Application of Curcuminoids-loaded Nanoemulsion for Cancer Therapy

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**ABSTRACT**

**Introduction:** Curcuminoids and oleic acid, reported in the literature as antioxidant and antitumor agents, were combined in nanoemulsion, using Pluronic F127 as a stabilizer.

**Methods:** The main objective is to investigate its cytotoxic activity against cancer cells.

**Results and Discussion:** The nanoemulsion show values of the particle size (251.4 ± 4.6 nm), zeta potential (-34.8 ± 1.0 mV), and pH (4.8) classifying it as stable and non-irritating for pharmacological applications. The main results indicate that, in the toxicity test in fish (Zebrafish), the nanoformulation proved to be biosafe and the evaluation of the antioxidant action (by DPPH) showed excellent antioxidant activity. Furthermore, in the in-vitro cytotoxicity studies, the nanoemulsions showed good cytotoxic activity, ideal for fighting Colorectal, glioblastoma, and Leukemia cancers and more effectively in prostate cancer.

**Keywords:** Anticancer activity, Curcuminoids, Nanoemulsion, Oleic acid, Prostate Cancer.

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**INTRODUCTION**

The International agency for research on cancer estimates that there are more than 19 million new cases of cancer in 2020, worldwide, for both sexes and all ages. The most prevalent cancers are those of breast (12.5%), lung (12.2%), colorectum (10.7%), prostate (7.8), stomach (6%), liver (5%), cervix uteri (3.3%) and other cancers (42.5%). Meanwhile, cancer mortality rates are still high, almost 10.0 million cancer deaths occurred in 2020.¹

Several treatment strategies aim to inhibit specific cancer cell development, progression, and tumor metastasis without causing serious side effects.²³ In addition to synthetic anticancer agents, several vegetable species have been explored for the extraction of anticancer compounds with different modes of action. Some examples are Taxus brevifolia, Catharanthus roseus, Betula alba, Cephalotaxus species, Erythroxylum previllei, Curcuma longa, and many others.⁴ Among the vegetable species, we highlight Curcuma longa L. (turmeric), from which curcuminoids (curcumin, desmethoxy curcumin, and bisdesmethoxy curcumin) can be extracted from its rhizomes.⁵

Curcumin, as well as other curcuminoids present in turmeric, have received immense attention in the last two decades due to its bio-functional properties, such as antitumor, antioxidant, and anti-inflammatory activities.⁶ Many scientific works report the structure activity relationship of curcumin and other curcuminoids to improve their physicochemical and biological properties. The antitumor activity of curcumin and curcuminoids in breast cancer, lung cancer, squamous cell carcinoma of the head and neck, prostate cancer, and brain tumors has been verified² showing their ability to target multiple cancer cell lines. Despite all the mentioned advantages, the applications of curcumin and curcuminoids are limited due to their low water solubility, which results in low oral bioavailability and also low chemical stability.⁶

The low cellular absorption of curcumin and curcuminoids is related to the hydrophobicity of the molecules. Curcuminoid molecules tend to penetrate the cell membrane and bind to the fatty acyl chains of membrane lipids through hydrogen bonding and hydrophobic interactions, resulting in their low availability within the cytoplasm.⁸⁹

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Nanoemulsion drug delivery systems are a shows potential tool for delivering and improving the bioavailability of hydrophobic drugs and bioactive food components present in the blood fluid. To overcome these obstacles and improve the overall anticancer activity of these molecules, this work aims to incorporate curcuminoids in nanoemulsion, containing oleic acid as the oil phase and Pluronic F127 as a stabilizer, for increasing their solubility and bioavailability, to use this formulation in the treatment against the colorectal, glioblastoma, prostate and leukemia cancers.

MATERIALS AND METHODS

Materials
Pluronic F127 was purchased from Sigma-Aldrich (San Luis, EUA). The 2,2-Diphenyl-1-picryl-hydrazine (DPPH) was purchased from Honeywell International Inc. (Charlotte, EUA). Analytical grade methanol (99.9 %), ethanol (99.5 %), acetone (99.9 %), hexane (99.9 %), dichloromethane (99.9 %), 2,6-bis(1,1-dimethyl ethyl)-4-methyl phenol (BHT) and dimethylsulfoxide (DMSO-d₆) were obtained from Merck (Darmstadt, Alemanha).

Extraction of curcuminoids
For curcuminoids extraction, 20.0 g of turmeric was heavy in a beaker. Then were added 50 mL of dichloromethane and the solution was heated on a hot plate until boiling (40°C). The system remained under stirring for 30 minutes. The mixture was filtered, and the procedure was repeated three times. Then, 50 mL of hexane was added to the obtained powder and stirred for 30 minutes. The solvent was removed by rotary evaporation. The curcuminoids were obtained as an orange powder in a 2.4% yield. The perchlorination method was used. The ¹H Nuclear Magnetic Resonance (¹H-NMR) spectra of the obtained curcuminoids were performed using the Bruker Avance DPX-500 Spectrometer (Billerica, EUA). The samples were dissolved in deuterated dimethylsulfoxide (DMSO-d₆).

Nanoemulsion Preparation
The nanoemulsion (E1) was prepared to contain 0.02 g of the curcuminoids, 2 mL of ethanol, and 1.5 g of the oleic acid (OA). These mixtures were heated in a sand bath at 40°C until the complete evaporation of ethanol. In this way, only curcuminoids solubilized in the oil phase were obtained. Afterward, 8.48 g of 5% (w/w) aqueous solutions of pluronic F127 were added, respectively, to complete 10.0 g of each emulsion. To obtain the nanoemulsion, the formulation was placed in a probe ultrasound, model W-450D Branson Sonifier (Danbury, EUA). The mixture was submitted to ultrasonic irradiation with amplitude of 70% for 1 min (the 30s on/10s off). The empty nanosystem was prepared in the same way described, but without the presence of curcuminoids.

Particle size, polydispersity index, and zeta potential
Dynamic light scattering measurements were performed on a Zetasizer Nano, Malvern, ZS ZEN 90 (Worcestershire, UK). The analyzes were performed by diluting the nanoemulsion in distilled water at a ratio of 1:500 (v/v) (nanoemulsions: H₂O). The measurements were made at 25°C and were investigated through automatic scans with 60 s time to stabilize the sample. The analyses were performed in triplicate and the values express the arithmetic mean of the data obtained.

pH measurements
pH was measured using a pH Quimis (Diadema, SP, Brazil) at 25 ± 1°C temperature. The analyzes were performed by diluting the nanoemulsion in distilled water at a ratio of 1:10 (w/w) (nanoemulsions: H₂O). Three successive readings with a difference of less than 0.05 were used.

Encapsulation efficiency
The encapsulation efficiency of curcuminoids in nanoemulsion was determined using a centrifugation filter (10,000 MWCO, Millipore). Approximately 1.0 g of nanoemulsion was added to the centrifuge filter and then centrifuged at 9000 rpm for 30 minutes. Then the solution containing the non-encapsulated curcuminoids was diluted with ethanol in a 5 mL volumetric flask sample were analyzed using UV-vis spectrometry at a wavelength of 425 nm according to with procedure reported in the literature with some modifications. The results obtained were applied to a previously constructed calibration curve for curcuminoids.

In-vitro release
The in-vitro release of the curcuminoids was carried out using a dialysis membrane with a cut molar mass of 2000 g/mol (Sigma-Aldrich). Approximately 1.0 g of the nanoemulsions were inserted separately in the dialysis membrane which was immersed in 100 mL of phosphate buffer (pH 7.4) and ethanol in the ratio of 70:30 phosphate buffer: ethanol. At different time intervals up to 24 hours, 3 mL aliquots were removed from the buffer containing the released curcuminoids, and the removed volume was reconstituted with the buffered solution (3 mL). The aliquots removed were analyzed in a UV-Vis spectrophotometer at a wavelength of 425 nm. The results obtained were applied to a previously constructed calibration curve for curcuminoids. The experiments were carried out for 24 hours in triplicate at 32°C with stirring of approximately 400 rpm. This procedure was adapted from another study.

Antioxidant assay for DPPH free radical capture
The antioxidant potential of the extract was evaluated by the DPPH radical scavenging capacity, according to the procedure described in the literature. The nanoemulsion was diluted in water to obtain 4 more diluted solutions. The real concentration of the nanoemulsions obtained was 0.8; 0.4; 0.2 and 0.1 mg mL⁻¹. A 0.1 mL aliquot of each nanoemulsion was added to test tubes and then 3.9 mL of the 0.06 mM DPPH methanolic solution was mixed with the solutions. The samples were protected from light and the kinetics of the reaction were determined every 10 minutes until 45 min of the reaction. The decrease in the absorbance
of each solution was then measured on a spectrophotometer Thermo scientific, Genesys 6 (Waltham, EUA), at a wavelength of 515 nm. The same procedure was performed with excipients separately, oleic acid and Pluronic® F127, in the absence of curcuminoids. The antioxidant capacity was calculated according to the equation 1,

\[
DPPH (\%) = \frac{[A_0 - A_t]}{A_0} \times 100 \quad \text{Equation 1}
\]

where \( A_0 \) represents the absorbance of the control and \( A_t \) absorbance in the presence of the sample. The results were plotted on a DPPH inhibition graph as a function of the concentration of curcuminoids and the IC\(_{50}\) values were estimated according to the curve equation based on a polynomial model. BHT was used as a positive control.

_in-vivo_ Toxicity (Adult Zebrafishes)

Adult wild zebrafish ( _Danio rerio_,) of both sexes, aged between 60-90 days, similar size 3.5 ± 0.5 cm and weight 0.4 ± 0.1 g, were obtained from Agroquímica: Comércio de Veterinary Products LTDA, located in Fortaleza (Ceará, Brazil). The fish used in the study were acclimated for 24 hours in 10 L glass aquariums (30 x 15 x 20 cm) with tap water previously treated with anti-chlorine (Proteclor®) and air pumps with filters submerged at 25 ± 2°C and pH 7.0, under simulation of alteration of the circadian cycle (14:10 hours of light/dark). All fish were fed ad libitum during the 24 hours preceding the experiment. After the experiments, the fish were sacrificed by immersion in cold water (2-4°C) for 10 minutes, until the loss of opercular movements. All experimental procedures were approved by the Animal Use Ethics Committee of the Federal University of Ceará (CEUA-UFC) under protocol number 1806202101/2021. This entire procedure is adapted from another scientific article. On the day of the experiment, fish were randomly selected, transferred to a wet sponge, and treated orally with study samples (p.o.). Then, the fish were transferred individually to beakers (250 mL) containing 150 mL of aquarium water and were released to rest. The nanoemulsion was administered using a 20 μL variable automatic pipette with sterile tips.

The open-field test was performed to assess whether the animals’ motor coordination was affected. Initially, the animals (n = 6 / group) were treated with samples E1 5%, E1 15% and E1 25% (20 μL; p.o.). A group of untreated animals was included (Naive). After 1 hour of sample administration, the animals were transferred to glass petri dishes (10 x 15 cm), containing the same aquarium water, marked with four quadrants, then the locomotor activity was analyzed by counting the number of crossings of the line (CL) for 5 minutes. Using the line crossing value from the naive group as the baseline (100%), the percentage of locomotor activity (LA%) was calculated for each group of fish. The results obtained were expressed as mean values ± standard error of the mean (SEM) for each group of 6 animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were submitted to analysis of variance (unidirectional ANOVA), followed by the Tukey test.

The acute toxicity study was performed against adult zebrafishes according to Principles of Laboratory Animal Care. The animals (n = 6 /each) were treated with pure samples of E1 5, E1 15, and E1 25% (20 μL; p.o.) and kept at rest for analysis of mortality for 96 hours after administration of the samples. The naive (untreated) group was used and kept under the same conditions. Animals were fed normally during the test. The toxicity of the samples was evaluated by the number of dead fish. Samples that cause the death of 50% of the population are considered toxic.

**MTT assay for determination of cytotoxicity**

The human tumor cell lines used in this study were HCT-116 (colon carcinoma), SNB-19 (glioblastoma), PC3 (prostate), and HL-60 (leukemia), which were provided by the National Cancer Institute (Bethesda, MD, USA). All tumor cell lines were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg mL⁻¹ streptomycin, at 37°C with 5% CO₂. For the MTT assays, cells were plated in 96-well plates (0.1 x 10⁴ cells/ well for PC3 and SNB-19; 0.3 x 10⁴ cells/well for HL-60 cells; and 0.7 x 10⁴ cells/well for HCT-116 cells). Curcuminoids (free-drug and nanoemulsion) were tested at increasing concentrations (highest concentration of 100 μg mL⁻¹ for free drug or 50 μg mL⁻¹ for all systems). The final concentration of dimethyl sulfoxide (DMSO) in the culture medium was always kept lower than 0.1 % v/v.

The cell viability was determined by the MTT assay, adapted from another study, which consists of the reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product by metabolically active cells (Mosmann, 1983). After the incubation time (72 h at 37°C/5% CO₂), plates were centrifuged, and the supernatant was replaced with a fresh medium (100 μL) containing 0.5 mg mL⁻¹ MTT. Three hours later, plates were centrifuged, the MTT formazan product was dissolved in DMSO (100 μL), and the absorbance was measured using a multplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, California, EUA) at 525 nm. The absorbances obtained were used to calculate the IC₅₀ values (concentration capable of inhibiting 50% of cell growth) of each sample by non-linear regression using GraphPad Prism® 8.0 program. All treatments were performed in triplicate in at least four independent experiments.

**RESULTS AND DISCUSSION**

**Nuclear Magnetic Resonance (¹H NMR)**

The ¹H NMR spectrum is in Figure 1 following.

Legend: Curcumin: R₁=R₂=OCH₃; Desmethoxycurcumin: R₁=OCH₃ and R₂=H; and Bisdesmethoxycurcumin: R₁=R₂=H.

The literature reports that curcuminoids in organic solvents such as dimethyl sulfoxide (DMSO) in the 1,3-diketone form are not detected. Thus, the proton at the position α of the curcuminoids molecule appears as a singlet with...
integration for hydrogen at 6.04 ppm for curcumin, 6.13 ppm for desmethoxycurcumin, and 6.06 ppm for bisdemethoxycurcumin. In Figure 1 it is possible to observe the peaks of the aromatic rings of the protons of curcuminoids between 6.77-7.57 ppm and also the protons related to the methoxy group of curcumin and desmethoxycurcumin, with a chemical displacement of 3.84 ppm, data similar to those found in another study.\(^\text{18}\)

**Particle size, polydispersity index, zeta potential, and pH**
The values of hydrodynamic diameter, polydispersity index, and zeta potential for E1 observed were, respectively, 251.4 ± 4.6 nm, 0.26 ± 0.08, and -34.8 ± 1.0 mV. These data characterize the E1 with good colloidal stability in solution.\(^\text{19,20}\) The nanoemulsion proposed in this study has a moderately polydisperse particle size distribution, according to reports in other articles that contain curcuminoids as actives.\(^\text{21-22}\)

The pH value for E1 is 4.8. The acidity of the nanoemulsion contributes to the stabilization of the encapsulated active principle (curcuminoids) because the stability of curcumin increases in medium acidic.\(^\text{23}\) The pH values ranging from 4.2 to 5.8 are generally observed for nanoemulsions, making them non-irritating for pharmacological applications.\(^\text{24}\)

**Encapsulation efficiency**
The nanoemulsion E1 achieved Encapsulation Efficiency (EE) above 99%. Other studies\(^\text{25,11}\) that prepare emulsions containing curcumin as an active ingredient obtained EE values similar to those presented in this study, ranging from 74 to 99%.

**In-vitro release**
Figure 2 shows the release profile for E1 nanoemulsion. The standard deviation found for the percentage of release was less than 0.02 at any time.

The maximum release profile reach for nanoemulsion E1 is 9.7%, after 24 hours after the start of the experiment. The relatively low level of curcuminoids release observed is mainly due to the strong hydrogen bonds between the oil phase and/or surfactant.\(^\text{13,26}\) In addition, the low ionic strength found in the receptor medium, at pH 7.4 (\(\mu=2.51\times10^{-7}\)), also hinders the attraction of active molecules to the receptor medium.

**Antioxidant assay for DPPH free radical capture**
The concentration at which the E1 nanoemulsion inhibits the DPPH concentration by 50% is 0.34 mg/mL while that for the BHT standard the value is 0.29 mg/mL.\(^\text{13}\) This reveals that the prepared nanoemulsion presents satisfactory results in terms of antioxidant action. The antioxidant action of E1 nanoemulsion is due to the synergistic effect caused by the presence of oleic acid and curcuminoids in the formulation, which is known, as an antioxidant agent. Although the studies do not definitively prove that antioxidants have no anticancer effect, it’s easy to find authors find evidence that one property favors the Other.\(^\text{27,28}\)

**In-vivo toxicity (Adult Zebrashes)**
Figure 3a revealed that the pure oleic acid caused an impact on the central nervous system of the animals. However, the concentration of this acid in the nanoemulsion is 15% (w/w), the that not caused impact on the nanoemulsion E1 and vehicle. In Figure 3b, for all tested groups, no deaths were observed, suggesting potential for use in vivo. Authors

![Figure 1: 1H NMR of Curcuminoids mixture (500 MHz DMSO-d6).](image1)

![Figure 2: The release profile of curcuminoids for E1 nanoemulsion.](image2)

![Figure 3a: Effect of nanoemulsion 5, 15, and 25% (w/w), vehicle, and oleic acid in the locomotor activity of adult zebrafish (Danio rerio) through the Open Field Test (0–5 min). Naive: untreated animals. Vehicle: nanoemulsion E1 without curcuminoids.](image3a)

![Figure 3b: Acute toxicity study (96 hours) in adult Zebrafish model.](image3b)
report that zebrafish are suitable model organisms to study the toxic effects of curcumin, validating the results of this present study.

**Results of MTT assay for determination of cytotoxicity**

The MTT assay was performed to investigate cytotoxicity and to calculate IC$_{50}$ values for free and encapsulated curcuminoids, against four cancer cell lines (Table 1). Data are represented in IC$_{50}$ values (µg mL$^{-1}$) and correspond to the mean ± SD of at least four independent experiments performed in triplicate.

The result of the MTT assay, in Table 1, indicates that all samples used exhibited cytotoxicity to all tumor cell lines tested. However, IC$_{50}$ values for curcuminoid nanosystems (E1) and empty nanosystems were lower than for free curcuminoids, indicating that the oleic acid present in both nanosystems acted synergistically in the inhibition of cancer cells. Previous studies report that oleic acid inhibited the growth and survival of tongue squamous, gastric, and breast cell carcinoma, but the mechanisms are not fully understood.

The literature further reported a method that uses oleic acid-conjugated polymeric photosensitizer to kill metastatic cells. The sample that showed the greatest antitumor activity in all strains tested was the curcuminoid system (E1), with great potential for applications against prostate cancer. The sample that showed the greatest antitumor activity in all strains tested was the curcuminoid system (E1), with great potential for applications against prostate cancer.

**CONCLUSIONS**

The nanoemulsion proposed in this study is stable, with ideal pH for pharmaceutical applications, encapsulation efficiency above 99% and proved to be efficient carriers, releasing curcuminoids in a controlled manner. In the toxicity test in fish (Zebrafish) the nanoformulations proved to be biosafe, as they did not kill the fish, and in the evaluation of the antioxidant action (by DPPH) showed excellent antioxidant activity. Furthermore, in the in vitro cytotoxicity studies, the nanoemulsions showed good cytotoxic activity, ideal for fighting of Colorectal, Glioblastoma, and Leukemia cancers and more effectively in prostate cancer.

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**REFERENCES**


