POLITHECNICA BUCHAREST NATIONAL UNIVERSITY OF SCIENCE AND TECHOLOGY
FACULTY OF CHEMICAL ENGINEERING ANF BIOTECHNOLOGIES
Doctoral School: Chemical Engineering anf Biotechnologies

SUMMARY OF the PhD THESIS

NANOENCAPSULATION OF NUTRACEUTICALS IN NANOSTRUCTURED LIPID SYSTEMS FOR OBTAINING ADVANCED FOOD SUPPLEMENTS

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Keywords: nanocapsulation, nanocarrier lipid systems (NLC), optimization, bioactive principles, morpho-structural characterization, therapeutic evaluation

Thesis content
(The numbering of chapters corresponds to the pagination related to the doctoral thesis)

PART I. BIBLIOGRAPHICAL RESEARCH

PART II. ORIGINAL RESEARCH

Introduction

In the context of the continuous modification of the eating habits, the need to supplement some nutrient deficiencies or the healing of certain diseases, nanotechnology, especially the nanocapsulation of active principles in efficient distribution systems, has become a broad field addressed by nanotechnology researchers. Intended to ensure the protection of the active principle of interest against the aggression of various factors, increase the encapsulation efficiency, ensure the release of the compound at the site of action, enhance its biological activity, nanocapsulation has become more and more widespread. With regard to the encapsulated compounds, the trend towards the choice of natural compounds, extracted from plant sources, can be highlighted, as they are meant to diminish the side effects (sometimes with toxic potential) of synthesis compounds. These compounds have a nutritional aspect and a pharmacological side, actively participating in the prevention and/or cure of certain diseases, which is why they were classified as nutraceuticals. In the literature are presented a series of nanostructured lipid systems, which served to encapsulate nutraceuticals, for example liposomes, niosomes, nanoemulsions, microemulsions or lipid nanoparticles. Lipid nanoparticles being solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have major advantages over other distribution systems. Some of the advantages of the lipid systems include the ability to capture hydro- and liposoluble compounds and improve absorption in the body (increased bioavailability of the active compound), high chemical and physical stability, the use of *in vivo* biodegradable ingredients, controlled release of active compounds, low toxicity and appropriate production methods, including at industrial level. An intensely debated aspect facing the menopausal women's population is whether hormone replacement therapy (with the administration of synthetic hormones, estrogen and/or synthetic progesterone), applied in the treatment of menopausal symptoms is or is not safe and effective. Although some studies prove the effectiveness of this replacement therapy, in others, associated side effects are noted, especially in women with high cardiovascular risk, thromboembolic disease or imminent cancer (breast, endometrial). In addition, the individuality of each woman, sociocultural perceptions may be other reasons why some refuse the ingestion of synthetic hormones, preferring alternative therapies.

In this context, the present doctoral thesis, *Nanoencapsulation of nutraceuticals in nanostructured lipid systems for the development of advanced food supplements*, aimed at identifying alternative therapeutic solutions, based on the validation of some vegetable active principles (present in nano-form in various lipid distribution systems) which have estrogenic and other complementary therapeutic actions for the development of dietary supplements. Thus, the thesis addresses the obtention, physico-chemical characterization, as well as the *in vitro* evaluation of the specific properties of some nanostructured distribution systems from the category of nanostructured lipid carriers (NLC), capable of encapsulating different natural principles from individual plant sources – or mixed (in the form of plant extracts). The aim is associated with improving the bioavailability of both plant extracts (by ensuring sustained and steady release of the two bioactive compounds), protecting them against gastrointestinal degradation (avoiding primary metabolism) and combining both types of therapeutic, anti-inflammatory and antioxidant effects. The studied plant principles, selected on the basis of the therapeutic benefits reported in various studies published in recent years (through the results

of *in vitro* assays, *in vivo* tests and preclinical studies) are: Diosgenin (**DSG**), wild yam (***Yam***) extract, Glycyrrhiza glabra (***GlyG***) extract, Polygonum cuspidatum (***PCus***) extract and Cymicifuga racemosa (***CymR***) extract.

The doctoral thesis formulated under the name „*Nanoencapsulation of nutraceuticals in nanostructured lipid systems for the development of advanced food supplements*", is structured on 10 chapters, divided into 2 parts, bibliographic research and original research.

PART I. Bibliographic research includes 3 chapters:

o ***Chapter 1*** presents the compositional aspects, structural features and advantages of NLC systems, as well as a „state of the art" on the efficiency of distribution systems in the delivery of natural or synthetic active ingredients.
o The applicative potential of NLC and SLN lipid nanoparticles in the food industry, respectively the benefits of these systems in the nutraceutical distribution, are detailed in ***Chapter 2.***
o ***Chapter 3*** covers the main aspects of plant compounds or phytochemical mixtures investigated in this doctoral thesis, namely: sources of production, chemical composition, biological effects and therapeutic benefits.

PART II-a. The original research begins with the mention of the research objectives, it continues with aspects of original contributions in the field of obtaining, physico-chemical characterization and *in vitro* evaluation of certain biological properties of some nanocarrier lipid systems that co-opt different active principles of plant origin and finalize with the conclusions related to the research undertaken.
o ***Chapter 4*** presents the techniques and methods used to characterise nanocarrier lipid systems incorporating the active principles selected in the research.
o ***Chapter 5*** exposes the preliminary studies of obtaining free NLC (without active principles content), of NLC encapsulating a single active principle, as well as NLC systems that co-opt different dual (mixed) active principles. Here are the optimization steps in terms of obtaining lipid nanosystems, *e.g.:* selecting the optimal ratio between lipids, surfactants and/or co-surfactants, active principle quantity, active principle quantity, operating parameters during high pressure homogenization processing (technique HPH), etc.
o ***Chapter 6*** presents the original results obtained from the preparation and characterisation of individual and mixed NLC systems (containing evening primrose oil and soybean oil), distribution systems including diosgenin (DSG) and Glycyrrhiza Glabra (*GlyG*) extract, as active principles. The physico-chemical characterization of NLC is complemented by *in vitro* evaluation of the therapeutic potential of NLC_*GlyG* (evening primrose oil/I and soy/II oil), NLC_DSG(I/II) and mixed systems, respectively, NLC co-encapsulating DSG and *GlyG* simultaneously. The partial conclusions are summarised at the end of the chapter.
o In ***chapter 7*** we find the results of the research applied to the individual NLC systems, which include DSG, respectively wild yam extract (*Yam*), but also dual vegetable principles, NLC_DSG_*Yam*. The research includes the physico-chemical characterization of free and loaded nanotransporters, as well as the results of *in vitro* analyzes to evaluate the cytotoxic potential, to determine the antioxidant and anti-inflammatory activity.
o ***Chapter 8*** assesses the adaptability of new constituent lipid matrices of NLC (containing milk thistle oil and linseed oil, associated with glyceryl monostearate and cocoa butter) to

host DSG, an extract of Polygonum cuspidatum (*PCus*, high Resveratrol content), respectively mixture of the two active principles. Newly formed systems were characterized dimensionally, morphologically and structurally. The research is complemented by the results of *in vitro* analyzes, e.g., cytotoxicity determination, release profile study, release profile study, antioxidant activity and anti-inflammatory effect.

o The synthesis and physico-chemical characterization of some nanocarrier systems that co-opt two other categories of plant active principles, diosgenin and black cohosh extract (Cymicifuga racemosa, *CymR*) are reunited in ***chapter 9***. The chapter also covers aspects associated with the evaluation of the cytotoxic potential of NLC, as well as the *in vitro* determination of antioxidant and anti-inflammatory properties.

o ***Chapter 10*** includes the final conclusions and the main original contributions.

The thesis ends with the enumeration of bibliography corresponding to the documentary research and that of original research, the list of abbreviations, figures and tables. The results of the research were capitalized by publishing the original results in 4 ISI-listed international journals, which cumulate a FIC = 16,144, respectively, the participation in an international conference (with three representative papers, two in the form of a poster and an oral presentation). In addition, a patent application was also submitted for the patenting of these nanotransporter systems, capable of co-opting several bioactive plant principles (A 2021/00220/29.04.2021).

II. Explanation of the research topics. Thesis aims

Given the current global trends, the use of herbal products to provide natural bioactive ingredients that exhibit multiple therapeutic effects and diminished or even non-existent side effects, opens new perspectives in pharmacotherapy of various diseases. Therefore, in the present thesis entitled „Nanoencapsulations of nutraceuticals in nanostructured lipid systems for the development of advanced food supplements", it was targeted the encapsulation in various lipid matrices of several types of active, lipophilic and hydrophilic principles to obtain distribution systems designed to increase the therapeutic efficiency of the compounds captured in the lipid core and/or/or in the hydrophilic shell, created by surfactants. The main objectives were:

I. Obtaining dual nanostructured lipid systems (multiple therapeutic response NLCs) by incorporating active vegetable principles that exhibit complementary and/or synergistic biological actions.

II. Morpho-structural characterization of the new nanostructured systems obtained, in order to demonstrate their efficiency and ability to co-opt both categories of vegetal, lipophilic and hydrophilic principles.

III. Comparative evaluation of *in vitro* therapeutic responses of NLC containing plant principles in order to identify the best therapeutic performance, by means of enhanced specificity and maximized antioxidant and anti-inflammatory actions.

5. Preliminary studies for NLC obtention

5.1. Identification of optimal composition for the obtention of stable NLC with preset dimensions

This chapter provides an overview study of the influence of the composition of a mixture of 3 surfactants and co-surfactants, hydrophilic and amphiphilic (Tween 20, Phosphatidylcholine and Poloxamer 188) in obtaining NLC with preset sizes. For the purpose of selecting optimal compositions of surfactants – co-surfactants – lipids, to ensure the formation of well-defined spherical nanoparticles with average diameters < 200 nm, several variations of the basic components were made (e.g., concentration and ratio between surfactants, vegetable oil type), but also of the operating parameters (e.g: number of homogenization cycles, homogenization pressure, number of *rpm*). Initially, the studies looked at varying the concentration of the mixture of surfactants by selecting two concentrations, 2% and 2.5%, as well as tracking the influence of the weight ratio between the three types of surfactants. As shown in Table 2, the predominant surfactant chosen was Tween 20 (Polisorbate 20/polioxyethylene (20) sorbitan monolaurate), with the specification that the mentioned % are reported to the total of 2% and 2.5% respectively, of the mixture of surfactants and co-surfactant used to obtain NLC (for 100g NLC aqueous dispersion). In addition to the mixture of surfactants and co-surfactants, a mixture of solid lipids (glyceryl monostearate (GMS) and cetyl palmitate (CP)) with liquid lipids was used to formulate the lipid core. The changes in the solid character of the lipid core was achieved by using two types of vegetable oils, evening primrose oil (for NLC_I), and, soybean oil (for NLC_II) with which 12 experimental variants were synthesized, detailed in Table 1. Subsequent to the synthesis, the nanotransporters were characterised in terms of mean lipid particle diameters and electrokinetic potential for stability validation over time.

Table 1. Different types of obtained NLC and composition in lipids, surfactans and co-surfactants

Crt.	NLC samples	Lipids, g				Surfactant and co-surfactant, g		
		GMS	CP	*I*	*II*	Tw20	Polx188	Fosf
Nanocarriers with evening primrose oil								
1.	NLC_I_2%_1	3.5	3.5	3.0	-	1.40	0.30	0.30
2.	NLC_I_2%_2	3.5	3.5	3.0	-	1.60	0.20	0.20
3.	NLC_I_2%_3	3.5	3.5	3.0	-	1.70	0.15	0.15
4.	NLC_I_2,5%_1	3.5	3.5	3.0	-	1.75	0.375	0.375
5.	NLC_I_2,5%_2	3.5	3.5	3.0	-	2.00	0.250	0.250
6.	NLC_I_2,5%_3	3.5	3.5	3.0	-	2.125	0.187	0.187
Nanocarriers with evening soybean oil								
7.	NLC_II_2%_1	3.5	3.5	-	3.0	1.40	0.30	0.30
8.	NLC_II_2%_2	3.5	3.5	-	3.0	1.60	0.20	0.20
9.	NLC_II_2%_3	3.5	3.5	-	3.0	1.70	0.15	0.15
10.	NLC_II_2,5%_1	3.5	3.5	-	3.0	1.75	0.375	0.375
11.	NLC_II_2,5%_2	3.5	3.5	-	3.0	2.00	0.250	0.250
12.	NLC_II_2,5%_3	3.5	3.5	-	3.0	2.125	0.187	0.187

Table 2. The ratios used between the 3 types of mixtures of surfactants and co-surfactants to optimize the process of obtaining nanotransporters

Crt.	Nanocarrier formulations	Tw20 (%, w/w)*	Fosf (%, w/w)*	Polx188 (%, w/w)*
1.	NLC_I/II_1	70	15	15
2.	NLC_I/II_2	80	10	10
3.	NLC_I/II_3	85	7.5	7.5

Note: *% of the total of 2% and 2.5% of the mixture of surfactants and co-surfactant used to obtain NLC (reported to 100g NLC dispersion)

Figures 1, 2 offer a narrow overview of the average particle diameters and potential zeta for synthesised systems with different surfactant concentrations (variable weight ratios, respectively, see Table 2), respectively distinct vegetable oils. The recorded results pointed out that a mixture of 2.5% surfactants and co-surfactant, found in a report, Tw20 : Polx188 : Fosf = 70 : 15 : 15, for both types of oils, evening primrose oil and soybean oil, it led to timely values, with average diameters below 150 nm, the degree of distribution of the lipid population is relatively narrow and high physical stability. In this manner:

- for NLC_I, Zave = 143.8 ± 1.74 nm, PdI = 0.23 ± 1.74 and ξ= -48.8 ± 0.95 mV,
- for NLC_II, Zave = 134.3 ± 1.65 nm, PdI = 0.18 ± 1.65 and ξ= -46.5 ± 0.35 mV.

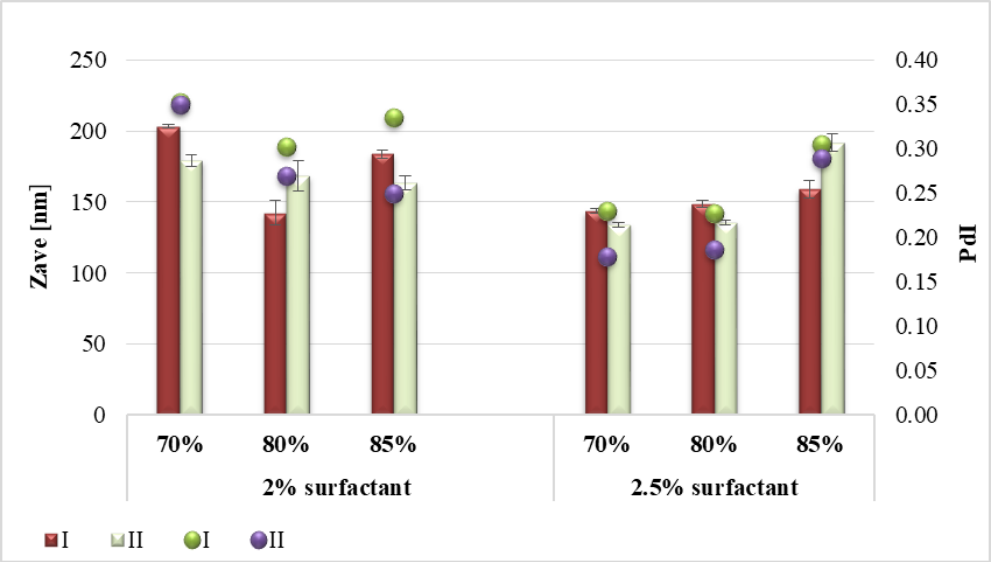


Figure 1. Comparative evaluation of Zave (■) and PdI (●) for NLC_I and NLC_II, prepared with 2% and 2.5% respectively mixture of surfactants and co-surfactant (different compositional reports, the majority being Tw20).

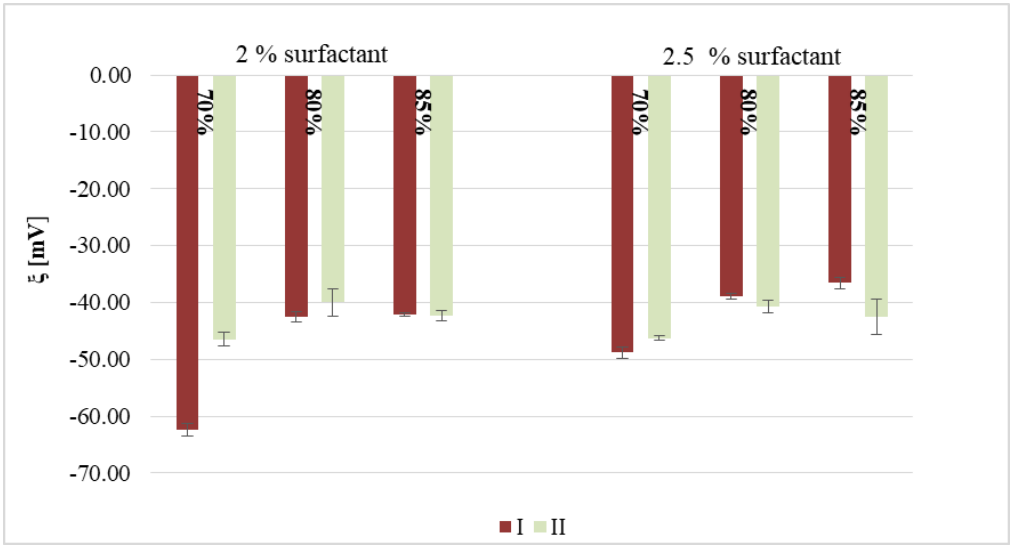


Figure 2. Comparative evaluation of the values ξ for the 3 compositions of the mixture of surfactants (which together cumulate a total of 2% and 2.5%, respectively), determined for the systems NLC_I and NLC_II

After identifying an appropriate composition with respect to the lipid core stabilized by the mixture of surfactants and co-surfactant, the optimization process continued with the modification of process parameters. Thus, they have undergone variations, 2 parameters in the process of high pressure homogenization: homogenization pressure (400 and 500 bar) and the number of cycles (4, 6, and, 8 homogenization cycles), respectively the change in the number of rotations/minut (rpm) (8,000 ÷ 17,000) in the shear homogenization process (HSH), respectively, for effective bursting of lipid drops (Iordache, T.-A., 2022).

According to the graphs represented below, although applying a greater number of homogenization cycles, e.g., 8 cycles leads to lower values of particle diameters, Fig. 3, the ideal values obtained by bringing together Zave – PdI – ξ , emphasized the superiority of the system characterized by applying 6 homogenization cycles to HPH processing, the, 500 Bar (corresponding to 3 min HPH processing time and 20 sec.); Zave = 180.9 ± 0.95 nm, PdI = 0.27 ± 0.02 and ξ > - 48 mV.

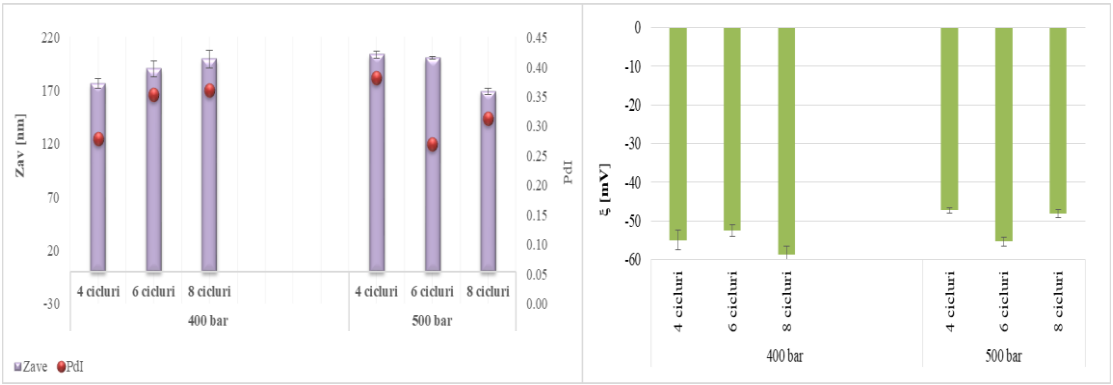


Figure 3. Comparison of Zave and PdI values obtained by varying the number of homogenization cycles and pressure change (processing stage by HPH); Electrokinetic potential variation

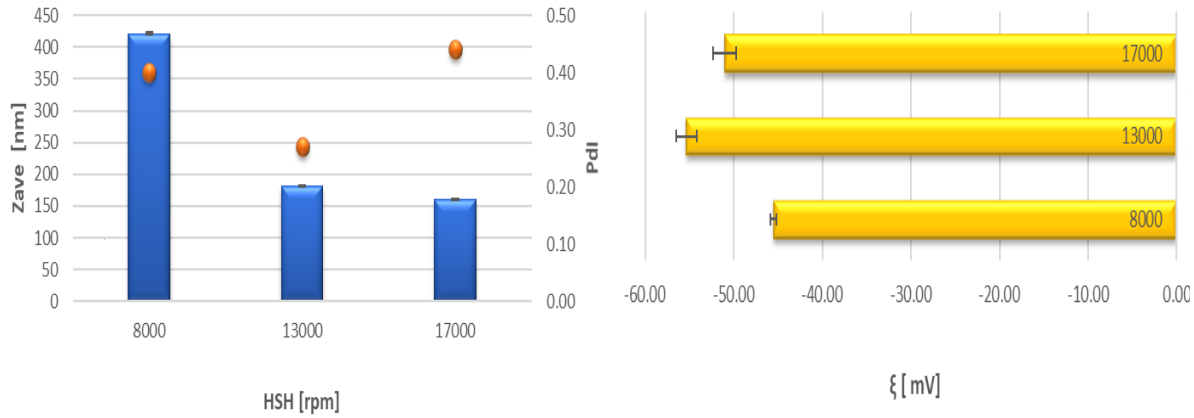


Figure 4. Mean particle size, polydispersity indices and electrokinetic potentials obtained from the variance in the number of *rpm* (processing stage by HSH)

Tracking the influence of *rpm* suggested a significant decrease in *Zave* as the number of rpm increased from 8,000 to 17,000 rpm. Despite the fact that the smallest average diameter was obtained in the case of the lipid system subjected to 17,000 rpm, however, the increase in the polydispersity index (PdI > 0.4) suggests the existence of a wide range of distribution of lipid nanotransporters, namely the detection of a high degree of polydispersity, which leads to the instability of the systems. This latter aspect, also correlated with the zeta potential value (Fig. 4) reinforces the idea of HSH operation in later stages, at a maximum value of 13,000 rpm (Iordache, T.-A., 2022).

5.1. Conclusions

By summarizing the information obtained in the process of optimizing the composition and the basic operational parameters, the ideal option for obtaining the subsequent lipid nanocarrier systems involves: i) the use of a mixture of 2.5% surfactants and co-surfactant (found in a compositional ratio of Tw20 : Polx188 : Phosph = 70 : 15 : 15, report to 100 g NLC aqueous dispersion); ii). operation in the HSH processing stage at 13,000 rpm (for 1 min.); iii) 6 homogenization cycles (corresponding to 3 min. time and 20 sec.), at 500 bar, for the HPH processing stage.

Chapter 6. The complex role of NLC encapsulating two individual/dual vegetable principles, DSG and *GlyG*, in enhancing therapeutical efficiences

6.1. Synthesis and characterization of NLC_I/II_DSG and NLC that co-opt DSG and *GlyG*

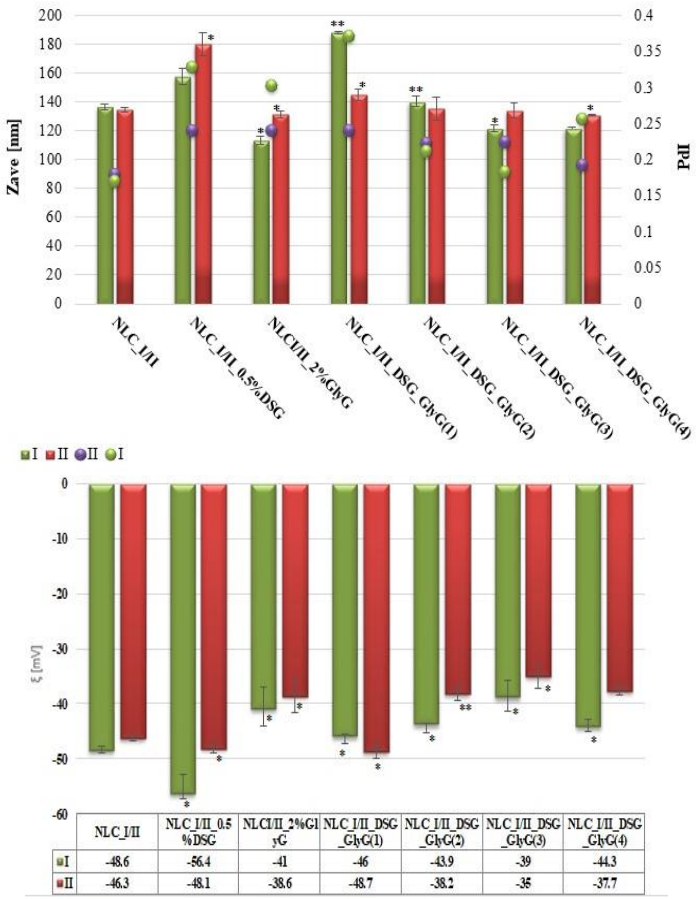
Within this chapter, the aim is to develop lipid nanotransporters containing different bioactive principles from plants (phytochemical mixtures selected from two categories of lipophilic and hydrophilic principles), and demonstration of their therapeutic effectiveness. The two vegetable principles of lipophilic and hydrophilic nature, co-opted in the same nanostructured lipid system are diosgenin, DSG (natural active, lipophilic, from wild yam extract, sp. Dioscorea villosa) and licorice extract (hydrophilic vegetable mixture, standardized in 10% glycyrrhizic acid.

The synthesis involved the existence of two phases, *an aqueous phase* (including surfactants and hydrophilic active principles, licorice extract/GlyG) and *a lipid phase* (containing the lipid mixture and the lipophilic, diosgenin/DSG principle), obtaining an emulsion (melting emulsion), and subsequently, its processing by high shear homogenization (HSH) and high pressure homogenization (HPH), 14 nanotransporter systems (Table 3) were obtained. The systems were characterized by the variation of the average diameter of the particles, the range of their size distribution, as well as the zeta potential.

Table 3. Lipids, surfactants and active principles from the composition of lipid nanocarriers with DSG, *GlyG*, respectively DSG and *GlyG*

Crt.	Lipid nanoformulations	Lipids, g				Surfactants, g			Active principles	
		GMS	CP	I	II	Tw 20	PC	Polx 188	DSG, g	*GlyG*, g
1.	**NLC_I**	3.5	3.5	3.0	-	1.75	0.375	0.375	**-**	**-**
2.	**NLC_I_DSG**	3.5	3.5	3.0	-				**0.5**	**-**
3.	**NLC_I_*GlyG(4)***	3.5	3.5	3.0	-				**-**	**2**
4.	**NLC_I_ DSG_*GlyG(1)***	3.5	3.5	3.0	-				**0.5**	**0.5**
5.	**NLC_I_ DSG_*GlyG(2)***	3.5	3.5	3.0	-				**0.5**	**1**
6.	**NLC_I_DSG_*GlyG(3)***	3.5	3.5	3.0	-				**0.5**	**1.5**
7.	**NLC_I_DSG_*GlyG(4)***	3.5	3.5	3.0	-				**0.5**	**2**
8.	**NLC_II**	3.5	3.5	-	3.0				**-**	**-**
9.	**NLC_II_DSG**	3.5	3.5	-	3.0				**0.5**	**-**
10.	**NLC_II_*GlyG(4)***	3.5	3.5	-	3.0				**-**	**2**
11.	**NLC_II_DSG_*GlyG(1)***	3.5	3.5	-	3.0				**0.5**	**0.5**
12.	**NLC_II_ DSG_*GlyG(2)***	3.5	3.5	-	3.0				**0.5**	**1**
13.	**NLC_II_DSG_*GlyG(3)***	3.5	3.5	-	3.0				**0.5**	**1.5**
14.	**NLC_II_DSG_*GlyG(4)***	3.5	3.5	3.0	-				**0.5**	**2**

*Note: NLC_I – systems obtained with evening primrose oil; NLC_II – systems containing soybean oil



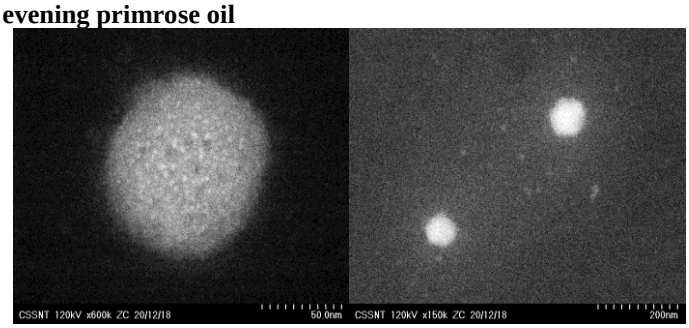
	NLC_I/II	NLC_I/II_0.5%DSG	NLCI/II_2%GlyG	NLC_I/II_DSG_GlyG(1)	NLC_I/II_DSG_GlyG(2)	NLC_I/II_DSG_GlyG(3)	NLC_I/II_DSG_GlyG(4)
I	-48.6	-56.4	-41	-46	-43.9	-39	-44.3
II	-46.3	-48.1	-38.6	-48.7	-38.2	-35	-37.7

Figure 5. Variation of Zave, PdI and zeta potential for nanocarriers encapsulating the two plant principles (*GlyG* and DSG), obtained with I/II vegetable oils;

The comparison between encapsulated nanotransporters with individual and dual plant active principles, respectively free ones, underlines that NLC_I/II has similar dimensions, ~135 nm and polydispersity indices around 0.17 – 0.18. Regarding encapsulated nanotransporters with vegetal active principles, the average lipid population sizes show a variation in the range 115 and 185 nm and polydispersity indices that are within the range 0.18 ÷ 0.37. The electrokinetic potential of the synthesized nanotransporters was found in the range - 39 ÷ - 56 mV for NLC_I, respectively - 35 ÷ - 48 mV for NLC_II. The high values underline the existence of a phenomenon of electrostatic repulsion, which prevents the occurrence of nanotransporters alteration phenomena, regardless of the nature of the encapsulated compound concerned.

Morphological analysis revealed the existence of distinct internal morphologies, depending on the type of vegetable oil used in nanotransporter synthesis. Thus, in the case of nanotransporters synthesized with soybean oil (Fig. 6B), spherical particles with diameters ranging from 50 ÷ to 180 nm were visualised, with no other changes in the internal structure, in contrast, for the second type of nanotransporter, the, in which evening primrose oil was used (Fig. 6A, inclusions of nanospheres with diameters < 5nm could be observed in the lipid core.

A. Dual NLC with evening primrose oil



B. Dual NLC with soybean oil

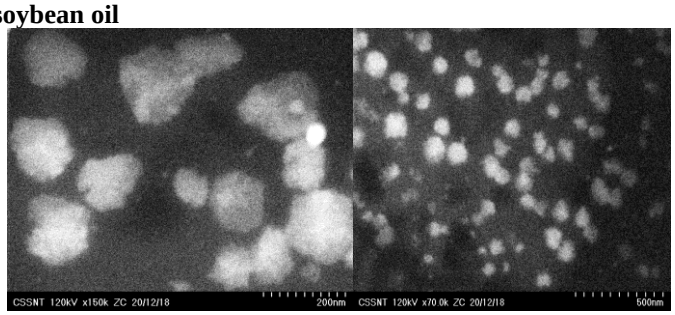


Figure 6. Morphological analysis of mixed nanotransporters obtained with the two vegetable oils

6.1.4. Structural evaluation of lipid core before and after the encapsulation of active principles

From the differential scanning calorimetry (Fig. 7) there are three lipid-appropriate endothermic peaks in the physical mixtures (GMS + CP + I and GMS + CP + II), 56.5 ÷ 62.5°C for GMS, respectively, 52.3°C for CP, respectively temperature range < 50°C, specific to unsaturated fatty acids from the vegetable oils composition. The same endothermic peaks were viewed in the NLC structure, but with a smaller amplitude and in a wider temperature range, 42 ÷ 60°C. This enlargement can be the cause of a much more complex and disorganized structure, which suggests the high compatibility between lipids used in the synthesis process and the amorphous structure of the lipid core. For NLC_I/II_*GlyG(4)*, a smaller widening of the endothermic bit is highlighted, which reinforces the idea of hydrophilic extract in the surfactant layer, which subsequently induces an almost insignificant alteration of the lipid core. At the opposite pole, the largest expansion of endothermic droplets was recorded for nanotransporter systems that encapsulate both types of active principles.

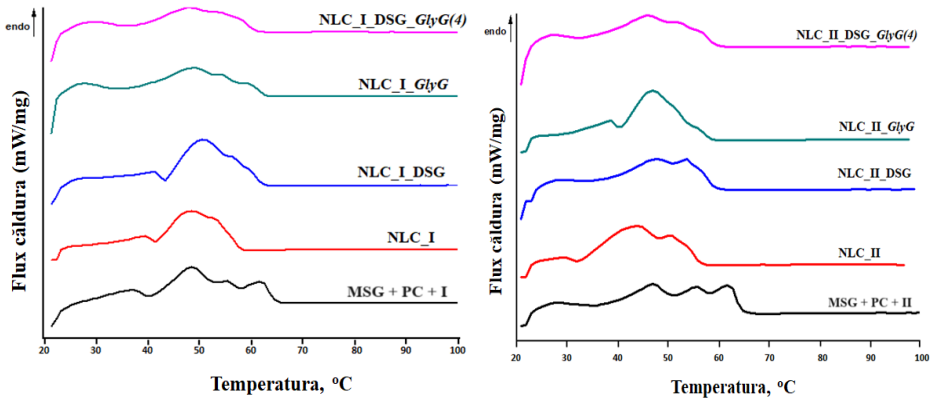


Figure 7. The comparative profile of thermal analysis (obtained by differential scanning calorimetry, DSC) for NLC_/NLC_II, free and those loaded with a single active principle, respectively mixture of DSG and *GlyG*

6.1.5. Determination of encapsulation efficiency and loading capacity with DSG and *GlyG*

The analysis of determining the encapsulation efficiency revealed the ability of the two types of nanostructured systems, synthesized with the two types of vegetable oils, I and II, to capture within the same nanocarrier system, both categories of bioactive compounds, with distinct affinities (compared to the lipid core, DSG or to the hydrophilic shell, created by surfactants, *GlyG*). Following the HPLC determinations, despite the hydrophilic nature of *GlyG*, very high encapsulation/capture (EE%) efficiencies have been achieved for glycyrrhizic acid, EE (NLC_I_*GlyG(4))*= 96.5% ± 0.57, were achieved, respectively EE NLC_II_*GlyG(4)*) = 95.8% ± 0.54. As regards the encapsulation efficiency of the lipophilic compound, the DSG, its affinity for the lipid mixture, in particular for NLC systems obtained with night light oil, is demonstrated by the determined values: EE NLC_I_DSG = 89.5 ± 2.55%, respectively EE NLC_II_DSG = 85.7 ± 3.38%.

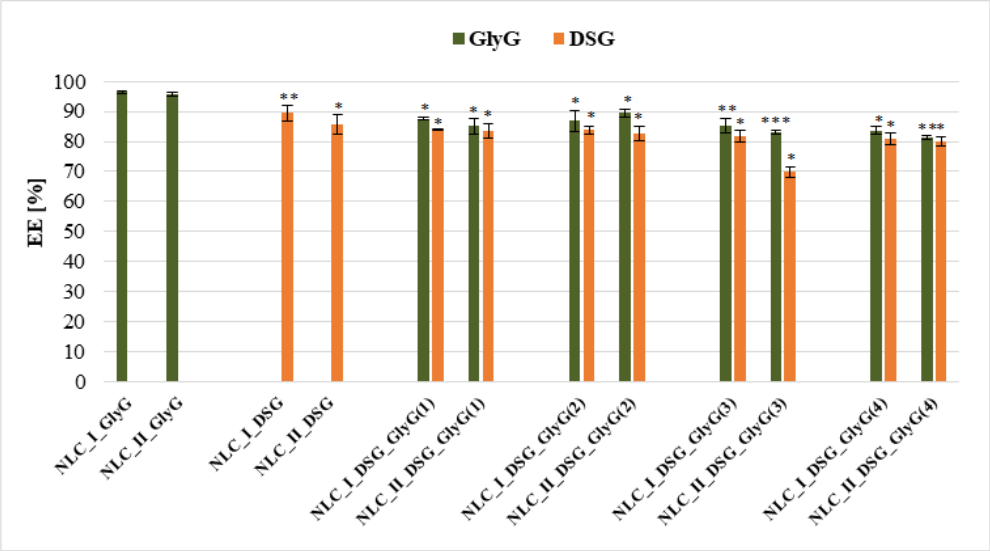


Figure 8. Values of encapsulation/capture efficiency of the active principles (GDS and glycyrrhizic acid) in NLCs prepared with I and II

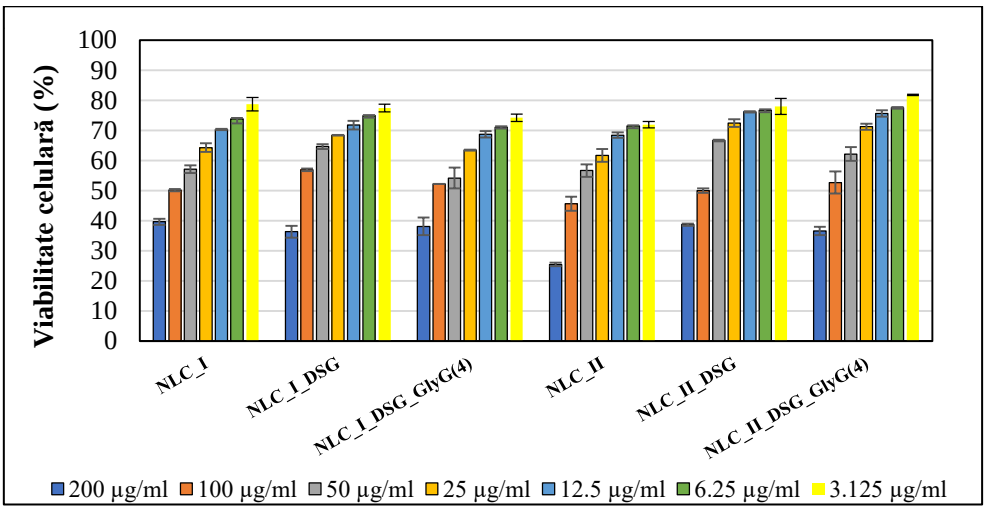
6.2. Evaluation of therapeutic potential of NLC_I/II_DSG, respectively of NLC_I/II_DSG_*GlyG(4)*

`6.2.1. *In vitro* evaluation of citotoxic potential (MTS and RTCA)

MTS technique consisted of treating HUVEC cell cultures for 24 hours and 48 hours with increased NLC concentrations, respectively, in the range of 3.125 ÷ 200 µg/ml. The results obtained showed that the viability of HUVEC endothelial cells depends on the applied NLC concentration, with a significant influence detected at concentrations of > 100 µg/ml. After the first 24-hour treatment, cell viability exceeded 65% at concentrations < 50 µg/ml, with a few exceptions, suggesting a low cytotoxic effect of NLC_I/II _DSG_*GlyG(*4). Extension of treatment by an additional 24 hours resulted in an improvement in cell viability, resulting in > values 80% in the concentration range 25 ÷ 50 µg/ml. This suggests the

presence of cellular regeneration phenomena, the most effective systems in terms of viability over cytotoxicity being dual NLCs; for example, for NLC_II_DSG_*GlyG(4);* for example, the survival rate of HUVEC endothelial cells was 81.9%.

A.



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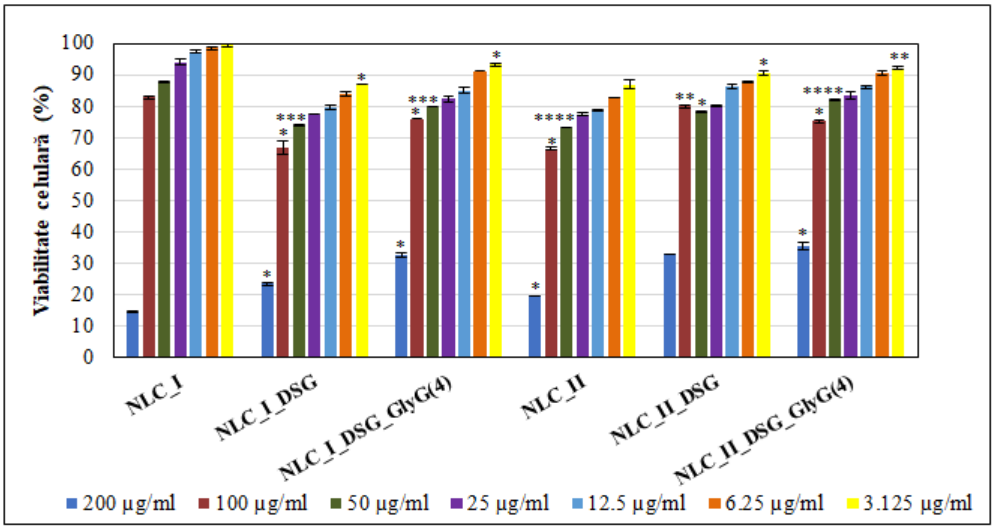


Figure 9. Assessment of the cytotoxicity of the obtained NLCs on the HUVEC cell line over a period of 24h (A) and 48h (B) respectively;

Real-time evolution of proliferation *vs.* cytotoxicity, generated by treatment with different concentrations of NLC (free and loaded with active principles, DSG and *GlyG*) was tracked by RTCA analysis, characterized by measuring a cellular index at any time, indicating the treatment time with the NLC concentration resulting in 50% viability/cytotoxicity (IC50). The use of this technique has served to identify a non-cytotoxic concentration range, ranging from 25 ÷ 100 µg/ml. IC50 values characteristic of mixed systems, NLC_I_DSG_*GlyG(4)*/NLC_II_DSG_*GlyG(4)* of 152.82 ± 2.14 and 159.92 ± 1.61 µg/mL pointed to the minimal cytotoxic effect they exerted.

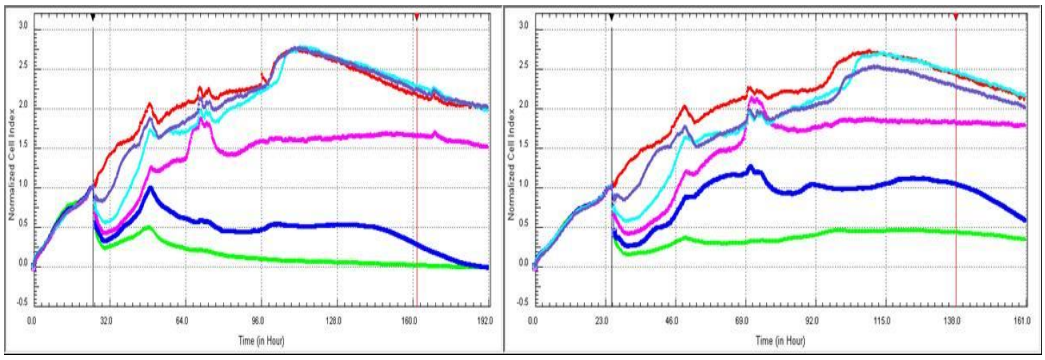


Figure 10. Cytotoxic *vs.* proliferative action induced by NLC_I/II treatment, free and loaded with DSG and *GlyG*, on normal endothelial cells (real time analysis, RTCA)

6.2.2. *In vitro* antioxidant activity

In the case of the spectrophotometric TEAC method aimed at inhibiting long-life radicals, for the NLC_I/II_*GlyG(4)* systems, an activity of inhibiting the cationic radical $ABTS^{\cdot+}$ of 64.38% ± 0.47, the, respectively 60% ± 0.87 was detected. On the opposite side are the systems synthesized with both types of oils and diosgenin, NLC_I/II_DSG, which exerts a modest inhibitory activity, of ~ 13.4%. However, despite this, by combining the two active principles within the same distribution system, a significant improvement is observed compared to free nanotransporters, NLC_I/II.

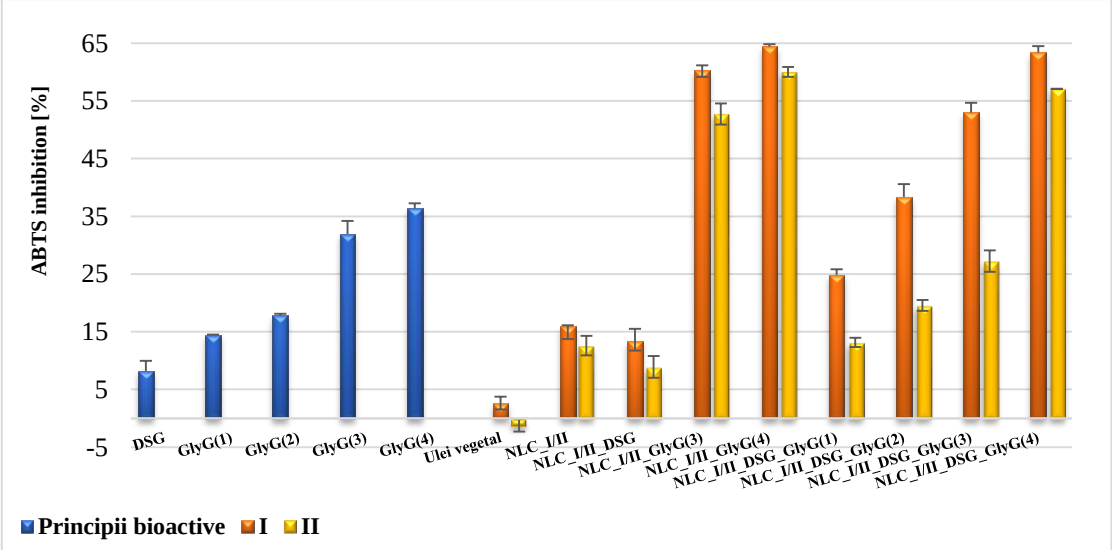


Figure 11. Variability of the inhibition capacity of the ABTS radical by NLC systems containing a single active principle (GlyG or DSG) vs. those which co-opt both active principles (*GlyG* and DSG);

As shown in Figure 12, although individual capture of DSG and GlyG in nanotransporters produces a moderate antioxidant effect, their simultaneous co-opting leads to a noticeable increase in the ability to capture oxygenated free radicals, reaching values of ~98%. If for free nanotransporters, NLC_I/II, the antioxidant activity values were around 59%, for NLC_I/II_DSG_GlyG(2, 3, 4), the ROS radical capture interval showed values between 91 ÷ 96%.

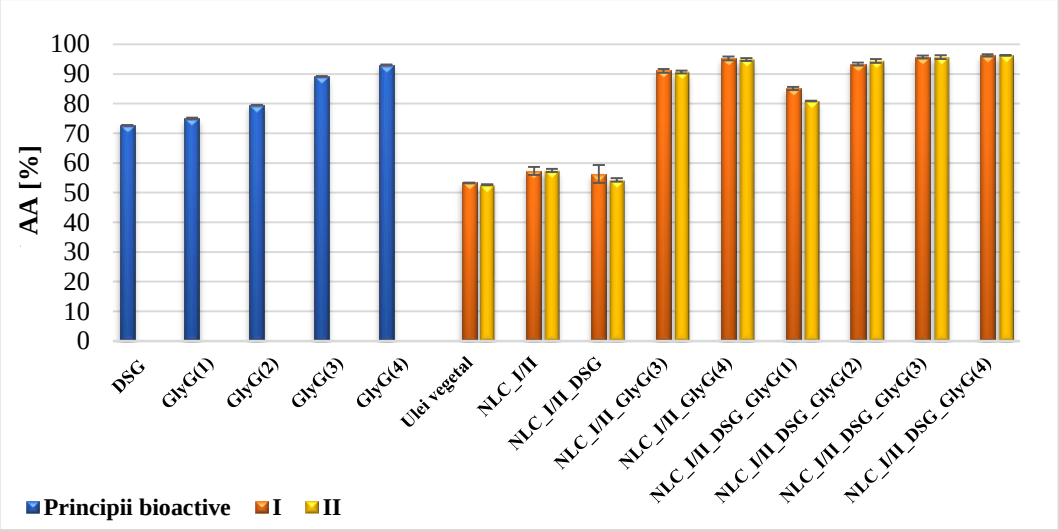


Figure 12. *In vitro* antioxidant activity of nanostructured systems, by chemiluminescence technique;

6.2.3. *In vitro* release studies of DSG and *GlyG* from lipid nanocarriers

The results obtained by studying the release profiles of the two bioactive components from nanotransporters revealed that for both categories of nanotransporters containing GlyG, NLC_I/II_GlyG(4), NLC, the release of glycyrrhizic acid occurred completely after 5h. The explanation lies in the preferential distribution of hydrophilic phytocompounds from the extract/GlyG to the surface of the NLC. Diosgenin, too, showed a much slower release trend, 50%, in a 5h interval, as the release of glycyrrhizic acid was achieved in maximum percentage, in the same interval. Slower release of GSD from NLC is directly influenced by DSG uptake into the nanocompartments in the core of the lipid nanospheres and by low solubility to the receiving environment.

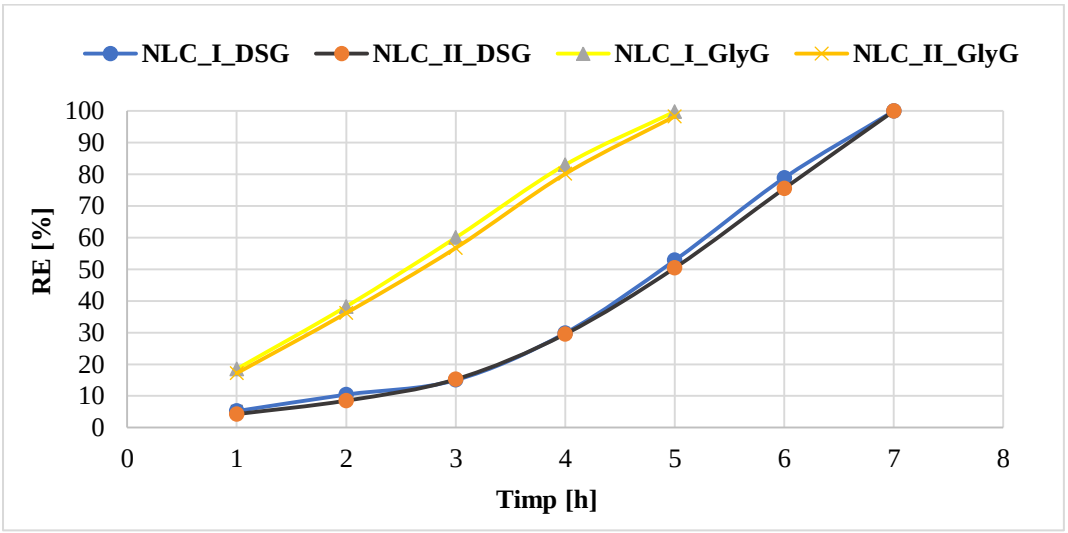


Figure 13. Controlled release profile of the active principles of individual nanotransporters, NLC_I/II_DSG/*GlyG(4)* of the two active principle categories, DSG and *GlyG*

In the case of dual NLC systems, NLC_I/II_DSG_*GlyG(4)*, a slower release of both lipophilic and hydrophilic bioactive components, e.g. DSG in 8h, and *GlyG* in 7h in NLC_I_DSG_*GlyG(4)*, Fig. 14.

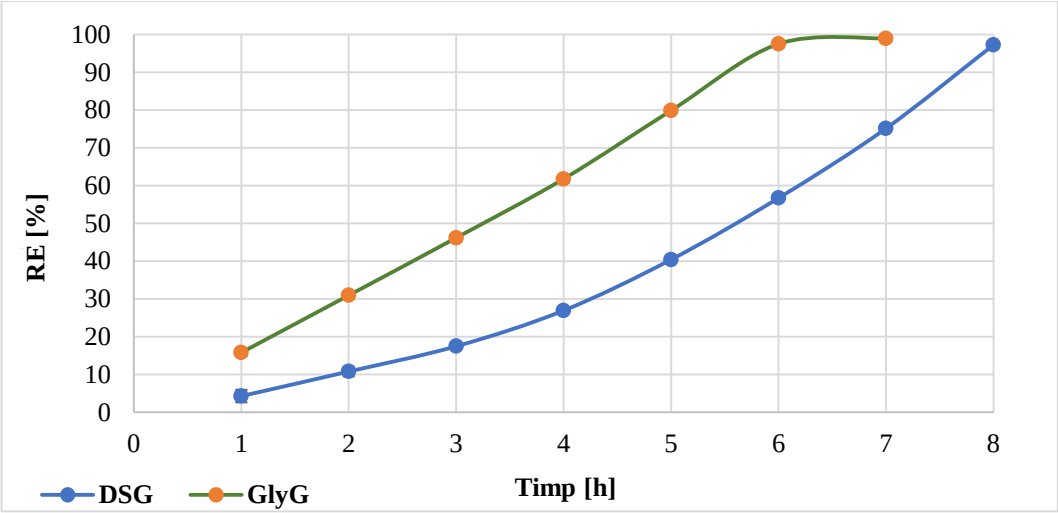


Figure 14. The release profile of the active principles of the mixed nanotransporter: NLC_I_DSG_*GlyG(4);*

6.2.4. *In vitro* assay of anti-inflammatory action

In vitro results from ELISA analysis, which evaluates the expression of proinflammatory cytokines TNF-F-6 and IL-6, after treatment of normal HUVEC cells with the two NLC concentrations (50, 200/ml), it showed the appearance of a strong anti-inflammatory effect, which was dependent on both the applied dose and the type of vegetable active principle encapsulated in NLC. Treatments with 200 µg/ml NLC resulted in a decrease in TNF-and IL-6 inflammatory markers compared to the 50 µg/ml dose, where there was a significant increase in the percentage of inhibition of both proinflammatory cytokines released following treatment. By comparing the anti-inflammatory effect of NLC on the release of cytokine TNF versus interleukin level 6 (IL-6), a significant difference was detected between percentages of IL-6 and TNF-F, respectively, much more effective is the inhibition of TNF-release, which has values of over 70% in the case of NLC loaded with dual principles.

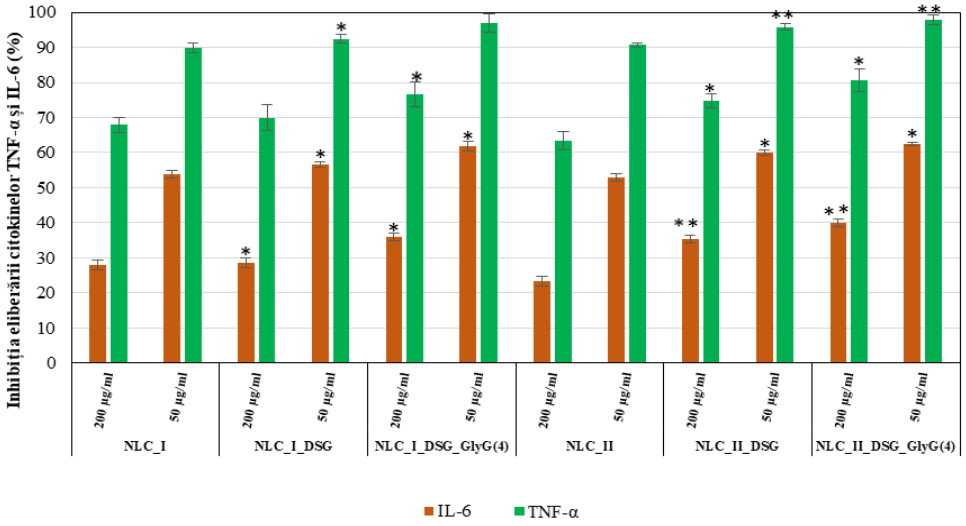


Figure 15. Evaluation by ELISA technique of the effect of NLC_DSG and NLC_DSG_*GlyG* treatments on the release of TNF-Cytokine compared to IL-6 by normal HUVEC cells

6.3. Partial conclusions

Following the studies carried out in this chapter of experiments, research aimed at combining diosgenin and Glycyrrhiza glabra extract with the two vegetable oils, similar values were obtained for the diameter of lipid particles, for both categories of free NLC_I/II nanotransporters.. In the case of those loaded, those with GlyG recorded slightly more opportune values. Physical stability was found to be superior in nanotransporters with night light oil over those with soybean oil.

The preferential distribution of the two bioactive principles was supported by the considerations resulting from differential scan calorimetry and in vitro release studies (DSG being captured in the lipid phase, while GlyG showed affinity for the hydrophilic coating, generated by surfactants). Chromatographic analysis highlighted the remarkable abilities of lipid nanotransporters to capture both types of plant principles, the efficiency of which proved to be more than 80% for DSG and close to 90% for *GlyG*, respectively. Safety in use and reduced toxicity were confirmed by MTS and RTCA techniques, with concentration range 25 ÷ 100 100 μg/mL proving non-toxic in HUVEC cell line treatments.

Both categories of co-encapsulated nanotransporters have demonstrated significant skills of annihilation/combating both types of radicals, long-life cationics (ABTS+), 63.4% ABTS•+ and short life oxygenated radicals (ROS), AA% ranging from 91 ÷ 96%.

The anti-inflammatory effect of the developed NLC was dose-dependent: treatments at 50/mL levels result in greater inhibition of IL-6 and TNF-alpha cytokine release compared to 200/gF/ml. As an example, TNF inhibition: 90.6% (NLC_II) versus 97.9% (NLC_II_DSG_GlyG(4)). For interleukin IL-6, NLC_II led to a 52.8% inhibition of it as opposed to 62.5% in NLC_II_DSG_*GlyG(4)*.

Chapter 7. Challenges in co-opting in the same lipid nanostructured system of two bioactive principles (lipophilic and hydrophilic), with the aim of raising bioavailability

In this chapter it is envisaged to obtain nanotransporter lipid systems, stabilized with a mixture of surfactants, predominantly ionic, for simultaneous and efficient integration of steroidal saponin, diosgenin (DSG) and wild yam extract (*Yam*) in the lipid core, but also in the hydrophilic coating generated by the mixture of surfactants, in order to improve the ***in vivo*** bioavailability of the two active principles of plant origin.

7.1. Optimization of nanocarrier lipid systems for co-opting DSG and *Yam*

The optimization phase aimed to prepare various compositions of surfactants in order to tilt the balance towards a mixture of predominantly ionic surfactants. To this end, one of the surfactants used in the previous system, phosphatidylcholine (amphiphilic), was substituted for another, sodium (anionic) cholate. The choice of the optimal variant was made after the dimensional and surface loads assessment for three NLCs obtained by operating % variations between surfactants/co-surfactant, namely: Sodium colate: Tween 20 : Poloxamer188: *50:35:15 (A); 70:20:10 (B); 80:15:5 (C)*, using as experimental vegetable oil-demonstrativ, soybean oil (2), keeping the total percentage of 2.5% surfactate and co-surfactant mixture in the composition of the aqueous formulation.

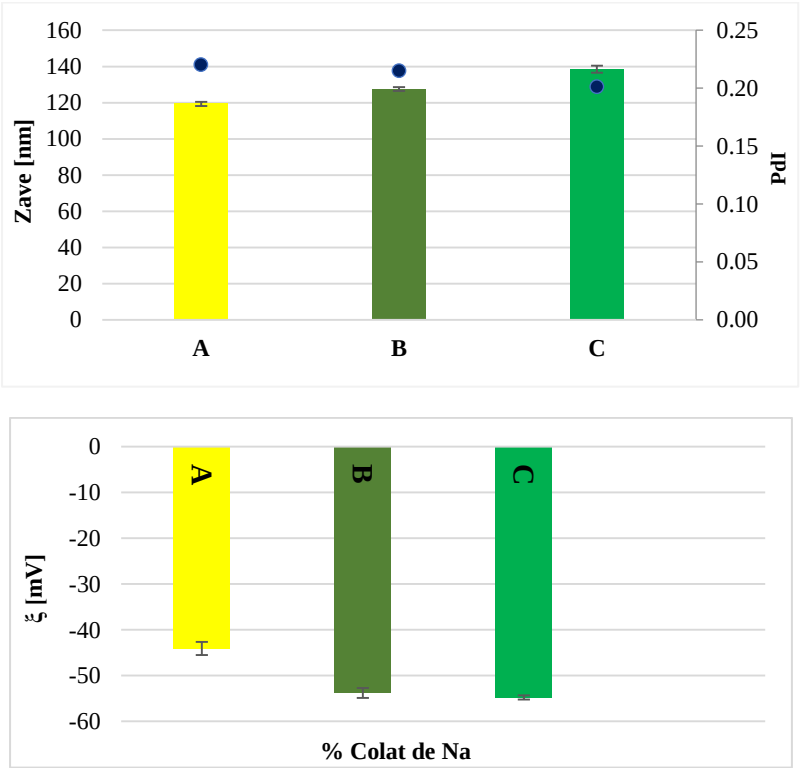


Figure 16. Change in mean size, polydispersity indices (●) and corresponding potential electrokinetics of lipid particles obtained by varying percentages between Tw20: ColNa : Polx188

In this manner, the average diameters 120 ÷ 140 nm, PdI ~ 0.2 and the electrokinetic potentials ranging between -43 ÷ -55 mV, pointed out the superiority of variant **A, 50:35:15** between Sodium Cholate: Tween 20: Poloxamer 188 for the synthesis of subsequent systems assembled in Table 4.

Table 4. Composition of synthesised nanocarriers, in terms of lipids, surfactants and active principles, DSG and *Yam*, g

Crt.	Nanoformulations	Lipids (10%), *g*			Surfactants blend (2.5%), *g*			Bioactive principles, *g*	
		GMS	CP	(1)/(2)*	Polx 188	Tw20	ColNa	DSG	*Yam*
1.	**NLC_1**	3.5	3.5	3	0.375	0.875	1.25	**-**	**-**
2.	**NLC_DSG_1**							**0.5**	**-**
3.	**NLC_*Yam*_1**							**-**	**0.5**
4.	**NLC_DSG_*Yam*_1**							**0.5**	**0.5**
5.	**NLC_2**							**-**	**-**
6.	**NLC_DSG_2**							**0.5**	**-**
7.	**NLC_*Yam*_2**							**-**	**0.5**
6.	**NLC_DSG_*Yam*_2**							**0.5**	**0.5**

Note: *(1)//2) – nanocarrier systems obtained with evening primrose oil oil/soybean oil

7.1.1. Stability over time of lipid coloidal dispersions. Morphological analysis of nanocarriers encapsulating DSG and *Yam*

Measurements targeting changes in lipid particulate evolution, size (Zave), polydispersity indices (PdI) and electrokinetic potential (ξ), were performed over a period of 2 months. Both categories of evening primrose oil and soybean oil systems recorded values of diameters < 142 nm, with a relatively narrow distribution of the lipid population, 0.21 - 0.28 and surface potential values in the range -40 mV ÷ -54 mV. However, systems prepared with soybean oil (2) have proven superior physical stability to those with evening primrose oil (1).

Regarding the morphological analysis, the electron transmission microscopy revealed the spherical shapes of the lipid particles, with diameters < 200 nm and at an advanced magnitude, the presence of nanospheres with dimensions below 5 nm, respectively, evenly distributed and homogeneous. These considerations suggest a unitary incorporation of the active principles into the nanocompartments created in the lipid core by vegetable oils.

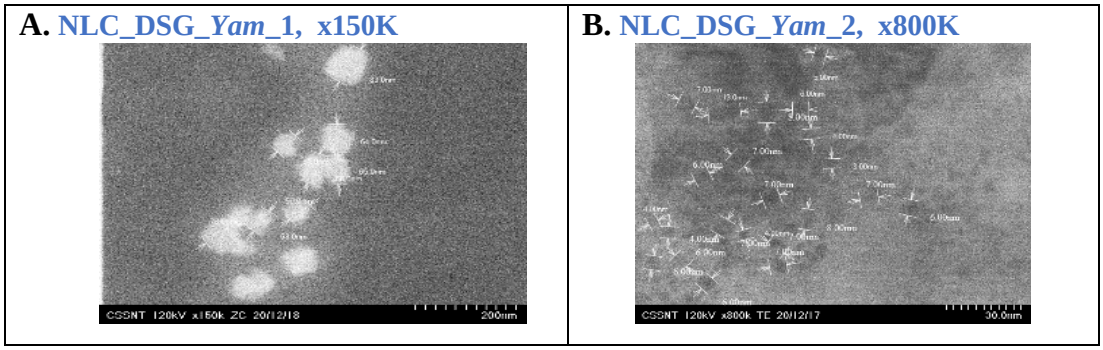


Figure 19. Morphological analysis of nanostructured systems with DSG and *Yam* in mixed systems

7.2. Thermal behavior of free and loaded nanocarriers using differential scanning calorimetry

According to the endothermic curves (Fig. 20) represented below, the two pure lipid mixtures have 3 endothermic peaks 48°C, 55°C and 62°C, component lipid caracetrics. The same endothermic peaks present in the physical blends were also found in NLC formulations, in the form of a wider range of melting range. This extension could be due to*: i.* changes in the lipid matrix generated by the presence of the 2 vegetable oils that produce an alteration of the crystalline structure of MSG and PC*; ii.* submicron dimensions of NLC_1 and NLC_2. This phenomenon can be attributed to the Gibbs – Thompson effect that the reduced particle diameter results in a drop in melting point due to improved free energy at the surface particle (Joudeh & Linke, 2022).

In dual systems, melting points decreased by approx. 1.5°C compared to free NLCs. Moreover, in the case of NLC_2 (Fig. 20), a significant narrowing of the melting range is observed, suggesting a more ordered lipid network, favored by the presence of unsaturated fatty acids in soybean oil. Within the same system, an amplification of the crystallinity degree was reported when capturing the two active principles.

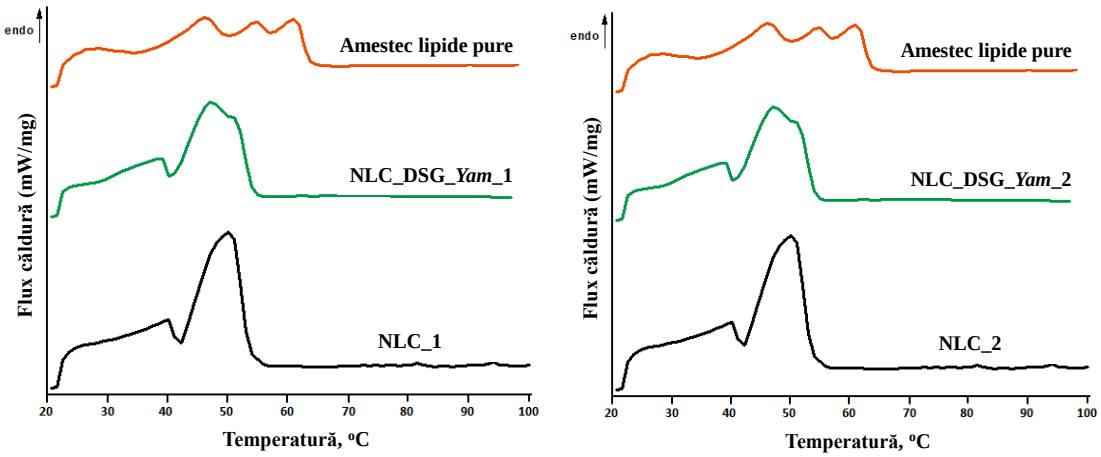


Figure 20. Calorimetric behaviour (via DSC) of lipid nanotransporters co-opting the two active principles (DSG and *Yam*) prepared with night light/soybean oil compared to NLC_1/NLC_2

7.3. Determination of encapsulation effciiency of the two active principles, DSG and *Yam*

The encapsulation efficiencies (EE%), > 80%, achieved for both types of systems, NLC_1 and NLC_2 emphasized the high compatibility of the mixture of lipids, surfactants/co-surfactant, with active principles selected in research. Altering the balance from predominantly non-ionic to ionic led to an increase in encapsulation efficiency. If for individual systems, encapsulation efficiencies for DSG and *Yam* highlighted values higher than 83%, for mixed systems, NLC_DSG_*Yam*_1/2 (EE *Yam* + DSG = 84% ± 3.54 in the case of NLC_DSG_*Yam*_1 and 81.2% ± 3.93 for NLC_DSG_*Yam*_2).

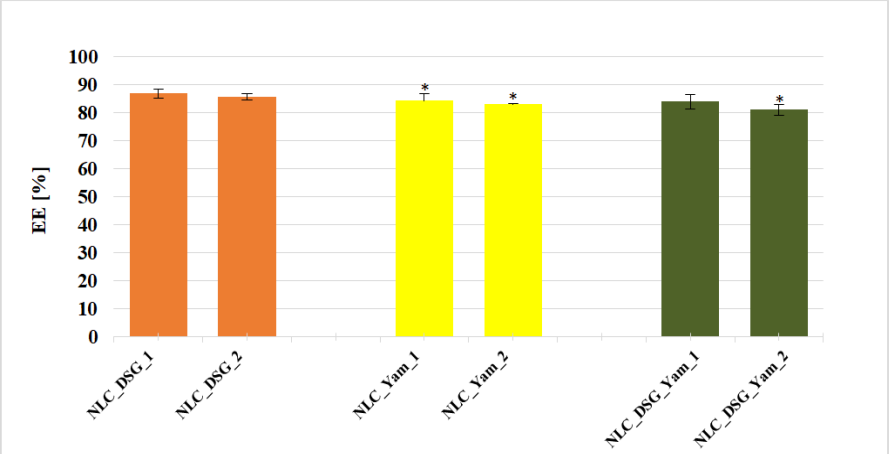
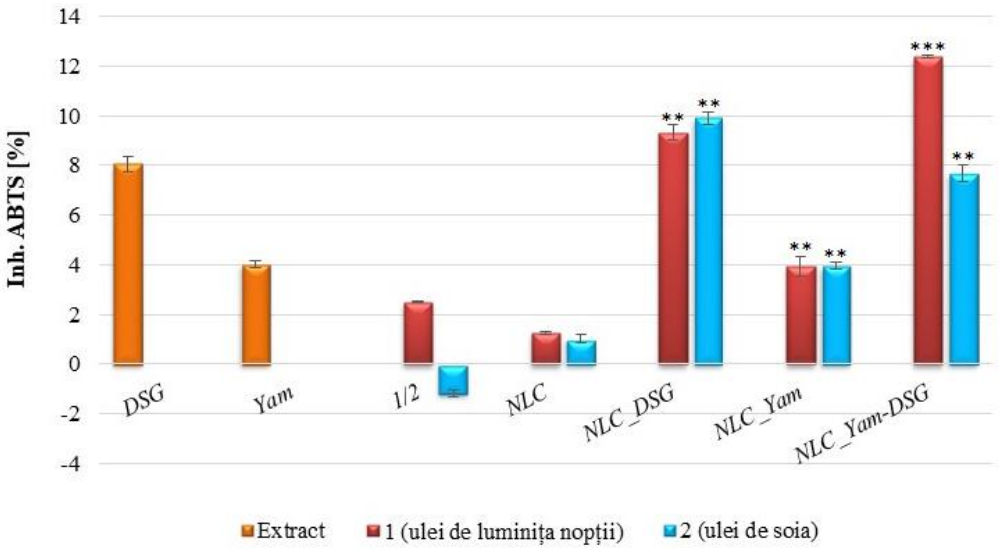


Figure 19. Encapsulation efficiency values for individual and mixed systems with DSG and *Yam*

7.4. *In vitro* assays, in proving the therapeutical properties of NLC_DSG_*Yam* systems

7.4.1. *In vitro* evaluation of antioxidant activity (TEAC and chemiluminiscence)

The results of experiments on the capacity of nanotransporter systems encompassing individual and dual active princes (DSG and *Yam*) to inhibit free radicals, they pointed out as a first aspect a low ability to inhibit ABTS cationic radicals, the maximum value being recorded for the NLC_DSG_*Yam*_1 system, 12.4%. Nanocapsulation has led to an increase in this activity by 1.5% for all categories of systems, (Fig. 22). By comparison, the oxygen free radical scavenging capacity proved superior, especially in the case of systems that simultaneously co-opt the 2 active principles, AA% values ranging between 82 ÷ 84% (ex. 82% ± 0.74 for NLC_DSG_*Yam*_2 and 84% ± 1.6 for NLC_DSG_*Yam*_1) (Iordache, T.-A., 2021).



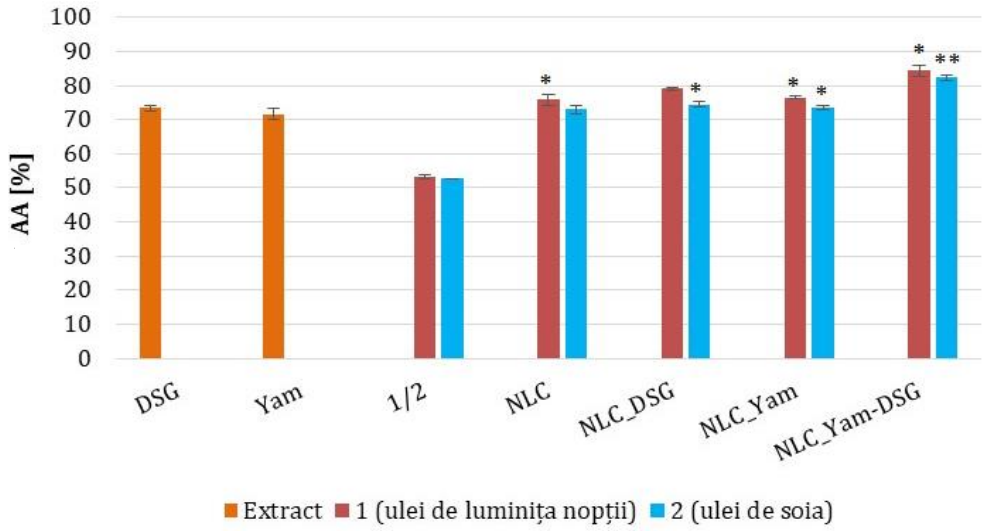


Figure 22. *In vitro* evaluation of the antioxidant activity of free and encapsulated NLC with vegetable active principles by TEAC and chemiluminescence mehods

7.4.2. Evaluation of *in vitro* controlled release of DSG from the NLC systems with DSG and *Yam*

Experiments that wanted to emphasize the release behavior of active principles co-opted in the same distribution system, pointed to a notable difference between the release profile of diosgenin in individual systems *vs.* dual ones. Thus, a high percentage of diosgenin released from the first hour was determined in the NLC-individual systems (e.g.: 10.86% in the NLC_DSG_1 system and 8.12% in the NLC_DSG_2 system), as well as in the NLC, compared to those containing both active principles where release has registered a sustained trend, during the 24 hours of study. Following the study, cumulative percentages revealed high values for DSG, 88.02 ± 1.68 % (NLC_DSG_*Yam*_1) and 85.5 ± 0.3% (NLC_DSG_*Yam*_2) (Iordache, T.-A., 2021).

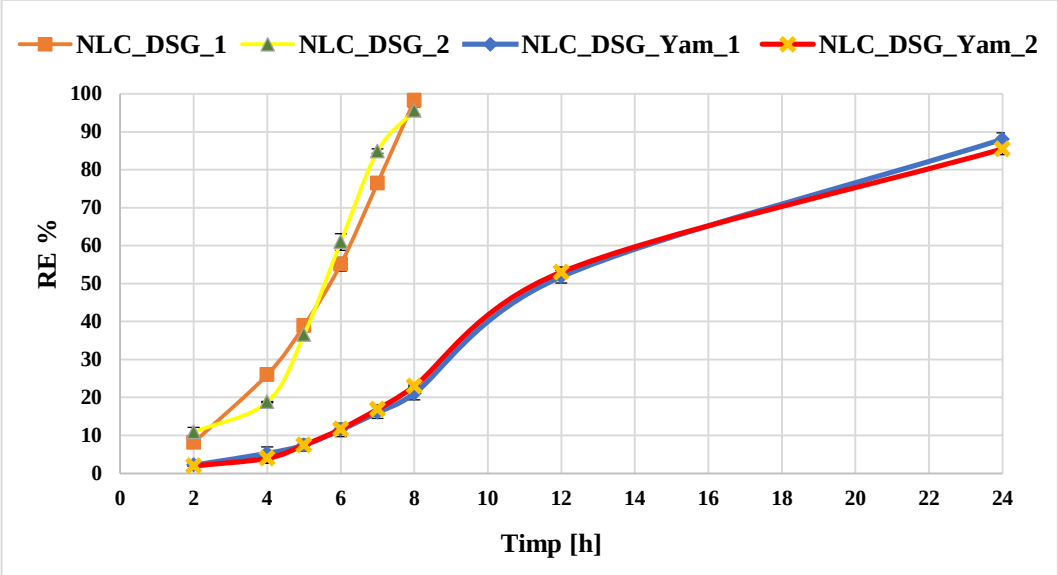


Figure 23. DSG release profiles from individual and dual systems (containing DSG, *Yam*, DSG and , respectively)

7.4.3. *In vitro* evaluation of citotoxicity of NLC systems

The viability of HUVEC endothelial cells was initially studied by MTS, spectrophotometric analysis.

Treatments with concentrations between 3 and 50 µg/mL ensure cellular viability that is maintained at > 70%, (Fig. 24). These results indicate a low cytotoxicity effect of the NLC_DSG_*Yam* system. By continuing treatment with an additional 24h, cellular viability saw an increase, > values 85% in the concentration range 50 ÷ 3.125 µg/mL, for the NLC_DSG_*Yam*_2 system. Increasing the survival rate of endothelial cells to prolonged treatment could be attributed to cell proliferation, a result of reduced toxicity of NLC, and, thus, the NLC_DSG_**Yam**_2 system can be classified as non-toxic for normal endothelial cells.

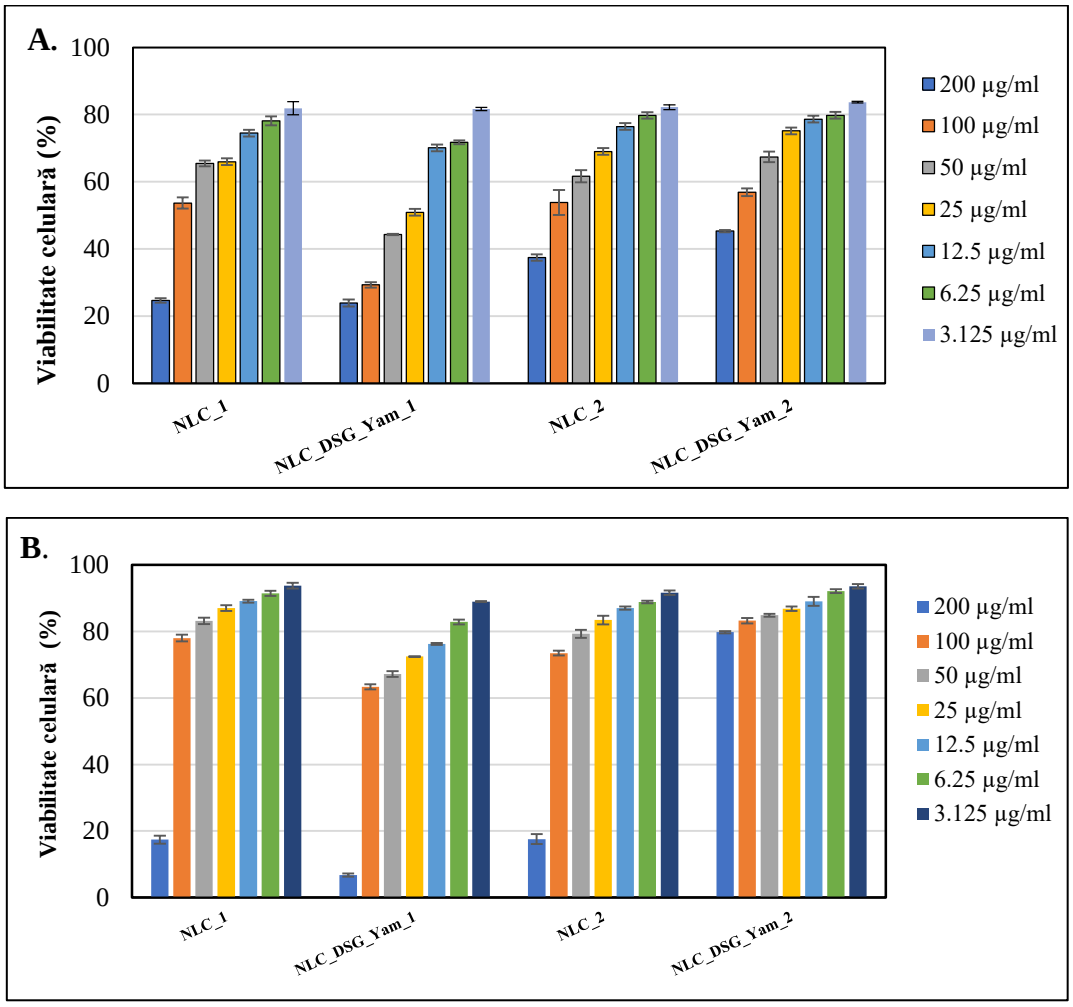


Figure 24. Effect of NLC_DSG_Yam on the viability of HUVEC normal cells for 24h (A) and 48h (B).

Results from the RTCA assay on HUVEC endothelial cells support data from the MTS analysis. Following the evaluation of cytotoxicity *vs.* proliferation of HUVEC endothelial cells treated with different concentrations of NLC, from 200 µg/ml to 25 µg/mL, it is observed that at high concentrations, 200 µg/mL, viability decreases significantly, indicating an increase in the cytotoxicity of NLC compounds at the respective concentrations (Fig. 25). Conversely, at concentrations below 100 µg/mL, cell viability is increased, comparable to untreated cells (control, red curve). The use of concentrations of 25 - 50 µL/mL NLC, with a

few exceptions, allows to obtain values comparable to those of untreated cells, indicating a lack of cytotoxic effects (Iordache, T.A., 2021).

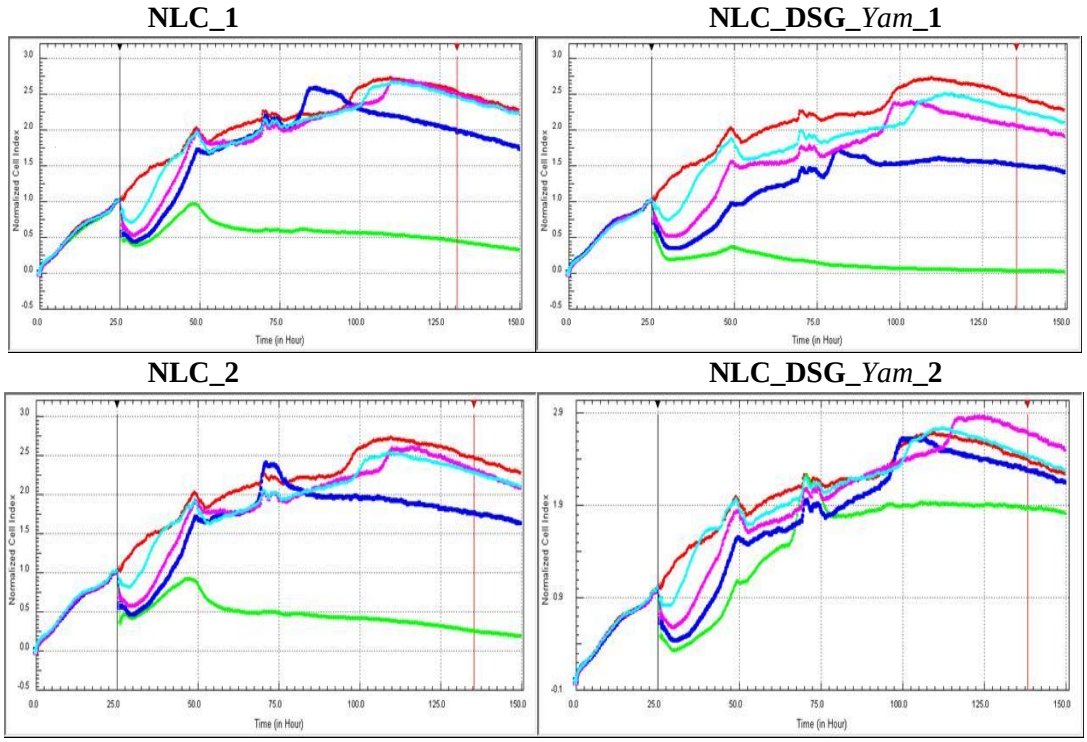


Figure 25. Cytotoxic *vs.* proliferative action induced by free NLC_1/2 systems and NLC_DSG_Yam_1/2 on normal HUVEC cells

7.4.4. *In vitro* determination of anti-inflammatory action of NLC systems

Assigning the potential anti-inflammatory effect of NLC_DSG_*Yam* systems was met by cuantifying the amount of proinflammatory citokines TNF-α and IL-6. It has been observed that by treating HUVEC cells with different concentrations of NLC, the production of proinflammatory cytokines TNF-α and IL-6 is strongly inhibited, the dose of 50 µg/mL of NLC being superior to 200 µg/mL, Fig. 26. This consideration can be explained by the results of the MTS and RTCA techniques that pointed the high cytotoxicity at high concentrations. High doses, 200 µg/mL produce cell denaturation that has a direct influence on proinflammatory cytokines release.

Another visible aspect is the more pronounced inhibition of TNF-α compared to IL-6, with all NLC analysed showing an inhibition of TNF-α greater than 80%, at a concentration of 50 µg/mL. In addition, by comparing the two types of NLC that contain evening primrose oil/1 or soybean oil/2, it could be seen that inhibition of the two cytokines is more pronounced with NLC_2 than with NLC_1. However, the most relevant example is the inhibition of cytokines produced by dual systems, NLC_DSG_*Yam*_1/2, which produce an inhibitory percentage of 98.2% ± 1.07 of TNF-α (NLC_DSG_*Yam*_2), respectively 90.42% ± 1.17 (NLC_DSG_*Yam*_1).

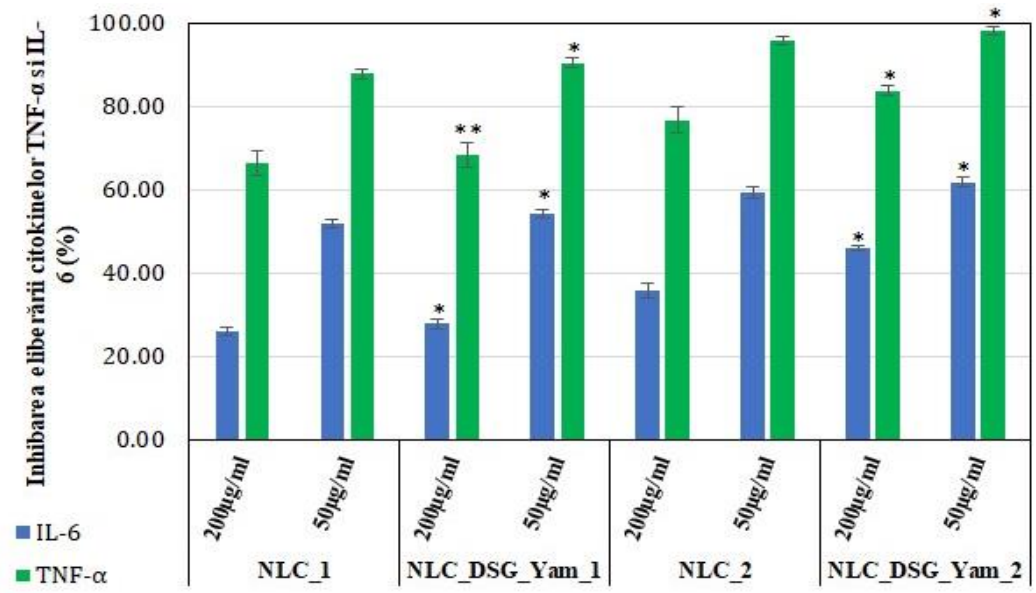


Figure 26. Evaluation by ELISA of the effect of NLC_DSG and NLC_DSG_*Yam* nanocarrier systems treatments on the release of TNF-α and IL-6 proinflammatory cytokines by normal HUVEC cells.

7.5. Partial conclusions

By using a mixture of 2.5% surfactants, predominantly ionic, in a compositional ratio of 50% ColNa: 35% Tw20: 15% Polx 188, the nanocarrier systems NLC_DSG_*Yam*_1 and NLC_DSG_Yam_2, have recorded high encapsulation efficiencies for DSG, timely physical stability, in terms of electrokinetic potential values, > -35 mV.

Secondly, although these nanocarrie systems possess a modest ability to capture long-life radicals, $ABTS^{\cdot+}$ (the maximum inhibition value is only 12.4%), the inhibition of ROS-type free radicals (generated by chemiluminescence), this ability is significantly improved (e.g. $AA_{NLC\_DSG\_Yam\_1/2} = 82 \div 84\%$).

The safety aspects of the obtained NLCs, monitored by *in vitro* MTS and RTCA tests, have shown that at concentrations below 50 µg/mL, no cytotoxic effects occur.

In the case of NLC_DSG_*Yam*_1/2 systems, a controlled release trend was noted, much slower compared to NLC_DSG, which extended over a 24-hour study period.

Regarding the anti-inflammatory effect of NLC_DSG_*Yam*, it can be noted that it was dependent on the dose applied to endothelial cells. Treatments with concentrations of 50 µg/mL resulted in greater inhibition of IL-6 and TNF-α cytokine release rather than 200 µg/mL. Simultaneous capture of natural principles within the same nanocarrier resulted in an enhanced anti-inflammatory effect, more pronounced in TNF-α inhibition: 95.9% (for NLC_2) versus 98.2% (for NLC_DSG_*Yam*_2).

Chapter 8. Influence of co-opting *Polygonum cuspidatum* with DSG in the same NLC, on antioxidant properties and anti-inflammatory effect

The research integrated in this chapter was aimed at preparing and characterizing nanostructured systems that integrate DSG and Polygonum cuspidatum extract (*PCus*) and monitoring the synergy of Polygonum cuspidatum extract in the presence of DSG, for its dual potential, anti-inflammatory and antioxidant.

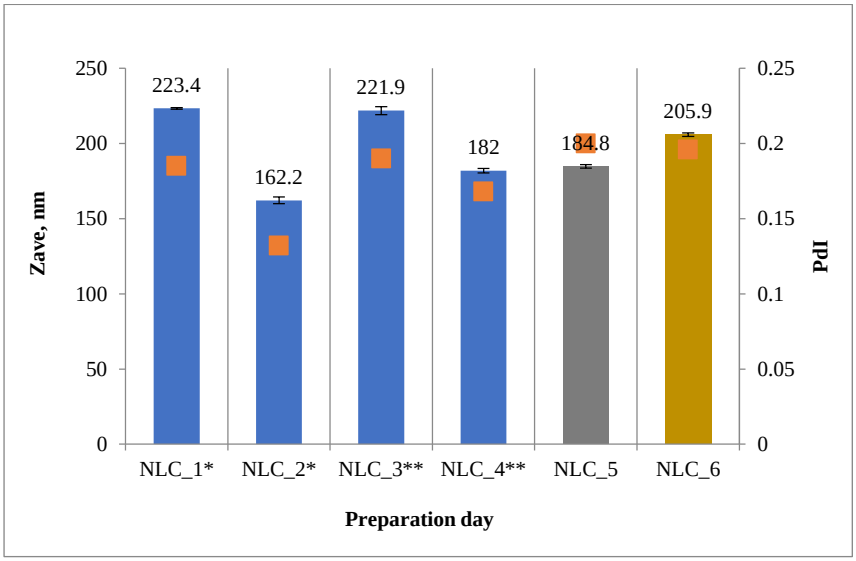
8.1. Improvement in the nanostructured systems composition

The need to ensure superior complementarity between the mixture of surfactants, lipids and active encapsulation principles led to the realization of various experimental variants that allowed to choose the ideal composition. Thus, a system of surfactants (Tween 20 and Span 80, in combination with Phosphatidylcholine or Poloxamer 188) was used in combination with a lipid mixture, glyceryl monostearate, and, cocoa butter and milk thistle oil (UAr), which led to the achievement of nanostructured systems, characterized dimensionally and in terms of electrokinetic potential.

Table 5.Composition of nanocarrier systems obtained with milk thistle oil

Nr. crt.	Denumire nanotransportori lipidici	Solid lipids, 10g			Surfactants, 2.5%			
		GMS	Cacao butter	UAr	Tw20	Sp80	Fosf	Polx188
1	NLC_1*	4	3	3	1.25	1.25	0	0
2	NLC_2*				1.7	0.8	0	0
3	NLC_3**				1.25	1.25	0	0
4	NLC_4**				1.7	0.8	0	0
5	NLC_5				1.59	0.72	0.19	0
6	NLC_6				1.59	0.72	0	0.19

Note: * Sp80 has been added to the aqueous phase; **Sp80 has been added to the lipid phase.



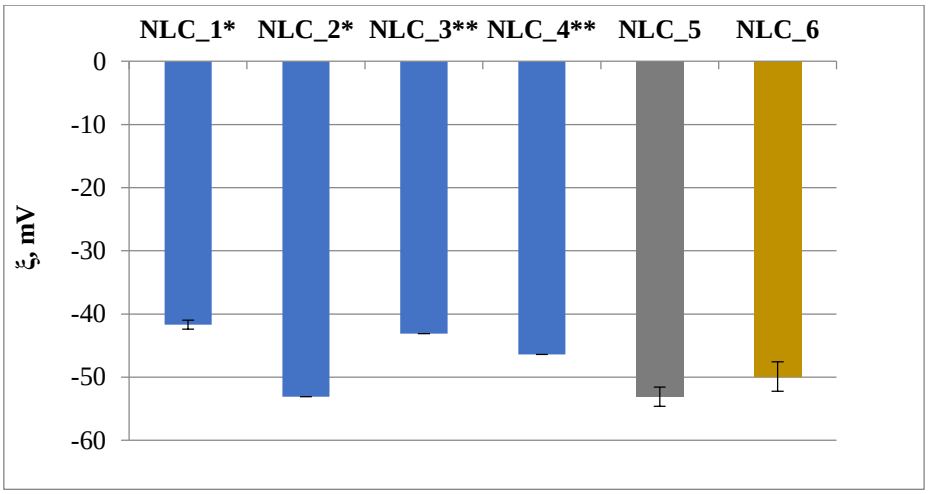


Figure 27. Comparative analysis of mean diameters, polydispersity indices (■) and electrokinetic potentials of NLC categories prepared using GMS lipid matrices, cocoa butter and UAr (DLS analysis)

Following the comparative analysis, the NLC_2 system resulted in obtaining optimal values in terms of Zave = 162.2 ± 2.196 nm, PdI = 0.132 ±0.015 and ξ = -53.1 ± 0.01 mV.

Subsequently, a variation was made based on the second vegetable oil, the linseed oil. Compositional variation for flaxseed oil samples considered the influence of co-polymer, poloxamer 188, and lecithin powder, on the size and stability of NLC formulations. The dimensional analysis carried out within 7 days highlighted that the most appropriate values were found in the NLC_7 variant: Zave =146.2 ± 0.15 nm, which was the most appropriate, PdI = 0.148 ± 0.013 and ξ = - 43.7 ± 1.35 mV.

Table 6. Composition of nanocarrier systems obtained with linseed oil

Crt.	Lipid nanoformulations	Solid lipids, 10g			Surfactants, 2.5%			
		GMS	Cacao butter	UIn	Tw20	Sp80	Lecitină pulbere	Polx188
1.	NLC_7	4	3	3	1.70	0.80	0	0
2.	NLC_8				1.75	0.65	0.1	0
3.	NLC_9				1.75	0.65	0	0.1

8.2. Preparation and morpho-structural characterization of NLC with DSG, *PCus* and mix of the two active principle

Utilizând compoziția optimă determinată în cadrul capitolului anterior, s-au sintetizat 8 categorii de nanotransportori, regăsite în tabelul 7.

Table 7. Detailed composition of individual/dual samples encapsulating both DSG and *PCus*

Denumire formulări nanostructurate	**Ingrediente formulări nanostructurate**						
	Lipide, g (10%)			Surfactanți, g (2.5%)		Principii vegetale active, g	
	MSG	**Unt de cacao**	**UAr/ UIn**	**Tw20**	**Sp80**	**DSG, g**	***PCus*, g**
Nanotransportori cu ulei de armurariu							
NLC_UAr	3.5	3.5	3	1.7	0.8	-	-
NLC_DSG_UAr						**0.5**	-
NLC_PCus_UAr						-	**0.2**
NLC_DSG_PCus_UAr						**0.5**	**0.2**
Nanotransportori cu ulei de in							
NLC_UIn	3.5	3.5	3	1.7	0.8	-	-
NLC_DSG_UIn						**0.5**	-
NLC_*PCus*_UIn						-	**0.2**
NLC_DSG_*PCus*_UIn						**0.5**	**0.2**

The lipid nanotransporters prepared with the two oils showed average diameters in the range of 150 ÷ 220 nm, with significant decreases in the NLC systems formulated with linseed oil. This may be due to the viscosity difference between the two oils or may be explained by the varying concentration in unsaturated fatty acids, corresponding to the two vegetable oils that can generate an internal rearrangement of the hydrophobic tails of the lipid core.

In terms of potential zeta, both for NLC systems where DSG, PCus, and those that co-opt both categories of bioactive principles, have been encapsulated, the values are between -46.5 mV and -64.2 mV. The strongly electronegative values of the electrokinetic potential demonstrate the existence of electrostatic repulsion phenomena between the aqueous suspended lipid particles, favorable situation for the prevention of coagulation and flocculation phenomena of lipid nanotransporters loaded with selected vegetable active principles. The existence of multiple pregnancies may be due to the distribution of free fatty acids (from the composition of the lipid mixture) at the oil/water interface by exposing the carboxyl groups to the surface of the nanotransporter.

Table 8. Dimensional parameters, Z*ave*, PdI and physical stability, determined for the 8 samples, individual and in mixture

Nr. Crt.	**Denumire nanotransportori (liberi și încărcați cu principii vegetale)**	**Z_{ave} ± stdev, nm**	**PdI ± stdev**	**ξ ± stdev, mV**
1.	**NLC_UAr**	170.1 ± 2.969	0.153 ± 0.026	-46.5 ± 0.400
2.	**NLC_DSG_UAr**	207.1 ± 3.400	0.169 ± 0.003	-47.8 ± 0.850
3.	**NLC_*PCus*_UAr**	184.2 ± 2.718	0.219 ± 0.004	-64.2 ± 0.720
4.	**NLC_DSG_*PCus*_UAr**	218.2 ± 0.888	0.114 ± 0.016	-54.8 ± 1.000
5.	**NLC_UIn**	150.5 ± 2.193	0.144 ± 0.004	-48.0 ± 1.600
6.	**NLC_DSG_UIn**	200.5 ± 1.715	0.171 ± 0.013	-47.9 ± 0.960
7.	**NLC_*PCus*_UIn**	164.3 ± 1.873	0.183 ± 0.014	-55.4 ± 2.210
8.	**NLC_DSG_*PCus*_UIn**	193.9 ± 2.951	0.152 ± 0.019	-52.8 ± 2.150

Morphological analysis (Fig. 28) performed on NLC-duali revealed the presence of nanotransporters with spherical morphology, with diameters ranging from 100 to 250 nm, without particle clumps. These diameters confirm the experimental results obtained by particle size analysis, DLS.

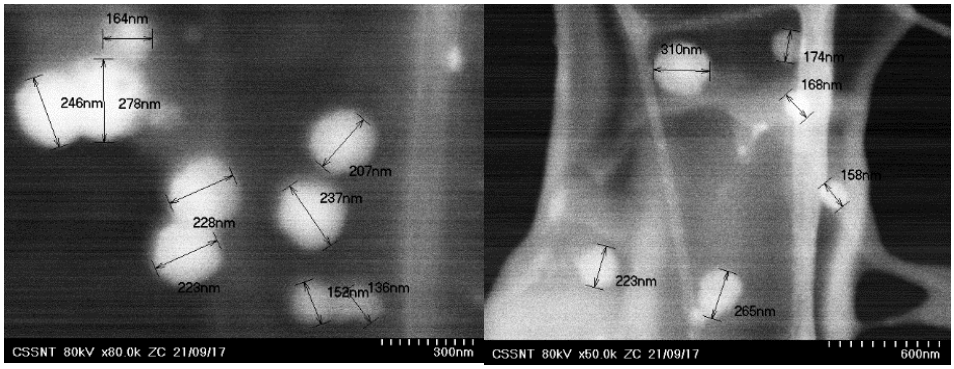


Figure 28. Morphological analysis of dual systems containing DSG *and* PCus, together with linseed oil (left) or milk thistle oil (right)

8.3. Structural alterations in NLC_UAr and NLC_UIn, following incorporation of active principles

The process of melting of lipids, part of the two lipid cores of NLC (preparations with UIn and UAr) takes place in an endothermic domain, corresponding to the temperature range, 60 ÷ 67 °C. According to the literature, MSG has a melting range ranging from 57 to 65 °C, while cocoa butter has an endothermic peak around 36°C. Cocoa butter, as is known, has 6 different polymorphic states, so a second bit visible between 28 ÷ 31.9 °C can signal its 5th form (Joshi, 2020). The same peak is found in nanostructured formulations, however, in a much wider melting field. These changes may be justified in addition to the compositional influence of cocoa butter, the formation of an imperfect internal structure by association with the two oils.

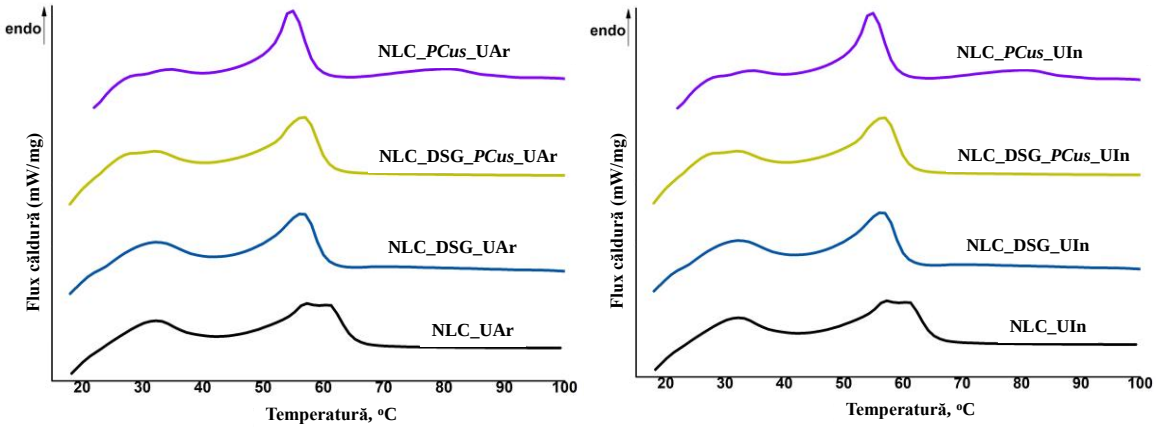


Figure 29. DSC analysis of individual and dual NLC prepared with UAr (left) and UIn (right), respectively

The NLC loaded with DSG and PCus led to a decrease in melting point (signed in the DSC of peaks ranging from 55 to 60 °C). Physical lipids (MSG, cocoa butter and UIn or UAr) had melting temperatures of 63.8°C and 62.7°C, respectively, while dual NLCs recorded

melting points of 57^{o}C, respectively, respectively 56^{o}C in NLC_DSG_PCus_UAr and NLC_DSG_PCus_UIn. The decrease in melting point, correlated with the decrease in the diameter of lipid particles, is widely debated in literature, e.g. Gokce et al. when preparing SLN-resveratrol (Gokce, 2012). The lipids brought to the nanoscale have a melting point below the melting temperature of the physical lipids (for example, a decrease in p.t. by approx. 1-5^{o}C), due to the higher ratio between the specific surface area and the volume of particles with a smaller size (Joudeh & Linke, 2022).

8.4. Encapsulation efficiency of the 2 bioactive principles associated in nanocarriers, individual and dual ones

The results of the quantitative determinations carried out by HPLC and UV-Vis revealed the higher affinity of diosgenin for the lipid mixture formed by the two solid lipids with flax oil, e.g: EE $_{NLC\_DSG\_UIn}$ = 91.64% ± 0.30, versus EE $_{NLC\text{-}DSG\_UAr}$ = 86.6% ± 2.50). A possible explanation can be attributed to the better solubility of DSG lipofil in linseed oil. Regarding the uptake of *PCus*, an influence of the oils used on the % encapsulation efficiency could be observed (ex: 64.10% ± 1.50 (NLC_PCus_UIn) versus 73.00% ± 1.30 (NLC_PCus_UAr)).

Table 9. EE% of individual and dual systems containing DSG and *PCus*

Crt.	Nanostructured formulations	Encapsualted active principles, % ± stdev	
		PCus	**DSG**
1.	**NLC_DSG_UAr**	0	86.59 ± 2.50
2.	**NLC_DSG_UIn**	0	**91.64 ± 0.31**
3.	**NLC_*PCus*_UAr**	73.0 ± 1.30	0
4.	**NLC_*PCus*_UIn**	64.1 ± 1.50	0
5.	**NLC_DSG_*PCus*_UAr**	63.3 ± 3.71	80.98 ± 0.15
6.	**NLC_DSG_*PCus*_UIn**	56.6 ± 1.21	**86.98 ± 1.10**

8.5. In vitro controlled release studies of DSG and a *PCus* from NLC_DSG, NLC_*PCus*, respectively NLC_DSG_*PCus*

In vitro release studies conducted by the dialysis bag method have pointed to the distinct distribution of the two active principles in the structure of the lipid nanocarrier. This recital applies both to mixed systems, Fig. 30A, as well as individual ones, Fig. 30B. Thus, the hydrophilic compound, *PCus*, has a much faster release profile than the lipophilic principle, DSG. Starting from the first hour of the release study, 15.3% and 17.7% resveratrol percentages were determined from NLC_*PCus*, the maximum release (in cumulative percentages), reaching after 5h. The explanation lies in the presence of *PCus* in the surfactant layer, with which it forms weak bonds (hydrogen bonds). On diosgenin, on the other hand, its firm uptake into the lipid-core nanocompartiments and low solubility in the receiving environment justify the slower release profile, with the maximum recorded being at 7h.

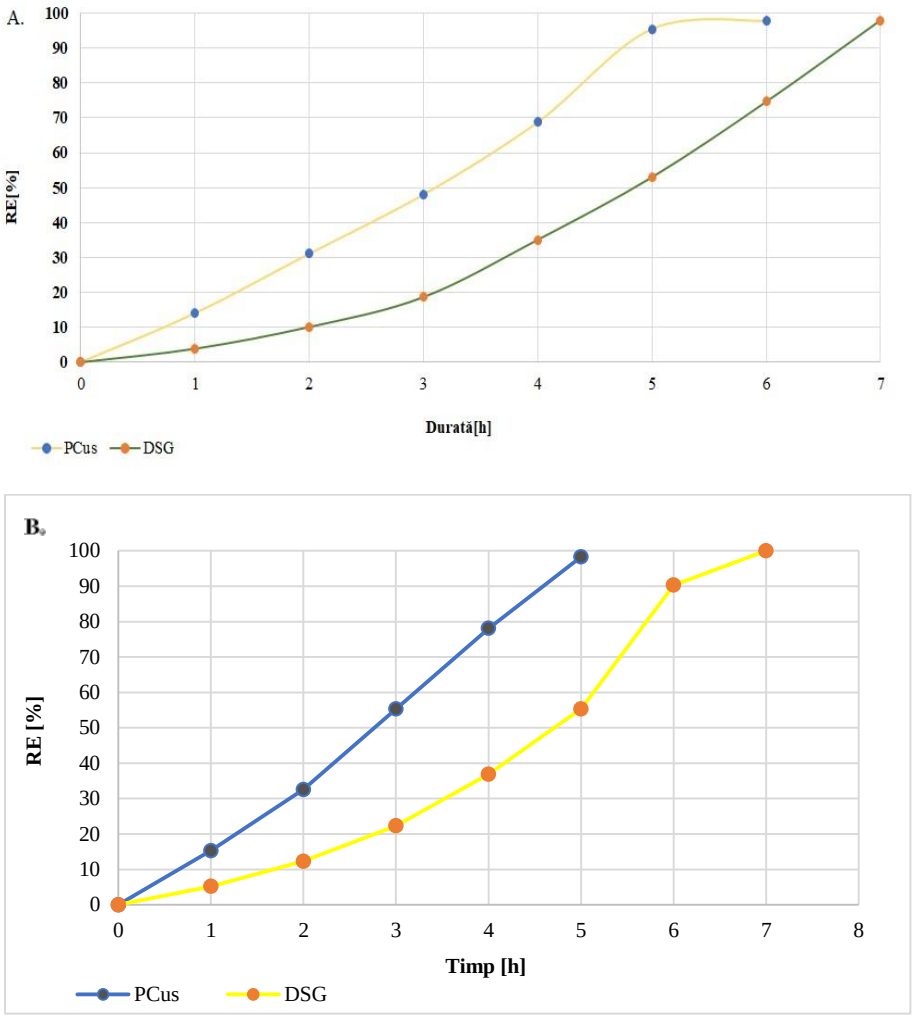


Figure 30. Sustained release of the two co-encapsulated plant principles in the dual nanocarriers, A. **NLC_DSG_*PCus*_UAr**; B. **NLC_DSG/*PCus*_UAr**

8.6. *In vitro* studies for the determination of antioxidant activity

Nanostructured systems of NLC_DSG type, obtained in this chapter, have proven an inhibitory activity of long life radicals type, $ABTS.^{+,}$ modest, manifesting an action to counter them in the interval 13 ÷ 16%. On the other hand, the systems that encapsulated the *PCus*, in an individual/mixed approach, showed a net superior ability to capture cationic radicals, their values ranging between 74.5 and 77.9%, Fig. 31. These values can be explained by the rich composition in a number of other phytocompounds with antioxidant properties, within the extract of *PCus*. Regarding the ability to remove oxygen lilber radicals generated in the chemiluminescent system, the antioxidant activity was enhanced in the case of individual systems with *PCus* (e.g. $AA_{NLC\_PCus\_UAr}$ = 95.65 ± 0.7%, respectively $AA_{NLC\_PCus\_UIn}$ = 97.44 ± 0.58%). In diosgenin systems, no improvements were detected by nanocapsulation, a sign that it has no effect in stopping free radicals.

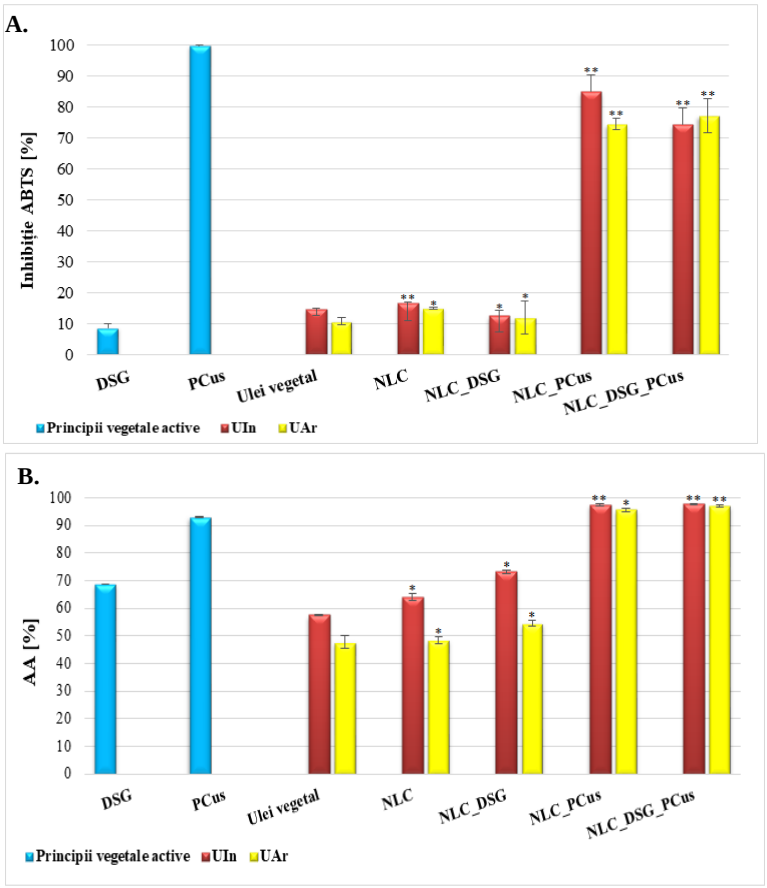
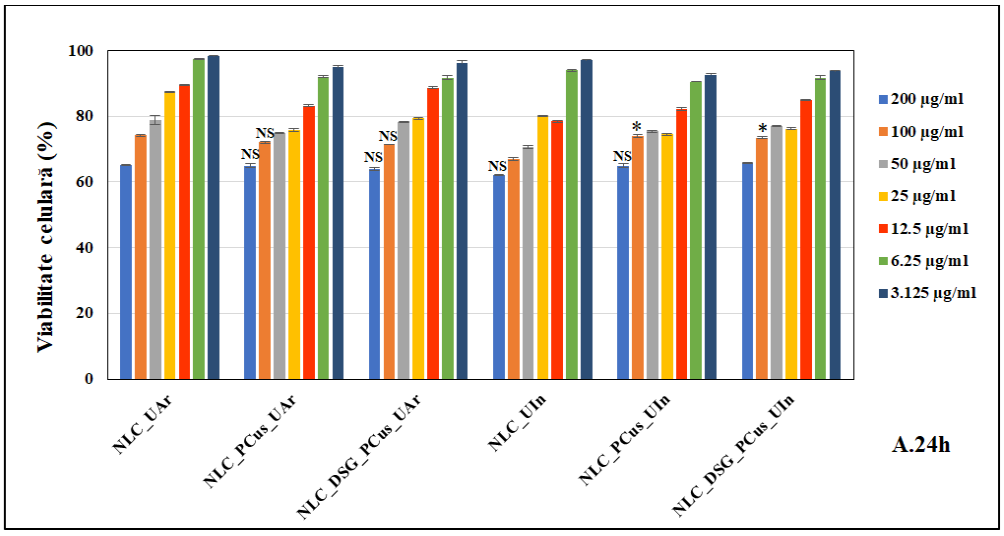


Figure 31. Determination of the long-life radical scavenging capacity of ABTS by NLC_free, individual-NLC and dual-NLC (teac method, A) and removal of short-lived radicals by chemiluminescence, B

8.7. *In vitro* assay of citotoxicity of individual and dual nanosystems

As with the previous systems, the MTS results revealed that by using concentrations ranging from 3.125 to 50 µL/mL NLC-phytochemicals, the cell viability was maintained at high values, between 70.6 and 98.3% for 24h, 72.1 and 96.7% treatment, corresponding to 48h, Fig. 32.



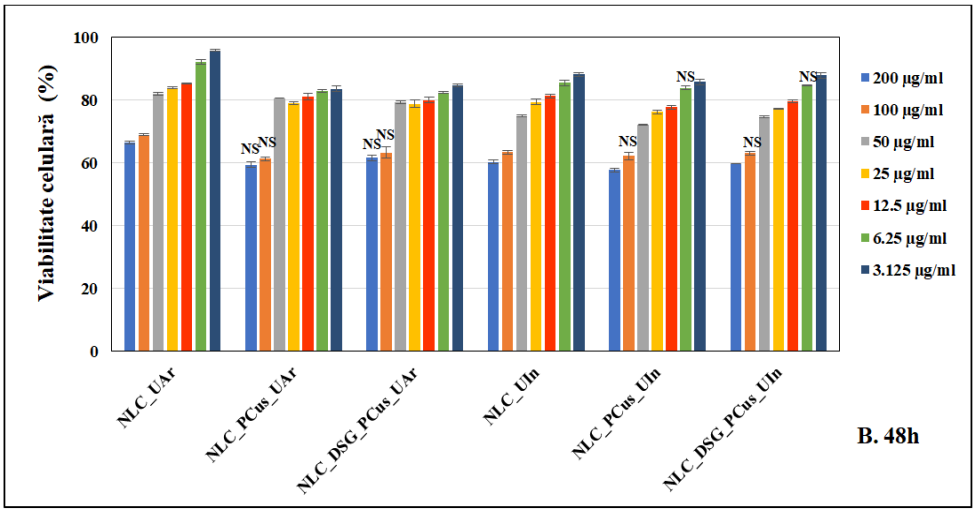


Figure 32. Effects of individual and dual systems on the viability of normal EA.hy926 cells after (A. 24h) and (B. 48h), respectively

RTCA assay aimed at real-time comparison of proliferative capacity *vs*. cytotoxicity of NLC_*PCus* and NLC_DSG_*PCus*, pointed out that at high concentrations, 400 µg/mL and in some cases 200 µg/mL, respectively, viability decreases significantly, indicating an increase in cytotoxicity. Conversely, concentrations below 100 µg/mL provide a comparable percentage of cell viability to untreated (witness) cells. This last aspect demonstrates the safety of concentrations between 25 and 100 µg/mL NLC, Fig. 33.

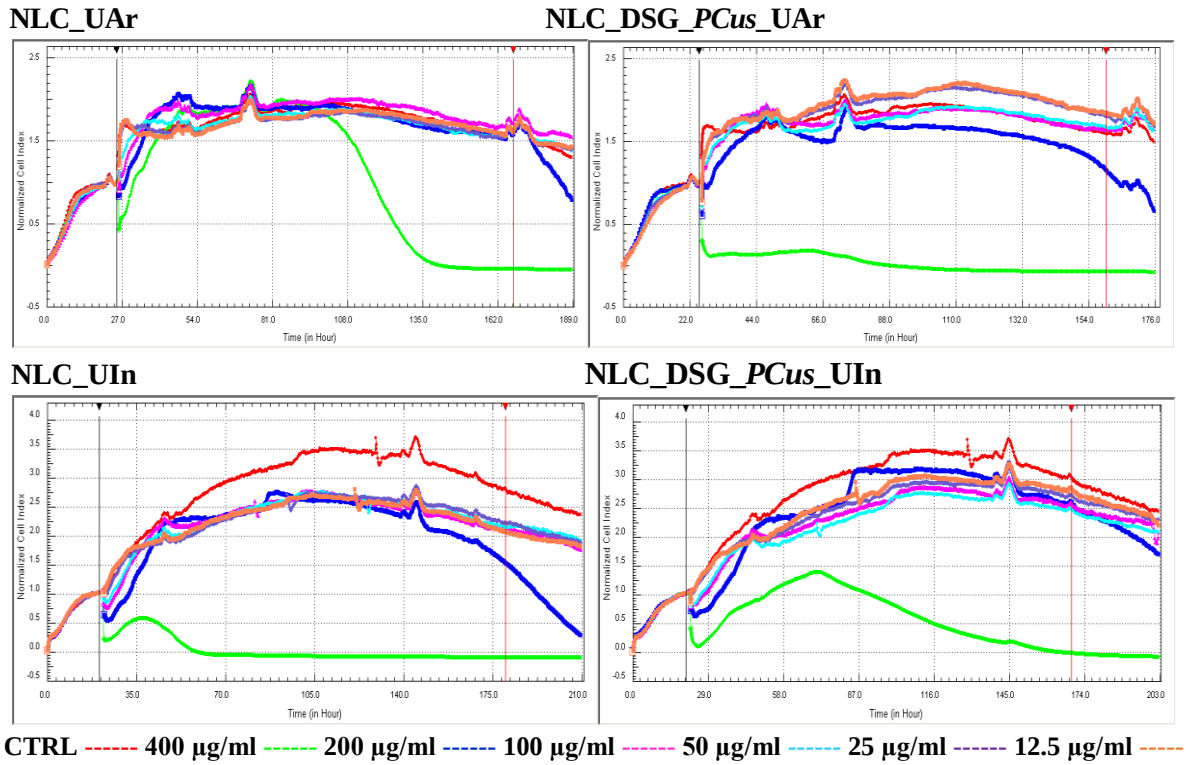


Figure 33. Proliferative *vs.* cytotoxic action results induced by free and dual nanocarriers (NLC_UAr/IUn and NLC_DSG_*PCus*_UAr/UIn)

8.8. Determination of anti-inflammatory effect for individual and dual-NLC (including DSG, *PCus* and a mixture of the active principles)

The anti-inflammatory activity of the DSG and *PCus* containing NLC systems, in terms of inhibition of IL-6 and TNF-α, scored at concentrations of 50/mL an IL-6 inhibition of up to 94.9% for milk thistle oil systems and 94.6% for linseed oil systems (24h treatment) (Iordache T., 2023).

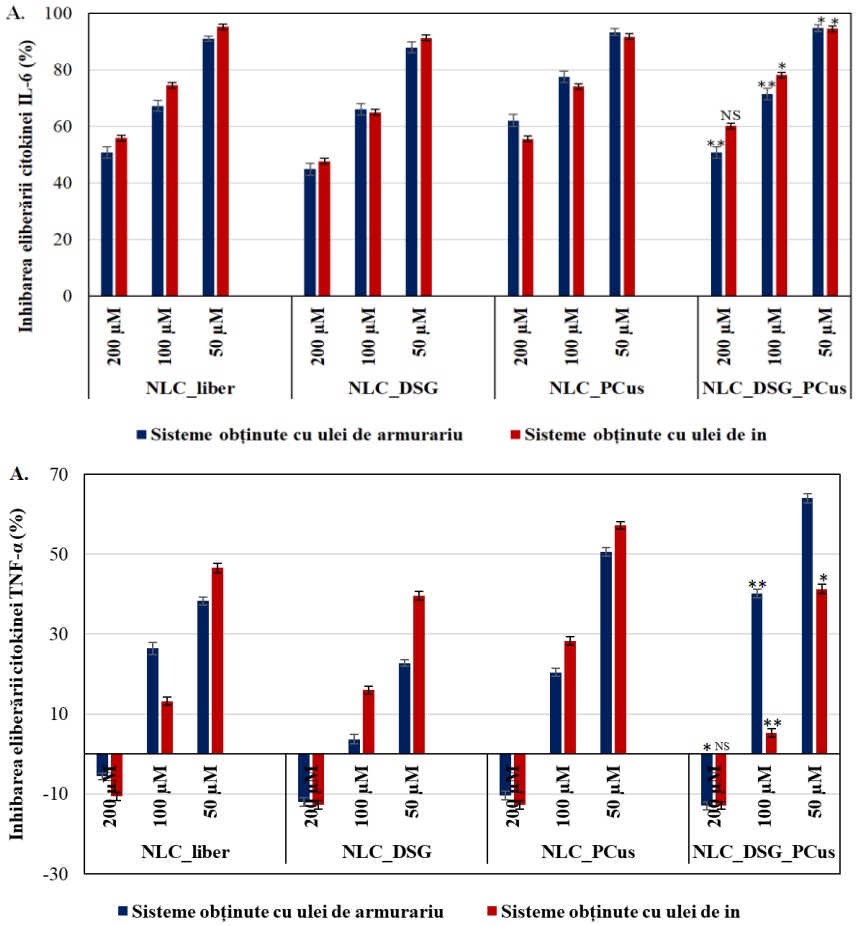


Figure 34. Assessment of inhibition of proinflammatory cytokines **IL-6 and TNF-α,** after treatment for **24h**

Regarding the inhibition of the second pro-inflmatory cytokine, by comparing the indivisible and dual NLC, the efficiency of those capturing both types of vegetal principles (e.g. NLC_DSG_*PCus*_UAr recorded a 73% inhibition of TNF-α or 40% inhibition for NLC_DSG_*PCus*_UIn after 24h, Fig. 34).

8.9. Partial conclusions

The lipid nanoparticles obtained within this chapter have recorded spherical morphologies, with dimensions ranging from 100 to 250 nm, with a uniform and restricted distribution of the lipid population (PdI ~ 0.15), competitive stability (> - 46.5 mV) and encapsulation efficiencies of: 65% for *PCus* and 87% for DSG.

Cytotoxicity tests (MTS and RTCA) proved the non-toxic effect of NLC_DSG_*PCus*_UAr on EA.hy926 line cells and increased antioxidant activity in capturing reactive oxygen species, > 97% for NLC_*PCus* and NLC_DSG_*PCus* and 74.5 ÷ 77.9% ABTS cationic radicals anihilation ability.

According to the ELISA analysis, the evaluation of treatments with dual NLC_systems, with DSG and *PCus*, showed a very high degree of inhibition of proinflammatory cytokines, IL-6, ranging from 91.9 to 94.9%. Regarding TNF-α inhibition, in this case, there has been a significant increase in it by using NLC with both vegetable principles.

Chapter 9. Superior functional properties obtained by association of Diosgenin and *Cimicifuga racemosa* extract in lipid nanocarriers

Dimensional, structural characterization and evolution of therapeutic efficacy of some lipid nanocarriers encapsulating two other categories of nutraceuticals – extract of Cimicifuga racemosa, *CymR* (black cohosh, standardized in 2.5% triterpene glycosides) and DSGs are the objective of this chapter.

9.2. Dimensional and morphological characterization of individual and dual nanocarrier systems

The recorded results showed lipid particle sizes between 150 and 210 nm. Indices of polydispersity, with values < 0.17, have strengthened the idea of the existence of relatively monodispersed and homogeneous lipid populations. As electrokinetic potential values, they vary between -43.2 ± 1.67 mV and -56 ± 1.53 mV, Fig. 35a.

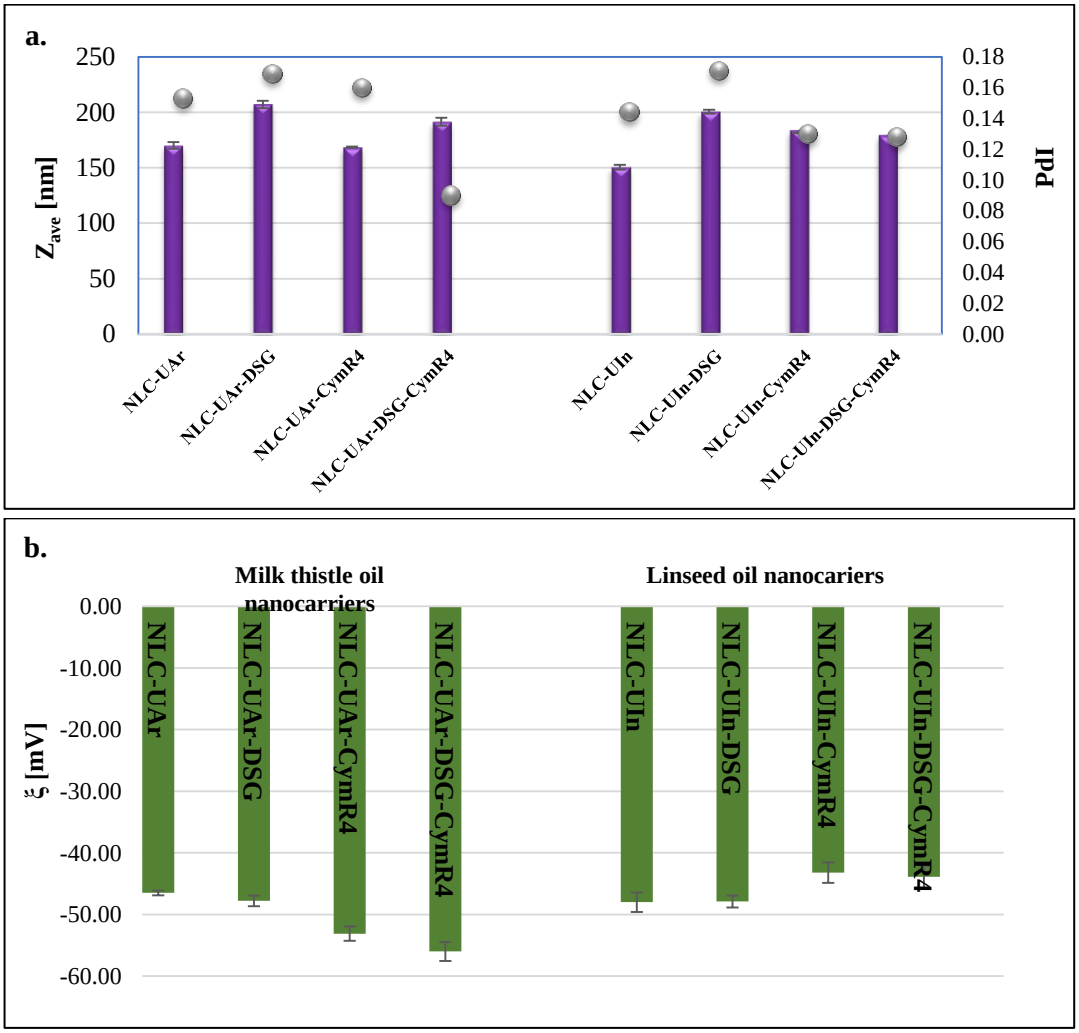


Figure 35. Variation of mean diameters, polydispersity indices (PdI), ***a*** and potential zeta, ***b***, depending on the type of encapsulated vegetal principle

9.4. Structural changes in NLC after capturing vegetable active principles, observed through differential scanning calorimetry

The results obtained by studying the behavior of NLCs subjected to a controlled heat treatment regime emphasized a high compatibility between the lipids selected for synthesis, the mixture of surfactants, involved in the stabilization of the lipid core, the two extracts. The peaks highlighted in the graphs shown in Fig. 36 suggest the presence of lipids, both solids and fatty acids that are components of vegetable oils. In dual nanocarriers, one can note the presence of the same peak, but deviated to lower temperature values, as a result of the presence of the two vegetable principles and internal changes in the lipid core arising from the capture of lipophilic compound. Conversely, in individual systems with *CymR*, the peak has a much narrowed profile that causes it to be retained in the surfactant shell, leading to a non-alteration of the lipid core structure.

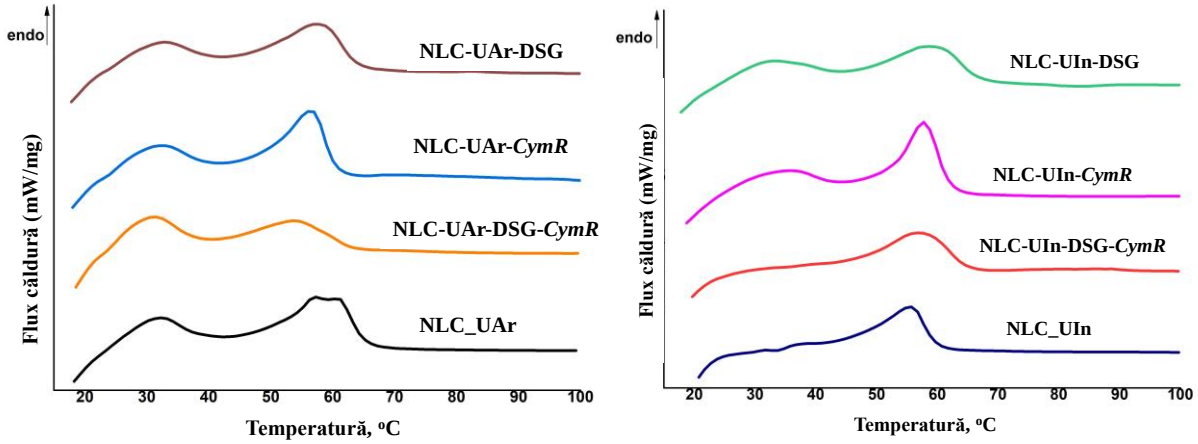


Figure 36. Comparative representation of endothermic curves of individual NLC-free systems

9.5. Determination of encapsulation efficiency of DSG anf *CymR* in lipid nanocarriers

In this set of experiments diosgenin was quantified by the chromatographic technique of high-performance liquids and for the Black Cohosh extract, the polyphenol-rich composition was taken into account, they serve to quantify the actual encapsulation efficiency. Quantitative determination of the latter was carried out using a spectrophotometric method. As shown in Fig. 37, the highest encapsulation efficiencies were determined for individual diosgenin-NLC-Un systems, in percentages of ~ 92%, while for *CymR*, the highest encapsulation efficiencies were determined, the maximum value is 76.5% in NLC-Un-dual. The explanation lies in the preferential affinity of the two principles for the two constituent phases of the system, lipid core and surfactant coating.

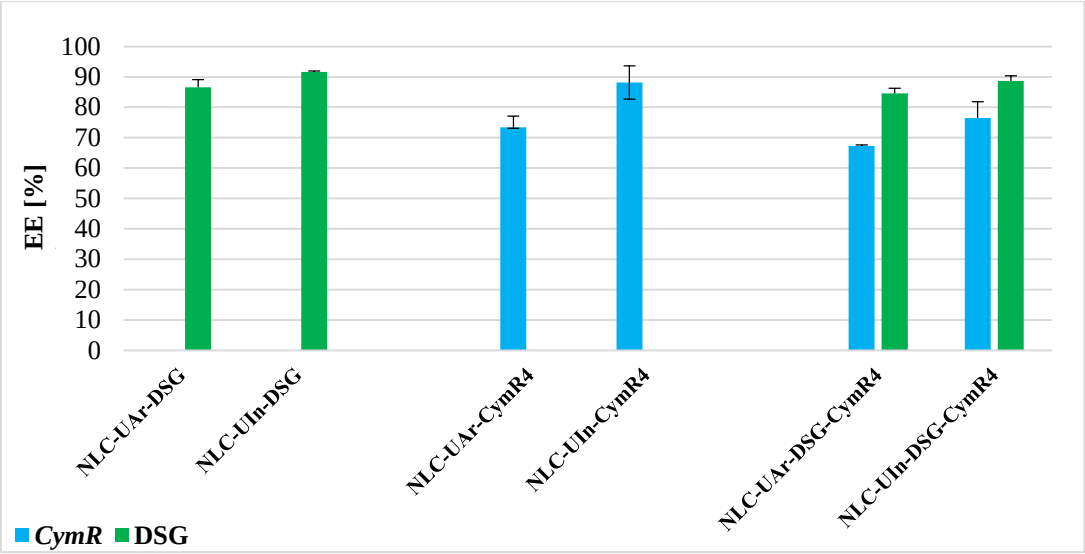


Figure 37. The encapsulation efficiency of DSG and black cohosh extract in NLC synthesized with the two vegetable oils

9.6. *In vitro* assay of controlled release of bioactive vegetable principle (DSG and *CymR*) from the obtained NLC

The *in vitro* release study aimed to quantify the polyphenols in the black cohosh extract by UV-Vis and the diosgenin from the receiving environment, by HPLC.

In the case of NLC-individual systems, a slower release trend was observed in the first 2 hours of study, followed by a rapid evolution, the maximum release rate reaches after 5h for *CymR* and 7h for DSG, respectively.

For mixed systems, on the other hand, an extension of the time-release period was observed for both categories of phytochemical principles, which were more pronounced in the case of DSG. The 100% release of polyphenols from *CymR* occurred at 4h and 5h, in the case of NLC-mixt, respectively, 5h in the case of nanotransporter systems with UAr and 6h in the case of NLC prepared with UIn, Fig. 39. For diosgenin, the allure of release is similar for both categories of systems.

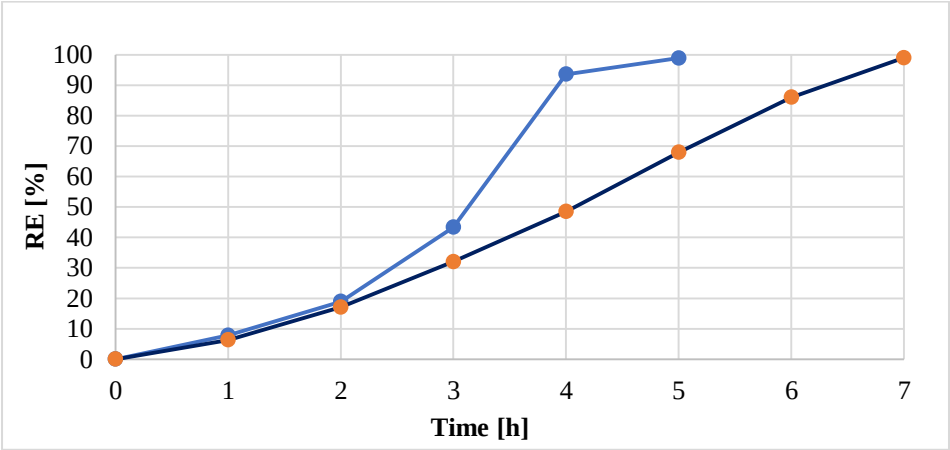


Figure 38. Release profile of DSGs and polyphenols (from *CymR*), present in individual NLC systems: NLC-UIn-DSG and NLC-UIn-*CymR*; blue – CymR, purple – DSG

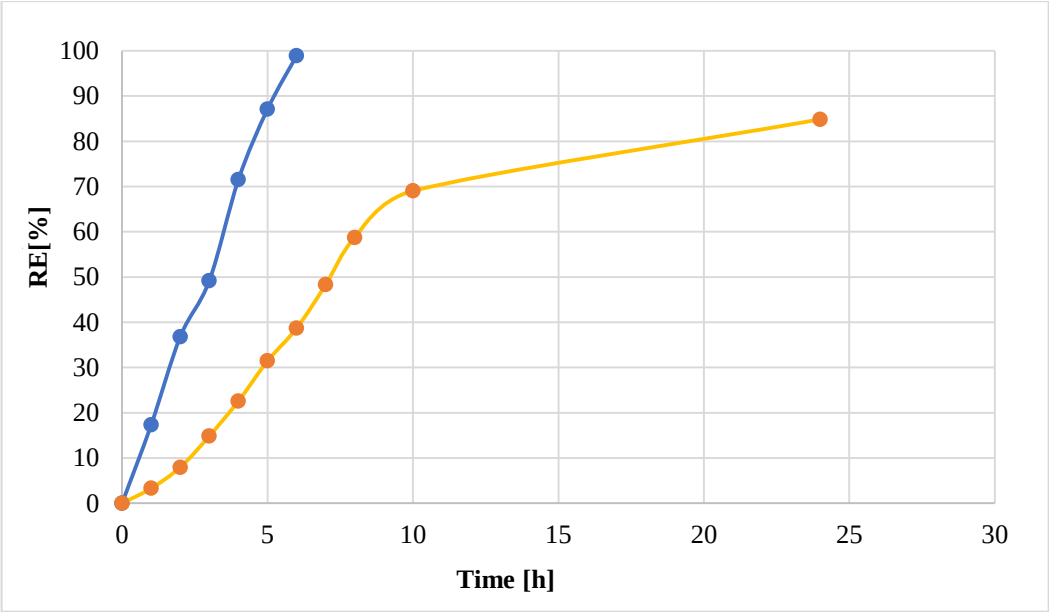


Figure 39. Release profiles of DSG and polyphenols (from *CymR*), present in mixed NLC systems: NLC-UIn-DSG-CymR; blue – CymR, orange – DSG

9.7. *In vitro* cuantification of the ability of capturing and inhibiting free radicals, shown by the individual and dual NLC systems

Systems that co-optase both diosgenin and black cohosh extract, recorded inhibitory values of 20-28% and 30% for systems with milk thistle oil, namely linseed oil, Fig. 40. The difference can be justified by the varied composition in fatty acids, components of the two vegetable oils.

By nanoencapsulation, both diosgenin and the second extract register a significant increase in the inhibitory percentage of long-lived radicals.

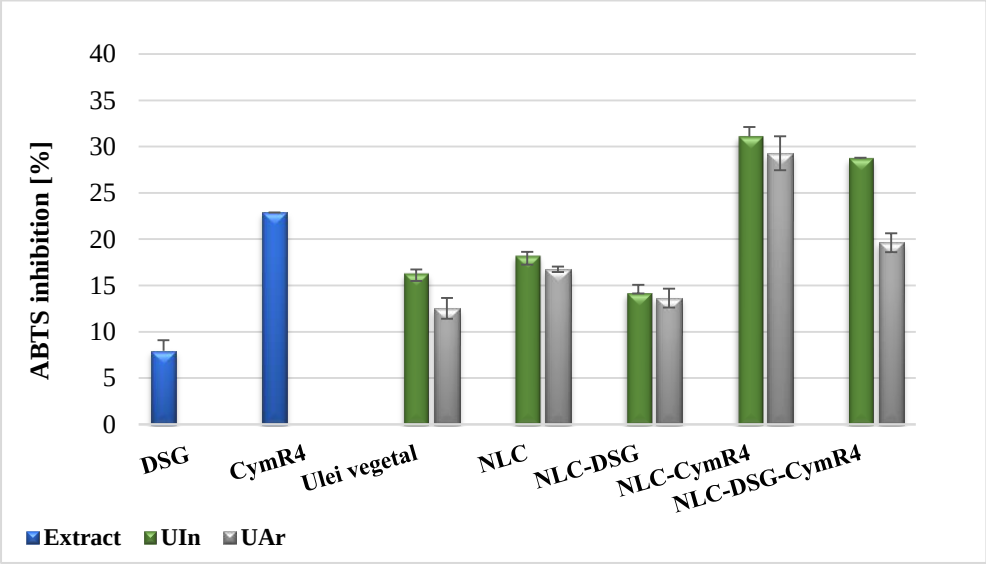


Figure 40. ABTS inhibition of pure extracts and vegetable oils, free nanocarriers, individual and mixed with DSG and *CymR*

Unlike the spectrophotometric method, the results recorded for the second technique, the chemiluminescence pointed to a better efficiency of the active principles, especially for the systems that co-encapsulate diosgenin and *CymR* extract, to capture short-lived radicals. As an example are the results: $AA_{NLC\text{-}UIn\text{-}DSG\text{-}CymR4}$ = 93.2% and $AA_{NLC\text{-}UAr\text{-}DSG\text{-}CymR4}$ = 88.9%.

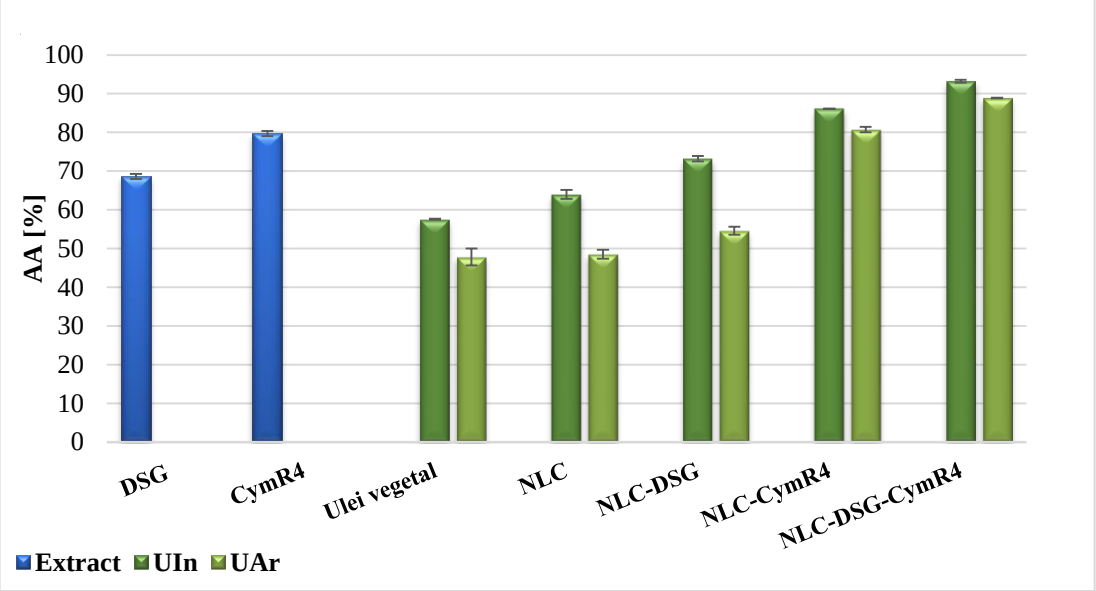


Figure 41. Representing the antioxidant potential of individual systems, NLC-IUn-DSG and NLC-UIn-*CymR4* and mixed systems, NLC-UAr/UIn-DSG-*CymR4* (chemiluminescence method)

9.8. *In vitro* monitoring of citotoxicity of individual and dual NLC systems, containing DSG, *CymR* and blend of the two active principles

In the study phase of cytotoxicity, nanostructured systems encompassing the two categories of active principles, systems prepared with UAr, respectively, UIn was found that milk thistle oil has a better biocompatibility than normal EA.hy926 cells, compared to linseed oil (*in vitro* MTS analysis). If for NLC-UAr systems, cell viability values ranged from 75.3 ÷ 80.7% (treatment 24h, with concentrations of 50 mg/mL NLC) and 80.2 ÷ 83.7% (treatment 48h, respectively, with concentrations of 50 µg/mL NLC), for NLC-UIn systems, cell viability suffered slight decreases: 70.6 ÷ 73.7% (treatment 24h, 50 µg/mL, 50, NLC-UIn individual and mixed) and 73 ÷ 76.1% (treatment 48h, 50 µg/mL, NLC-UIn individual and mixed). The above mentioned values of cell viability highlight that a prolonged treatment of 48 hours leads to the development of a cellular regeneration phenomenon possibly due to the phytoconstituent-rich composition of *CymR* extract.

Cytotoxic *vs.* proliferative action induced by individual *CymR*-NLC systems on normal EA.hy926 cells, assessed by real-time analysis, RTCA (Fig. 42), as measured by, reveals that treated with <100 µg/mL concentrations of NLC-individual/dual, recorded high cell viability values. This demonstrates safety in all NLC categories at concentrations between 25 and 100 µg/mL. It was also observed that at high concentrations of 400 µg/mL viability decreases significantly, indicating an increase in NLC cytotoxicity at those concentrations *e.g.* individual systems with *CymR* extract, NLC-UAr/UIn-*CymR*.

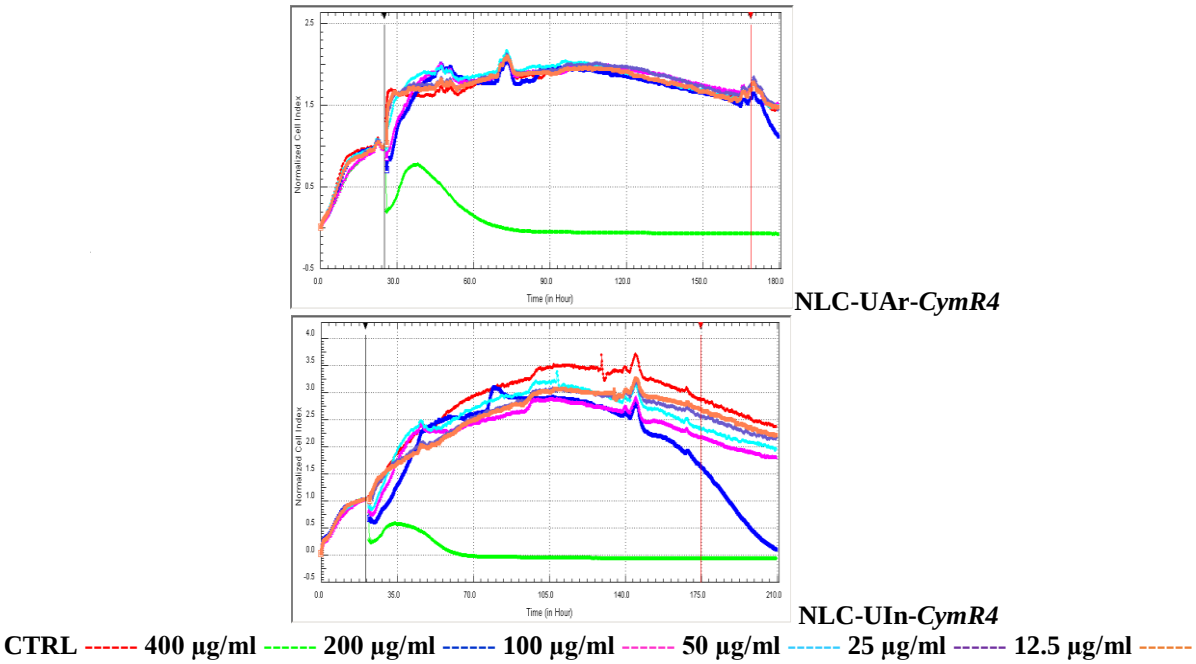
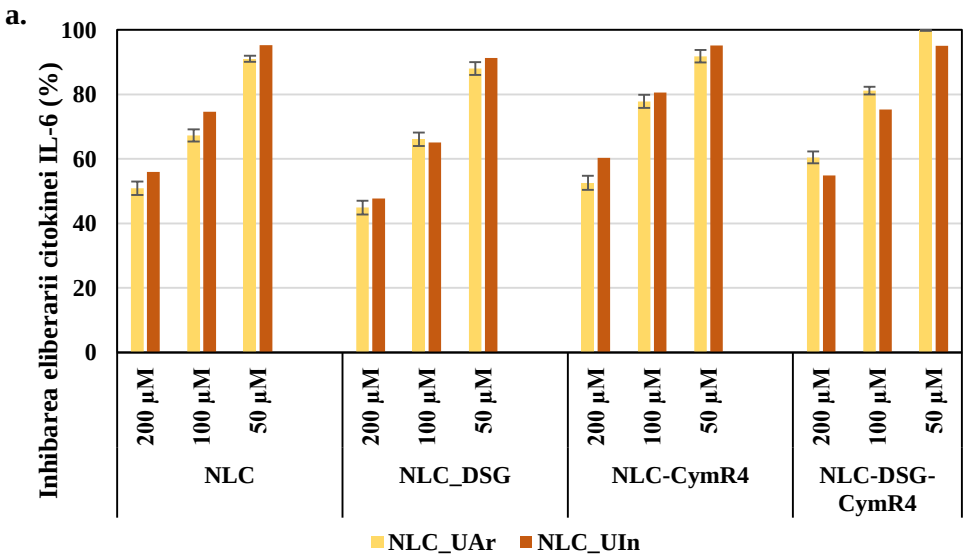


Figure 42. Cytotoxic *vs.* proliferative action induced by individual *CymR* NLC systems on normal EA.hy926 cells (RTCA analysis)

9.9. Study of the anti-inflammatory effect of individual NLC, NLC-UAr/UIn-DSG/*CymR4* and dual, NLC-UAr/UIn-DSG-*CymR4*

The results of the analysis of quantification of anti-inflammatory activity revealed the presence of a strong anti-inflammatory effect manifested by both categories of NLC systems, loaded with a single vegetable principle (*CymR*, DSG) or mixed vegetable principles (*CymR* and DSG). By performing NLC treatments on EA.hy926 cells, there was a strong inhibition of both categories of proinflammatory cytokines. For example, there are nanocarriers synthesized with milk thistle oil that have generated IL-6 proinflammatory cytokine inhibition ranging from 91.1 ÷ 99.8% (treatment of 24h) and 90.5 and 99.5% (treatment of 48h).



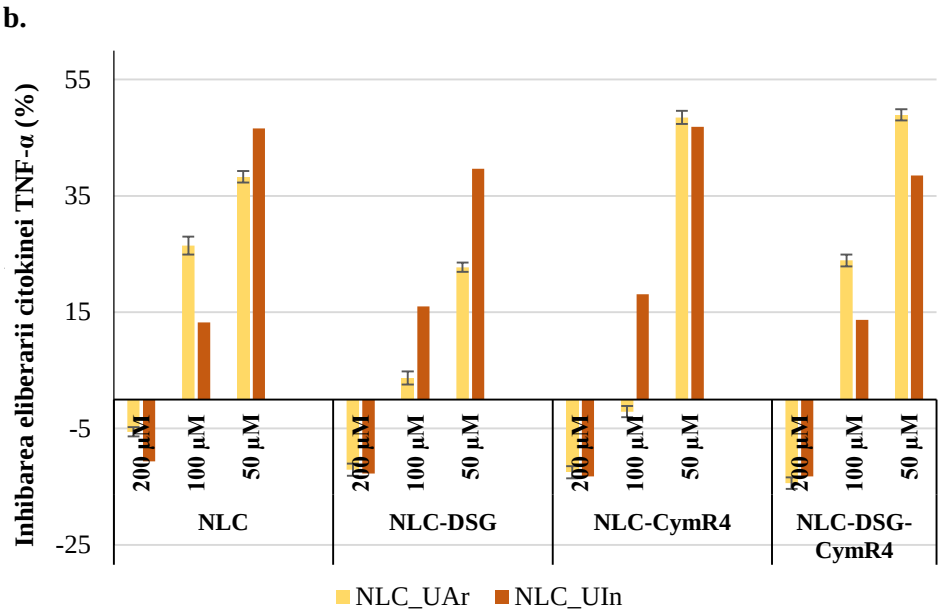


Figure 43. Evaluation by ELISA technique of the effect of treatments with NLC-DSG, NLC-*CymR* and NLC-DSG-*CymR* nanocarrier systems on the release of IL-6 cytokines (a.) and TNF-α (b.), by normal cells EA.hy926, after 24h of treatment

In the case of TNF-α proinflammatory cytokine inhibition, the anti-inflammatory potential of NLC was somehow more limited, in the sense that % inhibition did not exceed 60%, regardless of the type of vegetable oil used. In figure 43b, a maximum percentage of inhibition, generated by the mixed system with milk thistle oil, is observed at a concentration of 50 µg/mL, 54.6%.

9.10. Partial conclusions

The new lipid nanotransporter systems encapsulating diosgenin and/or Cimicifuga racemosa/*Cohosh Black* extract have shown suitable stability parameters associated with mean particle diameters < 200 nm, respectively, polydispersity indices < 0.17 and electrokinetic potentials more electronegative than - 43.2 mV.

The effective incorporation and compatibility of plant principles with the mixture of lipids and surfactants was revealed by quantitative determinations of encapsulated DSG and *CymR* (spectrophotometric and chromatographic/HPLC analyses). The preferential affinity of the two phytochemical principles for the two constituent phases of the nanotransporter system generated a capture of *CymR* in the outer layer of surfactants, obtained EE% being moderate for *CymR* (e.g. 76.5% for NLC-UIn-dual system). In contrast, the DSG affinity for the lipophilic core created by solid lipids together with UIn/UAr led to high encapsulation efficiencies (e.g., ~92% corresponding to individual systems with linseed oil).

Inhibition of long-life radicals, quantified by the ABTS technique, emphasized a relatively small inhibitory percentage, maximum 30% for mixed and individual NLC systems. At the opposite pole is the antioxidant activity manifested by these NLCs on the reactive oxygen species – ROS, short life, the, which highlighted superior free radical scavenging net values (ex: $AA_{NLC\text{-}UIn\text{-}DSG\text{-}CymR4} = 93.2\% \pm 0.40$; $AA_{NLC\text{-}UAr\text{-}DSG\text{-}CymR4} = 88.9\% \pm 0.05$).

Regarding the cytotoxic potential of increasing NLC concentrations, by MTS analysis, it was noted that NLC systems prepared with milk thistle oil have a better biocompatibility than normal cells, EA.hy926, compared to NLC prepared with linseed oil. Cell viability values after 24h treatment ranged from 75.3 ÷ 80.7% for 50 μg/mL treatment with individual and dual NLC-UAr systems, 70.6 ÷ 73.7% respectively for individual and dual NLC-UIn systems.

The RTCA test pointed out that at < 100 μg/mL concentrations, the viability of EA.hy926 normal cells is increased, comparable to that of untreated control cells.

The involvement of these distribution systems in the anti-inflammatory process has brought to the fore the efficacy of lipid nanotransporters at concentrations of 50 μg/mL, especially the dual ones. However, the inhibition of interleukin 6 was much higher, > 92%, as opposed to the second factor whose % inhibition did not exceed 64%, being visibly was dependent on the type of vegetable oil and the period of applied treatment.

10. FINAL CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

10.1. Final conclusions

The original research carried out in this thesis, materialized by the existence of **5 original chapters,** revealed the following:

Chapter 5 presents the optimization studies carried out in order to obtain the optimal variant for the subsequent synthesis of nanotransporters loaded with active principles. Variation studies (in terms of concentration or weight ratio between surfactants involved in the process, vegetable oils or operational parameters) have highlighted as appropriate the wording which: a) contains a mixture of 2.5% surfactants and co-surfactant (found in a compositional ratio of Tw20 : Polx188 : Phosph = 70 : 15 : 15, respectively, reported to 100 g NLC aqueous dispersion); b). involves operation at the HSH processing stage at 13,000 rpm (for 1 min.); c) involves the use of 6 homogenization cycles (corresponding to a time of 3 min. and 20 sec.) at 500 bar, in the HPH processing stage.

Experimental results in ***Chapter 6*** have demonstrated that by combining the compositional and structural characteristics of two solid lipids (glyceryl monostearate and cetyl palmitate) together with vegetable oils (evening primrose oil, sp. *Oenothera biennis*, soybean oil, sp. *Glycyne max*) and the mixture of surfactants (formed from Tween 20, Phosphatidylcholine and Poloxamer 188), nanotransporter lipid systems can be developed, favorable to the effective co-opting of two categories of phytochemical principles, a steroidal sapogenin (diosgenin, DSG) and a hydrophilic phytochemical mixture (extract of Glycyrrhiza glabra, *GlyG*).The obtained NLCs were presented in the form of spheres with diameters between 50 ÷ 200 nm, which include nanocompartments created by I/II oils. The preferential distribution of the two bioactive principles was argued by coupling the information from differential scanning calorimetry and *in vitro* release studies (DSG being captured in the lipid phase, while *GlyG* showed affinity for the surfactant coating).

The encapsulation efficiency parameter highlighted high values, > 80% for DSG and ~90% for *GlyG*. Safety in use and reduced toxicity in HUVEC endothelial cell line treatments have been confirmed for concentration range 25 ÷ 100 μg/mL, respectively, by conducting

two specific *in vitro* assays (MTS and RTCA).

The *in vitro* release profile showed that mixed NLC-phytochemicals allow sustained release, which gives these active phytocompounds to reach the inflamed cells over a certain period of time, where they have their therapeutic target.

Both categories of nanotransporters have proven significant abilities to combat long-life cationic radicals ($ABTS^{\cdot+}$) and short-lived oxygenated radicals (ROS). In addition, encapsulation of natural herbal mixtures resulted in an enhanced anti-inflammatory effect, which was much more pronounced in the inhibition of proinflammatory cytokine TNF-α: 90.6% (for NLC_II) *versus* 97.9% (for NLC_II_DSG_GlyG).

Chapter 7 aimed to obtain stabilised NLCs with a predominantly ionic surfactant mixture (sodium cholate being the primary emulsifier) for simultaneous and effective integration of steroidal saponin, DSG together with wild yam extract (*Yam*) to improve the *in vivo* bioavailability of the two phytochemicals. The NLC_DSG_*Yam* systems recorded a high physical stability in terms of electrokinetic potential values, more electronegative than -35 mV, mean diameters of 125 nm and high encapsulation yields, > 80% for DSG.

In vitro quantification of free radical scavenging percentages revealed a modest ability to attach long-life radicals, $ABTS^{\cdot+}$ (the maximum inhibition value being only 12.4%), while ROS free radical inhibition targeted significantly improved values, AA: 82 - 84%.

The safety aspects of the obtained NLCs, monitored by *in vitro* MTS and RTCA tests, demonstrated that at concentrations of 50 μg/mL NLC, at concentrations of, cell viability was maintained at > 80%, indicating a lack of NLC cytotoxicity on HUVEC endothelial cells.

Similar to the previous system, the anti-inflammatory action was dependent on the applied dose, in the sense that a more pronounced inhibition of the release of IL-6 and TNF-α cytokines occurred at concentrations of 50 μg/mL NLC. Capture of DSG and *Yam* in stabilised nanotransporters with predominantly ionic surfactant mixture led to more pronounced inhibition of proinflammatory cytokine TNF-α (ex: 98.2% ± 1.07), than IL-6 (ex: 62% ± 1.07), for the NLC_DSG_*Yam*_2 distribution system.

In ***Chapter 8,*** the effective simultaneous integration of an extract of Polygonum cuspidatum (*PCus*) together with the steroidal saponin DSG was achieved. The obtained NLCs showed spherical morphologies, ranging in size from 50 to 250 nm, with a uniform distribution of the lipid population (PdI ~ 0.15) and competitive physical stability. The rich composition of hydrophilic phytoconstituents in *Polygonum cuspidatum* extract resulted in some encapsulation percentages with moderate values (e.g.: 64 ± 1.5% for NLC_*PCus*_UIn and 73 ± 1.3% for NLC_*PCus*_UAr. For mixed NLC systems, EE % ranged from 91.6% to 87.0% for DSGs, while in the case of *PCus* the encapsulation efficiency values did not exceed 65%. Calorimetric analysis highlighted the significant change in the lipid core after capturing the two bioactive principles.

The non-toxic effect of mixed systems on EA.hy926 cells was demonstrated by the results of two *in vitro* cytotoxicity tests, MTS and RTCA.

The NLC systems that encapsulated *Polygonum cuspidatum* extract in individual or dual system showed an ability to capture cationic radicals of $ABTS^{\cdot+}$ type ranging from 74.5 to 77.9%, respectively%, the most effective systems being those based on linseed oil. By

simultaneously co-opting the two plant principles – the extract of *PCus* and DSG – in the nanotransporter lipid systems, a significant increase in the ability of NLC to capture oxygenated free radicals was observed, the antioxidant activity values being very close to the complete removal of ROS species, e.g.: 97-98%.

The *in vitro* release study revealed that there was a slower trend towards the release of lipophilic compound, DSG, trapped in the lipid core, compared to the *PCus* which was released in a percentage >65% after 3h.

In vitro evaluation of the anti-inflammatory potential of NLC-mixte systems containing DSG and *PCus* has demonstrated a very high degree of inhibition of IL-6 cytokines, between 91.9 and 94.9%. Regarding TNF-α inhibition, the values were relatively lower.

Chapter 9 presents the development and characterization of nanostructured systems capable of encapsulating diosgenin and/or extract of Cimicifuga racemosa/*Black cohosh*. Mean lipid particle diameters ranged from 140 ÷ 210 nm (according to DLS analysis), polydispersity indices < 0.17 and electrokinetic potentials were more electronegative than -43.2 mV.

The effective incorporation and compatibility of plant principles with the mixture of lipids and surfactants were revealed by quantitative determinations of encapsulated DSG and *CymR* (spectrophotometric and chromatographic/HPLC analyses), and, as well as differential scanning calorimetric analysis (DSC).

The preferential distribution of the two categories of active principles (*CymR* for surfactant coating and DSG for lipophilic core matrix) is in strict dependence with the rapid release of polyphenols from *CymR* extract, which is, as a maximum percentage, in the first 5 hours of the *in vitro* release study, while diosgenin follows a gradual release profile, extended over a period of 24h.

Inhibition of long-life radicals, quantified by the ABTS technique, emphasized a relatively low inhibitory percentage, maximum 30% for mixed and individual NLC systems with *CymR*, the study found, however, the ability to capture reactive oxygen species was noted by slightly more promising values (e.g.: $AA_{NLC\text{-}UI\text{-}DSG\text{-}CymR4}$ = 93.2%; $AA_{NLC\text{-}UAr\text{-}DSG\text{-}CymR4}$ = 88.9%).

The MTS and RTCA analyses pointed out that at < 100 μg/mL concentrations, the viability of EA.hy926 cells is increased, comparable to that of untreated control cells.

The involvement of these distribution systems in the anti-inflammatory process, by mediating the proinflammatory cytokine response, has brought to the fore the efficacy of lipid nanotransporters at concentrations of 50 μL/mL, especially the dual ones, ex. 99.80 ± 0.1% (for NLC-UAr-DSG-*CymR4*), IL-6 inhibition. The second factor targeted in the inflammatory process, TNF-α recorded somewhat more limited values, in the sense that the percentage of inhibition did not exceed 64%, regardless of the type of vegetable oil or plant principle concerned.

10.2. Original contributions

The original contributions, brought by this doctoral thesis, aimed at obtaining and characterizing nanotransporter lipid systems (NLC) that co-opt/co-encapsulate various plant mixtures, bioactive, and, which can provide a promising alternative to the controlled distribution and release of phytocompounds (by passive targeting in different specific areas of the body) for the purpose of preventing/treating inflammatory diseases, but not only.

These original research was carried out by:

1. Co-encapsulation of mixed active principles (DSG and *Yam*, *GlyG*, *PCus*, *CymR*) in lipid nanotransporters prepared by valorization of 4 vegetable oils (soybean oil, evening primrose oil, etc, linseed oil and milk thistle oil) in combination with solid lipids (glyceryl monostearate, cetyl palmitate or cocoa butter). Notable is the novelty of these simultaneous co-options of vegetable mixtures in the same nanostructured distribution system; there are no studies in the literature attesting the encapsulation of selected plant extracts and used in the doctoral thesis.
2. Obtaining an increased bioavailability and improved therapeutic potential of different plant active principles from various phytochemical classes (e.g., steroidal saponins, triterpene saponins, phenolic acids, etc.) by co-opting them into NLC-type lipid distribution systems.
3. The complex study of the evidence of *in vitro* release profiles of steroidal sapogenin (DSG), triterpene saponin (glycyrrhizic acid, present in *GlyG* extract), phytoalexin (resveratrol, present in the extract of *PCus*), but also polyphenols (from *CymR* extract).
4. Proof of the lack of cytotoxicity of NLCs developed on target cell lines, HUVEC and EA.hy926, at concentrations < 50 μg/mL.
5. *In vitro* monitoring of the antioxidant action by TEAC and chemiluminescence.
6. Prove the anti-inflammatory efficacy of lipid nautransporters by involving these NLC distribution systems in mediating the proinflammatory response of IL-6 and TNF-α.

10.3. Future directions for the application of this research

The results obtained in the doctoral thesis *called „Nanoencapsulations of nutraceuticals in nanostructured lipid systems for the development of advanced food supplements*" will be able to serve as a starting point in further experiments on scale applicability wide range of these nanosystems addressed to a specific group of subjects (e.g., women in pre/post-menopausal periods). Moreover, a first step was achieved through a collaboration of the Faculty of Chemical Engineering and Biotechnologies (FICBI), of the National University of Science and Technology POLITEHNICA Bucharest (UNSTPB), Bucharest, with a private partner (A.C. Helcor S.R.L., Baia Mare) within a POSDRU project „Complishing an innovative dietary supplement for menopausal women's health". In this context, representative samples of lipid nanotransporters were conditioned by the pharmaceutical company A.C. Helcor S.R.L. (Fig. 44) in the form of solid capsules (with excipients of the fujisil type and magnesium stearate), for the further development of dietary

supplements based on NLC-the main bioactive phytochemicals that have multiple health benefits associated with the female population.

Figure 44. Solid capsules containing vegetable NLC-oils-diosgenin-phytochemical mixtures (e.g., *Yam* extract)

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Representative publications in the field of the phD thesis

Scientific articles:

1. Teodora-Alexandra Iordache, Nicoleta Badea, Mirela Mihaila, Simona Crisan, Anca Lucia Pop, Ioana Lacatusu. *Polygonum cuspidatum* Loaded Nanostructured Lipid Carriers for Dual Inhibition of TNF-α- and IL-6 Cytokines and Free Radical Species, *Materials,* **2023**, 16(9), 3492, doi: 10.3390/ma16093492; **IF = 3.4.**

2. Teodora-Alexandra Iordache, Lucia Coc, Adriana Laura Mihai, Nicoleta Badea, Ioana Lacatusu, Aurelia Meghea. The influence of vegetable oil and self-organizing agents' composition on obtaining stable nanostructured lipid carriers, *U.P.B. Sci.Bull, Seria B,* **2022,** 84, 1; **IF = 0.5.**

3. Teodora-Alexandra Iordache, Nicoleta Badea, Mirela Mihaila, Simona Crisan, Anca Lucia Pop, Ioana Lacatusu, Challenges in Coopted Hydrophilic and Lipophilic Herbal Bioactives in the Same Nanostructured Carriers for Effective Bioavailability and Anti-Inflammatory Action, *Nanomaterials*, **2021,** 11, 3035. https://doi.org/ 10.3390/nano11113035; **IF = 5.719**.

4. Ioana Lacatusu, **Teodora Alexandra Iordache**, Mirela Mihaila, Dan Eduard Mihaiescu, Anca Lucia Pop, Nicoleta Badea. Multifaced Role of Dual Herbal Principles Loaded-Lipid Nanocarriers in Providing High Therapeutic Efficacity, *Pharmaceutics*, **2021,** 13, 1511. https://doi.org/10.3390/pharmaceutics13091511; **IF = 6.525.**

(Cerere brevet de invenție). Lăcătuşu Ioana, Badea Maria-Nicoleta, Pop Anca Lucia, **Iordache Teodora-Alexandra**, Pop Coriolan, Procedeu de încapsulare dual㠠a două categorii de principii vegetale bioactive în același sistem de distribuție nanostructurat; 2021/00220/ 29.04.2021.

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1. **Teodora Alexandra Iordache**, Simona Crisan, Anca Pop, Mirela Mihaila, Nicoleta Badea, Ioana Lacatusu, *Optimized approach for developing lipid nanocarriers loaded with active herbal extracts***,** 22nd Romanian Internaţional Conference on Chemistry and Chemical Engineering (RICCCE 22), 7-9.09.2022, Sinaia, România;
2. **Teodora Alexandra Iordache**, Simona Crisan, Anca Pop, Mirela Mihaila, Ioana Lacatusu, Nicoleta Badea, *Use of herbal ingredients for development of anti-inflammatory nanostructured delivery systems*, 22nd Romanian Internaţional Conference on Chemistry and Chemical Engineering (RICCCE 22), 7-9.09.2022, Sinaia, România;
3. Cristina Ott, Mihaela Tociu, **Teodora Iordache**, Simona Crisan, Anca Pop, Nicoleta Badea, Ioana Lacatusu, *Resveratrol and Diosgenin co-loaded lipid nanocarriers with effective anti-inflammatory action*, 22nd Romanian Internaţional Conference on Chemistry and Chemical Engineering (RICCCE 22), 7-9.09.2022, Sinaia, România;

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