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1 **Cineole-containing nanoemulsion: development, stability, and antibacterial activity**

2

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25

26 **Abstract**

27 1,8-cineole is a monoterpene commonly used by the food, cosmetic, and pharmaceutical  
28 industries owing to its flavor and fragrances properties. In addition, this bioactive  
29 monoterpene has demonstrated bactericidal and fungicidal activities. However, such  
30 activities are limited due to its low aqueous solubility and stability. This study aimed to  
31 develop nanoemulsion containing cineole and assess its stability and antibacterial activity  
32 in this context. The spontaneous emulsification method was used to prepare  
33 nanoemulsion (NE) formulations (F1, F2, F3, F4, and F5). Following the development of  
34 NE formulations, we chose the F1 formulation that presented an average droplet size (in  
35 diameter) of about 100 nm with narrow size distribution (PDI <0.2) and negative zeta  
36 potential ( $\sim -35$  mV). According to the analytical centrifugation method with  
37 photometric detection, F1 and F5 formulations were considered the most stable NE with  
38 lower droplet migration velocities. In addition, F1 formulation showed high incorporation  
39 efficiency (> 80 %) and TEM analyses demonstrated nanosized oil droplets with irregular  
40 spherical shapes and without any aggregation tendency. Antibacterial activity assessment  
41 showed that F1 NE was able to enhance the cineole action against *Staphylococcus aureus*,  
42 *Enterococcus faecalis*, and *Streptococcus pyogenes*. Therefore, using a simple and  
43 reproducible method of low energy emulsification we designed a stable nanoemulsion  
44 containing 1,8-cineole with improved antibacterial activity against Gram-positive strains.

45

46 **Keywords:** 1,8-cineole, Spontaneous emulsification, Nanoemulsion, Stability,  
47 Antibacterial activity.

48

49

50

## 51 **1. Introduction**

52 In nature, bioactive oils based on terpene (isoprene) and terpenoid compounds are  
53 synthesized by aromatic plants as secondary metabolites, commonly known as essential  
54 oils (EO) or volatile oils. Monoterpenes are formed by two isoprene units (10 carbon  
55 atoms) and constitute more than 80 % of EO composition (Asbahani et al., 2015; Bakkali  
56 et al., 2008; Bilia et al., 2014; Matos et al., 2019; Slamenova and Horvathova, 2013).  
57 According to each plant source, EO comprise widely varied monoterpenes,  
58 sesquiterpenes, and phenylpropenes combinations. There is a terpenic oxide among such  
59 monoterpenes, namely 1,8-cineole ( $C_{10}H_{18}O$ ; M.W. 154.25 g mol<sup>-1</sup>), which is a cyclic  
60 ether found as the main constituent in EO obtained from eucalyptus species. This  
61 monoterpene is a limpid and colorless oily liquid with a strong camphor-like odor and  
62 also called 1,8-epoxy-p-menthane, eucalyptol, and cajeputol (Adak et al., 2020; Ali et al.,  
63 2015; De Vincenzi et al., 2002; Salehi et al., 2019; Slamenova and Horvathova, 2013).  
64 Owing to its bactericidal and fungicidal activities as well as flavor and fragrances  
65 properties, 1,8-cineole has been used by food, cosmetic, and pharmaceutical industries.  
66 However, despite all these applications, the use of 1,8-cineole or EO containing this  
67 terpene is limited due to its limited thermal stability, substantial volatility, and low water  
68 solubility (Adak et al., 2020; Hammoud et al., 2019; Prakash et al., 2018a; Prakash et al.,  
69 2018b).

70 In order to prevent terpenes and phenylpropanoid derivatives volatilization or oxidation,  
71 as well as their degradation by light and for improving the chemical stability, the  
72 encapsulation of these compounds into nanostructured systems has been commonly used.  
73 Several nanocarriers have been well designed to entrap EO or their main compounds in  
74 this context. Among these carriers, the choice covers polymeric nanocapsules or  
75 nanoparticles, liposomes, phytosomes, nanoemulsions, microemulsions, solid lipid

76 nanoparticles, and nanostructured lipid carriers (Asbahani et al., 2015; Bilia et al., 2014;  
77 Harwansh et al., 2019; Matos et al., 2019; Prakash et al. 2018a; Prakash et al., 2018b;  
78 Zhao et al., 2016).

79 Oil-in-water nanoemulsion is the most often-used colloidal lipid carrier to incorporate  
80 EO. According to recent review conducted by Matos et al. (2019), almost half of all  
81 nanostructured systems containing EO was represented by nanoemulsified dispersions.  
82 Nanoemulsions (NE) are ultrafine dispersions constituted of two immiscible liquids  
83 (organic and aqueous phases) stabilized by surfactant(s). Despite being considered  
84 nonequilibrium systems (thermodynamically unstable), nanoemulsions are metastable or  
85 kinetically stable colloidal dispersions (Anton and Vandamme, 2011; Gupta et al., 2016;  
86 Helgeson, 2016; Komaiko and McClements, 2016; Singh et al., 2017; Solans and Solé,  
87 2012).

88 In general, nanoemulsions are produced using low or high-energy methods. High-energy  
89 emulsification approaches, i.e., work-based emulsification methods, usually require  
90 sophisticated and expensive devices, namely high-pressure homogenizers, ultrasound  
91 generators, and microfluidizers. On the other hand, low-energy methods or  
92 thermodynamic methods are easy production processes based on simple stirring and  
93 changing some formulation parameters, e.g., surfactant concentration and type, oil-water  
94 ratio, temperature, etc. Usually low-energy emulsification methods have been categorized  
95 as phase inversion composition, phase inversion temperature, emulsion inversion point,  
96 and spontaneous emulsification (Anton and Vandamme, 2009; Gupta et al., 2016;  
97 Helgeson, 2016; Komaiko and McClements, 2016; Safaya and Rotliwala, 2020; Singh et  
98 al., 2017; Solans and Solé, 2012).

99 The main reason for incorporating EO or their major constituents into the lipid core of  
100 nanoemulsion is directly related to bioactive molecules improving their stability and

101 subsequent enhanced bioefficacy (Asbahani et al., 2015; Bilia et al., 2014; Harwansh et  
102 al., 2019; Matos et al., 2019). Also, EO-based nanoemulsion formulations have received  
103 increasing attention as effective nanocarriers improving antimicrobial activity of  
104 bioactive components (Prakash et al., 2018a; Prakash et al., 2018b). Nevertheless,  
105 available information about preparation, stability, and biological activities of  
106 nanoemulsions containing isolated bioactive compounds from EO is still scarce, requiring  
107 more investigations. Therefore, this study aimed to develop cineole-containing  
108 nanoemulsion formulation using an isothermal low-energy emulsification method and  
109 assess its accelerated stability and antibacterial activity.

110

## 111 **2. Materials and methods**

112

### 113 **2.1 Materials**

114 1,8-Cineole (Eucalyptol 99 %,  $d = 0.921 \text{ g mL}^{-1}$  at  $25 \text{ }^\circ\text{C}$ ), sorbitan monooleate (SMO),  
115 polyoxyethylen-20 sorbitan monolaurate (SML-20), polyoxyethylen-20 sorbitan  
116 monooleate (SMO-20), sodium phosphate monobasic, and sodium phosphate dibasic  
117 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Medium-chain  
118 triglycerides (MCT,  $d = 0.94 \text{ g mL}^{-1}$  at  $20 \text{ }^\circ\text{C}$ ) oil was kindly supplied from Brasquim  
119 (Porto Alegre-RS, Brazil).

120

### 121 **2.2 Nanoemulsion preparation**

122 Nanoemulsions (NE) were prepared using a method based on a spontaneous  
123 emulsification procedure described by Saberi et al. (2014) with slight modifications.  
124 Briefly, spontaneous emulsification was carried out by addition of an organic phase  
125 consisting of carrier oil (MCT), 1,8-cineole, and surfactant(s), to an aqueous phase

126 (phosphate buffer solution pH 7.4) under magnetic stirring (200 rpm) and ambient  
 127 temperature kept constant throughout process. Initially, different ratios between phases  
 128 and surfactant mixtures were assessed to find more suitable formulations resulting in final  
 129 composition (wt. %) of the five nanoemulsions reported in Table 1. Thus, both carrier oil-  
 130 to-cineole and surfactant-to-oil weight ratios were kept constant at 1.0 and 2.0,  
 131 respectively. The proportion of surfactant:oil:water was set at 10:10:80 (wt. %),  
 132 respectively, and for F1, F3, and F5 formulations the surfactant combinations were  
 133 maintained at HLB 12 (Table 1). All formulations were prepared three times (n=15) and  
 134 stored at 4 °C ( $\pm 2$ ). Unloaded NE (F5 formulation) was obtained from organic phase  
 135 composed only of carrier oil (MCT) and surfactants using the same spontaneous  
 136 emulsification process.

137

138 **Table 1.**

139 Composition (wt. %) of NE formulations.

NE	SML-20	SMO-20	SMO	MCT	Cin	PBS
F 1	6.2	-	3.8	5.0	5.0	80
F 2	10	-	-	5.0	5.0	80
F 3	-	7.2	2.8	5.0	5.0	80
F 4	-	10	-	5.0	5.0	80
F 5	6.2	-	3.8	10	-	80

140 SML-20: polyoxyethylen-20 sorbitan monolaurate; SMO-20: polyoxyethylen-20 sorbitan  
 141 monooleate; SMO: sorbitan monooleate; MCT: medium-chain triglycerides; Cin: 1,8-  
 142 cineole; PBS: 10 mM phosphate buffer solution pH 7.4.

143

144 **2.3 Droplet size measurement**

145 The hydrodynamic diameter of droplets and polydispersity index (PdI) of the NE  
146 formulations were determined by photon correlation spectroscopy (PCS) at 25 °C and a  
147 scattering angle of 173° (Zetasizer® Nano ZS, Malvern PCS Instruments, UK). All  
148 samples were diluted (1/100) with ultra-purified water. The reported values are average  
149 ±SEM of three different batches of each colloidal dispersion.

150

#### 151 **2.4 Zeta potential measurement**

152 Zeta ( $\zeta$ ) potential of NE was measured by electrophoretic mobility using a Zetasizer®  
153 Nano ZS apparatus (Malvern PCS Instruments, UK). The Smoluchowski model was used  
154 to estimate  $\zeta$  potential from electrophoretic mobility. The analyses were conducted at 25  
155 °C, and the samples were appropriately diluted (1/100) with ultra-purified water. Values  
156 reported are average ±SEM of three different batches of each colloidal dispersion.

157

#### 158 **2.5 Forced stability study**

159 Stability study was performed using the analytical centrifugation method with  
160 photometric detection at 865 nm (LUMiSizer® 611, LUM GmbH, Germany). The  
161 samples of NE formulations were incorporated into polycarbonate cell and equilibrated  
162 to 25 °C before analyses. Measurements were carried out at 3,618 rpm for 4.5 h resulting  
163 in accelerated migration of the droplets and integration of transmission profiles within a  
164 selected region of the holding cell allowed to get information about dispersion stability.  
165 The analyses were carried out using specific cuvettes with a radius position of 129.5 mm  
166 and based on results, it was possible to predict the shelf life of the NE samples from  
167 following equation (1) (Goto et al., 2017):

168 Shelf life (seconds) = Measurement time x Relative Centrifugal Force (1)

169



170 **2.6 Incorporation efficiency**

171 1,8-cineole was determined using a UV spectrophotometric method described by Yin et  
172 al. (2021), with slight modifications. Calibration curves were constructed for 1,8-cineole  
173 in n-hexane at different concentrations (w/v) and their absorbances detected at 250 nm  
174 using an Even UV/VIS spectrophotometer (Ionlab, Paraná, Brazil). Total monoterpene  
175 amount in the F1 formulation was assessed with the addition of an aliquot of the  
176 formulation in n-hexane (1:3, v/v). This mixture was put in an ultrasonic bath at 30 °C  
177 for 30 min, and then centrifuged at 4,000 rpm for 20 min. Next, the amount of 1,8-cineole  
178 was determined in the upper phase using UV spectrophotometric method as mentioned  
179 above. Free 1,8-cineole amount was determined by measuring the non-incorporated  
180 monoterpene present in a clear ultrafiltrate obtained through separation of aqueous phase  
181 using an ultrafiltration/ultracentrifugation procedure (Microcon Ultracel YM-100,  
182 Millipore, Ireland) at 10,583 x g for 60 min at 4 °C (*novatecnica*, Microcentrifuge NT  
183 805, Piracicaba-SP, Brazil). The same procedure used to determine 1,8-cineole in NE  
184 formulation was applied to quantify free monoterpene fraction in the dispersion medium  
185 (aqueous phase). All analyses were performed in triplicate and the incorporation  
186 efficiency (IE %) of 1,8-cineole in F1 formulation was calculated from following equation  
187 (2) (Rodrigues et al., 2018):

188 
$$IE (\%) = (TCin - FCin/ThCin) \times 100 \quad (2)$$

189 Where, TCin is the total 1,8-cineole amount; FCin, free 1,8-cineole amount; ThCin,  
190 theoretical 1,8-cineole amount.

191

192 **2.7 Electron microscopy analysis**

193 NE formulation was analyzed using transmission electron microscopy (TEM) technique.

194 Aliquots (~ 100 µL) of cineole nanoemulsion (F1 formulation) were put onto copper TEM

195 grids and negatively stained with 1 % (w/v) sodium phosphotungstate solution; the excess  
196 sample was thoroughly removed by filter paper. After that, the samples were dried at  
197 room condition before analyses. Transmission electron micrographs of the samples were  
198 taken using a Microscope HT7700 Hitachi (Fukuoka, Japan) operated at accelerating  
199 voltage of 80 KV and emission 12  $\mu$ A.

200

## 201 **2.8 Antibacterial activity assay**

202 The minimal bactericidal concentration of free 1,8-cineole and cineole nanoemulsion (F1  
203 formulation) were determined by microdilution techniques in Mueller-Hinton broth  
204 (MHB) (CLSI, 2018). Briefly, serial twofold dilutions of free 1,8-cineole and F1  
205 formulation were carried out in MHB using 96-well microplates. Bacterial suspension  
206 ( $10^8$  CFU mL<sup>-1</sup>) cultured in TSB at 37 °C for 24 h were inoculated onto wells (200  $\mu$ L  
207 final volume). Microplates were incubated at 35 °C for 24 h. Bacterial growth control  
208 (MHB+ strain + DMSO 10 %), sterility control (MHB), and positive controls with  
209 gentamicin and chlorhexidine digluconate were performed simultaneously on assays. An  
210 aliquot of 10  $\mu$ L of each well was inoculated on Mueller-Hinton Agar (MHA) at 37 °C  
211 for 24 h for colonies growth. Minimal bactericidal concentration was defined as the  
212 lowest 1,8-cineole or F1 concentration that did not allow visible growth on MHA agar.  
213 Experiments were performed three times.

214

## 215 **2.9 Statistical analysis**

216 All experiments were performed three times and data are expressed as average value  
217 ( $\pm$ SEM). Statistical analysis was performed by One-way ANOVA followed by Tukey's  
218 pairwise comparisons using the Prism GraphPad Software. The statistical significance  
219 was set at  $p < 0.05$ .

220

### 221 **3. Results and discussion**

222 In this study, cineole nanoemulsions were prepared using the spontaneous emulsification  
223 method as an isothermal approach applied to volatile and hydrophobic organic  
224 compounds. In order to prepare colloidal dispersions containing 1,8-cineole, as aqueous  
225 phase was used PBS (pH 7.4) while organic phase consisted of a mixture of cineole and  
226 MTC (1:1 w/w), and different types of surfactants.

227 For emulsions obtained herein, there was a variation of visual appearance among the fluid  
228 nanoemulsified dispersions from slightly turbid with bluish reflection to milky opaque  
229 dispersion reflecting directly the dispersed phase formed in each colloidal formulation.  
230 As shown in Table 2, the main properties of nanoemulsified systems were dependent on  
231 both surfactant types and organic phase composition. The types of nonionic surfactants  
232 used in the formulations demonstrated a considerable influence over droplet average  
233 diameter and size distribution of the colloidal dispersions. Hence, the smallest droplets  
234 (<115 nm) and with the narrowest size distribution (PdI <0.2 and monomodal) were  
235 observed for F1 formulation ( $p < 0.05$ ), which was prepared containing SML-20 and SMO  
236 as pair of nonionic surfactants. In contrast, the largest droplets with broader size  
237 distribution were noticed from dispersion prepared with SMO-20 and SMO surfactants  
238 (F3 formulation). Likewise, for those formulations containing only SML-20 or SMO-20  
239 as a surfactant, i.e., F2 and F4 formulations, respectively, the droplet average sizes were  
240 higher than 150 nm and the droplet size distribution was bimodal with PdI values above  
241 0.2. Moreover, when the organic phase composition was altered to contain only MCT  
242 carrier oil, the droplet average diameter and size distribution were significantly larger  
243 than other NE formulations, as can be particularly observed comparing F1 and F5  
244 formulations ( $p < 0.05$ ). Similarly, the organic phase composition also influenced both

245 droplet size e PDI of NE formulations, e.g., F5 formulation, whose organic phase  
246 consisted entirely of MCT oil presented relatively large droplets ( $p < 0.05$ ). This result is  
247 in accord with earlier research developed by Saberi et al. (2013) in which the increasing  
248 MCT oil concentrations resulted in larger droplets from mini-emulsions. Such influence  
249 might be explained by the significant impact of the MCT carrier oil on surfactant  
250 spontaneous curvature and solubility affecting directly formation of small droplets  
251 (Komaiko and McClements, 2016; Saberi et al., 2014).

252 The mechanism assigned to the formation of small-sized droplets using spontaneous  
253 emulsification is directly connected to the fast displacement of surfactant(s) from organic  
254 phase into aqueous phase. Afterward, both phases come into contact; this mixture results  
255 in the rapid diffusion of surfactant(s) from organic to aqueous phase producing turbulence  
256 at oil/water interface and consequent formation of fine droplets (Anton and Vandamme,  
257 2009; Komaiko and McClements, 2016; Saberi et al., 2013). It is commonly recognized  
258 that some factors can affect the formation and stability of nanodroplets designed from  
259 spontaneous emulsification approach. Among such factors are organic phase  
260 composition, type and concentration of surfactant(s) and/or cosurfactant(s), and  
261 conditions related to emulsifying process (Helgeson, 2016; Komaiko and McClements,  
262 2016).

263 Zeta ( $\zeta$ ) potential reflects surface charge of droplets and it has been related to stability of  
264 colloidal formulations. High surface charge density may imply NE's long-term physical  
265 stability due to relevant repulsive electrostatic forces among the droplets preventing rapid  
266 aggregation and flotation of dispersed phase (McClements and Gumus, 2016; Singh et  
267 al., 2017). All NE formulations presented significant negative values of zeta potential ( $<$   
268  $-20$  mV, Table 2) which may be related to free ionized groups from components of  
269 organic phase. It is worth mentioning that additionally electrostatic stabilization of the

270 dispersed phase, the nanoemulsion physical stability is also a result of van der Waals,  
 271 hydrophobic, and steric interactions among droplets (Gupta et al., 2016; Helgeson, 2016;  
 272 McClements and Gumus, 2016; Singh et al., 2017).

273

274 **Table 2.**

275 Droplet size (hydrodynamic diameter), polydispersity index (PdI), and  $\zeta$  potential of NE  
 276 formulations (average  $\pm$ SEM, n = 3).

NE	Size (nm)	PdI	$\zeta$ potential (mV)
F1	111.5 ( $\pm$ 0.79) <sup>a</sup>	0.182 ( $\pm$ 0.007) <sup>b</sup>	- 36.75 ( $\pm$ 0.33)
F2	203.4 ( $\pm$ 6.68)	0.303 ( $\pm$ 0.017)	- 29.43 ( $\pm$ 0.54)
F3	212.8 ( $\pm$ 8.67)	0.223 ( $\pm$ 0.004)	- 38.60 ( $\pm$ 0.17)
F4	159.3 ( $\pm$ 1.28)	0.240 ( $\pm$ 0.015)	- 19.75 ( $\pm$ 0.36) <sup>c</sup>
F5	264.5 ( $\pm$ 1.17)	0.321 ( $\pm$ 0.014)	- 42.02 ( $\pm$ 0.42)

277 <sup>a</sup>p <0.05 compared to F2, F3, F4, and F5 formulations concerning size.

278 <sup>b</sup>p <0.05 compared to F2, F3, F4, and F5 formulations concerning PdI.

279 <sup>c</sup>p <0.05 compared to F1, F2, F3, and F5 formulations concerning  $\zeta$  potential.

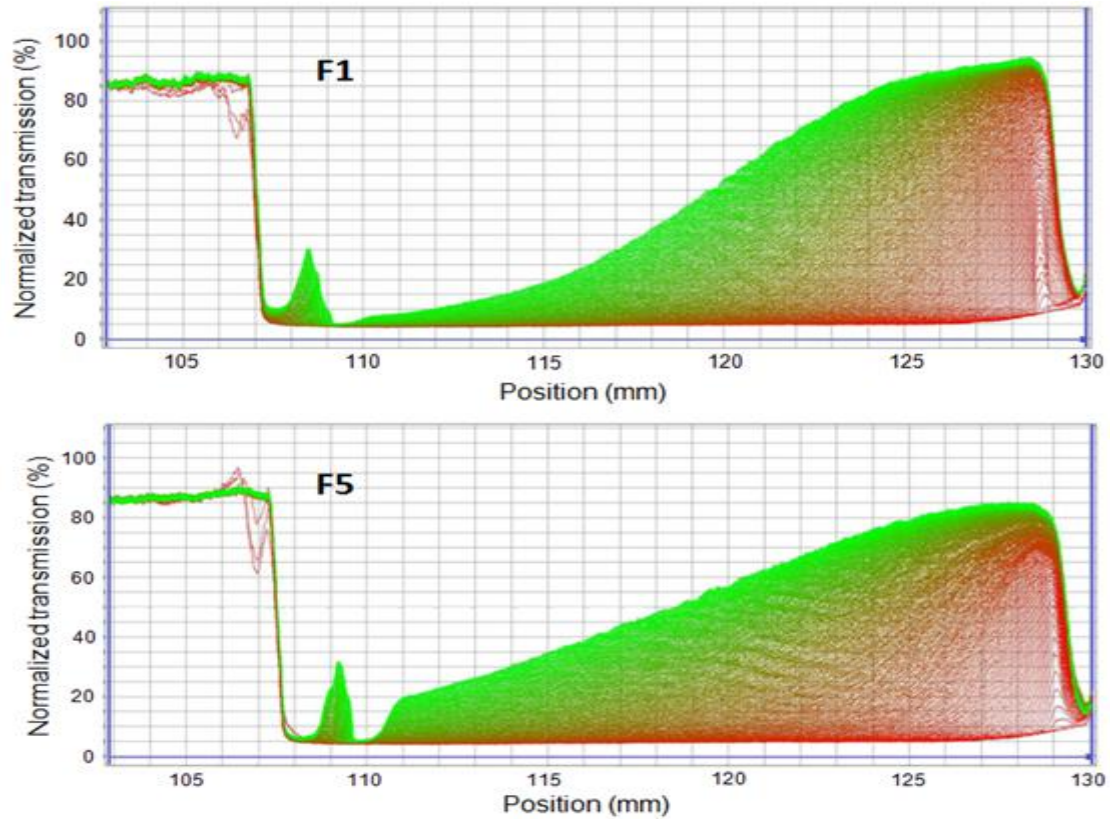
280

281 Once nanosized oil droplets are formed, it is essential that their stability can be assessed  
 282 throughout the formulation lifetime. In this study, centrifugal separation analysis with  
 283 photometric detection enabled physical stability assessment of the NE formulations. The  
 284 analytical photocentrifugation method is based on the measurement of droplet migration  
 285 by applying a centrifugal force and photometric detection using near-infrared (NIR) light.  
 286 This light passes throughout the sample and then the transmitted light intensity is detected  
 287 by sensors forming profiles of light transmission as a function of time and position. The  
 288 shape and gradual change process of the light transmission profiles through tested

289 samples allow the analysis of separation kinetics of dispersed phase from continuous  
290 phase in colloidal dispersions enabling a comparison of long-term stability among NE  
291 formulations (Fernandes et al., 2017). For each NE sample, 255 transmission profiles  
292 were recorded in intervals of 65 s, and flotation velocities of oil droplets from undiluted  
293 samples of the NE were determined at 25 °C (Table 3).

294 Fig. 1 shows NIR transmission profiles obtained for F1 and F5 formulations while Fig. 2  
295 shows transmission profiles for F2, F3, and F4 formulations. F1 and F5 formulations  
296 presented similar transmission profiles (Fig. 1) with the lowest clarification process, i.e.,  
297 minimal phase separation due to the dispersed phase's flotation. These results were  
298 confirmed by flotation velocity obtained for F1 and F5; for these two formulations, the  
299 flotation velocities were the lowest among all samples as well as a narrower velocity  
300 distribution was also observed with only slight speed variation of droplet migration  
301 between D10 % and D90 % as shown in Table 3. Thus, we considered that F1 and F5  
302 were the most stable NE formulations. Moreover, this result confirms that the inclusion  
303 of 1,8-cineole in the nanoemulsified system could not alter long-term formulation  
304 stability. Meanwhile, F2, F3, and F4 formulations showed a strong clarification behavior  
305 (Fig. 2) resulting in a significant creaming separation process and a wider distribution of  
306 droplet flotation velocity when compared with F1 and F5. These findings indicate that a  
307 substantial droplet aggregation might take place during the storage period for F2, F3, and  
308 F4 formulations resulting in more fast flotation due to larger oil droplets and/or their  
309 aggregates in the colloidal dispersion. In spite of different droplet sizes between F1 and  
310 F5 formulations, the packing of surfactant molecules adsorbed could result in a shielding  
311 coating in the oil droplets preventing aggregation of the dispersed phase and consequent  
312 improvement physical stability of these formulations. Therefore, such an outcome may  
313 be attributed to the surfactant monolayer's optimum curvature formed at oil/water

314 interface of droplets in the F1 and F5 nanoemulsions (Komaiko and McClements, 2015;  
315 Saberi et al., 2013).  
316



317  
318 **Fig. 1.** Normalized NIR transmission profiles obtained with cineole-containing NE (F1)  
319 and unloaded NE (F5) formulations by accelerated stability analysis during analytical  
320 centrifugation. The first and the last registered profiles are shown in red and in green,  
321 respectively.

322  
323 The main destabilization mechanisms of nanoemulsified systems are related to close  
324 contact among droplets which may result in flocculation, Ostwald ripening, coalescence,  
325 and lastly phase separation. Thus, any aggregation process of the dispersed phase may  
326 trigger such instabilities and, consequently, the coarsening of the colloidal formulations  
327 (Gupta et al., 2016; Helgeson, 2016; Singh et al., 2017). In this study, it seems reasonable  
328 to assume that the adsorbed layer of SML-20 and SMO surfactants on the oil droplets

329 increased the steric stabilization of F1 and F5 formulations avoiding oil droplet  
 330 aggregation. It is also important to note that the addition of MCT carrier oil to formulation  
 331 could prevent droplets' growth caused by instability primary mechanism of  
 332 nanoemulsions known as Ostwald ripening, which is characterized as the mass transfer  
 333 from smaller to the larger oil droplets (Gupta et al., 2016; Helgeson, 2016). Furthermore,  
 334 considering that there was no signal of instability for F1 and F5 formulations as well as  
 335 this analysis at 3,618 rpm for 4.5 h corresponded to a predicted shelf life of 12 months,  
 336 we could consider F1 and F5 NE as stable formulations for a such period of time.  
 337 Accordingly, in view of all results described above, concerning NE formulation having  
 338 the smallest oil droplets and narrow size distribution along with improved physical  
 339 stability, we selected F1 formulation for further studies. Moreover, as expected the  
 340 assessment of the incorporation efficiency of 1,8-cineole in NE formulation (F1)  
 341 confirmed high entrapment (86.3 %  $\pm$ 0.87) into dispersed phase mainly due to limited  
 342 water solubility of this monoterpene.

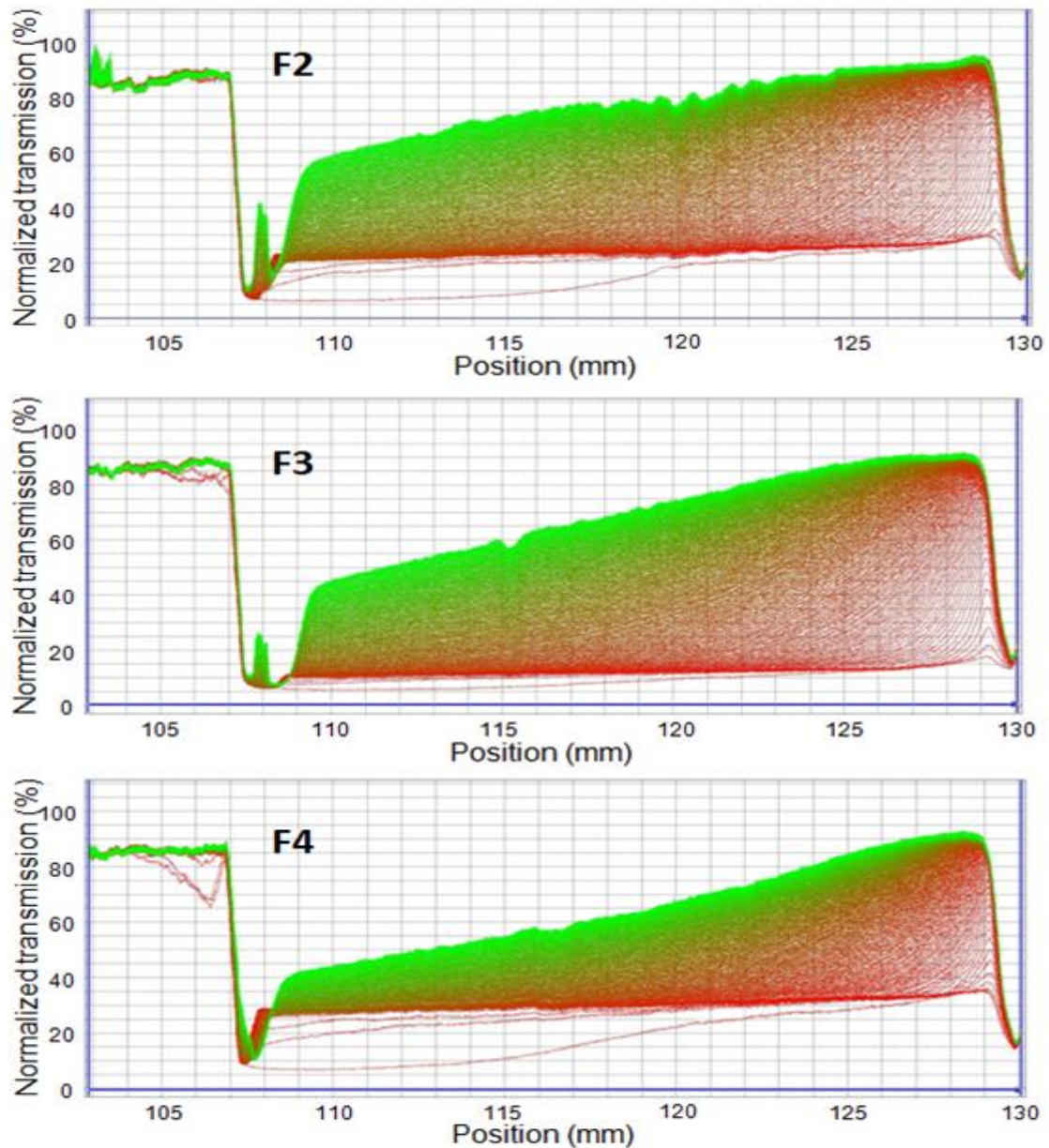
343

344 **Table 3.**

345 Distribution (D) of velocity ( $\mu\text{m s}^{-1}$ ) from droplet flotation of NE formulations measured  
 346 at 25 °C using analytical photoentrifugation.

NE	D10 % ( $\mu\text{m s}^{-1}$ )	D50 % ( $\mu\text{m s}^{-1}$ )	D90 % ( $\mu\text{m s}^{-1}$ )	Mean velocity ( $\pm$ S.D.)
F1	1.280	1.619	2.290	1.634 ( $\pm$ 0.377)
F2	1.605	89.190	260.551	4.462 ( $\pm$ 104.703)
F3	1.510	3.106	199.099	3.140 ( $\pm$ 82.126)
F4	1.820	168.326	268.696	8.540 ( $\pm$ 94.195)
F5	1.291	1.641	2.279	1.645 ( $\pm$ 0.364)





348

349 **Fig. 2.** Normalized NIR transmission profiles obtained with F2, F3, and F4 NE

350 formulations by accelerated stability analysis during analytical centrifugation. The first

351 and the last registered profiles are shown in red and in green, respectively.

352

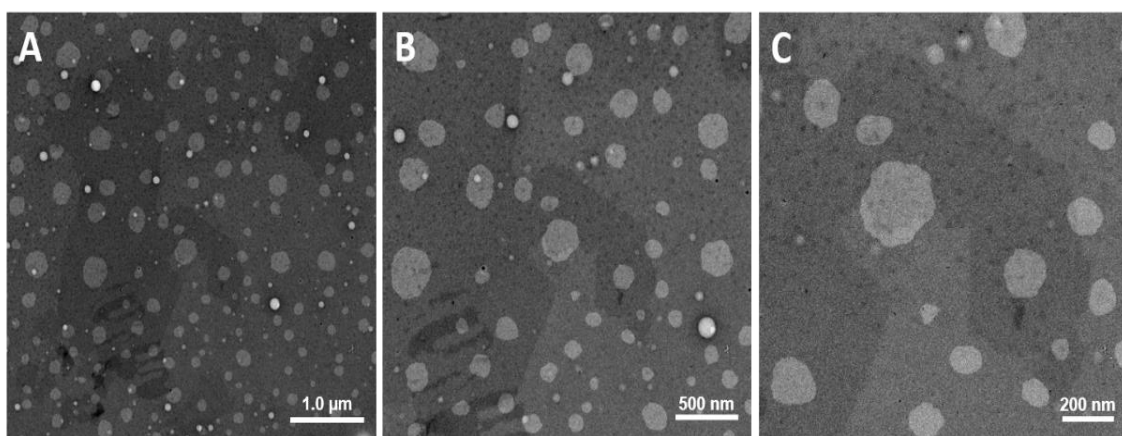
353 Transmission electron microscopy (TEM) has been considered a successful technique in

354 order to analyze size and shape droplets, size distribution, and agglomeration tendency of

355 the dispersed phase in nanoemulsified systems (Falsafi et al., 2020). TEM analyses were

356 carried out for F1 formulation and images are depicted in Fig. 3. As can be noted from  
357 photomicrographs, throughout F1 formulation non-aggregated oil droplets with irregular  
358 spherical shapes were observed. Furthermore, oil droplet sizes viewed by TEM technique  
359 were similar to those measured by dynamic light scattering analysis. All these findings  
360 confirm the presence of nanosized oil droplets with uniform size distribution in the F1  
361 formulation and its enhanced physical stability as previously related.

362



363

364 **Fig. 3.** TEM photomicrographs of cineole-containing NE (F1 formulation).  
365 Magnifications (A) x7.0K, (B) x12.0K, and (C) x20.0K.

366

367 The potential activity of free 1,8-cineole and cineole NE (F1 formulation) against  
368 *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pyogenes* strains was  
369 assessed using the broth microdilution method. As depicted in Table 4, the bactericidal  
370 concentrations of nanoemulsion containing 1,8-cineole were 2-fold lower than those  
371 found for free 1,8-cineole. More specifically, *S. aureus* and *E. faecalis* were more  
372 sensitive to the cineole NE when compared to free monoterpene, i.e., nanoemulsified  
373 cineole was the most effective in killing these two bacteria. Meanwhile, cineole  
374 nanoemulsion was only slightly more active against *S. pyogenes* in comparison with free  
375 cineole (Table 4). Both gentamicin ( $1.66 \text{ mg mL}^{-1}$ ) and chlorhexidine digluconate ( $1.2$

376 mg mL<sup>-1</sup>) were found to be more active than pure cineole or cineole-NE against Gram-  
377 positive bacteria.

378 Unlike these findings herein about free and nanoemulsified cineole against Gram-positive  
379 strains, Harkat-Madouri et al. (2015) found that the essential oil of *Eucalyptus globulus*,  
380 in which the major compound was 1,8-cineole, was more active against Gram-negative  
381 strains. Despite this, it is claimed that EO are relatively less active against Gram-negative  
382 than Gram-positive microorganisms due to the uppermost layer of lipopolysaccharide  
383 present on Gram-negative bacteria, thus limiting the diffusional process of lipophilic  
384 molecules (Harkat-Madouri et al., 2015; Prakash et al., 2018a). Furthermore, the  
385 enhanced antibacterial activity from nanoemulsified cineole could mainly be related to  
386 the increased surface area from ultra-small sized droplets in the nanoemulsion  
387 formulation allowing passive transport through the outer cell membrane (Prakash et al.,  
388 2018a).

389

#### 390 **Table 4.**

391 Minimal bactericidal concentrations of free cineole and cineole-containing nanoemulsion  
392 (F1 NE formulation) against Gram-positive bacteria.

Bacterial strains	Free cineole (mg mL <sup>-1</sup> )	Cineole-NE (mg mL <sup>-1</sup> )
<i>Staphylococcus aureus</i>	28.6	14.0
<i>Enterococcus faecalis</i>	28.6	14.0
<i>Streptococcus pyogenes</i>	28.6	24.0

393

#### 394 **4. Conclusions**

395 In summary, we have designed nanoemulsions containing 1,8-cineole using low energy  
396 emulsification, which is commonly known as spontaneous emulsification method. The

397 most stable nanoemulsion formulation (F1) consisted mainly of very small sized droplets  
398 with monomodal size distribution, and negative zeta potential. Stability study  
399 demonstrated that F1 formulation could be considered stable with lower droplet migration  
400 velocity. In addition, high incorporation efficiency of 1,8-cineole into F1 formulation was  
401 demonstrated and also the photomicrographs from TEM analysis confirmed that F1  
402 formulation was formed by nanosized droplets and no aggregation tendency.  
403 Antibacterial activity of cineole nanoemulsion against *S. aureus* and *E. faecalis* was  
404 enhanced (2-fold) compared to free 1,8-cineole. Hence nanoemulsified dispersions could  
405 be considered like a relevant lipid nanocarrier applied for inclusion of hydrophobic  
406 terpenic compounds. Moreover, these results encourage further studies about antibacterial  
407 activity of nanoemulsified cineole on preservation of food, cosmetic, and pharmaceutical  
408 products.

409

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412

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