed nicely measuring 60 cm compared to RM - the control rosemary, where the height of the batch was only 45 cm, but the chamomile, even if it started promisingly, dry during the 2 months of the study; differences were also found in the St. John's wort - celandine crop: SF- the St. John's wort from the phytosociological group reaches a height of 83 cm compared to the control - 68 cm; and ROF - the rostopasca from the phytosociological group also measured 29 cm in June, compared to ROM - the control celandine with a height of only 17 cm; although there are no such obvious differences related to height in the 2 months, those related to the horizontal extent in the marigolds - thyme crop are at the same time very significant. It shows, like the rest of the phytosociological groups, changes compared to the control: GF- marigolds from the phytosociological crop reach a height of 39 cm in June, compared to GM - marigolds from the control crop, where the height is 29 cm. In May, the values were 32 cm and 26 cm, respectively. Partial conclusions . This year also brought changes in the horizontal and vertical evolution of plants, these being directly influenced by the pedo-climatic differences compared to the previous year [14]. The amount of precipitation as well as the proportion of days with precipitation was lower in 2019, compared to 2018, also leading to certain changes in the crops studied.

5. ANATOMICAL AND MORPHOLOGICAL CHANGES OBSERVED IN VEGETABLE PRODUCTS FROM PHYTOSOCIOLOGICAL CROPS

Considering the previously obtained results, horizontal and vertical development differentiated depending on the type of phytosociological crop, we decided that the research should continue with the evaluation of the morphological and anatomical particularities of the plant products harvested from the plant materials that are the subject of this study. The analysis of plant raw materials (flowers, leaves) was carried out with the help of a stereomicroscope (ZEISS Stemi 508 Greenough Stereo Microscope with 8:1 Zoom, details up to 50× magnification) [14,15,16] .

Final conclusions. The changes occurring including in the morphological and anatomical structure of plant products are in direct correlation with the type of culture and the type of medicinal species grown in the phytosociological crop. It is very obvious that they are medicinal species that positively influence each other very well, they diversify the presence of certain anatomical tissues, a fact that should materialize in the secretion of secondary metabolites in larger quantities.

6. PHYTOCHEMICAL RESEARCH ON THE PHYTOSOCIOLOGICAL CROPS TAKEN INTO THE STUDY

This chapter presents the phytochemical research carried out on all types of plant products harvested from the phytosociological crops and compared with the control samples. We performed quantitative determinations using specific phytochemistry, spectrotometric methods, and in the next chapter the FT-ICR and UHPLC-MS methods will also be presented. The purpose of these determinations is to establish whether there is an influence regarding the biosynthesis of the active principles depending on the type of medicinal plants associated in the culture. There may be phytosociological groups in which we can observe a positive influence, in the sense of the secretion of a higher concentration of secondary metabolites, and why not, possibly even a negative one.

Final conclusions. Based on the study carried out during the years 2019 – 2021 we found: as in the case of flavones and PCAs, the type of alcohol used in the extraction is dependent on the nature of the plant raw material, although we are discussing the same type of active principle; there is a clear difference between the concentrations of total polyphenols determined in the samples from the phytosociological crops compared to the control crops; the dynamics of the content of total polyphenols is dependent on the type of plant raw material, the pedoclimatic conditions and the type of association in the phytosociological crops.

Phytochemical research on the ability to migrate active principles from *Menthae folium* in a 28-hour interval

In order to highlight the migration capacity of the active principles in a medicinal plant, we looked for mint leaves harvested from 3 different areas (leaves located in the basal area – brother base, leaves located in the central area of the plant – brother plants and leaves located at the top of the plant – leaf tip), the migration of active principles based on the spectrophotometric dosage of flavones, PCA – their ures and total polyphenols [17] **.** The working methodology applied was identical to that described for the analysis of different types of batches.

The influence of biostimulants on the content of active principles in the batch *Menthae - Melissae folium*

In this subchapter we have followed the influence of a chemical fertilizer (NPK) and an ecological fertilizer (BIO) on the biosynthesis of active principles in the mint - lemon balm batch. The research focused on soil analysis and the analysis of plant raw materials.

Final conclusion . Although the medicinal species from the chemically fertilized soil are more developed, this fact did not lead to a higher concentration of active principles.

7. OBTAINING VEGETABLE EXTRACTS AND DETERMINING THEIR QUALITY

This chapter includes the phytochemical research carried out on all the dry extracts obtained from the plant products harvested from the phytosociological crops and analyzed compared to the control crops.

Partial conclusions. The results obtained on the plant extracts were predictable, the behavior is similar to that of the plant raw materials from which they were obtained. The significantly large differences between the different types of plant products show the causal relationship that was established between the medicinal plants grown in the phytosociological group, a fact that confirms the positive influences between them. Of course, there are small exceptions, thyme together with marigolds, where there is a negative influence in the biosynthesis of certain types of active principles.

Identification and quantification of polyphenolic compounds by ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS)

After establishing the analysis method and evaluating the chromatograms for the standard compounds against which the report was made, the solutions obtained from the dry extracts from all medicinal species cultivated in 2021 were also injected. Table VII.1 shows all the determined polyphenolic compounds both in phytosociological and control crops.

Table VII. 1. Concentrations of polyphenols in plant extracts (μγ compound/g extract)

No. Sample	Name Sample	PRO [µg /g]	Ruth [µg/g]	CAFE [µg/g]	CHL [µg/g]	CLAY [µg/g]	KAE [µg/g]	gnawed [µg/g]	WHAT [µg/g]	ISO [µg/g]	tert [µg/g]	EOC [µg/g]
1	mint crop (MML)	48.62	68.00	345.45	75.42	87.03	0.94	51,027	116.38	170.67	M	20.78
2	lemon balm crop (MML)	98.54	205.82	1296.55	397.91	547.50	3.13	64,317	76.84	270.91	M	54.64
3	mint control (MML)	57.13	27.36	321.44	56.09	99.68	M	43,950	5.93	176.48	M	28.81
4	lemon balm control (MML)	78.82	28.74	84.15	73.99	121.19	1.22	48,591	4.62	170.67	M	10.32
5	rosemary crop (RC)	39.44	M	357.74	68.02	53.35	2.48	33,204	19.79	167.29	M	39.83
6	thyme crop (RC)	47.58	11.78	684.23	46.34	341.92	2.31	35,947	20.06	151.54	M	8.02
7	rosemary control (RC)	16.82	M	524.43	21.51	87.17	4.93	34,681	M	143.86	M	61.68
8	thyme control (RC)	32.74	12.73	356.00	23.87	29.28	1.49	27,485	18.93	85.78	M	5.17
9	St John's wort. crop (SM)	250.40	4278.16	26.24	F	40.71	41.12	M	11789.84	1813.92	24.00	1.20
10	lemon balm crop (SM)	60.14	112.00	254.67	101.07	137.45	F	62,492	178.63	263.83	M	F
11	St John's wort. control (SM)	83.33	4205.48	21.37	F	38.77	24.30	M	7632.44	1421.70	16.46	77.91
12	marygolds crop (CG)	50.02	491.32	167.98	3805.70	F	F	0.252	24.17	153.23	M	9.58
13	thyme crop (CG)	62.72	8.82	713.52	23.01	433.53	F	32,162	27.76	167.05	M	16.03
14	marigolds control (CG)	61.83	396.88	139.73	4008.29	F	F	0.095	45.74	156.12	M	M
15	chamomile crop (MCo)	94.22	9.60	47.65	4547.89	9.52	4.23	M	395.39	361.49	3236.80	32.40
16	yarrow crop (MCo)	21.97	341.16	77.59	4620.89	1000.62	3.48	0.073	131.28	320.36	M	F
17	chamomile control (MCo)	113.19	17.49	92.92	3909.07	52.93	4.45	M	110.33	281.53	2410.47	32.61

No. Sample	Name Sample	PRO [µg /g]	Ruth [µg/g]	CAFE [µg/g]	CHL [µg/g]	CLAY [µg/g]	KAE [µg/g]	gnawed [µg/g]	WHAT [µg/g]	ISO [µg/g]	tert [µg/g]	EOC [µg/g]
18	yarrow control (MCo)	27.32	438.95	121.32	5541.75	1352.84	4.55	M	220.27	516.98	M	9.37

Legend: PRO – Protocatechuic acid, RUT – Rutin, CAF – Caffeic acid, CHL – Chlorogenic acid, LUT – Luteolin, KAE – Kaempferol, ROS – Rosmarinic acid, QUE – Quercetin , ISO – Isoquercitrin, FER – Ferulic acid, COU – p-Coumaric acid; MML – phytosociological batch mint – melissa ; RC – phytosociological batch rosemary – thyme; SM – phytosociological group bellflower – lemon balm; CG – phytosociological batch thyme – g yellows ; MCo – phytosociological batch mu setel – tail of the mouse; F-found but not quantifiable, M-missing.

Partial conclusions. The applied analysis method allowed the identification of polyphenolic compounds, their concentration being dependent on the nature of the vegetable raw material from which the extract was obtained.

Identification of polyphenolic compounds by the FT-ICR MS method (Fourier - Transform Ion - Cyclotron-Resonance high-resolution mass spectrometer also equipped with a mass spectrum)

Two different methods were used depending on the type of ionization: positive ESI ionization (**ESI +**) **and** negative ESI ionization (ESI -) . To verify with this method also the presence of polyphenols in the extracts obtained from the phytosociological batches and their controls, we used both ionizations, both positive and negative ionization (also used in UHPLC - MS), and the results are presented in tables VII.2. and VII.3. What was interestingly obtained was that depending on the method chosen, some compounds will ionize and be seen in the spectrum, but some will not.

Table VII.2. Identification of the presence of polyphenols in plant extracts, ESI+

No. Sample	Name Sample	PRO	Ruth	CAFE	CHL	CLAY	KAE	gnawed	WHAT	ISO	tert	EOC
1	mint crop (MML)	-	-	+	+	+	+	+	+	+	+	-
2	lemon balm crop (MML)	-	+	+	+	+	+	+	+	+	+	+
3	mint control (MML)	-	-	+	+	+	+	+	+	+	+	-
4	lemon balm control (MML)	-	+	+	+	+	+	+	+	+	+	+
5	rosemary crop (RC)	+	+	+	+	+	+	+	+	+	+	-
6	thyme crop (RC)	+	+	+	+	+	+	+	+	+	m	+
7	rosemary control (RC)	+	+	+	+	+	+	+	m	+	+	-
8	thyme control	+	+	+	+	+	+	+	+	+	m	+

No. Sample	Name Sample	PRO	Ruth	CAFE	CHL	CLAY	KAE	gnawed	WHAT	ISO	tert	EOC
	(RC)											
9	St John's wort. crop (SM)	+	+	+	+	+	+	+	+	+	-	-
10	lemon balm crop (SM)	-	+	+	+	+	+	+	+	+	+	+
11	St John's wort. control (SM)	+	+	+	+	+	+	+	+	+	-	-
12	marygolds crop (CG)	+	+	+	+	+	+	+	+	+	m	-
13	thyme crop (CG)	+	+	+	+	+	+	+	+	+	m	+
14	marigolds control (CG)	+	+	+	+	+	+	+	+	+	m	m
15	chamomile crop (MCo)	+	+	+	+	+	+	+	+	+	+	+
16	yarrow crop (MCo)	-	-	+	+	+	+	+	+	+	+	F
17	chamomile control (MCo)	+	+	+	+	+	+	+	+	+	+	-
18	yarrow control (MCo)	+	+	+	+	+	+	+	+	+	+	-

Legend: PRO – Protocatechuic acid, RUT – Rutoside, CAF – Caffeic acid, CHL – Chlorogenic acid, LUT – Luteolin, KAE – Kaempferol, ROS – Rosmarinic acid, QUE – Quercetin, ISO – Isoquercitrin, FER – Ferulic acid, COU – p -Coumaric acid; MML – phytosociological batch mint – melissa ; RC – phytosociological batch rosemary – thyme; SM – bell phytosociological batch – lemon balm; CG – phytosociological batch thyme – g yellows ; MCo – phytosociological batch mu setel – tail of the mouse; M - is missing

Table VII.3. Identification of the presence of polyphenols in plant extracts, ESI-

No. Sample	PRO	Ruth	CAFE	CHL	CLAY	KAE	gnawed	WHAT	ISO	tert	EOC	PRO
1	mint crop (MML)	+	+	+	+	+	+	+	+	+	+	+
2	lemon balm crop (MML)	+	+	+	+	+	+	+	+	+	+	+
3	mint control (MML)	+	+	+	+	+	+	+	+	+	+	+
4	lemon balm control (MML)	+	+	+	+	+	+	+	+	+	+	+
5	rosemary crop (RC)	+	m	+	+	+	+	+	+	+	+	+
6	thyme crop (RC)	+	+	+	-	+	+	+	+	+	+	+
7	rosemary control	+	m	+	+	+	+	+	+	+	+	+

No. Sample	PRO	Ruth	CAFE	CHL	CLAY	KAE	gnawed	WHAT	ISO	tert	EOC	PRO
	(RC)											
8	thyme control (RC)	+	+	+	-	+	+	+	+	+	m	+
9	St John's wort. crop (SM)	+	+	+	+	+	+	m	+	+	+	+
10	lemon balm crop (SM)	+	+	+	-	+	+	+	+	+	+	+
11	St John's wort. control (SM)	+	+	+	+	+	+	m	+	+	+	+
12	marygolds crop (CG)	-	+	+	+	+	+	+	+	+	+	+
13	thyme crop (CG)	+	-	+	-	+	+	+	+	+	+	-
14	marigolds control (CG)	-	+	+	+	+	+	+	+	+	+	+
15	chamomile crop (MCo)	-	+	+	+	+	+	+	+	+	+	-
16	yarrow crop (MCo)	-	-	-	+	-	-	-	-	+	+	F
17	chamomile control (MCo)	-	+	+	+	+	+	+	+	+	-	-
18	yarrow control (MCo)	-	+	+	+	+	+	-	-	+	m	-

Figures 7.1 – 7.2 show the mass spectra for the phytosociological rosemary batch by both types of ionization.

ESI + ionization

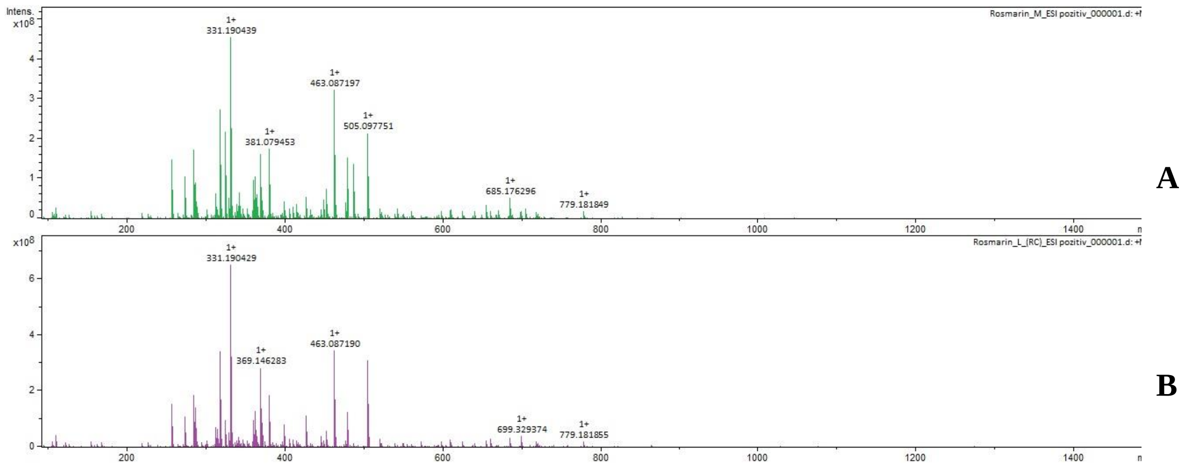

Figure 7.1. Mass spectrum, ESI+ ionization

(A) Rosemary Extract Control Batch, (B) Rosemary Extract Phytosociological Batch

ESI ionization -

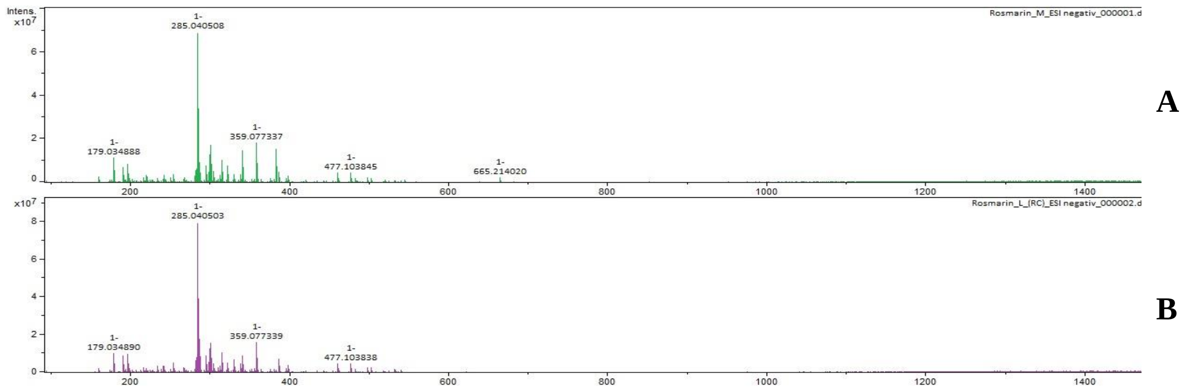

Figure 7.2. Mass spectrum, ionization ESI-

(A) Rosemary Extract Control Batch, (B) Rosemary Extract Phytosociological Batch

Identification of triterpenic acids by UHPLC MS and FT-ICR MS method (ESI+, ESI -)

The determination of pentacyclic triterpenes and their concentrations in the dry extracts from the crops in our research are presented in table VII.4.

Table VII.4. Quantification of pentacyclic triterpenes in plant extracts (mg compound/g extract)

Sample id	Sample name	nay [mg/g]	a [mg/g]	u [mg/g]
1	mint crop (MML)	F	5.66	5.64
2	lemon balm crop (MML)	0.30	5.21	9.78
3	mint control (MML)	F	3.38	3.47
4	lemon balm control (MML)	F	3.17	5.94
5	rosemary crop (RC)	8.75	8.51	6.22
6	thyme crop (RC)	m	1.95	4.91
7	rosemary control (RC)	10.97	11.45	11.67
8	thyme control (RC)	m	1.30	3.50
9	St John's wort. crop (SM)	F	m	F
10	lemon balm crop (SM)	0.13	4.89	9.56

Sample id	Sample name	nay [mg/g]	a [mg/g]	u [mg/g]
11	St John's wort. control (SM)	F	m	F
12	marygolds crop (CG)	m	m	m
13	thyme crop (CG)	m	3.72	8.70
14	marigolds contro (CG)	m	m	m
15	chamomile crop (MCo)	m	m	m
16	yarrow crop (MCo)	m	m	F
17	chamomile control (MCo)	m	m	m
18	yarrow control (MCo)	m	m	F

Caption: F-found but not quantifiable, M-missing.

8. DETERMINATION OF ANTIOXIDANT ACTION *IN VITRO* AND *IN SILICO*

Determination of antioxidant action *in vitro*

For the description of the antioxidant profile of freeze-dried extracts obtained from *Rosmarini folium* , *Thymi herba* , *Hyperici herba* , *Melissae folium* , *Menthae folium* , *Calendulae flores* , *Chamomillae flos* and *Millefolii herba* we used several antioxidant methods (DPPH, ABTS, FRAP), which assume different mechanisms and are very often used in determining the antioxidant profile of plant products [18].

Results and discussion . The antioxidant effects induced by the tested extracts were directly correlated with the concentration of secondary metabolites. The IC_{50} of vitamin C was determined using DPPH and its value is 0.0165 mg/mL. Comparing the data, it was found that the substances that generated the strongest antioxidant activities were found in MLF E from the phytosociological batch with St. John's wort (the lowest IC50 value by all three methods compared to the other extracts – table VIII.1.). The fact that the IC_{50} /$EC_{50\ values}$ for MLF E are, among all the extracts studied, substantially closer to the antioxidant values of the standard used, which underlines the superior antioxidant action of MLF E compared to the other samples, is particularly noteworthy.

Table VIII.1. Analysis of the antioxidant action of the extracts by the 3 methods

		ANTIOXIDANT METHOD IC_{50} [mg/ml]		
No.	MEDICINAL HERBS	DPPH	ABTS	FRAP
1	MINT CROP EXTRACT 2021	0.0563	0.0287	0.6198
2	MINT CONTROL EXTRACT 2021	0.0827	0.0371	0.8079
3	MELISSA CROP EXTRACT MM 2021	0.0424	0.0255	0.5914

4	MELISSA CONTROL EXTRACT 2021	0.0488	0.0270	0.7561
5	ROSEMARY CROP EXTRACT 2021	0.0620	0.0305	0.6206
6	ROSEMARY CONTROL EXTRACT 2021	0.0861	0.0464	0.8310
7	THYME CROP EXTRACT RC 2021	0.0773	0.0419	0.8208
8	THYME CONTROL EXTRACT 2021	0.0993	0.0546	0.9795
9	THYME CROP EXTRACT GC 2021	0.0848	0.0437	0.8959
10	MELISSA CROP EXTRACT SM 2021	0.0349	0.0244	0.5735
11	ST JOHN'S WORT CROP EXTRACT 2021	0.0495	0.0300	0.5961
12	ST JOHN'S WORT CONTROL EXTRACT 2021	0.0643	0.0312	0.6248
13	MARYGOLDS CROPS EXTRACT 2021	0.4834	0.2224	3.6837
14	MARYGOLDS CONTROL EXTRACT 2021	0.5205	0.2371	4.3640
15	CHAMOMILE CROP EXTRACT 2021	0.1995	0.089	2.5685
16	CHAMOMILE CONTROL EXTRACT 2021	0.2363	0.1178	3.2472
17	YARROW CROP EXTRACT 2021	0.2523	0.1208	2.0945
18	YARROW CONTROL EXTRACT 2021	0.3877	0.1335	3.3712

***In silico* studies .** Molecular docking simulations were performed for the identified compounds in order to evaluate potential biological activities on sirtuin isoforms. Crystal structures of human sirtuin 1 (PDB ID: 5BTR, 3.20 Å resolution [19]), sirtuin 5 (PDB ID: 4HDA, 2.60 Å resolution [20]) in complex with peptide substrates and the resveratrol activator, and of sirtuin 6 in complex with ADP-ribose and the activator quercetin (PDB ID: 6QCD, 1.84 Å resolution) and the inhibitor catechin gallate (PDB ID: 6QCJ, 2.01 Å resolution) respectively [21], were taken from RCSB PDB database. Since there are experimentally determined structures available for both activating and inhibitory polyphenolic derivatives of SIRT6, we chose to perform docking experiments on both receptor structures to differentiate between potential activators and inhibitors. A set of 11 polyphenolic ligands were docked into the binding sites of 3 sirtuin isoforms to evaluate the potential of these compounds to act as direct activators. The implemented docking protocol was successfully validated and the predicted binding positions superimposed over the experimental conformations are shown in Figure 8.1. Only small variations in ligand orientation were observed for all 4 positive controls. The SIRT1 and SIRT5 activator resveratrol showed a binding energy of -9.332 kcal/mol and a ligand efficiency of 0.549 for SIRT1 and a much higher binding energy of -5.220 for SIRT5 (0.307 ligand efficiency). The SIRT6 activator, quercetin, had a binding energy of -6.899 kcal/mol and a ligand efficiency of 0.314, while the SIRT6 inhibitor, catechin gallate, a quercetin derivative, had a docking score of -8.732 kcal /mol and a ligand efficiency of 0.273. Although catechin gallate is structurally similar to quercetin and shares the same binding site, previous studies have shown that differences in ligand and protein side chain orientation within the binding site are responsible for a complete change in biological activity [186]. Thus, the polyphenols in the studies were docked in both protein conformations, to discriminate between potential stimulatory and inhibitory activities. Binding energies and ligand efficiencies obtained from docking simulations are shown in Table VIII.1. Binding energies after docking on SIRT1 ranged from -9.827 kcal/mol to -5.858 kcal/mol, with an average value of -8.430 ± 1.337 kcal/mol. For SIRT5, docking scores ranged from −8.764 to −5.720 kcal/mol, with a mean value of −7.217 ± 1.104 kcal/mol. Binding energies after docking to the activator-specific conformation of SIRT5 ranged from -7.775 to -5.721 kcal/mol (-6.698 ± 0.602 kcal/mol), while energies for the inhibitor-specific receptor conformation ranged from -8.287 to -5.703 kcal/mol (-6.774 ± 0.860 kcal/mol).

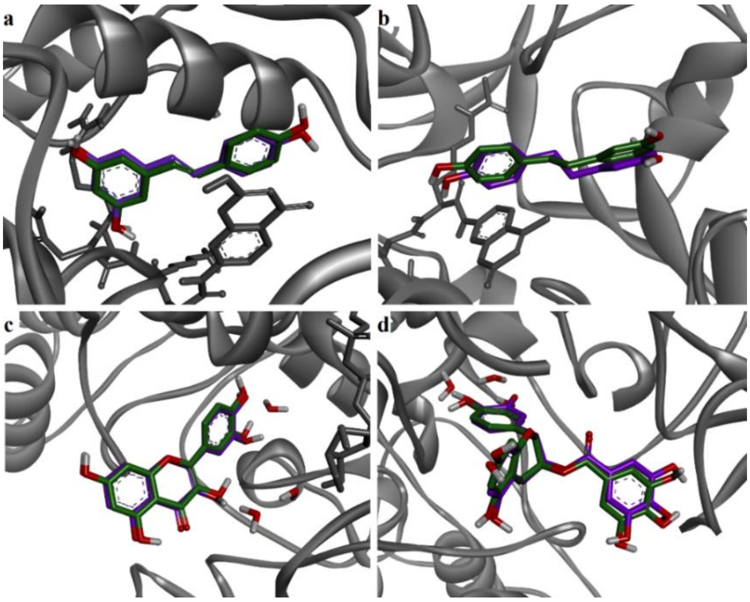

FIGURE 8.1. Overlay of predicted positions (purple) over initial conformations (green). (a) – SIRT1–resveratrol; (b) – SIRT5–resveratrol; (c) – SIRT6–quercetin; (d) – SIRT6-catechin gallate

Table VIII.1. Molecular docking results for specific sirtuin isoforms

	SIRT1 (activator)		SIRT5 (activator)		SIRT6 (activator)		SIRT6 (inhibitor)	
Ligand	**ΔG (kcal/mol)**	**they**	**ΔG (kcal/mol)**	**they**	**ΔG (kcal/mol)**	**they**	**ΔG (kcal/mol)**	**they**
Caffeic acid	-7,186	0.553	-6,224	0.479	-6,274	0.483	-6,411	0.493
Chlorogenic acid	-8,027	0.321	-7,212	0.289	-6,609	0.264	-7,262	0.291
Ferulic acid	-7,334	0.524	-6,057	0.433	-5,721	0.409	-6,172	0.441
Isoquercitrin	-8,995	0.273	-8,205	0.249	-6,460	0.196	-5,865	0.178
Kaempferol	-9,827	0.468	-7,583	0.361	-6,690	0.319	-6,787	0.323
Luteolin	-9,520	0.453	-7,962	0.379	-6,419	0.306	-7,279	0.347
p-Coumaric acid	-7,376	0.615	-5,720	0.477	-6,977	0.581	-5,900	0.492
Protocatechuic acid	-5,858	0.533	-5,750	0.523	-6,232	0.567	-5,703	0.519
Quercetin	-9,259	0.421	-7,554	0.343	-6,899*	0.314*	-6,912	0.314
Rosmarinic acid	-9,638	0.371	-8,357	0.321	-7,775	0.299	-8,287	0.319
rutozid	-9,709	0.226	-8,764	0.204	-7,623	0.177	-7,940	0.185

Resveratrol*	-9,332	0.549	-5,220	0.307	-	-	-	-
Catechin gallate*	-	-	-	-	-	-	-8,732	0.273

ΔG – bond energy; LE – ligand efficiency; * – positive control.

Regarding the molecular interactions between docked ligands and target proteins, we chose to discuss the predicted interactions for a particular compound, rosmarinic acid, which showed both good docking scores and good ligand conformations. Rosmarinic acid formed 4 hydrogen bonds with the SIRT1 binding site involving residues Asn226, Glu230 and Phe414. In addition, the ligand formed an additional hydrogen bond with Lys3, which is part of the peptide substrate, thereby stabilizing the sirtuin-substrate complex. Moreover, rosmarinic acid formed hydrophobic pi-alkyl interactions with Pro212, Leu215, Arg446 and Pro447 and Van der Waals interactions with other resistances (Figure 8. 2 .a – b). The complex between rosmarinic acid and SIRT5 is stabilized by hydrogen bonds with Gln140, Asp143 and His158. Two arginine residues are involved in a salt bridge with the carboxylic moiety and a relational interaction with the phenyl ring. Hydrophobic interactions such as pi-alkyl interactions and weak Van der Waals forces are observed with other residues in the binding site. Unfortunately, an unfavorable acceptor-acceptor interaction was formed between a hydroxyl group and Asp143 (Figure 8. 2 .c – d).

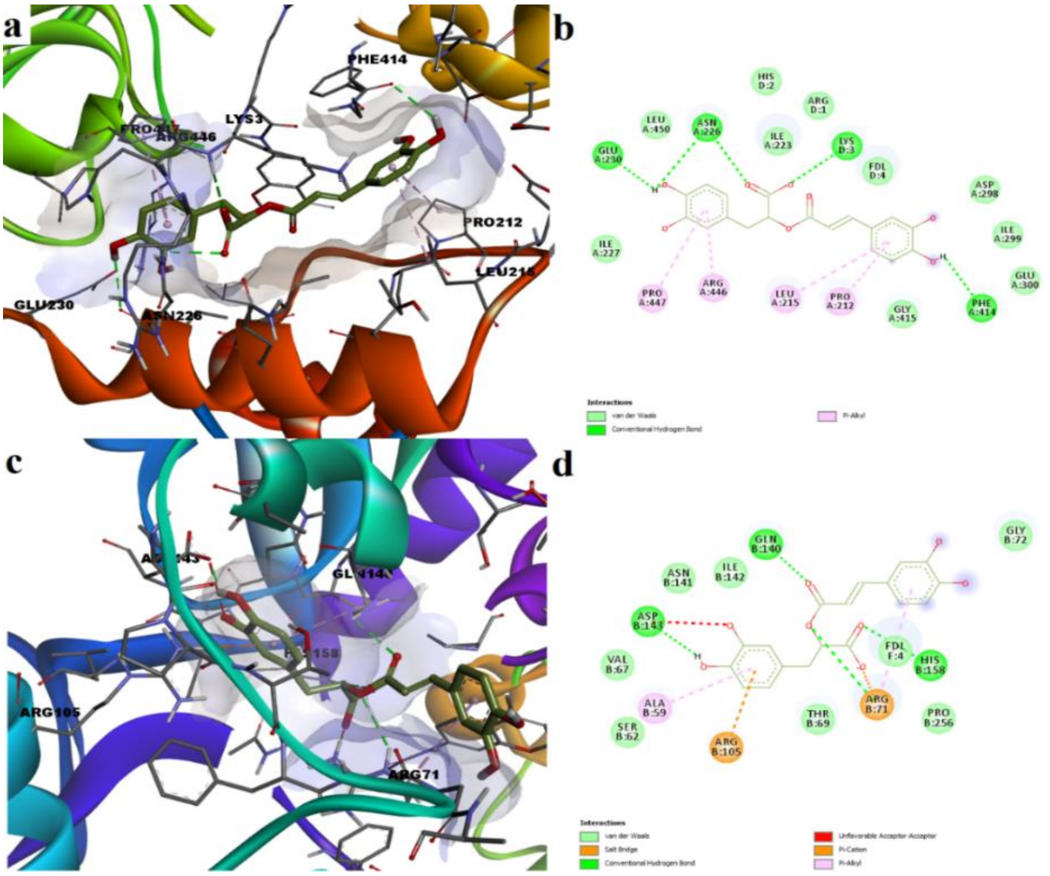

Figure 8. 2 . Ligand positions and predicted molecular interactions between rosmarinic acid and SIRT1 and SIRT5. (a) - 3D conformation of the predicted rosmarinic acid-SIRT1 complex; (b) - 2D representation of protein-ligand interactions

for the predicted rosmarinic acid-SIRT1 complex; (c) - 3D conformation of the predicted rosmarinic acid-SIRT5 complex; (d) - 2D representation of protein-ligand interactions for the predicted rosmarinic acid-SIRT5 complex

Partial conclusions . Molecular docking studies supported the hypothesis that the obtained extracts have the potential to directly activate SIRT1, 5 and 6 through several polyphenolic compounds, thus complementing the free radical scavenging activity with the potential stimulation of endogenous antioxidant defense mechanisms.

Final conclusions. The concentration of polyphenols is in direct correlation with the antioxidant action. The higher the concentration of polyphenols, the more representative the antioxidant action. *In vitro* studies are also supported by *in silico* results. The activation of sirtuins complements the antioxidant action of the extracts.

GENERAL CONCLUSIONS. ORIGINALITY OF RESEARCH RESEARCH PERSPECTIVES

GENERAL CONCLUSIONS

The research carried out on the plant products studied led us to the following conclusions:

- In our study over a period of 4 years we analyzed and followed how a group of medicinal plants can be influenced, being grown together, without intervening in any way for the common crop such as : enriching the soil with any type of fertilizer ânt , applying a different amount of water compared to the control, etc.
- We observed the appearance of some morpho-anatomical differences, in the frequency distribution, density, and their abundance in the common group compared to the control group.
- Most were medicinal species that developed harmoniously together, produced different amounts of plant product, but there were also medicinal species that self-destructed in phytosociological (common) cultures .
- The types of crops and medicinal species developed in the phytosociological group are directly correlated with the changes that occur in plant products, including those of their morphological and anatomical structures. It is clear that they are medicinal species with strong positive interactions with each other. They also diversify the presence of certain anatomical tissues, which should be manifested by increased secretion of secondary metabolites.
- Following the analyzes carried out to determine qualitatively and quantitatively the synthesized active principles, the following were revealed: the type of alcohol used in the extraction is dependent on the type of vegetable raw material, although we are

talking about the same type of active principle; there is a clear difference between the concentrations of flavones determined in the samples from the phytosociological groups compared to the control groups; the dynamics of flavone content is dependent on the type of vegetable raw material.

- There is a clear difference between PCA concentrations – hate determined in the samples from the phytosociological crops compared to the control crops; the dynamics of the content of phenolcarboxylic acids it is dependent on the type of plant raw material, the pedoclimatic conditions and the type of analysis; and, as in the case of flavones, the type of alcohol used in the extraction is dependent on the nature of the plant raw material, despite the fact that we are discussing the same type of active principle.
- There is a clear difference between the concentrations of total polyphenols determined in the samples from the phytosociological crops compared to the control crops; the dynamics of the content of total polyphenols is dependent on the type of vegetable raw material, on pedoclimatic conditions and, as in the case of flavones and PCAs – the type of alcohol used in the extraction is dependent on the nature of the plant raw material.
- Following the migration capacity of the active principles from *Menthae folium* over a 28-hour period led to interesting results: depending on the time and period of the day, they are found in a higher concentration in a different area of the plant and this process is cyclical.
- The influence of biostimulators on the content of polyphenolic compounds in the *Menthae* - *Melissae folium* group determined different amounts of synthesized active principles depending on the type of fertilizer. Although the medicinal species from the chemically fertilized soil are more developed, this fact did not lead to a higher concentration of active principles.
- The results obtained from the analysis of the plant extracts were predictable, as they behaved in a manner consistent with the behavior of the plants from which they were derived. The causal association established between the medicinal plants grown in the phytosociological crop is demonstrated by the particularly large differences between the different types of plant products; this fact shows the beneficial influences between them. There are, of course, some exceptions, such as in thyme and marigolds, where the biosynthesis of a certain type of active principle is negatively affected.

- The identification and quantification of polyphenolic compounds by ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) allowed the identification of polyphenolic compounds, their concentration being dependent on the nature of the plant raw material from which the extract was obtained.
- The identification of polyphenolic compounds by the FT-ICR MS method (Fourier-Transform Ion-Cyclotron-Resonance high-resolution mass spectrometer also equipped with a mass spectrum) led to the validation of the results obtained by the UHPLC-MS method, with differences, however, in the ionization positive (some compounds could be detected precisely by using a different ionization).
- The triterpenic acids identified in the samples by both methods could only be found in certain extracts.
- The determination of the antioxidant potential was tested by 3 distinct test methods following which an increase in the antioxidant capacity of the plants found in the phytosociological groups was observed.
- The theory that the extracts obtained have the potential to directly activate SIRT1, 5 and 6 through a number of polyphenolic compounds was supported by molecular docking studies. This would complement the extracts' ability to scavenge free radicals with the potential stimulation of endogenous antioxidant defense mechanisms.

ORIGINALITY OF RESEARCH

I believe that the originality of this thesis is given by:

- Establishment of phytosociological crops and their follow-up over a period of 4 years.
- Evaluation and analysis of co-growing behavior of certain medicinal plants belonging to different genera and from the same or different families in a pilot study.
- Establishing the quantitative chemical differences between all the crops studied by methods specific to phytochemistry;
- Studies on the migration of active principles over a period of 28 hours in mint leaves.
- In silico studies on the interaction between polyphenols and different types of sirtuins.

RESEARCH PERSPECTIVES

- Extending phytosociological studies to other types of medicinal plants.
- Expansion of the cultivation area of the phytosociological crops.
- Extension of in silico studies to other types of proteins/enzymes.

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