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1 **Development and *in vitro* cytotoxicity assessment of nanoemulsified lawsone**

2

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6

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Abstract

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Lawson is a biomolecule that belongs to the naphthoquinone class found in henna leaves. This quinone is widely used in cosmetic products being considered the oldest coloring agent applied for dyeing hair. Moreover, biological activities have been attributed to lawsone such as antibacterial, antifungal, and anticancer. However, these activities are mostly limited due to its low aqueous solubility and chemical instability. In this context, this study aimed to develop nanoemulsion containing lawsone (NE-law) and assess its *in vitro* cytotoxicity against cervical carcinoma cells. Emulsion phase inversion method was used to prepare nanoemulsion formulations (NE1, NE2, NE3, NE4, and NE-law). Following the formulation development, NE1 formulation was chosen to incorporate lawsone, presenting average droplet size about 45 nm with narrow size distribution (PdI <0.1) and negative zeta potential (≈ -13 mV). In TEM analyses, droplets were visualized with irregular spherical shapes, high homogeneity, and without aggregation. The incorporation efficiency of lawsone into NE was higher than 90 %, and *in vitro* release profile of this naphthoquinone from formulation was gradual and regular throughout the experiment. Nanoemulsified lawsone improved *in vitro* cytotoxicity on cervical carcinoma cells compared to free lawsone. Therefore, we prepared a stable lawsone-loaded NE using a reproducible emulsification method.

Keywords: Lawson, EPI method, Nanoemulsion, Cytotoxicity.

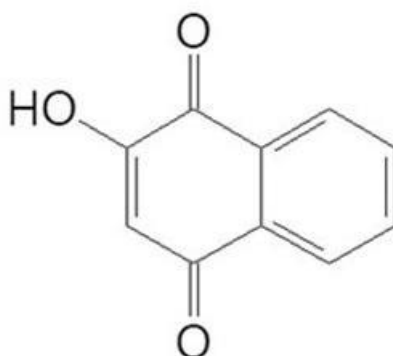
Introduction

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59

60 *Lawsonia inermis* L. is a small shrub-like tree commonly known as “Henna”. This
61 plant has been traditionally used for cosmetic and medicinal purposes in the eastern
62 Mediterranean, North and East Africa, the Middle East, the Arabian Peninsula, and South
63 Asia for centuries (Badoni Semwal et al. 2014; Singh et al. 2015). Phenolic compounds are
64 the main constituents of henna extracts and among these are naphthoquinones, coumarins, and
65 flavonoids. Naphthoquinones are a prevalent quinone class distributed in plants and
66 comprised of juglone, menadione, plumbagin, lawsone, and lapachol. Lawsone (2-hydroxy-
67 1,4-naphthoquinone, Fig. 1) is a bioactive naphthoquinone and the leading dye found in the
68 henna leaves being considered the oldest coloring agent used for dyeing hair (Petzel-Witta et
69 al. 2019; Singh and Luqman 2014). Beyond its dye qualities, several pharmacological
70 activities have been attributed to lawsone including antibacterial, antifungal, antiparasitic, and
71 antitumor (Adeli-Sardou et al. 2019; López et al. 2014; Singh and Luqman 2014; Singh et al.
72 2015). However, lawsone is described as a red-orange colored powder poorly soluble in water
73 and unstable upon exposure to light and oxygen, hindering its therapeutic application (Badoni
74 Semwal et al. 2014; Mahkam et al. 2014; Pandit et al. 2020; Singh and Luqman 2014).

75



76

77 **Fig. 1.** Chemical structure of lawsone ($C_{10}H_6O_3$, M.W. $174.15 \text{ g mol}^{-1}$).

78

79 In order to overcome these limitations, the lawsone encapsulation into a nanocarrier
80 emerges as an essential tool that can enhance its dissolution profile and bioavailability.
81 Regarding the molecule encapsulation into lipid-based delivery systems, nanoscale colloidal
82 emulsions have been considered as potential lipid carriers applied to hydrophobic molecules
83 due to improved drug dissolution, long-lived shelf life, versatility, and easy to prepare
84 (Elzayat et al. 2021; Fryd and Mason 2012; Panigrahi et al. 2019; Singh et al. 2017).

85 Nanoemulsions (NE) are metastable colloidal dispersions of immiscible liquids kinetically
86 stabilized by an interfacial layer of surfactant(s). Typically, emulsions are composed of
87 organic phase (synthetic and/or vegetable oils), aqueous phase, and emulsifying agent(s).
88 When constituted by oil droplets finely dispersed into a continuous aqueous phase, they are
89 referred to as oil-in-water (O/W) nanoemulsions (Gupta et al. 2016; Komaiko and
90 McClements 2016; Marhamati et al. 2021; Naseema et al. 2021; Panigrahi et al. 2019).

91 Nanoemulsions can be produced using either high or low-energy emulsification
92 methods. High-energy emulsification methods, also known as mechanical methods, are based
93 on work of different devices including high-pressure homogenizers, microfluidizers, and
94 ultrasonicators. However, these methods require substantial energy input density to form
95 small droplets, yet most mechanical energy generated may be dissipated as heat. On the other
96 hand, low-energy or thermodynamic methods rely on the spontaneous generation of
97 nanodroplets simply by changing environmental conditions (e.g., temperature) or some
98 formulation parameters, such as concentration and type of surfactant and/or oil, and oil-water
99 ratio (Guo et al. 2020; Gupta et al. 2016; Helgeson 2016; Lima et al. 2021; Meng et al. 2021;
100 Panigrahi et al. 2019). The main low-energy methods of nanoemulsion formation include
101 phase inversion composition, phase inversion temperature, emulsion phase inversion, and
102 spontaneous emulsification (Komaiko and McClements 2016).

103 In this regard, available information on the preparation and biological activities of
104 nanoemulsified systems containing hydrophobic naphthoquinones is still scarce, needing
105 more investigations. In addition, despite the widespread use of lawsone as a coloring agent in
106 several cosmetic products, the promising application of this naphthoquinone as a therapeutic
107 agent against cancer has been poorly explored. Therefore, this study aimed to produce a
108 nanoscale colloidal emulsion containing lawsone and assess its *in vitro* cytotoxicity on human
109 cervical carcinoma cells.

110

111

Experimental

112

113 *Materials*

114 Polyoxyethylene-40 hydrogenated castor oil (HCO-40), polyoxyethylen-20 sorbitan
115 monolaurate (SML-20), sorbitan monooleate (SMO), soybean oil (SO), and lawsone (law: 2-
116 hydroxy-1,4-naphthoquinone 97 %) were purchased from Sigma-Aldrich Co. (St. Louis, MO,
117 USA). Sorbitan isostearate (SIS) was kindly supplied from Croda do Brasil Ltda (Campinas,
118 SP, Brazil).

119

120 ***Nanoemulsion preparation***

121 Emulsion phase inversion method was used to prepare oil-in-water (O/W)
 122 nanoemulsion (NE) formulations as described by Rodrigues et al. (2018). Initially, the organic
 123 phase was prepared by mixing oil and surfactants under magnetic stirring (200 rpm) for 20
 124 min. After that, the aqueous phase (purified water) was added dropwise into to organic phase
 125 under continuous stirring at room temperature (≈ 25 °C). Firstly, a baseline formulation
 126 composed of constant surfactant-to-oil weight ratio at 2.0 and proportion of
 127 surfactant:oil:water at 10:5:85 (wt. %), respectively, was obtained. Then, different surfactant
 128 combinations were set at hydrophilic-lipophilic balance (HLB) 11 in order to achieve more
 129 suitable formulations resulting in the final composition (wt. %) of the NE described in Table
 130 1. Lawsone-loaded NE (NE-law) was prepared by adding this naphthoquinone directly in the
 131 organic phase and using the same preparation process for unloaded NE. All formulations were
 132 prepared three times (n=15) and stored at 4 °C (± 2).

133

134 **Table 1.** Composition (wt. %) of NE formulations

Formulation	HCO-40	SML-20	SMO	SIS	SO	PW
NE1	6.8	-	3.2	-	5.0	85.0
NE2	6.7	-	-	3.3	5.0	85.0
NE3	-	5.4	4.6	-	5.0	85.0
NE4	-	5.6	-	4.4	5.0	85.0
NE-law	6.8	-	3.2	-	5.0	85.0

135 HCO-40: polyoxyethylene-40 hydrogenated castor oil; SML-20: polyoxyethylen-20 sorbitan
 136 monolaurate; SMO: sorbitan monooleate; SIS: sorbitan isostearate; SO: soybean oil; PW:
 137 purified water; law: lawsone.

138

139 ***Droplet-size analysis***

140 Hydrodynamic diameter of droplets and polydispersity index (PdI) of the NE
 141 formulations were determined by dynamic light scattering at 25 °C and scattering angle of
 142 173° (Zetasizer[®] Nano ZS, Malvern PCS Instruments, UK). All samples were diluted with
 143 ultra-purified water. The reported values are average \pm SEM of three different batches of each
 144 formulation.

145

146 ***Zeta-potential determination***

147 The droplet surface electrokinetic or zeta (ζ) potential was determined by
148 electrophoretic light scattering using a Zetasizer[®] Nano ZS apparatus (Malvern PCS
149 Instruments, UK). The analyses were conducted at 25 °C, and the samples were appropriately
150 diluted with purified water. Values reported are average \pm SEM of three different batches of
151 each formulation.

152

153 *Electron microscopy analysis*

154 NE formulations were analyzed using the transmission electron microscopy (TEM)
155 technique. Aliquots (100 μ L) of nanoemulsion (NE1 and NE2) were put onto copper TEM
156 grids and negatively stained with 1 % (w/v) sodium phosphotungstate solution; the excess
157 sample was thoroughly removed by filter paper. After that, the samples were dried at room
158 condition before analyses. Transmission electron micrographs of the samples were taken
159 using a Microscope HT7700 Hitachi (Fukuoka, Japan) operated at accelerating voltage of 80
160 KV and emission 12 μ A.

161

162 *Lawsone quantification and incorporation efficiency into NE*

163 Lawsone concentration was determined using UV spectrophotometric method
164 developed by Rodrigues et al. (2018) with slight modifications. Total lawsone amount in NE
165 was assessed by diluting 50 μ L of the colloidal formulation in 5 mL of ethanol. Afterward,
166 lawsone concentration was determined by UV spectroscopy conducted on an Even UV/Vis
167 spectrophotometer (Ionlab, Paraná, Brazil) with detection at 290 nm. Free lawsone content
168 was determined by measuring the non-incorporated naphthoquinone present in a clear
169 ultrafiltrate obtained through separation of aqueous phase using an
170 ultrafiltration/ultracentrifugation procedure (Microcon Ultracel YM-100, Millipore, Ireland)
171 at 10,000 rpm for 60 min at 4 °C (Eppendorf, Centrifuge 5430 R, Hamburg, Germany). All
172 analyses were performed three times and the incorporation efficiency (IE) of lawsone into NE
173 was calculated from the following equation 1 (Rodrigues et al. 2018) (T_{law} is the total lawsone
174 concentration; F_{law} , free lawsone concentration; Th_{law} , theoretical lawsone concentration):

$$175 \quad IE (\%) = (T_{law} - F_{law}/Th_{law}) \times 100 \quad (1)$$

176

177 *In vitro release of lawsone from NE*

178 *In vitro* release assays were carried out using the dialysis technique for free and
179 nanoemulsified lawsone (Rodrigues et al. 2018). An aliquot (5.0 mL) of lawsone-loaded NE
180 (NE-law) or free lawsone dispersion was placed inside a dialysis bag (cellulose membrane,

181 molecular weight cut-off 14,000, Sigma-Aldrich Co., St. Louis, MO), sealed, and immersed in
182 a vessel containing 125 mL of 10 mM phosphate buffer solution (PBS pH 7.4). The releasing
183 system was maintained at 37 °C (± 1), under magnetic stirring (100 rpm). One-milliliter
184 aliquots were taken out of the dissolution medium at pre-determined time intervals, replaced
185 with fresh buffer solution (PBS), and analyzed for lawsone release by UV spectrophotometry
186 at 290 nm. Release profiles were expressed as cumulative the amount of released lawsone
187 (average \pm SEM) and plotted versus time. The experiments were carried out three times for
188 both free naphthoquinone and lawsone incorporated into NE.

189

190 *Assessment of in vitro cytotoxicity using Resazurin bioassay*

191 *In vitro* cytotoxicity of unloaded NE1 formulation, NE-law, and free lawsone
192 dispersion was assessed on a normal cell line, namely, mouse embryonic fibroblast cells
193 (NIH/3T3 - ATCC[®] CRL-1658[™]) and cytotoxic activity for NE-law and free lawsone was
194 assessed on human cervical carcinoma cell line (HeLa - ATCC[®] CRM-CCL-2[™]). One day
195 before the assays, NIH/3T3 or HeLa cells were seeded at a density of 5×10^3 cells/well onto
196 96-well plates, and allowed to attach overnight. Next, NIH/3T3 cells were incubated with
197 DMEM medium containing dilutions of unloaded NE1 (0.1, 0.5, 1.0, 2.5, and 5.0 % v/v), NE-
198 law (0.5, 1.0, and 2.5 % v/v), or free lawsone dispersion (1.0 and 2.5 % v/v) for 3 h while
199 HeLa cells were treated with NE-law (22, 44, and 110 μ M) or free lawsone (44 and 110 μ M)
200 during 3 h as well. After 24 h, 20 μ L of resazurin solution (25 μ g mL⁻¹) and 180 μ L of
201 DMEM without phenol red were added to each well and incubated for 4 h as protocol
202 described by Amantino et al. (2021). Afterward, the cell viability analyses were carried out in
203 the microplate reader EnSpire[®] (PerkinElmer, USA) with excitation at 540 nm and emission
204 at 590 nm. Control cells (CT) were incubated with culture medium alone (untreated cells) and
205 the percentages of viable cells were calculated from the following equation 2 (O.D._{sample} is
206 optical density from treated cells and O.D._{control} optical density from untreated cells):

$$207 \quad \text{Cell viability (\%)} = (\text{O.D.}_{\text{sample}} / \text{O.D.}_{\text{control}}) \times 100 \quad (2)$$

208

209 *Statistical analysis*

210 All the experiments were performed in triplicate and data are expressed as average
211 value (\pm SEM). Statistical analysis was performed by One-way ANOVA followed by Tukey's
212 pairwise comparisons using the Prism GraphPad Software. The statistical significance was set
213 at $p < 0.05$.

214

Results and discussion

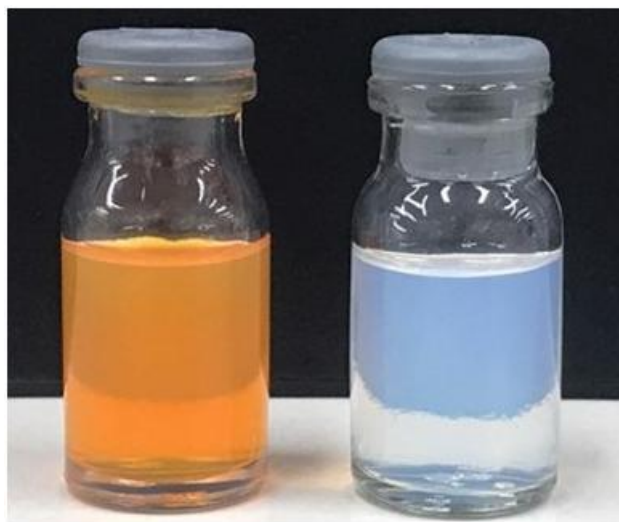
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217 Lawsone has been traditionally used for dyeing hair, skin, and nails in some African,
218 Middle Eastern, and Asian countries. However, the biological activities of this
219 naphthoquinone have been poorly explored due to its limited water solubility and chemical
220 stability (Adeli-Sardou et al. 2019; Pandit et al. 2020). In this context, colloidal formulations
221 based on O/W nanoemulsion may improve dispersions of hydrophobic drugs in aqueous
222 medium as well as protecting such molecules inside oil droplets. Nanoemulsion (NE)
223 formulation containing lawsone developed herein was obtained using a low-energy
224 emulsification approach, namely emulsion phase inversion method. Colloidal emulsion
225 systems with different combinations of nonionic surfactants (Table 1) at a constant HLB were
226 assessed in order to reach a stable NE formulation.

227 The visual appearance of the fluid nanoemulsified dispersions ranged from translucent
228 (NE1, NE2, and NE-law formulations) to highly turbid (NE3 and NE4 formulations), which
229 were closely related to droplet sizes formed in each NE formulation. In this regard, NE
230 formulations with very small oil-droplet sizes (< 50 nm) were translucent, while emulsion
231 systems consisting mainly of larger droplets (> 200 nm) showed an opaque appearance. As
232 depicted in Fig. 2, NE formulation containing lawsone (NE-law) showed a solid yellowish-
233 orange color and unloaded NE1 formulation was translucent with bluish reflection.

234



235

236 **Fig. 2.** Formulation photoimages of NE loaded with lawsone (NE-law, emulsion on the left)
237 and unloaded NE1 (emulsion on the right).

238

239 The average size, PdI, and zeta potential results of the NE formulations are reported in
 240 Table 2. The surfactant combinations employed to prepare the NE formulations influenced
 241 strongly the droplet average diameter and droplet-size distribution (PdI) of the resulting
 242 colloidal dispersions. As observed in Table 2, formulations composed of HCO-40 and SMO
 243 (NE1) or HCO-40 and SIS (NE2) surfactants showed the smallest droplet sizes (< 50 nm)
 244 both being characterized as finely dispersed emulsified systems. On the other hand,
 245 formulations containing SML-20 and SMO (NE3) or SML-20 and SIS (NE4) surfactants
 246 presented larger droplet sizes (> 200 nm). Likewise, droplet size distributions (PdI) were
 247 dependent of the surfactant combination present in each dispersion. NE1 and NE2
 248 formulations demonstrated a narrow droplet size distribution with PdI values below 0.2,
 249 whereas NE3 and NE4 had the widest size distributions (PdI > 0.45) comprising of polymodal
 250 distribution with larger and polydisperse droplets. Unlike the results mentioned above,
 251 electrokinetic potentials of the droplet surfaces from unloaded NE formulations (NE1, NE2,
 252 NE3, and NE4) were not influenced by surfactant combination (Table 2), as expected from
 253 nonionic surfactants.

254

255 **Table 2.** Droplet size (hydrodynamic diameter), polydispersity index (PdI), and ζ potential of
 256 the NE formulations (average \pm SEM, n = 3)

Formulations	Size (nm)	PdI	ζ potential (mV)
NE1	41.97 (\pm 0.71)	0.092 (\pm 0.03) ^b	- 31.75 (\pm 3.31)
NE2	38.03 (\pm 1.88)	0.188 (\pm 0.01)	- 28.43 (\pm 8.03)
NE3	241.7 (\pm 10.64) ^a	0.644 (\pm 0.02)	- 39.01 (\pm 0.55)
NE4	708.7 (\pm 40.07) ^a	0.452 (\pm 0.02)	- 42.34 (\pm 3.71)
NE-law	42.91 (\pm 0.94)	0.066 (\pm 0.01) ^b	- 12.7 (\pm 1.21) ^c

257 ^ap < 0.05 compared to NE1, NE2, and NE-law formulations concerning size.

258 ^bp < 0.05 compared to NE2, NE3, and NE4 formulations concerning PdI.

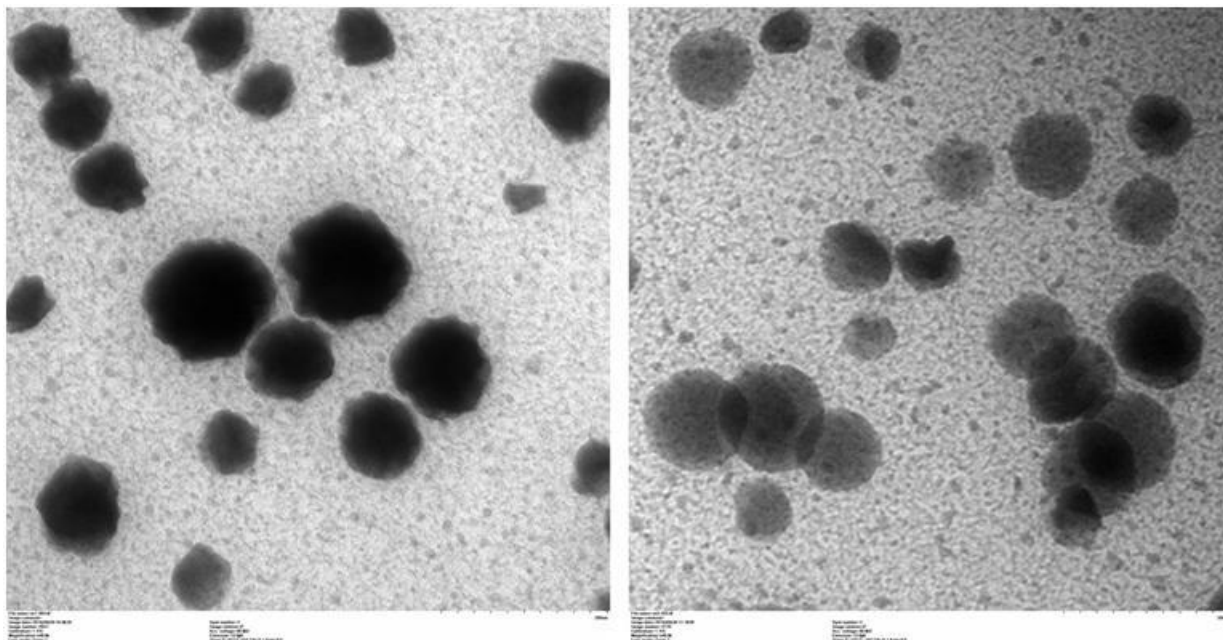
259 ^cp < 0.05 compared to NE1, NE2, NE3, and NE4 formulations concerning ζ potential.

260

261 Emulsion phase inversion (EPI) is an isothermal emulsifying method based on the
 262 addition of an aqueous phase into an organic phase under stirring. The formation of nanosized
 263 droplets in emulsified dispersions produced by EPI method is closely related to the surfactant
 264 monolayer adsorbed at oil-water interface. Thus, the more compact the surfactant layer
 265 around dispersed oil droplets, the lower interfacial tension of the dispersion (Komaiko and
 266 McClements 2016; Ravera et al. 2021). The EPI method mechanism of nanodroplet formation

267 is attributed to the gradual addition of aqueous phase to the organic phase leading to an
268 intermediate phase of bicontinuous microemulsion. Next, this intermediate stage with ultra-
269 low interfacial tension drastically breaks down in small oil droplets owing to the increasing
270 amount of aqueous phase in the dispersion (Komaiko and McClements 2016; Meng et al.
271 2021; Ravera et al. 2021; Rodrigues et al. 2018). Therefore, the combinations of HCO-40 and
272 SMO (NE1) or HCO-40 and SIS (NE2) surfactants were able to form nanodroplets with
273 robust interfacial layers resulting also in narrow size distributions, particularly for NE1
274 formulation with highly monodisperse droplet-size distribution ($PdI < 0.1$). This result
275 primarily shows that HCO-40 and SMO surfactants contributed to near-complete
276 solubilization of the organic phase into aqueous phase by forming small-sized oil droplets
277 with very homogeneous size distribution. Furthermore, there was no observation of oil
278 droplets agglomeration tendency, such as flocculation or creaming, for NE1 and NE2
279 formulations indicating enhanced physical stability of these nanoscale colloidal emulsions
280 (Gupta et al. 2016; Lima et al. 2021; Marhamati et al. 2021; Ravera et al. 2021). Such finding
281 could be explained by a better packing of HCO-40 and SMO or HCO-40 and SIS at oil-water
282 interfaces leading to a higher interfacial surfactant load and consequently optimum curvature
283 and lower interfacial tension. This result might be mainly related to the enhanced
284 accommodation of HCO-40 at interface as compared to SML-20 owing to distinct hydrophilic
285 head group sizes in both molecules promptly realized by the different HLB values of these
286 surfactants (Marhamati et al. 2021; McClements and Jafari 2018; Ravera et al. 2021).

287 Also, we were able to characterize size, shape, dispersity, and agglomeration tendency
288 of droplets in the colloidal dispersions using transmission electron microscopy (TEM) (Falsafi
289 et al. 2020). TEM analyses were carried out for NE1 and NE2 formulations and images are
290 shown in Fig. 3. As observed from photomicrographs (Fig. 3), both the dispersed phases of
291 the dispersions were formed by oil droplets with irregular spherical shapes, high
292 homogeneity, and without aggregation among droplets. These results clearly confirm the
293 dispersed phase behavior of NE1 and NE2 described before showing no indication of
294 flocculation and/or creaming tendency in these dispersions. In addition, droplet sizes observed
295 by TEM for the NE1 and NE2 formulations are in accord with droplet average sizes
296 established previously by dynamic light scattering technique.



298

299 **Fig. 3.** TEM photomicrographs of NE1 (photo on the left) and NE2 (photo on the right)
 300 formulations (Magnifications x40.0 K).

301

302 Accordingly, in view of the findings mentioned earlier, concerning well-designed NE
 303 formulation containing small oil droplets and narrower size distribution, we selected NE1
 304 formulation for further studies. In this regard, the lawsone incorporation into NE formulation
 305 did not alter the droplets' average diameter and size distribution compared with unloaded NE1
 306 formulation ($p < 0.05$). By contrast, the zeta potential of NE-law was slightly affected by
 307 naphthoquinone loaded into the formulation ($p > 0.05$) (Table 2).

308

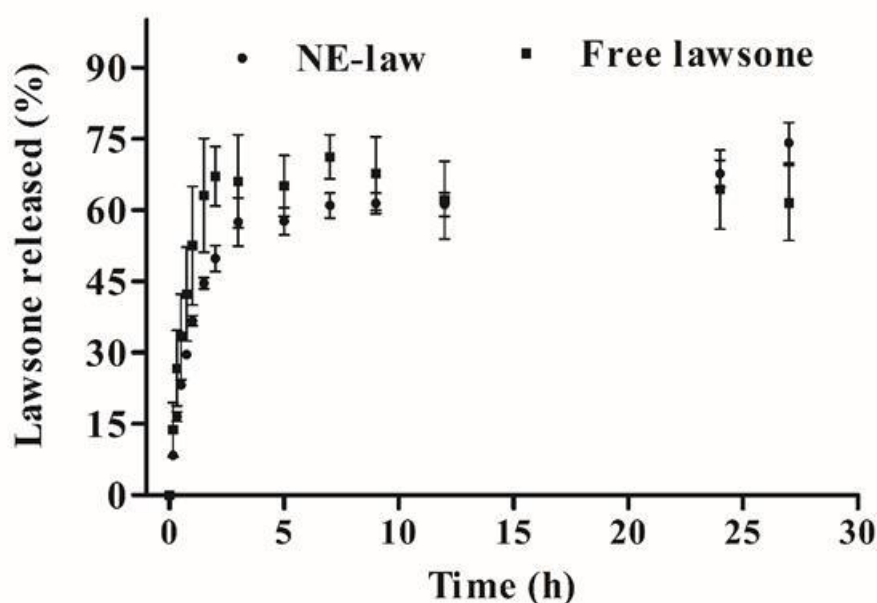
Quantitative analyses using a spectrophotometric method were carried out to assess
 309 the total amount, incorporation efficiency (IE) into NE, and in vitro release of lawsone. The
 310 total concentration of lawsone found for NE-law formulation was $0.52 \text{ mg mL}^{-1} (\pm 0.05)$ and
 311 IE of this naphthoquinone into NE reached $94.4 \% (\pm 0.99)$. This high IE of lawsone into the
 312 oily core of NE could be attributed directly to an intrinsic property of this biomolecule, i.e. its
 313 hydrophobicity. Such finding is in accord with Barani et al. (2018) that recently reported
 314 entrapment efficiency about 70 % for lawsone in niosome formulation. Likewise, in recent
 315 research conducted by Rodrigues et al. (2018), lapachol, also a hydrophobic natural
 316 naphthoquinone, was efficiently incorporated into nanoemulsion reaching IE about 90 %.

317

The in vitro release profiles for lawsone from NE formulation and coarse dispersion
 318 were compared using the dialysis bag diffusion technique. Despite both in vitro release
 319 profiles being slightly similar (Fig. 4), free lawsone was erratically released with wide
 320 variation as compared to its nanoemulsified form. In general, free lawsone was released 1.5-

321 fold faster than lawsone incorporated into NE. Hence, after 1 h, about 50 % of free lawsone
 322 amount have been released, reaching a maximum release of 67.2 % (± 6.3) at 2 h. On the other
 323 hand, lawsone was gradually released from NE-law in a regular way throughout experiment.
 324 In the first 1 h, about 35 % of lawsone have been released from NE formulation and after 12 h
 325 the naphthoquinone amount released reached 61.2 % (± 2.5). The maximum amount of
 326 lawsone released from NE formulation was 74.2 % (± 4.3) after 27 h of experiment. Such
 327 results could be related to considerable aggregation of free lawsone in aqueous medium
 328 hindering its dissolution due to a decrease in the exposed surface area. In contrast,
 329 nanoemulsified lawsone would be fully dispersed in the oil phase improving the release
 330 process. Similar results were found by Rodrigues et al. (2018) when lapachol was slowly
 331 released from NE formulation reaching the maximum amount of naphthoquinone released
 332 about 60 % after 24 h.

333



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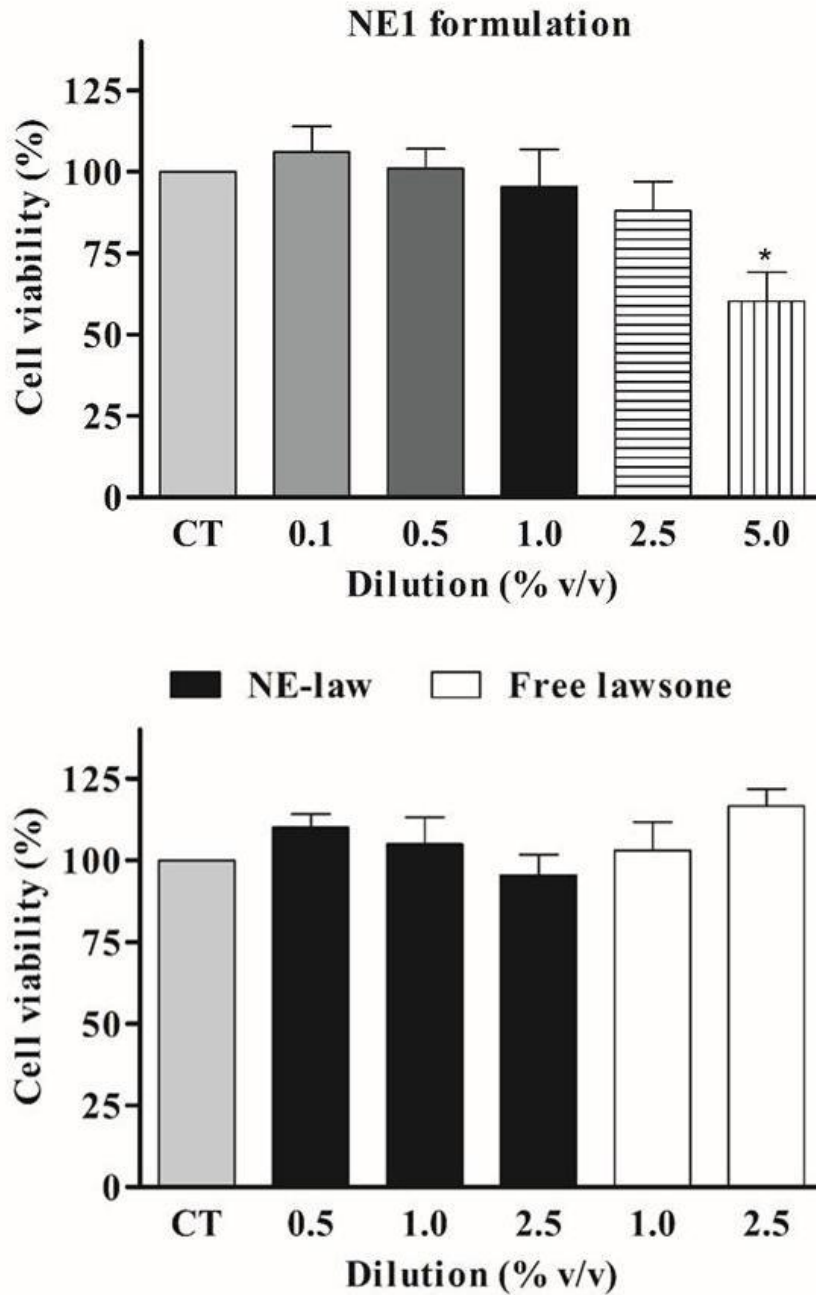
335 **Fig. 4.** *In vitro* release of free and nanoemulsified lawsone in PBS pH 7.4 (average \pm SEM,
 336 $n=3$).

337

338 Firstly, different dilutions of unloaded NE1 in culture medium were tested on
 339 NIH/3T3 cells to assess the intrinsic cytotoxicity from unloaded NE formulation. As shown in
 340 Fig. 5, dilutions between 0.1 and 2.5 % (v/v) did not show cytotoxic effect on NIH/3T3 cells
 341 with NIH/3T3 cell viability ranging from 88.0 % (± 9.0) to 106.1 % (± 7.9). On the other hand,
 342 the highest NE1 formulation concentration (5.0 % v/v) demonstrated an important cell

343 viability reduction about 40 % when compared to control cells ($p < 0.05$). In accordance with
344 ISO 10993-5:2009 (ISO 2009), reductions in cell viability higher than 30 % should be
345 considered cytotoxic effects, thus dilutions of NE1 formulation tested herein below 2.5 %
346 (v/v) could be seen as cell-friendly. Additionally, corresponding dilutions of NE-law and free
347 lawsone dispersion in DMEM medium were tested to assess the cytotoxicity on NIH/3T3
348 cells. As depicted in Fig. 5, cell viability ranging from 95.4 % (± 6.4) to 116.6 % (± 5.3), thus
349 there was no evidence of toxic effect from lawsone-loaded NE (NE-law) and free lawsone on
350 NIH/3T3 cells for any of the tested dilutions ($p > 0.05$). These results of free and
351 nanoemulsified lawsone along with those found for unloaded NE1 formulation confirm the
352 biocompatibility of all dispersion components. In addition, these results support the
353 performance of cytotoxic activity assays for both NE-law and free lawsone.

354



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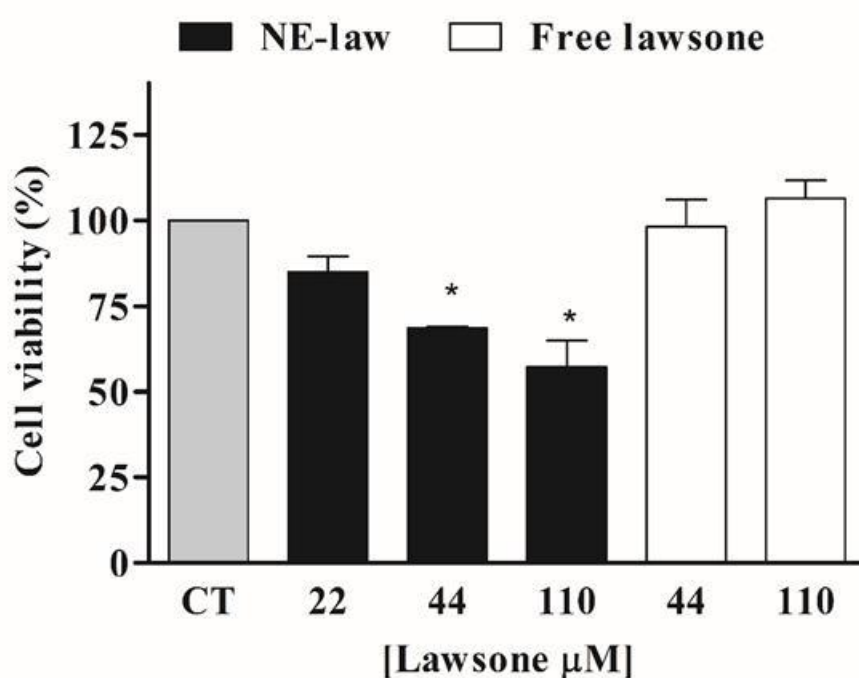
356 **Fig. 5.** *In vitro* cytotoxicity of unloaded NE1, NE-law, and free lawsone dispersion on
 357 3T3/NIH fibroblast cells. Data are presented as average \pm SEM of three independent
 358 experiments (CT = control, * $p < 0.05$).

359

360 *In vitro* cytotoxic activity of NE-law and free lawsone was tested on human cervical
 361 carcinoma cells (HeLa). The lowest concentration of NE-law (22 μ M) and both
 362 concentrations of free lawsone (44 and 110 μ M) did not show cytotoxic effect on HeLa cells
 363 as shown in Fig. 6. On the other hand, concentrations of nanoemulsified lawsone at 44 and
 364 110 μ M showed an essential decrease on HeLa cell viability reaching about 50 % ($p < 0.05$) at

365 the highest concentration of NE-law. Thus, we could assume an *in vitro* cytotoxicity of NE-
 366 law in a dose-dependent manner against HeLa cervical carcinoma cells. Such improved
 367 cytotoxic activity of nanoemulsified lawsone could be explained by a better interaction and
 368 internalization of this colloidal emulsion system with HeLa cells resulting from large surface
 369 area of nanoscaled droplets (Singh et al. 2017). Furthermore, it is considered that nanodevices
 370 have different biological interaction mechanisms and the pinocytosis mechanism is
 371 predominantly characteristic of nanostructured colloidal systems for enhancing cellular uptake
 372 of drugs (Murugan et al. 2015).

373



374

375 **Fig. 6.** *In vitro* cytotoxic activity of NE-law and free lawsone on HeLa human cervical
 376 carcinoma cells. Data are presented as average \pm SEM of three independent experiments (CT =
 377 control, * $p < 0.05$).

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Conclusions

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381 In summary, we have successfully achieved a lawsone-loaded NE formulation with
 382 well-controlled physical properties using an environmentally-friendly and cost-effective
 383 emulsification process. NE formulation was monodispersed and formed by small sized oil
 384 droplets confirmed by DLS and TEM analyses. In addition, NE-law formulation reached an
 385 incorporation efficiency of lawsone about 95 % and *in vitro* release assay demonstrated that

386 this naphthoquinone could be regularly released from NE. Moreover, *in vitro* cytotoxic effect
387 of nanoemulsified lawsone against HeLa human cervical carcinoma cells was improved
388 compared to free molecule. Therefore, these findings encourage further studies on
389 nanoemulsified phenolic compounds in different therapeutics protocols against neoplastic cell
390 lines.

391

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