Nanostructured lipid carriers (NLC) are new colloidal systems having submicron particles with spherical shape and average diameters ranging from 50 to 500 nm [1, 2]. NLC are solid at body temperature being made by a blend of solid and liquid lipids dispersed in an aqueous medium and stabilized by surfactants [3, 4]. The lipid core is able to encapsulate lipophilic drugs [5-8], particularly between the imperfections of the crystal lattice [9], offering protection for chemical unstable compounds [10, 11]. As drug delivery systems, they present many more advantages such as physical stability, biodegradability, biocompatibility and controlled drug release [12-14]. Because of their versatility, NLC are very promising systems in many applications (e.g. parenteral, oral and dermal formulations) [15].

Natural products are valuable sources of bioactive compounds that can be used for multiple therapeutic purposes. The disadvantage of natural products is their low bioavailability and various studies were performed in order to valorize and to increase their efficacy by tailoring these complex systems at nanometer size [16-18]. Vegetable oils and selective plant extracts used for the development of lipid nanoparticles demonstrated an enhancement of their bioactive properties [19].

Nystatin (Nys) is also a natural product of Streptomyces noursei that has been used as a topical fungicidal drug since 1950s. It is active against Candida, Cryptococcus, Histoplasma, Blastomyces and Aspergillus spp [20]. Because of its toxicity, the use of Nys is limited to topical application and several studies were done in order to reduce its toxicity while preserving the antifungal activity by incorporation into liposomes [21] and solid lipid nanoparticles [22]. The aim of the present study is to incorporate Nystatin into NLC prepared based on Savory oil in order to develop formulations with enhanced antifungal properties and reduced toxicity. In this respect, the synthesis parameters of Nys-loaded NLC by high pressure homogenization method have been systemically controlled and optimized in order to obtain a suitable carrier system.

**Experimental part**

*Materials and methods*

Polyoxyethylene sorbitan monolaureate (Tween 20) and Polyoxyethylenesorbitan monooleate (Tween 80) were purchased from Merck (Germany). Poloxamer 407 (block copolymer of polyethylene and polypropylene glycol) was supplied by (Germany) and Soybean lecithin by (Germany). Emulgade (a mixture of Glycerol Stearate, Ceteareth-20, Ceteareth-12, Cetearyl Alcohol, Cetyl Palmitate) and Glycerol monostearate (GM) were supplied by (Romania) and 3-amino-phthalhydrazide (Luminol) was purchased from Sigma Aldrich Chemie GmbH.

*Synthesis of lipid nanocarriers*

Nanostructured lipid carriers (NLCs) were prepared using a combination of high shear and high pressure homogenization techniques. The lipid phase, in a concentration of 10 or 12% and consisting in Em, GS and savory oil in various ratios (4.5:4.5:1, 4:4:2, 3.5:3.5:3 w/w), was heated under stirring at 85°C. Nys was added into the lipid phase and stirred until a clear molten solution was formed. The aqueous phase containing 2.5 or 3% surfactants mixture, Tween/Lecithin/Block copolymer in a ratio of 8:1:1, was also heated under stirring at the same temperature (85 °C). The formed pre-emulsion was further homogenized using a high-shear homogenizer (PRO250 type; 0~28.000 rpm; power of 300 W , Germany) and it was further subject to a high-pressure homogenization (APV 2000 Lab Homogenizer, Germany) at various pressures (600 and 800 bar) and number of cycles (6 and 9). The nanoemulsions were cooled at room temperature for obtaining solid lipid
nanocarriers. In order to remove the excess of water, the dispersions were frozen at -25 °C overnight and they were lyophilized for 72 h at -55 °C, using an Alpha 1-2 LD Freeze Drying System (Germany).

Characterization of nanostructured lipid carriers

Particles size measurements

The mean particle diameter and size distribution of NLC were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (United Kingdom). The mean particle size (Zav) and the polydispersity index (PdI) of the aqueous lipid nanodispersions were measured at a scattering angle of 90° and at a temperature of 25°C. Before measurements, the dispersions were diluted with deionized water to an adequate scattering intensity. The particle size data were evaluated using intensity distribution. The average diameters (based on Stokes-Einstein equation) and the polydispersity index were given as average of three individual measurements.

Zeta potential analysis

The Zeta potential (ZP) was determined by measuring the electrophoretic mobility of the nanoparticles in an electric field, by using the Helmholtz-Smoluchowski equation. Prior to the measurements, which were performed with the appropriate accessory of Zetasizer Nano ZS (U.K.), the nanostructured lipid carriers in dispersion were diluted with a sodium chloride solution (0.9%, w/v), to adjust the conductivity to 50 μS/cm. All measurements were performed at 25°C in triplicate and the mean value was reported.

Determination of Nystatin entrapment efficiency

For the investigation of the entrapment efficiency of Nys into the lipid core, the concentration of Nys in the lyophylized samples was evaluated using UV-VIS spectroscopy. The entrapment efficiency of Nys into NLC has been calculated according to the relation:

$$\text{EE} = \frac{C_{\text{Nys}} - C_{\text{unloaded Nys}}}{C_{\text{Nys}}} \times 100$$

where, $C_{\text{Nys}}$ is the concentration of Nys, which was initially added into lipid nanocarriers and $C_{\text{unloaded Nys}}$ is the UV-Vis analyzed concentration of free Nys.

Evaluation of the antioxidant activity (AA %)

The in vitro antioxidant activity of loaded-NLCs and free-NLCs has been determined by chemiluminescence method (CL) using a Chemiluminometer Turner Design TD 20/20, USA. Luminol has been used as light amplifying substance. H₂O₂ in Tris-HCl buffer solution (pH 8.6) has been used as generator system for free radicals. The samples were subject to ultrasonication for 3 min before the antioxidant activity determination. The antioxidant activity (percentage of free radical scavenging) of unloaded- and loaded-NLC was compared with the same concentration of free (unloaded) Nys and savory oil individual solutions and was calculated by using the relation [23]:

$$\text{AA} = \frac{I_0 - I_s}{I_0} \times 100$$

where $I_0$ is the maximum CL for standard at t = 5 s; $I_s$ is the maximum CL at t = 5 s, for sample.

Results and discussions

Optimization of process parameters for synthesis of lipid nanocarriers

The synthesis of nanostructured systems obtained by combining solid lipids (Em and GM) with liquid lipids (SO), which was stabilized with a surfactants mixture, has firstly involved an optimization stage of the homogenization parameters. In this context, keeping constant the concentration of the lipid mixture (10% of the total weight), a number of lipid carriers has been synthesized by varying the pressure and the number of the homogenization cycles. Table 1 gives an overview on the produced formulations. The size and zeta potential values of all synthesized unloaded lipid nanocarriers are presented in figure 1 and 2, respectively.

By analyzing the results obtained from DLS, lipid nanocarriers with diameters smaller than 200 nm and variable polydispersity index can be observed. The increased pressure applied in the synthesis procedure has led to a decrease in the average diameters of the resulted nanoparticles. The same effect, with few exceptions, was observed by increasing the number of cycles from 6 to 9.

The two concentrations of surfactants used, 2.5 and 3%, did not determine notable differences between the sizes of NLC. Instead, as it was expected, the type of surfactant influences the size of NLC, thus the NLCs prepared with Tween 80 present smaller particle sizes than those prepared with Tween 20. Regarding the ZP, in general, the values were around -20 mV, which reflects a poor stability of the

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Table 1

<table>
<thead>
<tr>
<th>Main surfactant</th>
<th>Tween 20</th>
<th>NLC 1</th>
<th>NLC 2</th>
<th>NLC 3</th>
<th>NLC 4</th>
<th>NLC 5</th>
<th>NLC 6</th>
<th>NLC 7</th>
<th>NLC 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>NLC 9</td>
<td>NLC 10</td>
<td>NLC 11</td>
<td>NLC 12</td>
<td>NLC 13</td>
<td>NLC 14</td>
<td>NLC 15</td>
<td>NLC 16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surfactants concentration</th>
<th>2.5%</th>
<th>3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPH Pressure</td>
<td>600 bar</td>
<td>800 bar</td>
</tr>
<tr>
<td>HPH cycle</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

All samples were prepared with 10% (w/w) lipid blend, in a ratio of Em:GM:SO = 4:5:4:5:1, and with the surfactant mixture in a ratio of Tween/Lecithin/Block copolymer = 8:1:1.

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Fig. 1. Particle size (Zav) and polydispersity index (PdI) of unloaded NLC
unloaded NLC. Figures 3 and 4 show the particle size distribution and zeta potential of some selected lipid nanoparticles.

By correlating all the processes and synthesis parameters, the best results, in terms of size, were obtained by applying a pressure of 800 bar and 6 cycles of homogenization (e.g. 93.5 nm and PdI of 0.214 for NLC 7 prepared with 3% surfactants mixture and 88.7 nm and PdI of 0.189 for NLC 11 prepared with 2.5% surfactants mixture).

**Particles size analysis of Nystatin loaded lipid nanocarriers prepared with various concentrations of lipid blends**

Starting from the optimized systems, which were synthetized with Tween 80/Lecithin/Block copolymer in a concentration of 2.5% and using the HPH parameters of 800 bar and 6 cycles, some NLCs were developed for an effective encapsulation of Nys. In this sense, the Nys concentration, the lipid blend composition and Savory oil concentration were varied in order to obtain efficient nanocarriers with appropriate size distribution and physical stability. The characteristics of the developed Nys-loaded lipid nanocarriers are shown in figures 5 and 6, while figures 7 and 8 exemplify the particle size distribution and zeta potential of 0.5% Nys-loaded lipid nanoparticles based on 12% lipids, of which 3% are SO.

Even the average hydrodynamic diameters of the lipid nanocarriers prepared with 10% lipid blend (in a weight ratio of solid lipids to savory oil of 9:1, 8:2 and 7:3) were lower than those prepared with 12%, an optimum ratio between $Z_{av}$ and PdI was obtained for the last composition (e.g. 121.4 nm and PdI of 0.237 for NLC prepared with 12% lipid blend versus 119.1 nm and 0.345 for NLC prepared with 10%).

The results obtained by varying the savory oil content (1%, 2% and 3% of the total weight of the nanodispersion) indicated the formation of nanostructures with main diameters ranging between 112 and 168 nm (for a lipid blend of 10%) and between 116 and 196 nm (for a lipid blend of 12%). The influence of savory oil concentration on $Z_{av}$ has revealed a constant decrease of the average size as the oil content has been increased (e.g. 149.1 nm for NLC prepared with 10% SO and loaded with 0.5% Nys versus 121.4 nm for NLC prepared with 30% SO). These results may be related to lower viscosity samples and therefore a better homogenization can be ensured.

Regarding the effect of Nys encapsulation on $Z_{av}$ and PdI, no significant changes have been observed by increasing the concentration of the antifungal compound from 0.5 to 1% (fig. 5). These results underline the efficiency of Em, GM and SO in formation of a lipid core with an appropriate network for the encapsulation of various active concentrations.

**Physical stability of the aqueous dispersion of lipid nanocarriers**

The physical stability of the lipid nanocarriers has been evaluated by zeta potential measurements. The zeta potential values obtained for several Nys-loaded lipid nanocarriers meet the required conditions for a good stability in time under the investigated condition. Interesting is the antifungal agent influence on physical stability of the synthesized lipid nanodispersions. The free nanocarriers show low physical stability, with zeta potential values higher in absolute value than -17 mV, while the Nys incorporation leads in almost all prepared nanocarriers to more electronegative zeta potentials, e.g. from -21 mV to -30 mV.
The zeta potential distribution for selected lipid nanocarriers loaded with antifungal agent is exemplified in figure 6. The most stable nanocarriers have been prepared with 12% lipid blend and loaded with 0.75% Nys, with a mean potential value of -25.4, -28.4 and -30.4 mV, respectively. In general physical stability increased with increasing the amount of savory oil from 10 to 30%. These results confirm the distribution of the antifungal agent in the outer shell of the lipid core, which led to a change in surface charge.

**Determination of Nystatin encapsulation**

In order to assess the solubilization capacity of the antifungal agent in the lipid nanocarriers, the entrapment efficiency (EE%) was determined (fig. 9). Contrary to expectations, increasing the loading of the antifungal agent resulted in an increase of the encapsulation efficiency. For instance, a load of 0.5% Nys has led to an entrapment between 40 and 82.6%, while the encapsulation of 1% active increased significantly the EE% up to 95.5%. Regarding the influence of savory oil on the encapsulation efficiency no major changes have been reported depending on the oil content.
Antioxidant activity of NLC

The antioxidant activity (%) of NLC, which is mainly ensured by SO, is shown in figure 10. AA values of different concentrations of SO (1, 2, 3%) encountered in NLC were compared with those of unloaded (free) SO. It can be observed that increasing the oil concentration from 1 to 3%, the AA is improved for both unloaded-lipid nanocarriers and individual oil solution systems. This behaviour is also observed for a low concentration of Nys (0.5%) loaded into NLC prepared with 10% or 12% lipids, while for higher concentration of Nys (0.75% and 1%) the AA of NLC drops with increasing the oil content.

Conclusions

The antifungal lipid nanocarriers with main average diameters ranging between 114 and 200 nm are obtained by using high pressure homogenization method. The zeta potential values of the nanodispersions (< –30 mV) revealed a moderate stability in time of the NLC. The presence of savory oil in low concentration has led to a decrease of the particle size, while the physical stability was better in case of NLC prepared with 12% lipid blend and loaded with 0.75% Nystatin. The prepared lipid nanocarriers offer an increased encapsulation of Nys for an initial 1% loading in comparison with 0.5% loading (e.g. entrapment efficiency reached 90% for 1% Nys and 82% for 0.5%, respectively). The antioxidant activity of Savory oil is increasing byincorporation into lipid nanocarriers, reaching up to 90% of oxygen free radicals scavenged.

The results obtained in the present study demonstrated that lipid nanocarriers based on emulgate, glyceryl monostearate and savory oil could be successfully used for the encapsulation of Nystatin in order to obtain antifungal formulations.

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References


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