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Multicomponent nanosystems with applications in pathologies with cardio-cerebro-vascular risk

PhD THESIS ABSTRACT

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Keywords: polymeric nanosystems, lipidic nanosystems, liposomes, transfersomes, ethosomes, cardio-cerebro-vascular pathologies, amlodipine besylate, valsartan, S*ambucus ebulus*, plant extracts

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INTRODUCTION

The aim of the Phd thesis, entitled ***Multicomponent nanosystems with applications in pathologies with cardiovascular risk***, is to obtain and characterize nanosystems with polymeric and lipid matrix loaded with synthetic substances, respectively, a standardized plant extract for biomedical applications, with a special reference to their administration in the therapy of diseases with cardiovascular risk.

The originality of these studies can be revealed from several perspectives:

- obtaining polymeric nanosystems from PEG/PLGA in which a combination of two active substances from different pharmacodynamic classes was incorporated, but with complementary actions (amlodipine - calcium channel blocker, and valsartan - angiotensin II receptor antagonist), in a fixed dose of 1:16 (mg) - AML: VAL;
- obtaining three types of lipid nanosystems (liposomes, ethosomes, transferosomes) in which a standardized plant extract from the leaves of *Sambucus ebulus* was incorporated;
- addressing the qualitative relationship between the obtained nanosystems and normal cells of murine fibroblasts (NIH3T3, L-929), human fibroblasts (PCS-201-012) and breast epithelial cells (MCF-12A) and the effects they produce;
- investigating the behavior of polymeric nanosystems once inside the cell through a microscopic laser scanning technique;
- investigating the release profile of the active principles from obtained nanosystems using them later as controlled transport systems to increase patient compliance.

The objectives related to the general purpose of the paper pursued a series of aspects, such as:

1. The selection of a suitable polymer for biomedical applications (especially for the therapy of diseases with cardio-cerebrovascular risk): the polymeric material must be characterized by an increased biocompatibility and biodegradability; the products resulting from the process of polymer metabolism must be free of any toxicological potential; have good cytotoxicity in order not to be more easily eliminated from the body, eg. to lead to a good clearance of the active substance encapsulated in the polymeric material; the structural properties that confer the hydrophilicity/hydrophobicity of the polymer, as well as the degree of crosslinking of the polymeric material will determine the release of hydrophilic or hydrophobic therapeutic agents from these systems.
2. The selection of a standardized plant extract containing a large amount of total polyphenols, as strong antioxidant properties could beneficially influence the evolution of diseases with cardiovascular risk.
3. The selection of the optimal method for the preparation of polymeric and lipid nanosystems, resulting in particles as small as possible for intravenous administration, for example, but at the same time large enough not to be purged too easily from the blood system.
4. Obtaining polymeric and lipid nanosystems that have a high entrapment efficiency of the active ingredients, small particle size, uniform distribution and a moderate degree of stability, as well as a release profile of the active ingredients that will allow a subsequent administration in a controlled manner.
5. The development of working protocols for the evaluation of intracellular nanosystems to investigate their cytotoxic profile.

The Phd thesis is structured in two main parts, namely, *The current state of knowledge* (part I) and *Original contributions* (part II).

The bibliographic study comprises 3 chapters, summarizing the current state of research on the incidence, allopathic and natural treatment of diseases with cardio-cerebro-vascular

risk, the use of nanostructured systems in the delivery of active substances, as well as on the physico-chemical and in vitro methods most often used in this case.
The second part of the thesis, extended on 4 chapters, represents the personal contributions/researches.

Chapter 1 ***Global aspects regarding cardio-cerebrovascular pathology*** presents statistical data on the incidence of cardiovascular diseases in Romania and Europe, information on the current treatment based on active substances, as well as on plant extracts of cardio-cerebrovascular diseases. vascular.

Chapter 2 ***Global aspects regarding the development* of *nanotechnology*** discusses key notions about the evolution of nanotechnology and the current state of nanosystems loaded with active synthetic substances and plant extracts.

Chapter 3 ***Development of multicomponent nanosystems with cardiovascular substances*** discusses the obtaining of polymeric multicomponent nanosystems (pharmacodynamics and pharmacokinetics of active principles, nanosystem components, methods of production), obtaining lipid nanosystems (composition and potential potentem, and about the most common physico-chemical and in vitro methods used to characterize nanosystems that have biomedical applicability.

Part II of this doctoral thesis develops the general and specific objectives and reproduces the original contributions in 4 chapters, finalizing this study by the general conclusions that are presented in Chapter 8.

Chapter 4 ***Obtaining and characterization of polymeric multicomponent nanosystems loaded with amlodipine besylate and/or valsartan*** presents the materials and methods used in this study to obtain a series of polymeric nanosystems loaded with either a single active substance or a combination of two substances, evaluation of the entrapment efficiency, determination of the particle size, the polydispersity index and the zeta potential, and the study of the *in vitro* release of the active substances used in the obtained nanosystems. Following this study, the optimal nanosystem was selected, subsequently subjected to further investigations.

Chapter 5 ***Obtaining and characterization of a multicomponent nanosystem based on PLGA loaded with amlodipine besylate and valsartan*** discusses the characterization of the optimal polymeric nanosystem, regarding morphology, evaluation of intracellular distribution and cytotoxicity.

Chapter 6 ***Obtaining and characterization of standardized plant extracts from plant species: Sambucus ebulus, Lycium barbarum, Armoracia rusticana and Echinacea purpurea*** presents the materials and methods used to obtain standardized plant extracts, and their characterization in terms of polyphenol content, antioxidant activity and cytotoxic potential. At the end of this study, a plant species was selected, implicitly the extract that showed the highest amount of polyphenols, in order to be incorporated into lipid matrices.

Chapter 7 ***Obtaining and characterization of lipid nanosystems with standardized plant extract of Sambucus ebulus*** presents the materials and methods for obtaining lipid nanosystems with standardized plant extract from the leaves of Sambucus ebulus and their characterization in terms of entrapment efficiency, particle size determination, the polydispersity index and zeta potential, and the behavior of in vitro release of polyphenols from the obtained nanosystems was investigated, as well as the effect of pretreatment with free extract and incorporated in nanosystems against H2O2-induced toxicity on cells.

This Phd thesis concludes with Chapter 8, **Conclusions**, which summarizes the main original results discussed during the thesis and which led to the improvement of the field of treatment of diseases with cardiovascular risk, original contributions and prospects for further development.

This thesis contains 164 pages, general aspects and original contributions being recorded in 102 tables and figures and 302 bibliographical references.

Chapter 4. Obtaining and characterization of multicomponent polymeric nanosystems loaded with amlodipine besylate and/or valsartan

4.1.1Obtaining polymeric nanosystems with a combination of APIs

The combination of active substances AML-VAL that was incorporated into the polymeric nanosystems was selected following the critical study of the literature data, having a fixed mass ratio AML: VAL of 1:16 (mg). The method for obtaining these nanosystems was nanoprecipitation. [1]

Table 1. Formulation of binary nanosystems AML:VAL –PEG matrix

No.	Sample Code	$m_{AML:VAL}$ (mg)	m_{PEG} (mg)	m_{F127} (mg)	$V_{CH3-CO-CH3}$ (mL)	V_{H2O} (mL)	Drop rate (mL/min)	Stirring rate (rpm)	Stirring time (min)
1	F1’	1:16	5(PEG 6000)	10	5	15	0,5	1200	60
2	F2’	1:16	7.5(PEG 6000)	10	5	15	0,5		
3	F3’	1:16	5(PEG 4000)	10	5	15	0,5		
4	F4’	1:16	7.5(PEG 4000)	10	5	15	0,5		

Table 1. Formulation of binary nanosystems AML:VAL – PLGA matrix

No.	Sample Code	$m_{AML:VAL}$ (mg)	m_{PLGA} (mg)	m_{F127} (mg)	$V_{CH3-CO-CH3}$ (mL)	V H_2O (mL)	Drop rate (ml/min)	Stirring rate (rpm)	Stirring time (min)
1	F1	1:16	5	10	5	15	0,5	1200	60
2	F2	1:16	7.5	10	5	15	0,5		
3	F3	1:16	10	10	5	15	0,5		
4	F4	1:16	17	10	5	15	0,5		
5	F5	1:16	34	10	5	15	0,5		
6	F6	1:16	51	10	5	15	0,5		
7	F1”	1:16	5	10	5	15	0,5	2400	60
8	F2”	1:16	7.5	10	5	15	0,5		
9	F3”	1:16	10	10	5	15	0,5		
10	F4”	1:16	17	10	5	15	0,5		
11	F5”	1:16	34	10	5	15	0,5		
12	F6”	1:16	51	10	5	15	0,5		

4.3Results and discussions

4.3.1Obtaining polymeric nanosystems and evaluation of entrapment efficiency of the AML: VAL mixture into the polymeric matrix

All PLGA formulations showed high EE (%) for both APIs. By increasing the amount of PLGA (5; 7.5; 10; 17; 34; 51 mg), the nanoparticle suspension became increasingly cloudy, reaching a maximum entrapment efficiency for both active substances in the formulation of F3 with PLGA 10 mg. The increased amount of PLGA polymer may lead to the incorporation of the hydrophobic drug to a greater extent. Thus, for the formulation F3, the maximum degree of incorporation was observed for both active principles, namely 91.98 ± 0.45% for VAL and 82.54 ± 0.40% for AML, with minor differences from F1 and F2, which used a smaller amount of PLGA (5 and 7.5 mg, respectively). A high stirring rate causes smaller droplets and therefore the total surface area of the particles increases. [2-4] This leads to additional space for the polymeric matrix to encapsulate several molecules of the substance; thus the EE (%) for VAL was slightly improved. However, comparing the stirring rates applied to the preparation method: 1200 and 2400 rpm, respectively, it was concluded that the

variation of this parameter influences (not substantially) the entrapment efficiency of polymeric nanosystems.

4.3.2 Dimensional analysis of multicomponent polymeric nanosystems

Table 3. Characteristics of multicomponent nanosystems with AML: VAL - PLGA matrix

No.	Sample Code	Size (nm)	Zeta potential	PDI
1	F1	124.70 ± 1.15	-30.86 ± 0.31	0.095 ± 0.050
2	F2	135.60 ± 1.30	-32.46 ± 0.31	0.089 ± 0.020
3	F3	140.40 ± 1.34	-33.07 ± 0.22	0.108 ± 0.030
4	F4	158.40 ± 1.15	-34.45 ± 0.52	0.091 ± 0.040
5	F5	171.10 ± 1.30	-38.34 ± 0.21	0.048 ± 0.030
6	F6	204.10 ± 1.34	-39.34 ± 0.21	0.102 ± 0.050
7	F1”	123.90 ± 1.13	-30.44 ± 0.32	0.105 ± 0.010
8	F2”	125.60 ± 1.21	-31.76 ± 0.22	0.099 ± 0.030
9	F3”	130.80 ± 1.53	-34.24 ± 0.20	0.101 ± 0.040
10	F4”	132.10 ± 1.31	-37.45 ± 0.24	0.104 ± 0.080
11	F5”	130.10 ± 1.30	-38.72 ± 0.23	0.098 ± 0.020
12	F6”	152.70 ± 1.29	-40.45 ± 0.33	0.097 ± 0.060

4.3.3In vitro release study of active substances AML, VAL from polymeric nanosystems with PEG matrix

AML-VAL was completely dissolved in 0.1 M PBS at pH 7.4, reaching a maximum after 6 hours for AML and 7 hours for VAL, respectively, while PEG-based nanosystems provided much slower release of active substances. The *burst effect* was drastically reduced in nanosystems (CDR - cumulative drug release below 20% for AML and 38% for VAL were released in the first 30 minutes for all PEG-based nanosystems).

It was observed that both the molecular weight of the polymer used (4000; 6000) and the amount of PEG (5; 7.5) influenced the release of AML and VAL. The formulation with the smallest amount of polymer (5 mg) and molecular weight 6000 (F1 ') had a higher CDR, with a maximum release of 63.20 ± 2.03% for AML and 60.30 ± 1.74% for VAL after five days.

As the molecular weight of PEG decreased, there was a decrease in the CDR of the formulation, e.g., release from F3 'reaching a maximum of 52.90 ± 1.54% for AML and 44.10 ± 1.67% for VAL in the same time frame. Also, as the proportion of polymer increased, drug release decreased, for example, release from F2 reached a maximum of 49.50 ± 0.99% for AML and 38.10 ± 0.94% for VAL in the same time frame.

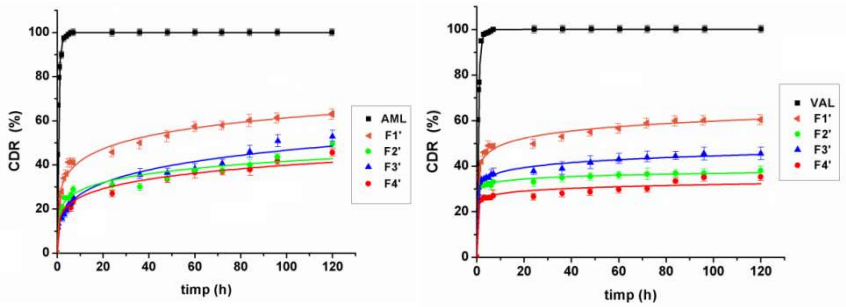


Fig. 1. In vitro release profile of AML and VAL from PEG-based polymeric nanosystems

The *in vitro* release results were subjected to a kinetic analysis to determine the mechanism of release of the active substance (AML, VAL). Therefore, the experimental data was fitted using six models, Weibull, Korsemeyer-Peppas, Higuchi, zero order, first order and Hixson-Crowel. The correlation coefficient (R^2) was chosen to distinguish between models. For this criterion, a value closer to 1 means a better fit. The results are presented in **Table 4** and **Table 5.**

Table 4. Correlation coefficient for various mathematical models - free versus entrapped AML release in PEG-based polymeric nanosystems

No	Sample	Correlation coefficient (R^2)					
		Ordin zero	Ordin unu	Higuchi	Korsmayer-Peppas	Hixson-Crowell	Weibull
1	AMLfree	0.8514	0.9680	-*	-*	0.9345	0.9957
2	F1'	0.9921	0.8303	0.8904	0.9724	0.8170	0.9861
3	F2'	0.8559	0.8874	0.9310	0.9708	0.7391	0.9707
4	F3'	0.9006	0.9300	0.9631	0.9715	0.9153	0.9529
5	F4'	0.9152	0.9282	0.9207	0.9725	0.9712	0.9695

Table 5. Correlation coefficient for various mathematical models - free versus entrapped VAL release in PEG-based polymeric nanosystems

No	Sample	Correlation coefficient (R^2)					
		Zero Order	First Order	Higuchi	Korsmayer-Peppas	Hixson-Crowell	Weibull
1	VALfree	0.7207	0.9762	-*	-*	0.9548	0.9987
2	F1'	0.7023	0.7675	0.8476	0.9710	0.7463	0.9864
3	F2'	0.7987	0.8135	0.9146	0.9719	0.8086	0.9944
4	F3'	0.7654	0.7904	0.8812	0.9713	0.7905	0.9876
5	F4'	0.9237	0.9266	0.9199	0.9703	0.9258	0.9502

* condition for application of Korsemeyer-Peppas and Higuchi model is not met (Mt / M (∞) <2/3)

For polymeric nanoparticles it was observed that the release of AML and VAL from all samples is best described by the Weibull model and the Korsmeyer-Peppas model (R^2> 0.95). For all samples, the diffusion exponent, *n*, is less than 0.5, indicating a pseudo-Fickian release mechanism. Also parameter b is less than 0.75 revealing a Fickian diffusion.

4.3.4. Study of in vitro release of active substances AML, VAL from polymeric nanosystems with PLGA matrix

In the case of PLGA-based polymeric nanosystems, the amount of active substances released was lower compared to the free substance, especially in the case of formulations with a large amount of PLGA. In the case of formulations with a small amount of PLGA after approximately 24 hours, approximately the entire amount of loaded AML and VAL was released. It can be observed that the solubility curve of AML-VAL showed a *burst effect*, while in the case of active substances loaded in polymeric nanosystems the burst effect was reduced.

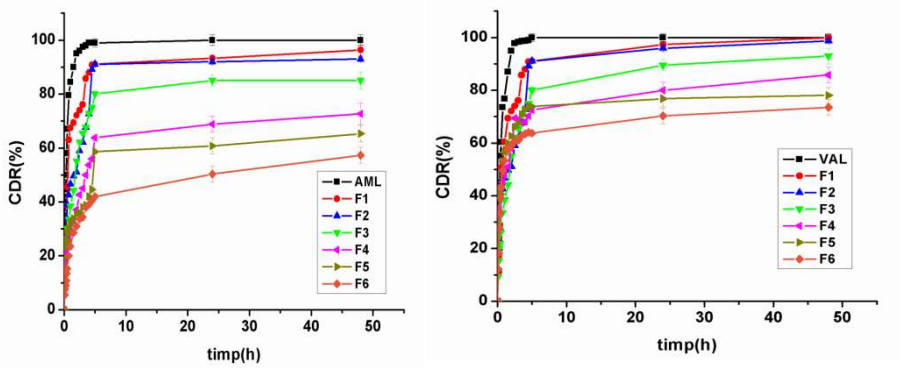


Fig. 2. *In vitro* release profile of AML and VAL from F1-F6 nanoformulations

To evaluate the release profiles of active substances *in vitro*, various kinetic models have been used to describe the release kinetics of AML and VAL from PLGA-based polymeric nanosystems. Korsmeyer-Peppas, Higuchi, Zero order, First order, and Hixson-Crowell kinetic models were applied. The correlation coefficient (R^2) was chosen to compare the models. For this criterion, a value closer to 1 means a better correlation. The results are presented in **Table 6** and **Table 7**.

Table 6. Correlation coefficient for various mathematical models - free versus entrapped AML release in PLGA-based polymeric nanosystems

No.	Sample	Correlation coefficient (R^2)				
		Zero Order	First Order	Higuchi	Korsmayer-Peppas	Hixson-Crowell
1	AML liber	0,8514	0.9680	-*	-*	0.9345
2	F1	0.5681	0.8729	0.9921	0.9805	0.9810
3	F2	0.6704	0.8760	0.9646	0.9765	0.7441
4	F3	0.8531	0.8801	0.9834	0.9854	0.8682
5	F4	0.9348	0.9509	0.9856	0.9836	0.9465
6	F5	0.9106	0.9633	0.9759	0.9776	0.9410
7	F6	0.8964	0.9282	0.9842	0.9833	0.9200

Table 7. Correlation coefficient for various mathematical models - free versus embedded VAL release in PLGA type polymer nanosystems

No.	Sample	Correlation coefficient (R^2)				
		Zero Order	First Order	Higuchi	Korsmayer-Peppas	Hixson-Crowell
1	VAL liber	0.7207	0.9762	-*	-*	0.9548
2	F1	0.8949	0.9638	0.9695	0.9837	0.9363
3	F2	0.9613	0.9508	0.9647	0.9765	0.9609
4	F3	0.9378	0.8768	0.9725	0.9719	0.9165
5	F4	0.8780	0.8654	0.9555	0.9879	0.8320
6	F5	0.6534	0.7413	0.9645	0.9886	0.7929
7	F6	0.8890	0.8264	0.9677	0.9618	0.7129

* condition for application of Korsemeyer-Peppas and Higuchi model not met (M (t) / M (∞) < 2/3)

The solubility curve of both amlodipine and valsartan can best be described by an exponential equation (first order), with $R^2 = 0.9680$ for AML and $R^2 = 0.9762$ for VAL. For PLGA-based polymeric nanosystems, it was observed that the release of AML and VAL from all samples is best described by the Higuchi model and the Korsmeyer-Peppas model ($R^2 > 0.95$). For samples with a high PLGA content (17-51 mg), the coefficient *n* is lower than 0.5,

indicating a Fickian diffusion, while for the other samples *n* is between 0.5 and 1, indicating a non- Fickian diffusion. Also, formulations with high PLGA content showed a lower value for k_H than other polymeric formulations, indicating a less intense *burst effect*.

Chapter 5. Obtaining and characterization of the multicomponent nanosystems based on PLGA loaded with amlodipine besylate and valsartan

5.3. Results and discussions

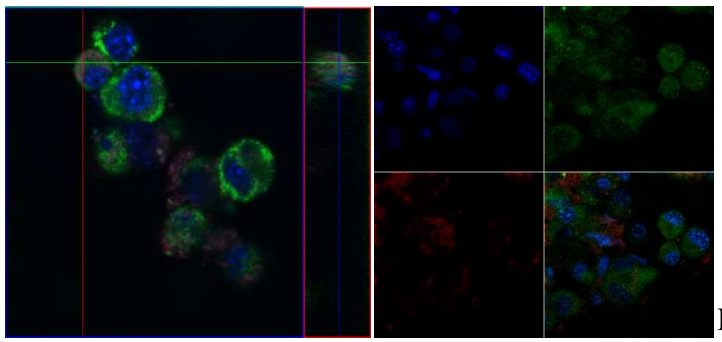
5.3.1 Determination of compatibility between active substances and polymeric matrix and determination of polymeric nanosystems morphology

The results obtained by FT-IR confirmed the presence of functional groups characteristic of the active substances and the polymeric component PLGA in the obtained nanosystems. The results of the FT-IR analysis indicate that the incorporation of the active substances into the polymeric particles has been achieved, with no interactions at the molecular level between the therapeutic agents or between the therapeutic agents and the polymer.

At the same time, SEM images of nanoparticles loaded with AML: VAL were recorded. The analysis was performed for the optimal sample - F3 (PLGA: AML: VAL – 10:1:16 mg) in terms of particle size, distribution and zeta potential, determined by DLS, and entrapment efficiency. SEM images of polymeric nanosystems loaded with AML:VAL showed larger particles than in the case of DLS analysis, probably due to the aggregation of nanoparticles during the lyophilization process in the SEM analysis.

5.3.2.Evaluation of the intracellular distribution of the optimal polymeric nanosystem

In this study, confocal microscopy was used to determine the biodistribution and localization at the cellular level of PLGA-based polymeric nanoparticles loaded with AML: VAL. The cell line chosen for this assay was the normal mouse fibroblast line (NIH3T3). In the case of 48h+ samples (NIH3T3 that remained for 48h with NP-Fc), a cytoplasmic internalization could be observed, and also a greater tendency to form conglomerates compared to the other two categories of samples (24h +; 24h +/-).



A **B**

Fig. 3. Localization of NP into NIH3T3 cells (48 +) - A and (24 +/-) - B by confocal microscopy

red-fluorescence of phthalocyanine - marks nanoparticles, green-fluorescence of Rhodamine B - stains the cell membrane, blue-fluorescence - given by Hoechst that stains the nucleus

Regarding the 24h +/- samples (cells that stood with the NP-Fc suspension for 24h, then being washed with phenol-free medium and kept in the incubator for another 24h, after which they were stained and visualized microscopically), it was observed a much lower intracellular incorporation than in the first category of samples.

Due to the fact that NP-Fc entered the cell after only 24h of incubation (24h +) and that the highest percentage of their internalization was obtained at this incubation time, argues for incorporation through the endocytosis mechanism. [5]

5.3.3.Evaluation of the intracellular cytotoxicity of the optimal polymeric nanosystem

Five different concentrations were used in this experiment: 150, 100, 50, 10 and 5 μg / mL which were incubated with the aforementioned cells. The results were expressed as a percentage of viable cells. The colorimetric signal is proportional to the activity of mitochondrial reductases of viable cells, there is a simultaneous contribution of two parameters, namely: cell number and state of mitochondrial enzymatic activity.

Thus, regarding the situation of cells - human fibroblasts (ATCC-PCS-201-012), viability values between 98.54 ± 2.01% corresponding to the 5 μg / mL concentration of F3 and 96.50 ± 0.98 corresponding to the 150 μg / mL concentration of F3 were observed.

Following the *in vitro* evaluation of the polymeric nanosystem by the MTT method, the absence of cytotoxicity was found. Taking into account the high concentrations (the maximum concentration, by correlation with the pharmacokinetic parameters is the equivalent of a dose of more than 5 g / kg body weight) it can be considered that F3 does not present a health risk.

Chapter 6. Obtaining and characterizing standardized plant extracts from plant species:*Sambucus ebulus, Lycium barbarum, Armoracia rusticana, Echinacea purpurea*

6.2.Materials and methods

Plant material: The leaves of EP (*Echinacea purpurea*), LB (*Lycium barbarum*), AR (*Armoracia rusticana*) and SE (*Sambucus ebulus*) were harvested at maturity in Dâmbovița County, Romania (latitude: 45 ° 18'15.4 " N, longitude: 25 ° 23'28.4 " E) and identified by the team of the Extractive Biotechnologies Department within the National Institute for Chemical-Pharmaceutical Research-Development (INCDCF) - ICCF, Bucharest, Romania.

6.3Results and discussions

6.3.1.Obtaining standardized plant extracts

In this study, a series of extractions were performed in 50% (v/v) ethanol, a solvent that ensures the extraction of polyphenols [6] for 4 plant species. Following the analysis of the experimental data, it is found that the hydroalcoholic extract from the leaves of *Sambucus ebulus* had the highest content of polyphenols, 25.55 ± 0.01 mg GAE / g dry material, according to literature data.

6.3.2 Identification and quantification of polyphenolic compounds in standardized plant extracts

Chromatograms of the HPTLC analysis showed the presence of polyphenolic compounds in the case of standardized plant extracts: caffeic acid, chlorogenic acid, rutin and hyperoside.

Also, the obtained plant extracts were investigated using reverse phase HPLC analysis, using 19 reference phenolic compounds. Of all the reference phenolic compounds for SE extract, four compounds were detected and quantified: chlorogenic acid (14.389 ± 0.018 mg / g extract), caffeic acid (2,997 ± 0.004 mg / g extract), rutin (0.564 ± 0.001 mg / g extract) and quercetin (0.073 ± 0.001 mg / g extract). In the case of *Echinacea purpurea* extract, 5 polyphenolic compounds were identified and quantified, mainly chicoric acid (19.528 ± 0.004 mg compound / g extract) and caftaric acid (15.423 ± 0.018 mg compound / g extract). The chromatogram of *Armoracia rusticana* extract reveals the presence of three phenolic compounds, all with low concentrations: trans-ferulic acid, p-coumaric acid,

kaempferol. Kaempferol showed the highest concentration in horseradish leaf extract, namely 0.050 ± 0.002 mg compound / g extract. For the *Lycium barbarum* extract, 4 polyphenolic compounds were identified and quantified. The compound found in the highest proportion is chlorogenic acid, with a concentration of 18.740 ± 0.031 mg / g extract. Caffeic acid was also present in a significant amount, namely 1.773 ± 0.002 mg / g extract.

6.3.4.Assessment of the cytotoxic and antiproliferative effects of standardized plant extracts

Cytotoxicity assays use a standardized procedure, which includes exposing cells to different concentrations of the samples. Thus, a cell line was selected, namely the murine fibroblast cell line - L929 (ATCC-CRL-6364) and the cytotoxic potential was investigated by the method of assessing the metabolic viability of cells (test for colorimetric evaluation of mitochondrial enzyme activity based on formazan - MTS). It is observed that with the increase of the concentration of the plant extract, the cell viability decreases, but not so much as to affect the total cellular integrity. The MTS test did not show significant cytotoxic or antiproliferative effects for any of the plant extracts, the IC_{50} values being between 64.120 ± 0.288 µg / mL (EP extract) and 91.910 ± 0.292 µg / mL (LB extract), as these doses are difficult to achieve *in vivo*. Also, taking into account the high concentrations (the maximum concentration, by correlation with pharmacokinetic parameters is the equivalent of a dose of more than 5 g / kg body weight), it can be considered that all studied plant extracts do not present a health risk.

Chapter 7. Obtaining and characterizing lipid nanosystems with standardized plant extract of Sambucus ebulus

7.2.2.Obtaining lipid nanosystems loaded with Sambucus ebulus extract

Obtaining liposomes and transferosomes loaded with standardized Sambucus ebulus extract was done using the method of hydrating the lipid film by manual agitation [7, 8]. Ethosomes loaded with *Sambucus ebulus* extract were obtained using the cold preparation method [9]. All formulations were sonicated for 20 minutes, using an ice-filled sonication bath with automatic frequency adjustment system and amplitude set at 20%. Subsequently, all vesicles were extruded 5 times successively through 0.4 µm membranes and 5 times successively through 0.22 µm membranes. Liposomes, transfersomes and empty ethosomes were also prepared as control. All samples were obtained in triplicate and stored in the refrigerator at 8 °C for a maximum of 48 hours before use for analytical and pharmacological characterization.

7.3.Results and discussions

7.3.1.Obtaining lipid nanosystems - the study of the influence of preparation parameters

The first phase of the study had as objective the formulation of various lipid nanosystems (liposomes, transfersomes, ethosomes) loaded with SE plant extract. In this regard, the study of the influence of various parameters on the obtaining of lipid nanosystems with SE was performed using the OFAT method (one-factor-at-a-time experiment). Thus, a single parameter was varied, such as the ratio PC: cholesterol, the ratio PC: sodium colate, the amount of extract, the stirring speed, the evaporation temperature for liposomes and transfersomes, and the ratio ethanol: water, the ratio PC: extract SE for etosomes, while the other parameters were kept constant and the influence of the varied parameter on the entrapment efficiency was observed

7.3.2.Characterization of lipid nanosystems loaded with SE plant extract

Lipid nanosystems loaded with SE plant extract were characterized by entrapment efficiency (EE%), particle size, polydispersity index (PDI) and zeta potential. **The evaluation**

of the entrapment efficiency was determined spectrophotometrically using the Folin-Ciocâlteu method. **Particle size and PDI** of lipid vesicles were determined by dynamic light scattering (DLS) using a particle size analyzer. **The zeta potential values** of SE-loaded lipid vesicles were determined using a Zetasizer analyzer. **The AFM analysis** was performed in collaboration with Mr. CS II Dr. Mihai Anastasescu (Institute of Chemistry-Physics "Ilie Murgulescu") using an XE-100 microscope, in non-contact mode. **Dark field microscopy** was performed in collaboration with Ms. Assoc. Mona Mihailescu (Faculty of Applied Sciences, UPB) using a CytoViva system. **SEM analysis** was performed with a Tescan Vega 3 LMH microscope.

The increase in the size of lipid nanosystems (SE@L, SE@T, SE@E) compared to empty lipid nanosystems - without plant extract (L, T, E) was caused by the incorporation of SE extract phytoconstituents in the vesicle structure. The size of SE-loaded lipid nanosystems increases in the following order: liposomes < transfersomes < ethosomes. All lipid nanosystems showed a narrow size distribution (PDI < 0.21), proving good homogeneity and reduced tendency to aggregate. Incorporation of SE extract improved homogeneity, while empty lipid vesicles had a larger size distribution than SE-loaded vesicles. The values of the zeta potentials were in the range 43.00 ± 1.03 mV – 37.00 ± 0.23 mV, all samples indicating a moderate stability. [10]

Table 8. Characteristics of lipid nanosystems

ID probă	Formulare	EE (%)	Dimensiune (nm)	ξ-potential	PDI
SE@L	PC: Colesterol: SE= 10:1:2 (w/w)	80.05 ± 0.51	123.01 ± 2.50	-43.01 ± 1.03	0.18 ± 0.01
SE@T	PC: Sodium colate: SE= 8:2:2 (w/w)	75.10 ± 1.12	155.12 ± 3.31	-39.17 ± 0.50	0.16 ± 0.02
SE@E	PC: SE= 8:2.5 (w/w); H_2O:Ethanol = 7:3 (v/v)	85.10 ± 1.50	190.10 ± 2.53	-37.04 ± 0.23	0.20 ± 0.01
L	PC: Cholesterol= 10:1 (w/w)	-	49.09 ± 1.52	-20.11 ± 1.23	0.44 ± 0.01
T	PC: Sodium colate = 8:2 (w/w)	-	105.03 ± 0.23	-25.10 ± 0.89	0.37 ± 0.02
E	PC = 8 mg; H_2O:Ethanol = 7:3 (v/v)	-	65.12 ± 0.51	-30.19 ± 1.15	0.44 ± 0.01

AFM analysis of SE-loaded samples revealed randomly distributed quasi-spherical particles for all types of lipid vesicles, with dimensions of tens of nm. Therefore, the size of lipid vesicles obtained by AFM analysis are smaller than the hydrodynamic diameters measured by DLS or the size determined by SEM, but the same order of magnitude was observed for lipid vesicle sizes: liposomes < transfersomes < ethosomes. Hyperspectral images of the SE@E sample were obtained using enhanced dark field microscopy and it was observed that the nanoparticles are isolated from each other and the light scattering spectra have similar maximum intensities, indicating a narrow size distribution of lipid vesicles.

The entrapment efficiency of SE extract in all lipid vesicles was over 75% and increased in the order of liposomes < transferosomes < ethosomes, the applied preparation methods being efficient. Similar results reported for entrapment efficiency for *Polygonum aviculare* [11], *Glycyrrhiza glabra L.* [12] and *Artemisia arborescens* [13]. SE-loaded lipid vesicles were stable for at least three months, with approximately the same amount of incorporated phytocompounds after one month (loss of extract compounds <0.65%) and after 3 months (loss of extract compounds <3.35%)

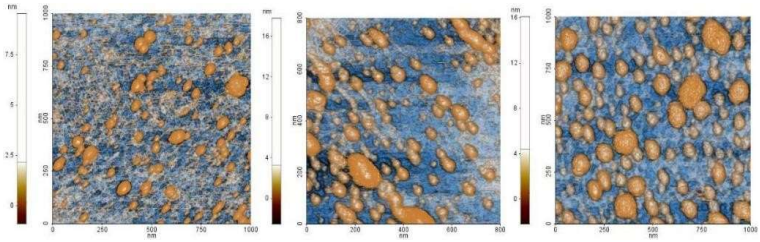


Fig. 4. AFM images of lipid vesicles loaded with SE extract: liposomes (A); transfersomes (B) and ethosomes (C)

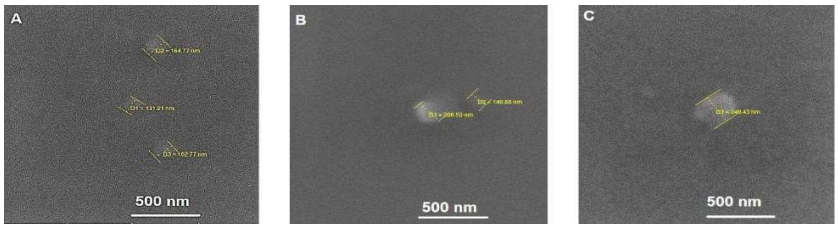


Fig.5. SEM images of lipid vesicles loaded with SE extract (vacuum dried)

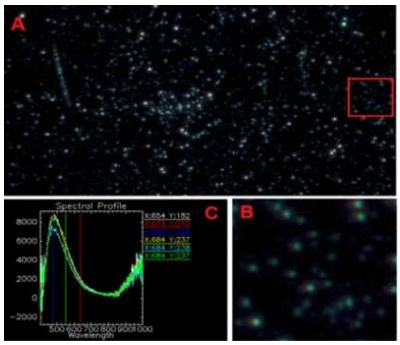


Fig.6. Image recorded by dark field microscopy using Cytoviva (A); magnification of the area marked in Figure A corrected by the spectrum of the lamp (B) and the spectra collected at the isolated points (C)

7.3.3Study of in vitro release of polyphenols from lipid nanosystems

The release of polyphenols from the free extract showed a "burst effect", while in the case of SE extract incorporated in the lipid vesicles the release of phytoconstituents was slower. The release of polyphenols from the plant extract was complete after 10 hours (98.51 ± 1.07%), while the release of polyphenols from all lipid vesicles had a much lower release rate (below 70%).

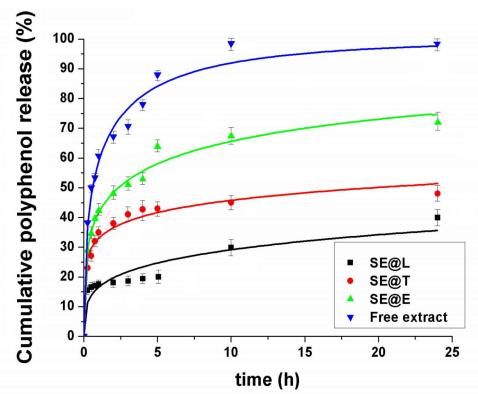


Fig. 7. *In vitro* release profile of polyphenols in lipid vesicles loaded with SE extract compared to free SE extract (fit with Weibull model)

To evaluate the mechanism of polyphenol release, three mathematical models were applied: Weibull, Korsmeyer-Peppas, and Higuchi. The best correlation of the coefficients was obtained for the Weibull equation, in all lipid formulations, parameter b < 0.75, revealing a Fickian diffusion. The Korsmeyer-Peppas and Higuchi models were applied only for the first 60% polyphenols released with correlation coefficients higher than 0.96 and 0.92, respectively (**Fig. 8.**). For all samples, the coefficient *n* is less than 0.5, indicating a Fickian diffusion, a mechanism according to the Weibull model. SE-loaded liposomes showed a lower value for kH than the other lipid formulations, indicating a less intense effect, probably due to their cholesterol content, which increased the rigidity of the lipid bilayer, changing its permeability.

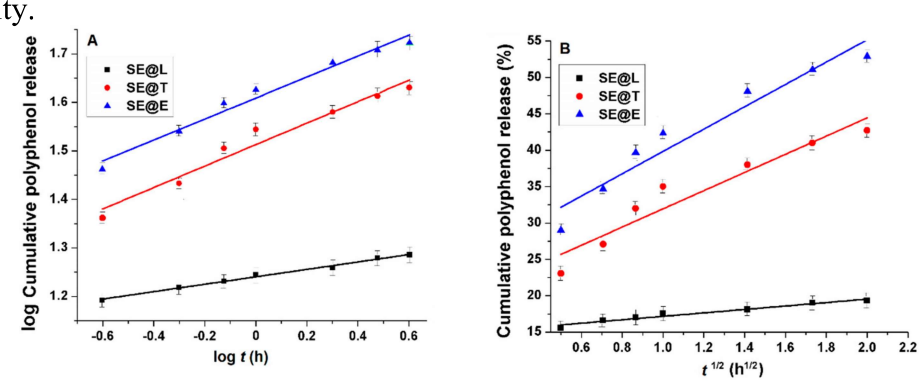


Fig.8. Polyphenol release profiles from lipid nanosystems loaded with SE extract fitted with Korsmayer-Peppas (a) and Higuchi (b) kinetic models.

5.4.4Effect of pretreatment with free and SE plant extract entrapped into lipid nanosystems against H_2O_2-induced toxicity on the L-929 cell line

The viability of L-929 mouse fibroblast cells was evaluated after 4 hours of exposure to different concentrations of aqueous hydrogen peroxide solution in the range of 1-100 mM, its cytotoxicity being dose dependent. Following the results obtained, the concentration of 50 mM H_2O_2 was selected for the following studies, a concentration that reduces cell viability to 50%. Three concentrations (5, 10, 25 µg / mL) of SE extract and SE-loaded lipid vesicles were selected, which are non-toxic and could prevent H_2O_2-induced cytotoxicity. Therefore, L-929 fibroblast cells were pretreated with free SE extract and SE-loaded lipid vesicles before adding the hydrogen peroxide solution with appropriate IC_{50} concentration. Pretreatment administered to the fibroblast cell line improved viability (**Fig. 9**), thus suggesting a significant cytoprotective effect against ROS for both the free extract and the one incorporated in lipid nanosystems. However, the effect was not present in the case of cells exposed in the short term to test products, which suggests that the exercise requires the intracellular presence of compounds and is dependent on altered expression of specific enzymes and antioxidant substrates. All samples were able to protect L-929 cells against H_2O_2-induced cytotoxicity, probably due to their polyphenol content. These in vitro experiments showed that the polyphenols in the SE leaf extract have the ability to remove radicals resulting in a cytoprotective effect even at a low concentration of 5 µg / mL. It is known that the use of lipid vesicles as nanotransporters could help the cellular internalization of polyphenols through the process of endocytosis. [14] Therefore, incorporation of SE extract into lipid vesicles may be an effective method to improve the intracellular transport of polyphenols.

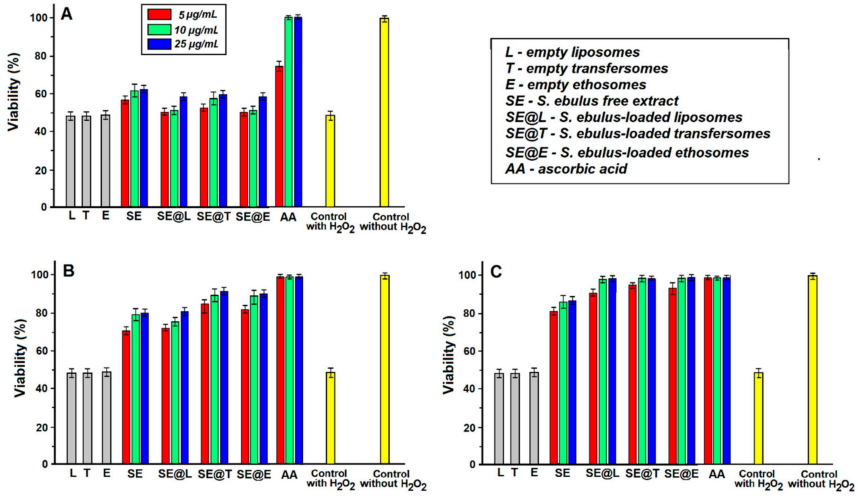


Fig. 9. Effect of H_2O_2 on L-929 cell viability after exposure.
Viability of L-929 cells after 1 hour (A), 12 hours (B), 24 hours (C) pretreated with free / entrapped SE leaf extract in lipid nanosystems prior to exposure to H_2O_2 solution

Evaluation of lipid nanosystems by the MTS test demonstrated a cytoprotective effect against hydrogen peroxide-induced toxicity on the mouse fibroblast cell line L-929 of the free extract of Sambucus ebulus leaves and loaded into lipid vesicles.

Chapter 8. Conclusions

8.1.*General conclusions*

1.Conclusions on the methods of obtaining and selecting the components of polymeric nanosystems

- The optimal method for obtaining polymeric nanosystems was the nanoprecipitation method (solvent evaporation), which proved to be a quick and easy method that leads spontaneously to the formation of nanoparticles, with a high degree of incorporation and high yield.
- In order to obtain an optimal multicomponent nanosystem, three types of experiments were performed in which the following parameters were varied: type of polymer (PEG, PLGA), molecular mass of the polymer in the case of PEG (PEG 4000, PEG 6000), amount of polymer: 5; 7.5 mg for PEG 4000 and PEG 6000, and for PLGA: 5; 7.5; 10; 17; 34; 51 mg, and agitation speed (1200, 2400 rpm) only for formulations that have demonstrated high entrapment efficiency (PLGA-based formulations).
- Nanosystems with PEG 6000 matrix proved slightly higher degrees of incorporation compared to those with PEG 4000 matrix, the molecular mass having an insignificant influence on the entrapment efficiency.
- PLGA-based polymeric nanosystems had higher degrees of incorporation than PEG-based ones, indicating that the nature of the polymer plays an important role in the formulation of these systems.
- Regarding the relation between the amount of PLGA polymer and the entrapment efficiency, there is a direct proportionality (the higher the concentration of the polymer, the higher the degree of incorporation) up to a certain threshold value, detected as the concentration of 10 mg PLGA.
- In the case of formulations with polymer matrix type PLGA, the stirring speed applied in this study (1200, 2400 rpm) led to a slight change in the entrapment efficiency.

- The entrapment efficiency of synthetic active substances (amlodipine besylate, valsartan) calculated on the basis of determinations by UV-VIS spectrophotometry was greater than 75%.
- Characterization by dynamic light diffusion (DLS) showed for all polymeric nanosystems based on PLGA nanometric dimension in the range 123.90 ± 1.13 - 204.10 ± 1.34 nm, potential negative zeta: (-) 40.45 ± 0.33 - (-) 30.86 ± 0.31, which means good stability of the formulations obtained, and a polydispersity index of less than 0.2, which demonstrates the uniformity of nanosystems.
- Regarding the study of the release of active substances from polymeric nanosystems, 5 kinetic models were applied: zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell. It has been observed that the release of amlodipine besylate and valsartan from PEG / PLGA polymeric nanosystems is best described by the Higuchi model and the Korsmeyer-Peppas model ($R^2 > 0.95$).

After analyzing the experimental data, the F3 formulation containing PLGA 10 mg, AML: VAL of 1:16 (mg), obtained by the nanoprecipitation method at a stirring speed of 1200 rpm and a dripping rate of 0.5 mL / min, was chosen as the optimal nanosystem, for which physico-chemical characterization studies and in terms of safety profile were continued.

2. Conclusions on the characterization of the chosen optimal nanosystem

- FT-IR structural analysis demonstrated good compatibility between the active substances (amlodipine besylate and valsartan) and the polymeric matrix (PLGA).
- High performance electron microscopy (SEM) analysis for the optimal multicomponent nanosystem (F3) showed polymer particles with smooth texture and round morphology.
- A working protocol was developed in order to evaluate the distribution of the optimal polymeric nanosystem, following which it was possible to observe the distribution of nanoparticles both extra- and intra-cellular for the nanosystem PLGA: AML: VAL (10:1:16 mg). Thus, by confocal microscopy, an intracytoplasmic localization of the polymeric nanoparticles could be highlighted, but it could not be detected if the nanoparticles were included in cytoplasmic organs.
- The MTS *in vitro* colorimetric test showed that the polymeric nanosystem PLGA: AML:VAL (10:1:16 mg) demonstrated a high biocompatibility, no cytotoxic potential at the tested concentrations (5, 10, 15, 50, 100, 150 µg / mL) on normal cell lines: human breast epithelial cells (MCF 12A ATCC CRL-10782) and human fibroblasts cells (ATCC-PCS-201-012).

3.Conclusions on the methods of obtaining and choosing the plant extract as an active ingredient in the treatment of diseases with cardio-cerebrovascular risk

- Four standardized hydroalcoholic extracts (g GAE / 100 g extract) were obtained from the aerial part (leaves) of some plant species: *Sambucus ebulus* (boz) - 2.550 ± 0.010 g GAE / 100 g extract, *Echinacea purpurea* (echinacea) - 1.470 ± 0.030 g GAE / 100 g extract, *Armoracia rusticana* (horseradish) - 2.160 ± 0.020 g GAE / 100 g extract, and *Lycium barbarum* (goji) - 1.83 ± 0.030 g GAE / 100 g extract. Thus, the plant species that showed the highest concentration of polyphenols was *S. ebulus*.
- HPTLC and HPLC analyzes have demonstrated the presence of certain phenolic compounds with an adjuvant role in the treatment of diseases with cardio-cerebrovascular risk. In the case of all plant extracts, by HPTLC analysis were observed 4 compounds: caffeic acid, chlorogenic acid, rutin and hyperoside, and by HPLC analysis was able to identify and quantify 4 polyphenolic compounds for *Sambucus ebulus* and *Lycium barbarum*, 5 polyphenolic compounds for *Echinacea purpurea*, and 3 polyphenolic compounds for *Armoracia rusticana*. Regarding the extracts of *S. ebulus* and *L. barbarum*, high

concentrations of chlorogenic acid (14.389 ± 0.018, respectively 18.740 ± 0.031 mg compound / g extract) were observed, in the case of *E. purpurea* extract chicoric acid and caftaric acid were predominant compounds (19.528 ± 0.004 mg compound / g extract, respectively 15.423 ± 0.018 mg compound / g extract), and *A. rusticana* extract showed the lowest concentrations in polyphenolic compounds, predominantly kaempferol (0.050 ± 0.002 mg compound / g extract)

- All the standardized plant extracts obtained were characterized in terms of antioxidant action by the DPPH method, showing a high anti-radical power of ROS. Thus, three extracts showed the IC_{50} value below 50 µg / mL: *S. ebulus* extract (10.330 ± 0.056 µg / mL), L. barbarum extract (11.330 ± 0.056 µg / mL) and *A.rusticana* extract - 20.09 ± 0.056 µg / mL, and a single extract showed an IC_{50} value above 50 µg / mL: *E. purpurea* extract – 56.890 ± 0.056 µg / mL

- The *in vitro* evaluation of cytotoxicity showed a high safety profile for all plant extracts, with IC_{50} values that support cellular integrity: 64.120 ± 0.288 µg / mL (*Echinacea purpurea* extract), 64.710 ± 0.322 µg / mL (*Sambucus ebulus* extract) , 70.400 ± 0.305 µg / mL *(Armoracia rusticana* extract), and 91.910 ± 0.292 µg / mL (*Lycium barbarum* extract).

Due to the high concentration of polyphenols, the increased antioxidant activity, the standardized extract of the species *Sambucus ebulus* L. was selected, in order to be incorporated in different types of lipid nanosystems.

4. Conclusions on obtaining and characterizing lipid nanosystems loaded with S. ebulus plant extract

- Three types of lipid nanosystems were developed and characterized - liposomes, transfersomes and ethosomes, in which a standardized plant extract of *Sambucus ebulus* was incorporated.

- A study was performed in which certain parameters were modified to establish the conditions for the preparation of lipid nanosystems. Thus, in the case of liposomes and transfersomes were studied: i) the influence of the PC / cholesterol (sodium colate) ratio on the entrapment efficiency, the best results being obtained for the 10/1 PC/cholesterol ratio for liposomes and the 8/2 PC ratio/sodium colate for transfersomes; ii) the influence of the evaporation temperature on the entrapment efficiency, the highest entrapment efficiency being observed for the formulations prepared at 35 °C; iii) the influence of stirring speed on the entrapment efficiency, the stirring speed of 200 rpm determining the best results for both lipid nanosystems, and iv) the influence of the amount of *S. ebulus* extract on the entrapment efficiency, the best results being obtained for 20 mg of *S. ebulus* extract for both lipid nanosystems. In the case of ethosomes loaded with *S. ebulus* extract, the influence of two factors (PC / SE extract ratio and water / ethanol ratio) on the entrapment efficiency was evaluated. The formulation with 8 / 2.5 ratio PC / SE extract and 3/7 water / ethanol ratio showed the best entrapment efficiency.

- The entrapment efficiency of phytoconstituents in the case of lipid nanosystems was higher than 75%. Thus, liposomes showed the entrapment efficiency of phytoconstituents 80.05 ± 0.51%, transfersomes - 75.10 ± 1.12%, and ethosomes - 85.10 ± 1.50%. Also, after evaluating the entrapment efficiency for three months, it was concluded that all lipid nanosystems showed high stability at 4 °C.

- Dynamic light diffusion analysis (DLS) showed for all lipid nanosystems loaded with *S. ebulus* a nanometric dimension (liposomes - 123.01 ± 2.50 nm, transfersomes - 155.12 ± 3.31 nm, ethosomes – 190.10 ± 2.53 nm), high uniformity of particle distribution, moderate stability revealed by zeta potential value (liposomes - (-) 43,00 ± 1.03, transfersomes - (-) 39.00 ± 0.50, ethosomes - (-) 37.00 ± 0.23).

- SEM measurements indicated a quasi-sphere morphology, with a smooth surface for all lipid nanosystems. The AFM analysis showed larger and more homogeneously distributed particles in the case of ethosomes, while in the case of liposomes, smaller compactly distributed particles were observed. Also, the vesicular nature of the nanosystems was confirmed by their spiky (noisy-like) profile. Microscopic analysis showed that lipid nanosystems have a narrow size distribution, with the smallest being liposomes that are more hydrophobic and less deformable than transfersomes and ethosomes, while the largest were ethosomes, which showed the highest entrapment efficiency.
- Hyperspectral images of lipid nanosystems (ethosomes) were obtained using dark field microscopy, showing a narrow size distribution of lipid vesicles.
- *In vitro* release studies were performed in PBS medium, pH = 7.4, at 37 ^{0}C and demonstrated the slower release of polyphenols entrapped in lipid nanosystems compared to the solubilization of polyphenols under the same experimental conditions. Thus, three mathematical models (Weibull, Korsmeyer-Peppas and Higuchi) were applied to evaluate the mechanism of polyphenol release. Liposome-type lipid nanosystems showed the lowest rate of polyphenol release, while transfersomes and ethosomes resulted in a much faster release of polyphenols.
- *In vitro* evaluation of the cytotoxicity of free plant extract and lipid nanosystems loaded with *S. ebulus* was performed by MTS method, which demonstrated a cytoprotective effect against hydrogen peroxide-induced toxicity on the mouse fibroblast cell line L-929. It was also observed that a short pretreatment (one hour) with *S. ebulus* extract did not show a significant cytoprotective action on cells, requiring at least 12 hours of pretreatment before exposure to hydrogen peroxide solution to observe significant results in terms of prevention of potential cytotoxicity.

8.2 Original contributions

An element of originality is that a combination of active substances with complementary mechanisms of action has been incorporated in a fixed dose (amlodipine: valsartan 1:16 mg) in polymeric nanosystems with PLGA, PEG 4000, PEG 6000 matrix type, in order to cumulate the therapeutic effects and reduce the side effects.

Also, the originality of this thesis derives from the developing of a protocol to evaluate the intracellular distribution of polymeric nanosystems loaded with AML:VAL with applications in the treatment of cardio-cerebro-vascular diseases on the murine cell line (NIH3T3). It has been shown that polymeric nanoparticles enter the membrane and correlated with data from the literature, a first step was taken in understanding the path of these nanosystems.

Another element of originality is the attempt to evaluate the safety profile on human fibroblast cell lines and mammary epithelial cells for polymeric nanosystems loaded with amlodipine and valsartan.

From the data literature, so far, no experiments have been performed regarding the incorporation of *Sambucus ebulus* leaf extract. Thus, this thesis brings as a novelty the obtaining and characterization of three lipid nanosystems - liposomes, ethosomes, transfersomes, in which the plant extract standardized in total polyphenols of *Sambucus ebulus* was incorporated.

At the same time, a protocol was developed to test the cytoprotective effect of lipid nanosystems, applying a pretreatment with a standardized extract of free *Sambucus ebulus* and incorporated into lipid nanosystems against H_2O_2-induced toxicity on the L-929 murine fibroblast cell line.

8.3. Perspectives for further development

Considering that this paper began the study of innovative nanometric systems with application in the treatment of cardio-cerebro-vascular diseases, and succeeded in locating polymeric nanosystems in murine fibroblasts, it is necessary to investigate the mechanism by which nanoparticles are transported in specific cell lines (cardiomyocytes). Starting in this direction, future studies should focus on quantifying the endocytocic mechanism of polymeric and lipid nanosystems:

- with the help of soluble reporter molecules that penetrate the cell in this way - if we speak of a fluid phase endocytosis (fluorophores such as Lucifer Yellow, TMA-DPH, albumin/dextrans/fluorescently labeled or radiolabeled polysaccharides, galactosidase, alkaline phosphatase, perosidase horseradish etc. which become visible through reaction products that occur when the cell is incubated with the enzyme substrate);
- by using pharmacological inhibitors (chlorpromazine, brefeldin A, intracytoplasmic potassium depletion, hypotonic shock - in the case of clathrin -mediated endocytosis; methyl-β-cyclodextrin, nystatin, philippine, etc. - in the case of caveolin-mediated endocytosis).

In order to achieve a complete characterization of a pharmaceutical nanosystem, a complex approach is required, comprising a suite of complementary experimental techniques. For this purpose, thermodynamic data are needed in the process of studying pharmaceutical solids. Thus, future studies will focus on determinations by thermal analysis methods: DSC (Differential Scanning Calorimetry), TG (Thermogravimetry), DTA (Differential Thermal Analysis), thermomicroscopy (HSM-Hot Stage Microscopy).

It is also necessary to make determinations regarding the stability of nanosystems over a longer period of time.

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5. Ramona-Daniela Păvăloiu, **Fawzia Sha'at**, Lucia Pirvu, Cristina Hlevca, Mousa Sha'at, Gheorghe Nechifor, *Design and evaluation of innovative lipid nanovesicles containing Centaurea cyanus L. extract*, 4th International Conference on Natural Products Utilization: From Plants to Pharmacy Self, Albena, Bulgaria, May, 29 – June, 01, **2019**, Book of Abstracts, ISSN: 2682-9487, p.272, Poster.
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10. **Fawzia Sha'at***, Ramona-Daniela Pavaloiu, Cristina Hlevca, Mousa Sha'at, Daniela Berger, Gheorghe Nechifor, *Cardiovascular APIs encapsulated into biodegradable matrix: in vitro evaluation on drug release kinetics*, 21st Romanian International Conference on Chemistry and Chemical Engineering, Constanta - Mamaia, Romania, September, 4 – 7, **2019**, Poster, ***autor correspondent**

PROJECTS LIST (project manager/ team member) (selection)

1. Ctr. D no. 18 / 03.07.2019 - POC - *Topical products based on viper venom and active vegetal ingredients* **(project manager) -** July 2019 - June 2021
2. NUCLEU Program - PN 16-27-02-01, contract no. 6N / 2016 - *From generic to supergeneric: obtaining and characterizing nanostructured multicomponent mixtures with action in cardiovascular diseases* **(project manager) -** 2016 - 2018
3. NUCLEU Program - PN 19-41-04-01, contract no. 34 N/2019, *Nanostructured therapeutic systems based on microbial biopolymers with controlled/targeted release of antitumor substances* (**team member**) - 2019 – 2022
4. PN-III-P2-2.1-PED-2019-2125*, Innovative therapeutic formula for osteo-articular inflammations* (**team member**) - 2020- present
5. PCCDI, PN-III-P1-1.2-PCCDI-2017-0407, Component project no.39/2018 - *Design of bioconjugates with targeted action for the treatment of inflammatory bowel diseases* (**team member**) - 2018 - 2020
6. PCCDI - PN-III-P1-1.2-PCCDI-2017, *Development of advanced platforms for analysis and modeling of complex biological systems* (**team member**) - 2018 - 2020
7. NUCLEU - PN 1627-03-02, contract no. 6N / 2016, *Systems for transport and release of natural extracts with antioxidant potential based on lipid vesicles for topical administration*, (**team member**) - 2016 –2018
8. Contract D no.38/08.11.2017 POC, *Natural cosmetic preparations based on organic resins and ingredients of vegetable origin* (**team member**) - 2016 - 2019
9. ADER contract no. 14.1.2/06.10.2015, *Studies and research on the risks and benefits of consuming food supplements based on medicinal and aromatic plants* (**team member**) - 2015 - 2018.