UNIVERSITY OF MEDICINE AND PHARMACY

"CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL

DOCTORAL FIELD OF PHARMACY

Ph.D. THESIS

PhD Supervisor:

PROF. UNIV. DR. GÎRD CERASELA ELENA

PhD Student:

COSTEA LILIANA

2022

UNIVERSITY OF MEDICINE AND PHARMACY

"CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL

DOCTORAL FIELD OF PHARMACY

PRELIMINARY RESEARCH ON THE FORMULATION OF A PHYTOPREPARATION ASSOCIATED IN THE TREATMENT OF HEPATOPATHIES

Ph.D. THESIS SUMMARY

PhD Supervisor:

PROF. UNIV. DR. GÎRD CERASELA ELENA

PhD Student:

COSTEA LILIANA

2022

Table of Contents

List of published scientific papers

Articles published in ISI rated journals

1. **Liliana Costea**, Manuela Ghica, Teodora Costea, Cerasela Elena Gîrd. Spectrophotometric evaluation of flavonoids, phenolcarboxylic acids and total phenolic contents of several indigenous herbal products with potential hepatoprotective effect (2021). *Farmacia*, 69(6), 1176-1181, ISI indexed journal, with impact factor **1.433**, ISSN: 2065-0019 (*for the Online Edition*) and 0014-8237 (*for the Printed Edition*), link: https://doi.org/10.31925/farmacia.2021.6.23
2. **Liliana Costea**, Carmen Lidia Chițescu, Rica Boscencu, Manuela Ghica, Dumitru Lupuliasa, Dragoș Paul Mihai, Teodora Deculescu-Ioniță, Ligia Elena Duțu, Maria Lidia Popescu, Emanuela-Alice Luță, George Mihai Nițulescu, Octavian Tudorel Olaru, Cerasela Elena Gîrd. The polyphenolic profile and antioxidant activity of five vegetal extracts with hepatoprotective potential (2022). *Plants*, 11(13), 1680, ISI indexed journal, with impact factor **4.658**, ISSN: 2223-7747 (*for the Online Edition*), link: https://doi.org/10.3390/plants11131680

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

1. **Liliana Costea**, Cerasela Elena Gîrd. Preliminary research on formulation of a phytopreparation with antioxidant and hepatoprotective effects (2018). International Symposium - Priorities of chemistry for a sustainable development - PRIOCHEM XIV (ICECHIM), 14th edition, Bucharest, October 10-12, summary published in *Volume of summaries (Section 2: Bio-resources, biotechnologies and biorefining)*, ISSN: 2601 -4203 (*for the Online Edition*) and ISSN: 2601-4181 (*for the Printed Edition*)
2. **Liliana Costea**, Cerasela Elena Gîrd. Research on the formulation of a phytomedicine associated in the treatment of liver diseases. Obtaining and establishing the extraction procedure for vegetal extracts (2021). National Congress of Pharmacy XVIIIth Edition, Oradea, September 15-17, summary published in the *Congress Brochure: Pharmacy. From innovation to good pharmaceutical practice*, ISBN: 978-606-10-2144-4
3. **Liliana Costea**, Cerasela Elena Gîrd. Preliminary research on the chemical composition of a phytopreparation with antioxidant and hepatoprotective effects (2021). Symposium with international participation: Alternative and complementary therapies (Homeopathy/Phytotherapy), 4th edition, Constanța, March 26-27, summary published in *Book of abstracts*, ISSN: 2601-1476

Scientific works communicated at national events

- **Liliana Costea**, Elena Moroșan, Ana Corina Ioniță, Cerasela Elena Gîrd. Biochemical research on a mixture of vegetal extracts with potential use in the treatment of liver disease (2021). Congress of the University of Medicine and Pharmacy "Carol Davila", IXth edition, Bucharest, November 25-27, scientific paper presented during the *Young Researcher Session - Pharmacy Field* (certified with *Diploma of Excellence*).

INTRODUCTION

The liver, an accessory gland of the digestive tract, is considered one of the largest and most important organs in the human body. Due to its location, the liver represents a true functional "hemodynamic and metabolic sluice" through which a balance is permanently achieved between what the body metabolizes or eliminates and what the body receives from the outside through food, medication, or exogenous substances. It is the only organ in the body that possesses the remarkable capacity of self-regeneration but is also the second organ, after the brain, that can be attacked by free radicals, being considered, from many points of view, the "central laboratory" of the body due to its primordial importance in the normal development of essential vital processes. The pathogenesis of liver diseases is very complex and studied, and the most important factors involved are the resulting metabolic products (lipid, acid-base) and energy-dynamic, endocrine-metabolic, or neurovegetative disturbances, which generate severe changes at the level of the main functions and organs. However, the identification of hepatoprotective compounds that prevent these alterations or completely regenerate the affected functions and structures is still in a full process of continuous expansion by testing different constituents and searching for sources that offer products with obvious hepatoregenerative effect but free of side effects or adverse reactions.

The justification of the research topic consists of the existence on the pharmaceutical market of numerous insufficiently tested herbal preparations, which are not properly standardized so that the patient is guaranteed the quality conditions of the phytopreparation and can increase his confidence in the obvious therapeutic results that will appear during the selected adjuvant treatment. Also, the identification and research of some compounds with an antihepatotoxic role is of real use in the adjuvant therapy of liver diseases and not only, and phytotherapeutic extracts represent a rich source of such compounds because the solvent used for the extraction of the active principles can determine a much better solubilization of them and, implicitly, a much improved absorption in the body with the appearance of the desired therapeutic effect, the essential criterion being their standardization, as well as the quantification of the therapeutic actions in different categories of patients. In the current clinical context, in which the patient already presents the associated polypathologies and lives in a highly polluted environment (air, water, food), is subject to polypharmacy, adjacent polymedication, and later addition to the dosage regimen and food supplements non-standardized and insufficiently tested and characterized chemically, the liver cell is exposed to permanent oxidative stress. So, hepatic cells have to perform their functions in hostile and altered conditions biologically and enzymatically, and their detoxification capacities of endogenous or exogenous products can be far exceeded, which outlines an altered structural, cellular, and biohumoral picture with multiple clinical afflictions and severe metabolic

derangements. Thus, the need for research and standardization of a phytomedicine that can optimally intervene at the hepatocytic level, performing beneficial adjuvant therapeutic effects for preventing the occurrence of liver diseases, decreasing their frequency in the population, or ameliorating or curing them, creates the premises of this scientific path in which the topic addressed is fully justified and at the end of which lies the good and health of the patient. Starting from all these aspects, the purpose of this paper is to carry out a pharmacognostic and phytopharmacological analysis of a new phytopreparation proposal based on plant extracts with hepatoprotective, antihepatotoxic, hepatoregenerative, and antioxidant action.

The objectives of the research are: the selection of plant raw materials as a potential source of compounds with a hepatoprotective, antihepatotoxic, hepatoregenerative, and antioxidant role; establishing the phytochemical profile of the selected plant materials through profile analyses; drawing up the methodology for obtaining plant extracts, in the context of determining the type of solvent and the extraction process that allows the quantification of a large amount of bioactive principles; determining the stability of plant extracts at different time intervals; outlining the phytochemical profile through phytochemistry-specific analyses (spectrophotometric and HPLC dosages); alternative methods for determining extract cytotoxicity; testing the antioxidant activity in the acellular system as an important marker in evaluating the hepatoprotective and antiradical effects *in vitro* of the extracts; establishing the biochemical profile of the extracts through domain-specific analyses to evaluate the influence on the characteristic biochemical parameters following administration to experimental animals; assessment of the antioxidant capacity and redox damage at the liver tissue level by analyzing liver tissue homogenates; evaluation of the beneficial effects at the level of the hepatocyte through histological analysis of the liver samples taken from the laboratory animals subjected to the tests; highlighting the effects induced by the extracts on the cell lines hepatocellular carcinoma HepG2; establishing the pharmacotechnical parameters for the design of some pharmaceutical forms based on the analyzed plant extracts; dissemination of research results in specialized journals from the international scientific flow.

CURRENT STATE OF KNOWLEDGE

1. Theoretical data on liver diseases

This chapter contains information about: the causes of liver diseases; categories of patients at risk; specific symptoms; clinical consequences; treatment; diet and hepatic steatosis (causes, etiological mechanisms, symptoms, diagnosis, prognosis, and treatment).

2. The hepatoregenerative, hepatoprotective, and antihepatotoxic actions of some vegetal products / extracts – A literature study

This chapter contains information on the chemical compositions as well as the beneficial effects on the liver cells induced by different plant sources/extracts.

PERSONAL RESEARCH

3. Establishing the quality standard of the selected vegetal products

Based on the data from the scientific literature, five plant products were selected (*Cynarae folium*, *Rosmarini folium*, *Agrimoniae herba*, *Taraxaci herba,* and *Cichorii herba*), and the pharmacognostic analysis was used as a working method. Flavones, PCAs, and total phenolic content were measured by spectrophotometric methods (table III.1).

Table III.1. Results of spectrophotometric assays expressed as mean ± SD [1]

Flavones (FL) (g rutin/100 g dry herbal product)			
SA70	SA50	SA20	SAA
1.1745 ± 0.0293	1.0332 ± 0.0836	0.9447 ± 0.1638	0.6453 ± 0.0496
SCH70	SCH50	SCH20	SCHA
0.4771 ± 0.0348	0.5596 ± 0.0482	0.4350 ± 0.0116	0.3814 ± 0.0312
SCY70	SCY50	SCY20	SCYA
0.7382 ± 0.0590	0.7497 ± 0.0244	0.4361 ± 0.0476	0.4121 ± 0.0277
SR70	SR50	SR20	SR
1.0271 ± 0.1130	1.2771 ± 0.0684	0.7991 ± 0.1469	0.1156 ± 0.0099
ST70	ST50	ST20	STA
0.6737 ± 0.0192	0.6497 ± 0.1479	0.9154 ± 0.1138	0.7808 ± 0.1655
Phenolcarboxylic acids (PCAs) (g chlorogenic acid/100 g dry herbal product)			
SA70	SA50	SA20	SAA
4.1655 ± 0.2108	4.7469 ± 0.1683	6.5580 ± 0.4671	3.1846 ± 0.1978
SCH70	SCH50	SCH20	SCHA
1.8201 ± 0.0503	1.4614 ± 0.0766	1.4840 ± 0.0273	1.4974 ± 0.1054
SCY70	SCY50	SCY20	SCYA
0.6572 ± 0.1201	0.5643 ± 0.0401	0.6170 ± 0.0697	0.3802 ± 0.0428
SR70	SR50	SR20	SR
5.6123 ± 0.2365	5.6733 ± 0.7740	4.9732 ± 0.4402	0.8151 ± 0.0582
ST70	ST50	ST20	STA
2.0383 ± 0.1333	2.1674 ± 0.1824	2.6529 ± 0.0438	2.8350 ± 0.0867
Total phenolic content (TPC) (g tannic acid/100 g dry herbal product)			
SA70	SA50	SA20	SAA
8.0232 ± 1.9744	8.5634 ± 0.2243	8.2553 ± 1.1594	4.5732 ± 0.5401
SCH70	SCH50	SCH20	SCHA
2.0884 ± 0.3213	2.1534 ± 0.0303	1.9186 ± 0.0769	1.6393 ± 0.0746
SCY70	SCY50	SCY20	SCYA
1.4222 ± 0.1590	1.1984 ± 0.2579	1.1228 ± 0.1149	0.8756 ± 0.0125
SR70	SR50	SR20	SR
8.8233 ± 1.1743	8.1027 ± 0.6782	5.1887 ± 0.1836	0.8604 ± 0.0592
ST70	ST50	ST20	STA
2.8415 ± 0.2213	2.9509 ± 0.0486	3.3647 ± 0.3786	3.34 .1058

Considering the obtained results, 50° ethanol ensured the optimal extraction of phenolic compounds for all the plant products studied, except for dandelion, for which 20° ethanol was the best extraction solvent.

4. Obtaining dry extracts and establishing the control methodology

In order to formulate a phytopreparation aimed at the treatment of hepatopathies, it was necessary to obtain some extracts ennobled in chemical constituents of therapeutic interest. Lyophilization was selected as obtaining technique because it allows the integrative preservation of therapeutically active constituents. In order to establish the control methodology of the obtained dry extracts, their organoleptic characteristics were analyzed and the quantification of the chemical constituents of therapeutic interest was also pursued by performing the dosages of active principles for each individual dry extract. The determinations carried out immediately after obtaining the extracts revealed that the extracts of rosemary and agrimony are the richest in flavones, PCAs, and total polyphenols, and for chicory, dandelion, and artichoke the concentration values are much lower, but they have uniform and constant increases, which is relevant for the therapeutic action being researched. In order to be able to quantify the assurance of the therapeutic effectiveness of dry plant extracts over time and the stability of the pharmaceutical forms that will contain these dry extracts, the active principles of therapeutic interest from the extracts must present a stable concentration over time that remains within the same constant variation limits. Thus, it can be evaluated whether there are factors that would cause the active principles content of dry extracts to decrease from the optimal level to a much lower level, without clinical efficiency. For this purpose, dosages of active principles (PCAs, flavones, and total polyphenols) from the dry vegetal extracts were carried out over a period of one year, monitoring the stability of concentrations and performing quantitative determinations at 3 months, 6 months, and 12 months after obtaining them (the results are presented in fig. 4.1. - 4.3.). Considering the interdependence between the plant product and the active principles contained, the dry extracts analyzed presented optimal concentrations of PCAs, flavones, and total polyphenols, constituents of essential therapeutic importance, which proved to be phytochemically stable during the monitoring period. The stability study of the dry extracts emphasized the activity of the chemical constituents from extracts even during storage, observing fluctuations in concentrations from one time interval to another, as the active principles of plant origin have an atypical behavior and are totally distinct from that of synthetic active substances. However, following statistical interpretation, the only statistically significant difference was obtained for the content of PCAs in the rosemary extract, which was considerably reduced by almost half after 12 months, but this behavior was expected considering that phenolcarboxylic acids are the most unstable active principles over time and the most labile, which can be influenced by numerous factors. What was surprising at this stage of the research

was, in fact, the evolution of PCAs in the other dry vegetal extracts, with small and insignificant variations being recorded, despite their chameleonic profile. From a pharmacognostic and phytochemical point of view, the total flavones and polyphenols from the dry extracts proved to be stable, maintaining the optimal therapeutic level throughout the 12 months for all the studied extracts.

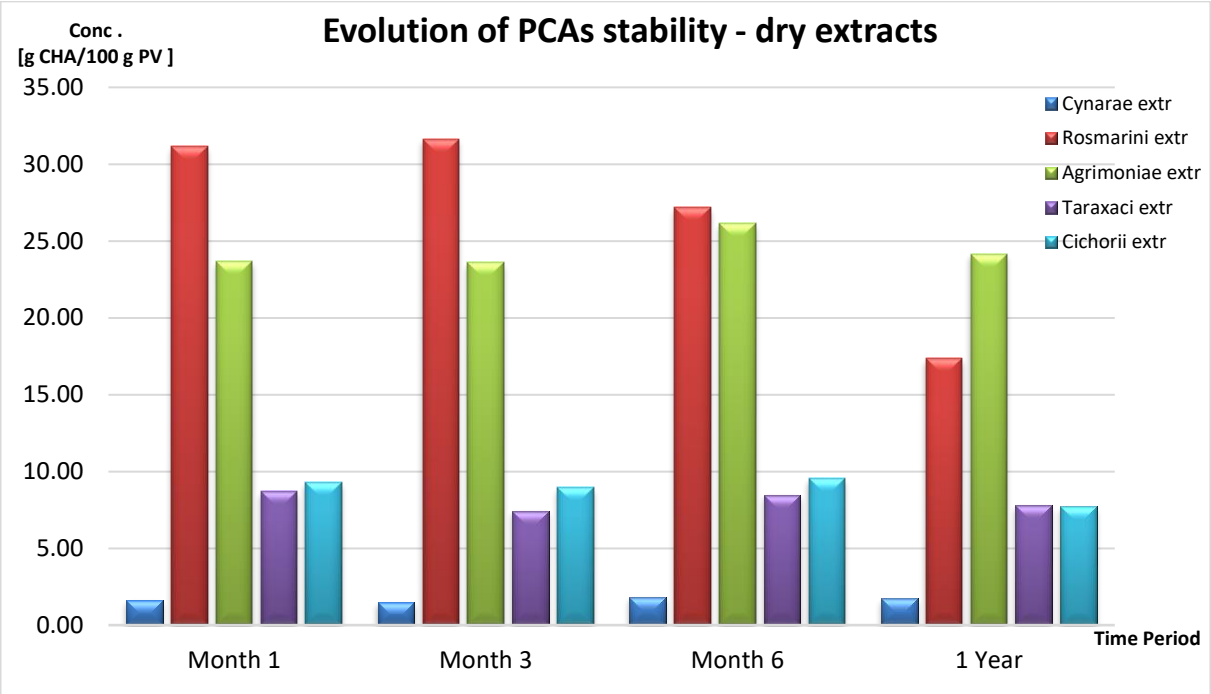


Fig. 4.1. Evolution of PCAs concentration in dry extracts

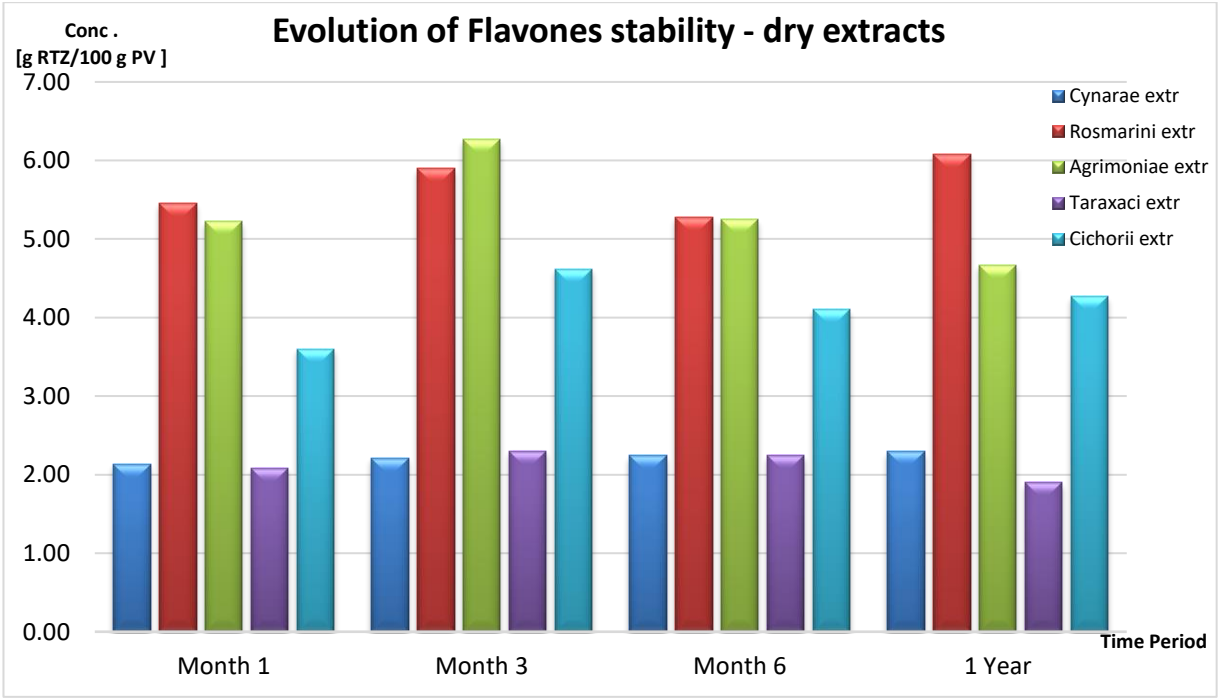


Fig. 4.2. Evolution of flavone concentration in dry extracts

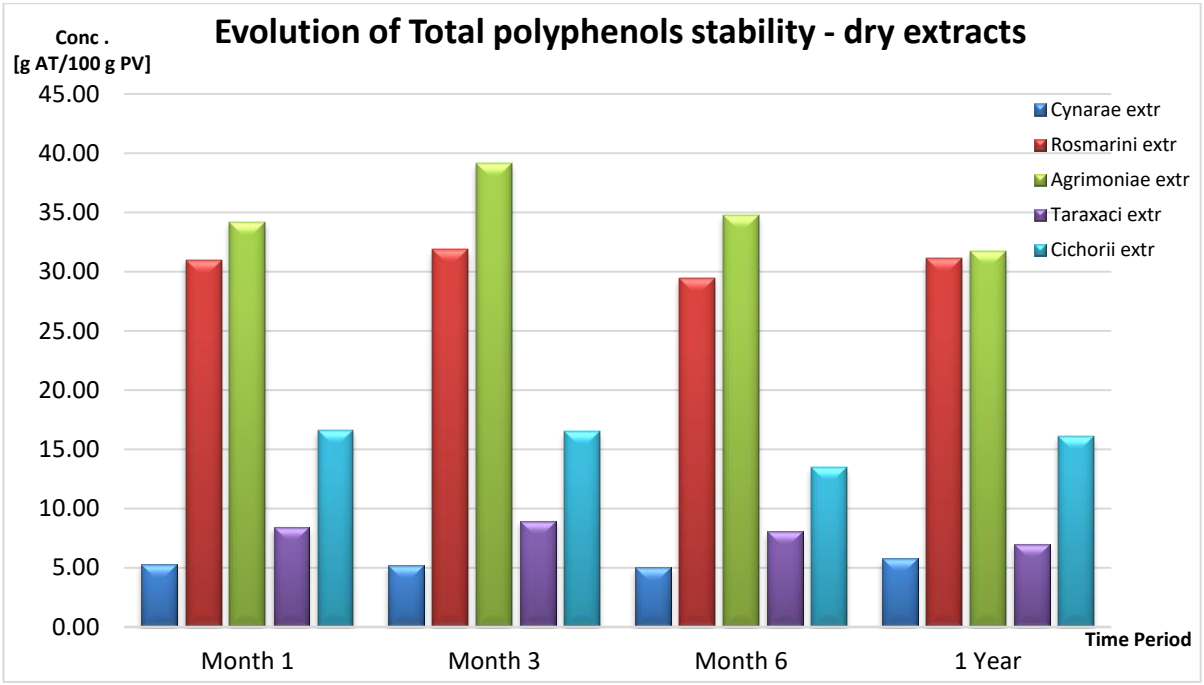


Fig. 4.3. Evolution of total polyphenols concentration in dry extracts

5. Chromatographic analysis of polyphenolic compounds from dry extracts, comparative cluster analysis of plant extracts, and molecular docking studies

The spectrum of compounds identified and quantified by UHPLC-HRMS/MS is dependent on the type of plant extract used, with the compounds identified being part of the class of flavonoids, phenolcarboxylic acids, and sesquiterpene derivatives. Thus, 28 compounds were identified in *Cynarae extractum (CE), in Rosmarini extractum* (RE) – 48 compounds, in *Taraxaci extractum* (TE) – 39 compounds, in *Cichorii extractum* (CHE) – 43 compounds, and in *Agrimoniae extractum* (AE) – 31 compounds. From the analysis of the obtained results, rutoside was identified in all extracts except AE, apigenin and kaempferol in all types of extracts, chlorogenic acid and caffeic acid in all extracts, except CHE (where only chlorogenic acid was identified along with one of its isomers), vitexin was identified in CE, TE, CHE, and AE, cynarine, scolimoside, and cynaropicrin were identified in CE, cichorin in CE, RE, and CHE, cynaroside and cynarotrioside in TE, CHE, and AE, catechin and epicatechin were identified in CHE and AE, azelaic acid was identified in all extracts, biochanin A was identified in RE, CHE, and AE, genistin and daidzin in CHE, genistin in TE and AE, and condensed procyanidins (proanthocyanidins) in TE, CHE, and AE. The compounds identified and quantified in the analyzed plant extracts are part of the polyphenolic profile cited in the scientific literature. The identification of pinocembrin in large quantities (43.1 µg/g) in *Rosmarini extractum* is consistent with the literature data, as this compound was also identified in rosemary honey. The chemical profile of the compounds identified in TE is also consistent with reported studies from the scientific literature [2,3].

The artichoke extract composition's dendrogram (Figure 2) shows the disposition of the identified compounds in four clusters. The first cluster contains a group of 9 compounds of the flavonoid class, the second one contains a group of 14 compounds represented by flavonoid aglycones (in this group there are 2 diterpene derivatives - carnosol and rosmanol, and 1 sesquiterpene compound - cynaropicrin), the third one contains a group of phenolic compounds such as chlorogenic acid and 2 coumarin derivatives, and the last one contains 2 phenolic acids - caffeic and azelaic acid.

The Cluster analysis performed for the five plant extracts showed a general heterogeneous distribution in a variable number of clusters for all extracts, the results obtained being in direct correlation with the compounds identified by the UHPLC-HRMS/MS method. Thus, in CE, the 28 compounds identified were distributed in four groups; in RE, TE, and AE, although a variable number of compounds were identified, they were distributed into five groups each; and in CHE, where 43 compounds were identified, they were classified into six groups. Hierarchical Clustering Analysis proved to be particularly useful for grouping the chemical constituents of the analyzed plant extracts, practically transforming the complicated classification algorithms based on statistical programs into a transparent "mirror" of the polyphenolic profile of the extracts, in which compounds present an organized and greatly simplified arrangement, taking into account the variation of similarity between them.

The determined polyphenolic compounds were evaluated by molecular docking as potential inhibitors of cytochrome P450 isoform 2E1 (CYP2E1) and tumor necrosis factor alpha (TNF-α), but also as allosteric activators of glutathione-peroxidase 4 (GPx4). CYP2E1 is an isoform responsible for the transformation of polyunsaturated fatty acids and exogenous compounds into toxic metabolites, and its inhibition can attenuate or prevent hepatotoxicity at the cellular and tissue level. TNF-α is a proinflammatory cytokine that generates inflammation, oxidative stress, and hepatocyte apoptosis, while GPx4 is an antioxidant enzyme that prevents hepatocellular destruction by suppressing lipid peroxidation and inflammation. So, polyphenolic phytocompounds from plant extracts can have a particularly important role in the allosteric regulation of many enzymes or biological structures essential for the proper functioning of the hepato-biliary tree. About 23 polyphenolic compounds identified in the analyzed samples were subjected to molecular docking simulations to predict their binding affinities and molecular interactions with potential targets involved in the hepatoprotective mechanism. The docking protocol was successfully validated by redocking positive controls into the active sites, with the redocked ligands showing little variation in positional configurations. The CYP2E1 inhibitor showed a binding energy of -7.776 kcal/mol and 0.1704 Å RMSD after superposition on the initial configuration. The TNF-α inhibitor showed a docking score of -8.867 kcal/mol and 0.1325 Å

RMSD, while redocking the allosteric activator GPx4 produced a binding energy of -6.978 kcal/mol and 0.2691 Å RMSD. Out of the 23 docked compounds, 2 ligands did not fit into the active site of CYP2E1, and binding affinity could not be determined due to their large molecular volume (naringin and rutin/rutoside). Binding energies after docking on CYP2E1 ranged from -8.942 kcal/mol to -0.884 kcal/mol, with an average value of -7.128 ± 1.855 kcal/mol. Pinocembrin showed the highest binding affinity, while hyperoside showed the lowest affinity for CYP2E1. The highest ligand efficiency was observed for cinnamic acid (0.7417; -8.159 kcal/mol). Ten polyphenolic compounds showed higher affinities than the positive controls (pinocembrin, chrysin, apigenin, formononetin, epicatechin, naringenin, catechin, cinnamic acid, *p*-coumaric acid, and abscisic acid). Binding energies for TNF-α ranged from -8.998 to -5.673 kcal/mol, with an average value of -7.382 ± 0.998 kcal/mol. The lowest binding energy was observed for rutoside and the highest energy for syringic acid. Moreover, cinnamic acid showed the highest ligand efficacy also for TNF-α (0.5436; -5.980 kcal/mol). Only one analyzed compound showed higher binding affinities than the positive control (rutoside), while three polyphenolic compounds showed slightly lower but comparable affinities (genistin, hyperoside, and naringin). Molecular docking targeting the GPx4 allosteric binding site produced binding energies between -7.023 and -4.637 kcal/mol (-6.198 ± 0.789 kcal/mol). Naringin showed the highest affinity for the GPx4 allosteric site, while syringic acid showed the lowest affinity. Only two ligands had lower binding energies than the positive control (naringin and genistin), while six other compounds had similar values (apigenin, rutoside, chlorogenic acid, hyperoside, epicatechin, and kaempferol). Interactions between CYP2E1 and cinnamic acid are particularly important because this particular compound shows strikingly high ligand efficiency. Cinnamic acid is involved in hydrogen bonding with Asn206 through its carboxyl moiety. The protein-ligand complex was further stabilized by a carbon-hydrogen bond with Val239, pi-pi interactions with Phe298, and van der Waals interactions with nine other residues in the active site (Fig. 5.1. A, B). Rutoside showed the highest binding affinity predicted for the TNF-α binding site. It also acted as a hydrogen bond donor for four residues (Ser60, Gln61, Tyr119) through several hydroxyl groups and formed a carbon-hydrogen bond with Leu120 (Fig. 5.12. C, D). Moreover, nonpolar interactions such as pi-alkyl (Tyr59) and van der Waals interactions were also responsible for binding to the active site. Apigenin had the third highest binding affinity for GPx4 among the phytochemicals analyzed, also showing a good ligand efficiency value. The binding potential of apigenin at the GPx4 allosteric binding site is supported by a hydrogen bond with Met102, 2 pi-anion interactions with Asp21 and Asp23, 2 pi-alkyl interactions with Val27 and Lyes90, and 11 van der Waals interactions (Fig. 5.1. E, F). Molecular docking studies in the field of phytochemistry are used to predict the preferred orientations of the analyzed compounds, but also their strength of association or binding affinity

towards different relevant biological targets in order to form a stable complex with a role in the transmission of the cellular signal involved in the dynamics of the biochemical or therapeutic effects (antagonism or synergism). Thus, an analogy of the behavior of phytoconstituents from plant sources in the body can be generated *in silico* and conformational changes, actions, or interactions at the cellular and tissue level can be predicted. Following *in silico* evaluations, polyphenolic compounds were found to have a hepatoprotective effect via interactions with CYP2E1, TNF-α, and GPx4. The reduction of oxidative stress, the annihilation of hepatocellular toxicity, the attenuation of fibrogenesis, as well as the regulation of key enzymes with an antioxidant and anti-inflammatory role are possible beneficial therapeutic actions of the extracts. These effects were outlined and confirmed with the help of computational molecular modeling techniques with an essential role in the rational design of phytomedicines [4].

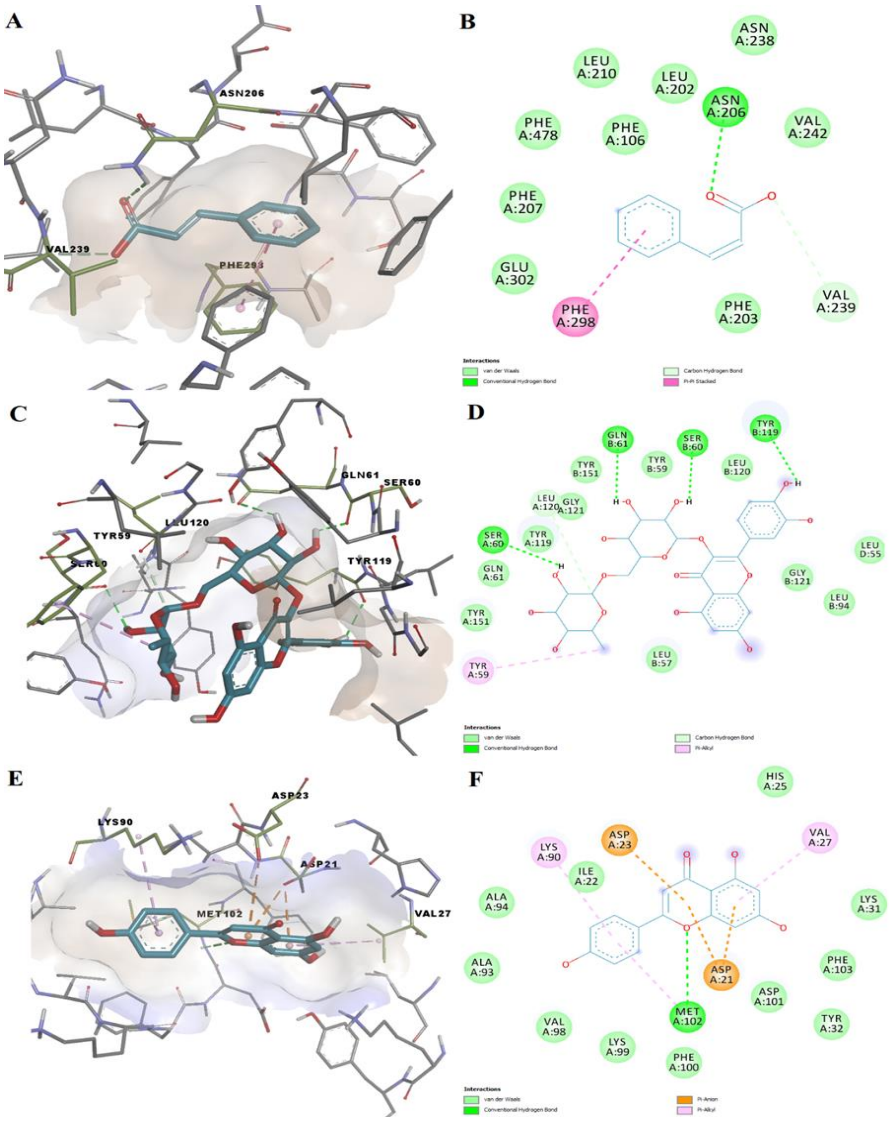


Fig. 5.1. Docking poses and molecular interactions between docked ligands and target proteins. (**A**) 3D conformation of predicted cinnamic acid-CYP2E1 complex; (**B**) 2D depiction of protein–ligand interactions for predicted cinnamic acid–CYP2E1 complex; (**C**) 3D conformation of predicted rutin–TNF-α complex; (**D**) 2D depiction of protein–ligand interactions for predicted rutin–TNF-α complex; (**E**) 3D conformation of predicted apigenin–GPx4 complex; (**F**) 2D depiction of protein–ligand interactions for predicted apigenin–GPx4 complex. [4].

6. The antioxidant activity of vegetal extracts in the acellular system

The antioxidant effects induced by the tested extracts are in direct correlation with the concentration of secondary metabolites. Comparing the results obtained by the three evaluated methods (Table VI.1.) it is found that the most intense antioxidant activity is induced by *Agrimoniae extractum* (the lowest IC_{50} value by all 3 methods, compared to the other extracts), a fact justified by the highest content of polyphenols in this type of extract (31.7017 g tannic acid/100 g dry extract). It can also be observed that, among all the analyzed extracts, the IC_{50} /EC_{50} values obtained for *Agrimoniae extractum* are much closer to the antioxidant values of the standard used (ascorbic acid), which accentuates the superior antiradicalar action of the agrimony extract compared to the other extracts.

Table VI.1. The antioxidant activity of vegetal extracts expressed by the IC_{50} value [4]

Vegetal extract	DPPH Method IC_{50} (mg/mL)	ABTS Method IC_{50} (mg/mL)	FRAP Method EC_{50} (mg/mL)
Cynarae extractum	0.6596	0.1588	0.5413
Rosmarini extractum	0.0900	0.0297	0.0537
Taraxaci extractum	0.3121	0.0752	0.2745
Cichorii extractum	0.1954	0.0539	0.2012
Agrimoniae extractum	0.0537	0.0147	0.0483

The antioxidant activity imprinted by these extracts showed a strong direct link with the polyphenol content evaluated in the analyzed extracts. Taking into account the results obtained by the 3 methods of quantifying the antioxidant effect (DPPH, ABTS, FRAP), it can be stated that all the extracts used in the work present a high level of antiradicalar action due to the plant compounds identified in the samples (PCAs, flavones, total polyphenols). The large variety of chemical compounds identified in the study by the UHPLC-HRMS/MS technique shows a much more appropriate evaluation of the antioxidant activity of plant extracts using at least 3 different determination methods for the comparative analysis to show accuracy. After evaluating the comparative analysis of the antioxidant capacity of the plant extracts used, different antiradicalar activities were obtained for each extract due to the varied types of active principles but also the high content of polyphenolic compounds and other phytochemical constituents in the composition. According to the results, the order of IC_{50} values was AE < RE < CHE < TE < CE. Thus, considering that a low IC_{50} value signifies a higher total antioxidant activity, the agrimony extract was identified as the most powerful antioxidant of all, followed by rosemary, chicory, dandelion, and artichoke. The inverse proportional relationship between the potency of the antioxidant effect and the IC_{50} value determined for each extract is also shown in Fig. 6.1., which highlights a minimum antioxidant activity for the artichoke extract (with a maximum IC_{50} value by all 3 methods) and a maximum antioxidant activity for the *Agrimoniae extractum*.

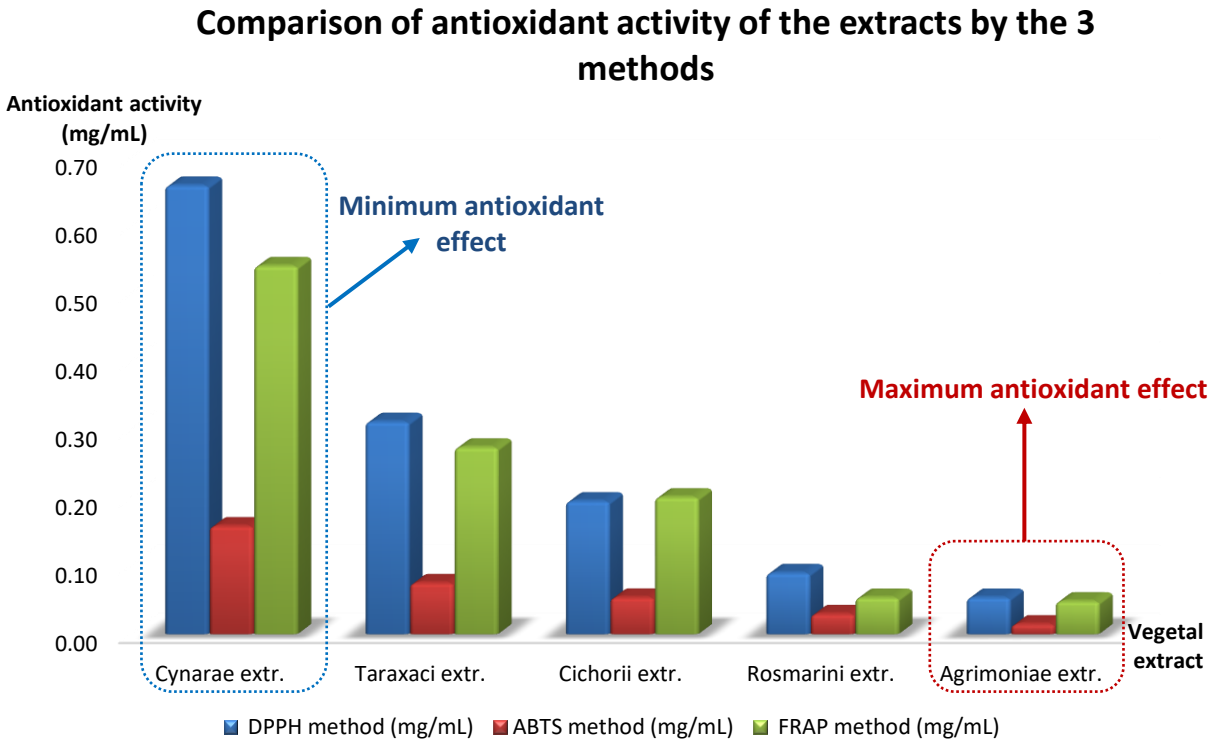


Fig. 6.1. The potency of the antiradicalar effect according to the IC_{50} value

After calculating the Pearson coefficient and the coefficient of determination, the very strong correlation existing not only between different antioxidant methodologies, but also between the active principles and the total antioxidant activity of the extracts was demonstrated. The antioxidant activity of the analyzed extracts, associated with a high content of polyphenolic compounds, can explain their hepatoprotective potential, since the link between hepatotoxicity and oxidative stress is especially the production of reactive oxygen species, which can damage the integrity and functionality of hepatocytes and contribute to the pathogenesis of many liver diseases.

7. Evaluation of cytotoxicity of extracts and mixture of extracts by alternative methods

The mixture of dry extracts was made, taking into account the pharmacognostic profile of each individual plant extract, the results obtained following qualitative and quantitative determinations by different methods, the antioxidant capacity that they can exert either by preventing lipid peroxidation of the hepatocyte membrane and removing stress oxidative, either by stimulating the antioxidant activity of endogenous antiradicals but also by the stability degree of the extracts (variation of the active principles concentration over time, the extraction solvent used, the consistency of the extract). The aim was to obtain a phytopreparation with an optimally improved phytotherapeutic profile, which presents considerable concentrations of active principles (table VII.1.) relevant from a clinical point of view but which does not have a major impact on the enzyme systems at the liver level, but to offer a balancing of port-hepatic processes, so as to be in

the area of therapeutic effectiveness without registering side effects and without attacking the liver cell or altering the redox balance in favor of pro-oxidants.

Table VII. 1. Dosage of the active principles from the mixture of extracts

MIXTURE	Concentration of active principles [g active principle/100 g dry extract]		
	PCAs	Flavones	Total Polyphenols
	8.5016 ± 0.3444	3.2961 ± 0.3045	13.9088 ± 0.8186

* Results are expressed as mean ± SD (n = 5).

Regarding the concentration of active principles, the mixture of dry extracts recorded average optimal values for all three categories of active chemical constituents, which are relevant from the phytopharmacological point of view and justify the therapeutic properties, as well as its possible use as an adjuvant in various liver diseases, aiming at prevention or clinical improvement. The mixture of extracts was subsequently tested *in vitro* and *in vivo* to determine the cytotoxicity but also to evaluate the therapeutic efficacy compared to that of the single extracts in order to quantify the safety profile and the influence on the essential biochemical parameters in rat. Following these determinations, the mixture of extracts can then be subjected to the procedures for inclusion in a pharmaceutical form intended for human administration if it meets the appropriate requirements for therapeutic use.

In vivo tests on animal models are frequently used, but for reasons of professional ethics, these determinations have started to be very limited, looking for efficient alternative methods and advantageous test models. Among the most effective and easy methods, frequently used today to evaluate the cytotoxicity of plant extracts, is the BSLA Test (Brine Shrimp Lethality Assay), considered a preliminary *in vivo* test, which is performed on larvae of *Artemia salina*, a crustacean species, used in toxicology, nanotoxicology [5], testing plant extracts, [6-9], etc. It was observed that, according to the Clarkson criteria, all the tested extracts are non-toxic (table VII.2.). The lethal effects recorded in 50% of the exposed organisms appeared only at high concentrations, above 1000 µg/mL (the concentration of 1200 µg/mL was considered the benchmark).

Table VII.2. Centralization of results from the testing of extracts on *Artemia salina*

Sample	Vegetal extract	Conc. lethal effects (µg/mL)	Mortality (%)	Mortality (%) at conc. 1200 µg/mL	Cytotoxicity classification
1	*Rosmarini extr.*	ND	0	0	non-toxic
2	*Agrimoniae extr.*	ND	0	0	non-toxic
3	*Cichorii extr.*	3732	50	0	non-toxic
4	*Cynarae extr.*	2990	50	0	non-toxic
5	*Mixture extr.*	800	2	7	non-toxic
6	*Taraxaci extr.*	800	7	7	non-toxic

ND: not detected.

The cytotoxicity of the analyzed extracts was determined by following their influence, at different concentrations carefully selected in an appropriate interval, on the development or behavior of the organisms of the genus *Daphnia sp.* The toxicity of the extracts on the invertebrate *Daphnia magna* suggests potential antiviral and antimicrobial actions, with possible clinical utility in liver diseases such as viral hepatitis. The extracts of rosemary and agrimony, which showed the highest cytotoxicity on *D. magna*, show antiviral and antibacterial properties known in the scientific literature [10,11–13]. Thus, the study to evaluate the cytotoxicity of the extracts on the *Daphnia model* led to the obtaining of results that are in close correlation with their antioxidant activity and with the concentration of determined active principles. Analyzing the LC50 values after 24 hours, it was observed that the artichoke and dandelion extracts were found to be non-toxic, the chicory extract showed low toxicity, the agrimony extract exerted medium toxicity, and the rosemary extract was recorded as being very toxic to *Daphnia magna*, with a maximum biological effect.

The effect of the analyzed extracts on HepG2 tumor cells was evaluated, and the results were expressed as cell viability as a percentage value. The viability of the control group was 100%, and the viability of the samples was calculated taking into account the absorbance values for the extract-treated group and the control group [14,15].

In Fig. 7.2. the effect of agrimony extract on HepG2 tumor cells is graphically represented. A significant reduction in tumor cell viability was observed at the sample concentration of 50 μg/mL (viability 84.1% ± 6.3% compared to the control group). At higher concentrations, a significant dose-dependent decrease in HepG2 viability was obtained (at 300 μg/mL cell viability was 44.3% ± 3.3% compared to the control group).

Figure 7.3. illustrates the effect of rosemary extract on HepG2 hepatocellular carcinoma cell line. A statistically significant decrease in tumor cell viability was obtained at all concentrations used, the most important reduction in cell viability being observed at the highest dose tested, 300 μg/mL (tumor cell viability was 7.2% ± 1.7% *vs.* control group).

The effect of inhibiting the viability of cancer cells exerted by the chicory extract is represented in Fig. 7.4. As can be seen in the graph, at concentrations starting at 25 μg/mL, the extract significantly reduces tumor cell viability, and at the maximum concentration of 300 μg/mL, the highest reduction in HepG2 cell viability was obtained (cell viability 59.3% ± 5.2% *vs.* control).

In Fig. 7.5. the effect of artichoke extract on HepG2 hepatocellular carcinoma cells is represented. The extract produced a decrease in tumor cell viability starting from the concentration of 25 μg/mL (viability 86.7% ± 5.6% compared to control). At the highest dose tested (300 μg/mL), tumor cell viability was 81.2% ± 9.5% compared to control.

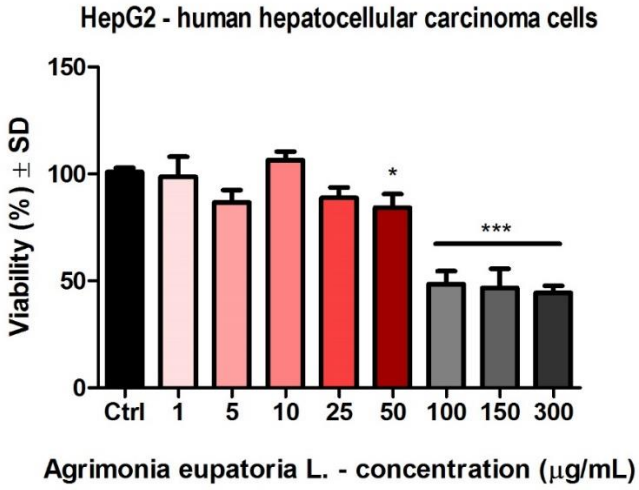


Fig. 7.2. The effect of agrimony extract (*Agrimoniae extractum*) on the viability of human hepatocellular carcinoma cells (* $p < 0.05$; *** $p < 0.001$);
Ctrl: control group

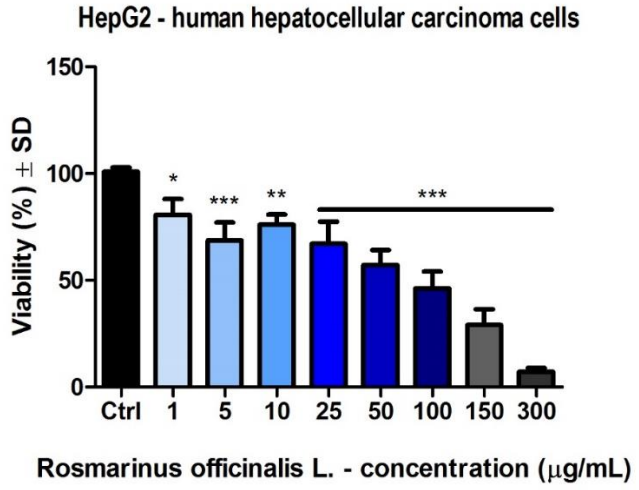


Fig. 7.3. The effect of rosemary extract (*Rosmarini extractum*) on the viability of human hepatocellular carcinoma cells (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$);
Ctrl: control group

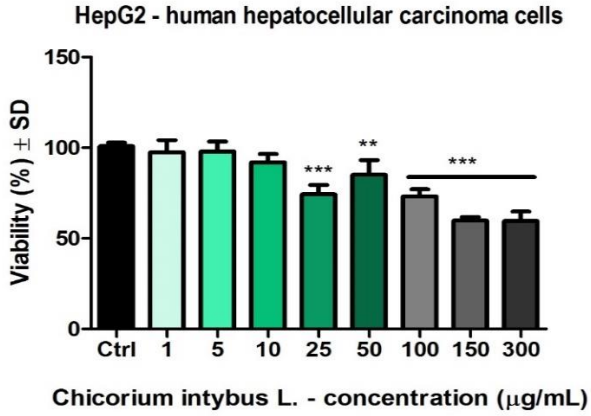


Fig. 7.4. The effect of chicory extract (*Cichorii extractum*) on the viability of human hepatocellular carcinoma cells (** $p < 0.01$; *** $p < 0.001$);
Ctrl: control group

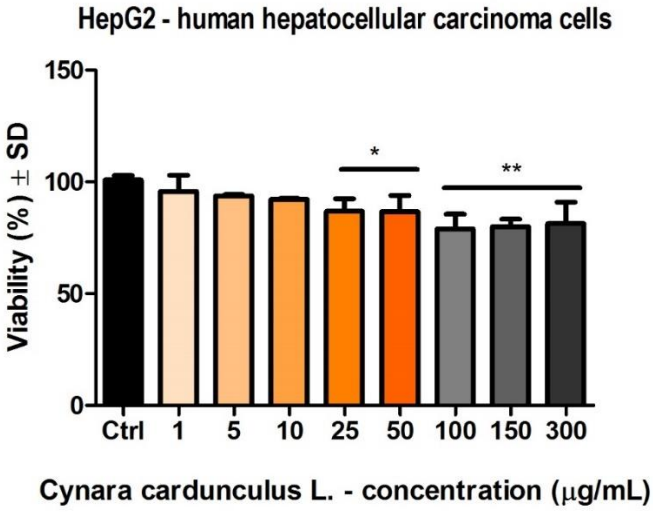


Fig. 7.5. The effect of artichoke extract (*Cynarae extractum*) on the viability of human hepatocellular carcinoma cells (* $p < 0.05$; ** $p < 0.01$);
Ctrl: control group

After incubation of HepG2 cells with dandelion samples, a significant dose-dependent decrease in HepG2 viability was observed starting from a concentration of 10 μg/mL (Fig. 7.6.). The most significant reduction in cell viability was reported at a concentration of 300 μg/mL (viability 77.6% ± 6.6% compared to control).

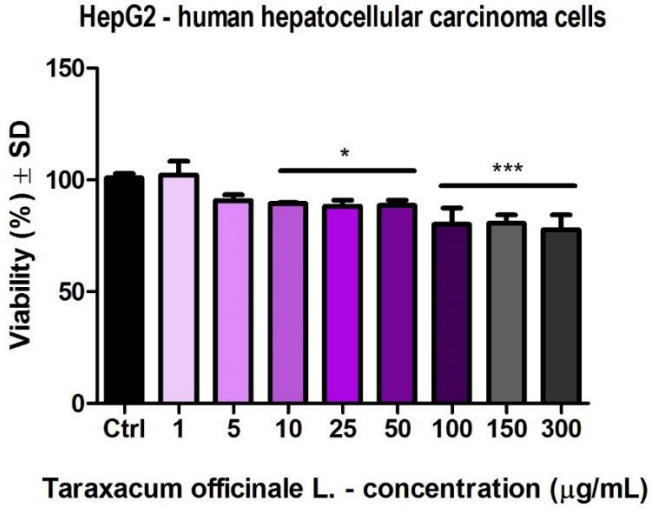


Fig. 7.6. The effect of dandelion extract (*Taraxaci extractum*) on the viability of human hepatocellular carcinoma cells (* $p < 0.05$; *** $p < 0.001$);
Ctrl: control group

The cytotoxic effect induced by the mixture of extracts on cancer cell lines is outlined in Fig. 7.7. The mixture was tested at the same concentrations as each individual plant extract. After applying the MTT method, a slight decrease in the viability of tumor cells was observed in a dose-dependent manner, the most significant effect being obtained at the concentration of 300 μg/mL (viability 78.5% ± 4.8% compared to the control group).

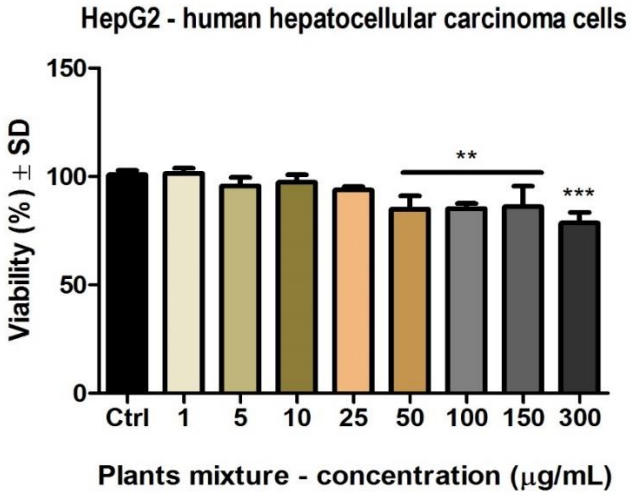


Fig. 7.7. The effect of the mixture of extracts on the viability of human hepatocellular carcinoma cells (** $p < 0.01$; *** $p < 0.001$);
Ctrl: control group

The IC_{50} values (µg/mL) for the extracts tested on human HepG2 cell cultures are shown in Table VII.3.

Table VII.3. IC_{50} values – *In vitro* toxicity test on cell lines

Agrimoniae extr. **IC_{50}**	***Rosmarini extr.*** **IC_{50}**	***Cichorii extr.*** **IC_{50}**	***Cynarae extr.*** **IC_{50}**	***Taraxaci extr.*** **IC_{50}**	***Mixture extr.*** **IC_{50}**
107.6 µg/mL	53.58 µg/mL	ND	ND	ND	ND

ND: not detected.

IC_{50} values could not be calculated for the extracts of chicory, artichoke, dandelion, and the mixture of extracts because they showed high viability throughout the analysis (between 59 – 100% viability for all concentrations and between 59 – 81% for the maximum tested concentration), the lethality percentage being extremely low for the tested concentrations (L% ≤ 40%). The results obtained from the analysis of the extracts on human cell cultures HepG2 are presented in Table VII.4.

Table VII.4. Overview of HepG2 cell viability assay results

Vegetal extract	**Maximum tested concentration (C_{max} - µg/mL)**	**Viability at C_{max} (%)**	**Lethality (L%)**
Agrimoniae extractum	300 µg/mL	44.3%	55.7%
Rosmarini extractum	300 µg/mL	7.2%	92.8%
Cichorii extractum	300 µg/mL	59.3%	40.7%
Cynarae extractum	300 µg/mL	81.2%	18.8%
Taraxaci extractum	300 µg/mL	77.6%	22.4%
Mixture of extracts	300 µg/mL	78.5%	21.5%

According to the cytotoxicity criteria, a cytotoxicity test on human hepatocellular carcinoma cell line HepG2 tumor cells revealed a marked cellular toxic effect for rosemary extract (IC_{50} = 53.58 μg/mL) and a medium toxicity for agrimony extract (IC_{50} = 107.6 g/mL). The results were also confirmed by the cell viability, which decreased significantly as the tested dose for these extracts was higher. The rest of the analyzed extracts (*Cichorii extractum*, *Cynarae extractum*, *Taraxaci extractum*, and the *Mixture of extracts)* showed a greatly reduced toxicity on tumor cells, indicating that they are not potent cytotoxic on HepG2 cells. Among them, artichoke extract and the mixture of extracts induced the lowest cell death (18.8% and 21.5%, respectively) and decreased viability in HepG2 cells in a predictable, stable, and dose-dependent manner, but the other extracts analyzed had a stronger effect, especially at the highest doses tested. Compared to the *in vivo* cytotoxicity studies performed on invertebrates (*Daphnia sp.* and *Artemia sp.*), the toxicity degrees of the extracts on the HepG2 tumor cell lines showed a very good correlation, the extracts of chicory, artichoke, dandelion, and the mixture being included as non-toxic extracts in all these methods. The differences in cytotoxicity that occur in the case of rosemary and agrimony extracts (non-toxic on *Artemia sp*.; highly toxic/with medium toxicity on *Daphnia sp.* and HepG2 cells) can be explained by the complexity of the chemical composition and the enhanced antioxidant profile of these extracts, which can trigger different biological behaviors and mechanisms of action at the cellular and metabolic level, depending not only on the characteristics of the exposed individual, but also on the distinct biochemical conditions in the cytoplasmic environment. The possible substrate specificity of *Rosmarini extractum* and *Agrimoniae extractum* can be highlighted as a result of the selective cytotoxic effect observed in the two toxicity studies (HepG2, *Artemia sp.*), proving direct cytotoxicity on HepG2 tumor cells but showing innocuousness towards the invertebrate model *Artemia salina*.

8. Biochemical and histological research

In vivo testing on laboratory animals for vegetal extracts is one of the experimental methods that provides the most important results, with an essential role in the preclinical path of research. After the application of alternative methods *in vivo* on invertebrates and *in vitro* on human tumor cell lines, in order to fully characterize the phytopharmacological profile of the extracts taken in the study, the determination of the specific blood biochemical parameters for the evaluation of liver function, after the exposure of the experimental animals, the biochemical analysis of the tissue homogenates, as well as the histological examination of the liver fragments taken from each individual animal, in addition to the evaluation of liver function by determining serum transaminases, the research aimed to determine blood glucose, total cholesterol, serum triglycerides, HDL-cholesterol, serum cholinesterase, and total proteins. The groups used in the test were: Group I - Normal Control Group: NaCl 0.9% in a dose of 10 mL/kg p.o. – 10 rats; Group

II - Intoxicated Control Group: CCl_4 in a dose of 0.2 mL/kg p.o. – 10 rats; Group III - Normal Reference Group: Silymarin 35 mg in a dose of 10 mL/kg p.o. – 10 rats; Group IV - Intoxicated Reference Group: Silymarin + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group V: *Cynarae extractum* (artichoke extract) + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group VI: *Rosmarini extractum* (rosemary extract) + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group VII: *Cichorii extractum* (chicory extract) + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group VIII: *Taraxaci extractum* (dandelion extract) + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group IX: *Agrimoniae extractum* (agrimony extract) + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats; Group X - Mixture Group: *Mixture of extracts* + CCl_4 (10 mL/kg p.o. + 0.2 mL/kg p.o. after one hour) – 10 rats.

The evolution of biochemical constants in acute and chronic intoxication was followed (fig. 8.1. – 8.3.).

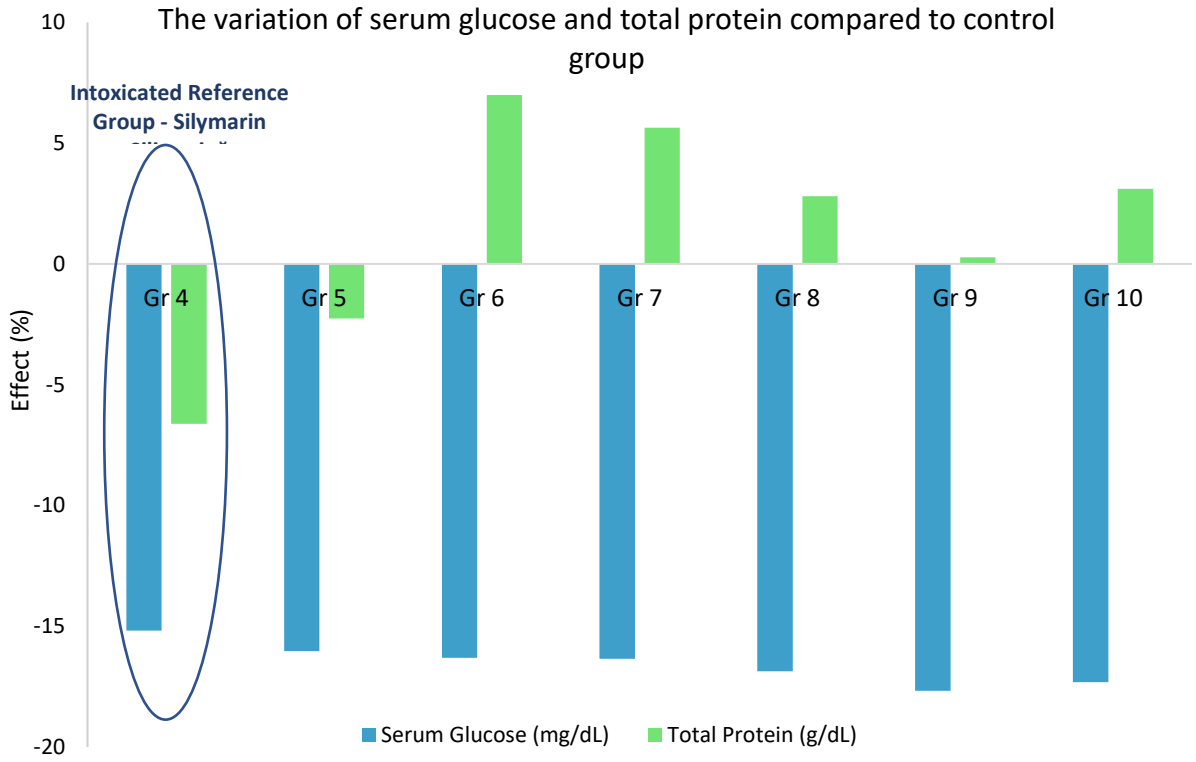


Fig. 8.1. Influence of extracts and mixture of extracts on blood glucose and total protein in rat

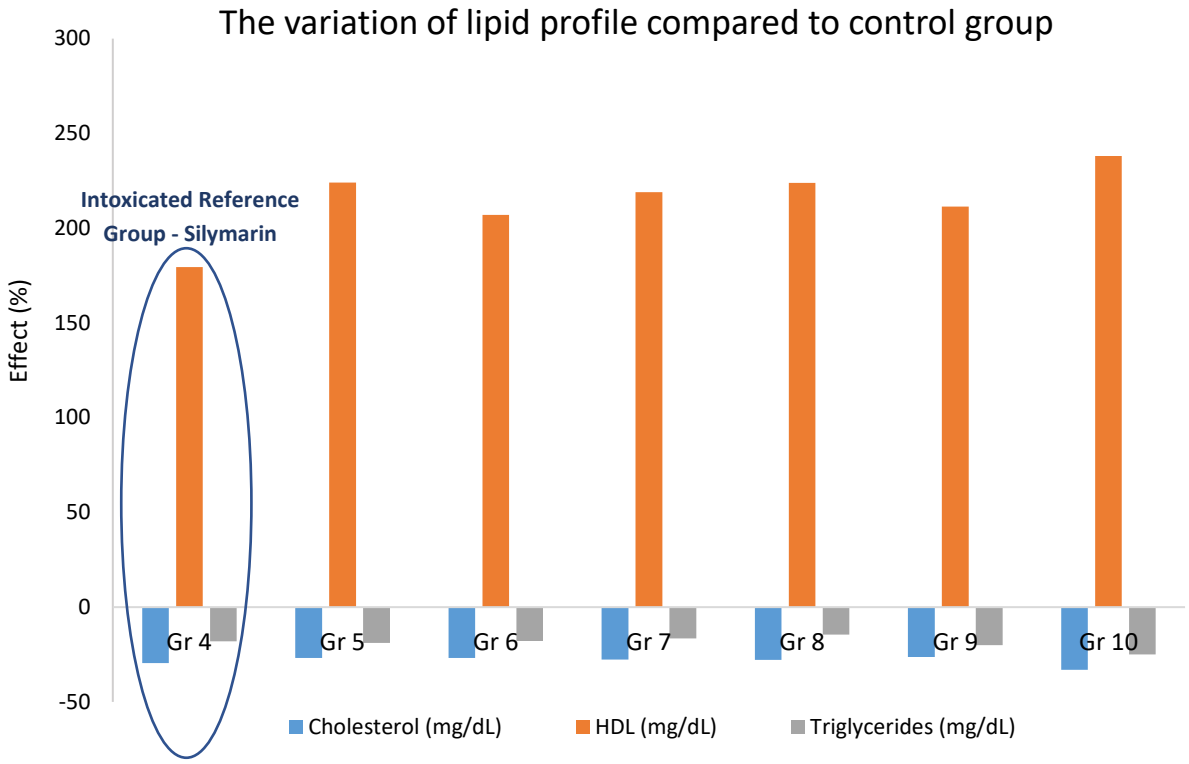


Fig. 8.2. Influence of extracts and mixture of extracts on lipid profile in rat

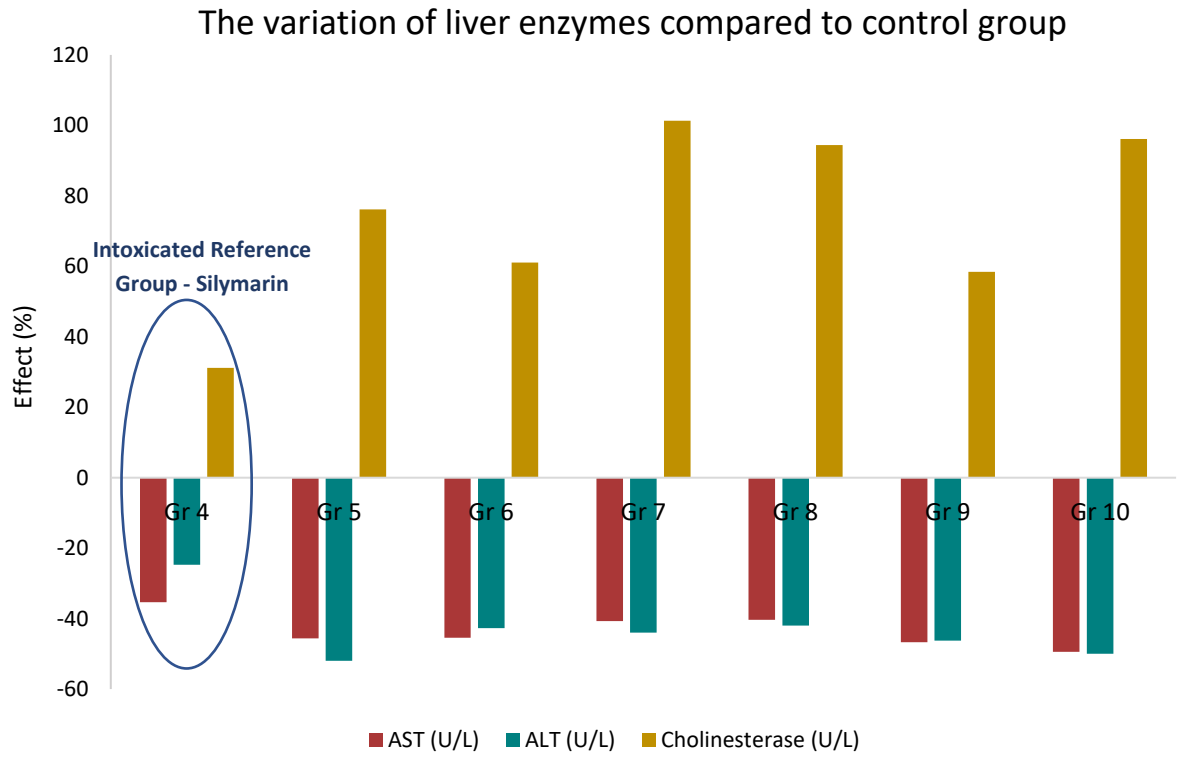
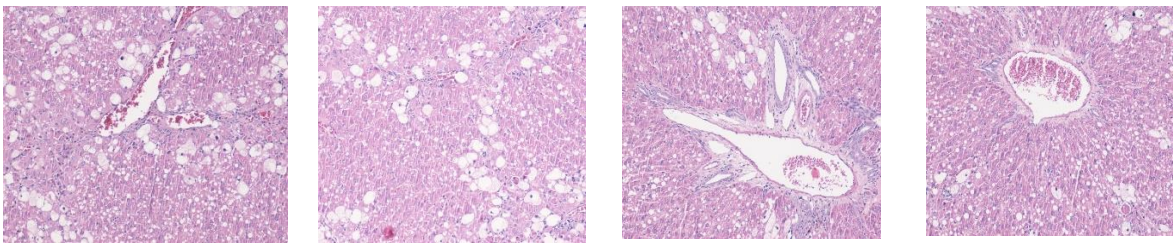


Fig. 8.3. Influence of extracts and mixture of extracts on liver enzymes in rat

The evaluated biochemical constants recorded improved or even normalized values with the attenuation of the toxicity induced by CCl_4, for the groups treated with the mixture of extracts or with the individual extracts, all extracts showing a positive influence on the mechanisms of defense

and hepatocellular regeneration. The serum glucose value, increased by the toxic agent, was reduced by the agrimony extract and the mixture of extracts, and the serum proteins were normalized by the extracts of rosemary, chicory, and the mixture of extracts. The mixture of extracts showed a remarkable ability to balance the lipid profile. The activity of serum transaminases was significantly decreased by the mixture of extracts and the artichoke extract, and the serum cholinesterase level was positively influenced by the extract of chicory and the mixture of extracts. From a histological point of view, we exemplify only the behavior of the liver cell under the action of the mixture of extracts and carbon tetrachloride: it did not show areas of cell necrosis or hepatocytes in apoptosis, unlike the other groups treated with the individual extracts. We could identify areas with vacuolar degeneration and branched cords of fibrosis that seem to be concentrated at the periphery of the lobule (Fig. 8.4.a and b). As a defense response to the aggression imprinted by the toxic agent, the pre-treatment with the mixture of extracts was able to provide protection by adapting the cell structures to the toxic conditions and thickening the cell walls in order to limit the hepatocytic destruction. Thus, it was observed that some portal spaces kept their normal architecture with the triad of portal vein, hepatic arteriole, and bile duct, but with a wall thicker than normal (Fig. 8.4.c.). Areas of microvacuolar degeneration were also noticed, mainly vessels with thickened walls, eosinophils and perivascular fibrin deposits (Fig. 8.4.d.).



a **b** **c** **d**

Fig. 8.4. Histological evaluation of liver samples for Group X (*Mixture of extracts* + CCl_4) 10x ob. (**a, b, c, d**) (original photos)

The examination of hepatocytic histopathology and liver cytology confirmed the hepatoprotective effects of the mixture of extracts by preserving, in a very high percentage, the normal liver architecture (including in the portal spaces) and by the absence of the phenomena of tissue necrosis or cell apoptosis.

Research conducted on liver homogenates revealed statistically significant differences when comparing tissue free radical scavenging capacities for both 7-day and 21-day exposure, for baseline assessment at 5 minutes ($p = 0.008$ and $p < 0.001$), 15 minutes ($p = 0.009$ and $p < 0.001$), and 30 minutes ($p = 0.009$ and $p < 0.001$). Catalase activity was similar between groups for both 7-day ($p = 0.756$) and 21-day ($p = 0.076$) treatment groups. Levels of total thiols were significantly different between groups at 7 days ($p = 0.002$), but not at 21 days ($p = 0.126$), as notable decreases were observed. Tissue redox insufficiency, determined by the FOX method, showed significant

differences between groups for both treatment periods: $p = 0.019$ (7 days) and $p = 0.033$ (21 days). Liver nitrite levels were significantly different between groups at 7 days ($p = 0.006$) and 21 days ($p < 0.001$). For total liver nitrites, no significant differences were found at 7 days ($p = 0.118$), but only at 21 days ($p = 0.024$).

9. Formulation, production, and pharmacotechnical control of plant extract chewable tablets

In order to formulate a pharmaceutical form with plant extracts rich in active principles with numerous benefits for the target categories of patients, the solid pharmaceutical form dosed for oral administration, as a chewable tablet, was selected, as it is a convenient alternative to conventional tablets.

According to all the favorable results obtained following the pharmacotechnical tests carried out, it is possible to appreciate the clear agreement between the properties of the obtained tablets and the quality requirements specified in the specialized literature.

The quality control parameters for the formulated chewable tablets fell within the limits and provisions of the specialized monographs, which justifies the optimal formulation selected, which will properly yield the active principles in the body.

The chosen formulation, through the many advantages of chewable tablets, can bring significant therapeutic benefits to patients in special categories such as children, the elderly, or people with increased sensitivity to swallowing.

The adjustment of the proposed pharmaceutical formula, so that it corresponds both to the formulation method and to the needs of patients, by adding different excipients (binders, lubricants, glidants, sweeteners, or flavorings), leads to the preparation of a final pharmaceutical product which can meet medical and pharmaceutical requirements, regardless of professional perspective (doctor, pharmacist, or manufacturer).

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

General Conclusions

Five vegetal products were included in the study: *Cynarae folium* (artichoke leaves), *Rosmarini folium* (rosemary leaves), *Agrimoniae herba* (agrimony aerial parts), *Taraxaci herba* (dandelion aerial parts) and *Cichorii herba* (chicory aerial parts), all presenting in the scientific literature data related to the beneficial actions on the hepato-biliary system, with the exception of agrimony, for which much less evidence was found about the hepatoprotective-hepatoregenerative effect, compared to the other products.

❖The extraction of active principles (phenolcarboxylic acids, flavones and total polyphenols) was evaluated in different concentrations of ethanol (20°, 50°, 70°), obtaining the highest concentrations of active principles in 20° ethanol for dandelion and in ethanol 50° for artichokes, rosemary, agrimony and chicory. Of all the hydroethanolic solutions analyzed, the extractive solutions of *Agrimoniae herba* and *Rosmarini folium* detected the highest amounts of flavones (1.17 g rutoside/100 g dry vegetal product for agrimony and 1.27 g rutoside/100 g dry vegetal product for rosemary), PCAs (6.55 g chlorogenic acid/100 g dry vegetal product for agrimony and 5.67 g chlorogenic acid/100 g dry vegetable product for rosemary) and total polyphenols (8.56 g tannic acid/100 g dry vegetal product for agrimony and 8.82 g tannic acid/100 g dry vegetal product for rosemary).

❖ After obtaining the dry extracts by the lyophilization method, using the appropriate extraction solvent, quantitative determinations of the compounds with possible action on the liver (PCAs, flavones, total polyphenols) were performed, and it was found that the richest extracts were also accessible for rosemary and agrimony, and for chicory, dandelion, and artichoke, the concentration values showed uniform and therapeutically relevant increases compared to the results from the extractive solutions. The stability study of dry plant extracts standardized in active principles, carried out over a one-year monitoring period, which involved quantitative determinations of PCAs, flavones, and total polyphenols at 3 months, 6 months, and 12 months after obtaining, highlighted the stability of flavones and total polyphenols, the therapeutic level remaining at optimal values throughout the 12 months for all the analyzed extracts. The only statistically significant difference was obtained for the content of PCAs in the rosemary extract, but this behavior was predictable considering the instability and lability of these compounds. On the other hand, for the other dry extracts, the evolution of PCAs over time registered small and insignificant variations, despite the influence of numerous conservation factors. Ultra-performance chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS/MS) analysis of polyphenolic compounds in dry plant extracts allowed their qualitative and quantitative quantification with high precision. Thus, 28 compounds were identified in *Cynarae extractum* (CE), 48 compounds in *Rosmarini extractum* (RE), 39 compounds in *Taraxaci extractum* (TE), 43 compounds in *Cichorii extractum* (CHE) and in *Agrimoniae extractum* (AE) – 31 compounds. From a quantitative point of view, after applying the UHPLC-HRMS/MS analysis, 13 compounds were quantified in CE, in RE – 17 compounds, in TE – 18 compounds, in CHE – 19 compounds, and in AE – 19 compounds, including rutoside, genistin, caffeic acid, chlorogenic acid, ellagic acid, and azelaic acid. Depending on the variables in the chromatographic system, the cluster analysis was performed by using algorithms to classify the constituents of the plant extracts in order to group them into homogeneous classes. The comparative multivariate examination of the

clusters demonstrated a general distribution in a variable number of clusters for all extracts, with the results obtained being in direct correlation with the compounds identified by the UHPLC-HRMS/MS method. Practically, each cluster obtained through the hierarchical grouping system can be considered a transparent "mirror" of the polyphenolic profile of the extracts, in which the compounds present an organized and simplified arrangement, depending on the variation of similarity between them, which is a much more suggestive graphic expression.

❖ The molecular docking studies had the role of predicting the possible molecular mechanism of action of the extracts by designing binding affinities to biological targets with an essential role in the dynamics of enzymatic and metabolic processes in which the liver is the main seat. *In silico* simulations evaluated the polyphenolic compounds determined in the extracts as potential inhibitors of cytochrome P450 isoform 2E1 (CYP2E1) and tumor necrosis factor alpha (TNF-α), but also as allosteric activators of glutathione-peroxidase 4 (GPx4), having a particularly important role in the allosteric regulation of many enzymes or biological structures essential for the proper functioning of the hepato-biliary tree. An *in silico* analogy of the behavior of phytoconstituents from plant sources in the body was generated and conformational changes, actions or interactions at the cellular and tissue level could be predicted. Following *in silico* evaluations, the hepatoprotective effect of polyphenolic compounds through their interaction with CYP2E1, TNF-α, and GPx4, and the reduction of oxidative stress, the annihilation of hepatocellular toxicity, the attenuation of fibrogenesis, as well as the regulation of key enzymes with an antioxidant and anti-inflammatory role were possible beneficial therapeutic actions of the extracts used, as these effects were outlined and confirmed with the help of computational molecular modeling techniques with an essential role in the rational design of phytomedicines.

❖ The antioxidant action of the plant extracts evaluated in the acellular system, through the 3 methods DPPH, ABTS, and FRAP, demonstrated the antioxidant effects induced by the plant extracts in direct correlation ($p < 0.05$) with the concentration of secondary metabolites, the most powerful antioxidant of all being *Agrimoniae extractum*, followed by *Rosmarini extractum*, *Cichorii extractum*, *Taraxaci extractum*, and *Cynarae extractum*.

❖ The mixture of plant extracts was formulated by establishing the optimal ratio between the individual dry extracts, taking into account the pharmacognostic profile of each extract, the results obtained from qualitative and quantitative determinations, but also the antioxidant action exerted *in vitro*. After dosing the active principles from the mixture of extracts, average optimal values were obtained for all 3 categories of chemically active constituents, the mixture being subsequently subjected to *in vitro* and *in vivo* testing together with the individual extracts.

❖ The evaluation of the cytotoxicity of the extracts and the mixture of extracts on alternative models highlighted, on the one hand, the safety profile and, on the other hand, also outlined some

substrate specificity of the analyzed extracts. After *in vivo* cytotoxicity testing on the *Artemia salina* model, it was found that all tested extracts are non-toxic according to Clarkson's criteria and do not influence the processes of cell division, growth, or differentiation of the larvae, the lethal effects appearing at high concentrations, over 1000 μg/mL. *In vivo* cytotoxicity studies in the *Daphnia magna* model revealed no toxicity for artichoke and dandelion extracts, low toxicity for chicory extract, medium toxicity for agrimony, and high toxicity for rosemary extract, suggesting potential antiviral and antimicrobial actions of rosemary and agrimony extracts, with possible clinical utility in liver diseases such as viral hepatitis. The cytotoxicity of the extracts was also determined *in vitro* on some liver cancer cell lines (HepG2), revealing a marked cellular toxic effect for the rosemary extract (IC_{50} = 53.58 μg/mL) and a medium toxicity for the agrimony extract (IC_{50} = 107.6 μg/mL). The remaining extracts exhibited much lower toxicity on tumor cells, indicating that they are not potent cytotoxic on HepG2 cells. In addition, a possible substrate specificity of *Rosmarini extractum* and *Agrimoniae extractum* was highlighted as a result of the selective cytotoxic effect observed in the two toxicity studies (HepG2, *Artemia sp.*), proving direct cytotoxicity on HepG2 tumor cells but showing innocuousness towards the invertebrate *Artemia salina*.

❖ *In vivo* research on laboratory animals led to the confirmation of the hepatoprotective action shown by plant extracts in rats intoxicated with CCl_4 for 7 days and 21 days, the protection evaluated being superior to the reference used in the study (silymarin). The evaluated biochemical constants (glycemia, serum proteins, lipid profile, liver enzymes) recorded improved or even normalized values with the improvement of toxicity induced by CCl_4, for the groups treated with the mixture of extracts or with the individual extracts, all extracts showing a positive influence on the mechanisms of defense and hepatocellular regeneration. The highest survival rate among animals was recorded for the group treated with the mixture of extracts. The macroscopic anatomical-pathological examination identified major tissue changes in all individuals intoxicated with CCl_4, the appearance of the organ being different from that of non-intoxicated controls. Compared to the intoxicated control, the livers taken from the rats treated with plant extracts show a healthier appearance and an improved red color, but the fatty infiltration of the hepatocytes remains detectable. In chronic intoxication, it was possible to detect the appearance of nodules of liver regeneration at the level of initial regions of cytolysis for all groups treated with the plant extracts. The histopathological analysis confirmed the hepatoprotective effects of the mixture of extracts by preserving, in a very high percentage, the normal liver architecture (including in the portal spaces), by annihilating cellular hypoxia and by the absence of the phenomena of tissue necrosis or cellular apoptosis, unlike the other samples. Regarding the effect of the extracts on the redox status in tissue homogenates and rat mitochondrial suspensions, it was discovered that both

individual extracts, but especially the mixture of extracts, cause the amplification of the protective response of the endogenous antioxidant systems, keeping total antioxidant levels within normal limits for the groups pretreated with the plant extracts or mixture of extracts, and escaping from antioxidant surveillance for the group treated with the toxic agent alone.

❖ The technical-pharmaceutical formulation of the chewable tablets, which contain the mixture of extracts as an active principle, embodied the therapeutic actions proven following research in a pharmaceutical form with numerous benefits for different categories of patients, meeting the quality requirements specified in the monographs in force and ensuring the corresponding yield in the body.

Degree of Originality

The original elements of the thesis are: the combination of selected plant products and the proposed percentage of each dry plant extract in order to formulate the final phytopreparation; the chewable solid pharmaceutical form intended for oral administration to obtain the hepatoprotective-hepatoregenerative, antioxidant and antihepatotoxic effect, as a more advantageous alternative to conventional tablets; the evaluation of the chemical composition and the behavior of the chemical constituents by various methods (pharmacognostic analysis, UHPLC-HRMS/MS method, *in vitro* antioxidant methods, the study of stability over time) for the component extracts of the phytomedicine, in order to standardize and quantify the preparation from phytochemical and pharmacokinetic perspectives; evaluating the phytopharmacological and toxicological impact of the newly developed phytomedicine on the animal cell by performing *in vivo* studies on invertebrates and laboratory animals, *in vitro* on HepG2 human carcinoma cell lines, *in silico* by molecular docking and biochemical modeling on the subcellular redox status in homogenates rat tissue and mitochondrial preparations.

Research Perspectives

In the future, we propose to bring the research from the preclinical stage to the clinical stage by carrying out a clinical study on healthy volunteers, prone to liver damage, but also on patients with established liver diseases in various stages, in order to quantify the therapeutic action in humans, the degree of improvement in the quality of life or the level of protection offered by the phytomedicine, taking into account the variability of the response in the presence of inter-individual factors, risk factors or environmental factors.

SELECTIVE BIBLIOGRAPHY

1. **Costea, L** .; Ghica, M.; Costea, T.; Gîrd, CE Spectrophotometric evaluation of flavonoids, phenolcarboxylic acids and total phenolic contents of several indigenous herbal products with potential hepatoprotective effect. *Pharmacy* 2021, *69*, 1176–1181. 10.31925/farmacia.2021.6.23.
2. Miłek, M.; Marcinčáková, D.; Legáth, J. Polyphenols Content, Antioxidant Activity, and Cytotoxicity Assessment of Taraxacum officinale Extracts Prepared through the Micelle-Mediated Extraction Method. *Molecules* 2019, *24*, 1025. 10.3390/molecules24061025.
3. Kenny, O.; J Smyth, T.; M Hewage, C.; P Brunton, N. Antioxidant properties and quantitative UPLC-MS/MS analysis of phenolic compounds in dandelion (Taraxacum officinale) root extracts. *Free Radicals and Antioxidants* 2014, *4*, 55–61. 10.5530/fra.2014.1.9.
4. **Costea, L** .; Chițescu, CL; Boscencu, R.; Ghica, M.; Lupuliasa, D.; Mihai, DP; Deculescu-Ioniță, T.; Go, LE; Popescu, ML; Luță, E.-A.; et al. The Polyphenolic Profile and Antioxidant Activity of Five Vegetal Extracts with Hepatoprotective Potential. *Plants* 2022, *11*, 1680. 10.3390/plants11131680.
5. Johari, SA; Rasmussen, K.; Gulumian, M.; Ghazi-Khansari, M.; Tetarazako, N.; Kashiwada, S.; Asghari, S.; Park, J.-W.; Yu, IJ Introducing a new standardized nanomaterial environmental toxicity screening testing procedure, ISO/TS 20787: aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using *Artemia sp* . nauplii. *Toxicol Mech Methods* 2019, *29*, 95–109. 10.1080/15376516.2018.1512695.
6. Braguini, WL; Alves, BB; Pires, NV Toxicity assessment of *Lavandula officinalis* extracts in Brine Shrimp (*Artemia salina*). *Toxicol Mech Methods* 2019, *29*, 411–420. 10.1080/15376516.2019.1567892.
7. Coe, FG; Parikh, DM; Johnson, CA; Anderson, GJ The good and the bad: Alkaloid screening and brineshrimp bioassays of aqueous extracts of 31 medicinal plants of eastern Nicaragua. *Pharm Biol* 2012, *50*, 384–392. 10.3109/13880209.2011.608077.
8. Hong, LS; Ibrahim, D.; Kassim, J. Assessment of in vivo and in vitro cytotoxic activity of hydrolysable tannin extracted from Rhizophora apiculata barks. *World J Microbiol Biotechnol* 2011, *27*, 2737–2740. 10.1007/s11274-011-0727-1.
9. Montanher Pimentel, AB; Pizzolatti, MG; Brighente Costa, IM An Application of the Brine Shrimp Bioassay for General Screening of Brazilian Medicinal Plants. *Acta Farm. Bonarense* 2002, *21*, 175–178.
10. Kwon, DH; Kwon, HY; Kim, HJ; Chang, EJ; Kim, MB; Yoon, SK; Song, EY; Yoon, DY; Lee, YH; Choi, IS; et al. Inhibition of hepatitis B virus by an aqueous extract of Agrimonia eupatoria L. *Phytotherapy Research* 2005, *19*, 355–358. 10.1002/for 1689.
11. Mancini, DAP; Torres, RP; Pinto, JR; Mancini-Filho, J. Inhibition of DNA virus: Herpes-1 (HSV-1) in cellular culture replication, through an antioxidant treatment extracted from rosemary spice. *Brazilian Journal of Pharmaceutical Sciences* 2009, *45*, 127–133. 10.1590/S1984-82502009000100016.
12. Nasr-Eldin, M.; Abdelhamid, A.; Baraka, D. Antibiofilm and Antiviral Potential of Leaf Extracts from Moringa oleifera and Rosemary (Rosmarinus officinalis Lam.). *Egypt J Microbiol* 2018, *52*, 129–139. 10.21608/ejm.2017.1439.1027.
13. Garber, A.; Barnard, L.; Pickrell, C. Review of whole plant extracts with activity against Herpes Simplex Viruses in vitro and in vivo. *J Evid Based Integr Med* 2021, *26*, 1–57. 10.1177/2515690X20978394.
14. Lin, X.; Bai, D.; Wei, Z.; Zhang, Y.; Huang, Y.; Deng, H.; Huang, X. Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. *PLoS One* 2019, *14*. 10.1371/journal.pone.0216711.
15. Suganya, K.; Poornima, A.; Sumathi, S.; Chigurupati, S.; Alyamani, NM; Ghazi Felemban, S.; Bhatia, S.; Al-Harrasi, A.; Sayed Moawad, A. Rutin induces endoplasmic reticulum stress-associated apoptosis in human triple-negative breast carcinoma MDA-MB-231 cells – In vitro and in silico docking studies. *Arabian Journal of Chemistry* 2022, *15*, 104021. 10.1016/j.arabjc.2022.104021.