International Journal of Pharmaceutics 643 (2023) 123195

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Curcumin or quercetin loaded nutriosomes as oral adjuvants for malaria infections

Federica Fulgheri [a], Matteo Aroffu [a], Miriam Ramírez [b,c,d], Lucía Román-Álamo [b,c,d], José Esteban Peris [e], Iris Usach [e], Amparo Nacher [e,f], Maria Manconi [a,*], Xavier Fernàndez-Busquets [b,c,d], Maria Letizia Manca [a]

[a] Dept. of Life and Environmental Sciences of the University of Cagliari, University Campus, Pad. A, S.P. Monserrato-Sestu Km 0.700, Monserrato 09042, CA, Italy
[b] Nanomalaria Group, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Baldiri Reixac 10-12, ES-08028 Barcelona, Spain
[c] Barcelona Institute for Global Health (ISGlobal, Hospital Clínic-Universitat de Barcelona), Rosselló 149-153, ES-08036 Barcelona, Spain
[d] Nanoscience and Nanotechnology Institute (IN2UB), University of Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain
[e] Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, 46100 Valencia, Spain
[f] Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

ARTICLE INFO

Keywords:
Liposomes
Nanomedicine
Nutriose® FM06
Plasmodium falciparum
Malaria infections
Oral administration

ABSTRACT

Artemisinin, curcumin or quercetin, alone or in combination, were loaded in nutriosomes, special phospholipid vesicles enriched with Nutriose FM06®, a soluble dextrin with prebiotic activity, that makes these vesicles suitable for oral delivery. The resulting nutriosomes were sized between 93 and 146 nm, homogeneously dispersed, and had slightly negative zeta potential (around −8 mV). To improve their shelf life and storability over time, vesicle dispersions were freeze-dried and stored at 25 °C. Results confirmed that their main physico-chemical characteristics remained unchanged over a period of 12 months. Additionally, their size and poly-dispersity index did not undergo any significant variation after dilution with solutions at different pHs (1.2 and 7.0) and high ionic strength, mimicking the harsh conditions of the stomach and intestine. An *in vitro* study disclosed the delayed release of curcumin and quercetin from nutriosomes (~53% at 48 h) while artemisinin was quickly released (~100% at 48 h). Cytotoxicity assays using human colon adenocarcinoma cells (Caco-2) and human umbilical vein endothelial cells (HUVECs) proved the high biocompatibility of the prepared formulations. Finally, *in vitro* antimalarial activity tests, assessed against the 3D7 strain of *Plasmodium falciparum*, confirmed the effectiveness of nutriosomes in the delivery of curcumin and quercetin, which can be used as adjuvants in the antimalaria treatment. The efficacy of artemisinin was also confirmed but not improved. Overall results proved the possible use of these formulations as an accompanying treatment of malaria infections.

1. Introduction

Malaria is an old parasitic disease that, despite being preventable and curable, remains a worrisome disease (Badmos et al., 2021). It caused around 247 million cases worldwide in 2021, most of them in Africa (World Health Organization, 2022). It mostly occurs in tropical and subtropical areas, and the causes of illness and death are directly related to poverty and economic inequality in low-income regions with poor access to medication, treatment, and healthcare (Ricci, 2012). Malaria is a life-threatening disease caused by *Plasmodium* parasites; whose most deadly form is caused by *Plasmodium falciparum*. *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* that can also cause moderate or severe symptomatology, although with a lower mortality rate (Geleta and Ketema, 2016).

Chloroquine was used as first line antimalarial for many years and continued to be an effective drug nearly 80 years later (Krafts et al., 2012), but the problem of resistance, along with the multiple side effects, has led researchers to look for alternative molecules. Artemisinin, a natural sesquiterpene lactone isolated from *Artemisia annua L.* (Asteraceae family), was found to exert a strong antimalarial effect

* Corresponding author.
E-mail address: manconi@unica.it (M. Manconi).

https://doi.org/10.1016/j.ijpharm.2023.123195
Received 13 March 2023; Received in revised form 28 June 2023; Accepted 29 June 2023
Available online 30 June 2023

without parasite resistance. Indeed, artemisinin and its derivatives are currently used as first-choice treatment in endemic countries (Arya et al., 2021). It is effective against the blood phase of parasites, preventing their development to more pathological mature stages, thus providing rapid clinical response in severe malaria (White, 2008). Current treatments combine artemisinin or its more potent derivative, dihydroartemisinin, with a second antimalarial drug that has a different mechanism of action and longer half-life (Alven and Aderibigbe, 2019; Banek et al., 2014). The combined treatments have continuously gained importance for their high efficacy level against malaria parasites, however, but reports on the failure of this combination strategy underline the need to improve the performances of current treatments, especially by increasing their poor bioavailability and short half-life and reducing drug resistance (Arya et al., 2021).

According to this purpose, in a previous study, curcumin was co-loaded in liposomes with artemisinin to ameliorate the efficacy of antimalaria treatment (Isacchi et al., 2012). Curcumin is a polyphenol traditionally used in the Chinese medicine and obtained from the rhizomes of turmeric. In the last few decades, it gained interest due to its promising beneficial activities especially when delivered in nanocarriers (Mehanny et al., 2016). In addition to its antioxidant and anti-inflammatory effects, it can exert anti-microbial activity as well, and inhibits the growth of a variety of pathogens, including *P. falciparum*, *Leishmania* and *Trypanosoma* (Koide et al., 2002; NOSE et al., 1998; Reddy et al., 2005). Quercetin is another polyphenol belonging to the class of flavonoids, having antioxidant, antiaging, anti-inflammatory, and antibacterial activities (D'Andrea, 2015), whose ability to inhibit the growth of *Plasmodium* has been previously confirmed (Ganesh et al., 2012). The combination of artemisinin with curcumin or quercetin appears promising especially if they are opportunely formulated in nanosystems, which can improve stability, bioavailability and biodistribution of the payloads facilitating the target to the parasite (Urban and Fernandez-Busquets, 2014). Among the different nanosystems, liposomes have been tested as carriers of different antimalaria drugs especially for parental administration (Isacchi et al., 2012; Rajwar et al., 2023). Recently, special phospholipid vesicles, called nutriosomes and enriched with a dextrin (Nutriose® FM06), have been tailored for oral administration, as they are more resistant than liposomes under the harsh conditions of the gastro-intestinal tract (Catalán-Latorre et al., 2018; Parekh et al., 2022). In a previous work, they were used to deliver curcumin and they were orally administered at 25 or 75 $mg \cdot kg^{-1} \cdot day^{-1}$ to mice infected with the lethal murine malaria parasite *Plasmodium yoelii* 17XL (Coma-Cros et al., 2018). The polyphenol was used in dispersion or loaded in Eudragit-hyaluronan liposomes or Eudragit-nutriosomes and only when it was loaded in Eudragit-nutriosomes, the viability of mice was significantly increased. Similarly, in a further work, curcumin-loaded Eudragit-nutriosomes were able to extend the life expectancy of *P. yoelii*-infected mice relative to that of animals treated with free curcumin and to the untreated controls (Manconi et al., 2019). The addition of Nutriose® FM06 to phospholipid vesicles permits the formation of gastrointestinal-resistant carriers more efficient than the corresponding liposomes in protecting the loaded molecules, improving their bioavailability and biodistribution (Allaw et al., 2020). Indeed, orally administered nutriosomes loaded with Nasco grape-pomace delivered the load phytochemicals to the central nervous system and protected against neurotoxicity of the dopaminergic system (Parekh et al., 2022). The dextrin used in nutriosomes acted also as a cryo-protector, preventing their breakage during a freeze-drying process (Catalán-Latorre et al., 2018).

Despite the different studies performed using nutriosomes as carriers for oral administration, they have never been tested for the delivery of first-choice antimalarial drugs alone or in combination with other phytochemicals. According to this, the aim of this study was to design a new oral formulation for malaria treatment loading artemisinin (as first-choice antimalarial drug), curcumin, or quercetin, alone or in association, in nutriosomes. Curcumin or quercetin were added since their previously confirmed effect against malaria and were expected to enhance the well-known efficacy of artemisinin. The physico-chemical and technological properties of the prepared vesicles were investigated along with their *in vitro* biocompatibility using two different cell lines (human colon adenocarcinoma epithelial cells and human umbilical vein endothelial cells). Moreover, their antiparasitic activity has been evaluated *in vitro* against 3D7 *P. falciparum*.

2. Material and methods

2.1. Materials

Soy phosphatidylcholine (Lipoid S75, S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Artemisinin was purchased from Carbosynth Ltd (London, United Kingdom). Nutriose® FM06, a soluble dextrin obtained from maize, was gently provided by Roquette (Lestrem, France). Curcumin, quercetin and other reagents and solvents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Glycerol was purchased from Carlo Erba (Milan, Italy). Water was purified through a Milli-Q system from Millipore (Milford, MA). All plastics and reagents for cells have been purchased from Sigma-Aldrich (Milan, Italy).

2.2. Quantification of artemisinin, curcumin, and quercetin

The phytochemicals were quantified using a high-performance liquid chromatograph (HPLC) Agilent equipped with a G1379A degasser, a G1310A pump, a G1329A 94 automatic injector and a G1314A variable wavelength spectrophotometric detector. The column was a Waters "Nova-Pack" C18 (4 μm, 3.9 mm × 150 mm) and the composition of the mobile phase was slightly different depending on the molecule to be analysed: acetonitrile, water, 60:40, v/v for artemisinin, acetonitrile; water, acetic acid, 49:50:1, v/v/v for curcumin, acetonitrile, water, acetic acid, 29:70:1, v/v/v for quercetin. Detection of artemisinin was performed at 216 nm, that of curcumin at 425 nm and that of quercetin at 380 nm. The injection volume was 25 μL, and the flow rate was 1 ml/min. Stock solutions (5 mg/mL) of each phytochemical were prepared using the mobile phase as solvent. From these, other working solutions at different concentrations (artemisinin from 5 to 500 μg/mL; curcumin from 0.05 to 20 μg/mL; quercetin from 0.1 to 100 μg/mL) were prepared by dilution. The calibration curve of each molecule was obtained plotting peak areas versus concentrations. The regression analysis gave a correlation coefficient value (R2) of 1 for artemisinin, 0.9998 for curcumin and 1 for quercetin (Fig. 2 SM).

Vesicles loading artemisinin, curcumin or quercetin or combinations of artemisinin and curcumin or artemisinin and quercetin were analysed after disrupting them with methanol (1/100 dilution). Alternatively, in the *in vitro* release assay, quantification was done measuring absorbance of artemisinin at 216 nm, that of curcumin at 425 nm and that of quercetin at 380 nm, as described in paragraph 2.6.

2.3. Vesicle preparation

Artemisinin (10 mg/ml) or curcumin (10 mg/mL each) or quercetin (10 mg/ml) or association of artemisinin and curcumin (10 mg/mL each) or association of artemisinin and quercetin (10 mg/mL each), were weighted in a glass vial together with Nutriose® FM06 (200 mg/mL) and S75 (260 mg/ml) and hydrated with an aqueous blend of phosphate buffered solution (PBS, pH 7.4) and glycerol (80:20) at 25 °C. The obtained dispersions were sonicated (10 cycles, 5 s on and 2 s off, 14 μ of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK). Empty vesicles (without artemisinin, curcumin or quercetin) were prepared as well, to evaluate the effect of these phytodrugs on vesicle assembling and characteristics. The dispersions were frozen at −80 °C and freeze-dried for 48 h, at −80 °C and 0.08 mbar, using a FDU-8606 freeze-dryer (Operon, Gimpo,

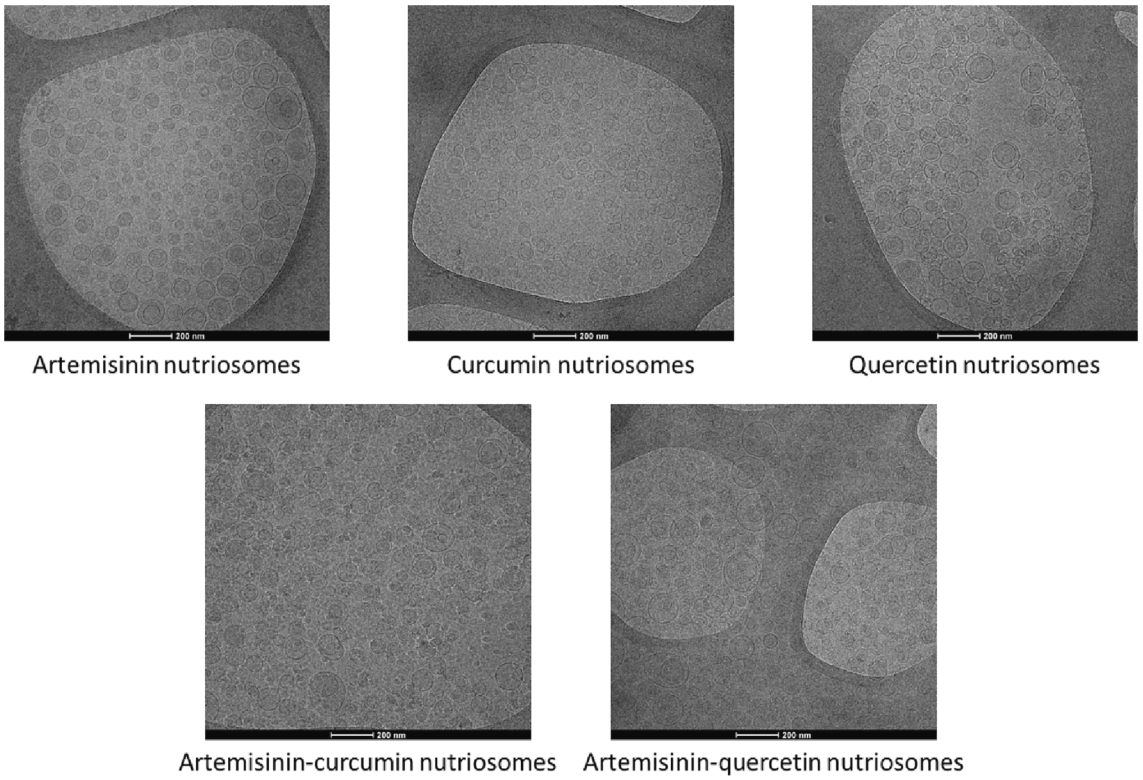

Fig. 1. Representative cryo-TEM images of freshly prepared artemisinin nutriosomes, curcumin nutriosomes, quercetin nutriosomes, artemisinin-curcumin nutriosomes and artemisinin-quercetin nutriosomes.

South Korea). Freeze-dried samples were then rehydrated with bi-distilled water at room temperature (25 °C) up to the initial volume (2 mL) by manually shaking them for 2 min.

The non-entrapped phytodrugs were separated from the vesicle dispersions by dialysis using water (4 L) as release medium. Each dispersion (1 mL) was transferred to a Spectra/Por® dialysis tube (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) and purified for 2 h at 25 °C under constant stirring, refreshing the medium after 1 h, aiming at ensuring the complete removal of the unentrapped molecules. The entrapment efficiency of payloads in vesicles was calculated as the percentage of their concentration found after dialysis versus that initially measured. The amount of artemisinin, curcumin and quercetin in each sample was determined as reported in paragraph 2.2.

2.4. Characterization of vesicles

Formation and morphology of vesicles were evaluated by cryogenic transmission electron microscopy (cryo-TEM). Sample (5 μL) was applied on a grid Lacey carbon film (Electron Microscopy Science, Hatfield, PA, USA). The grid was mounted on an automatic plunge freezing apparatus (Vitrobot FEI, Eindhoven, The Netherlands) to control humidity and temperature, immersed in liquid ethane, fast cooled from outside by liquid nitrogen, avoiding the formation of ice crystals. Observation was made at ~ -170 °C in a Tecnai F20 microscope (FEI, Eindhoven, The Netherlands) operating at 200 kV, equipped with a cryo-specimen holder Gatan 626 (Warrendale, PA, US). Digital images were recorded with an Eagle FEI camera, 4098 × 4098 pixels. Magnification between 20,000–30,000× and a de-focus range of 2–3 μm was used (Manca et al., 2019; Manconi et al., 2017).

Mean diameter and polydispersity index (a measure of the size distribution width) were evaluated by dynamic light-scattering measurements with a Zetasizer Ultra from Malvern Instruments (Worcestershire, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. Zeta potential was determined using the Zetasizer Ultra by using the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility. Before the analysis, each sample was diluted 100-folds with PBS to be optically clear and avoid the attenuation of the laser beam by the particles along with the reduction of the scattered light that can be detected (Allaw et al., 2021). Mean diameter, polydispersity index and zeta potential were evaluated before and after freeze-drying and rehydration with bi-distilled water.

2.5. Vesicle behaviour at pH 1.2 and pH 7.0, simulating the gastro-intestinal environment

A solution at pH 1.2 and high ionic strength was prepared dissolving 1.75 g of sodium chloride in 94 ml of distilled water and adding hydrochloric acid (0.1 M) up to 100 mL. A solution at pH 7.0 and high ionic strength was prepared by dissolving 0.726 g of disodium hydrogen phosphate, 0.356 g of sodium dihydrogen phosphate and 1.754 g of sodium chloride in distilled water and bringing the volume up to 100 mL. The pH was adjusted to 7.0 with a diluted solution of phosphoric acid.

To evaluate stability under the harsh conditions mimicking the gastro-intestinal environment, each vesicle dispersion was diluted 100-fold either with the solution at pH 1.2 and incubated at 37 °C for 2 h, or with the solution at pH 7.0 and incubated at 37 °C for 6 h. After incubation, the mean diameter, the polydispersity index, and the zeta potential were immediately measured, to detect possible variations connected with destabilization phenomena. Further, the amount of payloads still entrapped in the vesicles has been measured before and after dilution and incubation with the two different media to estimate their leakage.

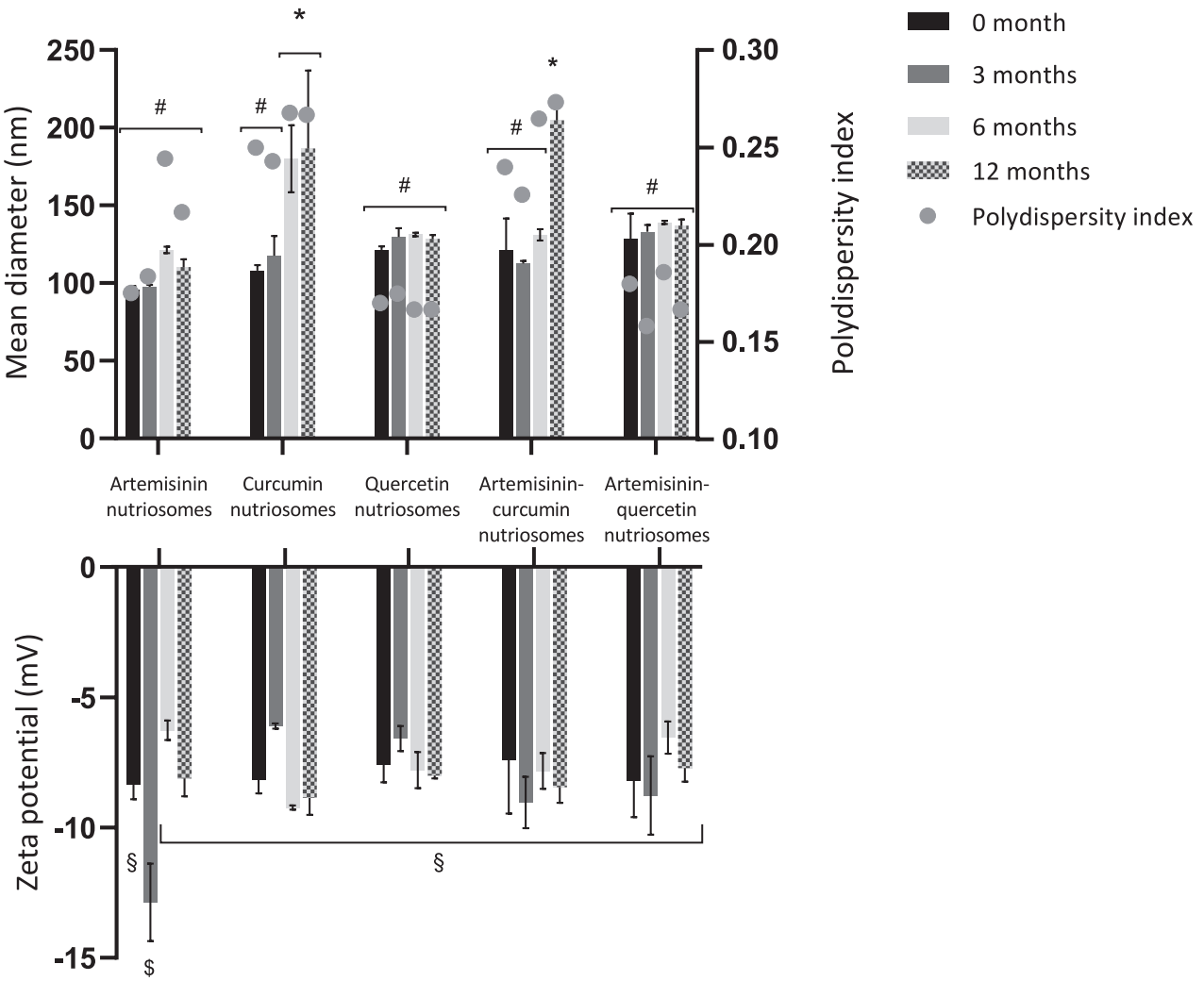

Fig. 2. Mean diameter, polydispersity index and zeta potential of nutriosomes stored for 3, 6 and 12 months at 25 °C and rehydrated before the analysis. Mean values (bars) ± standard deviations are reported (n = 3). The same symbols (*, #, §, $) indicate values that are not statistically different ($p > 0.05$).

2.6. In vitro release assay

A solution of either artemisinin, curcumin or quercetin was prepared by diluting 10 mg of the compound in a 100 mL flask with a mixture of methanol and water (50:50 v/v). Drug-loaded vesicles were diluted 1/100 using saline solution. Spectra/Por® dialysis membranes (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc) were placed between the donor and receptor compartments of Franz vertical cells. The receptor compartment was filled with saline solution (~6 mL), which was continuously stirred with a small magnetic bar and maintained at a constant temperature of 37 ± 1 °C. 100 µl of either the free drug in solution or loaded in nutriosomes opportunely diluted were placed in the donor compartment above the dialysis membrane. Every 12 h and up to 48 h, the receptor compartment's solution was replaced with an equal volume of a fresh solution. At the end of the experiment, the dialysis membrane was washed with 2 mL of methanol to recover the total amount of compound and, in the case of the nutriosomes, to break the vesicles and consequently release the eventual payload. Subsequently, the obtained solutions were assayed for curcumin and quercetin content using a plate reader and disposable 96-well plates by means of UV detection (425 nm for curcumin and 380 nm for quercetin). Artemisinin content was assayed by HPLC as reported in paragraph 2.2.

2.7. Cell viability assay

Cell viability was evaluated using two different cell lines: human colon adenocarcinoma epithelial cells (Caco-2) and human umbilical vein endothelial cells (HUVECs). Caco-2 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% of foetal bovine serum and 1% of penicillin and streptomycin. HUVECs were cultured in Medium 199 supplemented with 10% of foetal bovine serum and 1% of penicillin and streptomycin. Both Caco-2 and HUVEC cells were maintained at 37 °C with 5% carbon dioxide. Cells were subcultured every two days. For the viability test, cells were seeded in 96-well plates at a density of 10,000 cells/well for Caco-2 and 5000 cells/well for HUVECs. After a 24-h incubation, medium was removed, and cells were then exposed to different concentrations (40, 20, 10, 5, 2.5 µg/mL of each bioactive molecule) of either the drugs in dimethyl sulfoxide or loaded in vesicles. After 48 h, medium was renewed and 10 µL of resazurin solution (0.125 mg/ml) was added to each well (Rodríguez-Corrales and Josan, 2017). After 6 h, the fluorescent signal generated from the resorufin, proportional to the number of living cells in the sample, was measured at 530 nm excitation wavelength and 590 nm emission wavelength using a Tecan Infinity 200 Pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Cell viability was calculated as percentage of living cells in comparison to the untreated control cells (100% viability).

2.8. In vitro P. falciparum growth inhibition assay

P. falciparum 3D7 parasites were cultured in human B^+ erythrocytes at 37 °C under a gas mixture of 92.5% nitrogen, 5.5% carbon dioxide, and 2% oxygen, using complete Roswell Park Memorial Institute (RPMI) 1640 medium (supplemented with 2 mM L-glutamine, 50 μM hypoxanthine, 5 g/L Albumax II, 25 mM HEPES, pH 7.2). The medium was changed every two days maintaining 1% parasitaemia and 3% haematocrit. Parasitaemia was determined by observing under the microscope blood smears of the culture previously fixed with methanol and stained with Giemsa diluted 1:10 in Sorenson's buffer (pH 7.2) for 10 min. For the growth inhibition assay, sorbitol synchronization was performed to obtain ring forms, parasitaemia adjusted to 1.5% and haematocrit to 6% (Lambros and Vanderberg, 1979). 75 μL of the culture were seeded in 96-well plates with 75 μL of the vesicle dispersions or free drug solutions at different concentrations obtained by serial 1:2 dilutions (artemisinin solution and artemisinin nutriosomes from 0.250 to 0.002 μg/mL; artemisinin-curcumin and artemisinin-quercetin nutriosomes from 2.500 to 0.020 μg/mL; quercetin solution from 25 to 1.195 μg/mL; curcumin solution and quercetin nutriosomes from 50 to 0.391 μg/mL; curcumin nutriosomes from 75 to 0.586 μg/mL). Plates were incubated for 48 h as described above. The percentage of parasitaemia was calculated as the value found in erythrocytes treated with vesicle formulations or free drug solutions compared to that of untreated control erythrocytes. The growth inhibition graphs and IC50 values were obtained through sigmoidal fitting of growth data at different drug concentrations, analysed with the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

2.9. Ethical issues

The human blood used in this work was from voluntary donors and commercially obtained from the Banc de Sang i Teixits (https://www.bancsang.net). Blood was not collected specifically for this research; the purchased units had been discarded for transfusion, usually because of an excess of blood relative to anticoagulant solution. Prior to their use, blood units underwent the analytical checks according to the current legislation. Before being delivered to us, unit data were anonymized and irreversibly dissociated, and any identification tag or label had been removed to guarantee the non-identification of the blood donor. No blood data were or will be supplied, in accordance with the current Spanish Ley Orgánica de Protección de Datos and Ley de Investigacion Biomédica. The blood samples will not be used for studies other than those made explicit in this research.

2.10. Statistical analysis of data

Results are expressed as the means ± standard deviations. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between groups, while Student's *t*-test was used to compare two samples. Significance was tested at the 0.05 level of probability (p). Data analysis was carried out with the software package XLStatistic for Excel.

3. Results

3.1. Vesicle preparation and characterization

Vesicles have been prepared by direct sonication, which is a one-step easy method specifically used to produce small vesicles avoiding the use of organic solvents. Artemisinin (10 mg/ml), curcumin (10 mg/ml) or quercetin (10 mg/ml), alone or in association, were loaded in nutriosomes, which have previously demonstrated to be ideal phospholipid vesicles for oral administration of natural bioactive molecules (Rezvani et al., 2019). To effectively load the phytochemicals, a preformulation study was performed aiming at finding the most appropriate type and amount of phospholipid, the ideal amount of payloads to be entrapped and the type and amount of additives capable of ensuring a better stability of the final dispersions (Table 1-2SM). In the simplest cases, using phospholipid at lower concentration, the payloads, alone or combined, were not entrapped, and quickly precipitated forming two-phase dispersions. Due to the formation of large aggregates, it was not possible to measure mean diameter and polydispersity of these samples. The addition of Nutriose® FM06 among the solid components and glycerol in the aqueous phase as structuring and thickening agents, permitted the formation of more homogenous dispersions, but even with these additives the formation of a precipitate was observed as well, although in a longer time (after 2 weeks). To avoid these phenomena, especially the loss of playloads and to improve the stability of the dispersions, vesicles were freeze-dried and rehydrated just before the use, simply adding the correct amount of water (2 ml), and gently shaking them manually (for 2 min). Nutriose® FM06 acted as cryoprotectant, which avoided the break of vesicles during the freeze-drying process and allowed the immediate and simple rehydration. The effective formation of the vesicles and their morphology were confirmed by direct observation by means of cryo-TEM, which revealed the formation of uni- and oligolamellar spherical vesicles (Fig. 1).

Mean diameter, polydispersity index and zeta potential of vesicles were measured immediately after sonication and after freeze-drying and rehydration (Table 1 and Table 2SM).

Nutriosomes loading artemisinin were the smallest (~95 nm) and had a low polydispersity index (0.18); the lyophilization and rehydration process did not significantly modify the size (~93 nm). Before freeze-drying, curcumin loading nutriosomes were larger (~152 nm) than those loading artemisinin (~95 nm) and had the highest polydispersity index (~0.34), indicating a polydisperse and unstable dispersion. Freeze-dried and rehydrated curcumin nutriosomes were similar to those measured after sonication, and to all the other samples, as their size was ~ 108 nm. The simultaneous loading of curcumin and artemisinin did not affect the size of the vesicles (~111 nm) and the polydispersity index remained high (0.26) but slightly lower than that of curcumin loaded nutriosomes (~0.34). The rehydration of freeze-dried artemisinin-curcumin nutriosomes led to the formation of vesicles with a size (~121 nm) not statistically different from the others and with a similar polydispersity index (0.24). Before and after freeze-drying, quercetin loaded nutriosomes had the same size (~123) and a low polydispersity index (0.15 before and 0.17 after freeze-drying), indicating a monodispersed sample. The co-loading of quercetin with artemisinin did not affect the vesicle size (~119 nm) and the sample remained monodispersed (polydispersity index was 0.13). Empty nutriosomes after sonication were sized ~ 114 nm. The mean diameter of empty nutriosomes did not statistically increase after lyophilization and rehydration processes (~130 nm), while the polydispersity index increased from 0.16 to 0.24.

Before and after freeze-drying, the zeta potential was not affected by the payloads (~-9 mV) and the negative values were likely mostly due to the negative groups of phosphatidylcholines facing the inter-vesicle medium (Taylor et al., 2007).

The entrapment efficiency of artemisinin with or without curcumin or quercetin was the lowest (~19%). Entrapment efficiency of quercetin nutriosomes was ~ 45% and increased when it was co-loaded with artemisinin (~70%). Differently, the entrapment efficiency of curcumin nutriosomes was ~ 62%, which was the highest value among nutriosomes loading a single compound. The entrapment efficiency of curcumin decreased when it was co-loaded with artemisinin (~48%). There was no significant difference between the entrapment efficiency of each sample before and after freeze-drying (Table 1).

To evaluate the stability on storage, freeze-dried samples were stored at 25 °C, manually rehydrated by gentle shaking, at scheduled time points (3, 6 and 12 months) and analysed to measure their mean diameter, polydispersity index and zeta potential (Fig. 2). Parameters of quercetin and artemisinin-quercetin loaded nutriosomes remained

Table 1
Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of nutriosomes before and after freeze-drying. Entrapment efficiency (EE) of the samples before and after freeze-drying. Mean values ± standard deviations obtained from at least 3 samples are reported. The same symbols ($^{\circ}$, $^{\#}$, *, $^{+}$, §, $^{\&}$, $^{\$}$) indicate values that are not statistically different ($p > 0.05$).

Sample	Before freeze-drying				After freeze-drying			
	MD (nm)	PI	ZP (mV)	EE (%)	MD (nm)	PI	ZP (mV)	EE (%)
Artemisinin nutriosomes	$95^{*} \pm 3$	0.18	$-10^{*+} \pm 1$	$22^{\S} \pm 10$	$93^{*} \pm 2$	0.17	$-8^{+} \pm 1$	$16^{\S} \pm 14$
Curcumin nutriosomes	$152^{\#} \pm 48$	0.34	$-10^{*+} \pm 1$	$65^{\&} \pm 10$	$108^{\circ\#} \pm 4$	0.25	$-8^{+} \pm 1$	$62^{\&} \pm 21$
Quercetin nutriosomes	$125^{\circ\#} \pm 2$	0.15	$-11^{*} \pm 1$	$51^{\$} \pm 10$	$121^{\circ\#} \pm 2$	0.17	$-8^{+} \pm 1$	$45^{\$} \pm 8$
Artemisinin- curcumin nutriosomes	$111^{\circ\#} \pm 1$	0.26	$-8^{+} \pm 1$	$22^{\S} \pm 7$ $60^{\$} \pm 10$	$121^{\circ\#} \pm 21$	0.24	$-7^{+} \pm 2$	$20^{\S} \pm 17$ $48^{\$} \pm 15$
Artemisinin- quercetin nutriosomes	$119^{\circ\#} \pm 4$	0.13	$-8^{+} \pm 1$	$21^{\S} \pm 5$ $67^{\&} \pm 13$	$128^{\circ\#} \pm 16$	0.18	$-8^{+} \pm 1$	$21^{\S} \pm 18$ $70^{\&} \pm 25$
Empty nutriosomes	$114^{\circ\#} \pm 8$	0.16	$-10^{*+} \pm 1$	–	$146^{\circ\#} \pm 40$	0.24	$-10^{*+} \pm 1$	–

Table 2
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of the vesicles diluted and incubated at 37 °C, at pH 1.2 for 2 h (t_{2h}) and at pH 7.0 for 6 h (t_{6h}). Mean values ± standard deviation obtained from at least 3 replicates are reported. The same symbols (*, $^{\circ}$, $^{\$}$, $^{+}$, §, $^{@}$, $^{\#}$, $^{\&}$) indicate values that are not statistically different ($p > 0.05$).

Sample	Time	MD (nm)		PI		ZP (mV)		EE (%)	
		pH 1.2	pH 7	pH 1.2	pH 7	pH 1.2	pH 7	pH 1.2	pH 7
Artemisinin nutriosomes	$t_{2h/6h}$	$118^{*} \pm 5$	$123^{*\$} \pm 8$	0.15	0.19	$+5^{\#} \pm 4$	$-5^{\&} \pm 1$	19 ± 10	19 ± 12
Curcumin nutriosomes	$t_{2h/6h}$	$122^{*\circ} \pm 3$	$122^{*\$+} \pm 2$	0.20	0.18	$+7^{\#} \pm 3$	$-5^{\&} \pm 2$	65 ± 13	65 ± 18
Quercetin nutriosomes	$t_{2h/6h}$	$149^{\S} \pm 3$	$159^{\S} \pm 4$	0.20	0.17	$+6^{\#} \pm 3$	$-6^{\&} \pm 2$	49 ± 8	49 ± 10
Artemisinin-curcumin nutriosomes	$t_{2h/6h}$	$132^{\circ\$} \pm 5$	$134^{\circ\$+} \pm 5$	0.17	0.18	$+3^{\#} \pm 3$	$-4^{\&} \pm 3$	18 ± 6 61 ± 11	18 ± 10 64 ± 10
Artemisinin-quercetin nutriosomes	$t_{2h/6h}$	$187^{@} \pm 8$	$195^{@} \pm 8$	0.24	0.17	$+5^{\#} \pm 4$	$-5^{\&} \pm 2$	19 ± 4 63 ± 14	19 ± 8 63 ± 14

Table 3
IC50 values (μg/ml) of artemisinin, curcumin and quercetin in solution or loaded in nutriosomes against *in vitro P. falciparum* 3D7 cultures. Mean values ± standard deviations are reported (n = 3). The same symbols (*, $^{\#}$, §) indicate values that are not statistically different ($p > 0.05$).

Samples	IC50 (μg/ml)
Artemisinin solution	$0.006^{*} \pm 0.004$
Curcumin solution	$3.949^{\#} \pm 1.277$
Quercetin solution	$7.258^{\#\S} \pm 2.613$
Artemisinin nutriosomes	$0.070^{*} \pm 0.036$
Curcumin nutriosomes	$8.777^{\S} \pm 0.649$
Quercetin nutriosomes	$7.020^{\#\S} \pm 2.664$
Artemisinin-curcumin nutriosomes	$0.149^{*} \pm 0.057$
Artemisinin-quercetin nutriosomes	$0.062^{*} \pm 0.024$

constant for 12 months confirming their good stability probably related to a positive effect of quercetin on vesicle assembling and stability. In the case of artemisinin nutriosomes size remained constant (~106 nm) but the polydispersity index increased up to 0.24 and 0.22 at 6 and 12 months respectively. At 6 and 12 months, the size and polydispersity index of curcumin loaded nutriosomes significantly increased up to ~ 183 nm and 0.27. Artemisinin-curcumin nutriosomes followed the same path, but the increase of size was particularly evident at 12 months (~205 nm) while the polydispersity index reached higher values starting from 6 months (~0.27). Overall, quercetin and artemisinin-quercetin nutriosomes were the most stable formulations, being able to maintain low mean diameter and polydispersity index values over 12 months storage.

3.2. Nutriosome stability at pH 1.2 and 7.0

Nutriosomes were diluted with solution at pH 1.2 or 7.0 and high ionic strength and incubated at 37 °C to evaluate their possible behaviour in the stomach and intestine after oral administration. Indeed, a strong change in vesicle parameter indicates a possible break or aggregation of vesicles, which in turn is generally associated with the leakage of payloads. Artemisinin-curcumin nutriosomes were able to resist under these harsh conditions without breaking, since their size remained unvaried at 2 h in acidic solution or 6 h in neutral solution (~129 nm), confirming their potential stability in gastrointestinal fluids (Table 2). The other samples underwent a small increase in size at 2 and 6 h, but they remained homogenously dispersed, as the polydispersity index remained lower than 0.24.

At 2 h and pH 1.2, an inversion of zeta potential was observed for each formulation, due to the presence of H^{+} in the acidic medium, which coated the vesicle surface, while the zeta potential remained unchanged at 6 h and pH 7.0 (Obata et al., 2010). Nutriosomes were able to retain the payloads during the incubation period, at pH 1.2 and 7.0, as the entrapment efficiency of each sample after incubation was not statistically different from that of the corresponding freshly prepared sample (Table 2).

3.3. In vitro release assay

The amount of payloads released from nutriosomes was measured and compared with that released through the same membrane filled with the solutions of curcumin, quercetin or artemisinin at the same concentration (Fig. 3). Using the solution, the three bioactive molecules were almost completely released at 48 h (curcumin ~ 90%, quercetin ~ 95% and artemisinin ~ 100%). Using artemisinin-loaded nutriosomes, artemisinin was also completely released at 48 h ~ 100%). Artemisinin was already mostly released at 24 h, both in solution and loaded in nutriosomes (~86%). Differently, when curcumin and quercetin were loaded in nutriosomes, the release was significantly slowed as only ~ 53% of each bioactive molecule was released at 48 h, confirming the carrier ability to control the release of the loaded molecules. Using curcumin or quercetin nutriosomes, the first amount of payload was quickly released because, likely, it was not actually entrapped inside the vesicles, while the release of entrapped payload was retarded (curcumin ~ 62% and quercetin ~ 45%). Differently, the entrapment efficiency of

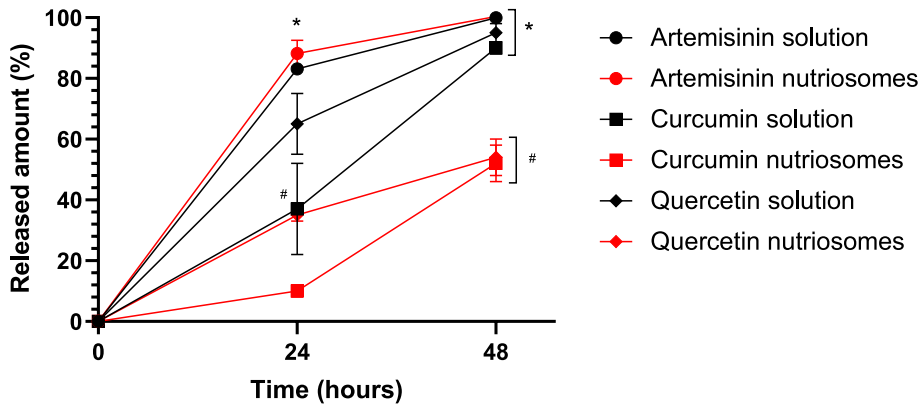

Fig. 3. Released amount (%) of artemisinin, curcumin, and quercetin through a dialysis membrane filled with these phytodrugs in water solution or in nutriosome dispersions over 48 h. Mean values ± standard deviations are reported (n = 3). The same symbols (*, #) indicate values that are not statistically different ($p > 0.05$).

artemisinin was very low (~19%) and its release was equal to that obtained with the drug in solution. These results indicates that nutriosomes seem to be optimal carriers for curcumin and quercetin but not for artemisinin.

3.4. Cell viability assay

Cell viability was evaluated using the resazurin assay, which is a simple, fast, and sensitive test (Rodríguez-Corrales and Josan, 2017). It permits to measure cell viability through the reduction, live cell mediated, of resazurin to the fluorescent product resorufin. Being the formulations designed for oral administration, Caco-2 cells were selected as a model of the intestinal epithelial barrier. HUVECs are very sensitive cells that are widely used for cytotoxicity assay and therefore were used as well for this assay. No significant toxicity has been observed neither treating Caco-2 nor HUVECs with curcumin-, quercetin- and artemisinin-loaded nutriosomes Fig. 4. Artemisinin solution at the highest concentration used (40 μg/mL) was highly biocompatible as the viability of Caco-2 cells was ~ 89% and that of HUVECs was ~ 99%. Using the lower concentrations, the viability was ≥ 100%. Treating Caco-2 cells with quercetin solution, at the highest concentration (40 μg/mL), the viability was lower (~73%) and increased up to ~ 100% using lower concentrations. When quercetin (alone or in combination with artemisinin) was loaded in the vesicles at the highest concentration (40 μg/mL) the viability was higher than 100%, probably because of the carrier ability to reduce the payload toxicity. On the other hand, when Caco-2 cells were treated with curcumin solution at 40 and 20 μg/mL, a high cytotoxicity was observed (viability ~ 1%); in contrast, when the cells were treated with curcumin and artemisinin-curcumin (40 and 20 μg/mL) loaded in nutriosomes, the viability was higher than 80%. The viability of HUVECs treated with quercetin solution at 40 μg/mL was ~ 46%, at 20 μg/mL was ~ 84%, and at lower concentrations was higher than 100%. When quercetin was loaded in nutriosomes, alone or with artemisinin, the viability was higher than 85%, irrespective of the used concentration. Similarly to Caco-2, treatment of HUVECs with curcumin solution provided the lowest cell viability values, which were lower than 20% using 40, 20, 10, 5 μg/mL and ~ 80% using 2.5 μg/mL. In a similar manner, HUVEC viability increased treating them with curcumin loaded in nutriosomes, with or without artemisinin, as the viability was ~ 68% using 40 μg/mL, ~93% using 20 μg/mL and higher than 100% at lower concentrations. These results underline that curcumin was toxic in solution at the highest concentrations, quercetin was less toxic, and

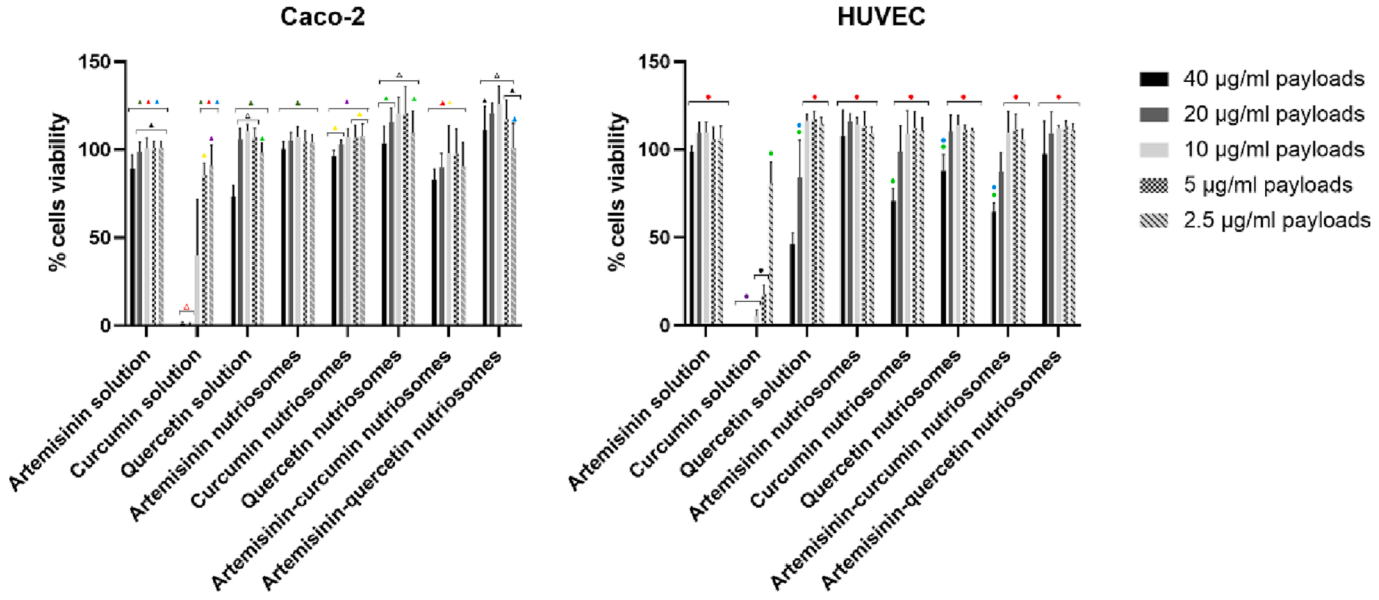

Fig. 4. Viability of Caco-2 (left panel) and HUVEC (right panel) cells, incubated with artemisinin, curcumin, or quercetin in solution or loaded in nutriosomes and diluted to reach 40, 20, 10, 5 and 2.5 μg/mL of each payload. Mean values (bars) ± standard deviations are reported. In each panel, the same symbols indicate values that are not statistically different ($p > 0.05$)..

artemisinin did not cause any toxicity, using the same concentrations. Anyways, their loading in nutriosomes reduced the toxicity of curcumin and quercetin also at the highest concentrations tested.

3.5. *P. falciparum* growth inhibition assay

The ability of formulations to inhibit the growth of *P. falciparum* cultured in human B + erythrocytes was evaluated (Table 3). As expected, the *in vitro* antimalarial activity of artemisinin was the highest and there was no statistical differences among the solution and the nutriosomes loading artemisinin alone or in combination with curcumin or quercetin, their IC50 ranged from 0.006 to 0.149 μg/mL ($p > 0.05$ among these values). The IC50 of curcumin solution was ~ 3.949 μg/ml and that of quercetin solution was ~ 7.258 μg/mL ($p > 0.05$ between the two values), confirming that their efficacy was lower than that of artemisinin, which is a potent antimalaria drug. The effect of quercetin was not affected by its loading in nutriosomes (IC50 ~ 7.020 μg/mL) while that of curcumin was slightly affected. Indeed, when curcumin was loaded in nutriosomes the IC50 was higher (~8.777 μg/mL, $p < 0.05$ versus the IC50 of curcumin and quercetin solutions) than that of free curcumin. The co-loading of curcumin or quercetin with artemisinin in nutriosomes had a negligible effect on parasite growth compared to that of artemisinin loaded nutriosomes, as their IC50 was ~ 0.149 and 0.062 μg/mL ($p > 0.05$ between the two values), since their effect was masked, thus unmeasurable, by that of the most potent artemisinin (Table 3).

4. Discussion

Curcumin, quercetin and artemisinin (10 mg/mL) were successfully loaded in nutriosomes alone or alternatively artemisinin was co-loaded with curcumin or quercetin. Nutriosomes are special and effective phospholipid vesicles enriched with Nutriose® FM06 and specifically tailored for oral administration, which have demonstrated to be ideal for the delivery of curcumin and other natural molecules or extracts (Allaw et al., 2020; Catalán-Latorre et al., 2018). Nutriose® FM06 is a water-soluble branched dextrin with high levels of fibre content, which did not form gelled dispersions in water. However, its addition to lamellar vesicles exerts a triple function since it improves the vesicle resistance during their passage through the gastro-intestinal environment, increases the loading of lipophilic payloads and facilitates the vesicle reforming after freezer-drying, acting as cryoprotectant (Marinho et al., 2021). Actually, nutriosomes were stable, small and monodispersed, except those loading curcumin (alone or in association with artemisinin), which were bigger and slightly polydispersed probably due to the impossibility to intercalate all the used payload (10 mg/mL) inside the bilayer. Indeed, curcumin is generally loaded at lower concentrations (10–100 folds lower) in phospholipid vesicles as it is a large molecule with a complex structure (Schmitt et al., 2020; Wang et al., 2021). Artemisinin is also very difficult to be loaded in liposomes in high amount and when loaded in liposomes, it was usually tested for parenteral administration at lower concentrations (Isacchi et al., 2011; Valissery et al., 2020). Nutriosomes were able to load a small amount of artemisinin (~19%), while curcumin and quercetin were loaded in higher amount, ~62 and ~ 45% when loaded alone, and ~ 48 and ~ 70% when loaded with artemisinin. The freeze-drying process did not lead to payload leakage, as there was no significant difference in terms of entrapment efficiency between each sample before and after freeze-drying.

Due to the lower amount of artemisinin entrapped in nutriosomes, these vesicles appeared to be more suitable for incorporating phenolic molecules and less for sesquiterpene tetracyclic endoperoxylactones, which are not as much polar and have a specific steric conformation (Jahan et al., 2021), whose characteristics drastically affected its intercalation inside the nutriosome bilayer thus reducing the entrapment efficiency. Results are in agreement with previous ones, in which the low entrapment efficiency was attributed to the size of prepared vesicles: the smallest the vesicles, the lowest the entrapment (Dadgar et al., 2013; Isacchi et al., 2011; Righeschi et al., 2014). Actually, artemisinin loaded nutriosomes were the smallest (~95 nm), and clearly the addition of curcumin or quercetin in artemisinin loaded nutriosomes modified this ratio, because the additional phytochemicals contribute to the enlargement of the vesicles. Indeed, the size of nutriosomes loading artemisinin and curcumin or quercetin was not statistically different from that of curcumin or quercetin loaded nutriosomes. These results suggested a key role of artemisinin in modulating the assembling of the phospholipids (which led to the formation of smaller vesicles) and confirmed the theory according to which its entrapment is highly affected by the mean size of the final system (Chen et al., 2015; Dadgar et al., 2013; Dwivedi et al., 2015; Jin et al., 2013).

The stability of vesicle dispersions on storage was inadequate, especially that of vesicles loading artemisinin, for this reason they were freeze-dried to obtain a stable powder easily re-dispersible just before use, by gentle manual shaking, avoiding the use of physical inputs, like sonication. In this way, freeze-dried formulations can be orally administered as capsules or tablets, or even as dispersion after their simple rehydration. The rehydrated vesicles had size and polydispersity index comparable or lower than those of the same vesicles before the freeze-drying, thanks to the presence of Nutriose® FM06, which acting as cryoprotectant avoided vesicle break and allowed their reforming (Karinawatie et al., 2008). In their dried state, vesicle dispersions were stable for 12 months, and after rehydration only the mean diameter and polydispersity index of nutriosomes loading curcumin, alone or in association with artemisinin, increased. The loading of such high amount (10 mg/mL) of artemisinin permits to easily reach the required dose, which is around 500 mg per day in adults (Ashton et al., 1998). It is important to note that the highest part of used drug was not actually entrapped inside the vesicles, as the entrapment efficiency was ~ 19% and this caused the immediate release of the drug from the vesicles (~86% at 24 h and ~ 100% at 48 h). On the contrary, as previously reported, nutriosomes are optimal carriers for polyphenol delivery, imparting a delayed release (~10% of released curcumin and ~ 35% of released quercetin at 24 h and ~ 53% of both at 48 h). This low entrapment efficiency and rapid release of artemisinin can negatively affect its oral delivery and systemic bioavailability. However, the vesicles were stable under the harsh conditions of the gastro-intestinal tract and their vesicle size, polydispersity index and zeta potential did not change after dilution in acidic and neutral media with high ionic strength, indicating that the destabilization phenomena are avoided (Wang et al., 2021). Nutriosomes kept their carrier structure intact, prevented the leakage of payloads, and could facilitate the passage through the intestinal membrane of the molecules loaded, such as curcumin and quercetin, and improve their systemic efficacy, as previously confirmed by *in vivo* studies performed with curcumin and other polyphenols extracted from grape by-products (Parekh et al., 2022). This advantage is expected to improve their intestinal absorption and bioavailability as previously confirmed for curcumin (Catalán-Latorre et al., 2018).

The efficacy of formulations was tested *in vitro*, measuring their ability to inhibit the growth of *P. falciparum* in human B + erythrocytes (Bouzón-Arnáiz et al., 2022). In these conditions, the performances of nutriosomes, which are oral carriers, were negligible and the effectiveness of artemisinin-loaded nutriosomes was lower than that of the drug solution while that of curcumin or quercetin nutriosomes was comparable to that of the corresponding free polyphenol solutions. Additionally, the co-loading of quercetin and curcumin with artemisinin in nutriosomes did not improve the formulation efficacy, but maintained the efficacy of artemisinin alone, probably because the effect of polyphenols was masked by that of the more potent artemisinin.

In previous works, curcumin loaded nutriosomes demonstrated to be effective in inhibiting the *in vivo* growth of *P. berghei* (Coma-Cros et al., 2018; Manconi et al., 2019). Nevertheless, in the light of the obtained results, nutriosomes do not seem to be suitable for the oral delivery of

artemisinin, as they did not actually entrap the drug. Nutriosomes seem to be effective carriers for polyphenols like curcumin and quercetin, especially the latter, which were usefully loaded and their release delayed. The loading in vesicles did not affect the *in vitro* efficacy of payloads, as expected being nutriosomes tailored for oral delivery and not suitable to be directly added to erythrocytes. Indeed, drugs must pass the intestinal barrier inside the carrier and are expected to arrive in the systemic circulation in the free form. Clearly, curcumin and quercetin even if loaded in nutriosomes, cannot be considered comparable to artemisinin but their oral administration can improve its effect, as previously found.

5. Conclusion

In the present work, nutriosomes incorporating 10 mg/mL of artemisinin, curcumin or quercetin, alone or in association, were successfully obtained, stored in de-hydrated form and rehydrated before the use by gentle manual shaking. Vesicles seemed to be resistant to gastrointestinal chemical stressors but unfortunately, only entrapped and retained a low concentration of artemisinin (~19%) and did not improve its *in vitro* efficacy against *P. falciparum*. Nutriosomes better loaded and retained curcumin and quercetin and their efficacy was comparable to that of free drug, the form in which the delivered payload can reach the systemic circulation. Overall results, underline good carrier ability of nutriosomes in the delivery of polyphenols but not in that of terpenic molecules, thus, curcumin and quercetin nutriosomes can be tested as adjuvants in malaria treatment while their co-loading with artemisinin in nutriosomes is not suitable.

CRediT authorship contribution statement

Federica Fulgheri: Investigation, Formal analysis, Data curation, Writing – original draft. **Matteo Aroffu:** Validation, Writing – review & editing. **Miriam Ramírez:** Methodology, Validation, Writing – review & editing. **Lucía Román-Álamo:** Methodology, Validation, Writing – review & editing. **José Esteban Peris:** Investigation, Methodology, Validation, Writing – review & editing. **Iris Usach:** Investigation, Methodology, Validation, Writing – review & editing. **Amparo Nacher:** Methodology, Validation, Writing – review & editing. **Maria Manconi:** Supervision, Project administration, Methodology, Validation, Writing – review & editing. **Xavier Fernàndez-Busquets:** Supervision, Methodology, Validation, Writing – review & editing. **Maria Letizia Manca:** Supervision, Project administration, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

Acknowledgement

The authors thank MIUR and PON R&I 2014-2022 for supporting the PhD grant number DOT1304004.

We acknowledge support from the grant CEX2018-000806-S funded by MCIN/AEI/ 10.13039/501100011033, and support from the Generalitat de Catalunya through the CERCA Program.

This research is part of the ISGlobal's Program on the Molecular Mechanisms of Malaria which is partially supported by the Fundación Ramón Areces.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.123195.

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