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Abstract

Nerolidol is a natural sesquiterpene alcohol with promising but limited application in food and pharmaceutical fields due to several factors including low photostability and low aqueous solubility. Recently, several carriers loading nerolidol were prepared and tested in fresh orange juice. Lipid vesicles loading nerolidol did not exhibit satisfactory organoleptic properties in this

beverage. Hence, DMPC/DHPC bicelles were prepared as a new phospholipid-based carrier for nerolidol at different molar ratios. The bicelle suspensions were characterized in terms of homogeneity, particles size, and morphology. The optimal formulation (phospholipid:nerolidol molar ratio 100:1) was selected based on transparent appearance, homogeneity, and particle size (~45 nm). Besides, it showed a high encapsulation efficiency of nerolidol and a high incorporation rate of phospholipids. Transmission electron microscopy analysis demonstrated the formation of bicelles. The bicelles membrane fluidity was assessed by 1,6-diphenyl-1,3,5-hexatriene fluorescence anisotropy and differential scanning calorimetry analysis. The membrane fluidity of bicelles appeared to increase in the presence of nerolidol in a concentration dependent manner. To our knowledge this is the first study dealing with the encapsulation of an essential oil component in bicelles.

Keywords: bicelles; differential scanning calorimetry; fluorescence anisotropy; membrane fluidity; nerolidol.

Highlights

- For the first time nerolidol, a sesquiterpene, was efficiently incorporated in bicelles
- Suspensions of bicelles loading nerolidol were transparent, homogenous, and stable at 4 °C
- Bicelles presented high encapsulation efficiency and high molar percentage of Ner
- High incorporation rate of phospholipids was also demonstrated for the optimal formulation
- Nerolidol increased the fluidity of bicelles in a concentration dependent manner

43 **Abbreviations:** DLS: dynamic light scattering; EE: encapsulation efficiency; HPLC: high
44 performance liquid chromatography; IR: incorporation rate; Ner: nerolidol; PdI: polydispersity
45 index; TEM: transmission electron microscopy.

46

1. Introduction

Bicelles are bilayered nanostructures composed of a mixture of long- and short-alkyl chain phospholipids. The long chain phospholipids form the bilayer of the bicelles whereas the short chain phospholipids form the rim (Dürr et al. 2012) (Fig 1). Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) are the most commonly used long chain phospholipids, and dihexanoylphosphatidylcholine (DHPC) is the most used short-chain lipid to prepare bicelles (Visscher et al. 2006). The bicellar system has been extensively employed as a membrane model to study the structure of membrane proteins and their interaction with phospholipids (Dürr et al. 2012). Bicelles are poorly studied for application in aqueous pharmaceutical preparations and the interest in bicelles for skin application as a replacement for liposomes and micelles has recently emerged (Rubio et al. 2010, 2011). Moreover, bicelles have been proposed as a delivery system for hydrophobic drugs such as the anticancer agent doxorubicin (Lin et al. 2016). To our knowledge, the development of bicelles for food application is not studied yet.

The bicellar system has a transparent aspect and combines the properties of classic micelles and liposomes. Compared to micelles, the advantages of bicelles are mainly related to the absence of surfactants (Rubio et al. 2010, 2011). They are generally smaller than liposomes and more stable than micelles, thus they may constitute a promising system for the delivery of active molecules in food, beverages, and pharmaceutical preparations enriched in water. Moreover, the use of liposomes in beverages, such as fruit juice, is challenging as they may change the organoleptic properties of the beverage (Marsanasco et al. 2015).

Nerolidol (Ner), 3,7,11-trimethyl-1,6,10-dodecatrien-3-ol, is a sesquiterpene alcohol which exhibits various biological and pharmacological activities, including a significant antimicrobial

activity against different microorganisms such as *Corynespora cassiicola* and *Staphylococcus aureus* (Chan et al. 2016). It has been reported that Ner is a photosensitive agent (Chan et al. 2016); however, its chemical and biological stability is not well documented in literature. Moreover, Ner is poorly soluble in water with a log Kow value of 5.68 and a water solubility of 1.538 mg/L at 25 °C (Chan et al. 2016); this encouraged its incorporation in carrier systems. Cyclodextrin/nerolidol inclusion complexes (Azzi et al. 2017), conventional liposomes, and drug-in-cyclodextrin-in-liposomes loading Ner were prepared, characterized and evaluated for their antimicrobial effects in fresh orange juice (Azzi et al. 2018a).

Cyclodextrins are natural oligosaccharides known for their capacity to encapsulate a large variety of hydrophobic compound. Cyclodextrins are widely used in the food and pharmaceutical industries to enhance the physico-chemical stability and aqueous solubility of hydrophobic active agents. Cyclodextrins are easy to process and are appreciated for their diverse properties including prebiotic action (α-cyclodextrin), non-toxicity, resistance to shear forces, and neutrality in terms of taste and odor (Moreira da Silva 2009). However, different authors discussed the limitations of the different forms of cyclodextrins used including a limited water solubility (such as β-cyclodextrin), mass limitations for oral dosage units, sensitivity to acidic conditions, and stability of cyclodextrin/guest inclusion complex (Miranda et al. 2011; Chen et al. 2014). Liposomes are microscopic spherical lipid vesicles used for the encapsulation of hydrophilic, lipophilic, and amphiphilic molecules. They are known for their low toxicity, bio-degradability, and bio-compatibility. When applied in water enriched food such as fruit juice, liposome formulations produce milky aspect thus altering the organoleptic property of fruit juices (Ephrem et al. 2019).

To the best of our knowledge, this work describes for the first time in literature the development of a DMPC/DHPC bicelles system to carry an essential oil component. Ner was added at different

phospholipids to Ner molar ratio, and the optimal formulation was chosen based on the appearance of the formulation, the homogeneity of the suspension, and the size and morphology of the particles. The selected formulation was further characterized in terms of Ner encapsulation efficiency (EE) and molar percentage in bicelles (%Ner), in addition to the incorporation rate of phospholipids (IR) in bicelles. Furthermore, the effect of Ner on the membrane fluidity of bicelles was studied by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and differential scanning calorimetry (DSC) analysis.

2. Materials and methods

2.2.Materials

Dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids (USA). Tetrahydrofuran, nerolidol (98%, mixture of *cis* (40%)/*trans* (60%) isomers), and ammonium molybdate were purchased from Sigma-Aldrich (USA). Potassium phosphate monobasic was purchased from Sigma-Aldrich (Mexico). 4-Amino-3-hydroxyl-1-naphthalene sulfonic acid was purchased from Sigma-Aldrich (India). Chloroform and methanol were purchased from Sigma-Aldrich (France). Sulfuric acid, hydrogen peroxide, and thymol (>99%) were purchased from Sigma-Aldrich (Germany).1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Across Organics (USA).

2.3.Preparation of blank- and nerolidol-loaded bicelles

Blank bicelles were prepared using the long chain phospholipid DMPC and the short chain phospholipid DHPC. The two phospholipids were mixed in chloroform at a DMPC to DHPC molar ratio of 2.0. The chloroform was then evaporated under reduced pressure at 35 °C until the formation of a lipid film. The film was hydrated with 1 mL of ultrapure water (35 °C) to yield a total lipid concentration of 20% (w/v). The obtained suspension was finally subjected to several

116 cycles of freeze-thaw and the size of the particles was monitored after each cycle by DLS analysis.
117 Freeze (0 °C) - thaw (room temperature) cycles were repeated until a transparent solution was
118 obtained.

119 Nerolidol-loaded bicelles were prepared as described for blank bicelles but with the drug dissolved
120 in the chloroform phase. Various phospholipids to Ner molar ratios (100:1, 50:1, 20:1, 10:1, and
121 4:1) were tested for Ner-loaded bicelles formulation.

122 **2.4.Characterization of blank- and nerolidol-loaded bicelles**

123 ***2.4.1. Determination of size, polydispersity index, and zeta potential***

124 The mean size and polydispersity index (PdI) values of the formulations were determined by
125 dynamic light scattering (DLS) using a Malvern Zetasizer nanoSeries (3600, Orsay, France). The
126 PdI is a measure of the width of the molecular weight distribution and thus is an indicator of the
127 suspension homogeneity. The zeta potential value was also obtained with a Malvern Zetasizer
128 nanoSeries (3600, Orsay, France) and it may be related to the stability of the system, as a low zeta
129 potential value may lead to the coagulation of the particles. Prior to each measurement, the sample
130 was diluted with ultrapure water at a temperature of 19 ± 1 °C or 25 ± 1 °C to obtain a count rate
131 between 150 and 300 kilo counts per second.

132 ***2.4.2. Transmission electron microscopy analysis***

133 The morphology of bicelles was determined by transmission electron microscopy (TEM) using
134 Philips CM120 microscope (Eindhoven, The Netherlands) at the "Centre Technologique des
135 Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). The analyses were
136 performed at an acceleration of 80 kV. An aliquot (10 μL) of each sample was placed on a carbon-
137 coated copper grid. After 2 min, the liquid excess was removed using a filter paper, to obtain a
138 thin liquid film on the grid. Negative staining was then made using a 1% sodium silicotungstate

139 solution. After 30 s, the excess sodium silicotungstate solution was removed with filter paper. The
140 sample was then left to dry at room temperature.

141 ***2.4.3. Separation of free and encapsulated components***
142

143 Prior to the determination of the encapsulation efficiency and loading rate of Ner and the
144 incorporation rate of phospholipids, free and encapsulated compounds were separated by
145 centrifugation. Therefore, an aliquot of the sample was centrifuged at 14,000 g for 30 min using
146 Vivaspin 500 centrifuge (3000 MWCO, Sartorius, United Kingdom).

147 ***2.4.4. Incorporation of phospholipids***
148

149 Bartlett's method was used to determine the total and free phospholipid concentrations in the
150 bicellar formulations (Bartlett 1959). Hence, aliquots were removed from the bicelles suspension,
151 filtrate, or standard solution. The calibration curve was determined using standard solutions of
152 potassium phosphate monobasic (KH_2PO_4) in ultrapure water with concentrations ranging from
153 0.064 to 0.416 mM. The procedure is well described is our previous publications (Azzi et al.
154 2018b).

155 The incorporation rate (IR) of phospholipids was determined according to the following equation:

156 $$\text{IR}\ (\%) = \frac{N_B - N_F}{N_I} \times 100$$

157 Where N_I is the initial number of moles of phospholipids and N_B and N_F are the total and free
158 number of moles of phospholipids determined in bicelle suspension, respectively.

159 ***2.4.5. Incorporation of nerolidol***
160

161 Ner was quantified in the bicellar formulations as previously described by Azzi et al. (2018a) using
162 an Agilent Technology 1200 series HPLC system. The mobile phase was a mixture of

methanol/water (75/25), the flow rate was set to 1 mL/min and the wavelength of the UV detector was 212 nm. The calibration curve of Ner was determined using standard solutions of Ner in methanol with concentrations ranging from 2.5 to 250 µg/mL. Thymol was used as an internal standard at a concentration of 100 µg/mL in methanol. An aliquot (100 µL) of Ner standard solution, filtrate obtained as described previously, or bicellar formulation with the adequate dilution was added to 200 µL of methanol and 100 µL of thymol solution. The samples were then sonicated for 10 min at 4 °C and centrifuged at 14,000 rpm for 30 min at 4 °C. An aliquot (20 µL) of the supernatant was then analyzed through a reversed-phase C18 Agilent column (15 cm × 4.6 mm, 5 µm).

The encapsulation efficiency (EE) of Ner was determined according to the following equation:

$$EE\ (\%) = \frac{M_T - M_F}{M_T} \times 100$$

Where M_T and M_F are the total and free mass of Ner determined in the bicelles suspension, respectively.

The molar percentage of Ner (%Ner) in bicelles was calculated as follows:

$$\%Ner = \frac{N_T - N_{Nf}}{N_B - N_F} \times 100$$

Where N_T and N_{Nf} are the total and free number of moles of Ner in the bicellar formulation, respectively, and N_B and N_F are the total and free number of moles of phospholipids determined in bicelle suspension, respectively.

2.5. Effect of nerolidol on membrane fluidity

2.5.1. Fluorescence anisotropy measurements

Steady state fluorescence polarization was used to determine the changes in bicelles fluidity using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). The fluorescent probe DPH was dissolved in tetrahydrofuran at a concentration of 1 mg/mL. Bicellar suspensions were incubated overnight in the dark at room temperature in presence of DPH at DPH:phospholipid ratio of 1:500. The incubation allowed the intercalation of the probe into the lipid bilayer; the final tetrahydrofuran concentration (< 0.01%) had no effect on the fluorescence anisotropy (Whiting et al. 2000). The experiments were performed at 20, 25, 40, and 60 ºC. The probe was excited at 360 nm with a manual polarizer accessory (Cary Eclipse Manual Polarizer, Agilent Technologies, Malaysia) using vertically polarized light. The emission intensities were measured at 450 nm at both parallel and perpendicular to the polarization vector of the exciting light. The Cary Eclipse Bio Software, delivered from the spectrophotometer, gives the fluorescence anisotropy (R), which is defined as:

$$R = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}},$$

Where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of the exciting beam.

2.5.2. Differential scanning calorimetry

Calorimetric analysis of the bicellar formulations were performed using a DSC Q200 scanning calorimeter (TA Instruments, France). An aliquot (30 µL) of each formulation was placed in a hermetically sealed standard aluminum DSC pans. The temperature was scanned at a rate of 1 °C/min over a range of 0 to 65 °C. The reference pan was filled with ultrapure water. The software "TA processor" was then used to determine the phase transition temperature (T_m) from the

maximum of the recorded heat, the transition enthalpy (ΔH) from the area under the peak, the respective pre-transition parameters (Tp and ΔHp), and the temperature width at half peak height (ΔT1/2). ΔT1/2 is inversely proportional to the cooperativity of the transition and thus will be taken as a measure of the latter (El Maghraby et al. 2005).

2.6. Statistical analysis

Statistical analysis was performed using Student t-test, and P values equal to or less than 0.05 were considered to be significant.

3. Results and discussion

3.1. Characterization of nerolidol-loaded bicelles

3.1.1. Appearance, size, polydispersity index, zeta potential, and morphology

Blank and nerolidol-loaded bicelles were prepared at 35 °C. At this temperature, the both lipids DHPC and DMPC would be in the disordered fluid phase.

Blank bicelles and bicelles loading 1% Ner became transparent at room temperature after 18 freeze-thaw cycles. Formulations containing 2 and 5% Ner appeared turbid at room temperature even at up to 40 freeze-thaw cycles. At higher Ner concentrations (10 and 25%), the formulations had a milky appearance even after 40 cycles. Many essential oils including Ner are reported to promote the enlargement of lipid particle size (Azzi et al. 2018a). Not surprisingly, the same effect was maintained with bicelles. Rubio et al. (2011) have previously reported a milky appearance of DPPC/DHPC bicellar system containing flufenamic acid (1% w/v) at 25 °C. In another study, the same group (Rubio et al. 2010) has reported no change in the appearance of DPPC/DHPC and DMPC/DHPC bicellar formulations following the addition of 1.16% (w/v) diclofenac. On the other hand, Barbosa-Barros et al. (2008) reported a milky aspect of DMPC/DHPC bicellar

227 formulations containing ceramide (10 and 20%) at temperatures above the T_m of DMPC. At
228 temperatures below the T_m of DMPC, the appearance of the formulation was dependent on the
229 content of ceramide (Barbosa-Barros et al. 2008).

230 The size and the PdI values of bicelles were determined by DLS (Table 1) at a temperature below
231 the transition temperature (T_m) (19 ± 1 °C) and slightly higher than the T_m (25 ± 1 °C) of DMPC.
232 At 19 ± 1 °C, two populations were obtained for all bicellar formulations. Blank bicelles showed
233 a good homogeneity (PdI = 0.264). Around 95% of particles in the blank formulations had a size
234 around 42 nm (Table 1) which is in agreement with literature data (Barbosa-Barros et al. 2012),
235 while the minor population contained micrometric structures (Table 1). The formulation
236 containing Ner (1%) did not influence the size nor the homogeneity of the particles (Table 1).
237 Whereas, the addition of Ner at higher concentrations (2 to 25%) to the formulations increased
238 drastically the size of the particles of the major population for more than 10 folds and altered the
239 homogeneity of the formulations. At 25 °C, all formulations were highly polydispersed and the
240 major population in each formulation had a size greater than 300 nm. Our results agree with the
241 results previously obtained elsewhere (Barbosa-Barros et al. 2008) which reported an increase in
242 the particle size and heterogeneity at temperatures higher than the T_m of DMPC (30 and 40 °C). In
243 fact, temperatures above the T_m of the phospholipid promote the transfer of DHPC from the rim
244 to the DMPC bilayer domain, leading to the increase in the bicelles size (van Dam et al. 2006). On
245 the other hand, it has been reported that the change of the bicellar size is dependent on the location
246 of the incorporated agent. Drugs that intercalate between the long chain phospholipids such as
247 flufenamic acid produced an increase in bicelle size (Rubio et al. 2011). Similarly, increasing the
248 long chain- to short chain- phospholipid molar ratio increased the size of the bicelles due to the
249 enlargement of the planar bilayer (Vold and Prosser 1996). In contrast, drugs that join the rims like

diclofenac induced a decrease in bicelle size. Ner may localize into the planar bilayer of the bicelle (Fig 1) between the acyl chains of DMPC, increasing their size. And, at high concentrations, Ner may promote the formation of other lipid structures, like liposomes, as it will be subsequently demonstrated by TEM analysis in this section.

On the other hand, blank bicelles had a zeta potential around -7 mV, which is close to the zeta potential value of drug-free DMPC/DHPC bicelles reported by Yamada et al. (2016). The zeta potential value was not greatly influenced by the addition of Ner at the different tested concentrations (Table 1).

TEM analysis supports the results obtained by DLS. Indeed, bicelles prepared in the presence of 1% Ner appeared similar to blank bicelles (Fig 2 A-B). The stacks of bicelles that appear in Figures 2A and B may explain the micrometric population revealed by DLS analysis. The bicellar formulation prepared in presence of Ner at concentrations higher than 1% led to the formation of multilamellar sheets and/or liposomes (Fig 2 C-F). These structures had been observed elsewhere. Fragments of multilamellar sheets and tubular structures, liposomes and elongated structures were observed in the freeze fracture electron microscopy images of flufenamic acid-containing DHPC/DPPC (2:1 molar ratio) bicelles formulation and TEM images of ceramid-containing bicelles formulation (Barbosa-Barros et al. 2008; Rubio et al. 2011).

Ner-loaded liposomal formulations were previously prepared by Azzi et al. (2018a) and Ephrem et al. (2019). The prepared suspensions had a phospholipid to Ner molar ratio of up to 10:1, particle size higher than 131 nm, a milky appearance (Azzi et al. 2018a; Ephrem et al. 2019) and a low homogeneity (PdI > 0.48) (Ephrem et al. 2019). Moreover, the addition of Ner-loaded liposomes to fresh orange juice has altered the organoleptic property of the juice (Ephrem et al. 2019). This

problem could be overcome by the transparent appearance of bicellar formulations, thus highlighting the importance of this system for the preparation of functional foods.

On the overall, the bicellar formulation prepared with Ner (1%) was retained for further characterization because of the reliability of the bicellar structure that was confirmed through particle size determination and morphological observation.

3.1.2. Encapsulation efficiency of nerolidol, incorporation rate of phospholipids and molar percentage of nerolidol

The bicelles preparation with Ner (1%) was characterized for the encapsulation efficiency (EE) of Ner, molar percentage of Ner in bicelles (% Ner), and incorporation rate of phospholipids (IR) in bicelles.

The quantification of phospholipids in the suspensions of blank bicelles showed that around 83.4 ± 1.2% are incorporated in the bicelles and the incorporation rate increased in the presence of 1% Ner (IR= 87.0 ± 1.9%) (data not shown). On the other hand, the bicellar formulations prepared with Ner 1% had an EE value of 100%. However, the molar percentage of Ner in bicelles was of 0.86%. The hydrophobicity of Ner and the length of its aliphatic chain (C_{12}) could explain the high encapsulation efficiency value. Indeed, Ner ($C_{15}H_{26}O$) could be easily incorporated between the DMPC molecules of acyl chains of 14 carbon atoms. Busch and Unruh (2011) previously reported the intercalation of farnesol ($C_{15}H_{26}O$) between DMPC molecules inside the lipid bilayer by aligning itself along the phospholipids molecules. On the other hand, it was proven that Ner may completely penetrate the lipid bilayer (Mendanha and Alonso 2015).

3.2. Effect of nerolidol on bicelles membrane fluidity

293 ***3.2.1. Fluorescence anisotropy results***

294 The effect of Ner on the membrane fluidity of bicelles was investigated by steady-state
295 fluorescence anisotropy of DPH (Lakowicz 2006). The anisotropy values decreased when the
296 temperature increased from 20 to 40 °C at all the tested Ner concentrations, but then increased
297 slightly when the temperature increased between 40 and 60 °C (Fig 3). This may be due to the
298 occurrence of only one phase transition of the system at a temperature (23 °C) close to the Tm of
299 pure DMPC bilayers (Khatun et al. 2013). Similar observations had been made elsewhere on
300 formulations containing mixtures of phospholipids and gangliosides; above 40 °C, a plateau was
301 observed with anisotropy values slightly above or below those obtained at 40 °C for these
302 preparations (Khatun et al. 2013). The authors observed similar decrease of the anisotropy values
303 at temperatures ranging from 17 to 40 °C with DMPC/CHAPS (3-[(3-cholamidopropyl)
304 dimethylammonio]-1-propanesulfonate) bicelles. On the other hand, the presence of Ner increased
305 the membrane fluidity in a concentration dependent manner (Fig 3) at the tested temperatures. Yet,
306 a minimal change in the membrane fluidity was observed at Ner 1%. The increase of the membrane
307 fluidity in presence of Ner was expected sincesesquiterpene alcohol has been previously reported
308 to increase the membrane fluidity of erythrocytes membranes (Mendanha et al. 2013) and plasma
309 membranes of parasites (Camargos et al. 2014).

310 ***3.2.2. Differential scanning calorimetry***

311 DSC is often used to study the thermodynamic properties of lipid bilayers. In fact, the
312 incorporation of an active agent can interfere with the packing of lipids thus changing the
313 thermodynamic parameters determined by DSC. The thermogram of DHPC exhibits no peak in
314 the studied temperature range (0 to 60 °C) (Rubio et al. 2011), thus the endotherms obtained most

likely arise from changes in the DMPC pre- and chain melting phase transitions at 9.3°C and 22.7° C, respectively, in the presence of the increasing concentration of Ner.

In the absence of Ner, the bicellar system displayed a poor enthalpic pre-transition (9.3 ± 0.54 °C) and a relatively sharp main transition (22.7 ± 0.40 °C) (Fig 4 and Table 2) which correspond respectively to the transitions of DMPC acyl chains from $L_{\beta'}$ (gel phase) to $P_{\beta'}$ (ripple phase), and from $P_{\beta'}$ to L_{α} (liquid crystalline phase) (Nagle 1980). The two peaks were previously reported by Sasaki et al. (2007) (Sasaki et al. 2007) at around 12 and 23.6 °C, respectively for DMPC/DHPC bicelles using DSC. On the other hand, the main phase transition enthalpy (ΔH, 9.21 ± 0.91 kJ/mol) was significantly higher than that of the pre-transition (0.13 ± 0.07 kJ/mol) and was close to that obtained by Sasaki et al. (2007) (1440 Cal/mol; 6.029 kJ/mol) (Sasaki et al. 2007). Small values of $\Delta T_{1/2}$ and thus peak sharpness indicate the phase transition cooperativity ($1/\Delta T_{1/2}$) which is determined by the purity of the lipid species being measured (Abboud et al. 2016). However, the bicellar system showed a lower sharpness in the transition peak and a ΔT1/2 value of 3.95 ± 0.28 °C, which arises from the mixture of two lipids (DMPC/DHPC) with a corresponding decrease in the chain melting phase transition cooperativity.

The pre-transition temperature decreases in presence of Ner and the peak is completely abolished at Ner concentrations above 2% (Fig 4 and Table 2). Previous studies have explained the loss of the pre-transition peak to the interaction of the added compound with the choline group of the phospholipid (Abboud et al. 2016). Also, the chain melting phase transition temperature decreases with the increasing Ner concentration, especially at Ner (25%) (Table 2). This could be explained by the length of nerolidol chain (C12) compared to that of DMPC side chain (C14). In fact, sterols with a chain length greater than the thickness of the bilayer increase the transition temperature of DPPC bilayers while sterols with short side chains decrease the transition temperature (McMullen

et al. 1995). However, the main transition enthalpy is not significantly influenced by the presence of 1, 2, and 5% Ner (Table 2). Whereas, at 25% Ner the ΔH increased (Table 2). On the other hand, the presence of Ner increased the $\Delta T_{1/2}$ and led to the broadening of the main transition peak (Fig 4 and Table 2).

The DPH fluorescence polarization measurements suggest that Ner is incorporated in the bicellar bilayer leading to an increase of the membrane fluidity and this is supported by the decrease in the chain melting phase transition cooperativity. Similarly, flufenamic acid increased the fluidity of DPPC/DHPC bicelles (Rubio et al. 2011). DSC analysis showed that Ner produced a disorder in the bicellar membrane. The smallest changes of thermodynamic parameters were obtained in the presence of Ner (1%), which is in accordance with the results obtained by DLS, TEM, and DPH anisotropy measurements

Conclusion

Ner was effectively incorporated in a bicellar system composed of DMPC and DHPC at a phospholipids to Ner molar ratio of 100:1. The formulation exhibited similar appearance, particles size range, PdI value, and morphology as the blank formulation. Moreover, it showed high EE (100%) of Ner, and high IR (87.0 ± 1.9%) of phospholipids. At higher concentrations, Ner altered the formulation characteristics. Finally, the fluidity of the bicelles bilayer increased in presence of Ner, especially at Ner concentrations higher than 1%.

This study examines for the first time the possibility of the incorporation of an essential oil component in a bicellar system. As a result, an optimized formulation was achieved which could be a promising candidate to enhance the efficacy of Ner in food and pharmaceutical preparations. Future trends could focus on the antimicrobial activity of Ner-loaded bicelles in culture media and food matrices where other encapsulating systems made of lipids failed to preserve the organoleptic

properties of foods, especially juices. Also, the effect of the incorporation of other essential oil components on the stability and characteristics of the bicellar system could be investigated.

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Figure captions

Fig.1 A schematic representation of an ideal bicelle

Fig.2 TEM images of bicelles containing A) 0, B) 1, C) 2, D) 5, E) 10, and F) 25% Ner

Fig.3 DPH anisotropy values for bicelles with or without Ner at phospholipid to Ner molar ratios of 100:1, 50:1, 20:1, 10:1, and 4:1 at 20, 25, 40, and 60 °C

Fig.4 DSC scans of blank bicelles and Ner-loaded bicelles at phospholipid to Ner molar ratios of 100:0, 100:1, 50:1, 20:1, 10:1, and 4:1

503 **Table 1: Size, polydispersity index, and zeta potential of bicellar formulations with and**
504 **without nerolidol.**

Ner %	19 ± 1 °C					
	Population 1		Population 2		PdI	Zeta potential (mV)
	%	Size (nm)	%	Size (nm)		
0	95.2 ± 4.6	42.2 ± 4.8	5.8 ± 4.9	3607 ± 1869	0.26 ± 0.04	-6.9 ± 1.0
1	96.8 ± 4.4	45.2 ± 5.5	4.9 ± 2.4	3715 ± 659*	0.19 ± 0.07	-9.6 ± 1.9
2	36.6 ± 2.4	41.1 ± 5.5	63.4 ± 2.4	624 ± 205*	0.69 ± 0.09*	-9.6 ± 3.5
5	40.2 ± 9.4	46.6 ± 7.1	59.8 ± 12.0	404 ± 172*	0.74 ± 0.20*	-6.1 ± 0.0
10	11.0 ± 3.4	128.5 ± 25.4*	89.0 ± 6.3	1532 ± 70*	0.73 ± 0.09*	-6.7 ± 0.5
25	88.5 ± 11.6	434.5 ± 2.5*	23.1 ± 12.2	2561 ± 703*	0.44 ± 0.01*	-12.2 ± 0.5
Ner %	25 ± 1 °C					
	Population 1		Population 2		PdI	Zeta potential (mV)
	%	Size (nm)	%	Size (nm)		
0	45.8 ± 23.3	48.7 ± 16.9	54.2 ± 23.3	515 ± 278	0.54 ± 0.19	-12.7 ± 6.3
1	42.7 ± 13.1	38.4 ± 7.85	56.3 ± 14.2	322 ± 68	0.53 ± 0.14	-14.1 ± 5.6
2	13.8 ± 4.3	170.9 ± 101.5	81.9 ± 8.7	1161 ± 470*	0.74 ± 0.26	-18.3 ± 5.2
5	9.8 ± 5.4	183.6 ± 33.8	70.5 ± 30.4	906 ± 358	0.82 ± 0.15	-10.9± 0.4
10	3.9 ± 3.8	61.9 ± 56.1*	96.1 ±3.8	560 ± 119	0.28 ± 0.03*	-8.8 ± 0.8
25	25.0 ± 5.1	148.4 ± 13.6*	85.0 ± 15.1	494 ± 78	0.61 ± 0.17	-12.2 ± 2

505 ** P values equal to or less than 0.05*

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514 **Table 2: Parameters with increasing Ner concentration in DMPC/DHPC (2:1 molar ratio)**
515 **bicelles.**

%Ner	T_p (°C)	ΔH_p (Kj/mol)	T_m (°C)	ΔH (Kj/mol)	$\Delta T_{1/2}$ (°C)
0	9.30 ± 0.54	0.13 ± 0.07	22.70 ± 0.40	9.21 ± 0.91	3.95 ± 0.28
1	7.74 ± 0.14	0.12 ± 0.01	22.30 ± 0.11	8.69 ± 0.61	4.32 ± 0.15
2	4.96 ± 0.78	0.16 ± 0.05	21.40 ± 0.02	9.91 ± 0.04	5.06 ± 0.11
5	-	-	20.50 ± 0.11	9.40 ± 0.36	6.03 ± 0.41
25	-	-	13.50 ± 0.02	11.67 ± 0.2	8.51 ± 0.18

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519 Figure 1

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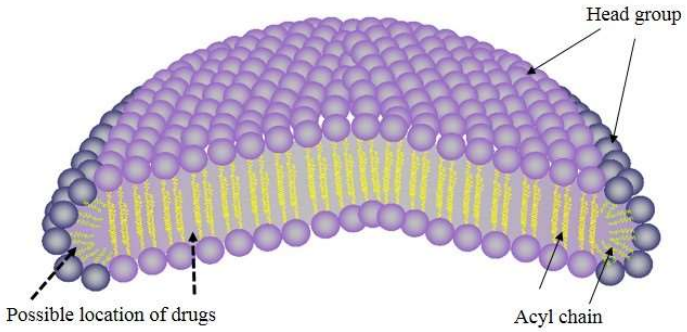

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527 Figure 2

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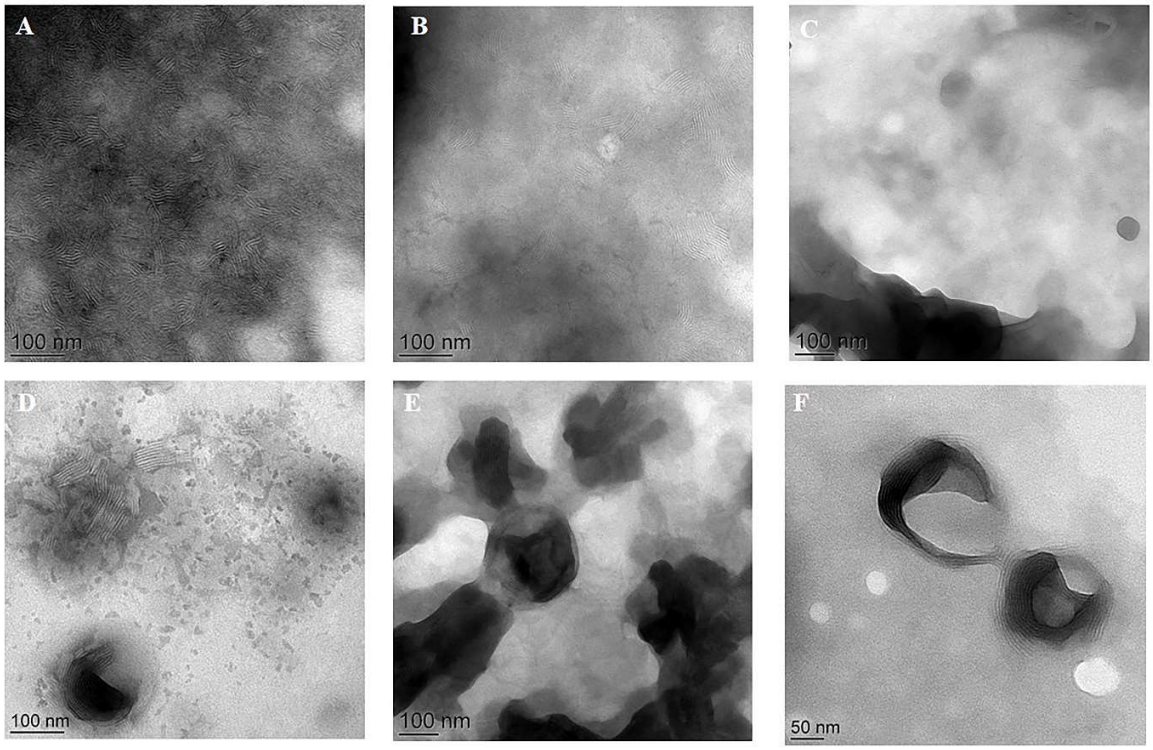

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533 Figure 3

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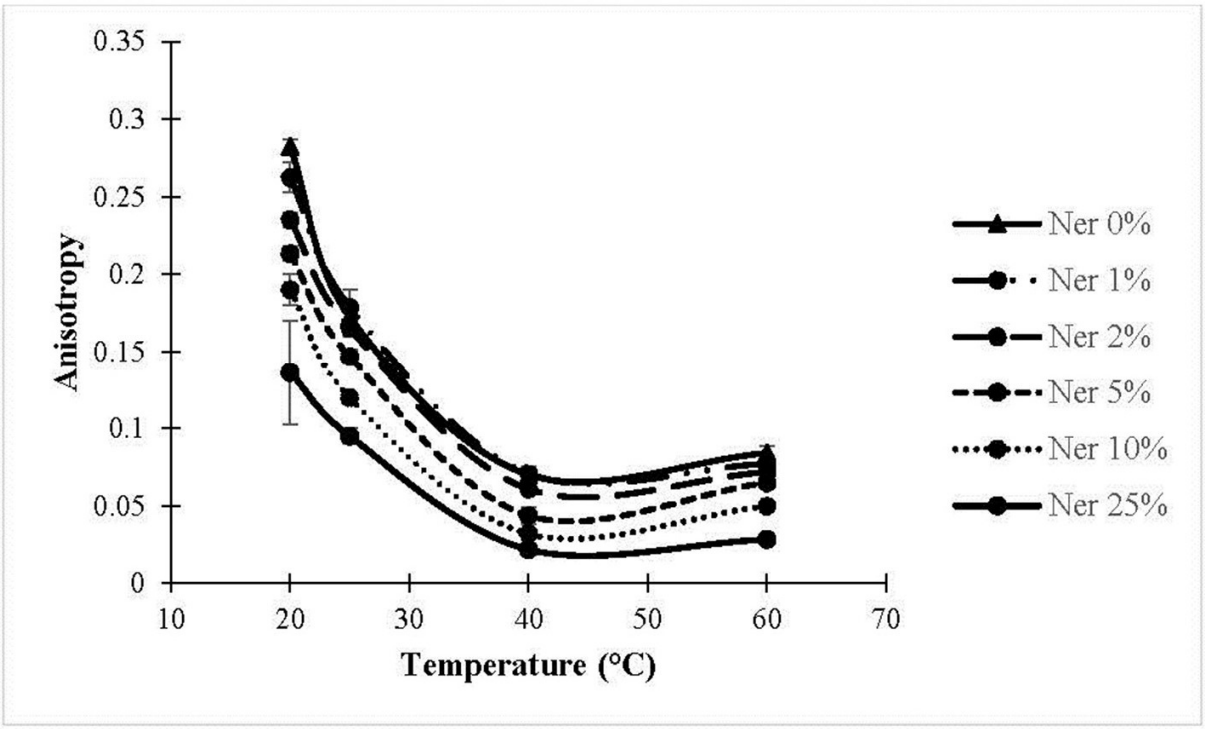

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539 Figure 4

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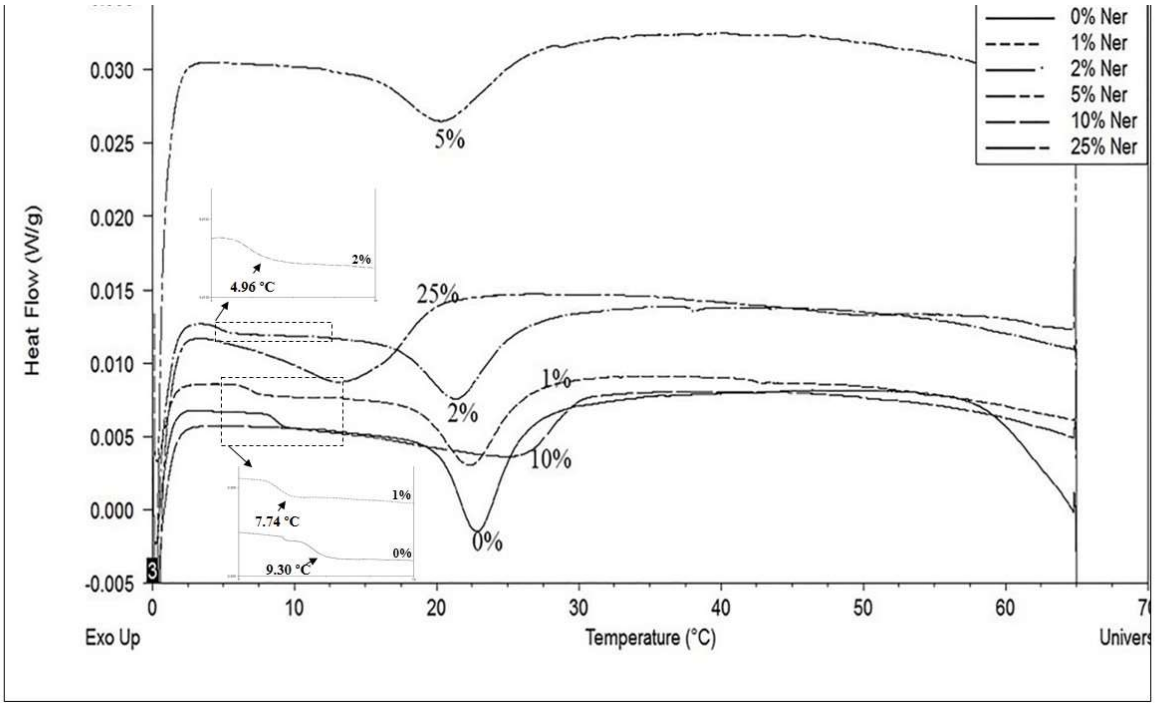

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