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PhD THESIS SUMMARY

NANOCARRIER SYSTEMS BASED ON NATURAL ACTIVES WITH ANTIOBESITY AND ANTIOXIDANT ACTIONS

SISTEME NANOTRANSPORTOARE PE BAZĂ DE PRINCIPII ACTIVE NATURALE CU ACȚIUNE ANTIOBEZITATE ȘI ANTIOXIDANTĂ

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The author

INTRODUCTION

The synthesis and characterization of nanostructured lipid systems such as nanostructured lipid carriers (NLCs) lead to efficient and safe pharmacologically systems.

Nanostructured lipid carriers are systems that contain a solid lipid matrix that protects and ensures a controlled release of active compounds [1]. These carriers contain a mixture of solid lipids and liquid lipids and are derived from SLN (*solid lipid nanoparticles*) systems, with the specification that SLN systems contain only solid lipids. NLC nanoparticles have a spherical shape, with an average particle size between 40 and 1000 nm, the lipid matrix being solid at body temperature. NLC systems may incorporate natural active ingredients with a biologically beneficial role in the treatment of certain diseases, a role enhanced by the association with vegetable oils and synthetic active substances.

The objective of the research presented in the doctoral thesis was to develop and synthesize NLC systems based on vegetable oils and natural extracts, in association with certain synthetic compounds, with antioxidant and anti-obesity properties. The studied nanocarrier systems have the ability to co-encapsulate and ensure a controlled release of natural active compounds (capsaicin, caffeic acid, curcumin, piperine) and synthesis active compounds (oleoylethanolamide, phenylalaninol oleamide) in order to obtain effective formulations.

The doctoral thesis is structured in two parts and contains 9 chapters:

PART I. The bibliographic research includes 3 chapters:

- ✓ **Chapter 1** presents general aspects of NLC systems, such as the structural features, advantages and disadvantages of these transport systems, types of NLC systems, the stability of NLC systems and methods of obtaining them.
- ✓ **Chapter 2** presents fundamental concepts on obesity, such as the mechanisms of action and biochemical parameters of obesity, but also the biological effects of some natural compounds with anti-obesity potential (capsaicin, piperine, linseed oil, etc.).
- ✓ **Chapter 3** presents examples of drugs and compounds used in the management of obesity, as well as examples of systems that include active compounds with anti-obesity and antioxidant action.

PART II. The experimental research presents the research objectives and the obtained experimental results, structured in 6 chapters, in the last part of the thesis being presented the final conclusions and the bibliography.

- ✓ **Chapter 4** presents the experimental methodology that includes the materials, the methods and the techniques used for the synthesis and characterization of the obtained NLC systems.

✓ **Chapter 5** presents the optimization of NLC systems by varying the concentration of the surfactant mixture and the evaluating the stability over time, as well as the synthesis and characterization of optimized NLC systems based on caffeic acid.

✓ **Chapter 6** presents the synthesis and characterization of NLC systems prepared with vegetable oil (linseed oil, respectively, argan oil), encapsulated with curcumin and co-encapsulated with phenylalaninol oleamide.

✓ **Chapter 7** highlights the synthesis and characterization of NLC systems based on piperine – natural active compound with many therapeutic effects.

✓ The synthesis and characterization of NLCs based on linseed oil and capsaicin in association with a synthetic active compound (oleoylethanolamide, phenylalaninol oleamide) are presented in the most extensive chapter of the thesis - **Chapter 8**. This chapter also presents *in vivo* studies regarding to **anti-obesity effect** of capsaicin-based nanocarriers.

✓ The doctoral thesis ends with **Chapter 9** entitled "Conclusions", a chapter in which the obtained experimental results, the originality of these studies, as well as the research perspectives are summarized.

The original results were capitalized by the publication of two scientific papers in ISI journals, one being in process of publication, and by the participation at an international scientific conference.

Keywords: nanostructured lipid carriers, antioxidant action, antiobesity action, caffeic acid, curcumine, piperine, capsaicin, linseed oil, argan oil, evening primrose oil, paraffin oil, oleoylethanolamide, phenylalaninol oleamide.

PART II
EXPERIMENTAL RESEARCH
THE PURPOSE AND THE OBJECTIVES OF THE RESEARCH

The research purpose

The main purpose of this research was the synthesis and characterization of nanocarrier systems based on natural active principles (caffeic acid, curcumin, piperine, capsaicin) in association with vegetable/ mineral oil (linseed oil, argan oil, evening primrose oil/ paraffin oil) and co-encapsulated with synthetic active principles. The endogenous lipids recognized by epithelial cells that were used, oleoylethanolamide and phenylalaninol oleamide, have the ability to distribute the active compound in an uniform and controlled manner.

The research objectives

✓ Synthesis and evaluation of the stability of NLC systems based on vegetable oil;

✓ Optimizing the concentration of surfactant mixture for NLC systems based on vegetable oil;

✓ Encapsulation of the active ingredient in NLC systems with the optimal concentration of surfactant mixture;

✓ Evaluation of the properties of NLC systems based on lipid mediators (oleoylethanolamide – OEA, phenylalaninol oleamide – FOA) co-encapsulated with natural

compounds (linseed oil – LO, argan oil – AO, evening primrose oil – ULN, caffeic acid – CA, curcumin – CRC, piperine – PIP, capsaicin – Cap);

✓ Evaluation of the biological properties (antioxidant properties, controlled release studies) of synthesized NLCs;

✓ Evaluation of the therapeutic response of NLC systems co-encapsulated with lipid mediators compared to the therapeutic response of unencapsulated natural extract.

CHAPTER 5

SYNTHESIS AND CHARACTERIZATION OF NLCs SYSTEMS LOADED WITH CAFFEIC ACID

5.2. CHARACTERIZATION OF FREE- AND CAFFEIC ACID-LOADED-NLCs SYSTEMS

Because one of the great advantages of NLC systems is that they assure an efficient controlled release of active ingredients, in this research we have developed NLC systems based on bioactive linseed oil. The ability to encapsulate a hydrophilic polyphenolic active compound with antioxidant, anti-inflammatory and antineoplastic potential – caffeic acid (CA) – and the ability to release this natural active agent were evaluated.

In this context, the main objective of this research was to encapsulate different amounts of caffeic acid in NLC systems, in order to determine the influence of caffeic acid concentration on antioxidant properties and on controlled release of caffeic acid. First, the influence of surfactant concentration on the most important characteristics of NLC systems, such as average particle size and zeta potential, was analyzed. Caffeic acid was encapsulated in nanoparticles with suitable dimensional characteristics. The CA-loaded NLC systems were evaluated in terms of size (average particle diameter, zeta potential) and encapsulation efficiency. The effectiveness of NLCs, as pharmaceutical formulations, has been *in vitro* characterized by the evaluation of antioxidant activity and by studies of controlled release of caffeic acid from NLC systems.

The research presented in this chapter aims to provide new perspectives on the association of two categories of natural active principles – hydrophilic and lipophilic – in the same lipid carrier system. Although there are several studies in which caffeic acid has been loaded into solid lipid nanoparticles (SLN) [217], its incorporation into lipid nanoparticles has not been reported so far. The novelty of this experimental part refers to the development of nanocarriers based on linseed oil loaded with caffeic acid, intensifying antioxidant activity and highlighting the synergistic effect of the two natural active principles – linseed oil and caffeic acid.

5.2.1. Synthesis of nanostructured lipid carriers (NLC)

In the first stage of the research presented in this chapter, we synthesized NLC systems based on linseed oil, using different concentrations of surfactant mixture (2%-3.5%). NLC systems were dimensionally characterized by the DLS method. Based on the optimal concentration of surfactants (2.5%), NLC systems based on linseed oil loaded with caffeic acid were synthesized, using the method of hot emulsification coupled with high shear homogenization and high pressure homogenization [218].

The composition of the synthesized NLC systems is shown in Table 5.1. Four different NLC formulations were obtained by varying the concentration of the surfactant mixture (2%, 2.5%, 3% and 3.5%), keeping contant the composition of glycerol monostearate (GMS), cetyl palmitate (CP) and linseed oil (LO). The main components of linseed oil are α-linolenic acid, oleic acid and linoleic acid

Table 5.1. Composition of NLC systems based on linseed oil

Formulation*	Surfactant mixture (g)	GMS (g)	CP (g)	LO (g)
NLC-LO1	2.0	3.5	3.5	3.0
NLC-LO2	2.5	3.5	3.5	3.0
NLC-LO3	3.0	3.5	3.5	3.0
NLC-LO4	3.5	3.5	3.5	3.0
*Mass of the synthesized sample = 100 g NLC aqueous dispersion *All NLC systems were prepared with 10% lipid phase, with a mass ratio of CP: GMS: LO = 1: 1: 1 and a mass ratio of Tween 20: poloxamer: L-α phosphatidylcholine = 70: 15: 15.				

5.2.2. Optimization of NLC systems and assessment of physical stability

In order to optimize the composition of NLC systems based on linseed oil, the dimensional characteristics of nanoparticles were evaluated by determining the average diameter (Zave) of nanoparticles and the polydispersity index (PdI), but also the physical stability of nanoparticles by determining zeta potential. Analyzing the experimental obtained data, it was shown that all NLC systems with different concentrations of surfactant mixture are stable over time.

The mean diameter particle size (Zave) and polydispersity index (PdI)

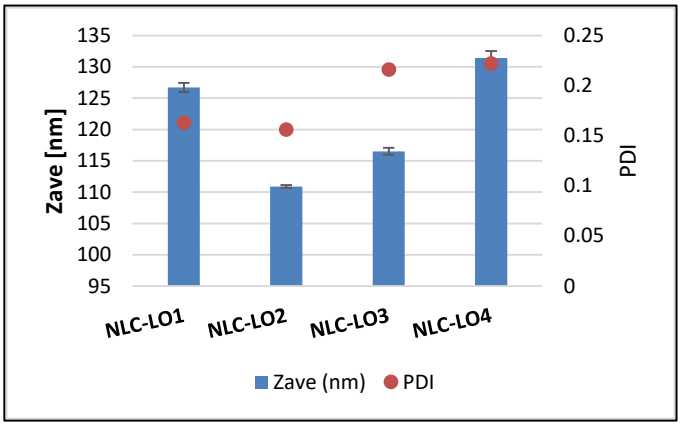


Figure 5.2. Mean diameter (columns) and PdI (circles) of NLCs prepared with variable content of surfactants

Dimensional characteristics (Zave and PdI) were evaluated by dynamic light scattering technique (DLS) [220]. The variation of Zave and PdI values of NLCs is shown in Figure 5.2.

By tracking the behavior of the four NLC formulations based on linseed oil, it was shown that all synthesized formulations have an adequate particle size distribution. The nanoparticle size ranged from 110.9 nm (for the NLC sample with 2.5% surfactant mixture) to 131.4 nm (for the NLC sample with 3.5% surfactant mixture). These results could be correlated with the efficiency of the surfactant mixture but also with the presence of the linseed oil, which contributes to the viscosity decrease of the lipid mixture, reducing the surface tension, with the formation of smaller and smoother surface particles [221]. Different values of Zave and PdI are influenced by the different

molecular weights of surfactants [222]. The best experimental data were obtained for the NLC-LO2 system with a surfactant concentration of 2.5%: Zave = 110.9 ± 2.3 nm and PdI = 0.156 ± 0.004.

In this experimental study it was noted that an optimal correlation between the average nanoparticle size and the polydispersity index was obtained in the case of the NLC-LO2 sample prepared with 2.5% surfactants mixture.

The zeta potential. The zeta potential (ξ) is one of the main characteristics that defines the behavior of nanoparticles in aqueous solution, which highlights the physical stability of NLC.

Prior to analysis, each NLC dispersion was diluted with ultrapurified water (in a ratio of NLC aqueous dispersion:ultrapurified water =1:0.01, v/v) and was adjusted by adding a minimum volume of sodium chloride 0.9% solution (e.g., to 20 mL of NLC aqueous dispersion was added 65µL NaCl solution, 0.9%) in order to reach a conductivity of 50 µS/cm (a value required in the electrophoretic light scattering technique). The zeta potential data of the synthesized NLCs are shown in Figure 5.3.

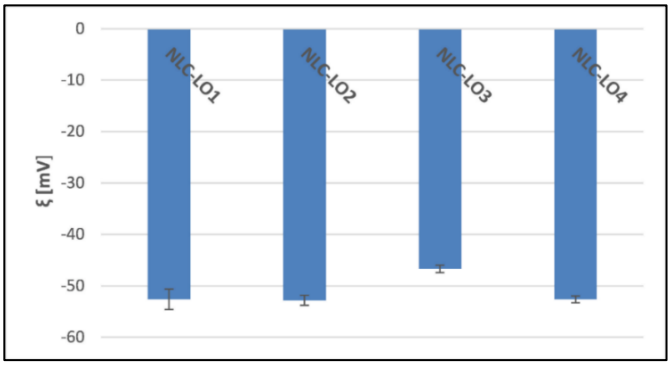


Figure 5.3. Evaluation of NLC stability by zeta potential

All the developed NLCs are stable, with the zeta potential distribution being between −52.8 mV and −47.6 mV. The negative charges of NLCs are due to surfactant mixture, with the best zeta potential result being obtained with 2.5% surfactant mixture, ξ = −52.8 mV ± 0.56 (Figure 5.3), which reveals a high stability of NLC and a low potential of aggregation of solid lipid nanocarriers suspended in aqueous media. Negative values of zeta potential indicate the presence of repulsions between nanoparticles, repulsions which prevent the nanoparticles aggregation.

It was noted that the best value of the zeta potential was obtained in the case of the NLC-LO2 sample, which has the best stability, which strengthens the previous considerations regarding to the average nanoparticle size and the polydispersity index, according to which the optimal concentration of surfactants mixtures is 2.5%.

5.2.3. Size characteristics and physical stability of CA-NLC systems

Table 5.7 shows different compositions of the synthesized CA-NLCs. Sample NLC-LO-CA1 contains the lowest concentration of caffeic acid (0.5%), sample NLC-LO-CA2 contains the intermediate concentration of caffeic acid (1%) and sample NLC-LO-CA3 contains the highest concentration of caffeic acid (1.5%).

Formulation	Surfactants mixture (g)	GMS (g)	CP (g)	LO (g)	Caffeic acid (g)
NLC-LO-CA1	2.5	3.5	3.5	3.0	0.5
NLC-LO-CA2	2.5	3.5	3.5	3.0	1.0
NLC-LO-CA3	2.5	3.5	3.5	3.0	1.5

The mean diameter particle size (Zave) and polydispersity index (PdI)

The NLC-LO2 system, with a small average nanoparticle diameter and optimal physical stability, was used to encapsulate the natural antioxidant – caffeic acid. The results obtained by the DLS technique showed that the NLC samples based on caffeic acid show a monomodal size distribution profile, with Zave values of 145.5 ± 0.8 nm for NLC-LO-CA1, 119.7 ± 0 .9 nm for NLC-LO-CA2 and 195.1 ± 5.3 nm for NLC-LO-CA3 (Figure 5.10 (a)). One example of size distribution is included in Figure 5.10 (b).

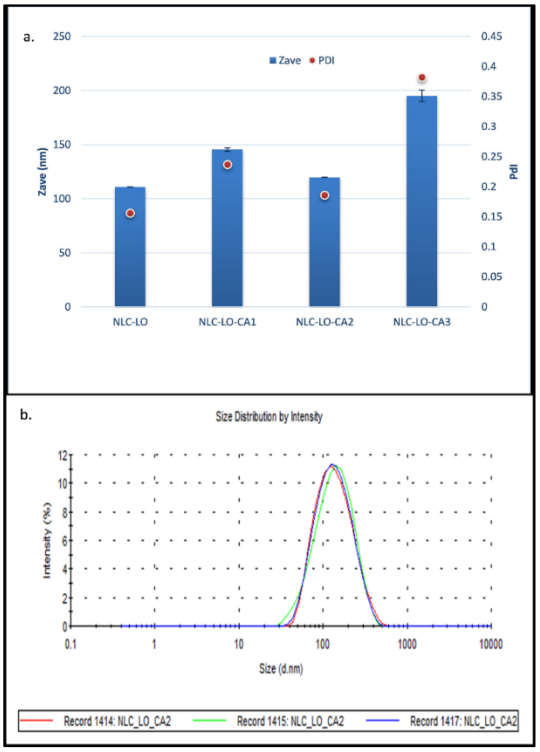


Figure 5.10. a) Evaluation of CA-loaded NLC stability by mean diameter and PdI
b) Exemplification of particle size distribution for NLC-LO-CA2

A drastic increase of Zave was observed for NLCs loaded with a high amount of CA, e.g., 195.1±5.3 nm for NLC loaded with 1.5% CA versus 119.7±0.9 nm for NLC loaded with 1% CA. This could be attributed to:

- ✓ the hydrophilic character of CA;
- ✓ the encapsulation of a limited concentration of caffeic acid in the surfactant coating;
- ✓ expulsion of caffeic acid excess outside the nanocarrier system.

The zeta potential

The results obtained after determination of electrokinetic potential showed that the NLC formulations loaded with CA are negatively charged (Figure 5.12).

As compared to the free NLC, there is a significant perturbation of electric surface after retention of caffeic acid. The addition of caffeic acid resulted in an increase in zeta potential, e.g., from -52.8 mV in free NLC to -48.2 mV and -34.9 mV in NLC-LO-CA1 and NLC-LO-CA2 (loaded with 0.5% and 1% CA, respectively). The presence of anionic active ingredient (CA) has led to a notable modification in zeta potential values because of surface charge rearrangements. A potential explanation may be related to masking of negative charges from phosphate groups (from phosphatidylcholine), due to the caffeic acid trapped by weak bonds in the surfactant coating or attached to the surfactant layer. These aspects justify a certain compensation of surface charges or a covering of negative charges from phosphatidylcholine.

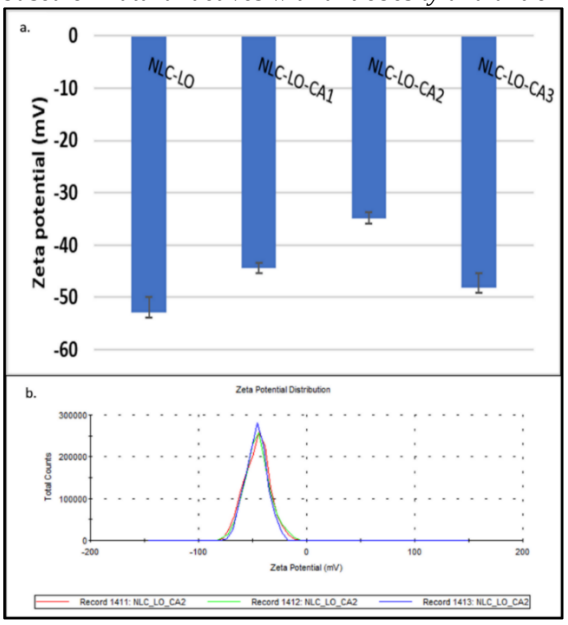


Figure 5.12. a) Zeta potential values of free and CA-loaded NLCs
b) Exemplification of zeta potential distribution for NLC-LO-CA2

5.3. Morphological characterization of NLCs loaded with caffeic acid

The lipid core of lipid nanocarriers is important to promote controlled release properties. The solid state of NLC systems was evaluated by differential scanning calorimetry [226], a thermal analysis that highlights possible changes in crystallinity in the lipid matrix. Morphological characterization of caffeic acid-based NLC systems, considering the optimal concentration of active substance (1%), was performed using the Atomic Force Microscope (AFM), 2D and 3D images (Figure 5.14 a), b)) provides an overview of the morphology of NLC systems, indicating an agglomeration of nanoparticles (the distance between the lowest peak and the highest peak is about 4µm).

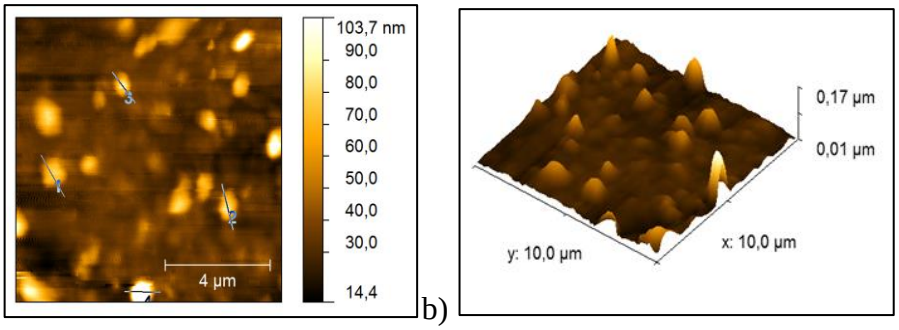


Figure 5.14. AFM morphological profile (2D-a) and 3D-b)) for NLC-LO-CA2

5.4. Entrapment Efficiency (EE%) of Caffeic Acid into NLC Systems

The results of EE% revealed that caffeic acid was efficiently encapsulated in NLC systems, mainly in the coating formed by the surfactants mixture and co-surfactants, obtaining EE% values higher than 95%. These results show a very good compatibility for a caffeic acid concentration of 1% (Figure 5.16).

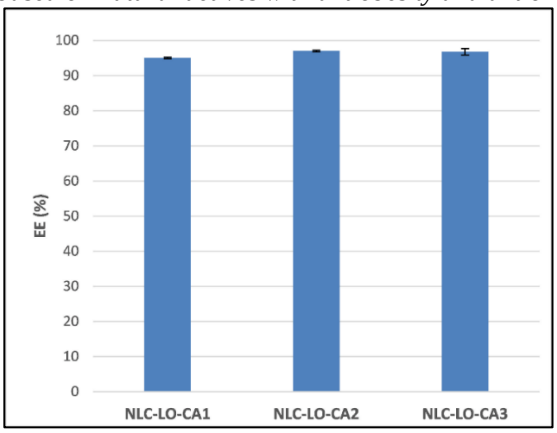


Figure 5.16. Entrapment efficiency of caffeic acid in NLCs

5.5. Evaluarea proprietăților biologice ale sistemelor NLC pe bază de acid cafeic

5.5.1. The *in vitro* determination of antioxidant activity

The ABTS method [228] highlights the antioxidant activity of complex biological systems and was used in our study to determine the scavenging activity of developed NLC-LO-CA 1/2/3 on $ABTS^{+*}$ radical, the measurements being made using a spectrophotometer at the wavelength of 734 nm and the final solution absorbance of 0.706 ± 0.01 [229].

The obtained data showed a higher antioxidant capacity for the CA-NLCs compared to native CA. The smaller concentration of the native CA (0.5%) shows a 21.5% ± 0.15 antioxidant capacity, while the encapsulation of CA into NLC revealed a radical $ABTS^{+*}$ inhibition activity of 47.2% ± 0.5 (Figure 5.17).

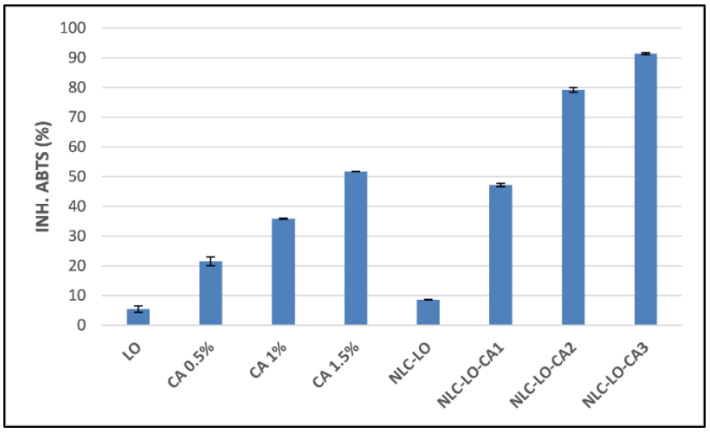


Figure 5.17. Comparative assessment of antioxidant activity of native CA, NLC-LO, and NLC-LO-CA

5.5.2. *In vitro* release of caffeic acid from NLC

The *in vitro* release of caffeic acid from loaded lipid nanocarriers in dispersion was evaluated using Franz diffusion [234].

The series of release profiles of CA from different NLCs loaded with 0.5%, 1%, and 1.5% CA showed a different behavior. Despite the similar sustained release pattern encountered for all NLC formulations in the first hours of release experiments, significant delimitation has been noted after 5 h as a function of the initial percent of CA loaded into NLC. Regarding the CA-NLCs, it was not evidenced a burst release, with a minimum CA release being found after the first hour of experiment (with about 8-10% of total CA released); a sustained release with a maximum degree of

about 65% CA was followed. It is interesting to note that during the entire release period, the amount of CA released from NLC loaded with 0.5 and 1% CA was almost similar (Figure 5.18).

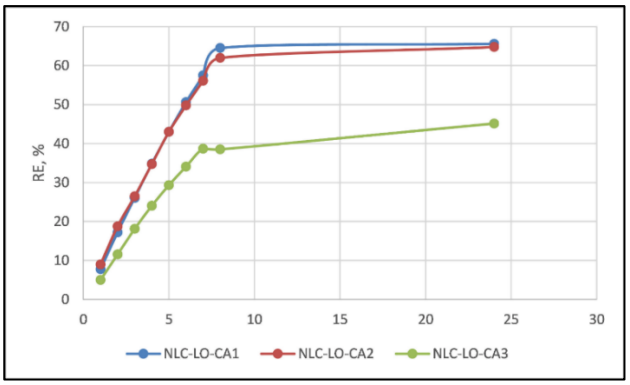


Figure 5.18. The influence of the lipid nanocarrier type on the release of caffeic acid

It is observed that the CA release from NLC is best reflected by the Korsmeyer–Peppas kinetics (R^2 > 0.994 in all cases studied). According to the release coefficient value (n = 0.5973, Table 2), NLC-LO-CA3 assures a release of CA following a non-Fickian diffusion model, while the release of CA% from NLC-LO-CA1 and NLC-LO-CA2 unfolds after a Fick diffusion mechanism (n < 0.5, Table 5.9) [44]. The almost constant trend of CA released remained constant over a period of 24 h, with a percentage of release of about 65% CA (from NLC-LO-CA1 and NLC-LO-CA2) and 45% in the case of NLC-LO-CA3. All experimental results demonstrate that LO from NLC influenced the controlled release of CA; this is a desired purpose in order to avoid the irritant effect of CA and to reach an effective plasmatic concentration of CA (after one hour). Indeed, the controlled release of CA, during a period of 24 h, could maintain an effective plasmatic concentration of CA

Table 5.9. Kinetic data obtained after release of CA from NLC prepared with linseed oil

Formulation	Zero order		First order		Higushi		Hixson-Crowell		Korsmeyer-Peppas		
	R^2	k_0	R^2	k_1	R^2	k_2	R^2	k_3	R^2	k_4	n
NLC-LO-CA1	0.9966	8.1046	0.9938	0.1353	0.9928	31.528	0.9897	0.0175	0.9967	2.0321	0.4754
NLC-LO-CA2	0.9946	7.5755	0.9972	0.1247	0.9946	29.528	0.9948	0.0161	0.9972	1.8555	0.4462
NLC-LO-CA3	0.9701	5.0423	0.9812	0.0667	0.9905	19.859	0.9823	0.0083	0.9941	2.1039	0.5973

CHAPTER 6
SINTHESIS AND CHARACTERIZATION OF NLCs SYSTEMS LOADED WITH CURCUMIN

6.2. CHARACTERIZATION OF FREE- AND CURCUMIN LOADED-NLCs SYSTEMS

The main objective of this chapter was to synthesize and characterize NLC systems prepared with vegetable oils (linseed oil – LO and argan oil – AO) that co-encapsulate curcumin (CRC) – natural active ingredient – and phenylalaninol oleamide (FOA) – synthetic active principle [263], in order to develop new safe and efficient NLC systems. In this study, we synthesized curcumin (CRC)-based lipid nanocarriers using a mixture of solid lipids (composed of glycerol monostearate and cetyl palmitate) in association with a lipid mediator (phenylalaninol oleamide) and a vegetable oil (linseed oil, respectively, argan oil).

6.2.1. Sinthesis of nanostructured lipid carriers based on curcumin

In the first stage of the study presented in this chapter, we synthesized NLC systems based on vegetable oil (linseed oil, respectively, argan oil), using the optimized concentration of surfactant mixture (2.5%); we obtained free vegetable NLC systems, which were characterized in terms of average nanoparticle size, polydispersity index and zeta potential using DLS method. We synthesized six different NLC formulations, the compositions being presented in table 6.1.

Table 6.1. Composition of NLCs based on linseed oil (LO)/argan oil (AO) and loaded with curcumin (CRC) and phenylalaninol oleamide (FOA)

Formulatiom	Surfactants mixture (g)	GMS (g)	CP (g)	LO (g)	AO (g)	FOA (g)	CRC (g)
NLC-LO	2.5	3.0	3.0	3.0	-	-	-
NLC-AO	2.5	3.0	3.0	-	3.0	-	-
NLC-LO-CRC	2.5	3.0	3.0	3.0	-	-	0.1
NLC-AO-CRC	2.5	3.0	3.0	-	3.0	-	0.1
NLC-LO-FOA-CRC	2.5	3.0	3.0	3.0	-	1.0	0.1
NLC-AO-FOA-CRC	2.5	3.0	3.0	-	3.0	1.0	0.1

6.2.2. Dimensional characteristics and physical stability of NLCs loaded with curcumin

The mean diameter particle size (Zave) and polydispersity index (PdI)

All the NLC systems formulations presented small particle size, with Zave values less than 200 nm. For example, Zave values of NLC-LO-PO-CRC and NLC-AO-PO-CRC are 129.7 nm and 127.6 nm, respectively, while those of NLC-LO-CRC/NLC-AO-CRC and NLC-LO/NLC-AO are of 149.1 nm/ 130.3 nm and 130.9 nm/ 157.6 nm (Figure 6.2). A reorganization of the lipid core, obtaining smaller diameters was observed when encapsulating curcumin in NLC. The result of Zave decrease can be attributed to a loss of viscosity (which helps in HPH processing, with smaller particles), by combining PO with the other lipids selected in the preparation.

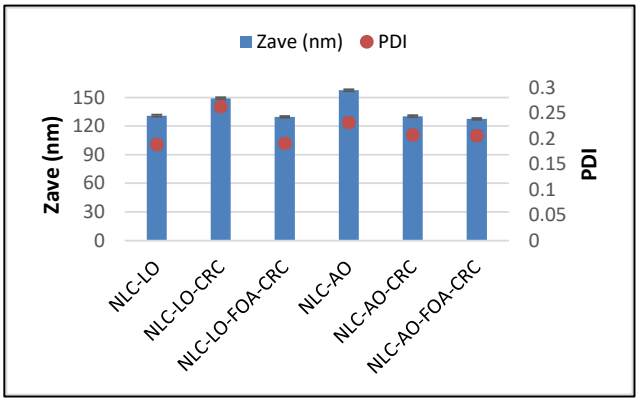


Figura 6.2. Mean particle size (nm) and PdI of NLC-LO/AO-FOA-CRC dispersions

Physical stability of CRC-NLC systems

The experimental results showed that all Curcumin-loaded NLCs are stable over time, with a zeta potential average between -46.6 mV (for NLC-AO-PO-CRC) and -55.3 mV (for NLC-LO-CRC), the entrapment of CRC in the lipid core explaining the small difference between the zeta potential values of NLC-AO and Curcumin-loaded NLCs (Figure 6.4).

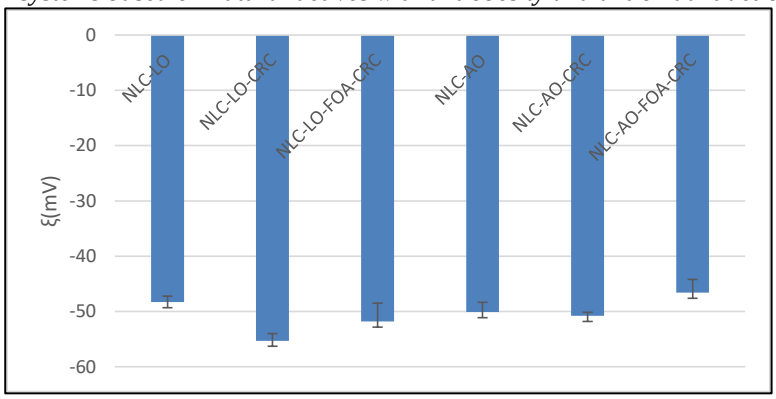


Figure 6.4. Electrokinetic potential distribution in case of free NLCs versus CRC-loaded NLCs

For all of the developed NLCs, the zeta potential values were lower than < 40 mV, so the obtained NLC systems are expected to manifest a long-term physical stability (Figura 6.7).

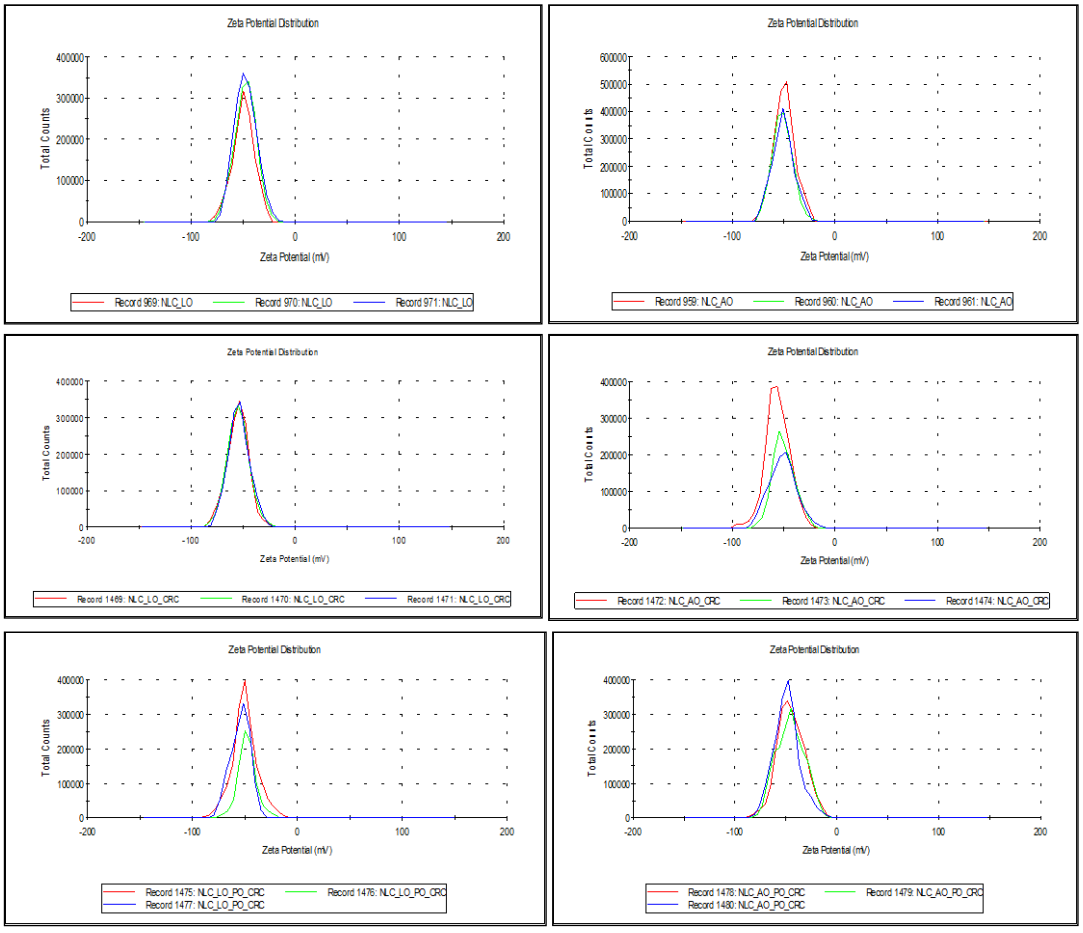


Figure 6.7. Zeta potential distribution for free and CRC-loaded NLCs

6.3. DETERMINATION OF ENTRAPMENT EFFICIENCY (EE%) OF CURCUMIN IN NLCs SYSTEMS

The entrapment efficiency (EE%) and loading capacity (LC%) of CRC within different prepared lipid nanocarriers based on linseed/argan oil are shown in Figures 6.10 and 6.11.

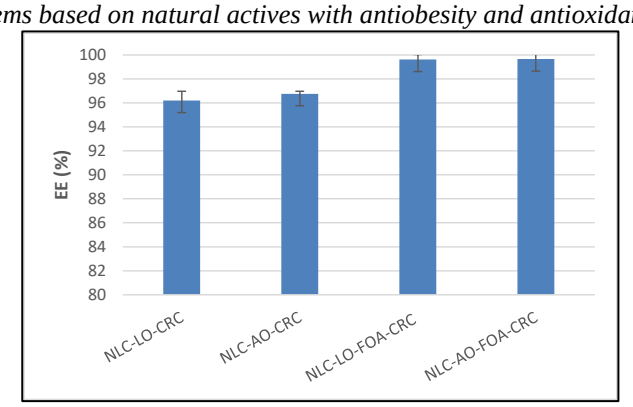


Figura 6.10. Entrapment efficiency (EE%) of CRC into NLCs based on linseed/argan oil

The loading capacity of curcumin into lipid phase is well correlated to the entrapment efficiency values, from 7.05% (NLC-LO-CRC) to 8.62% (NLC-LO-PO-CRC) (Figure 6.11).

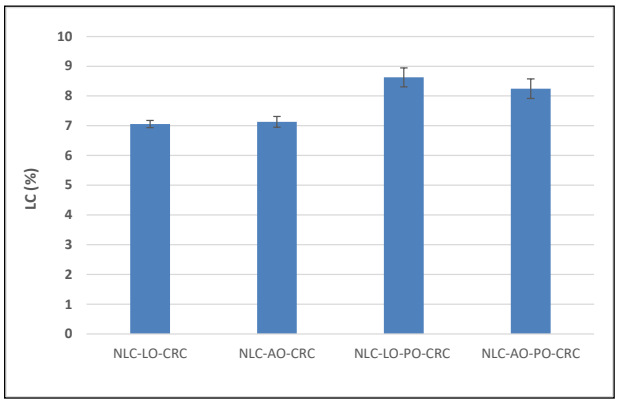


Figure 6.11. Loading capacity (LC%) of CRC into NLCs based on linseed/argan oil

The values of EE% of curcumin in NLC samples were higher than 96%, these results being explained by the efficient entrapment of curcumin in the lipid network formed by phenylalaninol oleamide in combination with vegetable oils, the values varying from 96.2% (in the case of the NLC-LO-CRC sample) to 99.66% (in the case of the NLC-AO-FOA-CRC sample).

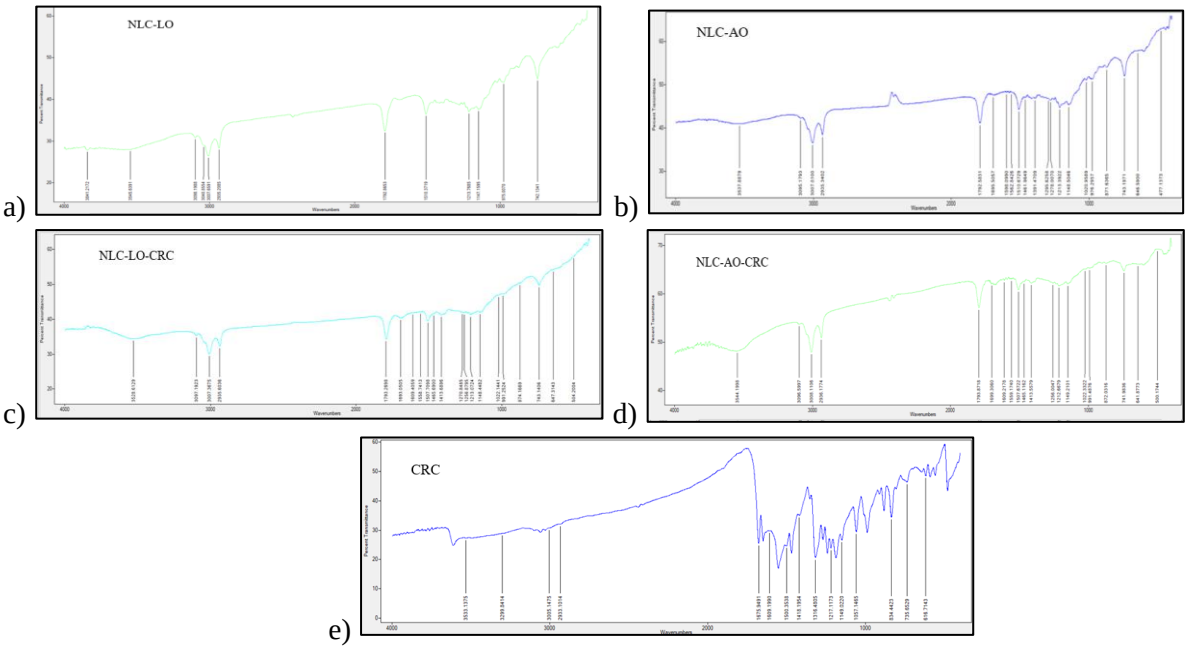


Figure 6.12. FT-IR spectra of CRC, NLC-LO/AO and NLC-LO/AO-CRC systems

The study on the encapsulation of curcumin in synthesized NLC systems led to optimal results of entrapment efficiency. In case of these NLC systems loaded with curcumin and co-loaded with phenylalaninol oleamide, the good results of the EE% can be explained by the high compatibility of the two active principles, curcumin and phenylalaninol oleamide, with the lipid mixture consisting of linseed oil / argan oil, glycerol monosterate and cetyl palmitate.

The encapsulation of curcumin in NLC systems based on linseed oil/argan oil was highlighted by Fourier Transform Infrared Spectroscopy using a Jasco FT-IR 620 spectrophotometer; the obtained FT-IR spectra show the structural changes as a result of curcumin encapsulation (Figure 6.12).

6.4. EVALUATION OF THE BIOLOGICAL PROPERTIES OF NLCs LOADED WITH CURCUMIN

6.4.1. *In vitro* testing of antioxidant properties

Vegetable oils used in the new NLC systems, linseed oil and argan oil, are important sources of antioxidants.

In our research, in order to determine the free radical scavenging activity of curcumin, FOA, linseed oil, argan oil, free NLC, NLC-CRC and NLC-FOA-CRC, a spectrophotometric ABTS assay was used. This method is helpful to quantify the free radical trapping capacity of antioxidants [228].

The activity against in situ obtained $ABTS^{+*}$ cation radicals showed the antioxidant potential of the Curcumin in association with argan/linseed oil and with FOA. The antioxidant effect of free NLC may be related to the high level of α-linolenic acid in the linseed oil and of linoleic acid in the argan oil. The presence of the native curcumin with linseed/argan oil in the same NLCs induces an improvement of antioxidant action, e.g. 61.62% inhibition of $ABTS^{+*}$ in case of NLC-LO-CRC and 64.2% in case of NLC-AO-CRC. The presence of both CRC and FOA has led to a higher ability of developed NLC to inhibits $ABTS^{+*}$ cation radicals, with inhibition percentage of 69.37% for NLC-AO-PO-CRC and 75.73% for NLC-LO-PO-CRC (Figure 6.13).

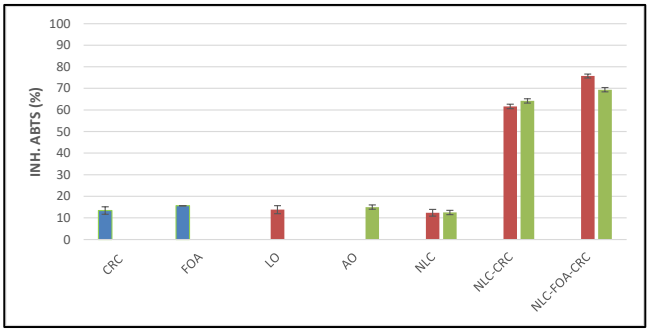


Figure 6.13. Antioxidant activity of developed-NLC *versus* native curcumin and vegetable oils (ABTS assay)

A comparative look of the data represented in Figure 6.14 demonstrates the synergistic effect manifested by CRC-linseed oil/argan oil, both captured in the same lipid delivery system. The percentage of ABTS inhibition is 6-fold higher in the case of NLC-CRC and NLC-PO-CRC, compared to NLC-free and native-CRC and LO/AO.

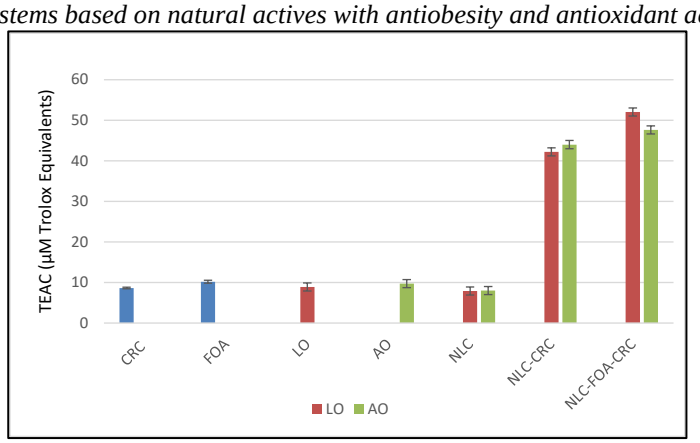


Figura 6.14. Comparative assessment of antioxidant activity of native curcumin, lipids, free- and CRC loaded-NLCs, by TEAC method

6.4.2. *In vitro* controlled release of curcumin

According to the release profiles (Figure 6.15), in the group of curcumin-loaded NLCs it was observed a slow release of curcumin; after 24 h of the *in vitro* experiments 17.1% of curcumin was released in case of NLC-AO-PO-CRC and 32% in case of NLC-LO-PO-CRC.

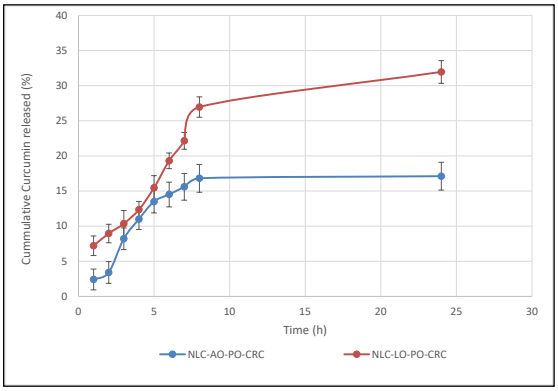


Figure 6.15. Influence of the lipid nanocarriers type on the release of curcumin

The curcumin release from NLC-LO/AO-FOA-CRC exhibited a controlled-release profile, which revealed a two-stage process: the initial release (during 8h of experiments) might be related to release of a small amount of curcumin adsorbed to the surface layer of NLC and to the passive diffusion of curcumin. After 8h, 16.8% of curcumin was released in case of NLC-AO-FOA-CRC and 26.9% of curcumin in case of NLC-LO-FOA-CRC. Moreover, the prolonged release profile after 8 h should be associated to the curcumin incorporated into the NLC inner core that is slowly released with the degradation of NLC. A possible explanation for the slow release of curcumin from the NLC-AO-FOA-CRC delivery system as compared with those of NLC-LO-FOA-CRC, might be an increased affinity between curcumin, FOA and AO due to π-stacking of phenyl moieties which causes a delayed release of the drug from the lipid network.

Table 6.4. Kinetic data obtained at controlled release of curcumin from NLC prepared with linseed oil

NLC formulations	Zero order		First order		Higushi		Hixson-Crowell		Korsmeyer-Peppas		
	R^2	k_0	R^2	k_1	R^2	k_2	R^2	k_3	R^2	k_4	n
NLC-AO-FOA-CRC	0.9423	2.181	0.95	0.0242	0.9698	8.6244	0.9511	0.0029	0.9513	2.0502	0.4877
NLC-LO-FOA-CRC	0.9298	2.669	0.9123	0.032	0.857	9.9885	0.9095	0.0039	0.8918	1.2032	0.8311

According to the correlation coefficients, it was observed that the release of curcumin from synthesized NLC is performed after a zero-order kinetics in case of NLC-LO-PO-CRC (R^2=0.9298) and after a Higuchi model in case of NLC-AO-PO-CRC (R^2=0.9698). After 8h, in case of NLC-AO-PO-CRC the rate constant (k=8.62) is higher than that of NLC-LO-PO-CRC (k=2.18), which strengthens the previous assumption regarding faster release of curcumin from the LO-based NLC compared to AO-based NLC.

The results highlight that NLC based on linseed oil played an important role in controlled release of CRC, this phenomenon could be efficient to avoiding the potential irritant effect of CRC on the gastrointestinal tract and could be useful to get an effective plasmatic concentration of CRC – which can be maintained for a long period of time by the sustained release during 24h.

CHAPTER 7

SINTHESIS AND CHARACTERIZATION OF NLCs SYSTEMS LOADED WITH PIPERINE

7.2. CHARACTERIZATION OF FREE- AND PIPERINE LOADED- NLCs SYSTEMS

In this chapter, the main objective was to synthesize and characterize lipid nanocarriers prepared with vegetable/mineral oil (evening primrose oil – ULN/paraffin oil – UP) that encapsulate piperine (PIP) as a natural active ingredient, in order to develop new pharmaceutical formulations useful to treat many diseases. The present research study was based on the synthesis of piperine-based NLC systems using a mixture of solid lipids (consisting of glycerol monostearate and cetyl palmitate) in combination with bioactive vegetable/mineral oil (evening primrose oil/paraffin oil).

7.2.1. Sinthesis of nanostructured lipid carriers based on piperine

For the synthesis of lipid nanocarriers we used a lipid phase consisting of a mixture of glycerol monostearate and cetyl palmitate in a mass ratio of 1:1 and vegetable / mineral oil (evening primrose oil/paraffin oil) and an aqueous phase consisting of a mixture of surfactants – Tween 20: L-α-phosphatidylcholine: Poloxamer Sinperonic PE / F68 in a mass ratio 75:15:15. We synthesized six different NLC formulations, the composition of the new NLC systems being presented in table 7.1

Table 7.1. Composition of NLCs based on evening primrose oil (ULN)/paraffin oil (UP) and loaded with piperine (PIP)

Formulation	Surfactants mixture (g)	GMS (g)	CP (g)	UP (g)	ULN (g)	PIP (g)
NLC-UP	2.5	3.0	3.0	3.0	-	-
NLC-ULN	2.5	3.0	3.0	-	3.0	-
NLC-UP-PIP 1%	2.5	3.0	3.0	3.0	-	1.0
NLC-LN-PIP 1%	2.5	3.0	3.0	-	3.0	1.0
NLC-UP-PIP 1,5%	2.5	3.0	3.0	3.0	-	1.5
NLC-LN-PIP 1,5%	2.5	3.0	3.0	-	3.0	1.5

7.2.2. Dimensional characteristics and physical stability of NLCs loaded with piperine

The main purpose of this characterization was to evaluate as accurately as possible the dimensional characteristics of the synthesized NLC systems. The determinations of the average

diameter (Zave) of the nanoparticles, the polydispersity index (PdI), but also the zeta potential that provides information about the physical stability of the nanoparticles are presented.

The mean diameter particle size (Zave) and polydispersity index (PdI)

The NLC-UP/LN-PIP formulations showed optimal nanoparticle sizes, with Zave values less than 200 nm. For example, the Zave values of the NLC-UP-PIP 1% and NLC-LN-PIP 1% are 122.9 nm and 121.9 nm, respectively, while the NLC-UP-PIP 1.5% formulations are / NLC-LN-PIP 1.5% and NLC-UP / NLC-ULN are 119.2 nm / 124.3 nm and 129.2 nm / 137.7 nm (Figure 7.3). A reorganization of the lipid core was observed, resulting in smaller diameters by encapsulating piperine in NLC systems. The result of the decrease of the average particle size can be attributed to a decrease of the viscosity (which helps to process HPH with smaller particles) by combining vegetable oils with other lipids used for NLC synthesis.

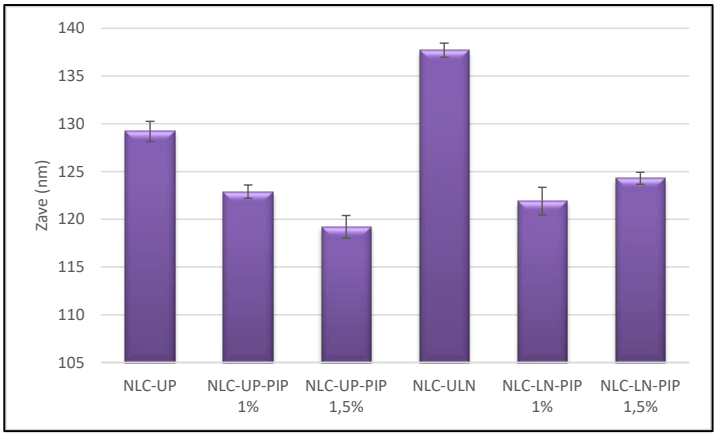


Figure 7.3. Mean particle size (nm) distribution in case of NLC-LO/AO-FOA-CRC dispersions

On the other hand, the values of the polydispersity index are optimally correlated with the values of the average nanoparticle size for all piperine-based NLC samples. The PdI values of the NLC-UP-PIP 1% and NLC-LN-PIP 1% samples are 0.173 and 0.169, respectively, while the NLC-UP-PIP 1.5% / NLC-LN-PIP 1.5% and NLC-UP / NLC-ULN formulations are 0.157/0.184 and 0.223/0.199 (Figure 7.4). The lowest value of the polydispersity index (0.157 ± 0.014) was attributed to NLC systems synthesized with paraffin oil and piperine 1.5%, and the highest value (0.223 ± 0.006) was attributed to free NLC based on paraffin oil. NLC samples based on vegetable oil and piperine 1% show similar values of the polydispersity index (0.173 ± 0.006 for NLC-UP-PIP 1% and 0.169 ± 0.006 for NLC-LN-PIP 1%).

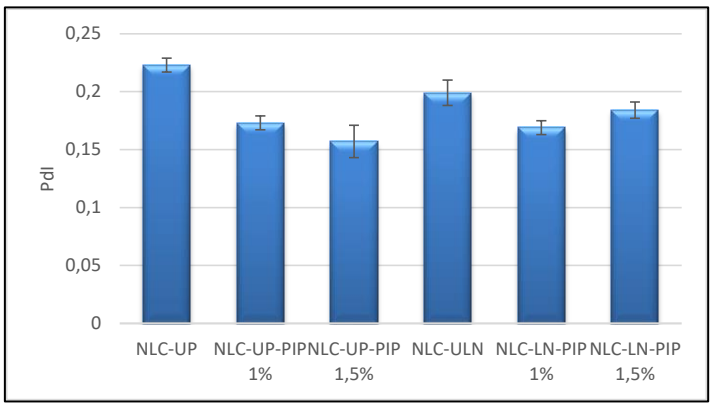
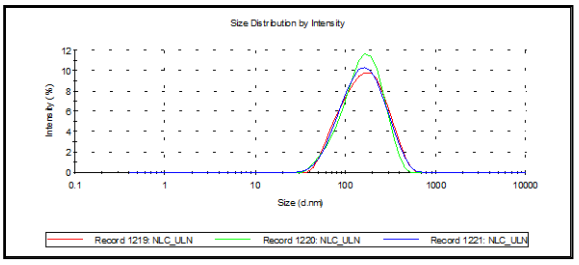


Figure 7.4. PdI distribution in case of NLC-LO/AO-FOA-CRC dispersions

Piperine-loaded NLC systems based on paraffin oil have a higher polydispersity index than in the case of piperine-loaded NLC systems based on evening primrose oil, with the exception of nanoparticles

with a piperine content of 1,5% (0,157 ± 0,014 for the sample NLC-UP-PIP 1.5% *versus* 0.184 ± 0.007 for sample NLC-LN-PIP 1.5%).

Free NLC systems prepared with evening primrose oil/paraffin oil have an optimal average nanoparticle size and a PdI polydispersity index less than 0.25 (Figure 7.5 a, b), the difference between the average size of nanoparticles prepared with paraffin oil (129.2 nm ± 1.1) and the average size of nanoparticles prepared with evening primrose oil (137.7 nm ± 0.7) can be explained taking into account the different chemical composition of both oils, evening primrose oil being enriched in unsaturated fatty acids (mainly linoleic acid and γ-linolenic acid). NLC systems prepared with evening primrose oil/paraffin oil not encapsulated with piperine have an optimal average nanoparticle size and a PdI polydispersity index with values less than 0.25 (Figure 7.5 a, b), stating that the difference between the average size of of nanoparticles prepared with paraffin oil (129.2 nm ± 1.1) and the average size of nanoparticles prepared with evening primrose oil (137.7 nm ± 0.7) can be explained on the basis of the different chemical composition of the two oils, evening primrose oil being enriched in unsaturated fatty acids (mainly linoleic acid and γ-linolenic acid).

a) 

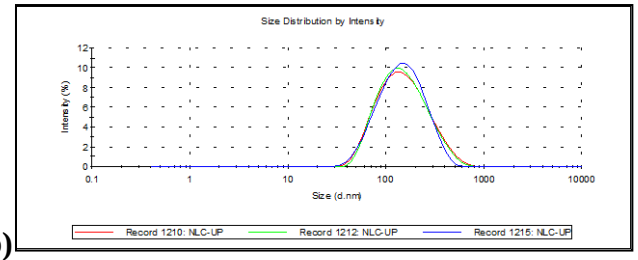
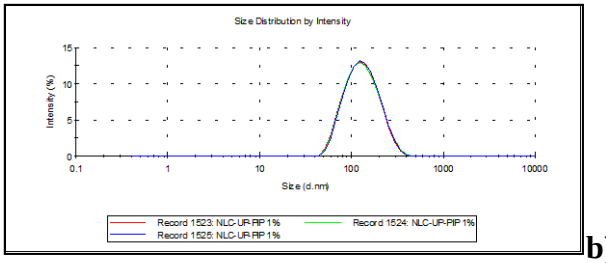
b) 

Figure 7.5. Mean particle size (nm) distribution
a) for NLC-ULN
b) for NLC-UP

NLC systems based on evening primrose oil/paraffin oil encapsulated with piperine of 1% concentration have an optimal average nanoparticle size and a PdI polydispersity index with values less than 0.25 (Figure 7.6 a, b), with a small difference between the average size of the nanoparticles prepared with paraffin oil (122.9 nm ± 0.7) and the average size of the nanoparticles prepared with evening primrose oil (121.9 nm ± 1.4). **It has been observed that encapsulation of piperine (1%) in the lipid matrix of nanocarriers based on evening primrose oil/paraffin oil has improved the average nanoparticle size.**

a) 

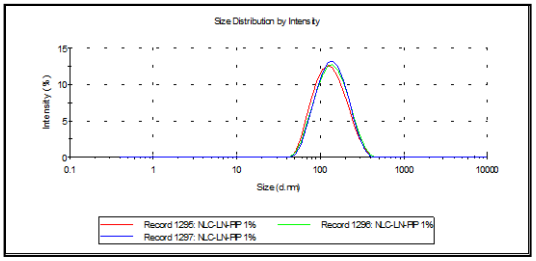
b) 

Figure 7.6. Mean particle size (nm) distribution
a) for NLC-UP-PIP 1%
b) for NLC-LN-PIP 1%

In the case of NLC systems based on evening primrose/paraffin oil encapsulated with piperine of 1.5% concentration, optimal values of the average nanoparticle size and the PdI polydispersity index were also obtained (Figure 7.7 a, b), with an insignificant difference between the average size of the nanoparticles prepared with paraffin oil (119.2 nm ± 1.1) and the average size

of the nanoparticles prepared with evening primrose oil (124.3 nm ± 0.6). **It was observed that encapsulation of piperine in higher concentration (1.5%) in the lipid matrix of nanocarriers based on evening primrose oil/paraffin oil further improved the average nanoparticle size.**

In conclusion, the encapsulation of a larger amount of piperine showed a more pronounced decrease in the average size of NLCs based on paraffin oil/evening primrose oil, a phenomenon explained by the affinity of piperine for the lipid core and by the rearrangement of fatty acid chains from the structure of vegetable oils that allow the incorporation of a larger amount of active compound, thus causing the decrease of the average size of NLC systems.

Physical stability of PIP-NLC systems

The experimental results showed that all NLCs encapsulated with piperine show physical stability over time, the average value of the zeta potential being ranged between -45.3 mV (for NLC-UP-PIP 1% sample) and -57 mV (for NLC-ULN sample), the encapsulation of piperine in the lipid core explaining the small difference between the values of the potential zeta of NLC-UP and NLC-ULN systems loaded with piperine. The negative loads of NLCs are due to the mixture of surfactants and the composition of the oils used. The distributions of potential zeta of developed NLCs, free NLCs and piperine-loaded NLCs, are shown in Figure 7.7.

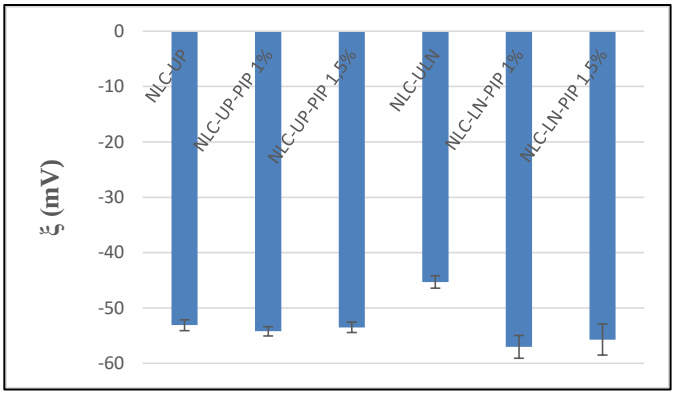
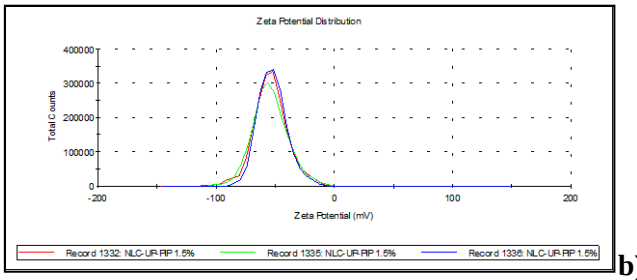


Figura 7.7. Electrokinetic potential distribution in case of free NLCs versus PIP-loaded NLCs

The encapsulation of piperine in the considered NLC systems led to an increase of sample stability, zeta potential values showing that NLC samples encapsulated with 1% piperine show the highest stability (-54.2 mV ± 0.84 for NLC-UP-PIP 1% and -57 mV ± 2.06 for NLC-LN-PIP 1%) (Figure 7.9). For NLC systems encapsulated with the highest amount of piperine (1.5%), stability can be demonstrated by the values of the zeta potential (-53.5 mV ± 0.929 for NLC-UP-PIP 1.5% and -55.7 mV ± 2.81 for NLC-LN-PIP 1.5%) (Figure 7.10 a, b).

a)



b)

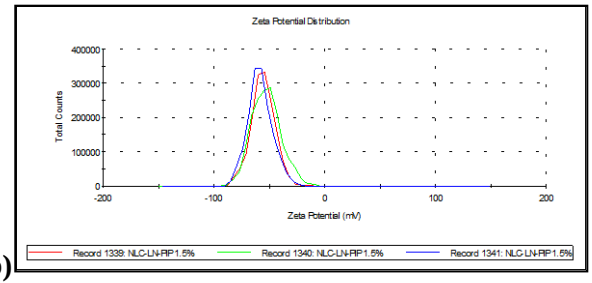


Figure 7.10. Electrokinetic potential distribution in case of a) NLC-UP-PIP 1,5%, b) NLC-LN 1,5%

The experimental data presented above show that the nature of the active substances encapsulated inside the lipid matrix of the NLC system has a strong influence on the stability of colloidal particles; the potential zeta values of the synthesized NLC systems more electronegative than -50 mV (except for the NLC-ULN sample, with a value of -45.3 mV ± 1.12) demonstrate the excellent stability over time of the synthesized NLCs.

7.3. DETERMINATION OF ENTRAPMENT EFFICIENCY (EE%) OF PIPERINE IN NLCs SYSTEMS

The encapsulation efficiency (EE%) of piperine in NLC systems based on paraffin oil/evening primrose oil is shown in Figure 7.13.

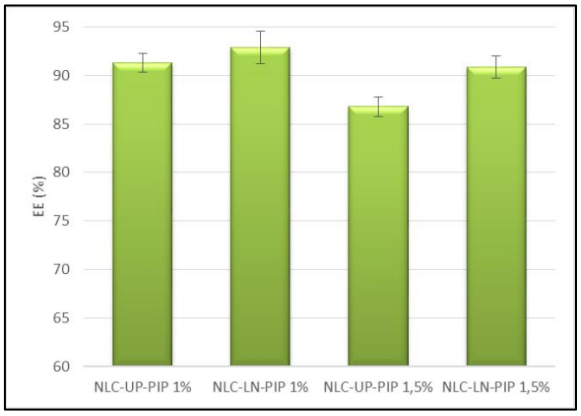


Figure 7.13. Entrapment efficiency (EE%) of piperine NLC systems based on paraffin oil/evening primrose oil

The EE% values were higher than 86%, these results being explained by the efficient encapsulation of piperine in the lipid network based on vegetable oils, the values varying from 86.77% (for NLC-UP-PIP 1.5%) to 92.89% (for NLC-LN-PIP 1%). It was shown that the NLC sample based on paraffin oil encapsulated with 1% piperine had a slightly lower encapsulation efficiency than the NLC sample based on evening primrose oil, no significant differences between the two formulations were. The encapsulation of a higher amount of piperine (1.5%) resulted in entrapment efficiency values of 86.77% for NLC-UP-PIP 1.5% and 90.85% for NLC-LN -PIP 1.5%; the difference between the entrapment efficiencies can be explained by the excess piperine (0.5%) which could no longer be encapsulated in the lipid matrix and remained outside the lipid core.

The study regarding the encapsulation of piperine in synthesized NLC systems led to optimal results of entrapment efficiency, which can be explained by the high compatibility between the active ingredient – piperine – and the lipid mixture consisting of paraffin oil/evening primrose oil, glycerol monostereat and cetyl palmitate.

7.4. EVALUATION OF THE BIOLOGICAL PROPERTIES OF NLCs LOADED WITH PIPERINE

The antioxidant activity of piperine-loaded NLC systems and the controlled release of piperine from NLC systems were evaluated by *in vitro* studies, as presented below.

7.4.1. *In vitro* testing of antioxidant properties

In vitro evaluation of antioxidant activity by ABTS method

The antioxidant activity of free NLC systems can be attributed to the chemical composition of the both oils used in the synthesis step. The encapsulation of piperine in NLC systems prepared with paraffin oil or evening primrose oil led to an improvement in antioxidant activity, the values of the ABTS^{+*} inhibition percentages being 27.99% for NLC-UP-PIP 1%, 31.73% for NLC-UP-PIP 1.5%, 34.3% for NLC-LN-PIP 1% and 38.49% for NLC-LN-PIP 1.5%. The presence of higher amounts of piperine in the synthesized NLC systems led to an increase in antioxidant activity, the values of the inhibition percentages for samples with 1.5% piperine being higher than in the case of NLC samples with only 1% piperine. A stronger antioxidant effect was also observed in the case of NLC samples based on evening primrose oil compared to the antioxidant effect of samples based on paraffin oil, regardless of the concentration of piperine used in the synthesis; this result can be explained by the evening primrose oil composition - rich in unsaturated fatty acids (linoleic acid, γ-linolenic acid, oleic acid) (Figure 7.15).

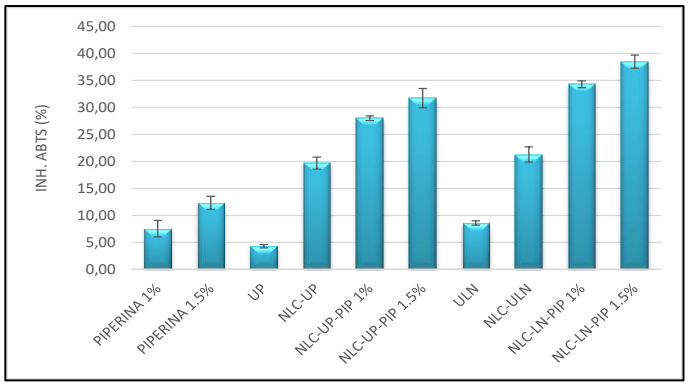


Figure 7.15. Antioxidant activity of NLC-UP/LN-PIP *versus* native piperine, paraffin oil and evening primrose oil (ABTS assay)

Another important step in the research was the evaluation of the antioxidant activity of non-encapsulated piperine, paraffin oil, evening primrose oil, free NLC systems compared to the antioxidant activity of piperine-loaded NLC systems, using the TEAC (Trolox Equivalents Antioxidant Capacity) method. A comparative look at the data shown in Figure 7.16 demonstrates the synergistic effect of piperine and evening primrose oil/paraffin oil, both natural active ingredients encapsulated in the same system of lipid nanocarriers. The percentage of ABTS^{+*} radical inhibition is 5-Fold higher in the case of the NLC-LN-PIP 1% compared to that obtained in the case of piperine 1%.

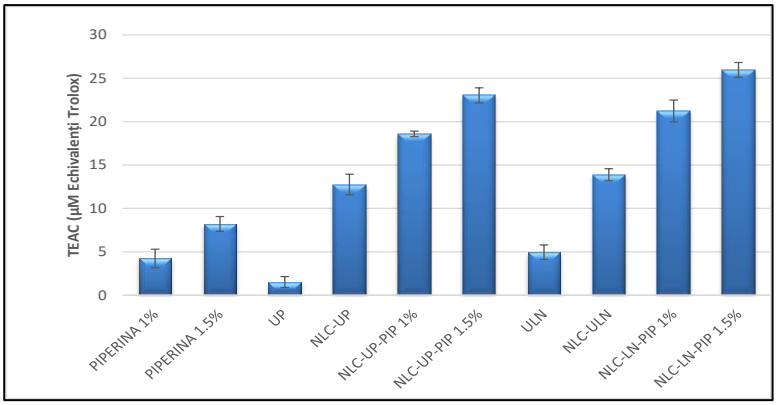


Figura 7.16. Comparative assessment of antioxidant activity of native piperine, evening primrose oil/paraffin oil, free- and PIP-loaded-NLCs (ABTS method)

The ability to scavenge the $ABTS^{+*}$ cationic radicals in case of PIP-loaded NLC varied in a piperine concentration dependent manner, so the most pronounced antioxidant activity was observed in case of NLCs with a content of 1.5 % piperine.

***In vitro* determination of antioxidant activity by chemiluminescence method**

The antioxidant activity evaluated by the chemiluminescence method led to results similar to those obtained by the ABTS method, so all NLC samples that encapsulate 1.5% piperine have the highest capacity to scavenge short-lived oxygen radicals (ROS) (76.11% for NLC-UP-PIP 1.5% and 82.62% for NLC-LN-PIP 1.5% compared to free NLCs (39.83% for NLC-UP and 41.40% for NLC-ULN) or with individual active compounds (46.99% for piperine 1%, 62.46% for piperine 1.5%, 16.05% for UP and 24.92% for ULN) (Figure 7.17). **It should be noted that the synthesized NLC samples have a higher affinity for scavenging short-lived radicals involved in oxidative processes than long-lived radicals.**

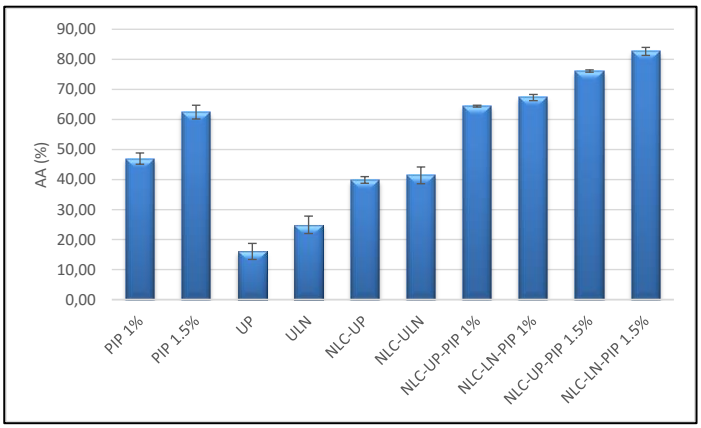


Figure 7.17. Antioxidant activity of NLC-UP/LN-PIP *versus* native piperine, paraffin oil and evening primrose oil (chemiluminescence method)

The encapsulation of piperine in association with an active lipophilic compound (evening primrose oil or paraffin oil) in the same nanocarrier system has led to enhanced antioxidant activity, with a wide range of action for both short-lived radicals (ROS) and long-lived radicals ($ABTS^{*+}$), so the developed NLC systems can be used successfully in the treatment of certain conditions such as obesity.

7.4.2. *In vitro* controlled release of piperine

As shown in Figure 7.18, the both categories of nanocarriers behave quite differently in terms of piperine release. It is interesting to note that the NLC system prepared with paraffin oil shows a rapid release of the active, reaching a release rate of more than 65% in the first 8 hours of experiments, compared to NLC systems prepared with evening primrose oil, for which the percentage of release did not exceed 20%. The rapid release of piperine from NLC system prepared with paraffin oil can be attributed to a much faster mass transfer of piperine, which occurs due to a structural incompatibility of piperine between the linearity conferred by alkanes in the paraffin oil, low solubility of piperine in paraffin oil, but also of a lower viscosity of the lipid matrix formed by the solid lipids associated with paraffin oil. In contrast, the two NLC systems based on evening primrose oil show the model of lipid core enrichment with active principle, due to the ability of lipid

mixtures to form highly disordered networks, with multiple imperfections between triacylglycerols hydrocarbon chains in the composition of evening primrose oil.

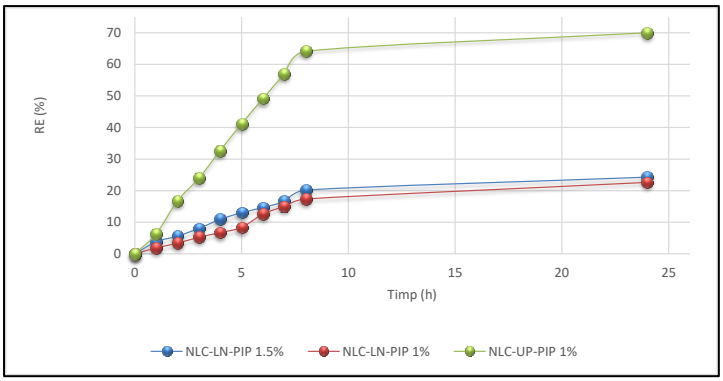


Figure 7.18. Influence of the lipid nanocarriers based on evening primrose oil /paraffin oil on the release of piperine

These results highlight a clear barrier between the influence of a mineral oil and that of a vegetable oil in the design of an efficient nanocarrier system, with a sustained release by active principles. **Thus, this study proved the relevance of the use of vegetable oils, to the detriment of mineral oils.**

According to the correlation coefficients, it was observed that the release of piperine is achieved according to a Korsmeyer-Peppas kinetics in the case of NLC samples based on piperine evening primrose oil (R^2 = 0.9992 for NLC-LN-PIP 1.5% and R^2 = 0.9838 for NLC-LN-PIP 1%) and zero kinetics for the sample based on piperine and paraffin oil (R^2 = 0.9984 for NLC-UP-PIP 1%) (Table 7.5).

Table 7.5. Kinetic data obtained after release of piperine from NLCs

NLC formulation	Zero order		First order		Higushi		Hixson-Crowell		Korsmeyer-Peppas		
	R^2	k_0	R^2	k_1	R^2	k_2	R^2	k_3	R^2	k_4	n
NLC-LN-PIP 1,5%	0.9935	2.2793	0.9916	0.0259	0.9722	8.7889	0.9911	0.003	0.9992	1.6153	0.619
NLC-LN-PIP 1%	0.976	2.2669	0.9702	0.0251	0.9247	8.6006	0.9693	0.003	0.9838	2.1732	0.469
NLC-UP-PIP 1%	0.9984	8.2036	0.9889	0.1351	0.9883	31.815	0.9837	0.0174	0.9928	2.1802	0.458

The NLC systems based on evening primrose oil showed a release according to the Korsmeyer-Peppas mechanism, having n = 0.62 (NLC-LN-PIP 1.5%) and n = 0.47 (NLC-LN-PIP 1%). The mechanism of piperine release is anomalous (0.43 <n <0.85), lipid nanoparticles showing a spherical morphology [274]. "Non-fickian" (or anomalous) diffusion involves the modification of the matrix in the first stage, followed by the dissolution of the layer of surfactants and co-surfactants of the lipid matrix and the diffusion of the active substance; in this case the release of piperine depends on diffusion phenomena.

The release of NLC-UP-PIP 1% system has a zero order kinetics, with a higher release than in the case of evening primrose oil-based systems, with a sustained release compared to evening primrose-based NLC. This behavior is explained by the different composition of the two oils. Release according to zero order kinetics is characteristic for the release of an active substance at a constant rate independent of the initial active substance concentration, which is the purpose of all controlled drug release mechanisms. This leads, in principle, to a better control of the active plasmatic concentration and offers several benefits, including improved patient comfort and reduced frequency of drug administration.

CHAPTER 8
SINTHESIS AND CHARACTERIZATION OF NLCs SYSTEMS LOADED WITH CAPSAICIN

8.2. CHARACTERIZATION OF FREE- AND CAPSAICIN LOADED- NLCs SYSTEMS

In the present study we envisage a novel concept for obesity therapy in which the anti-obesity herbal active – capsaicin (Cap) and an endogenous lipid regulator of appetite – oleoylethanolamide (OEA) or a structural analogue of Phenilalaninol with oleic acid – Phenylalaninol oleamide OEA (FOA), are simultaneously integrated within the same nanostructured lipid carriers (NLC), considering that the association of numerous advantages of natural active compounds (from vegetal and herbs) and endogenous lipid in the same delivery system is a straightforward approach for the development of safe and better tolerated obesity therapy with target applicability.

Our working hypothesis envisage three innovative strategies devoted to solve the main drawbacks underlined in the literature and could lead to the development of new formulas with improved gastric tolerability and enhanced specificity in adipose endothelial cells targeting: (1) reducing the toxicity of synthetic anti-obesity drugs, by using endogenous lipid – OEA and FOA, in association with the main natural actives from red pepper extract (Cap) and from linseed oil (α-linolenic acid); (2) achieving an increased bioavailability of selected anti-obesity actives by encapsulation into lipid nanocarriers prepared with a vegetable oil with anti-inflammatory and hypotriglyceridemic properties – linseed oil; (3) inducing an improved anti-obesity effect by entrapping both, herb and vegetal actives, and providing a gradual release of capsaicin.

8.2.1. Synthesis of capsaicin-loaded NLC systems

Lipid nanocarriers based on vegetable oil were encapsulated with different concentrations of capsaicin (1%, 2%), keeping constant the concentration of the surfactant mixture (2.5%) and the concentration of lipid mediator (1%) (Table 8.1).

Table 8.1. Composition of lipid nanocarriers based on linseed oil, OEA/FOA and Cap

Formulation	Surfactants mixture (g)	GMS (g)	CP (g)	LO (g)	OEA (g)	FOA (g)	Cap (g)
NLC-LO	2.5	3.0	3.0	3.0	-	-	-
NLC-OEA	2.5	3.0	3.0	3.0	1.0	-	-
NLC-OEA-Cap1	2.5	3.0	3.0	3.0	1.0	-	1.0
NLC-OEA-Cap2	2.5	3.0	3.0	3.0	1.0	-	2.0
NLC-FOA	2.5	3.0	3.0	3.0	-	1.0	-
NLC-FOA-Cap1	2.5	3.0	3.0	3.0	-	1.0	1.0
NLC-FOA-Cap2	2.5	3.0	3.0	3.0	-	1.0	2.0

8.2.2. Dimensional characteristics and physical stability of NLCs loaded with capsaicin

The mean diameter particle size (Zave) and polydispersity index (PdI)

The NLC-LO-OEA/FOA-Cap formulations presented relatively small particle size, with mean diameters (Zave) values under 230 nm (Figure 8.2). These nanometer-sized particles are suitable for oral administration since they allow an uptake by enterocytes and lead to an extended action of both actives. All NLC revealed a polidispersity index value ranging from 0.16 (for NLC-

LO) to 0.22 (for NLC-LO-FOA-Cap1) and 0.26 (for NLC-LO-FOA-Cap2), which represents a narrow dispersion around mean size and suggests an adequate unimodal behavior.

The addition of Capsaicin in NLC based on OEA led to a slight decrease in NLC-loaded capsaicin diameters. When the differences are so small, e.g. from 154 nm for NLC-OEA to 145 and 150 nm for NLC-OEA-Cap 1 and 2, two aspects are relevant with respect to the Zave values obtained: 1. statistical determination of Zave for the Dynamic Light Scattering measurement method; 2. occurrence of structural rearrangement phenomena of the lipid network, after accommodating capsaicin; 3. formation of crystallization/nucleating germs during solidification of the lipid melt. Owing to different saturation levels and dissolution velocity of Capsaicin, lipid nucleation occurs randomly. By this random nucleation, the size distribution of capsaicin loaded-NLC cannot be controlled and this leads to crystallization of lipid nanocarriers with different size distribution.

Regarding the two concentrations of anti-obesity active used, 2% Cap gave significantly higher particle size when compared to that of 1% Cap, in case of NLC-FOA-Cap. This could be attributed to the bigger content of Cap and its higher melting point (when compared to that of the lipids blend, e.g. 45-50°C *versus* 62–65°C for Cap) resulted in a more viscous dispersed phase, making difficult the mutual dispersion of the phases, with occurrence of larger particles.

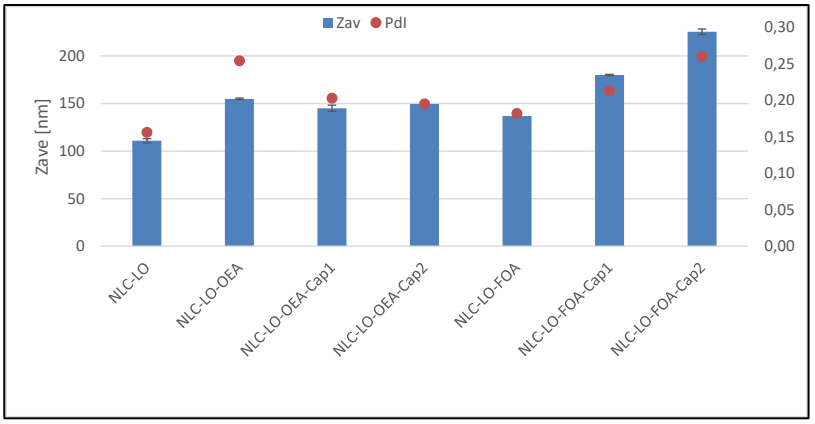


Figure 8.2. The size characteristics of lipid nanocarriers co-loaded with Cap and bioactive lipid– OEA/FOA. Mean particle diameter and polydispersity index

In conclusion, the encapsulation of a smaller amount of capsaicin showed a decrease in the average size of linseed oil-based NLCs, a phenomenon explained by the increased affinity of capsaicin for the lipid core and the rearrangement of fatty acid chains in the structure of linseed oil (50.59% α-linolenic acid, 24.33% oleic acid, 14.34% γ-linoleic acid, 5.19% palmitic acid, 4.34% stearic acid, 0.8% cis-vaccenic acid, 0.11% α-linoleic acid, 0.09% arachic acid, 0.09% 11-eicosaenoic acid) which allow the incorporation of an optimal amount of active compound, thus reducing the average size of NLC systems.

Physical stability of Cap-NLC systems

The results showed that the two types of NLC formulations co-loaded with Cap and OEA/FOA were both negatively charged, with zeta potentials of −42.8 mV and −58.5 mV, which were below the critical value. These negative zeta potential values are not an impediment to NLC-capsaicin uptake by adipocyte cells (Figure 8.5).

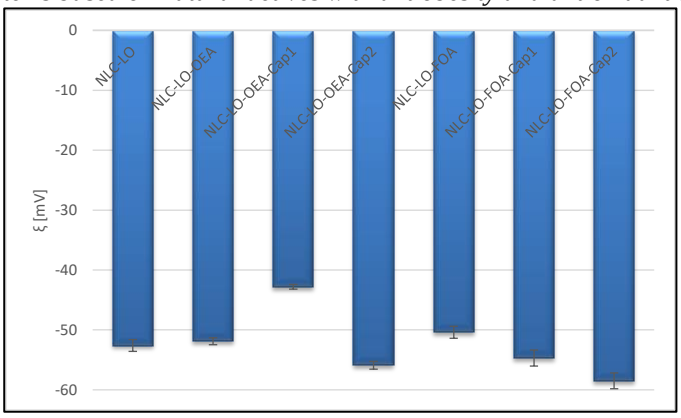


Figure 8.5. The zeta potential values of free- and co-loaded-Cap and OEA/FOA-NLCs

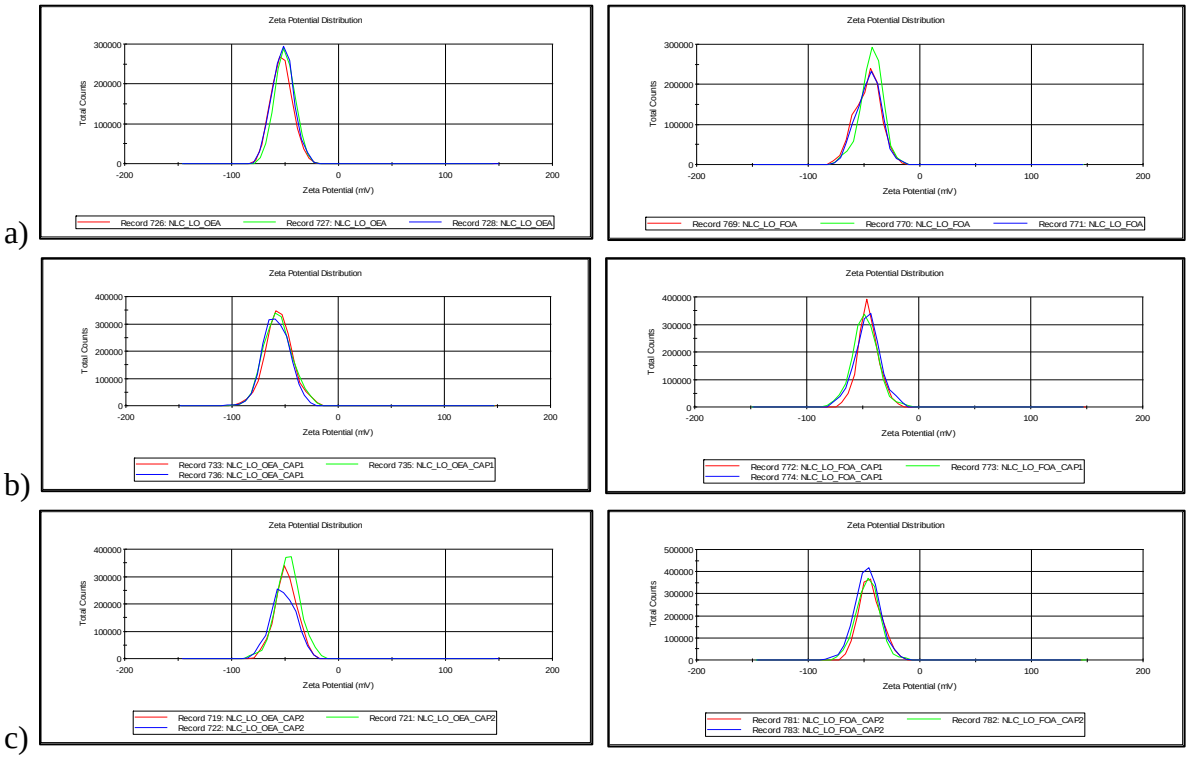


Figure 8.6. Electrokinetic potential distribution in case of:
a) NLC-LO-OEA / FOA
b) NLC-LO-OEA / FOA-Cap1
c) NLC-LO-OEA / FOA-Cap2

In the case of NLC systems based on linseed oil encapsulated with endogenous lipid OEA / FOA and capsaicin of different concentrations (1%, 2%), the profiles of the zeta potential (Figure 8.6 a, b, c) show a unimodal distribution and a perfect overlap of the peaks, **reinforcing the claim that NLC samples encapsulated with endogenous lipid and capsaicin show an excellent stability over time.**

8.3. THERMAL STABILITY CHARACTERIZATION OF NLCS LOADED WITH CAPSAICIN

The lipid core of lipid nanocarriers is important to assure the controlled release properties.The physical characteristics of the developed-NLC formulations and their crystalline or amorphous behavior were investigated by *differential scanning calorimetry* (DSC) [336].

The thermograms of bulk lipids, NLC-free and NLC co-loaded with Cap and OEA/FOA are depicted in Figures 8.7 and 8.8, being highlighted the perturbation of the lipid network after the encapsulation of lipid mediators and capsaicin.

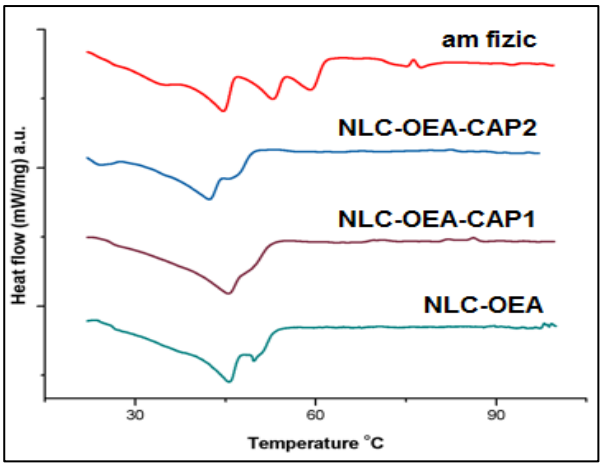


Figure 8.7. The DSC thermograms of NLC-LO-OEA-Cap1/Cap2 systems

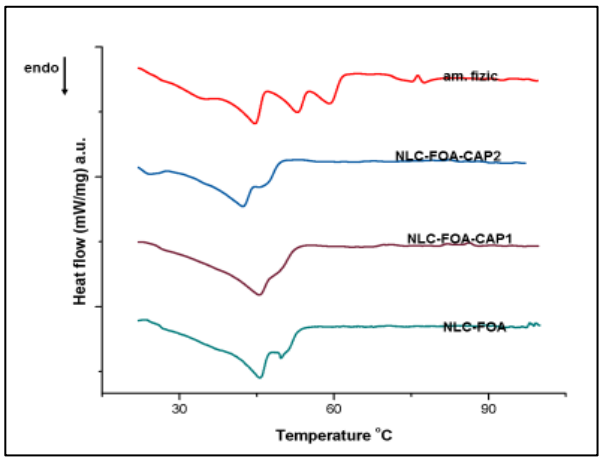


Figure 8.8. The DSC thermograms of NLC-LO-FOA-Cap1/Cap2 systems

The complexity of the lipid core containing solid lipids blended with linseed oil is evidenced by the appearance of three endothermic peaks at 45, 53 and 61°C in the physical blend of lipids, attributed to both – cetyl palmitate (54°C) and glycerolmonostearate (57–65°C). These relatively sharp melting endothermic peaks of bulk lipids indicated that the starting materials were almost crystalline. The use of linseed oil to form the complex lipid core of NLC, has led to nanostructured carriers that present a highly disordered lipid core, highlighted by the emergence of some endothermic events: Cap and OEA/FOA loaded-lipid nanocarriers showed two melting events, a wide endothermic region with a peak localized at 45°C and a shoulder around 50°C. Broad endothermic events between 40 and 55°C indicate characteristic amorphous nature of the developed NLC.

8.4. DETERMINATION OF ENTRAPMENT EFFICIENCY (EE%) AND LOADING CAPACITY (LC%) OF CAPSAICIN (Cap), OLEOYLETHANOLAMIDE (OEA) AND PHENYLALANINOL OLEAMIDE (FOA) IN NLCs SYSTEMS

The HPLC method was used for determination of OEA, FOA and Cap entrapment efficiency (EE%) into lipid nanocarriers. An amount of 0.05 g lyophilized NLC-OEA/FOA-Cap was dispersed into 1.5 mL acetonitrile, gently mixed and after that centrifuged for 15 min at 15000 rpm (Sigma 2K15, Germania). The resulted supernatant containing the unloaded-active compounds was analyzed using a Jasco 2000 liquid chromatograph equipped with an UV detector at λ = 220 nm and Nucleosil C18 column (25 × 0.4 mm). The mobile phase was composed by ACN:H_2O (70:30) and the flow rate was 1 mL/min; the retentions time were 3.93 min for Cap, 17.5 min for OEA and 26.5 min for FOA, respectively.

Owing to the low water solubility of Cap, OEA and FOA, there was a competition between the accommodations of both actives into the lipid core of NLC. All NLC formulations exhibited high EE% values, most of which exceeding 92% for OEA/FOA, corresponding to drug loadings close to the theoretical concentrations. These results revealed that both oleoylethanol amide and phenylalaninoyl oleamide were efficiently captured in the lipids core of NLC, resulting in the encapsulation efficiency ranging between 90.9 ± 0.37 and 92.3% ± 0.15 for OEA and 94.8% ± 0.29 and 96.4% ± 0.78% for FOA (Figure 8.9). These data can be strongly related to the high compatibility of OEA and FOA with the lipids blend formed by linseed oil, glycerolmonostearate and cetyl palmitate.

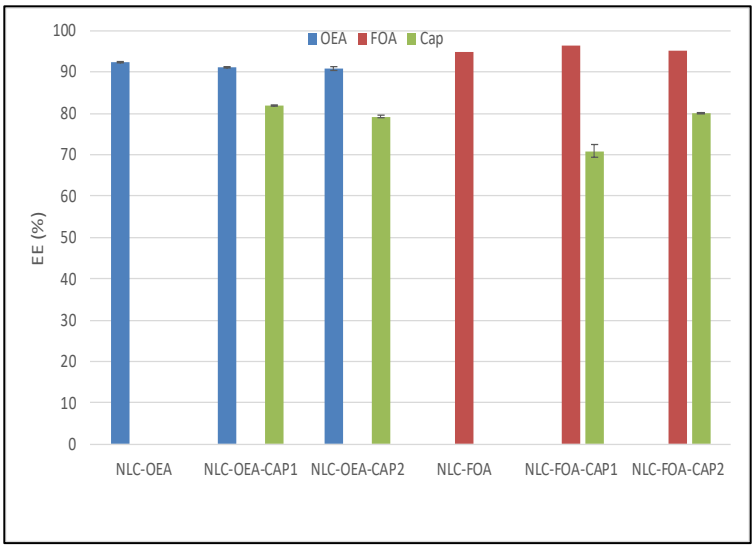


Figure 8.9. Entrapment efficiency of Cap, OEA and FOA inside the lipid nanocarriers based on linseed oil

Suitable entrapment efficiency was also determined for Capsaicin, but at lower levels than those obtained in case of endogenous lipid-OEA and its analogue-FOA; it was found to vary from 70.9 ± 1.57 to 80 ± 0.1, for NLC-FOA-Cap 1 and 2, and from 79.2 ± 0.27 to 81.9 ± 0.18 for NLC-OEA-Cap 1 and 2, respectively.

Two main aspects could be assigned with the values of EE% determined for Cap: 1. the low aqueous solubility of Cap (Cap has a water solubility of 10.3 mg/L, at 25°C) which provides suitable drug entrapment; 2. the capture of > 90% of the lipid mediator – OEA or FOA that has a rather bulky structure, has not left enough space to accommodate a significant amount of capsaicin.

The study on the encapsulation of capsaicin in synthesized NLC systems led to optimal results of encapsulation efficiency, which can also be explained by the high compatibility of the natural active ingredient – capsaicin – with the lipid mixture consisting of linseed oil, glycerol monosterate and cetyl palmitate.

8.5. *IN VITRO* EVALUATION OF THE BIOLOGICAL PROPERTIES OF NLCs LOADED WITH CAPSAICIN

8.5.1. *In vitro* testing of antioxidant properties

The activity against $ABTS^{\bullet+}$ generated *in situ* confirmed the significant antioxidant potential of the Cap-loaded NLC-OEA/FOA and the moderate antioxidant activity of NLC-OEA/FOA. The free NLC shows a low antioxidant action, demonstrating the ability to counteract only 9.4% ± 1.1 $ABTS^{\bullet+}$. The antioxidant effect of free NLC may be related to the high level of linolenic acid, ω-3, in the linseed oil. The presence of the two lipid mediators – OEA and FOA with linseed oil in the same nanostructured delivery system induces an improvement in antioxidant action, especially in the case of NLC-FOA, e.g. 16.7% ± 0.39 inhibition of $ABTS^{\bullet+}$.

In the presence of Capsaicin or NLC-OEA/FOA-Cap, $ABTS^{+*}$ inhibition proceeded in a capsaicin concentration dependent manner and is dependent on the type of NLC formulations as shown in Figure 8.11. The obtained results demonstrated that the ability to scavenge free radicals was statistically higher for the Cap-loaded NLC when compared to native capsaicin. For instance, the native Cap show an antioxidant activity of 45% ± 1.20, while the same concentration loaded into lipid OEA based–NLC exhibited a remarkable radical-scavenging activity against $ABTS\%^{+*}$, e.g. 76.1% ± 2.18.

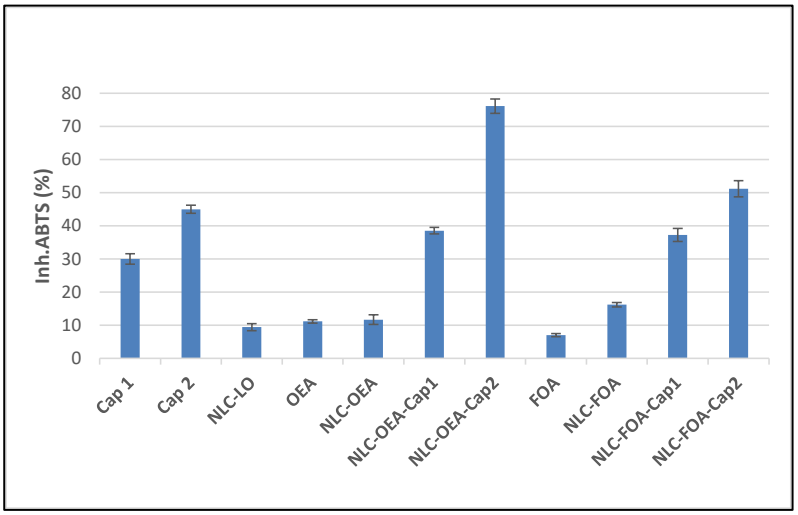


Figure 8.11. Comparative assessment of antioxidant activity of native Capsaicin, NLC-OEA/FOA and NLC-OEA/FOA-Cap (by ABTS assay).

The observed free-radical-scavenging potential of NLC-OEA/FOA-Cap compared to Cap might be attributed to the size effect induced by nanoencapsulation and to different types of functional groups from capsaicin that are responsible for the reduction and capture of the cation radicals or can donate hydrogen to free radicals and thus break the chain reaction of free radicals at the first initiation step [340]. *In vivo* and *in vitro* studies showed that capsaicin protects endothelial cells and macrophage against LDL (low-density lipoprotein) oxidation by direct antioxidant action [341, 342] and exhibited protective effects against oxidative damage by reducing the formation of cellular oxide species and increasing antioxidant enzymes such as superoxide dismutase, catalase,

glutathione-S-transferase, catalaza, glutation-S-transferaza [343, 344]. In terms of size effect, it is well known that the reduction of bioactive compound size has pronounced effects on the physical and biological properties that may be significantly different from the corresponding native form [345].

8.5.2. *In vitro* controlled release of capsaicin

From an overview of the release profiles shown in Figure 8.12, it can be seen that the developed lipid nanocarriers subjected to the in vitro release tests have ensured a slow release of Cap, e.g. 40% Cap was released after 24 h of *in vitro* experiments from NLC-OEA-Cap 1,2 and only 9% from NLC-FOA-Cap 1,2.

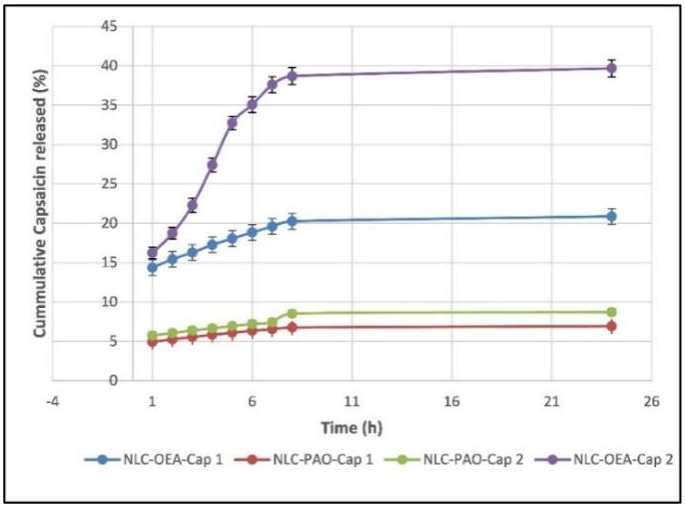


Figure 8.12. The influence of the lipid nanocarriers type on the release of capsaicin

The observed behavior can be attributed to the bulky structure of the lipid mediator – OEA and its analogue – FOA, which significantly hampers the migration of Cap through NLC. Interestingly, OEA-based-NLC showed a faster release of Cap compared to FOA-based-NLC, a phenomenon that may be closely correlated with the branched structure of FOA. First, a burst release at initial stage of release, caused by the dissolution or adsorption of the drug on the surface of nanoparticles. Second, the concentration gradient between the nanoparticle and the release medium caused the diffusion of drug. Third, the corrosion and degradation of nanoparticle materials, in which the drug incorporated in nanoparticles is released sustained. In the current study, in the group of Cap-FOA-co-loaded – NLC, the burst drug release was not observed; a faster drug release was detected for NLC-OEA-Cap 1 and 2. The NLC-OEA/FOA-Cap exhibited a controlled-release profile, which revealed a two-stage process. In this biphasic drug release pattern, capsaicin release evolution could be illustrated as follows: the initial release (during 1 and 8 h) might be related to double effects via both the release of small amount of Cap entrapped in the surface layer of NLC and the passive diffusion of Cap. Moreover, the prolonged release profile after 8 h should be caused by the Cap incorporated into the NLC inner core that is slowly released with the degradation of NLC.

The capsaicin concentration in the FOA-based NLC group moderately influenced the release rate (Figure 8.13). The NLC-FOA-Cap 1 released 6% Cap after 8 h, and the Cap-2 released 8%. Also, after 24 h, it was observed that the slowest release degree was obtained for the FOA-based NLC containing the higher amount of capsaicin. In contrast, in the case of Cap-OEA-co-loaded-NLC, a large amount of released Cap was detected, especially in the case of NLC-OEA-Cap 2: 16% of total Cap released (after the first hour of experiment), then followed by a sustained release with

about 39% of the Cap released in 24 h. As previously mentioned, a possible explanation for the slow release of Cap from the NLC-based FOA delivery systems as compared with those of NLC-based OEA, might be an increased affinity between Cap and FOA due to π-stacking of phenyl moieties which causes a delayed release of the drug from the lipid network.

Table 8.4. Kinetic data obtained at controlled release of Capsaicin from NLC prepared with linseed oil

NLC formulation	Zero order		First order		Higushi		Hixson-Crowell		Korsmeyer-Peppas		
	R^2	k_0	R^2	k_1*10^2	R^2	k_2*10^2	R^2	k_3*10^2	R^2	k_4*10^2	n
NLC-OEA-Cap1	0.995	0.837	0.996	1.01	0.992	3.94	0.944	3.44	0.996	1.44	0.224
NLC-OEA-Cap2	0.972	3.515	0.978	4.91	0.969	19.04	0.912	16.51	0.976	6.63	0.225
NLC-FOA-Cap1	0.993	0.259	0.995	0.28	0.994	1.08	0.952	0.94	0.994	0.41	0.222
NLC-FOA-Cap2	0.933	0.346	0.935	0.37	0.885	1.41	0.804	1.2	0.934	0.55	0.219

It is observed that the release of capsaicin for synthesized NLC is performed after a first order kinetics (according to the correlation coefficients) which denotes the presence of controlled diffusion processes. In case of NLC-OEA-Cap 1 the rate constant is higher than that of NLC-FOA-Cap 1 and 2, which strengthens the previous assumption with regard to faster release of Cap from the OEA-based NLC. These results demonstrate that NLC based on linseed oil played an important role on the delay of Capsaicin dissolution; this slow release of Cap encountered in the present research could be a desired phenomenon in order to avoid the potential irritant effect of Cap on the intestinal mucosa. Moreover, this behavior may help to reach an effective Capsaicin concentration in plasma (after 1 h), whereas the controlled release during a period of 24 h might maintain the effective concentration of Cap in plasma for a long time.

8.6. *IN VITRO* ASSESSMENT OF THE ANTIOBESITY PROPERTIES OF NLCs LOADED WITH CAPSAICIN

For evaluating the anti-obesity properties of synthesized NLC systems, the influence of treatment with lipid nanoparticles based on Cap, OEA / FOA, was determined in an experimental model of obesity performed on laboratory animals. The experimental animals used were *Albino Swiss* male mice, procured from the Biobase of University of Medicine and Pharmacy "Carol Davila" from Bucharest. The animals, kept in standard laboratory conditions, received food twice a day and water *ad libitum*. The experiments were carried out in accordance with the Directive no. 63/2010/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and with the Ordinance no. 37 of the Government of Romania from 2/02/2002.

Albino Swiss mice weighing 18 ± 3 g received hypercaloric (high-fat and sugar) diet for 3 weeks. After 3 weeks, only the obese animals were considered for the study [349, 350]. These animals were randomly distributed in 7 batches (n = 10) for NLC treatment and one obese batch (n = 10) as control. In parallel, a normal weight control batch (n = 10) kept in same conditions was fed with standard food twice a day and received water *ad libitum* for 3 weeks.

The animals were orally treated for 10 days. The NLC formulations doses used for the study correspond to the recommended therapeutic p.o. doses of oleoylethanolamide (10 mg/kg bw). The oral dose of FOA (12.5 mg/kg bw) is equimolecular with the OEA recommended p.o. dose. The

hypercaloric diet was maintained during the 10 days of treatment with NLC and for the obese group, except for the normal weight control group which received standard food. The body weight was monitored in days 1, 3, 5, 7 and 10 of the treatment. The effect of the treatment on food intake was determined after 3 h from the NLC administration comparing to the obese and normal weight groups. After 2 h from the last administration, the animals were anesthetized, and the blood was collected for the biochemical analyses of total serum cholesterol, blood triglycerides and glycaemia. The blood analyses were performed using a Cormay biochemistry analyzer with specific reagents. Data in figures were expressed as mean value ± standard deviation (SD) for three measurements. The statistical significance of the experimental data was determined using the ANOVA test and the Dunnett multiple compression test and was performed using the GraphPad Prism software. The results were considered statistically significant at $p < 0.05$ and insignificant statistically at $p > 0.05$.

8.6.1. Variation in body weight of experimental animals after administration of lipid nanocarriers that contain OEA/FOA and Capsaicin

The Albino Swiss mice groups treated with NLC-OEA/FOA-Cap showed fluctuations in body weight during treatment as compared to both control groups which present a constant weight increase during the 10-days treatment (Figure 8.13).

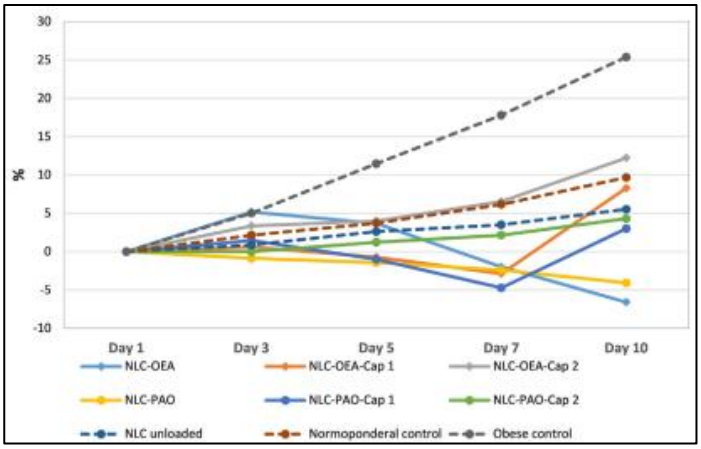


Figure 8.13. Body weight variation after administration of NLC formulations in experimental Albino Swiss animals, as compared to day 1

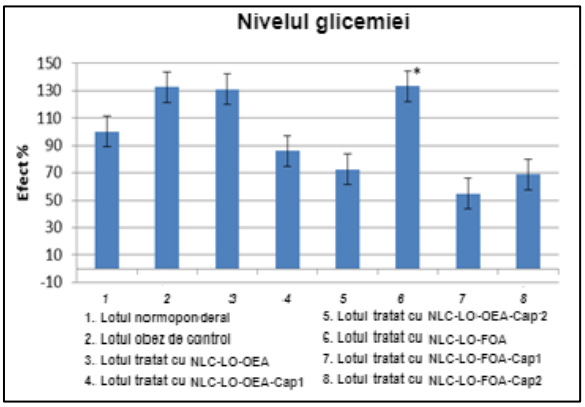
The treatment with NLC-OEA/FOA and NLC-OEA/FOA-Cap resulted in a body weight decrease of Albino Swiss mice as compared to the batch of obese animals. Interesting results have been observed in case of mice batch treated with lipid nanocarriers that contain only the lipid mediators – OEA and FOA. **Significant decreases ($p < 0.05$) in body weight under baseline weight (day 1 of treatment) were recorded after 3 days of administration and the effect was maintained during the 10 days of the study.**

8.6.3. Influence of NLC-OEA/FOA-Cap on lipid and blood glucose profile in experimental animals

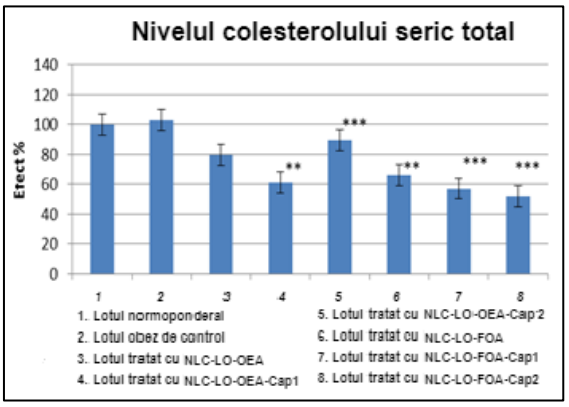
Following treatment with the synthesized NLC formulations, it was noted that co-encapsulation of the Capsaicin along with OEA/FOA into lipid nanocarriers improved the plasma lipid profile compared to the administration of NLC without Capsaicin. The results obtained are dose-dependent; increased concentrations of Cap have led to a significant decrease in cholesterol and triglycerides as well as glucose level. The results are presented as effect comparing to normoponderal control batch (Figure 8.15 a, b and c).

All these results demonstrate that NLC based on linseed oil, lipid mediators – OEA, FOA and a natural active – Capsaicin, have together played an important role in achieving an improved anti-obesity action, having a positive impact on weight lowering effect, decreasing the cholesterol, triglycerides and glucose levels, and provided the advantages of overcoming low gastric tolerability and reported drugs side effects problems.

a)



b)



c)

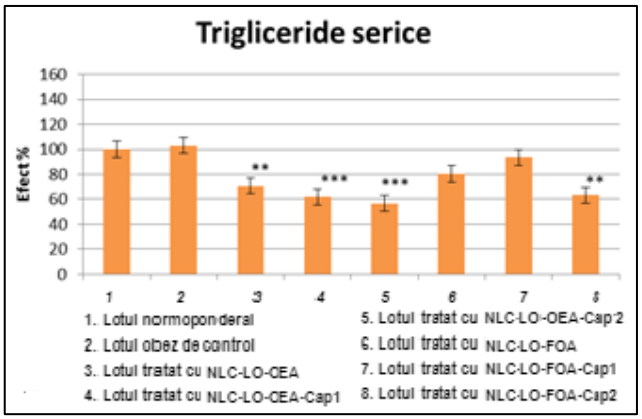


Figure 8.15. The influence of NLC treatment on: a) glycaemia; b) total serum cholesterol; c) serum triglycerides in experimental animals

CHAPTER 9. CONCLUSIONS

9.1. GENERAL CONCLUSIONS

The original results of the research steps performed are presented below:

✓ For the synthesis of the 26 free and co-encapsulated lipid nanocarriers systems with the considered active principles, we used the hot emulsification technique coupled with the high shear homogenization technique (HSH) and the high pressure homogenization technique (HPH).

✓ The synthesized NLC systems were physico-chemical and morphological characterized (Zave, PdI, zeta potential, DSC, UV-VIS) and were evaluated in terms of biological properties by *in vitro* studies evaluating the antioxidant activity, by *in vitro* studies of the controlled release of the active substance and by *in vivo* studies evaluating the anti-obesity effect.

✓ All NLC formulations based on vegetable oils, free or encapsulated with actives, showed optimal average nanoparticle sizes, with Zave values less than 200 nm, and good physical stability over time.

✓ For the synthesis of NLC systems we used a mixture of solid and liquid lipids (vegetable oils), the concentration of the lipid mixture influencing the size and stability of the nanoparticles.

✓ The lipid matrix consisted of the solid lipids glycerol monstearate (GMS) and cetyl palmitate (CP) and the liquid lipids represented by the oils considered in this study (linseed oil/argan oil/evening primrose oil/paraffin oil). The association of solid lipids with a liquid lipid led to a highly disordered crystalline network, which allowed the encapsulation of an optimal amount of active substance, a phenomenon highlighted by the average size of nanoparticles and the stability of NLC systems.

✓ For the synthesis of lipid nanocarriers we used a mixture of surfactants consisting of Tween 20, α-Phosphatidylcholine and Poloxamer 188. In the optimization phase, we used four different concentrations of the surfactant mixture (2%, 2.5%, 3% and 3, 5%), the optimal concentration being 2.5%, for which we obtained the best results of the average nanoparticle diameter, of the polydispersity index and the zeta potential.

✓ The nanoencapsulation of the active principles in the lipid structure of NLC systems did not lead to significant changes in the size of the average diameter, compared to free NLC systems, which highlights the effectiveness of developed systems.

✓ For the *in vitro* evaluation of the antioxidant activity, the ABTS method and the chemiluminescence method were used, the values of inhibition of free radicals of long / short life being higher than 30% / 76%. NLC systems loaded with active compounds were found to have enhanced antioxidant activity compared to free NLC systems or free active compounds.

✓ *In vitro* evaluation of the entrapment efficiency was performed by UV-VIS or HPLC methods. Very good entrapment efficiency values were obtained, higher than 80%.

✓ The optimal correlation between active substance loading capacity and the entrapment efficiency could be explained by a good accommodation of the active substance in the lipid matrix.

✓ The *in vivo* anti-obesity effect evaluation of Cap-loaded NLCs was performed on Albino Swiss laboratory mice, the study showing a optimal decrease in body weight, serum triglyceride level, blood glucose level and serum cholesterol level.

✓ The complex chemical composition of vegetable oils used in combination with natural active compounds, but also with active synthetic principles explains the stability over time of the new synthesized NLC systems. The new synthesized formulations can be used for the development of effective and safe pharmaceutical formulations that can be used in anti-obesity therapy.

✓ The NLC formulations encapsulated with the active principles (caffeic acid, curcumin, piperine, capsaicin) are an attractive prospect for the future development of innovative oral pharmaceutical formulations for proper long-term weight management.

9.2. ORIGINAL CONTRIBUTIONS

The most important original contributions of the doctoral thesis entitled "**Nanocarriers systems based on natural actives with antioxidant and anti-obesity actions**" can be summarized as follows:

❖ Synthesis, characterization and evaluation of the properties of new nanocarriers systems based on vegetable oils (linseed oil, argan oil, evening primrose oil, paraffin oil) in combination with a suitable mixture of solid lipids (glycerol monostearate and palmitate cetil).

❖ Encapsulation of natural actives (caffeic acid, curcumin, piperine, capsaicin) in new NLC systems and development of the efficient NLCs that have the role of improving the biological properties of these actives.

❖ Co-encapsulation of natural active principles with synthetic active principles (oleoylethanolamide – OEA and phenylalaninol oleamide – FOA) with antioxidant and antiobesity effect, in order to increase the therapeutic effects by synergy, but especially in order to reduce the toxicity of synthetic active principles.

❖ Nanoencapsulation of hydrophilic or weakly lipophilic actives in lipid nanocarriers systems prepared with vegetable oils, in order to increase the bioavailability of the actives accompanied by the amplification of their therapeutic activity.

❖ Nanoscale of vegetable extracts and synthesis active principles.

❖ Development of new nanocarriers systems that have an improved *in vitro* antioxidant effect compared to the antioxidant effect of non-encapsulated actives.

❖ Development of new nanocarriers systems that have a promising *in vivo* anti-obesity effect, linseed oil-based NLC systems encapsulated with capsaicin in combination with oleoylethanolamide / phenylalaninol oleamide being precursors of safe and effective pharmaceutical formulations that could be used in obesity therapy.

9.3. RESEARCH PERSPECTIVES

The main research perspectives can be formulated as follows:

➢ Development of new nanocarriers systems able to encapsulate other natural actives with antioxidant and anti-obesity effects (eg. bromelain), with optimal encapsulation properties, excellent physical stability (storage, transport) and controlled release properties.

➢ Co-encapsulation of natural actives and other synthetic active substances with anti-obesity effect, in order to reduce the side effects of synthetic compounds.

➢ Development of lipid matrices with high biocompatibility, encapsulated simultaneously with several active principles, and *in vitro* and *in vivo* testing of biological properties in order to enhance the activity of active principles by synergistic effect.

LIST OF THE PUBLISHED PAPERS IN THE FIELD OF DOCTORAL THESIS AND PARTICIPATION AT INTERNATIONAL CONFERENCES

1. I. Lacatușu, N. Badea, D. Udeanu, **L. Coc**, A. Pop, C. Cioates Negut, C. Tanase, R. Stan, A. Meghea, "Improved anti-obesity effect of herbal active and endogenous lipids co-loaded lipid nanocarriers: Preparation, *in vitro* and *in vivo* evaluation", Mater. Sci. Eng. C, vol. 99, pp. 12-24, 2019; **FI: 6,259/2019;**

2. L. M. C. Coc, I. Lacatusu, N. Badea, M. E. Barbinta-Patrascu, A. Meghea, "Effective Lipid Nanocarriers Based on Linseed Oil for Delivery of Natural Polyphenolic Active", J. of Nanomaterials, vol. 3, pp. 1-9, 2021; **FI: 2,44/2021;**

3. T. A. Iordache, **L. Coc**, A. L. Mihai, N. Badea, I. Lacatusu, A. Meghea, "The influence of vegetable oil and self-organizing agents' composition on obtaining stable nanostructured lipid carriers", U.P.B. Sci. Bull., Seria B (acceptată);

4. **L. Coc**, I. Lacatusu, N. Badea, D. Udeanu, A. Pop, C. Negut, I. Tanase, R. Stan, A. Meghea, "Nanostructured carriers based on lipid mediators and their therapeutic effects", 20th Romanian International Conference on Chemistry and Chemical Engineering (RICCCE), Sinaia, Romania, 6-9 September, 2017 (Certificate of Presentation for excellent oral presentation).

CUMULATED IMPACT FACTOR : 6,259 + 2,44 + 0,492 = 9,191

The present summary contains in a concise form the contents of chapters 5-8 of the original contributions. The numbering of chapters, subchapters and tables corresponds to that of the thesis. The significant bibliographic references used in the paper are presented.

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