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

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## Abstract

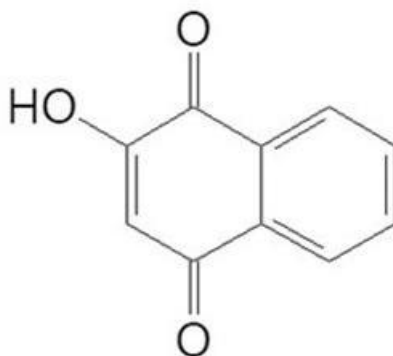
Lawson is a biomolecule that belongs to the naphthoquinone class found in henna leaves. This quinone is widely used in cosmetic products being considered the oldest coloring agent applied for dyeing hair. Moreover, biological activities have been attributed to lawson such as antibacterial, antifungal, 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 lawson (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 lawson, presenting average droplet size about 45 nm with narrow size distribution (PdI <0.1) and negative zeta potential ( $\approx -13$  mV). In TEM analyses, droplets were visualized with irregular spherical shapes, high homogeneity, and without aggregation. The incorporation efficiency of lawson into NE was higher than 90 %, and *in vitro* release profile of this naphthoquinone from formulation was gradual and regular throughout the experiment. Nanoemulsified lawson improved *in vitro* cytotoxicity on cervical carcinoma cells compared to free lawson. Therefore, we prepared a stable lawson-loaded NE using a reproducible emulsification method.

**Keywords:** Lawson, EPI method, Nanoemulsion, Cytotoxicity.

## Introduction

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



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**Fig. 1.** Chemical structure of lawsone (C<sub>10</sub>H<sub>6</sub>O<sub>3</sub>, M.W. 174.15 g mol<sup>-1</sup>).

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

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

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

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

## Experimental

### Materials

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

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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 ( $\approx 25$  °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 ( $\pm 2$ ).

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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.

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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.

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146 ***Zeta-potential determination***

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

### ***Electron microscopy analysis***

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

### ***Lawson quantification and incorporation efficiency into NE***

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

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

### ***In vitro release of lawson from NE***

*In vitro* release assays were carried out using the dialysis technique for free and nanoemulsified lawson (Rodrigues et al. 2018). An aliquot (5.0 mL) of lawson-loaded NE (NE-law) or free lawson 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 ( $\pm 1$ ), 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.

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#### 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<sup>®</sup> CRL-1658<sup>™</sup>) and cytotoxic activity for NE-law and free lawsone was  
 194 assessed on human cervical carcinoma cell line (HeLa - ATCC<sup>®</sup> CRM-CCL-2<sup>™</sup>). One day  
 195 before the assays, NIH/3T3 or HeLa cells were seeded at a density of  $5 \times 10^3$  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  $\mu$ M) or free lawsone (44 and 110  $\mu$ M)  
 200 during 3 h as well. After 24 h, 20  $\mu$ L of resazurin solution (25  $\mu$ g mL<sup>-1</sup>) and 180  $\mu$ 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<sup>®</sup> (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.<sub>sample</sub> is  
 206 optical density from treated cells and O.D.<sub>control</sub> optical density from untreated cells):

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

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#### 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  $p < 0.05$ .

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## 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.

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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).

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

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

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

<sup>a</sup> $p < 0.05$  compared to NE1, NE2, and NE-law formulations concerning size.

<sup>b</sup> $p < 0.05$  compared to NE2, NE3, and NE4 formulations concerning PdI.

<sup>c</sup> $p < 0.05$  compared to NE1, NE2, NE3, and NE4 formulations concerning  $\zeta$  potential.

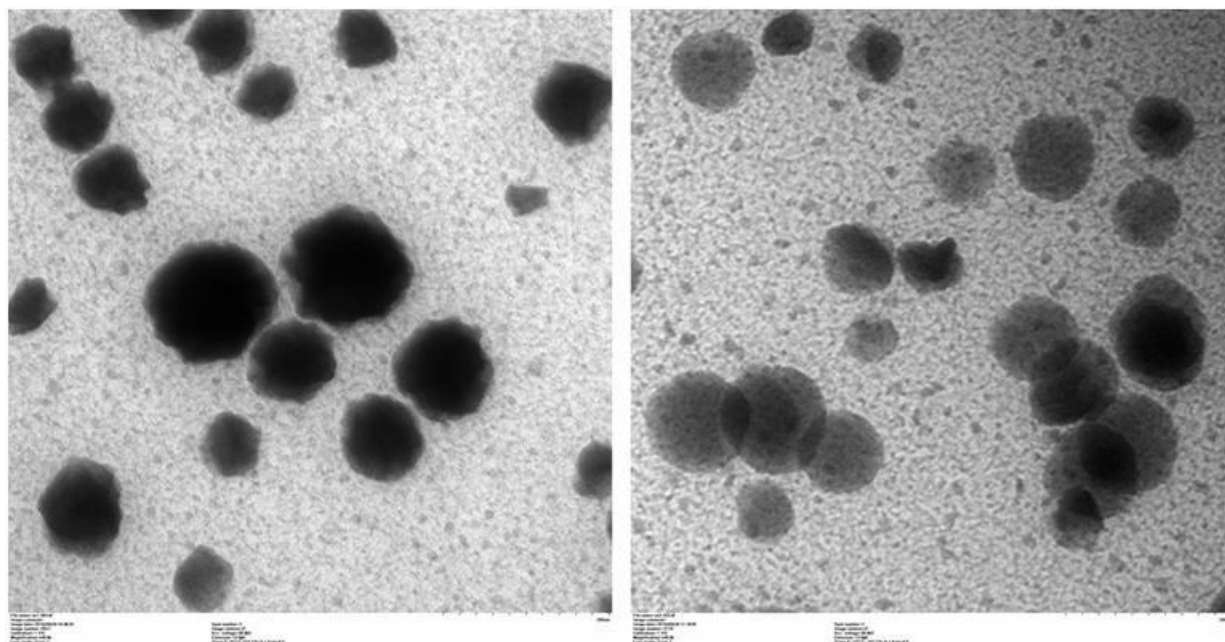
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Emulsion phase inversion (EPI) is an isothermal emulsifying method based on the addition of an aqueous phase into an organic phase under stirring. The formation of nanosized droplets in emulsified dispersions produced by EPI method is closely related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

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

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

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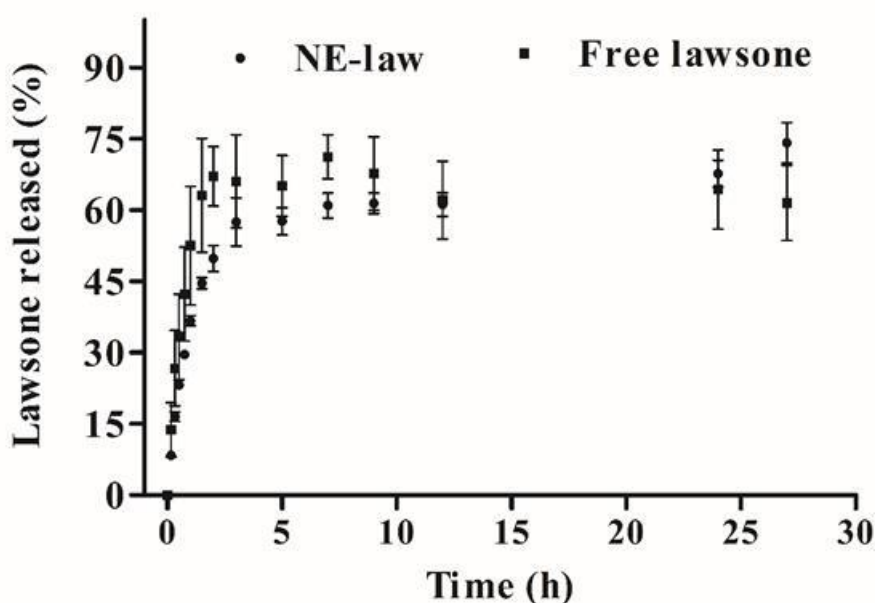
**Fig. 3.** TEM photomicrographs of NE1 (photo on the left) and NE2 (photo on the right) formulations (Magnifications x40.0 K).

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

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

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

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

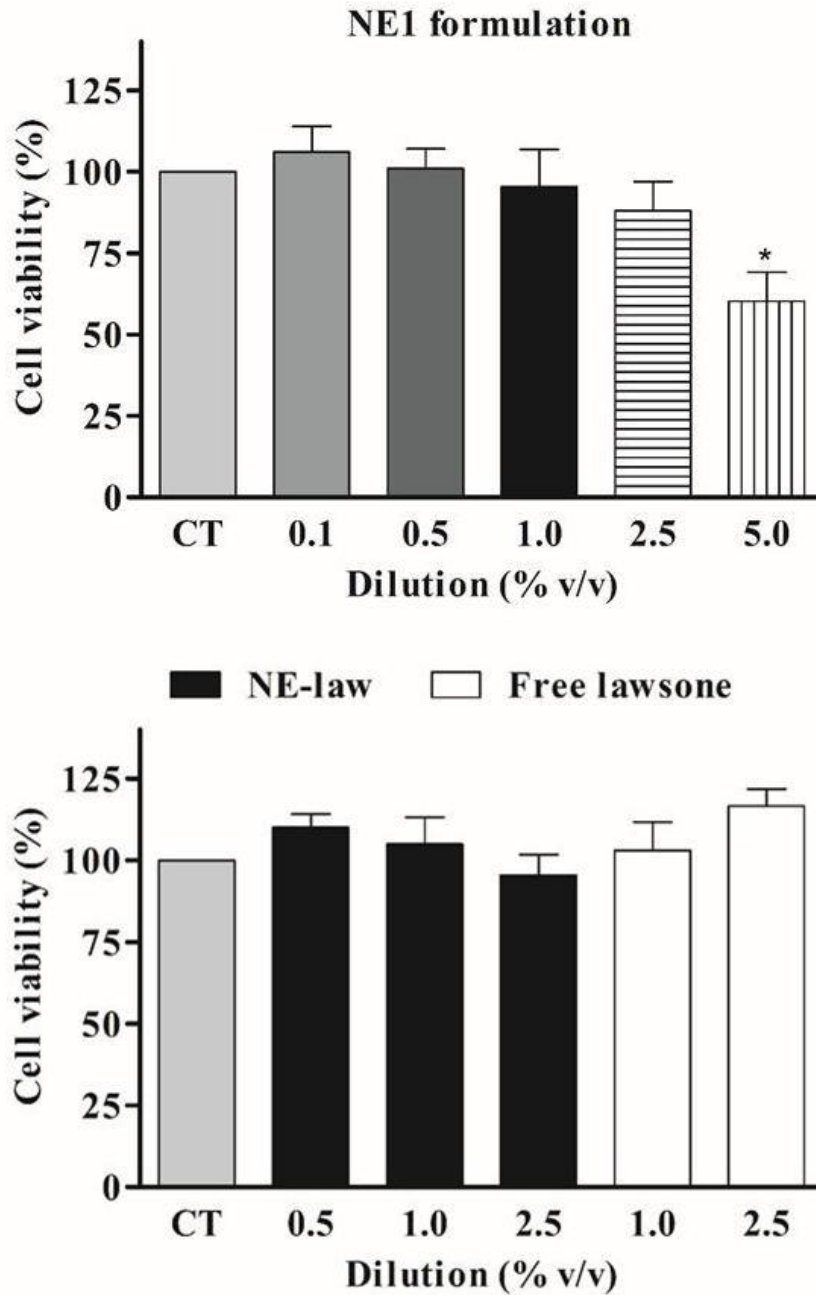


**Fig. 4.** *In vitro* release of free and nanoemulsified lawsone in PBS pH 7.4 (average  $\pm$ SEM,  $n=3$ ).

Firstly, different dilutions of unloaded NE1 in culture medium were tested on NIH/3T3 cells to assess the intrinsic cytotoxicity from unloaded NE formulation. As shown in Fig. 5, dilutions between 0.1 and 2.5 % (v/v) did not show cytotoxic effect on NIH/3T3 cells with NIH/3T3 cell viability ranging from 88.0 % ( $\pm 9.0$ ) to 106.1 % ( $\pm 7.9$ ). On the other hand, 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 % ( $\pm 6.4$ ) to 116.6 % ( $\pm 5.3$ ), 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.

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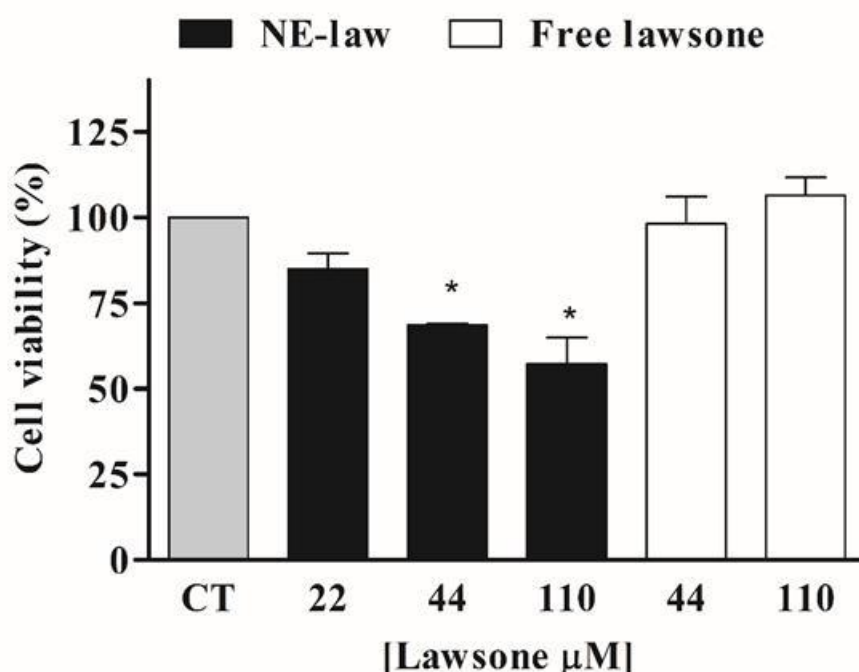
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356 **Fig. 5.** *In vitro* cytotoxicity of unloaded NE1, NE-law, and free lawsone dispersion on  
 357 3T3/NIH fibroblast cells. Data are presented as average  $\pm$ SEM of three independent  
 358 experiments (CT = control, \* $p < 0.05$ ).

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  $\mu$ M) and both  
 362 concentrations of free lawsone (44 and 110  $\mu$ 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  $\mu$ M showed an essential decrease on HeLa cell viability reaching about 50 % ( $p < 0.05$ ) at

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



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

## Conclusions

In summary, we have successfully achieved a lawsone-loaded NE formulation with well-controlled physical properties using an environmentally-friendly and cost-effective emulsification process. NE formulation was monodispersed and formed by small sized oil droplets confirmed by DLS and TEM analyses. In addition, NE-law formulation reached an 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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