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# Nanotoxicity and Dermal Application of Nanostructured Lipid Carrier Loaded with Hesperidin from Orange Residue

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**Abstract.** Flavonoids are a class of polyphenolic compounds that can be extracted from citrus fruits. Because of their features, they have been considered as new bullets in cancer therapy. Flavonoids present anti-oxidant, anti-inflammatory and anti-cancer properties and some studies are pointing that their higher intake involves lower risks of cancer. The nanostructured lipid carriers (NLC) are drug delivery systems composed from a mixture of solid and liquid lipids that increase physical stability and promote the sustained release of the drug. So, the main aim of this research was to combine the potential of the flavonoid as a natural anti-oxidant compound with the nanostructured lipid carriers to form an efficient system for flavonoid delivery into the cells. These NLC were prepared through high pressure homogenization technique and were evaluated in function of several standard properties. The samples exhibited good stability through time and temperature, with sizes of around 215 nm and zeta potentials of -35 mV during a period of 105 days. The encapsulation and the loading efficiency were respectively 96% and 2.25% up to 105 days of storage. *In vitro* release assays showed a burst process for NLC-L pointing to their shell structure with flavonoid situated in the contour of the particles. The cell viability tests showed that flavonoid, either free or loaded into NLC, did not present *in vitro* toxicity to the melanoma cells tested: A375, CHL01 and SKMEL147 through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red and crystal violet assays. Hesperidin at 45  $\mu\text{mol L}^{-1}$  was able to reduce significantly the radical forming up to  $92.58 \pm 0.82\%$  in DPPH assay. On the other hand, the produced NLC were applied in a skin lotion formulation, which showed a good stability and opened up a new application for the produced nanoparticles as in anti-aging and moisturizing cosmetics. Finally, the *in vitro* cytotoxicity tests were carried out using several human culture cells. The flavonoid loaded and empty nanocarriers have been used to treat the cells. All data suggested that, at the level studied, non-significant toxicities on cells were observed.

**Keywords**—*nanostructured lipid carrier, flavonoid, melanoma*

## 1. Introduction

Nanostructured lipid carriers (NLC) are a type of drug delivery system, derived from the solid lipid nanoparticles (SLN), which came up around the end of the 20<sup>th</sup> century. The biggest advantage of NLC over SLN is their structure. NLC are composed from a mixture of solid and liquid lipids which avoid lipid recrystallization providing spaces to host the active compound [1,2]. Nowadays, nanocarriers have been applied in pharmaceuticals and cosmetics due to their several advantages as drug delivery systems. For example, NLC avoid drug degradation due to the lipid protection and promotes drug-controlled release. Also, intestinal permeability is increased by the surfactants and the lipid nanoparticles are also muco-adhesive [2]. Flavonoids extracted from citrus fruits have been considered new bullets in cancer therapy due to their properties, such as, antioxidant, anti-inflammatory and antiproliferative [3]. Anticancer potential of flavonoids have been reported for the treatment of prostate, pancreatic, breast, ovarian and mouse melanoma cancer. There are different anticancer mechanisms involving flavonoids, for example,



some of them can target caspase cascade to induce cellular death, others are able to enhance cell death receptors, or inhibit angiogenesis and so on [3,4]. Despite all these characteristics, the bioavailability of flavonoids is low, which lower their body absorption [4]. Therefore, the main objective of this work is to combine NLC with natural flavonoids in order to produce a new system, NLC loaded with flavonoid, and evaluate its cytotoxicity to normal and cancer cells in order to apply as skin lotion formulation, as an anti-aging and moisturizing cosmetic product as safety nanomaterial to human.

## 2. Material and methods

The lipids *cupuaçu* butter and *buriti* oil were obtained from Amanter Group; anhydrous lanolin was courtesy from Croda Brazil; and, pluronic F68 and the flavonoid (> 80%) were acquired from Sigma Aldrich.

The method for production of NLC was adapted from Lima et al. [1]. Samples were prepared with 10% of lipids. For the loaded particles (NLC-L), flavonoid was dispersed in the mixture of melted lipids under ultra-sonication at a final formulation concentration of 4 mmol L<sup>-1</sup>. Empty particles (NLC-E) had the same amount of lipids and were used as control particles. The amount of incorporated flavonoid into NLC-L samples was quantified using HPLC (methanol and phosphoric acid 1%) and a Zorbax Eclipse XDB C18 (Agilent, USA). A certain amount of NLC-L formulation was filtrated through a qualitative filter paper of 14 µm pore size - just after preparation - to remove the flavonoid that wasn't loaded into the particles. The filtrated sample was used in the cellular viability assays.

The NLC characterization by measuring average particle size, polydispersity index (PDI) and Zeta potential using dynamic light scattering (Nano ZS ZetaSizer Malvern Instruments) was done. Samples were diluted in Milli-Q® water and 1 m mmol L<sup>-1</sup> KCl at 1 to 60 ratio for Zeta potential and size measurements. The morphology of the samples was analyzed using cryo-TEM (Talos F200C, FEI).

The *in vitro* release tests were done based on the work of Bose et al. [5] as to profile flavonoid release from of the particles. The release medium used was a 35% (v/v) aqueous solution of methanol. A membrane of Fisherbrand® regenerated cellulose dialysis with a cut size of 3,500 Da was performed in a jacketed vessel to maintain the temperature at 37 °C with stirring at 110 rpm.

Antioxidant activity tests were carried out using DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Sigma Aldrich). All solutions were prepared in dimethyl sulfoxide (DMSO PA, Neon) in order to get a final concentration of DPPH 250 µmol L<sup>-1</sup> and flavonoid at 0, 5, 15 and 45 µmol L<sup>-1</sup>. A 12-well plate was used to obtain a triplicate. The absorbance was read at 517 nm with a 2-minute gap, for 15 h at 37 °C. The equipment used was the plate reader Cytation 5 software Gen5 (BioTek, USA).

Cellular viability tests were performed using neutral red assay (Sigma Aldrich). Three human melanoma cell lines were used: A375, CHL01 and SKMEL147. The cells were plated in 96-well plate in a concentration of 0.4 x 10<sup>4</sup> cells/well using DMEM-High containing 10% of fetal bovine serum. After 24 h, cell lines were treated with the NLC in order to obtain a final concentration of flavonoid 0, 5, 15 and 45 µmol L<sup>-1</sup>. A control with the same amount of water was performed for each concentration. The cells were also treated with flavonoid in DMSO (less than 0.1%) at the same final concentrations. After 24 h of treatment, the cellular viability was estimated by neutral red assay (adapted from Repetto, G. [6]). Cells were incubated with 100 µL of neutral red 0.04 mg mL<sup>-1</sup>. After 2 h of incubation, absorbance was read at 570 nm.

## 3. Results and discussion

### 3.1. NLC Characterization

The total lipid concentration in both particles was 10%. The flavonoid concentration in the filtered loaded sample (NLC-L\*) was quantified by HPLC as 1520.68 ± 32.76 µg mL<sup>-1</sup>. For *in vitro* release assays (data not shown), a release profile with a burst process for NLC-L, enabled to propose a shell structure for these particles with the flavonoid situated in the contour of the particles.

### 3.2. Dynamic Light Scattering Analysis (DLS)

Size and zeta potential of the particles were measured throughout 105 days of storage. No significant changes were observed, which indicate that NLC were stable in this period.

**Table 1.** Size and zeta potential of empty (NLC-E) and loaded (NLC-L) NLC nanoparticles in function of the time.

	NLC-E	NLC-E	NLC-L	NLC-L
<i>Time (days)</i>	<i>Size average (nm) (PDI)</i>	<i>Zeta Potential (mV)</i>	<i>Size average (nm)(PDI)</i>	<i>Zeta Potential (mV)</i>
0	267.40 ± 29.82 (0.162)	-37.43 ± 1.27	284.03 ± 28.15 (0.142)	-32.53 ± 0.95
7	208.47 ± 6.18 (0.171)	-33.07 ± 0.21	229.20 ± 5.32 (0.139)	-33.66 ± 1.40
30	207.07 ± 9.58 (0.196)	-37.00 ± 1.06	212.93 ± 13.17 (0.193)	-37.40 ± 1.00
105	205.93 ± 13.83 (0.210)	-29.43 ± 0.64	217.66 ± 7.08 (0.198)	-33.06 ± 0.75

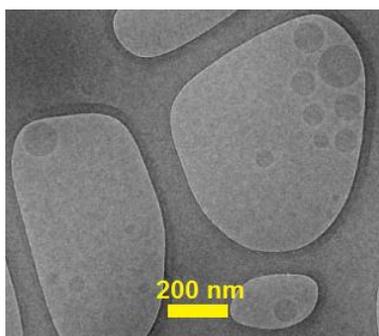
The work of Saupé et al. [7] showed that for NLC formulations, which were produced under similar conditions as in this work (by homogenization of high pressure: 500 bar and three cycles of homogenization), had an average diameter of 202 nm and were stable for one year.

### 3.3. Cryo-Transmission Electron Microscopy (Cryo-TEM)

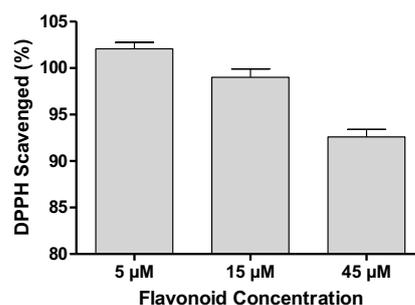
The spherical morphology of the nanocarriers (Fig. 1) and average diameters of NLC of  $101.6 \pm 78.6$  nm ( $n = 100$ ) were observed in cryo-TEM micrographs, which allowed to observe samples in almost natural conditions [8].

### 3.4. Antioxidant Activity

Flavonoid antioxidant activity (Fig. 2) was evaluated at the same concentration as used for the cellular viability assays. Flavonoid was able to reduce DPPH activity to  $92.58 \pm 0.82$  % after a period of 15 h only when its highest concentration ( $45 \mu\text{mol L}^{-1}$ ) was used. One-way ANOVA was performed to study the significance of these data in function of control, pure DMSO. It was found that the flavonoid concentrations of  $5 \mu\text{mol L}^{-1}$  and  $15 \mu\text{mol L}^{-1}$  were not effective to scavenge the radical ( $P > 0.05$ ). However, other references also used DMSO as a solvent to evaluate the antioxidant activity of flavonoid, only with proportions of DPPH to the flavonoid in the ratio 1 to  $1.5 \text{ mmol L}^{-1}$ . In that case, the reduction of DPPH by flavonoid was  $36.65 \pm 0.56\%$  [9]. It appears that the chosen solvent (DMSO) influenced the antioxidant activity of the flavonoid tested.



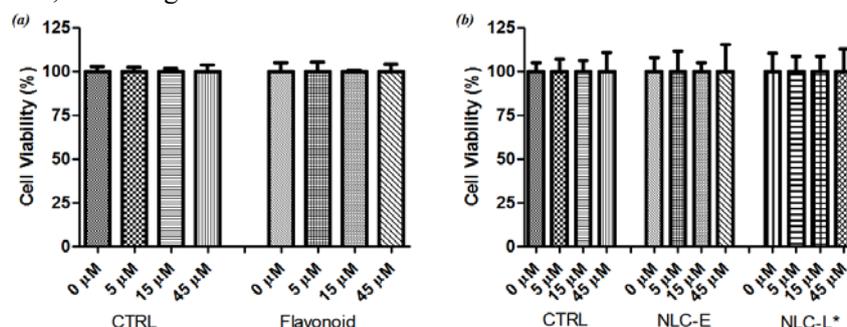
**Fig. 1.** Cryo-TEM of NLC-L sample.



**Fig. 2.** Percentage of radical scavenged by flavonoid.

### 3.5. Cellular Viability Assay

Cellular viability assays (Fig. 3) showed that either flavonoid and NLC-L were not cytotoxic to A375 cells. The same non-cytotoxic behaviors (data not shown) for flavonoid and NLC-L were observed against CHL01 and SKMEL147 cell lines. Statistical analysis was performed by one-way ANOVA and Bonferroni post-test, with a significance of  $P < 0.05$ .



**Fig. 3.** Neutral red assay with A375 melanoma cell lines after a 24-hours-treatment with (a) flavonoid and (b) empty (E) and loaded (L) nanostructured lipid carriers (NLC). Control (CTRL) refers to same test performed with addition of water (same amounts as other samples tested).

## 4. Conclusions

Herein, NLC were prepared and found to be stable up to 105 days of storage at room temperature. The flavonoid showed antioxidant activity at high concentrations ( $> 45 \mu\text{mol L}^{-1}$ ), however, at the amounts used for cellular viability assays its antioxidant potential was low. Assays performed *in vitro* showed an active release profile of the flavonoid from the NCL-L with the burst, thus, evidencing a shell NCL-L structure with flavonoid located at the peripheries of the particles. This is related to the structure of the particles, because when the active ingredient (drug) is on the outside of the structure, particles show a faster drug release at the beginning and a sustained drug release in the coming hours. Finally, it was possible to see that neither the flavonoid alone nor the NLC-E/L presented toxicity to melanoma cell lines tested after 24-hour-treatment. This indicates that NLC are safe and could be used for many applications, such as in cosmetics.

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