UNIVERSITY OF MEDICINE AND PHARMACY
"CAROL DAVILA", BUCURESTI
DOCTORAL SCHOOL
PHD IN PHARMACY

DOCTORAL THESIS

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

PhD student:
IONIȚĂ ELENA-IULIANA

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THE ROLE OF NATURAL COMPOUNDS IN THE MANAGEMENT OF HYPERLIPIDAEMIA PHD THESIS SUMMARY

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

PhD student:
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List of published scientific papers

Articles published in ISI-listed journals:

1. **Ilie (Ioniță) Elena Iuliana**, Olaru OT, Pițuru MT, Pogan AC, Moroșan E, Văleanu A, Corbu AR, Nica AE, Țăpoi DA, Tănăsescu MD, Mincă A, Gîrd CE. In vivo evaluation of the effects induced by hawthorn (Crataegi sp.) extract in rats, in two experimental diet models. *Farmacia*. 2024 Apr; 72(2): 305-314, ISI indexed journal, with impact factor **1,2**, ISSN: 2065-0019 (*for the Online Edition*) and 0014-8237 (for the *Printed Edition*), link: https://doi.org/10.31925/farmacia.2024.2.9. The article was elaborated from chapters 7 and 8 of the PhD thesis.

2. **Ilie Elena Iuliana**, Popescu L, Luță EA, Biță A, Corbu AR, Mihai DP, Pogan AC, Balaci TD, Mincă A, Duțu LE, et al. Phytochemical Characterisation and Antioxidant Activity Evaluation for Some Plant Extracts in Conjunction with Pharmacological Mechanism Prediction: Insights into Potential Therapeutic Applications in Dyslipidemia and Obesity. *Biomedicines*. 2024 June; 12(7):1431, ISI indexed journal, impact factor **3.9,** ISSN: 2227-9059, link: https://doi.org/10.3390/biomedicines12071431. The article was developed from chapters 4 and 5 of the PhD thesis.

Scientific papers presented at international events:

- **Elena Iuliana Ilie**, Liliana Costea, Emanuela Alice Luță, Cerasela Elena Gîrd. Natural compounds with hypolipidaidemic action (2023). Phytochemical Society of Europe Meeting 2023: "Natural compounds as regulators of molecular mechanisms in health and disease", Seoul, South Korea, 19-21 May, abstract published in *Book of abstracts*.

INTRODUCTION

Hyperlipidemia, an increasingly common condition, is characterized by elevated serum cholesterol (hypercholesterolemia), triglycerides (hypertriglyceridemia) or both (mixed hyperlipidemia), due to a chaotic lifestyle, high levels of stress on the body and beyond, and genetic inheritance. The effects induced by this condition are devastating for the body, with the diversification of cardiovascular diseases being directly correlated with hyperlipidemia. The basic treatment focuses mainly on synthetic molecules, but natural compounds have been and will continue to be an alternative in treatment, as they are well tolerated, often without side effects, even in long-term treatment.

Of course, the current goal of therapies in this complex condition is to reduce the side effects induced by hyperlipidemia and to maintain biochemical constants at optimal values, especially in vulnerable categories of patients, and to ensure a treatment that is as gentle as possible on the body in terms of side effects. Although the pharmaceutical market offers a wide variety of medicinal products, including phytotherapeutics, the need for formulations containing plant extracts standardized in active principles and subjected to all biochemical tests may constitute a therapeutic advance.

In this context, the aim of the research is to formulate phytotherapeutic drugs based on combinations of plant extracts standardized in active principles, correlated with the determination of their antioxidant action, cytotoxicity on invertebrates, biochemical tests on experimental animals in association with various diet models, histological evaluations of target organs subject to pathology-specific damage and proposal of pharmaceutical forms.

The **research objectives** are: to select plant raw materials, based on data from the literature, with hypocholesterolemic, hypolipidemic, antioxidant action; to establish the quality of plant raw materials by profile analysis specific to the field of phytochemistry; to obtain plant extracts and determine their quality by assaying active principles by spectrophotometric and HPLC methods; determination of mineral content; determination of antioxidant action in vitro; establishing the link between the compounds identified and various biological targets on the basis of computational studies; phytotoxicity assessment; biochemical, histological and redox status assessment on different types of organs; obtaining solid pharmaceutical dosed forms for oral administration.

CURRENT STATE OF THE ART

1. The role of lipids in the body

This chapter covers the types of lipids, their digestion and absorption, the importance and regulation of lipid production, the structure and hormones of adipose tissue, hyperlipidemias, with the diseases they cause, the diseases from which they may arise, dyslipidemias and the treatment of hyperlipidemias.

2. Plant sources with hypolipidemic action

This chapter contains selective data from the literature on different plant sources that are used for their hypolipidemic and antioxidant action.

PERSONAL RESEARCH

3. Determination of the quality of plant raw materials

Pharmacognostic analysis was used to assess the quality of five selected plant products based on literature (Rosmarini folium, Cynarae folium, Cichorii herba, Allii cepae folium (fresh), Apii graveolentis folium var. dulcis (fresh). The active principles were assayed by spectrophotometric methods and the results are presented in Table III.1.

Table III.1. Results of quantitative determinations on plant products

	Polyphenols		**PCA**		**Flavones**	
Vegetal product	**Ethanol 50%**	**Ethanol 70%**	**Ethanol 50%**	**Ethanol 70%**	**Ethanol 50%**	**Ethanol 70%**
Green onion	0,1015	0,4354	ND	ND	0,1643	0,1374
Apio	0,0798	0,1034	0,0355	0,0644	0,0407	0,0626
Rosemary	6,3423	5,9178	7,7078	6,7835	0,8844	0,9544
Chicory	2,1362	1,2697	1,4107	1,1392	0,4912	0,3956
Artichoke	2,5959	2,4798	1,9187	2,0610	1,3868	1,3972

ND = not detected

In accordance with the results presented above, and taking into account the most uniform distribution of the data, 50% ethanol was used for chicory and artichoke, and 70% ethanol for rosemary, green onion and celeriac to obtain the plant extracts.

4. Obtaining and characterization of plant extracts

In order to obtain extracts intended for internal administration in the treatment of hyperlipidemia, lyophilization was used, a method by which the active chemical constituents of interest are not degraded. As the yield of celeriac extract was very low and the extract obtained did

not have the expected consistency and stability, hawththorn extract was also included in the work, qualitative and stable, with a good yield, and which, according to the literature, has hypolipidemic properties. Spectrophotometric assays were performed for the characterization of the plant extracts as shown in Table IV.1 [1].

Table IV.1. Chemical content and antioxidant values of plant extracts

Vegetal extract	TP (g tannic acid / 100 g DE)	FL (g rutin / 100 g DE)	PCA (g chlorogenic acid / 100 g DE)	IC50 ABTS (mg/mL)	IC50 DPPH (mg/mL)	EC50 FRAP (mg/mL)
ACE	3.77	2.03	ND	0.18	1.32	1.41
AGE	0.57	0.35	0.38	2.66	10.31	2.65
CE	7.74	4.38	4.47	0.20	0.37	0.06
CGE	25.93	5.32	14.05	0.03	0.11	0.13
CHE	30.51	3.64	6.15	0.15	0.34	0.26
RSE	39.62	3.71	22.05	0.04	0.11	0.15

DE: dry extract; TP: total polyphenols; FL: flavones; PCA: phenolcarboxylic acids; ND: not detected; ABTS: 2,2-azinobis-3-ethylbenzothiazolin-6-sulfonic acid method; DPPH: 2,2-diphenyl-1-picrylhydrazine method; FRAP: iron reducing antioxidant power method.

The results revealed that the highest concentrations of PT for RSE (39.62 ± 13.16 g tannic acid/100 g dry extract), CHE (30.51 ± 1.96 g tannic acid/100 g dry extract) and CGE (25.93 ± 1.10 g tannic acid/100 g dry extract) were statistically significant ($p < 0.05$) compared to those obtained for the other extracts. A significant difference between the plant extracts was also observed for the determination of AFC ($p < 0.05$), revealing higher values for RSE (22.05 ± 1.31 g chlorogenic acid/100 g dry extract) and CGE (14.05 ± 1.65 g chlorogenic acid/100 g dry extract). The chemical profile of FL showed that hawthorn extract was the richest, with a significant concentration of 5.32 ± 0.26 g expressed as rutozide ($p < 0.001$) [1].

The antioxidant capacity to reduce ferric ions was much better for artichoke extract, with EC recording the lowest IC50 value by this method (EC: EC50FRAP = 0.06 mg/mL). But the next extracts showing high antioxidant effect were also CGE (EC50FRAP = 0.13 mg/mL) and RSE (EC50FRAP = 0.15 mg/mL). The free radical scavenging capacity of RSE and CGE, evaluated by all three antioxidant techniques, was very close to the IC50 value of the reference (IC50 ascorbic acid = 0.0165 mg/mL). The lowest antioxidant effect obtained in this study was available for celery extract, with the highest IC50 value among all six extracts, regardless of the type of method (AGE: IC50ABTS = 2.66 mg/mL; IC50DPPH = 10.31 mg/mL; EC50FRAP = 2.65 mg/mL). Consistent with

the pyramidal behavior pattern, onion extract was the modest antioxidant agent considering the results of DPPH and FRAP methods (ACE: IC50DPPH = 1.32 mg/mL; EC50FRAP = 1.41 mg/mL). The plant extract with the lowest concentration of phytocompounds was AGE (FL: 0.35 ± 0.07 g rutozide/100 g dry extract; TP: 0.57 ± 0.06 g tannic acid/100 g dry extract; AGE: 0.38 ± 0.01 g chlorogenic acid/100 g dry extract). Modest, but statistically significant concentrations of FL (2.03 ± 0.12 g rutozide/100 g dry extract) and TP (3.77 ± 0.84 g tannic acid/100 g dry extract) were recorded for ACE, while the level of AFC in onion extract could not be detected spectrophotometrically [1].

In order to more easily and clearly highlight how closely related the variables analyzed are, the Heatmap Correlation Matrix (HCM) was created, shown in Figure 4.1. After applying the matrix setting, it can be seen that any cell with a correlation coefficient value of 0.4 would be colored blue, cells with a value of 0.7 would be colored white, and cells with a value of 1 would be colored red. A color gradient in the range of values [0.4-1] would be obtained, the shades of which would indicate the strength of the correlation, making the matrix much easier for the reader to understand. In the range of values, the minimum point is 0.4, the median point is 0.7, and the maximum point is +1. Any value between these points will have the color shade representing that value of the correlation coefficient [1].

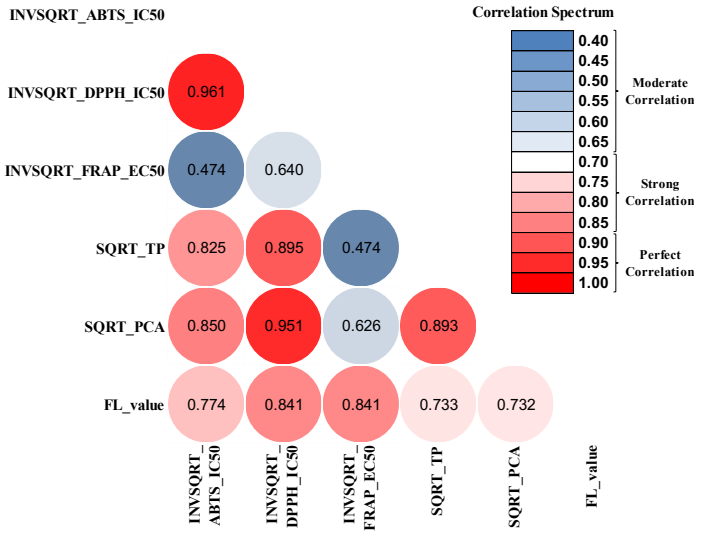


Figure 4.1. Heatmap correlation matrix and correlation spectrum (moderate correlation: [0.40 - 0.69]; strong correlation: [0.70 - 0.89]; perfect correlation: [0.90 - 1.00]; $|r|$ = absolute value of Pearson correlation coefficient; INVSQRT = inverse square root transformation of the data; SQRT = square root transformation of the data).

It can be easily seen in the HCM shown in Figure 4.5 that the correlations between DPPH_IC50 vs ABTS_IC50 ($|r| = 0.961$) and between PCA vs DPPH_IC50 ($|r| = 0.951$) are perfect correlations (very strong correlations), as they are the cells with the darkest red hue.

Likewise, the correlations between TP vs FRAP_EC50 ($|r| = 0.474$) and FRAP vs ABTS ($|r| = 0.474$) were found to be moderate correlations colored in the darkest shade of blue.

After the UHPLC-MS method was performed, it could be observed that chlorogenic acid and rosmarinic acid showed particularly high concentrations, indicating their abundance in the respective plant extracts. The presence and amount of these compounds contribute significantly to the antioxidant and potential health benefits of the extracts. The low concentrations of gallic acid, abscisic acid, ellagic acid and p-coumaric acid in all the samples analyzed, after quantification by UHPLC-MS method, can be attributed to several factors intrinsic to the nature of these compounds and the characteristics of the plant materials from which they were extracted [1].

5. Computational prediction research with biological targets

Based on the UHPLC-MS analysis, rosmarinic acid (RA) and chlorogenic acid (CGA) were selected for further in silico studies to predict the potential pharmacological mechanism involved in the hypolipidemic and anti-obesity activities. Predictions using the PASS algorithm revealed greater than 50 % probabilities for AR to exert antioxidant, free radical scavenging, lipid peroxidase inhibitor, hypolipidemic activities, cholesterol antagonist and peroxisome proliferator-activated receptor agonist, while for CGA the algorithm predicted antioxidant, reducing, free radical scavenging, lipid peroxidase inhibitor, lipid peroxidase scavenger, hypolipidemic and lipid metabolism regulating activities. Furthermore, the SwissTargetPrediction and SEA approaches predicted for both phytochemicals inhibitory activities on several carbonic anhydrase subtypes. In particular, both substances were predicted to inhibit the 5A and 5B isoforms of carbonic anhydrase, both of which are expressed at the mitochondrial level. Interestingly, carbonic anhydrase 5A (CA5A) has been shown to be involved in hepatic fatty acid synthesis and is a promising therapeutic target for the management of obesity [2]. Therefore, we chose CA5A as a putative molecular target for RA and CGA. Acetazolamide, a nonselective CA inhibitor, was also used as a positive control for both predictions. The positive control had a probability of 0.2915 to inhibit CA5A and a maximum Tanimoto's coefficient of 1 for the same target, emphasizing that the SEA prediction is more reliable in identifying true inhibitors in this case [1].

In addition, the YASARA structure was used to construct homology models of human CA5A, given that only the mouse homolog has been crystallized previously. Using two mouse CA5A structures as templates (1KEQ and 1DMY), two models with different conformations of the binding site were constructed, one with a catalytic water bound to Zn2+ (1KEQ) and another with acetazolamide (a nonselective carbonic anhydrase inhibitor) directly bound to catalytic Zn2+ (1DMY). Two models were chosen for molecular docking, considering that the selected phytochemicals could either bind the water molecule via phenolic hydroxyl groups or interact via attractive charges through the carboxyl moiety. Both models had exceptionally good collision scores, falling in the 100th percentile. Only three side chains had conformations that are not statistically feasible in the 1KEQ model, whereas only one weak rotamer was observed for the 1DMY model. In addition, model 1KEQ had 96.35% favored rotamers, while model 1DMY exhibited 97.72% favored side-chain conformations. The Z-score of the 1KEQ model was negative, while the score of the 1DMY model had a value very close to 0, suggesting that both structures had quality scores close to the average of the high-quality reference structures. Furthermore, none of the predicted structures had deviations of Cβ atom positions larger than 0.25 Å, while a very small proportion of bond lengths and angles were outside the ideal ranges. Ramachandran plot analysis showed that no residues were modeled as Ramachandran outliers, while 97.63% of the residues were considered Ramachandran favored for the 1KEQ-based model, and 98.72% were favored for the 1DMY-based model. The 1KEQ model had a MolProbity score closer to 1, highlighting the superior quality of this particular model [1].

Molecular docking simulations were performed using validated 3D structures of human CA5A. Caffeic acid (CFA) and acetazolamide (AZM) were docked as positive controls for comparison. We chose CFA as a positive control for the homologous model, which included the zinc-bound water molecule, considering the following two factors: it was previously experimentally validated as a CA5A inhibitor (Ki = 6.49 μM) [4] and was also identified in four of the six plant extracts evaluated. CFA exhibited a binding energy of -5,820 kcal/mol and had an docked position in the active site, reflecting the expected binding behavior, engaging in hydrogen bonding with the catalytic water molecule and relevant residues such as Thr236, Gln103 and Gln128. Hydrophobic interactions were also formed with residues His130, Val157 and Leu234 in the binding pocket. AZM was bound in the active site of the water-free structure and had a predicted binding energy of -7,140 kcal/mol. Not surprisingly, the sulfonamide moiety formed a metal bond with catalytic zinc and hydrogen bonds

with Glu142 and Thr235. In addition, the 1,3,4-thiadiazole ring engaged in pi-alkyl interactions with Leu234 and pi-sulfur and pi-pi T-shaped interactions with His130.

Docking studies showed that RA has a higher potential to inhibit CA5A activity by interacting with catalytic water bound to the Zn atom (template 1KEQ), whereas CGA was more likely to interact favorably with the inhibitor-bound active site conformation of CA5A by direct binding of catalytic Zn2+, similar to acetazolamide (template 1DMY). The different behavior of the two ligands is particularly interesting, given that both phytochemicals are condensation products of the known CA5A inhibitor caffeic acid. The docking calculations yielded a binding energy of -7.235 kcal/mol for RA and -7.465 kcal/mol for CGA, both values lower than those obtained for the positive controls. As shown in Figure 5.1. a,b, similar to the positive control CGA, RA formed one hydrogen bond with the zinc-bonded water molecule and four other hydrogen bonds with Thr98, Ser233 and Thr235 via phenolic hydroxyl groups. In addition, the carboxyl group engaged in hydrogen bonding with Gln128, while the ketonic oxygen formed a hydrogen bond with Gln103. The protein-ligand complex is further stabilized by hydrophobic interactions, such as pi-sigma interactions with Leu234 and pi-alkyl interactions with three valines. On the other hand, CGA interacted directly with catalytic Zn2+ via the carboxyl group through attractive charges, whereas the hydroxyl group in the α-carbon formed a hydrogen bond with Thr235 (similar to AZM). The phenolic hydroxyls also formed hydrogen bonds with Thr98, Gln128 and Tyr167 (Figure 5.1. c,d). Notably, Thr235 is a conserved residue among the CA subtypes that is thought to be involved in the binding of phenolic inhibitors [3].

Several potential pharmacological activities have been identified that are in line with the scope of the research, such as antioxidant, hypolipidemic, peroxisome proliferator-activated receptor-activated receptor (PPAR) agonist, and lipid metabolism regulating activities. Interestingly, rosmarinic acid has been previously shown to indeed exert its anti-inflammatory and cardioprotective activities through PPAR-gamma activation [4]. In addition, both rosmarinic acid and chlorogenic acid were predicted in the study as potential modulators of carbonic anhydrase 5 (CA5) isoforms. CA5 is the only mitochondrially expressed carbonic anhydrase and is involved in endogenous fatty acid biosynthesis and lipogenesis. The two isoforms of CA5, CA5A and CA5B, show different expression patterns, namely, CA5A is mainly expressed in hepatocytes, whereas CA5B has a broader tissue distribution [2, 5].

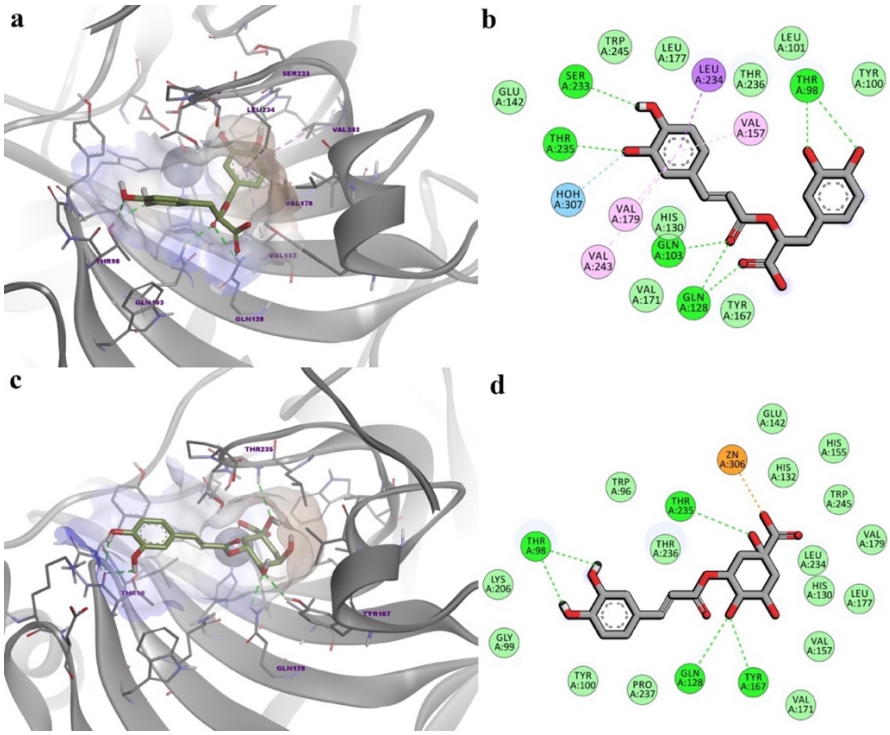


Figure 5.3. Predicted binding positions of RA and CGA in the active site of CA5A. (a) predicted conformation of the RA-CA5A complex; (b) 2D diagram of predicted interactions between RA and CA5A; (c) the predicted conformation of the CGA-CA5A complex; (d) 2D diagram of predicted interactions between CGA and CA5A. Green dots - hydrogen bonds, blue dots - hydrogen bonds with water molecules, orange dots - attractive charges, purple dots - pi-sigma interactions, pink dots - pi-alkyl interactions, light green circles - van der Waals interactions.

In silico findings suggest that the potential anti-obesity and hypolipidemic activities of artichoke, hawthorn and rosemary extracts could be partially supported by hepatic CA5A inhibition by rosmarinic acid and chlorogenic acid. In addition, both compounds have previously been shown to possess hypolipidemic and body weight and visceral fat mass lowering effects in several experimental settings, including animal models of obesity, through various molecular mechanisms [6-9].

6. Research on minerals in plant extracts

There are a number of possible correlations between certain micronutrients (zinc, magnesium, copper, chromium, selenium, calcium, vanadium, manganese, etc.) and lipid levels in the body. These correlations can be complex and variable depending on the specific context, including an individual's health status, diet and other environmental conditions.

Silicium is found in rosemary in the highest amount, followed by chicory and artichokes in similar amounts. Phosphorus has the highest concentration in chicory extract, followed by celeriac, artichoke, hawthorn and rosemary, and the lowest in green onion. Sulphur has variable amounts in the extracts analyzed, with high concentrations in chicory, artichoke and rosemary, and considerably lower amounts in green onion, celeriac and hawthorn. Potassium is present in very high amounts in chicory and rosemary extracts, followed by similar amounts in hawthorn, green onion and celeriac, the lowest amount being determined in artichoke extract. Calcium is present in varying amounts in all extracts, but in the highest concentration in rosemary extract. Chromium, nickel and manganese are present in rosemary and chicory, but absent in the other plant extracts. All extracts contain iron, but the highest amount is found in rosemary. Copper is present in hawthorn and chicory, but absent in the other extracts. Although present in all the extracts, zinc is more concentrated in chicory, while rubidium is higher in artichoke. Similar amounts of silver were determined in green onion and hawthorn. Magnesium is found in significant amounts in chicory, artichoke and celeriac, is detected in green onion samples, but is missing in hawthorn and rosemary. Aluminum is present in artichoke and celeriac samples, while selenium is found only in artichokes. Uranium was quantified in varying amounts in green onion, hawthorn and chicory samples, detected in rosemary and artichoke samples, and not detected in celeriac.

Elements such as titanium, tin, tin, stibium, tungsten, mercury, molybdenum, vanadium, cobalt and arsenic were not found in any of the plant extract samples analyzed.

The existence of elements that were found (detected) in only one or two of the three determinations made for each plant extract may suggest non-uniformity of the samples to be analyzed.

7. Phytotoxicity assessment of plant extracts

Toxicity tests using invertebrate species such as Artemia and Daphnia (D. magna and D. pulex) reduce the number of vertebrates used in experiments, aligning laboratory practices with the principles of reduction, replacement and refinement (the "3Rs" principles) promoted by global bioethics organizations [10].

For Artemia species, the viability of larvae at 24 h at tested concentrations ranging from 500 - 2500 µg/mL is very high, between 80-100%, as shown in Figure 7.1. The 20% reduction was recorded at 2500 µg/mL concentration of AG (artichoke) and RZ (rosemary) extracts, and can be correlated with phytochemical differences. Low viability below 50% is recorded for the 5000 µg/mL concentration of CI (chicory) and AG (artichoke) extracts.

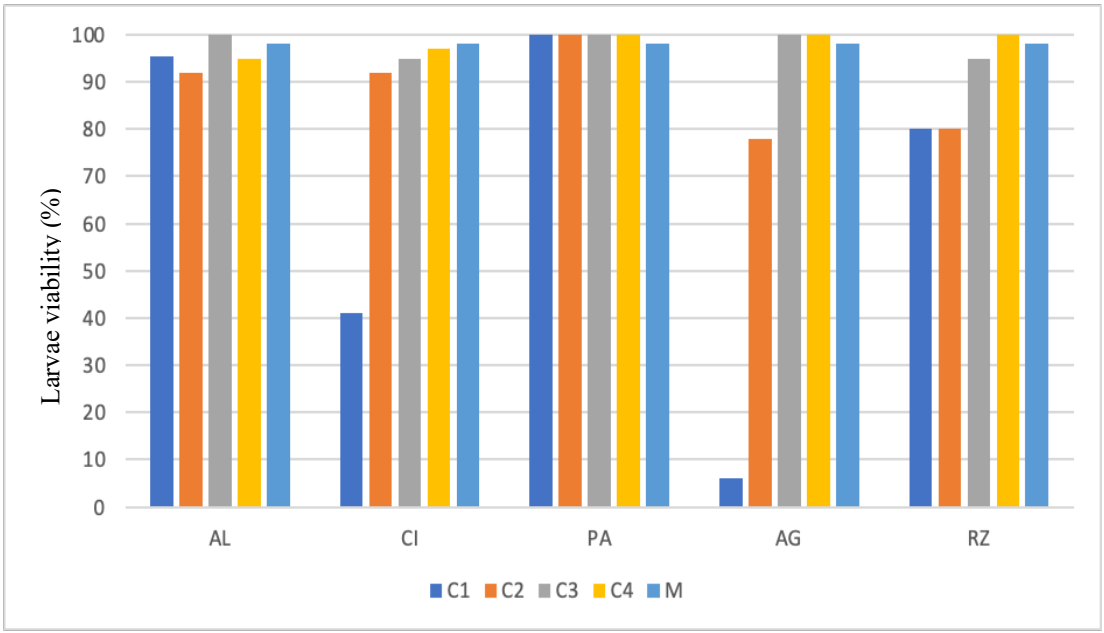


Figure 7.1. Viability (%) of Artemia salina larvae exposed to the tested extracts after 24h (acute toxicity test); C1 = 5000 µg/mL; C2 = 2500 µg/mL; C3 = 1000 µg/mL; C4 = 500 µg/mL; M = control (saline water)

According to these results, all extracts can be considered as non-toxic at concentrations up to 2500 µg/mL.

Toxicity tests on Daphnia are valuable because they allow rapid assessment of several parameters, including mortality, growth inhibition, and sublethal effects such as behavioral changes. Extracts were coded according to the abbreviated names used in Chapter 4, as follows: ACE - green onion extract; AGE - celery extract; CE - artichoke extract; CGE - hawthorn extract; CHE - chicory extract; RSE - rosemary extract.

The results of the percentage lethality determination (L%) for Daphnia magna (DM) are presented in Table VII.1.

Table VII.1. Lethality values (%) obtained in the D. magna test

Conc. (µg/mL)	**RSE**		**ACE**		**CE**		**CHE**		**AGE**		**CGE**	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
1000	0	50	0	5	80	100	0	25	10	15	10	35
750	5	40	0	5	55	100	10	20	0	10	5	30
500	5	20	0	5	25	95	0	5	25	35	0	15
250	0	15	0	0	0	5	0	10	0	0	0	20
100	5	10	0	15	0	0	0	0	10	15	0	15
50	0	0	0	10	0	0	0	5	0	0	0	10

DM exhibited high resistance to rosemary, with a maximum mortality of 50% observed at 1000 mg/L after 48 hours. The toxicity of onion extract showed resistance to DM, with low mortality at all concentrations tested, suggesting that the bioactive compounds in onion are not highly toxic to Daphnia. DM showed high sensitivity to artichoke extract, with 100% mortality at high concentrations. This highlights the superior toxic potential of artichoke compounds on aquatic organisms. DM showed high resistance to chicory extract, with a maximum of 25% mortality at 1000 mg/L after 48 hours. The results of celeriac extract for DM were weak, with minor effects on mortality. The susceptibility of DM to hawthorn extract was comparable to other extracts, with up to 35% mortality at 1000 mg/L. This reflects a moderate susceptibility of DM to compounds present in hawthorn.

Testing plant extracts on Daphnia magna embryos revealed varied effects, dependent on the type of extract and its concentration. Extracts of rosemary and celery slightly accelerated embryonic development while maintaining normal morphological characteristics. In contrast, onion at 250 µg/mL and chicory at the same concentration caused developmental retardation and embryotoxicity, with specific changes in the compound eye and antennae. Artichoke at 250 µg/mL showed significant embryotoxicity without morphological changes in surviving embryos. Lower concentrations of 100 µg/mL for onion, artichoke and chicory did not induce changes. Hawthorn stimulated development without adversely affecting embryo morphology [11].

8. Biochemical and histological evaluations

In addition to phytotoxicological investigations, preclinical evaluations in laboratory animals are also of interest. Thus, specific biochemical tests are needed to assess changes in serology induced by exposure of laboratory animals to different factors, histological examinations of several organs (heart, testis, liver) and biochemical determinations of the antioxidant capacity of tissue homogenates (heart, brain, liver, kidney and testis).

180 rats were divided into 18 groups of 10 subjects each as follows: Batch I = normal control 1 (MN1)* - normal laboratory chow, no treatment - 10 rats; Batch II = carbohydrate control 1 (GM1)* - hyperglycidic chow, no treatment - 10 rats; Batch III = lipid control 1 (LM1)* - hyperlipidic chow, no treatment - 10 rats (* lot sacrificed midway through the study, after 21 days); Batch IV = normal control 2 (MN2)** § - normal laboratory chow, no treatment - 10 rats; Batch V = carbohydrate control 2 (GM2)** § - hyperglycidic chow, no treatment - 10 rats; Batch VI = lipid control 2 (LM2)** § - hyperlipidic chow, no treatment - 10 rats; Lot VII = carbohydrate artichoke (GAN)** §- hyperglucidic

feed, and treatment with artichoke extract 2%, 5mL/kg body/animal - 10 rats; Lot VIII = carbohydrate onion (GCE)** § - hyperglucidic feed, and treatment with onion extract 2%, 5mL/kg body/animal - 10 rats; Batch IX = chicory carbohydrate (GCI)** § - hyperglucidic feed, and treatment with chicory extract 2%, 5mL/kg body/animal - 10 rats; Batch X = hawthorn carbohydrate (GP)** § - hyperglucidic feed, and treatment with hawthorn extract 2%, 5mL/kg body/animal - 10 rats; Lot XI = rosemary carbohydrate (GR)** § - hyperglucidic feed, and treatment with rosemary extract 2%, 5mL/kg body/animal - 10 rats; Lot XII = mixed carbohydrate (GA)** § - hyperglucidic feed, and treatment with mixed extract 2%, 5mL/kg body/animal - 10 rats; Batch XIII = artichoke lipid (LAN)** § - hyperlipidic feed, and treatment with artichoke extract 2%, 5mL/kg body/animal - 10 rats; Lot XIV = lipid onion (LCE)** § - hyperlipidic diet, and treatment with onion extract 2%, 5mL/kg body/animal - 10 rats; Lot XV = lipid chicory (LCI)** § - hyperlipidic diet, and treatment with chicory extract 2%, 5mL/kg body/animal - 10 rats; Lot XVI = lipid hawthorn (LP)** § - hyperlipidic feed, and treatment with hawthorn extract 2%, 5mL/kg body/animal - 10 rats; Lot XVII = lipid rosemary (LR)** § - hyperlipidic feed, and treatment with rosemary extract 2%, 5mL/kg body/animal - 10 rats; Batch XVIII = lipid mixture (LA)** § - hyperlipidic diet, and treatment with 2% extract mixture, 5mL/kg body/animal - 10 rats (** batch sacrificed at the end of the study, after 42 days; § - extracts administration was from day 22, and continued until day 42).

After blood collection following sacrifice, samples were centrifuged and serum determinations were performed: blood glucose, uric acid, homocysteine, homocysteine, triglycerides, total cholesterol, HDL-C, LDL-C, calcium, sodium, potassium, iron, magnesium, phosphorus, chlorides.

An increase in blood glucose was observed in the GM2 group for 42 days compared to the MN2 group, which received normal laboratory chow for the same period of time. The greatest decrease in blood glucose is recorded by the GA group compared to the GM2 group. However, all groups receiving individual plant extracts showed decreases in blood glucose. With respect to uric acid, it increased for batch GM2 compared to batch MN2, but showed decreases for all extract-treated batches compared to batch GM2. Thus, batch GA had the greatest decrease in uric acid, followed by GCI, GR and GP [11]. In some cases, the occurrence of diabetes leads to massive decreases in homocysteine for GM2 compared to MN2. In addition, treatment with plant extracts results in further decreases in homocysteine compared to GM2, with the largest decrease induced in the GR, GCE and GP batches. The smallest homocysteine decrease is induced in the GCI group. The increase in blood

glucose in LM2 rats is significantly lower than in GM2, but the decreases induced by the administration of individual extracts and mixture of extracts are smaller. Thus, LA shows the largest decrease compared to LM2, followed by LCE, LAN and LR, with the smallest decreases being generated by LCI and LP. Also, the changes in uric acid are not as large in the hyperlipidemic diet compared to the hyperglycidic diet. Thus, LM2 shows a very small increase compared to MN2 and the greatest decrease in uric acid occurs in the LA group. Decreases in homocysteine also occur in LM2, but not as massive as in GM2 compared to MN2. It can be seen that in the hyperlipidemic diet, the introduction of plant extracts produces greater decreases than those induced by the same extracts in the hyperglycidic diet.

Triglycerides were increased for GM2 compared to MN2, and showed modest decreases for all groups given plant extracts. Total cholesterol was increased in GM2 compared to MN2, but decreased in the groups treated with the plant extracts, despite continuing the same diet throughout the experiment. HDL-cholesterol levels decreased in GM2 compared to MN2, but increased significantly following the plant extracts. In comparison, an increase in LDL-cholesterol was observed for GM2 compared to MN2. However, administration of the extracts resulted in decreases in LDL values for all groups treated with plant extracts, with the greatest decrease in the GA group. Compared to GM2, LM2 showed almost two-fold increases in triglycerides and total cholesterol, but the decreases were also considerably greater following administration of plant extracts. In the case of HDL-cholesterol, LM2 showed decreases compared to MN2, but extracts administration resulted in a massive increase of up to 80% in the LA group. LDL-cholesterol increased in LM2 compared to MN2 and decreased in LA, followed by LR and LAN. Similar decreases were imprinted to the LP and LCI groups [11].

Calcium ions undergo increases both in GM2 compared to MN2 and in extract-treated batches compared to GM2. Sodium increases in GM2 compared to MN2. Compared to GM2, it decreases in all extract-treated cases. Potassium ions decrease in GM2 compared to MN2 and continue to decrease following plant extracts, with modest values for GAN and GCI, followed by GR and GCE as average values, and higher values for GP and GA, respectively. Calcium ions show no variation between MN2 and LM2, but increases occur in all cases of plant extracts administration. Sodium shows decreases in all rat groups. Potassium shows increases in all conditions.

Iron ions decrease in GM2 compared to MN2, but the use of plant extracts causes the values for the other groups to show upward variations. There are increases in magnesium values in all batches, with the largest changes in GAN and GR. As with the hyperglucic diets, the hyperlipidic diet

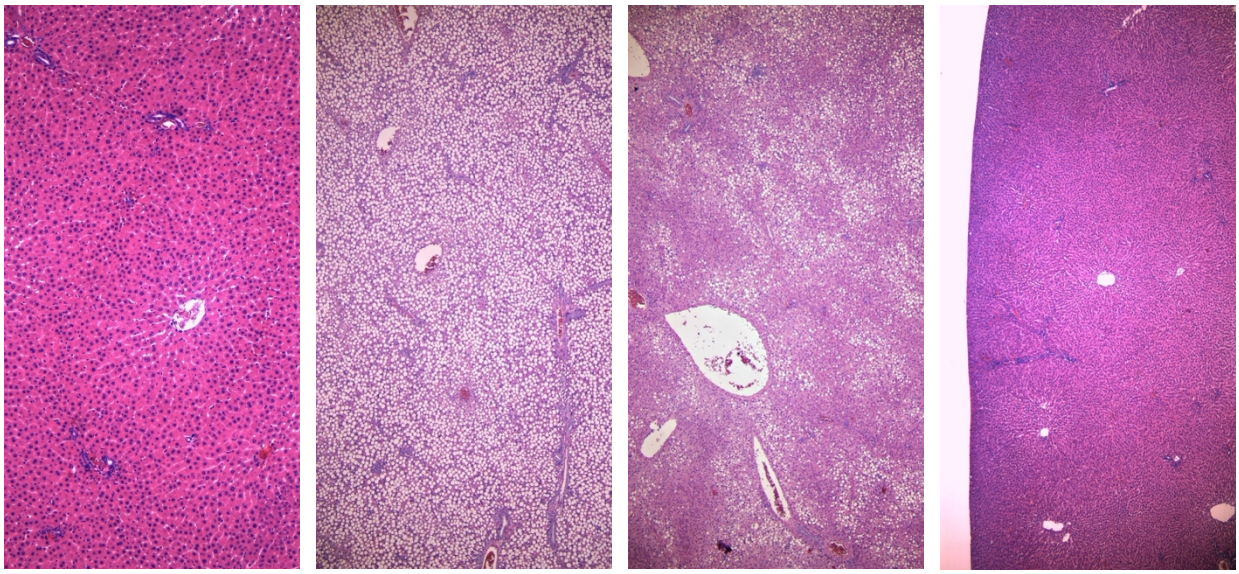
also causes the same type of change in iron ions, downward for LM2 compared to MN2, and upward for all batches treated with plant extracts compared to LM2. Magnesium shows upward variations for all batches with hyperlipidic diet.

Phosphorus ions show non-uniform variations within the groups fed hyperglucidic diets. GM2 shows an increase compared to MN2. Batch GP shows an increase compared to GM2, in contrast to the other extracts which either show insignificant increases (GAN) or decreases in phosphorus values. Chloride decreases in GM2 compared to MN2 and increases greatly following administration of the extracts. Phosphorus shows, as in the hyperglucidic diet groups, non-uniform variations, with decreases in LM2 compared to MN2, and decreases in the groups that received extracts. Chloride levels are low in LM2 compared to MN2, but show very large increases in all batches treated with plant extracts.

For the most complete and accurate evaluation of the effects of plant extracts on organs and systems, a histopathologic examination was necessary. Three organs were included in the study: liver, heart and testes.

On the basis of NAS activity score, liver sections from each of the batches sacrificed at 42 days were evaluated. Both hyperlipidemic LM2 and hyperglucidemic GM2 showed similar histopathologic features. Diffuse panlobular diffuse steatosis was observed in >66% of the samples examined (NAS score = 3). In addition, both groups developed a moderate chronic inflammatory response with approximately three foci/20x (NAS score = 2). Other less common changes included the presence of frequent apoptotic bodies and occasional Mallory-Denk bodies. However, fibrosis and balloonized hepatocytes were not observed. Taking all these features into account, on average, both groups scored 5 points out of 8 (equivalent to severe liver disease) on the NAS score, these results are consistent with the diagnosis of non-alcoholic steatohepatitis (NASH) [11].

An example of the appearance of the liver sections and morphologic changes can be seen in Figure 8.1.A-D for groups MN2, GM2, LM2 and LP.



A B C D

Figure 8.1. Morphologic changes of the liver in groups:

A - MN2 (10x); B - GM2 (4x); C - LM2 (4x); D - LP (4x).

The liver cytoarchitecture for the LP batch was fully restored and similar in appearance to the normal control 2 (MN2) [11].

The ability of plant extracts to influence the redox status of organs was analyzed by ABTS and GSH (total thiol assessment) methods. The result obtained for brain homogenates by the two methods [12-14] will be exemplified.

According to the results obtained from the ABTS test on brain homogenates processed from rats on hyperglycidic diet, the highest optical density, corresponding to the poorest antioxidant effect, is recorded by the GCI batch, followed by GP, GA, GM2, GR, GAN and MN2, while the best antioxidant effect is recorded by the GCE batch. In brain homogenates obtained from rats fed hyperlipidic diet, the lowest antioxidant capacity was associated with the LR batch, followed by the LP batch. With similar antioxidant activity were batches LA, LAN and LM2, followed by MN2 and LCI, with the lowest optical density being associated with LCE.

In brain homogenates evaluated by the GSH method, statistically significant differences were recorded for the LP, GCI and GR batches at $p<0.05$ compared to MN2, and for the LCE batch at the same level of significance compared to LM2.

9. Processing the mixture of plant extracts into solid dosage forms for oral administration

In order to obtain pharmaceutical forms for oral administration in adult patients, but also in children or persons with swallowing difficulties, it was decided to process the mixture of plant extracts with hypolipidemic action in the form of capsules with caps.

Pharmaceutical-grade materials were used for the formulation and preparation of the capsule filling material: a mixture of plant extracts standardized in active principles in equal parts: artichoke, green onion, chicory, hawthorn and rosemary extracts; excipients required for the formulation of the capsule filling mixture: ethyl alcohol (binding agent), polyplasdone XL (disaggregant), magnesium stearate (lubricant), colloidal silicon dioxide (glidant) [15]. The formulation of the capsule filling material is shown in Table IX.1.

Table IX.1. Formulation of the capsule filling material

Active ingredient	***Quantity (mg / capsule)***
Mixture of extracts	450
Excipients	
Ethylic alcohol	q.s.
Polyplasdone XL	8
Magnesium stearate	4
Colloidal silicon dioxide	5
Total mass	***467***

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

Conclusions

The research carried out in the PhD thesis aimed to identify some plant products with potential association in the treatment of hyperlipidemia following the evaluation of the therapeutic effects induced by extracts obtained from them on different experimental animal models.

- Based on literature data, plant sources (Cynarae folium, Cichorii herba, Rosmarini folium, Allii cepae folium, Apii graveolentis folium var. dulcis, Crategi sp.) with potential applicability in various combinations in the adjuvant treatment of hypercholesterolemia were selected;
- The quality of the plant raw materials was determined by evaluating the content of total polyphenols, phenolcarboxylic acids and flavones, using ethanol of 50 and 70% concentration respectively in the extraction; it was concluded that 50% ethanol should be used for chicory and artichoke, and 70% ethanol for rosemary, green onion and celeriac to obtain plant extracts;

- Dried plant extracts were obtained using the lyophilization method; the content of active principles was determined by spectrophotometric methods (considerably higher concentrations of total polyphenols were evaluated in extracts of rosemary, chicory and hawthorn compared to extracts of artichoke, green onion and celery; considerably higher concentrations of phenolcarboxylic acids in extracts of rosemary and hawthorn compared to extracts of chicory and artichoke, celery having the lowest amount of chlorogenic acid; higher concentrations of flavones in hawthorn and artichoke extracts compared to rosemary, chicory and green onion extracts, celeriac having the lowest amount of rutozide) and chromatographic (the main compounds evaluated in all samples in terms of their amount, based on data obtained by UHPLC-MS analysis, are: chlorogenic acid, rosmarinic acid, protocatechuic acid, caffeic acid, syringic acid, depending on the type of extract; as for the antioxidant action, it is in direct correlation with the content of active principles;
- Molecular docking investigations were performed to predict potential anti-obesity and hypolipidemic mechanisms for rosmarinic acid and chlorogenic acid, two phytochemical compounds evaluated in high amounts in the extracts investigated; The novelty of the extended computational study is illustrated by the observation that, although both chlorogenic acid and rosmarinic acid are esterification products between caffeic acid and quinic acid (CGA) or dihydroxyphenyl lactic acid (RA) and are chemically related polyphenols, the two metabolites have completely different in silico behaviors;
- Evaluation of mineral content is correlated with the therapeutic action of plant extracts;
- The viability of Artemia species larvae at tested concentrations ranging from 500 - 2500 µg/mL is very high, between 80-100%, which demonstrates that the extracts are not toxic at concentrations up to 2500 µg/mL; lysosomal evaluation indicates an intense metabolic activity; Allium extract allowed the growth and favored larval development, so that in the samples followed they reached the preadult stage (in 7 days); the results obtained on Daphnia species, indicate significant differences between the two species used in the test in terms of susceptibility of their behavior by exposure to the same extracts (Daphnia pulex appears to be more sensitive in general, with lower LC50 values compared to Daphnia magna for most extracts); embryotest on Daphnia magna embryos revealed varied effects, dependent on the type of extract and its concentration;
- Biological evaluations in experimental models in laboratory animals, aimed to determine all biochemical constants that are associated with hyperlipidemia: the greatest decrease in glycemia was for the group with the mixture of extracts (21 days), with respect to the group that received only

hyperglycemic feed; the groups that received individual extracts recorded decreases in glycemia of about 11% for artichoke and onion extracts, about 10% for chicory and hawthorn extracts, while the rosemary extract had the lowest effect, with a decrease of 9. 96%; the batch that received the mixture of extracts had the highest decrease in uric acid, with 20. 20%, followed by chicory and rosemary, which decreased the values by about 16%, and hawthorn by about 14%; treatment with plant extracts also generates decreases in homocysteine compared to the hyperglycidic control, the highest decrease being induced by rosemary, about 34%, closely followed by onion and hawthorn (32. 06% and 31.45% respectively); the mixture of extracts decreased triglyceride levels the most, by 5.25%, followed by chicory (4. 25%), rosemary and hawthorn (about 3%), while artichoke and onion imprinted almost negligible decreases; the mixture of extracts imprinted a decrease in total cholesterol by about 9%, chicory, hawthorn and rosemary by about 7%, followed by onion (6. 57%) and artichoke (3. 59%); the mixture resulted in 77. 61% increases in HDL-cholesterol compared to the hyperglycidic control, rosemary, chicory and hawthorn imprinted increases between 57-60%, and artichoke and onion had increases of about 40%; LDL-cholesterol decreased by 16. 41% for the batch treated with the mixture of extracts, with about 12% for artichoke and chicory extracts, and about 11% for hawthorn and rosemary; onion and artichoke extracts imprint increases of 13-14%, followed by rosemary and mixture, with 22-24%, the highest values being generated by hawthorn and chicory (34-35%) for calcium ion variations; on potassium ions, artichoke generates a decrease of 6. 42%, followed by onion, rosemary and chicory, with values ranging from 8 to 9%, while hawthorn and the mixture of extracts imprint decreases ranging from 11 to 12%; there are increases in the values of magnesium, iron, phosphorus ions, in all batches;

- Histological examinations on different types of organs are in direct correlation with the chemical composition of each type of extract tested, thus: in the liver from the hypergluccidic batches, treatment with hawthorn and rosemary led to complete disappearance of hepatic steatosis, with the exception that the persistence of rare isolated inflammatory cells was observed; in the hyperlipidemic groups, following treatment with hawthorn and a mixture of extracts, the cytoarchitecture of the liver was completely restored, with no pathological aspects identified; treatment with hawthorn extract and a mixture of extracts resulted in a complete restoration of myocardial histology in the hyperglucidemic groups, with no pathological aspects; in the hyperlipidemia model, following treatment with rosemary, myocardial histology was almost entirely restored, with only very rare muscle fibers with hyperchromatic, polylobated nuclei; microscopic examination of testicular tissue

showed an improvement depending on the type of plant extract administered; the tests performed on the different batches of organ homogenates are in direct correlation with the chemical composition of the extracts tested;

- A pharmaceutical formulation containing the tested mixture of plant extracts was proposed, subjected to pharmaco-technical tests specific to the field of Pharmaceutical Technology.

Personal contributions

- All the research objectives have been met and the entire research is original, starting from the selection of plant raw materials, determination of their quality, obtaining dried plant extracts, quantitative evaluation, determination of antioxidant action, molecular docking studies, determination of trace elements content, phytotoxicity evaluation, research on experimental models in laboratory animals, histological evaluations on different types of organs, evaluation of redox status on different types of homogenates, proposal of a pharmaceutical formulation.

Research perspectives

- Evaluation of UHPLC and other classes of chemical constituents involved in therapeutic response;
- Stability studies of plant extracts;
- Proposal of other types of pharmaceutical formulations;
- Pharmacokinetics studies

Selective bibliography

1. **Ilie EI**, Popescu L, Luță EA, Biță A, Corbu AR, Mihai DP, Pogan AC, Balaci TD, Mincă A, Duțu LE, et al. Phytochemical Characterization and Antioxidant Activity Evaluation for Some Plant Extracts in Conjunction with Pharmacological Mechanism Prediction: Insights into Potential Therapeutic Applications in Dyslipidemia and Obesity. *Biomedicines*. 2024; 12(7):1431. doi: 10.3390/biomedicines12071431.
2. Supuran, C.T. Anti-Obesity Carbonic Anhydrase Inhibitors: Challenges and Opportunities. *J. Enzyme Inhib. Med. Chem.* 2022, *37*, 2478–2488. https://doi.org/10.1080/14756366.2022.2121393.
3. Karioti, A.; Carta, F.; Supuran, C. Phenols and Polyphenols as Carbonic Anhydrase Inhibitors. *Molecules* 2016, *21*, 1649. https://doi.org/10.3390/molecules21121649.
4. Han, J.; Wang, D.; Ye, L.; Li, P.; Hao, W.; Chen, X.; Ma, J.; Wang, B.; Shang, J.; Li, D.; et al. Rosmarinic Acid Protects against Inflammation and Cardiomyocyte Apoptosis during Myocardial Ischemia/Reperfusion Injury by Activating Peroxisome Proliferator-Activated Receptor Gamma. *Front. Pharmacol.* 2017, *8*, 456. https://doi.org/10.3389/fphar.2017.00456.
5. Aspatwar,A.;Supuran,C.T.;Waheed,A.;Sly,W.S.;Parkkila,S.MitochondrialCarbonicAnhydraseVAand VB:Propertiesand Roles in Health and Disease. *J. Physiol.* 2023, *601*, 257–274. https://doi.org/10.1113/JP283579.
6. Pimpley,V.;Patil,S.;Srinivasan,K.;Desai,N.;Murthy,P.S.TheChemistryofChlorogenicAcidfromGreenC offeeandItsRole in Attenuation of Obesity and Diabetes. *Prep. Biochem. Biotechnol.* 2020, *50*, 969–978. https://doi.org/10.1080/10826068.2020.1786699.
7. Ohishi,T.;Fukutomi,R.;Shoji,Y.;Goto,S.;Isemura,M.TheBeneficialEffectsofPrincipalPolyphenolsfro mGreenTea,Coffee, Wine, and Curry on Obesity. *Molecules* 2021, *26*, 453. https://doi.org/10.3390/molecules26020453.
8. Kumar, R.; Sharma, A.; Iqbal, M.S.; Srivastava, J.K. Therapeutic Promises of Chlorogenic Acid with Special Emphasis on Its Anti-Obesity Property. *Curr. Mol. Pharmacol.* 2020, *13*, 7–16. https://doi.org/10.2174/1874467212666190716145210.
9. Alam,M.A.;Subhan,N.;Hossain,H.;Hossain,M.;Reza,H.M.;Rahman,M.M.;Ullah,M.O.Hydroxycinna micAcidDerivatives: A Potential Class of Natural Compounds for the Management of Lipid Metabolism and Obesity. *Nutr. Metab.* 2016, *13*, 27. https://doi.org/10.1186/s12986-016-0080-3.
10. Silva, J. D. S., Rocha, I. K. B. D. S., Freitas, L. C. D., Pereira, N. J., & Carvalho Neta, R. N. F. (2015). Bioethical principles applied to aquatic ecotoxicological studies. *Revista Bioética*, *23*, 409-418.
11. **Ilie (Ioniță) EI**, Olaru OT, Pițuru MT, Pogan AC, Moroșan E, Văleanu A, Corbu AR, Nica AE, Țăpoi DA, Tănăsescu MD, Mincă A, Gîrd CE. In vivo evaluation of the effects induced by hawthorn (Crataegi sp.) extract in rats, in two experimental diet models. *Farmacia*. 2024 Apr; 72(2): 305–314. https://doi.org/10.31925/farmacia.2024.2.9.
12. Ungurianu, A.; Şeremet, O.; Gagniuc, E.; Olaru, O.T.; Guţu, C.; Grădinaru, D.; Ionescu-Tîrgovişte, C.; Margină, D.; Dănciulescu-Miulescu, R. Preclinical and clinical results regarding the effects of a plant-based antidiabetic formulation versus well established antidiabetic molecules. *Pharmacol Res* 2019, *150*. 10.1016/j.phrs.2019.104522.
13. Mihai DP, Ungurianu A, Ciotu CI, Fischer MJM, Olaru OT, Nitulescu GM, Andrei C, Zbarcea CE, Zanfirescu A, Seremet OC, Chirita C, Negres S. Effects of Venlafaxine, Risperidone and Febuxostat on Cuprizone-Induced Demyelination, Behavioral Deficits and Oxidative Stress. Int J Mol Sci. 2021 Jul 2;22(13):7183. doi: 10.3390/ijms22137183. PMID: 34281235; PMCID: PMC8268376.
14. Nitulescu G, Mihai DP, Nicorescu IM, Olaru OT, Ungurianu A, Zanfirescu A, Nitulescu GM, Margina D. Discovery of natural naphthoquinones as sortase A inhibitors and potential anti-infective solutions against Staphylococcus aureus. Drug Dev Res. 2019 Dec;80(8):1136-1145. doi: 10.1002/ddr.21599. Epub 2019 Sep 4. PMID: 31486108.
15. Lupuleasa Dumitru, Popovici Iuliana, Tehnologie Farmaceutica, vol III, Editura Polirom, Iasi, 2009.