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# Thymoquinone loaded lipid nanocapsule dispersion: two methods of preparation, characterization and *in vitro* evaluations for oral administration

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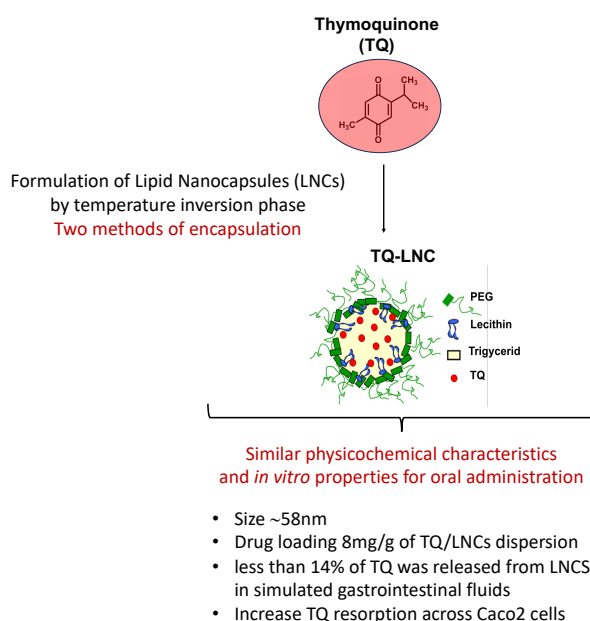
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## Abstract:

This work explores two methods to encapsulate Thymoquinone (TQ) into lipid nanocapsules (LNCs) for oral administration. TQ was added during the phase inversion temperature method (TQ-LNCs-1) or to unload LNCs dispersion (TQ-LNCs-2). LNCs were evaluated for mean diameter, polydispersity index (PDI),  $\zeta$ -potential, drug loading (DL), drop tensiometer, storage stability, *in vitro* stability in simulated gastrointestinal fluids (SGIF), and intestinal permeability across Caco-2 cells.

TQ-LNCs-1 and TQ-LNCs-2 produced NPs ( $58.3 \pm 3.7$  nm and  $61.5 \pm 3.5$  nm, respectively), with a DL of  $8.7 \pm 0.2$  and  $7.7 \pm 0.6$  mg/mL of suspension, respectively. For both, less than 14% of TQ was released in SGIF, and a minor increase in TQ intestinal permeability with LNCs compared to free TQ was observed. TQ-LNCs represented a promising formulation for oral delivery of TQ. Encapsulation of TQ by adding it at LNCs dispersion can be extended for further drugs.

## Graphical Abstract



30 **Keywords:** Lipid nanocapsules; Encapsulation, Thymoquinone; gastrointestinal stability; Caco-2  
31 permeability; oral drug delivery; nanomedicine.

## 32 1. Introduction

33 Lipid nanocapsules (LNCs) have garnered significant attention as effective nanocarriers to  
34 encapsulate different drugs (e.g. paclitaxel, 7-Ethyl-10-hydroxy-camptothecin (Sn38), ferrocifen,  
35 miltefosine, benznidazole) (Huynh *et al.*, 2009; Arrua *et al.* 2023) for notably cancerology and infectiology  
36 applications. LNCs were prepared by low-energy emulsification based on a phase inversion  
37 temperature process suitable for scale-up (Thomas and Lagarce, 2013) with Generally Recognized As  
38 Safe (GRAS) excipients (Heurtault *et al.* 2002). Two methods are currently available to encapsulate drugs  
39 or biological compounds. To encapsulate drugs into LNCs, the drug is first solubilized in an oily phase  
40 (with or without the assistance of organic solvent) and LNCs are formulated. To encapsulate biological  
41 compounds (DNA, miRNA, or siRNA) (David *et al.* 2012; Resnier *et al.* 2014 ), lipoplexes were  
42 formulated and added at the phase inversion zone temperature during the final temperature cycle.  
43 Thus, even if the LNCs process is organic solvent-free, very often to encapsulate active molecules,  
44 solvent use is added, making it impossible to classify the preparation method as organic solvent-free.  
45 Recently, Thymoquinone (TQ) (2-isopropyl-5-methyl-benzoquinone), (Figure. 1), a natural active  
46 product of *Nigella sativa*, with great promising pharmacotherapeutic effects including antioxidant, anti-  
47 inflammatory, anti-neoplastic, immunomodulatory and tissue-protective properties in a variety of  
48 medical disorders (Randhawa and Alghamdi, 2011; Darakhshan *et al.* 2015; Goyal *et al.* 2017). TQ has a  
49 logP of 2.2, and low water solubility which is a key problem for designing pharmaceutical forms and its  
50 administration (Ahmad *et al.*, 2018). To overcome this limit, TQ was successfully encapsulated into these  
51 nanoparticles. Among them, Lipid nanoparticles have gained interest because they are biodegradable,  
52 with low toxicity, and LNCs are prepared with a process devoid of solvent. Recently, intratumorally  
53 administration of TQ-LNCs in mice with subcutaneous colorectal cancer demonstrated a significant  
54 reduction in tumor size compared to the control group. Importantly, TQ-LNCs proved more effective  
55 in inducing tumor cell death than free TQ (Selmi *et al.* 2023). These findings highlight the potential of  
56 TQ-LNCs as a promising strategy for improving therapeutic outcomes in colorectal cancer treatment.  
57 Nevertheless, the oral route is the most preferred route over systemic and local drug delivery due to  
58 non-invasiveness, patient compliance, and convenience of drug administration. TQ's oral  
59 administration had been hampered by a low aqueous solubility (Salmani *et al.* 2014), poor intestinal  
60 permeability, and rapid elimination from blood circulation (Elmowafy *et al.* 2016; Kalam *et al.* 2017). To  
61 overcome these drawbacks, the encapsulation of drugs within organic nanocarriers was mainly  
62 demonstrated, but for TQ, a limited number of drug delivery systems to administer TQ orally were  
63 developed: liposomes, micelle nanoparticles, and chitosan nanoparticles (Rathore *et al.* 2019; Rahat *et al.*  
64 2021; Lodovichi *et al.* 2022). Considering LNCs, they are interesting candidates for oral drug delivery  
65 because they exhibit stability in terms of physicochemical characteristics within the gastrointestinal (GI)  
66 environments, and demonstrate an enhanced ability to facilitate the passage of substances like Sn38 and  
67 paclitaxel through a Caco-2 monolayer (Roger *et al.* 2009a; Roger *et al.* 2009b Roger *et al.* 2011) with a  
68 better diffusion in mucus (Groo *et al.* 2013), and increase the oral bioavailability of encapsulated drugs  
69 like paclitaxel and fondaparinux (Peltier *et al.* 2006; Ramadan *et al.*, 2011)). Accordingly, this present  
70 work aimed to develop a TQ-loaded LNC formulation for improved oral bioavailability.

71 Moreover, in preliminary experiments to monitor TQ within LNC, TQ powder was added to  
72 unloaded LNCs, and surprisingly, TQ appeared to be encapsulated in the LNCs (preliminary results).  
73 For this present work, this potential new method of encapsulation was also studied and compared with  
74 the available method described (drug solubilization in the oily phase before LNC preparation). LNCs

75 were prepared using two encapsulation methods, and their physicochemical and biopharmaceutical  
76 properties for oral administration were then assessed.

## 77 2. Materials and Methods

### 78 2.1. Materials

79 Thymoquinone ( $\geq 98\%$ ) was purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). All  
80 excipients that composed LNC are pharmaceutical grade reagents. Labrafac® WL1349, Labrafil®  
81 M1944CS, Peceol®, were supplied free of charge by Gattefossé (Saint-Priest, France), Ethyl Oleate®,  
82 Miglyol® 812, and Captex® 8000 were provided by Fluka (Buchs, Switzerland), Cremer Oleo Division  
83 (Hamburg, Germany) and Abitec Corp (Saint-Quentin Fallavier, France), respectively. Lipoid® S100  
84 (soybean Phosphatidylcholine, content  $\geq 94.0\%$ ) and Kolliphor® HS15 (consists of polyglycol mono- and  
85 di-esters of 12-hydroxystearic acid and of about 30% of free polyethylene glycol) were supplied from  
86 Lipoid GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. Sodium  
87 chloride was procured from Prolabo VWR International (Fontenay-sous-Bois, France). Deionized water  
88 was sourced from a Milli-Q System (Millipore, Paris, France). Acetic acid, acetonitrile, methanol and  
89 Water, HPLC grade were provided from Fisher Scientific (Loughborough, United Kingdom). Maleic  
90 acid, sodium monobasic phosphate anhydrous, pancreatin (8×USP specification) and  
91 Dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich. Pepsin (European Pharmacopeia),  
92 sodium taurocholate, and glyceryl monooleate were provided from Fluka (Buchs, Switzerland). Sodium  
93 oleate (purity  $> 82\%$ ) was obtained from Riedel-de Haen (Selze, Germany). Calcium chloride was  
94 provided from Prolabo.

### 95 2.2. TQ analysis by Ultra-violet Ultra-Performance Liquid Chromatography (UPLC-UV).

96 The analytical method previously developed (Selmi *et al.* 2023) was validated according to ICH Q2  
97 (Guideline, 2005). Briefly, TQ was diluted in methanol to obtain a 5-point calibration curve between 5  
98 and 50  $\mu\text{g/mL}$  (Selmi *et al.* 2023). Calibration curves were performed three times on 3 different days. The  
99 correlation coefficient ( $r^2$ ) of the mean calibration curve had to be  $> 0.999$ .

100 The accuracy of the method was assessed by making nine determinations of three concentrations (5, 25,  
101 and 50  $\mu\text{g/mL}$ ) three times a day, for 3 days. Accuracy was determined as the difference between the  
102 mean measured value and the accepted true value. Accuracy, for each concentration, had to be  $< 5\%$   
103 from the accepted true value to be accepted.

104 Repeatability was assessed by measuring a 5, 25 and 50  $\mu\text{g/mL}$  solution six times a day. Intermediate  
105 precision was evaluated by measuring these 3 solutions three times a day for 3 consecutive days.  
106 Repeatability and intermediate precision were measured using the standard deviation, which had to be  
107  $< 5\%$  to be accepted.

108 The limit of detection (LOD) and limit of quantification (LOQ) were based on the standard deviation of  
109 the response and the slope. LOD and LOQ calculations are shown in formulas 1 and 2.

110

111 **Formula 1 :** 
$$\text{LOQ} = \frac{3.3 \text{ Standard deviation of the response of 10 blanks}}{\text{slope of the calibration curve}}$$

112

113

114 **Formula 2:** 
$$\text{LOD} = \frac{10 \text{ Standard deviation of the response of 10 blanks}}{\text{slope of the calibration curve}}$$

115

### 116 2.3. LNCs Formulation

117 Lipid nanocapsule (LNC) formulations were prepared according to a phase inversion temperature  
118 process (Heurtault *et al.* 2002). The same composition as LNCs described by Selmi *et al.* (2023) was used.

119 To prepare unloaded LNCs, Captex® 8000 (13.12% w/w) and Lipoid® S100 (0.73% w/w) were heated at  
120 75 °C. Kolliphor® HS15, NaCl, and water (10.94% w/w, 0.80% w/w, and 19.73% w/w, respectively) were

121 added and mixed under magnetic stirring. Three cycles of progressive heating and cooling between 65  
122 and 90 °C were then carried out. During the last cooling cycle, tempering was induced at 78°C with 5  
123 mL 4°C water (54.68 %<sup>w/w</sup>). Finally, a gentle magnetic stirring was maintained for 5 min at room  
124 temperature.

125 To encapsulate TQ two methods were tested. The conventional method (TQ-LNC-1) was prepared  
126 as described by Selmi *et al.* (2023). Briefly, TQ was first solubilized in Captex® 8000 by magnetic stirring  
127 for 1 hour at a concentration of 633mg/g (corresponding to the maximum of TQ solubility into  
128 Captex®800: 523.1 mg/mL). Then, TQ dispersed in Captex® was used and LNC formulation was  
129 prepared as described above.

130 For the second method (TQ-LNC-2), 10 mg of TQ (as powder) was added to 1150 mg of unloaded  
131 LNCs dispersion, and magnetic stirring was maintained for 5 min. This quantity of TQ and LNCs  
132 dispersion allows to obtain the same theoretical Drug loading (~ 8,7mg of TQ / g of LNCs dispersion)  
133 as the conventional method above.

134 Subsequently, each formulation was filtrated using a Millex-LG 0.2 µm filter (Merck KGaA,  
135 Darmstadt, Germany) to eliminate non-soluble residual components, and then stored at 4 °C.

#### 136 2.4. Characterization of LNCs Formulation

##### 137 2.4.1. Particle Size, Polydispersity Index (PDI) and Zeta Potential (ζ-potential)

138 Particle size (measured in Intensity), PDI, and ζ-potential were assessed using dynamic light  
139 scattering (DLS) and electrophoretic mobility on a Zetasizer® Nano series DTS 1060 (Malvern  
140 Instruments S.A., Worcestershire, UK). The formulations were diluted 1:400 (v/v) in deionized water and  
141 three measurements were conducted.

##### 142 2.4.2. Drug Loading and Entrapment Efficiency

143 To separate free and encapsulated TQ in the LNCs formulation, two purification methods were  
144 evaluated: filtration using a Millex-LG filter unit (0.22 µm) and ultracentrifugation at 55.000 g for 15 h  
145 at 20 °C in an Optima TLX ultracentrifuge (Beckman, Villepinte, France), equipped with a TLS 55 rotor.  
146 Following filtration, TQ-loaded LNCs were present in the filtrate. After 15h-ultracentrifugation, tubes  
147 were frozen with liquid nitrogen, and three fractions were collected; TQ-loaded LNCs were found in  
148 the top of the tube (Roger *et al.* 2023).

149 Samples were then diluted by 200 in methanol (solvent used for the calibration curve) and analyzed by  
150 UPLC-UV method. TQ was quantified by comparison between observed peak area ratios of TQ of the  
151 samples and a calibration curve performed using the same conditions. The experimental and theoretical  
152 drug loading calculation formulas are shown in formulas 3 and 4, respectively. The entrapment  
153 efficiency calculation formula is shown in Formula 5.

154 Formula 3:

155  
156 Experimental Drug loading (mg of TQ/g of LNCs dispersion) = 
$$\frac{\text{Quantified TQ (mg)}}{\text{Total of LNCs dispersion (g)}}$$

157  
158 Formula 4:

159 Theoretical Drug loading (mg of TQ/g of LNCs dispersion) = 
$$\frac{\text{Weight of TQ (mg)}}{\text{Weight of LNCs dispersion (g)}}$$

160  
161 Formula 5:

162 Entrapment efficiency (%) = 
$$\frac{\text{Experimental drug loading (mg/g)}}{\text{Theoretical drug loading (mg/g)}} \times 100$$

163  
164

##### 165 2.4.3. Storage Stability Studies

166 The stability of TQ-loaded LNCs and post-inserted LNCs was assessed during storage at 2–8 °C.  
167 Each dispersion was filtered using a 0.2 µm filter and then the mean size of nanocapsules and drug  
168 loading were determined by DLS and UPLC-UV methods, respectively.  
169

#### 170 2.4.4. Drug localization within LNCs

##### 171 2.4.4.1 Drug Transfer Experiment

172 According to Bastiat *et al.* (2013), TQ transfer from LNCs to a lipophilic compartment (LC) was  
173 conducted to assess drug release into a hydrophobic medium. Briefly, LNC dispersion was mixed (1:1,  
174 v/v) with an oily phase (Captex® 8000) acting as LC. The mixture was vortexed for 15 s at room  
175 temperature and centrifuged for 30 min at 103822g (4000rpm, Micro 200 R, Villepinte, France). Once  
176 separation was obtained, mean size, and PDI were measured by DLS for the LNCs fractions. TQ  
177 concentrations were quantified by UPLC-UV in both LNCs and LC fractions. The ratio of TQ in each  
178 collected fraction was calculated from the initial drug loading of LNCs. The experiment was conducted  
179 in quadruple.

##### 180 2.4.4.2 Drop Tensiometer Study

181 Drop tensiometer evaluation was carried out with Tracker equipment (ITConcept, Longessaigne,  
182 France). Briefly, a 6 µL drop of LNCs dispersion or of an aqueous solution of 0,87% w/v of TQ was formed  
183 in the air using a stainless microsyringe (Prolabo, Paris, France). Interfacial tension measurements of  
184 the formed drop were digitized by a CDD camera that was coupled to a video image profile digitizer  
185 board (Imaging Technology, model PCVision Plus) and processed by a WINDROP software package.  
186 Experiments were performed at room temperature (around 21 °C) and repeated four times for unloaded  
187 LNCs, TQ-loaded LNCs, and post-inserted LNCs. Experiments were also performed on the Captex®-  
188 water interface, in the presence or not of TQ in Captex®, (at a 6.67% w/w concentration) with the water  
189 phase placed in an optical glass cuvette (Hellma, France) and the Captex® drop rising in the water. All  
190 the experiments were performed for 4 h, considering the obtention of an interfacial tension value at a  
191 pseudo-equilibrium. The final interfacial tension values after 4 h, noted  $\gamma_F$ , were used for the  
192 interpretation of the results.  
193

#### 194 2.5. In vitro evaluations for oral administration

##### 195 2.5.1 Stability in Simulated Gastro-Intestinal Fluids (SGIF)

196 The stability of TQ-LNCs was evaluated in Fasted State Simulated Gastric Fluid (FaSSGF), Fasted  
197 State Simulated Intestinal Fluid (FaSSIF-V2), and Fed State Simulated Intestinal Fluid (FeSSIF-V2)  
198 (Vertzoni *et al.* 2004; Jantratid *et al.* 2008), prepared according to Table 1. LNC dispersions were diluted  
199 at a final concentration of 10% v/v and incubated at 37 °C in the respective media. Sampling was  
200 conducted at specified time points, including 0, 0.5, 1, 2, and 3 h in FaSSGF and 0, 1, 2, 3, and 6 h in  
201 FaSSIF-V2 and FeSSIF-V2. To eliminate TQ released, samples were either filtered using a 0.2 µm Millex  
202 filter for FaSSGF or centrifuged for 5 minutes at 13.600 × g for FaSSIF-V2 and FeSSIF-V2. Subsequently,  
203 all samples were characterized in terms of mean size, PDI, and drug release. Drug release was calculated  
204 by difference with the theoretical drug payload, and profiles (percent release versus time) were plotted.

##### 205 2.5.2. Intestinal resorption across Caco-2 cells

###### 206 2.5.2.1. Cells Culture

207 The human colorectal adenocarcinoma cell line (caco-2) was acquired from the American Type  
208 Culture Collection (Manassas, USA) and used within passages 25 and 35. Cells were cultured in a high-  
209 glucose D-MEM medium supplemented with 20% v/v fetal bovine serum, 1% v/v non-essential amino

210 acids, 1% v/v sodium pyruvate, and 1% v/v Penicillin/Streptomycin (1 × 10<sup>4</sup> UI/mL penicillin, 10 mg/mL  
211 streptomycin,) and the cell cultures were maintained in a humidified atmosphere containing 5 % CO<sub>2</sub>  
212 at 37 °C.

213 Cells were seeded at 0.1 × 10<sup>6</sup> cells/well onto polycarbonate membrane filters (0.4 μm pore size, 1.12  
214 cm<sup>2</sup> growth area) inside Transwell® cell culture chambers (Corning Costar, Cambridge, MA). The apical  
215 and basolateral sides were supplemented with culture medium (DMEM) and were replaced daily  
216 during the first two weeks, followed by daily replacements in the third week. Cellular differentiation  
217 occurred after 21 days. Before conducting experiments, cell monolayers were rinsed twice with Hank's  
218 buffered salt solution (HBSS) for 15 min at 37 °C. The integrity of the monolayer was assessed by  
219 measuring the transepithelial electrical resistance (TEER) before and after each experiment by using a  
220 Millicell®-ER system (Millipore Corporation, Bedford, MA). Only cell monolayers with TEER values  
221 over 250 Ω cm<sup>2</sup> were included in the study.

#### 222 2.5.2.2. TQ resorption across Caco-2 Cells

223 The intestinal resorption of various TQ formulations, free TQ and both TQ-loaded LNCs, was  
224 investigated in the apical to basolateral direction using a differentiated Caco-2 monolayer model. TQ  
225 formulations were diluted at a final concentration of 1% v/v (corresponding to 80 μg/mL of TQ) in HBSS.  
226 The Experiments started with the addition of 0.5 mL of test solution at the apical side and 1.5 mL of  
227 HBSS at the basolateral side. After 2 h of incubation at 37 °C, samples were collected from both apical  
228 and basolateral sides and TQ content was determined by UPLC-UV. The apparent permeability  
229 coefficient (P<sub>app</sub>), expressed in centimeters per second, was calculated according to Grès *et al.* 1998  
230 (formula 6).

231

232 Formula 6:

$$233 \text{ Apparent Permeability (P}_{app}\text{)} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

234

235 Where dQ/dt represents the rate of drug appearance on the basolateral side (μg.s<sup>-1</sup>), C<sub>0</sub> is the initial  
236 concentration over the apical side (μg.mL<sup>-1</sup>) and A refers to the surface area of monolayer (cm<sup>2</sup> ).

237

#### 238 2.6. Statistical Analysis

239 Results are expressed as mean values ± SD. Multiple comparisons were made using two-way  
240 ANOVA analysis. The data were computed using GraphPad Prism® 8.4.2 (679) software. Differences  
241 were considered to be significant if the p-value was <0.01.

242

### 243 3. Results and discussion

#### 244 3.1. Validation of UV-UPLC- Method

245 The development of TQ-LNC formulations requires a validated analytical method to characterize  
246 drug loading and perform *in vitro* evaluations. The UPLC-UV method previously developed was  
247 validated according to the ICH Q2 R1 (Guideline, 2005). The chromatogram shows a symmetric and  
248 well-defined peak of TQ at 1.57 min (retention time of TQ). The linearity of the mean calibration curve  
249 of the method from the range 5 to 50 μg/mL on 3 different days was  $y = 28799x - 7759.1$ , where  $y$  is the  
250 area under the curve and  $x$  is the amount of TQ. The regression coefficient (r<sup>2</sup>) is systematically inferior  
251 to 0.999, confirming the linearity of the method. The LOD and LOQ were 0.10 and 0.14 μg/mL,  
252 respectively. The Accuracy of the method is ranged from 1.1% to 2.1%. The repeatability and  
253 intermediate precision were inferior to 1.1% and 0.4%, respectively. Accuracy, repeatability, and  
254 intermediate precision are inferior at 5% in line with the requirement of ICH-Q2 R1. This UPLC-UV  
255 method for TQ was sufficiently sensitive for drug loading as well as *in vitro* experiments.

256 3.2. TQ-LNCs formulation

257 Different nanocarriers were proposed in the literature to encapsulate TQ (Vignesh Kumar *et al.* 2017;  
258 Rani *et al.* 2018; Ramzy *et al.* 2020; Zafar *et al.* 2020), but all of them were prepared using organic solvent.  
259 Recently, TQ has been encapsulated in Lipid nanocapsules (LNC) (Selmi *et al.* 2023) prepared by a well-  
260 known low-energy emulsification process: the phase-inversion temperature method (Heurtault *et al.*  
261 2002) a free-organic solvent process. Interestingly, this composition of LNCs (notably Captex®8000  
262 chosen as the oily core of the formulation) was already used in LNC formulations to encapsulate active  
263 lipophilic pharmaceutical ingredients, such as SN38, Paclitaxel, and demonstrated interesting  
264 properties for oral administration (Roger *et al.* 2009a; Roger *et al.* 2009b; Roger *et al.* 2011; Kaeokhamloed  
265 *et al.*, 2021).

266 As a novelty, in comparison with the conventional method of encapsulation largely used within LNCs,  
267 another method of encapsulation was tested: adding TQ as a powder in an unloaded-LNCs dispersion  
268 (TQ-LNC-2). This new method of encapsulation can have the advantage of facilitating the production  
269 of LNCs. Indeed, a scale-up process to produce unloaded LNCs has already been proposed (Thomas &  
270 Lagarce, 2013). As a consequence, it will be easier to manage the mixing step of LNCs and drugs as  
271 powder, than a solubilization step in the oily phase before LNCs production. Moreover, adding drugs  
272 at the beginning of the process will implicate more difficult cleaning of the pilot but also potential drug  
273 degradation during temperature cycling.

274 Adding drugs as powder could limit potential degradation during the temperature cycle and modify  
275 drug localization into LNCs and consequently, LNCs properties. It could be expected that TQ was  
276 adsorbed at the surface or added to the shell of LNCs and not encapsulated into the oily core by this  
277 last method.

278 The mean size (in intensity), PDI, and  $\zeta$ -potential of LNC formulations are presented in Table 2. No  
279 modifications were observed between the unloaded and both TQ-loaded LNCs. All formulations  
280 formed a monodispersed suspension with a narrow distribution (PDI < 0.2), and a mean diameter above  
281 60 nm. For conventional and new encapsulation methods, reproducible results were obtained. With  
282 these specific quantities of oil, surfactants, and water, LNC size within the 50 nm range was expected  
283 and was demonstrated as the optimal size for oral delivery of LNCs LNCs (Roger *et al.* 2009a). Indeed,  
284 it is well-established that a size not exceeding 100-200 nm improves significantly cellular uptake and  
285 intestinal permeation while maintaining acceptable diffusivity through mucous layers (des Rieux *et al.*  
286 2005; Dizaj *et al.* 2015). The role of size in cellular uptake is crucial, as smaller nanoparticles can  
287 efficiently interact with cell membranes, facilitating internalization (He *et al.* 2010; Bannunah *et al.* 2014;  
288 Razavi and Hosseinzadeh, 2014). Finally, a relatively neutral charge zeta potential was obtained (Table  
289 2). This neutral charge zeta can be explained by the presence of PEG chains at the surface conferring  
290 steric stabilisation. No significant shift was observed. Hence the TQ loading process did not influence  
291 the surface charge of TQ-LNCs. In oral delivery, having a neutral surface charge have an interest  
292 because it is known to significantly enhance the cellular uptake of nanoparticles and enhance  
293 bioavailability (He *et al.* 2010; Bannunah *et al.* 2014; Razavi and Hosseinzadeh, 2014). These properties  
294 make TQ-LNCs suitable for application in oral delivery and could improve bioavailability.

295 Finally, TQ-LNCs-1 were already prepared for an *in vivo* study, and the same sizes, PDI, and potential  
296 zeta were obtained, demonstrating the reproducibility of this formulation (Selmi *et al.* 2023).

297 To separate unloaded and loaded drugs, different methods of purification (e.g. filtration, ultrafiltration,  
298 centrifugation, ultracentrifugation, tangential filtration) are commonly used depending on nanocarrier  
299 composition and drug properties (notably solubility in aqueous media for nanocarrier in aqueous  
300 dispersion).

301 Salmani *et al.* 2014 show that TQ has a solubility from 566 to 669  $\mu\text{g}/\text{mL}$  in aqueous solutions (water,  
302 Phosphate buffer pH 5 or pH 7.4) corresponding to 6-7% of the theoretical drug loading. A higher  
303 quantity of TQ in water is expected to precipitate, and filtration could theoretically unload TQ in  
304 aqueous media. To confirm, a quantification of TQ in a control (TQ in water at the same amount as in  
305 formulation) was performed, and after filtration, an amount of  $\approx 400 \mu\text{g}/\text{mL}$  ( $n=1$ ) was obtained,  
306 corresponding to less than 4% of the theoretical drug loading. Thus, the filtration can be used as a  
307 method of purification within a known error of 4%.

308 Recently, Roger *et al.* demonstrated that residual swollen micelles are also presented in LNC dispersion.  
309 They are notably able to separate swollen micelle from LNC by ultracentrifugation at 55.000g for 15h  
310 (Roger *et al.* 2023). Applying this method to TQ-loaded LNC dispersion, the amount of TQ encapsulated  
311 in micelle and LNCs was determined. The drug loading and entrapment efficiency determined after  
312 applying filtration and ultracentrifugation methods are compared in Table 4. Similar results were  
313 obtained with both methods of purification. Moreover, a very low amount of TQ was encapsulated in  
314 swollen micelles (less than 0.1mg/g of dispersion). Face to these results: no TQ encapsulated into swollen  
315 micelles, similar results of drug payload with both methods (eliminating the known error of 4% with  
316 filtration), and because the ultracentrifugation method is destructive (Roger *et al.* 2023), the filtration  
317 method was used for the following experiments to separate unencapsulated TQ from encapsulated TQ.  
318 In conclusion, an effective drug loading of about 8 mg/g was obtained for both formulations,  
319 corresponding to an encapsulation efficiency higher than 85%. This entrapment efficiency is higher than  
320 one obtained with other lipids nanoparticles (liposome, lipid nanoparticles) that allow a maximum EE  
321 of 88% (Mostafa *et al.*, 2018; Rathore *et al.*, 2019).

322 In terms of efficacy, in previous work, Selmi *et al.* have tested TQ-LNCs prepared with the conventional  
323 encapsulation method (with a similar drug loading) and after intratumorally administration at a dose  
324 of 5 mg  $\text{kg}^{-1}$  (corresponding to a volume of 80–100  $\mu\text{L}$  injected in mice), a significant reduction in tumor  
325 size was obtained. Moreover, thymoquinone is the main constituent (30-48%) of *Nigella sativa* (*N. sativa*)  
326 that demonstrated different preclinical and clinical effects on various diseases: anti-inflammatory effects  
327 in allergic encephalomyelitis, rheumatoid arthritis; immunomodulatory effects in patients with allergic  
328 diseases as eczema; antimicrobials effects; antitumor effects including lung cancer, breast cancer, renal  
329 adenocarcinoma, colon cancer, osteosarcoma cell, and cervical squamous carcinoma; effects on  
330 metabolic disorders as antidiabetic effects, antihyperlipidemic and antimetabolic syndrome effects;  
331 hepatoprotective effects; gastrointestinal protective effects; effects on neurological disorders; effects on  
332 cardiovascular disorders as antihypertensive effect; effects on respiratory disorders. Depending of the  
333 clinical studies, *N sativa* was used at a dose of 15mg/kg to 1g/kg (Gholamnezhad *et al.*, 2016).  
334 Considering that TQ corresponds to 30-48% of *N sativa*, the active dose would correspond to a dose of  
335 4.5 to 480mg/kg of TQ and for a person of 70kg, a dose of 315 mg to 33,6g should have an effect. Even if  
336 this effective dose of TQ is huge and dependent on the intended effect, it corresponds to a volume of  
337 35mL of dispersion of LNCs (acceptable to patients) to 4L of dispersion of LNCs. This volume could be  
338 important, so improving the drug loading or presenting TQ-LNCs as a powder to concentrate them and  
339 administrate them in solid pharmaceutical forms will be interesting. Moreover, more clinical  
340 pharmacological dose studies have to be determined.

341  
342 Finally, considering the new encapsulation method (adding TQ as a powder in an unloaded-LNCs  
343 dispersion (TQ-LNC-2)) allows obtaining the same physicochemical characteristics and a similar drug  
344 loading. Because one limitation of LNC preparation was the high temperature during the phase  
345 inversion process, adding drugs at LNCs dispersion open new perspective of use and notably, for drugs

346 unstable at temperature superior at 60°C, as biomolecules (peptides, SiRNA,...). Moreover, with the  
347 conventional method, it is mandatory to solubilize drug in the oily phase. By this new method, drugs  
348 that is not soluble in oily phase, such as amphiphilic drugs, can be encapsulated. Thus, with this new  
349 encapsulation method, the encapsulation potential of LNCs was enlarged to biomolecules, thermo-  
350 sensitive molecules, amphiphilic molecules...

351 To confirm the interest of this new encapsulation method, a more comprehensive characterization,  
352 notably on storage stability and TQ localization, among their evaluation for oral administration was  
353 then performed.

354

### 355 3.3. Storage Stability Studies

356 The Stability of TQ-loaded LNCs dispersions was assessed at 4 °C. After 35 days, the mean particle  
357 size of LNCs is still unchanged with a value around 60 nm (Figure 2A), demonstrated the  
358 reproducibility of the formulation. As shown in Figure 2B, a decrease of above 20% in the entrapment  
359 efficiency ratio was observed after 35 days indicating the instability of the formulation at the dispersion  
360 state. These profiles of entrapment efficiency ratio have not demonstrated a difference between TQ-  
361 LNCs-1 and TQ-LNCs-2. To address this decline and enhance the stability of these nanoparticles, many  
362 researchers explored the effectiveness of a freeze-dried method, and previous studies demonstrate the  
363 possibility of freeze-drying LNCs to preserve their physical characteristics as well as their drug loading  
364 (Saliou *et al.* 2013).

### 365 3.4. TQ Localization within Nanoparticles

366 The structure of LNCs comprises an oily core surrounded by an organized arrangement of lecithin  
367 and PEGylated surfactants suspended in water (Heurtault *et al.* 2002). To discern the drug's location,  
368 within the core or the shell or on the nanocarrier's surface, two methods were performed. Firstly,  
369 tensiometry measurement was performed. Indeed, the drop tensiometer technique allows to assess the  
370 rheological properties of oil–water and LNCs-air interface mimicking the surface structure of LNCs.  
371 The elastic nature of interfaces representing LNCs was notably characterized by this technique  
372 (Hirsjärvi *et al.*, 2012). The final  $\gamma_F$  were presented in Figure 3. First, the decrease of the  $\gamma_F$  value from  
373 around 72.8 mN.m<sup>-1</sup> for the pure water/air interface to around 35 mN.m<sup>-1</sup> for the water-TQ/air interface  
374 at the same temperature was obtained, suggesting an amphiphilic behavior of TQ. The Captex/water  
375 interface and the TQ solubilized in Captex/water interface showed  $\gamma_F$  of 5.5 mN.m<sup>-1</sup> and 6.5 mN.m<sup>-1</sup>,  
376 respectively. Despite the small difference between these two values, this result still suggests an  
377 amphiphilicity of TQ at the Captex®/water interface. Finally, for TQ- LNCs-1 and the TQ- LNCs-2,  $\gamma_F$   
378 against air, significantly decreased in comparison with unloaded LNCs. Considering this significantly  
379 smaller value of  $\gamma_F$  in the presence of TQ, an interfacial rearrangement with a different quality of  
380 interfacial films was formed in the presence of TQ, proving the association of TQ within LNCs as  
381 demonstrated by Minkov *et al.* (Minkov *et al.* 2005a, 2005b).

382 TQ transfer was also conducted following an innovative protocol established by Bastiat *et al.* (2013) .  
383 After separation, the LC (corresponding to the oily phase) remained at the top, while the aqueous LNCs  
384 dispersion was settled at the bottom of the tube. The characterization of the LNCs phase displayed no  
385 modifications in size, PDI, and  $\zeta$ -potential. TQ concentration was determined in the two resulting  
386 phases by UPLC-UV. Results revealed that ~80% of TQ were transferred from TQ loaded- LNCs to LC,  
387 (Figure. 4) (Bastiat *et al.* 2013). According to Bastiat *et al.* (2013), this result suggests that TQ was located  
388 inside the core without interacting with the surfactant shell. This localization can improve drug release  
389 within biological lipid reservoirs, e.g. the plasmic membrane of cells or intracellular lipid compartment

390 (Simonsson *et al.* 2016). Moreover, it suggests a lipophilic behavior of TQ with a small amphiphilicity,  
391 supported by a moderate Log P value of around 2.2. No significant difference was demonstrated  
392 between TQ-LNCs-1 and TQ-LNCs-2, suggesting no impact of the process on the TQ localization.

### 393 3.5. In Vitro Stability and TQ release in Simulated Gastro-Intestinal Fluids (SGIF)

394 Given the importance of drug delivery systems in enhancing the bioavailability and efficacy of TQ  
395 following oral administration, our study aimed to evaluate the potential of LNCs as an oral drug  
396 delivery system and the impact of the encapsulation method. In this context, the *in vitro* GI stability of  
397 the different formulations was performed in different SGIFs. In the FaSSGF characterized by a pH of  
398 1.2, the size of the LNCs remained unaffected after 3 h of incubation (Figure 5A). Interestingly, only a  
399 release of 10% of the initially entrapped TQ from both TQ-loaded LNCs was observed after 3 h of  
400 incubation (Figure 5B). Subsequently, the stability of TQ-LNCs was performed in two simulated  
401 intestinal fluids (FaSIF-V2 and FeSSIF-V2) (Figure 6). After 6 h, a release of 5% and 12% of TQ initially  
402 encapsulated in LNCs was observed with both formulations after incubation in the FaSIF-V2 and  
403 FeSSIF-V2 media, respectively (Figure 6B). Consistent with these findings, administering the  
404 formulation in pre-prandial state administration could be preferable. Therefore, more than 88% of TQ  
405 remains encapsulated in LNCs after incubation in the various SIGFs. Several works have also reported  
406 the efficiency of LNCs as nanocarriers for oral drug delivery, including for 7-Ethyl-10-hydroxy-  
407 camptothecin (sn38) and Paclitaxel showing high drug loading capacities with a limited sustained  
408 release (Roger *et al.* 2009a; Roger *et al.* 2011). A release of 20% of the initial amount of Sn38 was measured  
409 after 3h in FeSSIF and FaSSIF whereas a release of above 6,5% and 30% of paclitaxel in FaSSIF and  
410 FeSSIF, respectively were obtained. The difference in release could be explained by the drug's properties  
411 and consequently, their location in LNCs (shell, core, or core-shell interface).  
412 Nevertheless, the low release of TQ from LNCs and their physicochemical stability in SIGFs suggest  
413 that LNCs have promising potential to prevent burst release and the degradation of TQ after oral  
414 administration. Once again, the encapsulation method does not modify the properties of the LNCs in  
415 these SIGFs. Interestingly, TQ-LNCs exhibited a lower drug release compared to TQ-loaded chitosan-  
416 modified nanoparticles (CPLNPs) (Rahat *et al.* 2021). With LNCs, 10% of TQ was released after 3h,  
417 whereas 20% were released from CPLNPs after 2h in gastric fluid. In intestinal fluid, 5% after 6h versus  
418 40% after 2h were released from LNCs and CPLNPs, respectively (Rahat *et al.* 2021). LNCs are therefore  
419 more attractive than CPLNPs. Thus, LNCs have an interest to protect TQ with low release in SIGFs.

### 420 3.7. TQ resorption across the Intestinal Epithelium

421 LNCs were also able to improve *in vitro* drug permeability. For example, the permeability of  
422 paclitaxel across Caco-2 cell monolayers was increased by a factor of 3.5 in comparison with Taxol®  
423 dispersion (Roger *et al.* 2009a). Thus, the permeability of TQ across the caco-2 cells model was  
424 determined. Indeed, the Caco-2 cells model is well-established and used to study the intestinal  
425 permeability of drugs in the pharmaceutical sector (Artursson and Borchardt, 1997; Balimane and  
426 Chong, 2005), and it presents a strong correlation with *in vivo* models to predict oral resorption. Free  
427 TQ, and both TQ-loaded LNCs, were diluted at a final concentration of 80 µg/mL (corresponding to  
428 LNC dilution of 1% v/v in HBSS). After 2 h at 37 °C, the amount of TQ was quantified and the Papp was  
429 calculated (Figure 7). No difference was observed between both methods of encapsulation. A minor  
430 increase in TQ permeability with LNCs ( $2.0 \pm 0.2 \times 10^{-5} \text{ cm s}^{-1}$  and  $2.2 \pm 0.2 \times 10^{-5} \text{ cm s}^{-1}$ ) compared to  
431 free-TQ ( $1.7 \pm 0.3 \times 10^{-5} \text{ cm s}^{-1}$ ). After encapsulation into LNCs, a drug permeability improvement was  
432 already obtained with Paclitaxel, SN38 or decitabine ((Briot *et al.*, 2017; Roger *et al.*, 2009, 2011). On the  
433 other hand, for TQ, Lodovichi *et al.* obtained a Papp ( $1.5 \times 10^{-5} \text{ cm s}^{-1}$ ) across the Caco-2 cells model

434 after 2 h incubation with TQ encapsulated within polymeric micelles (Lodovichi *et al.* 2022). After oral  
435 administration, few LNCs can cross intact the intestinal barrier (less than 0,3%), by a consequence, no  
436 systemic particle toxicity could be expected (Kaeokhamloed *et al.*, 2021). Moreover, after 5-days  
437 repeated intravenous administration no toxic effect was demonstrated (Hureaux *et al.*, 2010).

438 Finally, the transepithelial resistance (TEER) was superior to 250  $\Omega$ .cm<sup>2</sup>, and no decrease was  
439 observed during the experiment. Accordingly, TQ-LNCs are unable to open tight junctions and do not  
440 allow paracellular transport (Hatiboglu *et al.* 2018). This result is under previous results that reported  
441 that LNCs are mainly transported via active endocytic processes, particularly via clathrin-dependent  
442 and caveolae-dependent transport mechanisms (Roger *et al.* 2009a).

#### 443 **4. Conclusion**

444 The results of this study confirm the potential use of LNCs as a nanosized delivery system for oral  
445 administration of TQ. The low release of TQ in GI fluids demonstrates the strong protection of LNCs,  
446 and the TQ resorption across the intestinal epithelium when loaded into LNCs, demonstrates the ability  
447 of LNCs to allow TQ transport across the intestinal barrier. As a perspective, further studies to evaluate  
448 the pharmacokinetic and therapeutic effects of TQ-LNCs after oral administration should be considered.  
449 Additionally, a new encapsulation method has been proposed that does not alter these properties. This  
450 method consists of adding TQ in unloaded LNCs dispersion. This method presents several advantages  
451 as no step of TQ solubilization in the oily phase, a critical step for industrial scale-up, the drugs do not  
452 undergo the temperature cycling that can degrade them (as for temperature-unstable drugs like  
453 biomolecules), and easier cleaning and limit cross contamination of the pilot of production.  
454 Consequently, this method opens a new way to produce and encapsulate drugs into LNCs.

455

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681 **Figure 1.** Chemical structure of Thymoquinone

682 **Figure 2.** Physiochemical behavior of Thymoquinone-loaded Lipid Nanocapsules (TQ-LNCs-1 and  
683 TQ-LNCs-2) stored at 2-8°C for 35 days (n=8, mean ± SD). (A) LNCs particle size (in intensity) and (B)  
684 ratio of the entrapment efficiency of Thymoquinone (TQ) with 100 % corresponding to the initial  
685 encapsulation efficiency of TQ. Statistical analysis was conducted, revealing a P-value greater than  
686 0.01. This indicates that there is no significant difference between day 0 and day 35 for both  
687 formulations.

688 **Figure 3.** Interfacial tension ( $\gamma_F$ ) measurements of unloaded Lipid Nanocapsules (LNCs),  
689 Thymoquinone-loaded Lipid nanocapsules (TQ-LNCs-1, TQ-LNCs-2), and controls (n = 4, mean ± SD);  
690 (\*\*p < 0.01 in comparison to unloaded LNCs).

691 **Figure 4.** Proportions of Thymoquinone (TQ) in Lipid Nanocapsules (TQ-LNC) (white part bar) and in  
692 the lipophilic compartment (LC) (black part bar) of TQ-LNCs-1 and TQ-LNCs-2 after transfer method  
693 (n = 4, mean ± SD).

694 **Figure 5.** Physiochemical behavior of Thymoquinone-loaded Lipid Nanocapsules (TQ-LNCs-1 and TQ-  
695 LNCs-2) in Fasted simulated gastric fluid (FaSSGF) at 37°C for 3h. (A) LNCs particle size (in intensity)  
696 and (B) Ratio of the entrapment efficiency of Thymoquinone (TQ) with 100 % corresponding to the  
697 initial encapsulation efficiency of TQ. (n = 4, mean ± SD). Statistical analysis was conducted, revealing a  
698 P-value greater than 0.01. This indicates that there is no significant difference between both  
699 formulations.

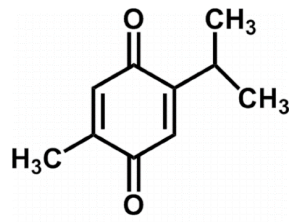
700 **Figure 6.** Physiochemical behavior of Thymoquinone-loaded Lipid Nanocapsules (TQ-LNCs-1 and TQ-LNCs-  
701 2) in Fasted and Fed simulated intestinal fluids (FaSSIF-V2 and FeSSIF-V2, respectively) at 37°C for 6h.  
702 (A) LNCs particle size and (B) Ratio of the entrapment efficiency of Thymoquinone (TQ) with 100 %  
703 corresponding to the initial encapsulation efficiency of TQ. (n = 4, mean ± SD). Statistical analysis was  
704 conducted, revealing a P-value greater than 0.01. This indicates that there is no significant difference  
705 between both formulations.

706 **Figure 7.** Apparent permeability ( $P_{app}$ ) coefficients of Thymoquinone (TQ) after 2 h of incubation at  
707 37°C with free TQ, TQ-loaded Lipid Nanocapsules (TQ-LNCs-1 and TQ-LNCs-2), diluted in buffered  
708 HBSS at 80µg/mL TQ to apical from basolateral side of Caco-2 cell monolayers (n = 3, mean ± SD) "\*\*\*\*" <  
709 0.01 in comparison to free TQ.

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Figures

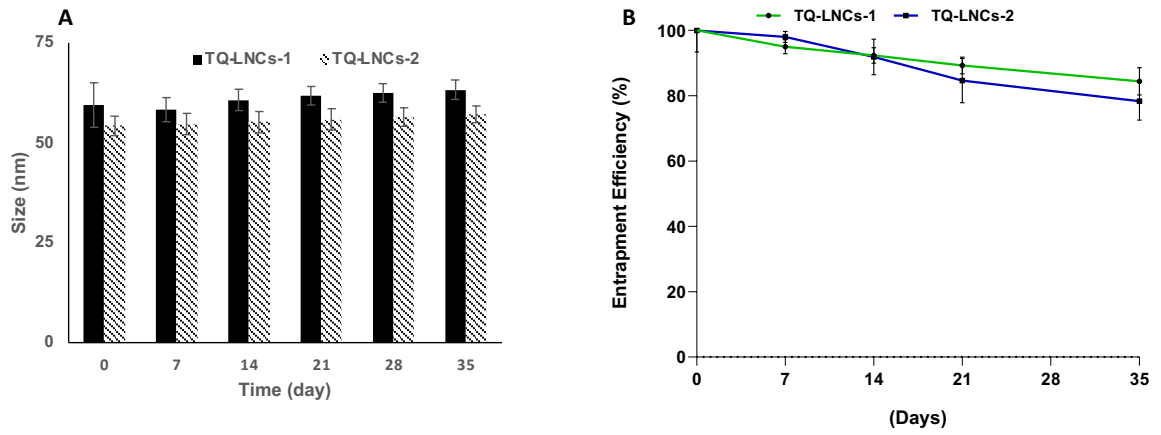


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Figure 1

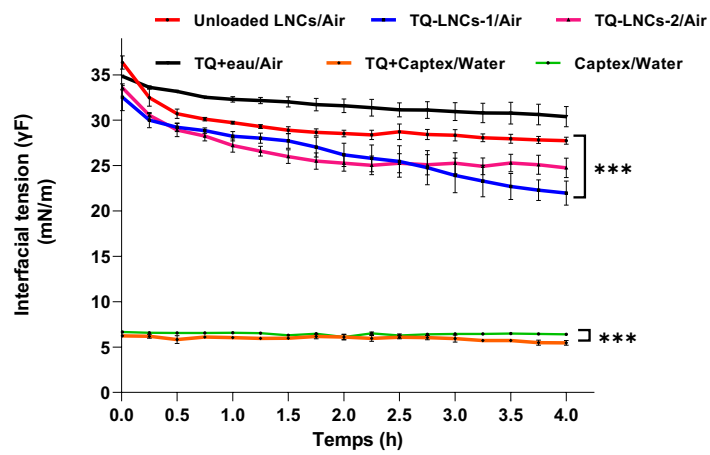


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Figure 2



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Figure 3

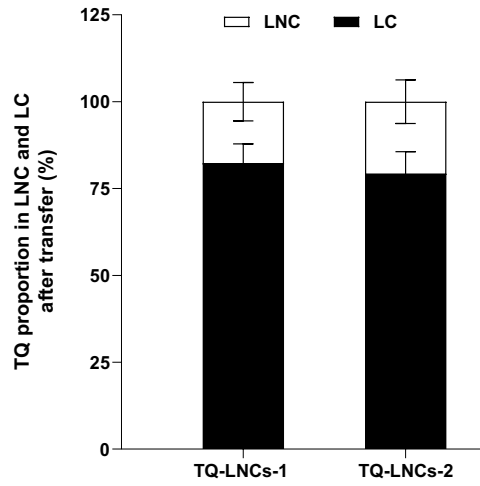


Figure 4

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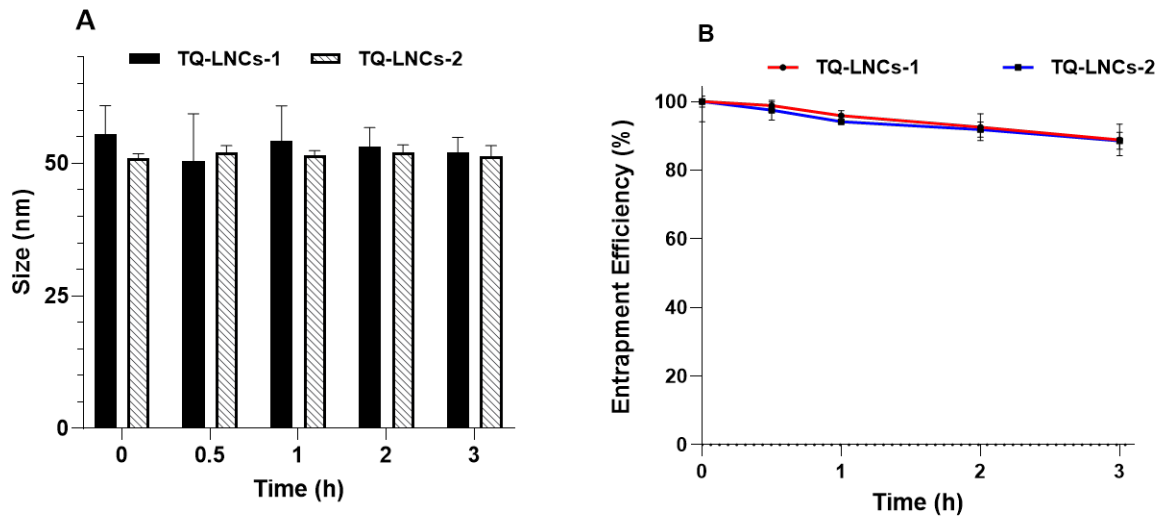


Figure 5

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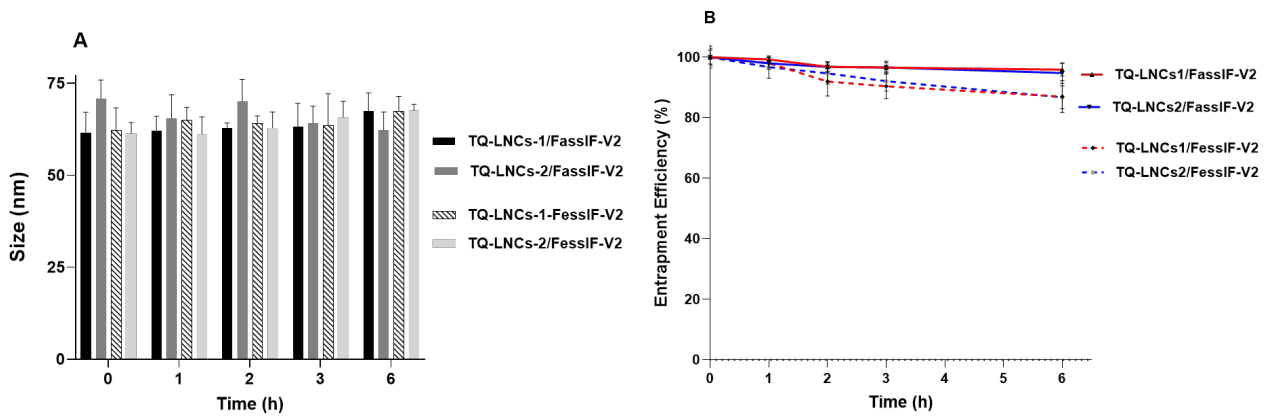


Figure 6

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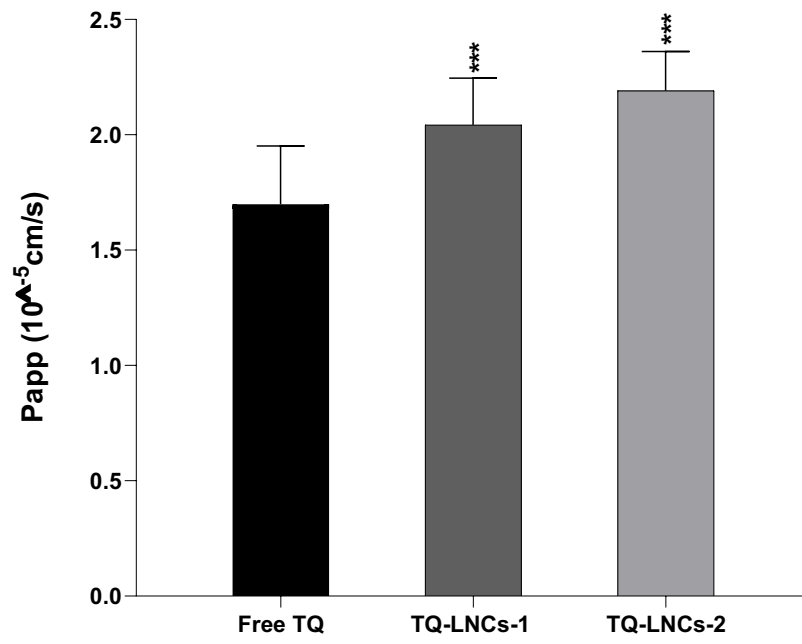


Figure 7

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735 **Table 1.** Composition of gastrointestinal simulated fluids: Fasted Simulated Gastric Fluid (FaSSGF),  
 736 Fasted Simulated Intestinal Fluid (FaSSIF-V2), and Fed Simulated Intestinal Fluid (FeSSIF-V2)  
 737 (Jantratid *et al.* 2008).

<b>Composition/medium</b>	<b>FaSSGF</b>	<b>FaSSIF-V2</b>	<b>FeSSIF-V2</b>
Sodium taurocholate	80 $\mu$ M	3 mM	10 mM
Lecithin	20 $\mu$ M	0.2 mM	2 mM
Pepsin	0.1 mg/mL	-	-
Maleic acid	-	19.12 mM	55.02 mM
Glyceryl monooleate	-	-	5 mM
Sodium oleate	-	-	0.8 mM
Sodium chloride	34.2 mM	68.6 mM	125.5 mM
Sodium hydroxide		34.8 mM	81.7 mM
Pancreatin	-	-	100 unit/mL
Calcium chloride	-	-	5 mM
pH	1.6	6.5	5.8

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742 **Table 2:** Mean particle size (in intensity), polydispersity index (PDI), zeta potential ( $\zeta$ -potential) values  
743 of unloaded LNCs, TQ-loaded LNCs (n = 3, mean  $\pm$  SD).

	<b>Size (nm)</b>	<b>PDI</b>	<b><math>\zeta</math>-potential (mV)</b>
<b>Unloaded LNCs</b>	57.5 $\pm$ 3.7	0.04 $\pm$ 0.01	-3.6 $\pm$ 0.5
<b>TQ-LNCs-1</b>	58.3 $\pm$ 3.7	0.05 $\pm$ 0.02	-3.8 $\pm$ 1.4
<b>TQ-LNCs-2</b>	61.5 $\pm$ 3.5	0.06 $\pm$ 0.03	-4.4 $\pm$ 1.7

744 \*Note: Statistical analysis was conducted, revealing a P-value greater than 0.01. No significant difference  
745 between the two encapsulation methods was obtained.

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748 **Table 3:** Drug loading (DL) and entrapment efficiency (EE) of Thymoquinone in lipid nanocapsules  
 749 (LNCs) after filtration and in LNCs and swollen micelles after ultracentrifugation for Thymoquinone-  
 750 Lipid Nanocapsules (TQ-LNCs-1 and TQ-LNCs-2) formulations (n=3, mean±SD).

Method of purification	Filtration		Ultracentrifugation			
	LNCs		LNCs		Swollen micelles	
	DL (mg/g)	EE (%)	DL (mg/g)	EE (%)	DL (mg/g)	EE (%)
TQ-LNCs-1	8.7 ± 0.2	95 ± 2	8.5 ± 1.3	91 ± 8	0.06 ± 0.08	0.6 ± 0.8
TQ-LNCs-2	7.7 ± 0.6	87 ± 6	8.0 ± 0.2	88 ± 3	0.04 ± 0.06	0.5 ± 0.7

751 \*Note: Statistical analysis was conducted, revealing a P-value greater than 0.01. This indicates that there  
 752 is no significant difference between the two encapsulation methods concerning DL and EE, and there is  
 753 no significant difference between the two methods of purification (ultrafiltration and  
 754 ultracentrifugation).

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