Journal of Drug Delivery Science and Technology 107 (2025) 106828

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

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

Preliminary evaluation of the *in vitro* and *in vivo* efficacy of a novel nanovesicle-doped nanoemulsion co-loading artemisinin and quercetin as a promising strategy to improve the oral malaria therapy

Federica Fulgheri [a], Miriam Ramírez [b,c], Lucía Román-Álamo [b,c], Paolo Gasco [d], Maria Manconi [a], Matteo Aroffu [a,*], Rita Abi Rached [a], Biancamaria Baroli [a], Xavier Fernàndez-Busquets [b,c], Maria Letizia Manca [a]

[a] Deptartment of Life and Environmental Sciences, University of Cagliari, University Campus, S.P. Monserrato-Sestu Km 0.700, Monserrato, 09042, CA, Italy
[b] Barcelona Institute for Global Health (ISGlobal, Hospital Clínic-Universitat de Barcelona), Rosselló 149-153, ES-08036, Barcelona, Spain
[c] Nanomalaria Group, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Baldiri Reixac 10-12, ES-08028, Barcelona, Spain
[d] Nanovector S.r.l., Via Livorno, 60-10144, Torino, Italy

ARTICLE INFO

Keywords:
Hybrid nanosystems
Flavonoids
Plasmodium falciparum
Caco-2 cells
HUVEC
Malaria infection

ABSTRACT

Since malaria is still a major problem for human health, especially in developing countries, the association of the most effective antimalarial drugs with complementary adjuvants and their loading in suitable delivery nanosystems seems promising to improve therapy effectiveness and minimize side effects. In this study, nanovesicle-doped nanoemulsions were developed and tested for the oral co-delivery of artemisinin and quercetin, using an easy, low-cost, and reproducible method along with cheap and naturally occurring components. The same formulations loading artemisinin or quercetin alone were prepared and used as comparison. Cryogenic transmission electron microscopy confirmed the formation of hybrid dispersions in which nanosized oil droplets coexisted with lamellar vesicles; both were sized ~61 nm and had a negative zeta potential (~-47 mV). Formulations were stable when diluted at gastrointestinal pHs and high ionic strength and had low *in vitro* cytotoxicity against Caco-2 and human umbilical vein endothelial cell lines (<15 %) when the lower concentrations were used (20, 10, 5, and 2.5 μg/mL). Nanovesicle-doped nanoemulsions co-loading artemisinin and quercetin effectively inhibited *in vitro* the growth of the 3D7 strain of *Plasmodium falciparum* with a half-maximal inhibitory concentration of 9 ng/mL. Following their oral administration *in vivo* to *Plasmodium yoelii yoelii* 17XL-infected mice, they improved the survival rate with respect to the animals treated with only the artemisinin solution. Results were promising since 33 % of mice treated with the nanovesicle-doped nanoemulsions co-loading artemisinin and quercetin completely recovered after clearing parasitaemia below detectable levels and developed immunity to malaria (no parasites were detected in blood upon reinfection without treatment). All in all, this study demonstrates the potential of nanovesicle-doped nanoemulsions as an effective oral delivery strategy for artemisinin-based combination therapies.

1. Introduction

Notwithstanding significant progress in malaria control over the last two decades, which has led to a reduction in case incidence and mortality rates, this disease remains a major global threat in developing countries, accounting for around 263 million cases and 597,000 estimated deaths worldwide in 2023 [1]. Artemisinin, extracted from the Chinese herbal plant *Artemisia annua* L. (Qinghao), has been widely used in traditional Chinese medicine. The Nobel Prize-winning scientist Youyou Tu first reported its antimalarial activity, leading to its development as the frontline treatment for uncomplicated malaria caused by *Plasmodium falciparum* (*P. falciparum*) [2]. Artemisinin and their derivatives (i.e., artesunate, artemether) – also known globally as artemisinins – are sesquiterpene lactones containing an unusual peroxide bridge, which is essential for their antimalarial activity [3]. This activity is characterized by a reduction in parasitaemia that occurs almost

* Corresponding author. Dept. Life and Environmental Sciences, Italy.
E-mail address: matteo.aroffu@unica.it (M. Aroffu).

https://doi.org/10.1016/j.jddst.2025.106828
Received 17 December 2024; Received in revised form 14 March 2025; Accepted 16 March 2025
Available online 17 March 2025

immediately after administration [4]. Following oral, parenteral, or rectal administration, these compounds are rapidly transformed into dihydroartemisinin, the active metabolite responsible for their therapeutic effect [5–7]. The current frontline treatments for uncomplicated malaria caused by *P. falciparum* consist of a 3-day oral administration of *Artemisia annua*-derived compounds combined with synergistic antimalarial drugs possessing a longer half-life [7–12]. These so-called artemisinin-based combination therapies are needed since artemisinin derivatives reach concentration peaks in 1–2 h after administration and are inactivated through hepatic metabolism within 0.5 and 3 h [13,14]. Intravenous or intramuscular artesunate remains the treatment of choice in case of severe malaria from *P. falciparum* until the patient can tolerate oral treatment and complete the therapy using an artemisinin-based combination therapy [4,15]. In the last 20 years, artemisinins have been extensively used for the clinical treatment of malaria caused by *P. falciparum*, but some safety concerns appeared due to acute haemolytic anaemia reactions [16,17]. Additionally, the last decade has also witnessed the spread of some cases of artemisinin-resistant malaria in Africa and Southeast Asia [18,19]. Therefore, there is an urgent need for safer therapies, especially those less susceptible to resistance. Currently, no group of drugs can replace artemisinins due to their unmatched high efficacy against malaria [20]. Moreover, if combined with a proper partner drug, artemisinin-based combination therapies can retain their effectiveness against parasites with reduced susceptibility to artemisinins [21]. Therefore, a good strategy to counteract emerging drug resistance might be exploring new combination therapies that exploit unconventional drug partners that can synergize the activity of artemisinins. Medicinal plants and edible foods are important sources of active ingredients. Recently, particular attention has been directed to flavonoids such as morin, quercetin, catechin, and quercitrin as novel natural antimalarial candidates [22]. Among them, quercetin, widely used in traditional Chinese medicine as an antioxidant, anti-inflammatory, and antiviral agent, has shown antiplasmodial activity both *in vitro* (in the 3D7/K1 *P. falciparum* line) and *ex vivo* (in fresh *P. falciparum* field isolates from Bangladesh) and represents, therefore, a valid candidate for new, unconventional combination therapy [23,24]. Using easily obtainable and low-cost companion drugs or adjuvants is crucial not only to reduce resistance evolution but also for reducing treatment expenses and enhancing therapy accessibility to low-income populations [25]. However, one of the biggest problems concerning natural compounds is often their low water solubility, which in turn can compromise their bioavailability and, ultimately, their efficacy once administered *in vivo* [26]. To address this problem, numerous strategies can be applied to deliver poorly water-soluble compounds [27]. Among them, nanosystems are cutting-edge approaches to improve pharmacokinetics and reduce the likelihood of resistance to malaria therapy [28]. However, choosing the most suitable nanosystem for a specific drug is not always easy, and its structure, physico-chemical properties, and administration route must be considered [29,30]. Looking at the current literature, the most used nanosystems in malaria therapy include nanoemulsions, micelles, lipid nanocarriers (e.g., liposomes), polymeric nanocarriers (e.g., PLGA nanoparticles), peptide nanocarriers (e.g., albumin nanoparticles), and, lastly, carbon-based, metallic, inorganic and magnetic nanoparticles [31–33]. Nanoemulsions and liposomes have emerged as successful nanosystems for the delivery of antimicrobials for treating infectious diseases, including malaria [34,35]. For example, orally administered primaquine-loaded nanoemulsions were more effective than a conventional oral dosage form in inhibiting *Plasmodium berghei* infection in Swiss albino mice [36], and dihydroartemisinin-loaded surface-modified lipid nanoemulsions were found to clear parasitaemia while avoiding haemolysis in *in vivo* studies [37]. Lastly, liposomes were explored by Fulgheri et al. as carriers for the oral co-delivery of artemisinin and quercetin, highlighting their suitability, especially for quercetin delivery as an adjuvant [38]. On these bases, this study led to the development of a hybrid system - a nanovesicle-doped nanoemulsion - for oral malaria therapy, which takes advantage of the strengths of both systems (nanoemulsions and liposomes). Similar hybrid systems have been used against cancer but never as malaria therapy. In that case, the emulsion was encapsulated within the vesicles [39]. In contrast, in this study, the nanoemulsion coexists with vesicles, making it, to date, one of a kind in malaria treatment. Artemisinin and quercetin, alone or in combination, were loaded into nanovesicle-doped nanoemulsions. They were fully characterized, and their biocompatibility was evaluated in human colon adenocarcinoma cells (Caco-2) and human umbilical vein endothelial cells (HUVEC) as representative of the intestine and the blood vessels, respectively. The ability of loaded nanovesicle-doped nanoemulsions to inhibit the growth of *P. falciparum* was evaluated *in vitro*. Lastly, the *in vivo* antimalarial efficacy was preliminarily assessed on *Plasmodium yoelii yoelii 17XL*-infected mice.

2. Materials and methods

2.1. Materials

Soy lecithin (Lecinova®) was purchased in a local market store (Cagliari, Italy). Artemisinin was obtained from Biosynth Ltd. (Compton, UK). Unless otherwise specified, quercetin, castor oil, Kolliphor® RH40, sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate and other analytical-grade reagents were acquired from Sigma-Aldrich (Milan, Italy). Dimethyl sulfoxide and 37 % hydrochloric acid were obtained from Carlo Erba (Milan, Italy). Water was purified through a Milli-Q system from Millipore (conductivity: 18.2 MΩ cm^{-1} at 25 °C; Milford, MA, USA). Unless otherwise specified, plastic labware and reagents for cell culture (e.g., Dulbecco's Modified Eagle Medium, Medium 199, foetal bovine serum, and penicillin/streptomycin) were purchased from Life Technologies Europe (Monza, Italy).

2.2. Sample preparation

Artemisinin (5 mg/mL final concentration), quercetin (5 mg/mL final concentration), or both (5 mg/mL each, final concentration), castor oil (100 mg/mL final concentration), Kolliphor® RH40 (100 mg/mL final concentration), and soy lecithin (25 mg/mL final concentration) were weighted in a glass vial, heated at 50 ± 0.5 °C in a thermostatic water bath, and maintained under stirring for 20 min, to obtain a homogeneous phase. Water was heated at 50 ± 0.5 °C as well and gently poured over the oil phase. Immediately, the obtained milky dispersions were sonicated at room temperature without any further temperature control (5 s on, 2 s off, 20 cycles, 13 μm of probe amplitude, probe frequency max 23 kHz) with a high-intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK). Sonication settings were selected during a preliminary study performed by varying the number and duration of cycles, amplitude and probe frequency, with the aim of obtaining small and homogeneous droplets and transparent systems. Nanovesicle-doped nanoemulsions were stored at 4 °C, protected from light, until further characterization. At least 6 batches were produced for each formulation. The chemical integrity of artemisinin and quercetin after preparation (e.g., heating, sonication) and treatment (e.g., high ionic strength and pH) was verified elsewhere [40–42]. Finally, formulations were not sterilized, but their bioburden was contained by manipulating them in a laminar flow biosafety cabinet and using sterilized glassware.

2.3. Characterization of nanovesicle-doped nanoemulsion

The morphology of particles contained in dispersions was observed using a cryogenic transmission electron microscope (cryo-TEM). A thin film of each sample was formed on a holey carbon grid and vitrified by plunging (100 % humidity and room temperature) into liquid ethane, which was maintained at its melting point using a Vitrobot (FEI

Company, Eindhoven, The Netherlands). The vitreous films were transferred to a cryo-TEM (Tecnai F20, FEI Company), and samples were observed (1 grid per dispersion batch). 5–6 images per grid were acquired at −173 °C, using low-dose imaging conditions (200 kV). The hydrodynamic mean diameter (nm), polydispersity index, and zeta potential (mV) of the studied nanovesicle-doped nanoemulsions were measured at 25 °C using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK). Mean diameters and polydispersity indexes were acquired in sextuplicate (6 batches) using the non-invasive backscattering technology at 173° angle (at least 3 repetitions per batch). Zeta potentials were measured in sextuplicate using the Mixed Mode Measurement-Phase Analysis Light Scattering (at least 3 repetitions per batch). Before the analysis, 10 μL of each sample was diluted with 990 μL of water. The proportion of nanovesicles and nanodroplets in the samples was quantified based on the cryo-TEM images, determining the percentage of each nanoparticle present in each formulation. Additionally, measurements of the diameters of the droplets and vesicles were performed using cryo-TEM images, with at least 60 measurements for each type of particle within the formulations.

2.4. Behaviour of nanovesicle-doped nanoemulsions at gastrointestinal pH

A solution at pH 1.2 and high ionic strength was prepared with sodium chloride (1.75 g), water (94 mL), and hydrochloric acid (0.1 M, 6 mL) to reach pH 1.2. A second solution at pH 7.0 and high ionic strength was prepared with disodium hydrogen phosphate (0.726 g), sodium dihydrogen phosphate (0.356 g), sodium chloride (1.754 g), water, and a solution of phosphoric acid to adjust the pH to 7.0 [43]. Each freshly prepared formulation (n = 6) was diluted 100-fold with the solution at pH 1.2 and incubated under mild stirring at 37 °C for 2 h or diluted 100-fold with the solution at pH 7.0 and incubated for 6 h. At the end of the incubation time, the mean diameter and polydispersity index of samples were measured at 37 °C.

2.5. In vitro cytotoxicity studies

Human colon adenocarcinoma (Caco-2) cells and human umbilical vein endothelial cells (HUVEC) were cultured at 37 °C, 100 % humidity, and 5 % carbon dioxide in, respectively, Dulbecco's Modified Eagle Medium (high glucose) or Medium 199 (M199), both supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin (10,000 units), and 10 mg/mL streptomycin. The medium was refreshed every two days. Freshly passaged Caco-2 and HUVEC cells were seeded at respective densities of 10,000 and 5000 cells/well in a clear flat-bottom 96-well plate and incubated overnight to allow cell adherence. Afterward, the medium was removed and replaced with serial dilutions of artemisinin and quercetin, as a nanovesicle-doped nanoemulsion (alone or in combination) or as a solution in the required cell medium. The final volume per well was 100 μL and the dimethyl sulfoxide concentration was always below 0.5 %. The final concentrations of artemisinin or quercetin in the wells were 40, 20, 10, 5, 2.5, and 0 μg/mL. Cells were incubated for 48 h, the medium was changed, and cell viability was evaluated using the resazurin assay, a fast and simple fluorometric test [44]. Briefly, 10 μL of resazurin sodium salt solution (0.125 mg/mL) was added to each well, and after 4 h, fluorescence was measured (λ ex/em: 535/590 nm) using a microplate reader (Tecan Infinite M Plex; Tecan Group Ltd., Männedorf, Switzerland). Three independent experiments were performed at different times, each time in triplicate. The viability (%) of treated cells was reported in comparison with the untreated control cells (100 % viability).

2.6. In vitro P. falciparum growth inhibition assay

P. falciparum 3D7 culture was maintained at 1 % parasitaemia and 3 % haematocrit in a hypoxia incubator (ESCO Lifesciences, Singapore) with a gas mixture containing 92.5 % nitrogen, 5.5 % carbon dioxide, and 2.0 % oxygen. The parasites were cultured in B+ red blood cells in 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 (complete RPMI). Before the assay, parasites were synchronized to the ring stage through 5 % (w/v) sorbitol lysis [45]. Serial dilutions of artemisinin and quercetin, alone or in combination, solubilized in dimethyl sulfoxide (10 mg/mL each) or loaded in nanovesicle-doped nanoemulsion (5 mg/mL each bioactive), were prepared in complete RPMI. The concentrations of artemisinin solution, artemisinin nanovesicle-doped nanoemulsion, and artemisinin-quercetin nanovesicle-doped nanoemulsion ranged from 0.500 to 0.004 μg/mL; that of quercetin solution from 50 to 2.39 μg/mL; that of quercetin nanovesicle-doped nanoemulsion from 20 to 0.156 μg/mL. Each diluted sample (75 μL) was added to at least three wells of 96-well plates, each one containing 75 μL of *P. falciparum* 3D7 culture at 1.5 % parasitaemia and 6 % haematocrit. The final volume per well was 150 μL and the dimethyl sulfoxide concentration was always below 0.5 %. Three additional wells containing 75 μL of the complete RPMI and 75 μL of *P. falciparum* 3D7 culture were prepared. The wells were incubated for 48 h and, diluted to 0.03 % parasitaemia with phosphate-buffered saline (PBS) containing 0.1 μM Syto11 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Parasitaemia percentage was determined by flow cytometry, using a four-laser LSRFortessa cytometer (BD Biosciences, San Jose, CA, USA) and recorded using the BD FACSDiva software (BD Biosciences). Three independent experiments were performed in triplicate. Growth inhibition was calculated as the percentage of parasitaemia measured after treatment versus the parasitaemia measured in the untreated control. The half-maximal inhibitory concentration (IC50) and the concentration that kills 90 % of parasites (IC90) were calculated using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) from the dose-response curves of the triplicate means for each sample.

2.7. In vivo antimalarial effect of nanovesicle-doped nanoemulsions

Dimethyl sulfoxide solutions of artemisinin, quercetin, or their combination (10 mg/mL each) were diluted with PBS to reach the IC90, which was previously determined in a *P. falciparum in vitro* growth inhibition assay. Each nanovesicle-doped nanoemulsion was diluted in PBS to obtain the corresponding IC50 and IC90. The *in vivo* antimalarial activity of artemisinin, quercetin, or their combination, using the above-mentioned dilutions, was assessed using a 4-day blood suppressive test as described elsewhere [46]. Briefly, BALB/c mice (three animals per group) were inoculated with 2×10^7 red blood cells (100 μL) from *Plasmodium yoelii yoelii* 17XL-infected mice by intraperitoneal injection. At 4 h (day 0), 28 h (day 1), 52 h (day 2), and 76 h (day 3) after the inoculation, 100 μL of each sample or 100 μL of PBS were orally administered to the mice. Each day for 11 days, Giemsa-stained blood smears were used to monitor the parasitaemia. At the end of the experiment, animals treated with artemisinin-quercetin nanovesicle-doped nanoemulsion at IC50 were reinfected with 2×10^7 red blood cells (100 μL) from *Plasmodium yoelii yoelii* 17XL-infected mice by intraperitoneal injection and weight, temperature, physical activity, and parasitaemia were observed for three weeks.

2.8. Ethical issues

The human blood used in this work was from voluntary donors and commercially obtained from the Banc de Sang i Teixits (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 the anticoagulant solution. Before their use, blood units underwent the analytical checks specified in the current legislation. Before being delivered, unit data were anonymized and irreversibly dissociated, and any identification tag or label was removed to

guarantee the non-identification of the blood donor. No blood data were or will be supplied, following the current Spanish Ley Orgánica de Protección de Datos and Ley de Investigación Biomédica. The blood samples will not be used for studies other than those made explicit in this research.

Mice (18–20 g) were maintained under standard environmental conditions: 20–24 °C and 12/12 h light/dark cycle, with ad libitum access to a semi-solid diet and water during the experiments. In the presence of toxic effects including, among others, >20 % reduction in animal weight, aggressive and unexpected animal behaviour, or the presence of blood in faeces, animals were immediately anesthetized using a 100 mg/kg Ketolar plus 5 mg/kg Midazolam mixture and sacrificed by cervical dislocation. The animal care and use protocols adhered to the national and international guidelines specified in the Spanish Royal Decree 53/2013, based on the European regulation 2010/63/UE. The studies reported here were performed under protocols reviewed and approved by the Ethical Committee on Clinical Research from the Hospital Clínic de Barcelona (Reg. HCB/2018/1223, January 23, 2019).

2.9. Statistical analysis of data

Results are expressed as the means of at least 3 independent experiments ± standard deviations. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between groups, while paired Student's t-test was used to compare two samples. Significance was tested at the 0.05 level of probability ($p < 0.05$). Data analysis was carried out using the software package XL Statistic for Excel.

3. Results

3.1. Preparation and characterization of nanovesicle-doped nanoemulsion

A preformulation study was performed to find the more suitable oil phase capable of forming stable and effective nanoemulsions. Different oils, surfactants, or cosurfactants in various ratios were tested until the more suitable combination could load artemisinin and quercetin and create a transparent and homogeneous nanoemulsion (data not shown). Castor oil, Kolliphor® RH40, and soy lecithin were selected as the main components of the lipid phase and combined in the most appropriate ratio (1:1:0.4). This composition was able to load the highest amount of artemisinin and quercetin (5 mg/mL) together and prevent hydrodynamic size increase and system phase separation, which occurred above this concentration.

The morphology of nanovesicle-doped nanoemulsions was analysed by cryo-TEM (Fig. 1), which revealed that the prevalent particles were oil droplets, which coexisted with small lamellar vesicles. Particles were easily distinguishable since oil droplets appeared as grey spheres. In contrast, vesicles were less electron-dense and delimited by a single layer, most likely composed of phosphatidylcholine molecules (indicated by arrows in Fig. 1). Therefore, the formulation could be described as a combined dispersion of oil nanodroplets and nanovesicles or, more easily, nanovesicle-doped nanoemulsion. The percentage of nanovesicles in each sample varied with the loaded molecules, being ~9 %, ~26 % and ~51 % in artemisinin, quercetin and artemisinin-quercetin nanovesicle-doped nanoemulsions, respectively. This suggests that the high amount of oil phase used favoured the organization of the dispersion into oil nanodroplets surrounded by surfactants, rather than the incorporation of oil into the lamellar membrane of the vesicles. Images were used to randomly measure the diameter and assess the relative percentage of oil nanodroplets and nanovesicles (at least 60 of each) within formulations (Table 1). Results indicated that the nanovesicles prepared without quercetin were smaller than those prepared with quercetin alone or in association with artemisinin. This effect is probably related to the intercalation of quercetin in the double layer of phospholipid vesicles [47].

The mean hydrodynamic diameter, polydispersity index, and zeta potential of nanovesicle-doped nanoemulsions prepared without bioactive molecule (empty) or loading artemisinin, quercetin, or their combination were measured immediately after sonication (Table 2). The hydrodynamic diameter of empty nanovesicle-doped nanoemulsion was ~65 nm ($p < 0.05$ versus other values), the polydispersity index was ~0.189, and the zeta potential was ~-45 mV ($p > 0.05$ versus the zeta potential of artemisinin nanovesicle-doped nanoemulsions; $p < 0.05$ versus other values). The addition of artemisinin or quercetin or even their combination did not considerably affect the mean hydrodynamic diameter and polydispersity index of loaded nanovesicle-doped nanoemulsions (≈60 nm, $p > 0.05$ among the three values). Zeta potential results, together with cryo-TEM images and the low water-solubility of artemisinin and quercetin, corroborate the hypothesis of their incorporation in the lipidic core of nanodroplets and, to a minor extent, in the lipidic bilayer of nanovesicles.

After 30 days at 4 °C (Fig. 2), the hydrodynamic diameter of artemisinin and artemisinin-quercetin nanovesicle-doped nanoemulsions maintained the same average values. However, the systems suffered some rearrangements as indicated by the decrease of their polydispersity index, and modification of their zeta potential, which became less negative in the artemisinin-quercetin nanovesicle-doped nanoemulsion ($p < 0.05$). In contrast, the size and polydispersity of quercetin nanovesicle-doped nanoemulsion increased, while zeta potential became less negative ($p < 0.05$).

Overall, differences in measured parameters (Table 2 and Fig. 2) can be considered minimal when biological applications, discussed further on, are considered.

3.2. Behaviour of nanovesicle-doped nanoemulsions at gastrointestinal pHs

To evaluate the stability of nanovesicle-doped nanoemulsions in the gastrointestinal fluids, these systems were diluted with solutions having high ionic strength and pH of 1.2 or 7.0, and further compared with freshly prepared systems for mean diameter and polydispersity index (Table 3). The dilution in the solutions mentioned above did not affect

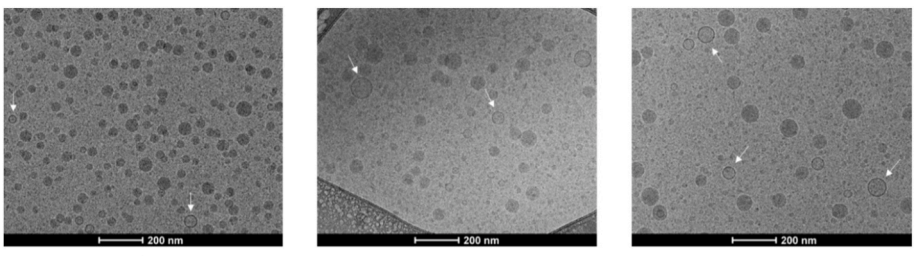

Fig. 1. Representative cryo-TEM images of freshly prepared nanovesicle-doped nanoemulsions loading artemisinin (A), quercetin (B), or artemisinin-quercetin (C). The white arrows indicate the nanovesicles contained in each nanovesicle-doped nanoemulsion.

Table 1
Mean diameter and relative percentage of nanodroplets and nanovesicles in nanovesicle-doped nanoemulsions loading artemisinin, quercetin, or their combination retrieved from cryo-TEM analysis. Mean values ± standard deviations are reported (n = 60).

Sample	Oil nanodroplets (nm)	Nanovesicles (nm)	Oil nanodroplets (%)	Nanovesicles (%)
Artemisinin nanovesicle-doped nanoemulsion	54 ± 16	33 ± 11	91	9
Quercetin nanovesicle-doped nanoemulsion	61 ± 22	41 ± 16	74	26
Artemisinin-quercetin nanovesicle-doped nanoemulsion	63 ± 16	46 ± 16	49	51

Table 2
Mean diameter (MD), polydispersity index (PI), and zeta potential (ZP) of freshly prepared nanovesicle-doped nanoemulsions loading artemisinin, quercetin or their combination. Mean values ± standard deviations are reported (n = 6). The same symbol (°, *,§,#) indicates values that are not statistically different ($p > 0.05$).

Sample	MD (nm)	PI	ZP (mV)
Empty nanovesicle-doped nanoemulsion	65 ± 1	0.189* ± 0.016	$-45^{\S}$ ± 1
Artemisinin nanovesicle-doped nanoemulsion	62° ± 1	0.192* ± 0.007	$-42^{\S}$ ± 3
Quercetin nanovesicle-doped nanoemulsion	58° ± 1	0.199* ± 0.009	$-51^{\#}$ ± 3
Artemisinin-quercetin nanovesicle-doped nanoemulsion	60° ± 3	0.176* ± 0.015	$-49^{\#}$ ± 3

the mean diameters of nanovesicle-doped nanoemulsions. The major changes were observed in the polydispersity index, indicating some structural rearrangement. Nonetheless, formulations were considered stable as these little fluctuations in the polydispersity index are not indicative of non-homogeneous systems, and upon visual inspection, they appeared still transparent and uniform.

3.3. Cell viability assay

The biocompatibility of artemisinin, quercetin or their combination, solubilized in dimethyl sulfoxide or loaded in nanovesicle-doped nanoemulsions, was evaluated using Caco-2 and HUVEC cells (Fig. 3). Caco-2 cells were used to evaluate the potential effect of the formulation on the colon, where artemisinin and quercetin are expected to be absorbed. HUVECs were selected as model of epithelial cells during blood vessel transit. All data and statistics are reported in Fig. 3, where it is possible to note that most formulations and doses did not considerably affect the viability of both cell lines. However, HUVEC cells appeared more sensitive to the hybrid systems than Caco-2 cells. Indeed, when HUVEC cells were exposed to loaded nanovesicle-doped nanoemulsions, the resulting viability was below 100 % in 9 out of 15 treatments (different per formulation and dose). By contrast, with Caco-2 cells, only 2 nanovesicle-doped nanoemulsion treatments (quercetin and artemisinin-quercetin nanovesicle-doped nanoemulsions at 40 μg/mL) led to a viability below 100 %. At the highest dose (40 μg/mL), the quercetin solution was more toxic than the artemisinin solution for both cell lines. When artemisinin was loaded in the nanovesicle-doped nanoemulsion, biocompatibility was improved only in Caco-2 cells at all concentrations. When quercetin instead was loaded into the

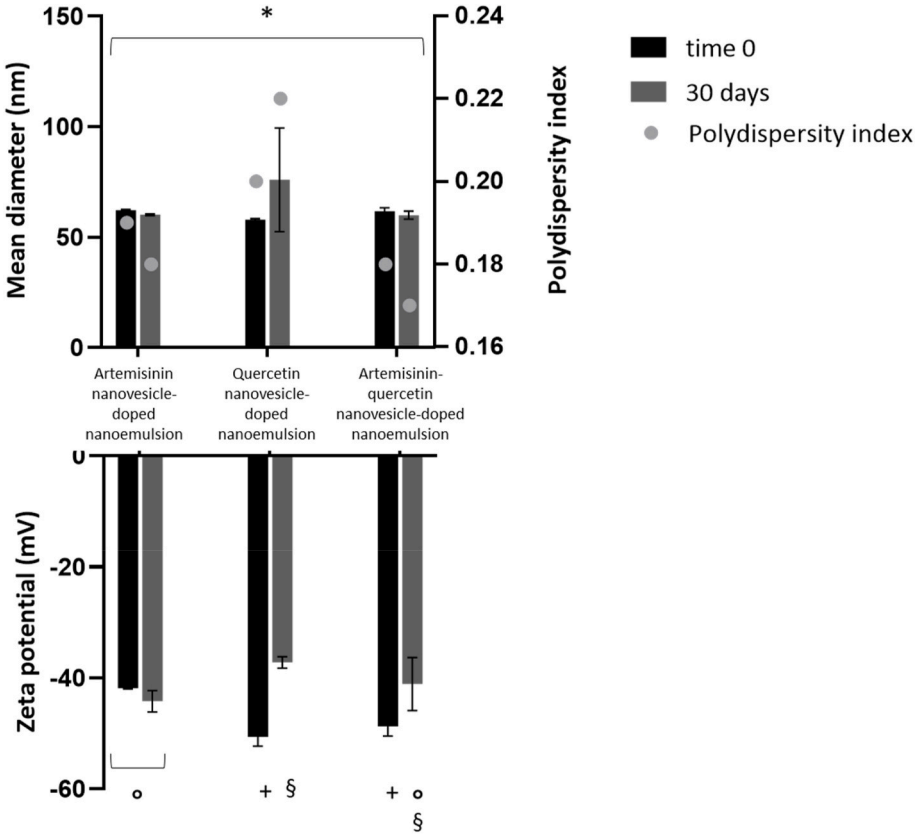

Fig. 2. Mean diameter, polydispersity index, and zeta potential of nanovesicle-doped nanoemulsions loading artemisinin, quercetin, or their combination, stored for 30 days at 4 °C. Mean values (bars) ± standard deviations are reported (n = 3). The same symbol (*, °, +, §) indicates values that are not statistically different ($p > 0.05$), whereas different symbols indicate statistically different values ($p < 0.05$).

Table 3
Mean diameter (MD) and polydispersity index (PI) of nanovesicle-doped nanoemulsions loading artemisinin, quercetin, or their combination incubated for 2 h at pH 1.2 or 6 h at pH 7.0. Mean values ± standard deviations are reported (n = 6). The same symbol (°,§, *) indicates values that are not statistically different ($p > 0.05$), whereas different symbols indicate statistically different values ($p < 0.05$).

Sample		MD (nm)	PI
Artemisinin nanovesicle-doped nanoemulsion	Freshly prepared	62° ± 1	0.192 ± 0.007
	2 h at pH 1.2	61° ± 2	0.122 ± 0.009
	6 h at pH 7.0	63° ± 3	0.116 ± 0.007
Quercetin nanovesicle-doped nanoemulsion	Freshly prepared	58§ ± 1	0.199 ± 0.009
	2 h at pH 1.2	57§ ± 1	0.104 ± 0.008
	6 h at pH 7.0	59§ ± 1	0.115 ± 0.007
Artemisinin-quercetin nanovesicle-doped nanoemulsion	Freshly prepared	60*° ± 3	0.176 ± 0.015
	2 h at pH 1.2	53* ± 4	0.109 ± 0.010
	6 h at pH 7.0	56*° ± 3	0.105 ± 0.011

nanovesicle-doped nanoemulsions, alone or in combination with artemisinin, the biocompatibility towards the highest dose was improved in Caco-2 cells but not in HUVECs, which were more sensitive. At the concentrations tested from 20 to 2.5 μg/mL), the co-loading of artemisinin and quercetin in nanovesicle-doped nanoemulsions improved the biocompatibility with respect to the artemisinin solution in both cell types, highlighting the suitability of this novel hybrid system as a safe oral treatment for malaria.

3.4. Growth inhibition assay

The growth inhibition of the 3D7 strain of *P. falciparum* caused by artemisinin and/or quercetin in solution or loaded in nanovesicle-doped nanoemulsion was assayed, and the IC50 and IC90 were calculated (Table 4). The IC50 of the artemisinin solution was ~0.019 μg/mL, and its IC90 was ~0.065 μg/mL. Both values did not statistically change when they were loaded in nanovesicle-doped nanoemulsions as the IC50 was ~0.018 μg/mL ($p > 0.05$ when compared to the solution IC50) and the IC90 was ~0.061 μg/mL ($p > 0.05$ between the two IC90s), suggesting that the antimalarial efficacy of artemisinin was not affected by its loading into nanovesicle-doped nanoemulsions. With respect to the artemisinin solution, the IC50 and IC90 values of the quercetin solution were significantly higher: the IC50 was ~4.301 μg/mL ($p < 0.05$ versus all other IC50) and the IC90–17.847 μg/mL ($p < 0.05$ versus all other IC90). This difference is probably due to the different mechanisms of action, as underlined in the discussion. Nonetheless, loading quercetin in the nanovesicle-doped nanoemulsion boosted its activity, leading to a decrease of IC50 of ~77 % (corresponding to ~1.004 μg/mL), which was statistically different from the IC50 of artemisinin both in solution or loaded into the nanovesicle-doped nanoemulsion ($p < 0.05$). Similarly, the IC90 of the quercetin nanovesicle-doped nanoemulsion decreased as well (~75 %) to ~4.545 μg/mL, yet it was still higher than those of artemisinin solution or artemisinin-loaded nanovesicle-doped nanoemulsion ($p < 0.05$). The co-loading of quercetin and artemisinin slightly modified the values obtained when artemisinin was tested

Table 4
IC50 and IC90 (μg/mL) of artemisinin, quercetin or their combination, in solution or loaded in nanovesicle-doped nanoemulsions, measured against the 3D7 strain of *P. falciparum*. Mean values (bars) ± standard deviations are reported (n = 3). The same symbol (°, *) indicates values that are not statistically different ($p > 0.05$), whereas different symbols indicate statistically different values ($p < 0.05$).

Sample	IC50 (μg/mL)	IC90 (μg/mL)
Artemisinin solution	0.019° ± 0.002	0.065* ± 0.003
Quercetin solution	4.301 ± 0.078	17.847 ± 1.439
Artemisinin nanovesicle-doped nanoemulsion	0.018° ± 0.012	0.061* ± 0.030
Quercetin nanovesicle-doped nanoemulsion	1.004 ± 0.162	4.545 ± 0.136
Artemisinin-quercetin nanovesicle-doped nanoemulsion	0.009 ± 0.001	0.041 ± 0.002

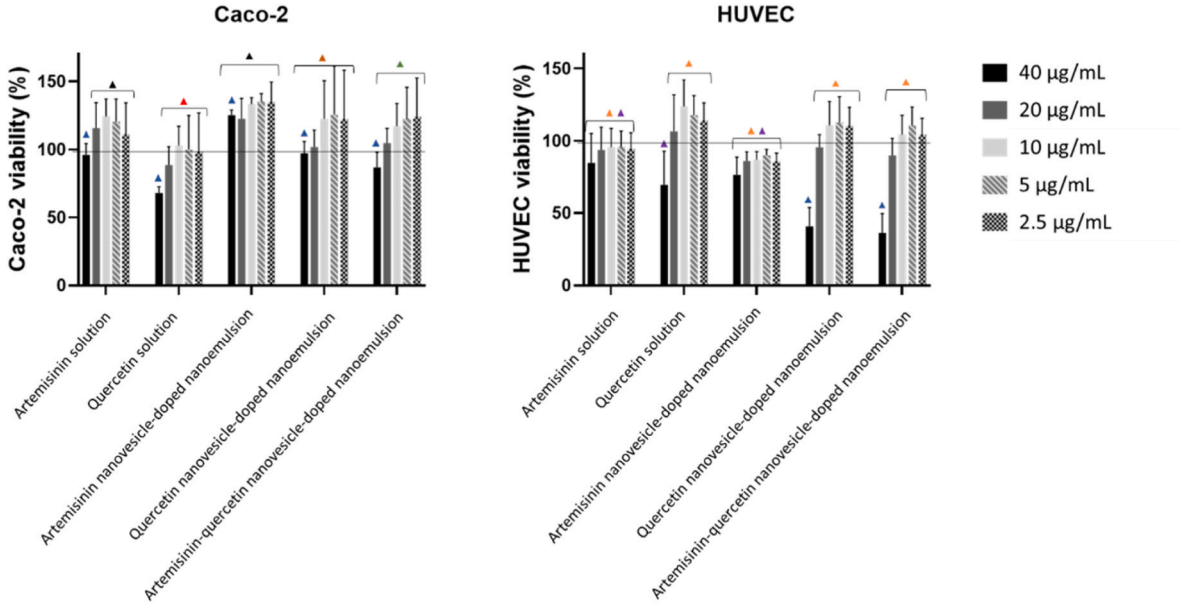

Fig. 3. Viability of Caco-2 (left panel) and HUVEC (right panel) cells, treated with artemisinin, quercetin, or their combination, in solution or loaded in nanovesicle-doped nanoemulsions, properly diluted to a concentration of 40, 20, 10, 5, and 2.5 μg/mL. Mean values (bars) ± standard deviations are reported (n = 3). In each panel, the same-coloured symbol indicates values that are not statistically different ($p > 0.05$), whereas different coloured symbols indicate statistically different values ($p < 0.05$).

alone, as the IC50 was ~0.009 μg/mL ($p < 0.05$ versus the IC50 of artemisinin solution and artemisinin nanovesicle-doped nanoemulsion). The IC90 was ~0.041 μg/mL ($p < 0.05$ versus the IC50 of artemisinin solution and artemisinin nanovesicle-doped nanoemulsion).

3.5. *In vivo* antimalarial assay

The *in vivo* antimalarial efficacy of artemisinin and/or quercetin loaded in nanovesicle-doped nanoemulsions was assessed by oral administration in mice infected with *P. yoelii yoelii 17XL* (Fig. 4). For this preliminary assay, samples were administered at two different concentrations (IC50 and IC90). The mean survival of the infected and untreated control animals was 5 days. Treatment with the IC90 of artemisinin or quercetin solution enhanced the survival of animals until day 9, while their combination in solution achieved reduced survival only up to day 6. The treatment with the IC50 and IC90 of artemisinin and quercetin nanovesicle-doped nanoemulsion also addressed the death of animals on day 6. Differently, the co-loading of artemisinin and quercetin in nanoemulsion led to an increased animal survival up to day 11, with one animal per group alive at the end of the experiment (day 11), irrespective of the concentration tested. In particular, the animal treated with IC50 of artemisinin-quercetin nanoemulsion, at the end of the experiment, did not have detectable parasitaemia upon microscopic examination, suggesting a complete recovery from the infection. One month later, the same animal was reinfected with 2×10^7 red blood cells (100 μL) from a *Plasmodium yoelii yoelii* 17XL-infected mice by intraperitoneal injection. The mouse did not develop malaria symptoms nor change in weight, temperature, or physical activity, and parasitaemia remained at 0 % for three weeks, suggesting a possible immunization following the treatment.

4. Discussion

The present study explored the potential of a nanovesicle-doped nanoemulsion as an innovative delivery system for a new natural drug combination rationally designed for oral malaria therapy. To this end, artemisinin and quercetin were selected as payloads due to their proven efficacy against *P. falciparum* [2,24]. To effectively load them, alone or in combination, at a suitable concentration, a preformulation study was carried out combining oils, surfactants, and cosurfactants in different ratios until a transparent, stable, homogenous, and nano-sized system was obtained [48]. Different vegetable oils were used (e.g., sweet almond oil, corn oil, coconut oil, lemon oil), as well as various surfactants (e.g., Tween 80, Span 20, Labrasol®, Labrafac®), while maintaining soy lecithin as the co-surfactant at different concentrations. Additionally, different preparation methods were tested (i.e., Ultra-Turrax alone, sonication alone, and a combination of both) to optimize the system for the effective co-loading of artemisinin and quercetin. Among the different preparation methods tested, sonication proved to be the most promising approach. Thus, various sonication parameters, including the number of cycles, pulse duration, and probe amplitude, were evaluated to obtain a small and homogeneous dispersion. From this initial screening, castor oil, Kolliphor® RH40, and soy lecithin were selected as oil, surfactant, and co-surfactant, respectively. While other tested combinations led to immediate phase separation, drug precipitation or highly polydisperse systems, this combination ensured small, homogeneous droplet and vesicle size and prolonged physical stability and was therefore preferred over other alternatives. Essentially, this combination was both innovative and fundamental as 1) it led to the development of a new hybrid nanosystem (nanovesicle-doped nanoemulsion) and 2) it allowed the co-loading of the highest amounts of artemisinin and quercetin, ensuring their compatibility and stability within the system. Castor oil is a vegetable oil with emollient and preservative (anti-fungal and anti-yeast) properties, extensively used in the food industry [49]. Recently, it has been tested as an anti-inflammatory agent and for its ability to interact with the immune and lymphatic systems [50]. Additionally, being composed of fatty acids, it may act as a penetration enhancer, like other fatty acids [51]. Moreover, it was chosen for its good solubilization ability for lipophilic drugs [52]. The solubility of artemisinin and quercetin in this oil has been reported to be ~1.8 mg/mL and ~1.6 mg/mL, respectively [53,54]. Kolliphor® RH40 is the polyethoxylated form of castor oil and was used as a non-ionic solubilizer and emulsifier, allowing to increase the concentration of both payloads to 5 mg/mL and reducing the risk of phase separation. Soy lecithin is a complex mixture of phospholipids that spontaneously assemble in bilayered vesicles when added to water. It is likely responsible for the formation of the unilamellar vesicles that coexist with the oil nanodroplets in the resulting hybrid system (nanovesicle-doped nanoemulsions) [55], while simultaneously acting as a co-surfactant and enhancing the interfacial stability of the nanodroplets. Interestingly, the loaded compounds influenced not only the percentage of vesicles in the nanovesicle-doped nanoemulsions but also their dimensions. Overall, the formulations containing quercetin (alone or in combination) had nanovesicles with slightly bigger diameters than those containing only artemisinin as a probable consequence of its disposition in the bilayers [47]. Nonetheless, regardless of the loaded compound, nanovesicle-doped nanoemulsions had comparable hydrodynamic diameters (~60 nm) due to the fact that the oil droplets, which are larger, are present in these systems in higher percentages (50 % or more) than the nanovesicles. These dimensions make them suitable for adsorption in the gastrointestinal tract via pinocytosis. To confirm their suitability for oral administration, the stability of the developed systems (i.e., artemisinin, quercetin, and artemisinin-quercetin nanovesicle-doped nanoemulsions) was evaluated in solutions that simulate the harsh conditions of the gastrointestinal tract. The mean diameter of freshly prepared nanovesicle-doped nanoemulsions was not altered upon dilution and incubation for 2 h at pH 1.2 or 6 h at pH 7.0 and high ionic strength, suggesting that no aggregation and/or fusion phenomena occurred, thus highlighting the potential positive attitude for these systems to be administered orally. The good colloidal stability, typically described for nanoemulsions, is therefore retained by the nanovesicle-doped nanoemulsions despite their hybrid nature [56,57].

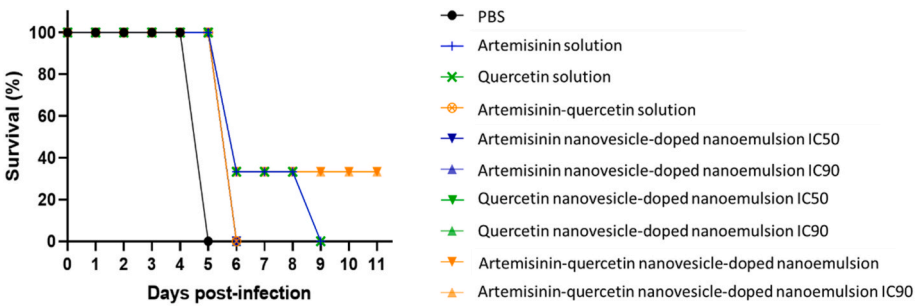

Fig. 4. Kaplan-Meier plot for the *in vivo* assay of the effect of artemisinin, quercetin, and their combination, in solution or loaded in nanovesicle-doped nanoemulsions, orally administered to mice infected with *P. yoelii yoelii*. Mean values ± standard deviations are reported (n = 3).

In addition to the good stability, all the nanovesicle-doped nanoemulsions were also highly biocompatible *in vitro* when tested on significant cell lines such as Caco-2 and HUVEC, with HUVEC being more sensitive, probably due to their type (normal human cells) and the ratio of dose/number of cells. The *in vitro* antimalarial activity was assessed using the same range of dilutions that led to the highest biocompatibility in both cell lines (10, 5, and 2.5 μg/mL). With respect to the solution, nanovesicle-doped nanoemulsions strongly boosted the activity of quercetin. Among the several activities it has, quercetin can modulate the expression of inflammation mediators [58]. In particular, it may inhibit glycogen synthase kinase-3β, an enzyme able to modulate the inflammatory response activated by pathogenic microorganisms [59]. This key activity might help make it possible to control the imbalance of cytokine production during malaria infections, making quercetin a potential therapeutic for this disease [60]. As for artemisinin, nanovesicle-doped nanoemulsions did not mask its activity, allowing it to achieve values of IC50 and IC90 comparable to its solution. However, it must be underlined that all the solutions were prepared in dimethyl sulfoxide. In contrast, the hybrid systems did not require such a solvent that might pose toxicological concerns after administration [61]. Valissery et al., in their study, were not able to boost the antimalarial activity of artemisinin with their nanoformulation but concluded that enhancing its solubility was a crucial step [62]. This approach is today widely accepted by many authors and might be the key factor that led to the high activity registered for the nanovesicle-doped nanoemulsions containing artemisinin [63]. When artemisinin and quercetin were co-loaded, the antimalarial activity of artemisinin was slightly improved, probably due to the synergistic effect provided by this flavonoid [64]. This phenomenon appears directly linked to the differences in the mechanisms of action of the two bioactives, which, however, have not yet been fully elucidated. In the case of artemisinin, it is believed to be cleaved inside infected erythrocytes, producing highly reactive carbon-centered radicals and reactive oxygen species that collectively kill the parasites [65]. In contrast, quercetin appears to inhibit fatty acid biosynthesis and target functional biomolecules (e.g., proteins, DNA), ultimately affecting the survival of the malaria parasite. In addition, it has been theorized that its phenolic groups may be converted to phenoxy anions under oxidative stress, potentially enhancing the irreversible damage inflicted by artemisinin on the cellular components of the parasites [66]. These promising *in vitro* results achieved with the combination of artemisinin and quercetin loaded in nanovesicle-doped nanoemulsions were confirmed *in vivo* in a preliminary study aimed at evaluating the efficacy of these systems for oral malaria therapy. While the survival of animals treated with quercetin or artemisinin, either in solution or loaded in nanovesicle-doped nanoemulsions led to a survival no longer than 9 days, the co-loading of artemisinin and quercetin in nanovesicle-doped nanoemulsions increased animal survival up to day 11, ensuring the complete recovery of one animal and even preventing its re-infection upon challenge. This interesting result requires new studies with a larger number of animals in order to confirm the observed effect. Quercetin reinforced the antimalarial effect of artemisinin only when co-delivered in nanovesicle-doped nanoemulsions, probably because 1) the oil nanodroplets (~49 %), acting as penetration enhancer, increased their bioavailability and favoured the rapid onset of the antimalarial effect, while 2) lamellar vesicles (~51 %), acting as carrier, prolonged the bioavailability of both phytochemicals and the interaction with cells and parasites.

Compared to existing antimalarial delivery systems, it seems that the double nature of nanovesicle-doped nanoemulsions, dual-drug loading, and superior stability in the gastrointestinal environment set them apart from existing nanosystems and stand out as key elements of their promising success. In the current literature, significant efforts have been devoted to developing nanosystems to overcome challenges such as poor bioavailability, short half-life, and resistance to artemisinins [67,68]. Most studies focused on monotherapies comprising artemisinin or its derivatives, and only in a few cases the dual-drug loading with a flavonoid was investigated, leading to advancements in malaria treatment and thus aligning with the encouraging results of this study [38,69,70]. Due to the demonstrated stability of these hybrid systems under simulated gastric conditions, it was possible to test them *in vivo* in mice through the route of choice for artemisinin combination therapy (oral administration). Therefore, these findings highlight the potential of this system as a next-generation antimalarial therapy, particularly in resource-limited settings where oral administration is preferred.

5. Conclusions

Despite being only preliminary data, the results obtained suggest the potential of nanovesicle-doped nanoemulsions for the co-delivery of artemisinin and quercetin in the oral treatment of malaria. The smart combination of castor oil, Kolliphor® RH40, and soy lecithin enabled loading effectively high concentrations of both bioactives in an innovative hybrid system. *In vitro* and *in vivo* results showed that the nanovesicle-doped nanoemulsions improved the efficacy of quercetin and provided significant antimalarial effects when co-delivered with artemisinin, highlighting a synergistic interaction. All in all, this study underscores the promise of phytopharmaceuticals, like quercetin, in enhancing the therapeutic effects of conventional antimalarial treatments and supports the integration of innovative formulations into traditional medicine to improve treatment outcomes. These preliminary yet promising results will be further investigated in depth, involving a bigger group of animals, and proposed in a future article.

CRediT authorship contribution statement

Federica Fulgheri: Writing – original draft, Investigation, Formal analysis, Data curation. **Miriam Ramírez:** Writing – review & editing, Validation, Methodology. **Lucía Román-Álamo:** Writing – review & editing, Validation, Methodology. **Paolo Gasco:** Validation, Methodology, Investigation. **Maria Manconi:** Writing – review & editing, Validation, Supervision, Project administration, Methodology. **Matteo Aroffu:** Writing – review & editing, Validation, Methodology. **Rita Abi Rached:** Writing – review & editing, Validation, Methodology. **Biancamaria Baroli:** Writing – review & editing, Validation, Methodology. **Xavier Fernàndez-Busquets:** Writing – review & editing, Validation, Supervision, Methodology. **Maria Letizia Manca:** Writing – review & editing, Validation, Supervision, Methodology.

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.

Acknowledgments

The authors thank MIUR and PON R&I 2014–2022 for supporting the PhD grant number DOT1304004. X.F.-B. was supported by grants (i) PID2021-128325OB-I00, *Ministerio de Ciencia, Innovación y Universidades/Agencia Estatal de Investigación* (MCIU/AEI/10.13039/501100011033), which included FEDER and European Union NextGenerationEU/PRTR funds; and (ii) 2021-SGR-00635, *Generalitat de Catalunya*, Spain (http://agaur.gencat.cat/). ISGlobal and IBEC are members of the CERCA Programme, *Generalitat de Catalunya*. We acknowledge support from MCIN/AEI through the *"Centro de Excelencia Severo Ochoa 2019–2023"* Program (CEX2018-000806-S). This research is part of ISGlobal's Program on the Molecular Mechanisms of Malaria which is partially supported by the *Fundación Ramón Areces*. The cryogenic transmission electron microscopy work was done at the Electron Cryomicroscopy Unit of the Scientific and Technological Centers of the University of Barcelona.

Data availability

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

References

[1] World Health Organization, World malaria report 2024. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2024, 2024 (accessed January 8, 2025).
[2] Y. Tu, The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine, Nat. Med. 17 (2011) 1217–1220, https://doi.org/10.1038/nm.2471.
[3] J. Yang, Y. He, Y. Li, X. Zhang, Y.-K. Wong, S. Shen, T. Zhong, J. Zhang, Q. Liu, J. Wang, Advances in the research on the targets of anti-malaria actions of artemisinin, Pharmacol. Ther. 216 (2020) 107697, https://doi.org/10.1016/j.pharmthera.2020.107697.
[4] G.A. Balint, Artemisinin and its derivatives: an important new class of antimalarial agents, Pharmacol. Ther. 90 (2001) 261–265, https://doi.org/10.1016/S0163-7258(01)00140-1.
[5] R.N. Price, Artemisinin drugs: novel antimalarial agents, Expet Opin. Invest. Drugs 9 (2000) 1815–1827, https://doi.org/10.1517/13543784.9.8.1815.
[6] J. Rautio, N.A. Meanwell, L. Di, M.J. Hageman, The expanding role of prodrugs in contemporary drug design and development, Nat. Rev. Drug Discov. 17 (2018) 559–587, https://doi.org/10.1038/nrd.2018.46.
[7] World Health Organization, Guidelines for the Treatment of Malaria, n.d.
[8] G.M. Keating, Dihydroartemisinin/Piperaquine, Drugs 72 (2012) 937–961, https://doi.org/10.2165/11203910-000000000-00000.
[9] J.Y. Doua, J. Matangila, P. Lutumba, J.-P. Van geertruyden, Intermittent preventive treatment: efficacy and safety of sulfadoxine-pyrimethamine and sulfadoxine-pyrimethamine plus piperaquine regimens in schoolchildren of the Democratic Republic of Congo: a study protocol for a randomized controlled trial, Trials 14 (2013) 311, https://doi.org/10.1186/1745-6215-14-311.
[10] F. Nosten, M. van Vugt, R. Price, C. Luxemburger, K. Thway, A. Brockman, R. McGready, F. ter Kuile, S. Looareesuwan, N. White, Effects of artesunate-mefloquine combination on incidence of Plasmodium falciparum malaria and mefloquine resistance in western Thailand: a prospective study, Lancet 356 (2000) 297–302, https://doi.org/10.1016/S0140-6736(00)02505-8.
[11] M. Makanga, S. Krudsood, The clinical efficacy of artemether/lumefantrine (Coartem®), Malar. J. 8 (2009) S5, https://doi.org/10.1186/1475-2875-8-S1-S5.
[12] S.B. Sirima, A. Gansané, Artesunate–amodiaquine for the treatment of uncomplicated malaria, Expet Opin. Invest. Drugs 16 (2007) 1079–1085, https://doi.org/10.1517/13543784.16.7.1079.
[13] V. Navaratnam, S.M. Mansor, N.-W. Sit, J. Grace, Q. Li, P. Olliaro, Pharmacokinetics of artemisinin-type compounds, Clin. Pharmacokinet. 39 (2000) 255–270, https://doi.org/10.2165/00003088-200039040-00002.
[14] T. Dai, W. Jiang, Z. Guo, Y. Xie, R. Dai, Comparison of in vitro/in vivo blood distribution and pharmacokinetics of artemisinin, artemether and dihydroartemisinin in rats, J. Pharm. Biomed. Anal. 162 (2019) 140–148, https://doi.org/10.1016/j.jpba.2018.09.024.
[15] M. van Agtmael, Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication, Trends Pharmacol. Sci. 20 (1999) 199–205, https://doi.org/10.1016/S0165-6147(99)01302-4.
[16] T. Gordi, E.-I. Lepist, Artemisinin derivatives: toxic for laboratory animals, safe for humans? Toxicol. Lett. 147 (2004) 99–107, https://doi.org/10.1016/j.toxlet.2003.12.009.
[17] K. Rehman, F. Lötsch, P.G. Kremsner, M. Ramharter, Haemolysis associated with the treatment of malaria with artemisinin derivatives: a systematic review of current evidence, Int. J. Infect. Dis. 29 (2014) 268–273, https://doi.org/10.1016/j.ijid.2014.09.007.
[18] B. Balikagala, N. Fukuda, M. Ikeda, O.T. Katuro, S.-I. Tachibana, M. Yamauchi, W. Opio, S. Emoto, D.A. Anywar, E. Kimura, N.M.Q. Palacpac, E.I. Odongo-Aginya, M. Ogwang, T. Horii, T. Mita, Evidence of artemisinin-resistant malaria in Africa, N. Engl. J. Med. 385 (2021) 1163–1171, https://doi.org/10.1056/NEJMoa2101746.
[19] C.J. Woodrow, N.J. White, The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread, FEMS Microbiol. Rev. 41 (2017) 34–48, https://doi.org/10.1093/femsre/fuw037.
[20] A.M. Dondorp, S. Yeung, L. White, C. Nguon, N.P.J. Day, D. Socheat, L. von Seidlein, Artemisinin resistance: current status and scenarios for containment, Nat. Rev. Microbiol. 8 (2010) 272–280, https://doi.org/10.1038/nrmicro2331.
[21] C. Nsanzabana, Resistance to artemisinin combination therapies (ACTs): do not forget the partner drug, Trav. Med. Infect. Dis. 4 (2019) 26, https://doi.org/10.3390/tropicalmed4010026.
[22] S. Abu-Lafi, M. Akkawi, F. Al-Rimawi, Q. Abu-Remeleh, P. Lutgen, Morin, quercetin, catechin and quercitrin as novel natural antimalarial candidates, Pharm. Pharmacol. Int. J. 8 (2020) 184–190, https://doi.org/10.15406/ppij.2020.08.00295.
[23] D.X. Dang, S. Cho, H. Wang, W.J. Seok, J.H. Ha, I.H. Kim, Quercetin extracted from Sophora japonica flower improves growth performance, nutrient digestibility, cecal microbiota, organ indexes, and breast quality in broiler chicks, Anim. Biosci. 35 (2022) 577–586, https://doi.org/10.5713/ab.21.0331.
[24] D. Ganesh, H.-P. Fuehrer, P. Starzengrüber, P. Swoboda, W.A. Khan, J.A. B. Reismann, M.S.K. Mueller, P. Chiba, H. Noedl, Antiplasmodial activity of flavonol quercetin and its analogues in Plasmodium falciparum: evidence from clinical isolates in Bangladesh and standardized parasite clones, Parasitol. Res. 110 (2012) 2289–2295, https://doi.org/10.1007/s00436-011-2763-z.
[25] M.P. Singh, K.B. Saha, S.K. Chand, L.L. Sabin, The economic cost of malaria at the household level in high and low transmission areas of central India, Acta Trop. 190 (2019) 344–349, https://doi.org/10.1016/j.actatropica.2018.12.003.
[26] J. Yang, K. Li, D. He, J. Gu, J. Xu, J. Xie, M. Zhang, Y. Liu, Q. Tan, J. Zhang, Toward a better understanding of metabolic and pharmacokinetic characteristics of low-solubility, low-permeability natural medicines, Drug Metab. Rev. 52 (2020) 19–43, https://doi.org/10.1080/03602532.2020.1714646.
[27] J. Zhao, J. Yang, Y. Xie, Improvement strategies for the oral bioavailability of poorly water-soluble flavonoids: an overview, Int. J. Pharm. 570 (2019) 118642, https://doi.org/10.1016/j.ijpharm.2019.118642.
[28] F. Fulgheri, M.L. Manca, X. Fernàndez-Busquets, M. Manconi, Analysis of complementarities between nanomedicine and phytodrugs for the treatment of malarial infection, Nanomedicine 18 (2023) 1681–1696, https://doi.org/10.2217/nnm-2023-0116.
[29] I. Charlie-Silva, L.F. Fraceto, N.F.S. de Melo, Progress in nano-drug delivery of artemisinin and its derivatives: towards to use in immunomodulatory approaches, Artif. Cells, Nanomed. Biotechnol. 46 (2018) 611–620, https://doi.org/10.1080/21691401.2018.1505739.
[30] M. Aroffu, M.L. Manca, J.L. Pedraz, M. Manconi, Liposome-based vaccines for minimally or noninvasive administration: an update on current advancements, Expet Opin. Drug Deliv. 20 (2023) 1573–1593, https://doi.org/10.1080/17425247.2023.2288856.
[31] I. Charlie-Silva, L.F. Fraceto, N.F.S. de Melo, Progress in nano-drug delivery of artemisinin and its derivatives: towards to use in immunomodulatory approaches, Artif. Cells, Nanomed. Biotechnol. 46 (2018) 611–620, https://doi.org/10.1080/21691401.2018.1505739.
[32] L. Gujjari, H. Kalani, S.K. Pindiprolu, B.P. Arakareddy, G. Yadagiri, Current challenges and nanotechnology-based pharmaceutical strategies for the treatment and control of malaria, Parasite Epidemiol. Control. 17 (2022) e00244, https://doi.org/10.1016/j.parepi.2022.e00244.
[33] L. Neves Borgheti-Cardoso, M. San Anselmo, E. Lantero, A. Lancelot, J.L. Serrano, S. Hernández-Ainsa, X. Fernàndez-Busquets, T. Sierra, Promising nanomaterials in the fight against malaria, J. Mater. Chem. B 8 (2020) 9428–9448, https://doi.org/10.1039/D0TB01398F.
[34] M. Juneja, T. Suthar, V.P. Pardhi, J. Ahmad, K. Jain, Emerging trends and promises of nanoemulsions in therapeutics of infectious diseases, Nanomedicine 17 (2022) 793–812, https://doi.org/10.2217/nnm-2022-0006.
[35] R.J. Wilson, Y. Li, G. Yang, C.X. Zhao, Nanoemulsions for drug delivery, Particuology 64 (2022) 85–97, https://doi.org/10.1016/j.partic.2021.05.009.
[36] K.K. Singh, S.K. Vingkar, Formulation, antimalarial activity and biodistribution of oral lipid nanoemulsion of primaquine, Int. J. Pharm. 347 (2008) 136–143, https://doi.org/10.1016/j.ijpharm.2007.06.035.
[37] C.E. Umeyor, O. Obachie, R. Chukwuka, A. Attama, Development insights of surface modified lipid nanoemulsions of dihydroartemisinin for malaria chemotherapy: characterization, and in vivo antimalarial evaluation, Recent Pat. Biotechnol. 13 (2019) 149–165, https://doi.org/10.2174/1872208313666181204095314.
[38] F. Fulgheri, M. Aroffu, M. Ramírez, L. Román-Álamo, J.E. Peris, I. Usach, A. Nacher, M. Manconi, X. Fernàndez-Busquets, M.L. Manca, Curcumin or quercetin loaded nutriosomes as oral adjuvants for malaria infections, Int. J. Pharm. 643 (2023) 123195, https://doi.org/10.1016/j.ijpharm.2023.123195.
[39] Z. Liu, L. Zhao, X. Tan, Z. Wu, N. Zhou, N. Dong, Y. Zhang, T. Yin, H. He, J. Gou, X. Tang, S. Gao, Preclinical evaluations of Norcantharidin liposome and emulsion hybrid delivery system with improved encapsulation efficiency and enhanced antitumor activity, Expet Opin. Drug Deliv. 19 (2022) 451–464, https://doi.org/10.1080/17425247.2022.2063834.
[40] W. Wang, C. Sun, L. Mao, P. Ma, F. Liu, J. Yang, Y. Gao, The biological activities, chemical stability, metabolism and delivery systems of quercetin: a review, Trends Food Sci. Technol. 56 (2016) 21–38, https://doi.org/10.1016/j.tifs.2016.07.004.
[41] A.T. Sipahimalani, D.P. Fulzele, M.R. Heble, Rapid method for the detection and determination of artemisinin by gas chromatography, J. Chromatogr. A 538 (1991) 452–455, https://doi.org/10.1016/S0021-9673(01)88868-3.
[42] E. Horosanskaia, A. Seidel-Morgenstern, H. Lorenz, Investigation of drug polymorphism: case of artemisinin, Thermochim. Acta 578 (2014) 74–81, https://doi.org/10.1016/j.tca.2013.12.019.
[43] A. Catalan-Latorre, M. Ravaghi, M.L. Manca, C. Caddeo, F. Marongiu, G. Ennas, E. Escribano-Ferrer, J.E. Peris, O. Diez-Sales, A.M. Fadda, M. Manconi, Freeze-dried eudragit-hyaluronan multicompartment liposomes to improve the intestinal bioavailability of curcumin, Eur. J. Pharm. Biopharm. 107 (2016) 49–55, https://doi.org/10.1016/j.ejpb.2016.06.016.
[44] J. Rodríguez-Corrales, J.S. Josan, Resazurin live cell assay: setup and fine-tuning for reliable cytotoxicity results, in: Methods in Molecular Biology, Humana Press Inc., 2017, pp. 207–219, https://doi.org/10.1007/978-1-4939-7201-2_14.
[45] C. Lambros, J.P. Vanderberg, Synchronization of Plasmodium falciparum erythrocytic stages in culture. https://www.jstor.org/stable/3280287?seq=1&cid=pdf, 1979.
[46] D.A. Fidock, P.J. Rosenthal, S.L. Croft, R. Brun, S. Nwaka, Antimalarial drug discovery: efficacy models for compound screening, Nat. Rev. Drug Discov. 3 (2004) 509–520, https://doi.org/10.1038/nrd1416.
[47] D. Sanver, A. Sadeghpour, M. Rappolt, F. Di Meo, P. Trouillas, Structure and dynamics of dioleoyl-phosphatidylcholine bilayers under the influence of quercetin and rutin, Langmuir 36 (2020) 11776–11786, https://doi.org/10.1021/acs.langmuir.0c01484.

[48] V. Borhade, S. Pathak, S. Sharma, V. Patravale, Clotrimazole nanoemulsion for malaria chemotherapy. Part I: preformulation studies, formulation design and physicochemical evaluation, Int. J. Pharm. 431 (2012) 138–148, https://doi.org/10.1016/j.ijpharm.2011.12.040.
[49] V. Subramaniyan, Therapeutic importance of caster seed oil, in: Nuts and Seeds in Health and Disease Prevention, Elsevier, 2020, pp. 485–495, https://doi.org/10.1016/B978-0-12-818553-7.00034-6.
[50] A. Yeboah, S. Ying, J. Lu, Y. Xie, H. Amoanimaa-Dede, K.G.A. Boateng, M. Chen, X. Yin, Castor oil (Ricinus communis): a review on the chemical composition and physicochemical properties, Food Sci. Technol. 41 (2021) 399–413, https://doi.org/10.1590/fst.19620.
[51] S.A. Ibrahim, S.K. Li, Efficiency of fatty acids as chemical penetration enhancers: mechanisms and structure enhancement relationship, Pharm. Res. 27 (2010) 115–125, https://doi.org/10.1007/s11095-009-9985-0.
[52] M. Basar, M.I. Khan, M.F. Akhtar, F. Anwar, A. Saleem, A. Madni, Z. Ahmad, A. Sharif, B. Akhtar, U. Shakoor, A. Khan, Olive oil and castor oil-based self-nanoemulsifying drug delivery system of flurbiprofen can relieve peripheral pain and inflammation through reduction of oxidative stress and inflammatory biomarkers: a comprehensive formulation and pharmacological insights, Inflammopharmacology 33 (2025) 353–379, https://doi.org/10.1007/s10787-024-01632-7.
[53] S. Gao, J. Chen, W. Peng, Y. Yang, Y. Yang, L. Hua, Y. Guo, Y. Wang, X. Zhang, The preparation and relative bioavailability of an artemisin in self-emulsifying drug delivery system, Drug Deliv. 30 (2023), https://doi.org/10.1080/10717544.2023.2168794.
[54] D.P.C. de Barros, R. Santos, P. Reed, L.P. Fonseca, A. Oliva, Design of quercetin-loaded natural oil-based nanostructured lipid carriers for the treatment of bacterial skin infections, Molecules 27 (2022) 8818, https://doi.org/10.3390/molecules27248818.
[55] Y. Chang, D.J. McClements, Optimization of orange oil nanoemulsion formation by isothermal low-energy methods: influence of the oil phase, surfactant, and temperature, J. Agric. Food Chem. 62 (2014) 2306–2312, https://doi.org/10.1021/jf500160y.
[56] Z. Niu, A. Acevedo-Fani, A. McDowell, A. Barnett, S.M. Loveday, H. Singh, Nanoemulsion structure and food matrix determine the gastrointestinal fate and in vivo bioavailability of coenzyme Q10, J. Contr. Release 327 (2020) 444–455, https://doi.org/10.1016/j.jconrel.2020.08.025.
[57] R. Zhang, D.J. McClements, Characterization of gastrointestinal fate of nanoemulsions, in: Nanoemulsions, Elsevier, 2018, pp. 577–612, https://doi.org/10.1016/B978-0-12-811838-2.00018-7.
[58] F.M. Shorobi, F.Y. Nisa, S. Saha, M.A.H. Chowdhury, M. Srisuphanunt, K. H. Hossain, MdA. Rahman, Quercetin: a functional food-flavonoid incredibly attenuates emerging and Re-emerging viral infections through immunomodulatory actions, Molecules 28 (2023) 938, https://doi.org/10.3390/molecules28030938.
[59] H.M. Abdou, H.-T.A.E. Abd Elkader, The potential therapeutic effects of Trifolium alexandrinum extract, hesperetin and quercetin against diabetic nephropathy via attenuation of oxidative stress, inflammation, GSK-3β and apoptosis in male rats, Chem. Biol. Interact. 352 (2022) 109781, https://doi.org/10.1016/j.cbi.2021.109781.
[60] A.H. Ali, S. Sudi, N. Shi-Jing, W.R.M. Hassan, R. Basir, H.K. Agustar, N. Embi, H. M. Sidek, J. Latip, Dual anti-malarial and gsk3β-mediated cytokine-modulating activities of quercetin are requisite of its potential as a plant-derived therapeutic in malaria, Pharmaceuticals 14 (2021) 248, https://doi.org/10.3390/ph14030248.
[61] B. Kollerup Madsen, M. Hilscher, D. Zetner, J. Rosenberg, Adverse reactions of dimethyl sulfoxide in humans: a systematic review, F1000Res 7 (2018) 1746, https://doi.org/10.12688/f1000research.16642.2.
[62] P. Valissery, R. Thapa, J. Singh, D. Gaur, J. Bhattacharya, A.P. Singh, S.K. Dhar, Potent in vivo antimalarial activity of water-soluble artemisinin nano-preparations, RSC Adv. 10 (2020) 36201–36211, https://doi.org/10.1039/D0RA05597B.
[63] A. Bilia, M. Bergonzi, J. Boulos, T. Efferth, Nanocarriers to enhance solubility, bioavailability, and efficacy of artemisinins, World J. Tradit. Chin. Med. 6 (2020) 26, https://doi.org/10.4103/wjtcm.wjtcm_2_20.
[64] J.F.S. Ferreira, D.L. Luthria, T. Sasaki, A. Heyerick, Flavonoids from Artemisia annua L. As antioxidants and their potential synergism with artemisinin against malaria and cancer, Molecules 15 (2010) 3135–3170, https://doi.org/10.3390/molecules15053135.
[65] J. Yang, Y. He, Y. Li, X. Zhang, Y.K. Wong, S. Shen, T. Zhong, J. Zhang, Q. Liu, J. Wang, Advances in the research on the targets of anti-malaria actions of artemisinin, Pharmacol. Ther. 216 (2020), https://doi.org/10.1016/J.PHARMTHERA.2020.107697.
[66] N. Puttappa, R.S. Kumar, K. Yamjala, Artesunate-quercetin/luteolin dual drug nanofacilitated synergistic treatment for malaria: a plausible approach to overcome artemisinin combination therapy resistance, Med. Hypotheses 109 (2017) 176–180, https://doi.org/10.1016/J.MEHY.2017.10.016.
[67] T.K. Rajwar, D. Pradhan, J. Halder, V.K. Rai, B. Kar, G. Ghosh, G. Rath, Opportunity in nanomedicine to counter the challenges of current drug delivery approaches used for the treatment of malaria: a review, J. Drug Target. 31 (2023) 354–368, https://doi.org/10.1080/1061186X.2022.2164290.
[68] Y. Avalos-Padilla, X. Fernàndez-Busquets, Nanotherapeutics against malaria: a decade of advancements in experimental models, Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 16 (2024) e1943, https://doi.org/10.1002/WNAN.1943.
[69] M. Thakkar, S. Brijesh, Physicochemical investigation and in vivo activity of anti-malarial drugs co-loaded in Tween 80 niosomes, J. Liposome Res. 28 (2018) 315–321, https://doi.org/10.1080/08982104.2017.1376684.
[70] K. Velasques, T.R. Maciel, A.H. de Castro Dal Forno, F.E.G. Teixeira, A.L. da Fonseca, F. de P. Varotti, A.R. Fajardo, D.S. de Ávila, S.E. Haas, Co-nanoencapsulation of antimalarial drugs increases their in vitro efficacy against Plasmodium falciparum and decreases their toxicity to Caenorhabditis elegans, Eur. J. Pharmaceut. Sci. 118 (2018) 1–12, https://doi.org/10.1016/J.EJPS.2018.03.014.