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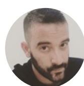

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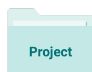

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RESEARCH PAPER

Anti-inflammatory Effect and Toxicology Analysis of Oral Delivery Quercetin Nanosized Emulsion in Rats

Gabriela Hädrich[1,2] • Gustavo Richter Vaz[1] • Michelle Maidana[1] • Jadel Muller Kratz[3] • Gecioni Loch-Neckel[4] • Daniely Cornélio Favarin[5] • Alexandre de Paula Rogerio[5] • Flávio Manoel Rodrigues da Silva Jr[1,2] • Ana Luiza Muccillo-Baisch[1,2] • Cristiana Lima Dora[1]

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ABSTRACT

Purpose This study evaluates the advantage of the quercetin encapsulation in nanosized emulsion (QU-NE) administered orally in rats in order to demonstrate its anti-oedematous and antioxidant effects as well as its toxicity.

Methods The nanocarriers were prepared using the hot solvent diffusion with the phase inversion temperature methods. The nanocarriers physicochemical properties were then investigated. The anti-edematous activity was tested using paw edema in rats. In addition, NF-kB expression in subcutaneous tissue of the paws was accessed by immunohistochemistry while the lipid peroxidation was analyzed in the liver by malondialdehyde reaction with thiobarbituric acid. Hematological, renal and hepatic toxicity as well as the genetic damage were also evaluated.

Results The results demonstrated that QU-NE exhibited pronounced anti-oedematous property comparable to drug diclofenac. This effect was associated with NF-κB pathway inhibition. The lipid peroxidation was also only reduced in rats treated with QU-NE. Besides this, no genetic damage, hematological, renal or hepatic toxicities were observed after administration of QU-NE.

Conclusions These results suggest that quercetin nanosized emulsion exhibits anti-oedematous and antioxidant properties and does not demonstrate toxic effects. This indicates that it has a potential application in the treatment of inflammatory diseases.

KEY WORDS anti-oedematous activity · antioxidant · inflammation · lipid nanocarriers · quercetin · toxicity

ABBREVIATIONS

ALT	Alanine-aminotransferase
AST	Aspartate-aminotransferase
CMC	Carboxymethylcellulose
CO	Castor oil
HBSS	Hank's balanced salt solution
HEPES	Sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate
HPLC	High pressure liquid chromatography
LY	Metoprolol tartrate and lucifer yellow
MDA	Malondialdehyde
MES	Methanesulfonic acid
MN	Micronucleus
NCEs	Normochromatic erythrocytes
NE	Unloaded nanosized emulsion
NSAID	Nonsteroidal anti-inflammatory drug
P_{APP}	Intestinal permeability coefficients
PCEs	Polychromatic erythrocytes
PDI	Polydispersed index
PEG 660-stearate	12-hydroxystearic acid–polyethylene glycol copolymer
PIT	Phase inversion technique

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QU	Quercetin
QU-NE	Quercetin nanosized emulsion
QU-SUSP	Free quercetin
ROS	Reactive oxygen species
TEER	Transepithelial electrical resistance

INTRODUCTION

Inflammation is a normal biological process in response to a tissue injury, microbial pathogen infection and chemical irritation. At the damaged site, inflammation starts with the migration of immune cells from blood vessels due to the release of mediators. The uncontrolled or exacerbated inflammation could cause increase of cells recruitment and pro-inflammatory mediators as well as oxidative burst (reactive oxygen species for example). This could lead to tissue damages as observed in several inflammatory diseases (1–4).

The most used drugs for the treatment of inflammatory diseases such as asthma, arthritis, glomerulonephritis and other are the steroidal (corticosteroids) and nonsteroidal anti-inflammatory drugs (NSAIDs). Although such drugs have pronounced pharmacological activity, they present severe adverse effects that preclude their long-term use (5). The search for new molecules and drugs carriers could contribute to the development of new anti-inflammatory drugs more effective and with less adverse effects (5,6).

Quercetin (3,3′,4′,5,7-pentahydroxyflavone – QU), a representative compound of the flavonoid family, is one of the most common dietary polyphenols. QU is widely spread in the plant kingdom, found in vegetables, fruits, medicinal herbs and red wine. Several studies have shown the multiple biological activities of QU. Among them, it is the pronounced anti-inflammatory property, an effect that is associated with the QU ability to block inflammatory mediators, inducible enzymes and nuclear transcription factor activation (7–9). QU could also reduce inflammation through the scavenge of free radicals that activate transcription factors to generate pro-inflammatory cytokines, which are often found in clinical patients suffering from chronic inflammatory diseases (10,11). However, the poor aqueous solubility, short biological half-life, and low oral bioavailability hamper the application of QU as a therapeutic agent (12–14).

Incorporating QU into lipid-based nanocarriers is an approach to deal with drugs poor water solubility, protect them from degradation, improve compound bioavailability, and control the drug release process. Lipid nanocarriers are typically composed of biocompatible lipid core and hydrophilic surfactant at the outer shell, which can entrap the lipophilic drugs in the inner lipid core and enhance their solubility in gastro-intestinal fluids, thereby promoting the intestinal absorption, enhanced bioavailability of the drug or sustained duration of action (15,16).

Previous studies of our research group have shown the feasibility of incorporating QU into lipid systems (17–19). Pharmacology studies carried out in a murine airway allergic inflammation model demonstrated that the orally administered nanosized emulsion with QU could reduce the asthma process. Such a reduction was not observed after the oral administration of a quercetin suspension at the same dose (20). Also, QU-NE demonstrates a protective role against neuro- and hepatoxicity induced by oxaliplatin (21). Following up on these results, here we investigate the advantage of the quercetin encapsulation in nanosized emulsion in order to prove the anti-oedematous and antioxidant effects of QU lipid nanocarriers administered orally in rats as well as its toxicity.

MATERIAL AND METHODS

Materials

Quercetin (QU), 12-hydroxystearic acid–polyethylene glycol copolymer (PEG 660-stearate/Solutol HS15®), castor oil (CO), λ-carrageenan, tiobarbituric acid were purchased from Sigma–Aldrich (Missouri, USA). Diclofenac was purchased from Novartis® (São Paulo, Brazil). Carboxymethylcellulose (CMC) was purchased from Natural Pharma (São Paulo, Brazil). Hydrogenated soybean lecithin (Phospholipon 80®) was purchased from Lipoid (Steinhausen, Switzerland). Alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), urea and creatinine were purchased from Labtest (São Paulo, Brazil). High-pressure liquid cromatography (HPLC) grade methanol was purchased from Pareac® (Barcelona, Spain) and HPLC grade water was prepared using a Milli-Q system from Millipore (Billerica, USA). Ethanol, acetone, phosphoric acid, and other chemicals used were analytical reagent grade. Hank's balanced salt solution (HBSS), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES), methanesulfonic acid (MES), trypsin, metoprolol tartrate and lucifer yellow (LY) were purchased from Sigma–Aldrich (Missouri, USA).

Preparation and Characterization of the Quercetin-Loaded Nanosized Emulsion and Quercetin Suspension

The nanosized emulsion (NE) was prepared using the hot solvent diffusion method with the phase inversion technique (PIT) (19). Briefly, a solution containing QU, castor oil (CO) and lecithin in acetone:ethanol (60:40, *v/v*) at 60°C was added to an aqueous phase containing surfactant PEG 660-stearate at 80°C, under magnetic stirring (700 rpm). The resulting colloidal suspensions were then cooled to room temperature, the organic solvent evaporated under reduced pressure, and the final volume adjusted to 20 mL. Finally, the colloidal

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suspensions were filtered through an 8 μm filter paper. QU was added to the organic phase of the formulation to prepare the nanosized emulsion (QU-NE). As for the biological experiments, free quercetin (QU-SUSP) was administered in a suspension form which has been prepared through the addition of equivalent QU amount to aqueous carboxymethylcellulose dispersion (0.5%; *p*/*v*).

Size and Zeta Potential Measurements

Particle size and zeta potential have been determined using light scattering and laser-doppler anemometry, respectively, using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Particle size measurements were performed at 25°C after appropriate dilution of the samples in distilled water. Each particle size analysis lasted for 300 s and it was performed with an angle detection of 90°. Stokes-Einstein's equation was used to determine the hydrodynamic radius. With respect to zeta potential measurements, the samples were placed in the electrophoretic cell with a potential of ±150 mV. The zeta potential values are calculated using Smoluchowski's equation as mean electrophoretic mobility values.

Determination of Quercetin Concentration and Entrapment Efficiency in the Lipid Nanocarriers

The analysis was carried out using a UFLC Shimadzu LC-20 AD system (Kyoto, Japan) equipped with a LC-20AT pump, SPD-20A UV detector, CMB-20A system controller, CTO-20 AC column oven, and the sample injection was performed through a Rheodyne 7125 valve with a 20 μL loop. The detector was set at 369 nm and peak areas were integrated automatically by computer using a LC solution® software program (Kyoto, Japan). The experiments were performed using a reversed-phase Zorbax ODS (Agilent Technologies, Wilmington, DE, USA) C18 column (150 mm×4.6 mm I.D., with a particle size of 5 μm), maintained at 40±1°C. The mobile phase consisted of a 1% phosphoric acid:methanol mixture (45:55 *v*/*v*; pH 2.7) and was eluted isocratically at a flow rate of 1 mL min^{-1}.

The QU content (total concentration) in the colloidal suspensions was calculated after determining the drug concentration in the mobile phase (in μg of QU/mL of suspension). The entrapment efficiency (%) was estimated as being the difference between the total concentration of quercetin in the supernatant obtained by ultrafiltration/centrifugation procedure using Ultrafree-MC membranes 10,000 NMWL from Millipore (Billerica, USA). All samples were analyzed in triplicates batches.

In Vitro Release Studies

For the experiment, 2 mL of the nanocarrier was placed into a dialysis bag MWCO 10,000 from Sigma-Aldrich® (Missouri, USA). The dialysis bags were placed into a becker containing 250 mL of ethanol:distilled water (35:75, *v*/*v*; pH 4.0) solution to maintain sink conditions. The release medium was maintained at 37°C under magnetic stirring at 75 rpm. Samples of the release medium were taken after 0.5, 1, 2, 4, 6, 8, and 24 h. The release medium was immediately replaced with fresh medium. The samples were analyzed through HPLC under the same conditions as described above. To evaluate the effect of the dialysis membrane on the drug release rate, a solution of quercetin in ethanol was placed into a dialysis bag and the quercetin diffusion through the membrane was assayed using the same conditions. All experiments were carried out in triplicates. The cumulative amounts of QU released (in %) were plotted against time (h).

In Vitro Intestinal Permeability

Caco-2 cells (ATCC:HTB-37) were maintained as previously described (22). For transport experiments, 1.0×10^5 cells (passages 121–123) were seeded on polycarbonate filter inserts (0.6 cm2, pore size 0.4 μm; Millipore, Billerica, MA, USA) and allowed to grow and differentiate for 21–22 days. The transepithelial electrical resistance (TEER) and the permeability of the paracellular marker Lucifer yellow (100 μg/mL) were used as monolayers integrity markers. Only monolayers with TEER values above 200 Ωcm^2 (before and after the experiments) were considered.

The intestinal epithelial permeability was determined from transport rates across Caco-2 cell monolayers (apical to basolateral). QU stock solution (5 mg/mL, DMSO) and all formulations were diluted to 50 μg/mL (equivalent amounts of QU) in HBSS pH 6.5. Before the experiments, cell monolayers were gently rinsed and equilibrated for 30 min at 37°C with the same blank buffer. The transport experiments were run for 90 min at 37°C under constant stirring (150 rpm). Receiver sides filled with HBSS pH 7.4 were sampled at appropriate times, and the amount of QU permeated was determined by HPLC analysis as described in "Determination of quercetin concentration and entrapment efficiency in the lipid nanocarriers" section. The apparent permeability coefficients (P_{app}, cm/s) were calculated from the equation 2:

$$P_{app} = \Delta Q/\Delta t \times 1/AC_0$$

where ΔQ/Δt is the steady-state flux (mol/s); C_0 is the initial concentration in the donor chamber at each time interval (mol/mL); and A is the surface area of the filter (cm^2) (23). Data are reported as the mean ± SD of three independent experiments.

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In Vivo Anti-oedematous Activity

Animals

Male Wistar rats (8 weeks old and weighing around 250–300 g) obtained from the Animal Facility of the Federal University of Rio Grande were used in this study. Animals were maintained in conventional cages, with normal temperature conditions (22 ± 2°C), relative humidity (60–80%) and light/dark cycle of 12 h, with food and water *ad libitum*. This experimental protocol was approved by the Animal Use Ethics Committee (CEUA) of the Federal University of the Rio Grande (FURG) (23116.006240/2012-68) in accordance with national and international Guidelines.

Experimental Design

Animals were divided into five groups (n = 15). The treatment saline solution (0.9%), quercetin suspension in CMC (QU-SP), unloaded-NE and QU-NE were given once a day, orally per 5 days before the test, by gavage with a volume (20 mg kg^{-1}) calculated according to the animal's weight. The experimental design was based on *in vitro* release test, once a day, since we have a sustained quercetin release over 24 h. Previous study showed that quercetin release profile followed a first order kinetics, indicating that QU exhibits controlled release when incorporated into the nanoemulsion (19). The doses of QU and QU-NE have been chosen based on previous data (20,24). The dose and treatment with Diclofenac (10 mg kg^{-1} - given once, intraperitoneally 90 min before the edema induction) follow the research literature on the use of this drug as a positive control for the carrageenan-induced hind paw edema test (25,26).

Carrageenan-induced Hind Paw Edema

The carrageenan-induced hind paw edema in rats was used to establish the anti-oedematous activity of the QU-NE, and it was conducted as described in (27), by injection of 100 μl of 1% ($w.v^{-1}$) λ-carrageenan, type IV, diluted in saline in the left hind paw pad. As control, 100 μl saline solutions were injected into that of the right hind paw. Paw edema was then determined using a water plethysmometer (PanLab, Barcelona, Spain) at times 0, 1, 2, and 3 h. In preliminary experiments we evaluated the effect of paw edema over a time period. The results have shown that carrageenan-induced hind paw edema is an acute model with peak of inflammation within 2–3 h. A slight decline in the paw edema was observed at the fourth hour. For this reason, T3 hours was chosen since we intended to evaluate the response of inflammation mediators during the inflammation peak (data not shown). After the 3rd hour animals were euthanized and the liver, bone narrow, hind paw tissue and blood were collected and properly stored until processing. The percentage inhibition of edema was calculated by the following equation (28):

$$\%\textbf{inhibition of edema} = [(\mathbf{V_t} - \mathbf{V_0})\ \textbf{Control} - (\mathbf{V_t} - \mathbf{V_0})\ \textbf{Treated}] / (\mathbf{V_t} - \mathbf{V_0})\ \textbf{Control} \times \mathbf{100}$$

Where V_t is the volume at different intervals after carrageenan injection and V_0 is the paw size initial volume recorded just before carrageenan injection.

Immunohistochemistry

The subcutaneous tissue of the paws was collected when euthanasia was done after the third hour of the edema measurement and fixed at 10% formol for 24 h, then kept in 70% ethanol until use. Immunohistochemical detection of p65 NF-κB was carried out using polyclonal rabbit anti-phospho-p65 NF-κB (#sc-109, 1:100) from Santa Cruz Biotechnology (California, USA). Tissues embedded in paraffin were cut into thick sections (5-μm). Slides were deparaffinized through a series of xylene baths and rehydrated through graded alcohol solutions. High temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98°C in 10 mmol/L trisodium citrate buffer pH 6.0, for 45 min. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus (DakoCytomation, Carpinteria, CA, USA), ready-to-use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by use of 3,3′-diaminobenzidine (DAB) (Dako) in chromogen solution and light counterstaining with Harris's haematoxylin solution. Images were obtained with a microscope (Eclipse 50i; Nikon, Melville, NY) and Digital Sight Camera (DS-Fi1; Nikon). Control and experimental tissues were placed on the same slide and processed under the same conditions. Nonimmune rabbit serum was used as a negative control.

Antioxidant Activity

Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS) (29). Liver homogenates were added to a reaction mixture made with 20% acetic acid, thiobarbituric acid (0.8%), Milli Q water and sodium dodecyl sulfate (SDS, 8.1%). Samples were heated at 95°C during 30 min and after cooling by 10 min, Milli Q water and n-butanol was added. After centrifugation (3000 g during 10 min at 15°C), the organic phase was placed in a microplate reader and the fluorescence registered after excitation at 515 nm and emission of 553 nm. The concentration of TBARS (nmol/mg of wet tissue) was calculated employing tetramethoxypropane (TMP) as standard.

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Toxicological Analysis

Biochemical and Hematological Analysis

To carry out analyzes of biochemical and hematological parameters, blood samples were collected and stored individually with heparin for hematological evaluation, or without heparin for biochemical analysis. Hematological evaluations were performed by manual measurement of lymphocytes, monocytes and segmented neutrophils levels. For the biochemical analyzes, blood samples were centrifuged at 3000 rpm at room temperature for 15 min, and the serum obtained was stored at −20°C for analysis. ALT and AST, as indirect indicators of liver damage, have been established according to the manufacturer instructions (Labtest, Brasil). Results were expressed as U/L. As indirect indicators of kidneys damage, urea and creatinine were measured according to the manufacturer's instructions (Labtest, Brasil).

Micronucleus Assay

The micronucleus test was performed according to OECD (2001) (30). Two slides of each animal were prepared and the ratio of polychromatic erythrocytes (PCEs) and normochromatic (NCEs) was recorded, the number of micronuclei per 1000 PCEs was counted.

Statistical Analysis

The data were reported as mean ± S.E.M. We used ANOVA to compare statistical significance among different treatments in each individual experiment. When significant differences were identified, individual comparisons were subsequently made with Tukey *post-hoc*'s. Values of $p<0.05$ were considered statistically significant.

RESULTS

Preparation and Characterization of the QU-NE

In the present study, a combination of the two low-energy methods, hot solvent diffusion and PIT, was employed to produce lipid nanocarriers containing QU. A summary of the physicochemical properties, QU content, and entrapment efficiency is presented in Table I. It has been observed mean size and polydispersed index (PDI) of approximately 20 nm and 0, 2, respectively. The zeta potential was about −4 mV. Unloaded nanocarriers were analyzed and the results were similar. Quercetin content, determined by HPLC, showed that it was possible to encapsulate 1.5 mg/ml of quercetin in the nanosized emulsion. The entrapment efficiency was evaluated and the QU incorporation into the nanocarriers was around 98%. Figure 1 shows the *in vitro* release kinetics of quercetin from QU-NE 8 in ethanol:distilled water (35:75, *v/v*; pH 4.0) at 37°C. The experiments were carried out in sink conditions, since the maximum concentration of QU that were reached in the release medium corresponded to 10% of its saturation concentration (1.8 mg/mL). Quercetin release profiles were characterized by biphasic kinetics, consisting of a quicker release in the first 8 h, followed by a sustained release of the drug over 24 h.

In Vitro Intestinal Permeability

The intestinal permeability coefficients (P_{app}) of QU in aqueous solution (from DMSO stock solutions), free QU or QU-NE (all samples corresponding to 50 µg/mL) were measured by Caco-2 cell assay. Permeability was obtained from unidirectional experiments in the absorptive direction (apical to basolateral). A P_{app} value of $8.43 \pm 2.60 \times 10^{-6}$ cm.sec^{-1} was obtained for the free aqueous solution of quercetin. Metoprolol, a low/high permeability boundary marker, showed a P_{app} value of $55.77 \pm 22.99 \times 10^{-6}$ cm.sec^{-1}. Lucifer yellow, a paracellular marker, showed a P_{app} value of $0.60 \pm 0.18 \times 10^{-6}$ cm.sec^{-1}. When incorporated into CMC suspensions, QU presented a permeability profile similar to dissolved QU ($P_{app} = 7.88 \pm 1.04 \times 10^{-6}$ cm.sec^{-1}). The P_{app} value obtained for the nanocarrier system was significant lower than both free QU systems, with a $P_{app} = 1.25 \pm 0.52 \times 10^{-6}$ cm.sec^{-1}.

In Vivo Anti-oedematous Activity

Intraplantar injection of carrageenan induced significantly greater rat paw volume. Rat treated with QU-NE or diclofenac presented significantly less pronounced oedematous responses to carrageenan, beginning at 1 h after carrageenan administration and continuing throughout the experimental period (*p* value <0.0001) (Fig. 2). Increase paw edema was observed in rats stimulated by carrageenan. Table II presents the delta of the paw edema and the percentage of inhibition of all treatments for all times tested calculated using equation 1. It is important to note the inhibition of QU-NE was 78.6, 78.8 and 90.6% in the first, second and third hour, respectively. At the third hour, the QU-NE treatment inhibited more effectively than treatment with diclofenac. We draw the attention that free QU administered with the same treatment protocol (20 mg kg^{-1}, 5 days orally) have not shown any anti-inflammatory effect in this model. No significant alteration on oedema was observed in the animals treated with vehicle, and injected with carrageenan, when compared to animals injected with carrageenan without treatment.

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Table I Physico-chemical Characteristics of Nanosized Emulsion

NE	Size (nm)	PDI	ζ-potential (mV)	QU content ($mg.mL^{-1}$)	Recovery (%)
NE	19.32 ± 0.41	0.14 ± 0.011	−5.12 ± 0.60	–	–
QU-NE	19.25 ± 0.20	0.20 ± 0.004	−5.34 ± 0.50	1.5 ± 0.02	99.5 ± 0.10

PDI Polydispersity index. The results were expressed by mean ± S.E.M. of 3 replicates

Effect of QU-NE on the NF-κB Activation in Rat Hind Paw Edema

Figure 3 shows the results of the imunohistochemical assay. The paw tissue injected with saline (control group) showed no fosforilation of p65 NF-κB (Fig. 3a). However, in the paw tissue of rats injected with carrageenan treated or not with vehicle it was detected the phosphorylated p65 subunit of NF-κB (Fig. 3b, and c). On the other hand, QU-NE given orally to mice, reduced the p65 NF-κB activation (Fig. 3e), and less reduction was observed in animals treated with QU-SUSP (Fig. 3d). Diclofenac similar to QU-NE also reduced the p65 NF-κB activation (Fig. 3f).

Antioxidant Activity

The anti-oxidant activity of the level of MDA was assessed as an index of lipid peroxidation in liver tissue. As can be seen in Fig. 4, the MDA level was increased in the animals injected with carrageenan treated or not with vehicle. QU-NE decreased significantly ($p < 0.001$) the lipid peroxidation when compared to group injected with carrageenan. The remarkable decrease in lipid peroxides in liver tissue when QU-NE was administrated indicates a reduction in the formation of reactive oxygen species (ROS), which plays a major role in cell injury indicating a better antioxidant activity of QU when loaded in nanocarriers. Free QU and diclofenac were unable to reduce the lipid peroxidation.

Toxicological Analysis

The results of haematological analysis, transaminases (AST and ALT), urea and creatinine levels are shown in Table III. No alteration in segmented neutrophils, lymphocytes and monocytes were observed during the administration of blank nanocarriers and QU-NE. The enzymes in the peripheral blood from the rats have not shown any statistically significant difference from the saline group. This demonstrates a normal function of the liver and kidneys after the test.

The micronucleus (MN) was used to evaluate genetic damage data for rat bone marrow cells. The treatment with QU-NE and with unloaded-NE did not show statistically significant increases in the mean number of MN frequency of micronucleus per 1000 cells for the groups threated (3.3 ± PNE) when compared with control (3.5 ± 0.8).

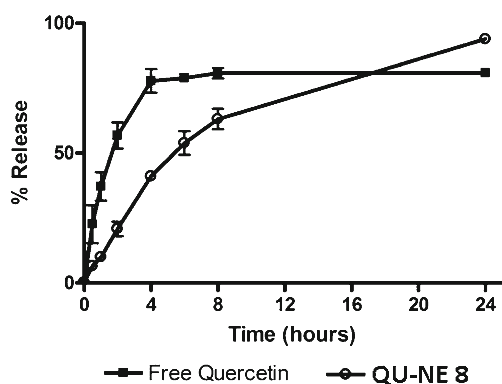

Fig. 1 Cumulative percentage of QU released from nanocarriers.

DISCUSSION

The main results in the present study are the demonstration that QU-NE exhibits pronounced anti-oedematous property when evaluated in carrageenan hind paw edema model in rats due to reduce the activation of NF-κB pathway. In addition QU-NE reduced the lipid peroxidation and does not demonstrate toxic effects.

Over the past few decades, drug delivery systems have provided tremendous strength in improving the reliability and safety of existing drugs. Quercetin, a representative compound of the flavonoid family, is one of the most common dietary polyphenols. Researches over the past decade have shown the compound to possess the preventive and therapeutic value against a wide variety of diseases (31,32). Despite its promising pharmacological activity, quercetin's low oral bioavailability remains a major hurdle. This makes quercetin a molecule of interest to drug delivery scientists (33).

To improve the oral efficacy, many edible delivery systems have been developed to enhance the bioavailability of quercetin through various mechanisms, including the complexation with cyclodextrins and liposomes (34,35). A major drawback of such technologies is the stability issue during sterilization and storage. On the other hand, nanoemulsion is one of the most oral delivery methods studied to protect against degradation, enhance solubility, and facilitate higher biological uptake of drugs. Specifically, oil-in-water nanoemulsions consist of oil droplets dispersed in an aqueous continuous phase, with the oil droplets being surrounded by a thin interfacial layer of emulsifier molecules. The lipophilic compounds, which have a low aqueous solubility, can be easily incorporated into emulsion-based delivery systems (36,37). Hence, the development of a safe, stable and efficient delivery method for

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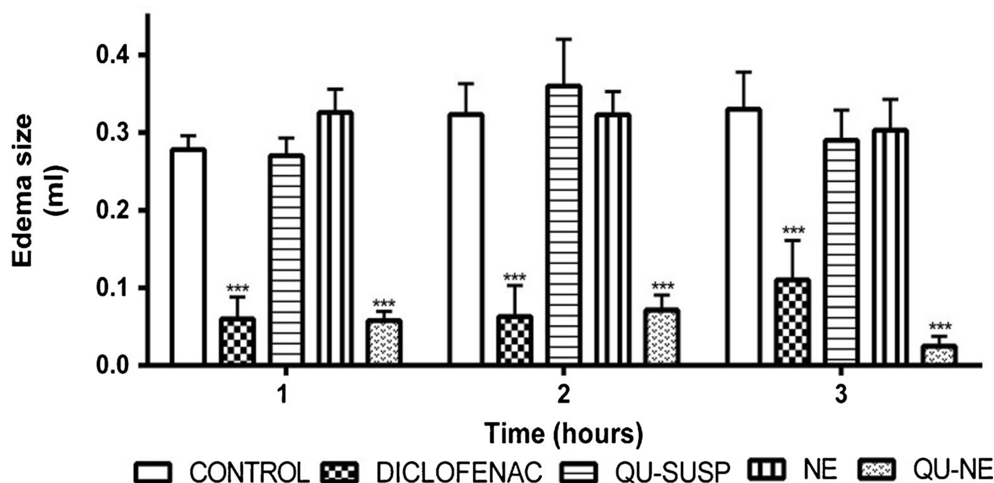

Fig. 2 Activity anti-edematous of diclofenac, free quercetin (QU-SUSP), unloaded nanosized emulsion (NE), and quercetin nanosized emulsion (QU-NE), on carrageenan-induced mouse paw oedema. Rats were evaluated for paw edema at 1, 2, and 3 h post-carrageenan injection. Results were expressed as the edema size (ml). Each point represents the mean ± SEM of six rats. ***Statistically significant compared with control group at $p < 0.001$.

quercetin encapsulation could present an interesting approach to investigate its potential therapeutic property.

Considering the potential of nanoemulsions as oral drug delivery system, we have evaluated the advantage of the quercetin encapsulation in nanosized emulsion administered orally in rats in order to prove its anti-oedematous and antioxidant effects as well as its toxicity.

Carrageenan induced hind paw edema is the standard experimental model of acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects. Moreover, the experimental model exhibits a high degree of reproducibility. Inflammation induced by carrageenan is acute, nonimmune, well researched and highly reproducible. It is characterized by releasing of several pro-inflammatory mediators such as cytokines, chemokines and oxygen-derived free radicals and consequently by increasing of vascular permeability, and cell migration mainly neutrophils (38,39).

Our results show that QU-NE exhibits pronounced oral anti-oedematous property. However, free quercetin (QU-SUSP) administered with the same treatment protocol did not have the effect. The anti-inflammatory activity of free quercetin was previously studied in carrageenan-induced rat paw edema (40). It was administrated orally (equivalent dose with 20 mg kg $^{-1}$ of quercetin) 30 min before the test and a slightly decrease (10%) in edema was observed. The anti-inflammatory activity of a quercetin-loaded nanostructured lipid carrier gel was evaluated by Chen-Yu *et al.* (2012) (41) in an acute inflammation model using the ear edema caused by xylene. The formulation was administrated topically at the site of inflammation (0.2 mg QU/ear). The results showed that the quercetin-loaded nanostructured lipid carrier gel inhibited 69.4% of the inflammation when compared to control and free QU decreases only 12%. Our results for QU-SUSP were similar, indicating the low bioavailability of this flavonoid when administered in free form.

These findings corroborate the idea that quercetin into a nanocarrier could have a higher anti-inflammatory effect, thus, representing an effective delivery system. In a previous study (20) we analyzed the oral absorption of quercetin nanosized emulsion in rats by HPLC-MS. In this study at 2 h and 3 h after the treatment it was detected quercetin metabolite in plasma of rats treated only with the nanocarrier

Table II Effect of Quercetin Nanosized Emulsion, Free Quercetin and Diclofenac on the Carrageenan-induced Paw Edema Size

Treatment	1 h		2 h		3 h	
	Δ Paw edema ± SEM (ml)	% inhibition	Δ Paw edema ± SEM (ml)	% inhibition	Δ Paw edema ± SEM (ml)	% inhibition
Saline	0.28 ± 0.02^{b}	6.3 ± 3.6	0.32 ± 0.04^{b}	10.9 ± 4.5	0.33 ± 0.05^{b}	12.9 ± 10.0
Diclofenac	$0.06 \pm 0.03^{a,c}$	77.8 ± 1.1	$0.06 \pm 0.04^{a,c}$	80.4 ± 12.3	$0.11 \pm 0.05^{a,c}$	65.1 ± 14.6
QU-SUSP	0.27 ± 0.02^{b}	7.0 ± 5.7	0.36 ± 0.06^{b}	13.4 ± 8.5	0.29 ± 0.04^{b}	19.6 ± 4.7
NE	0.33 ± 0.03^{b}	4.1 ± 4.1	0.32 ± 0.03^{b}	9.9 ± 4.3	0.30 ± 0.04^{b}	15.6 ± 8.1
QU-NE	$0.06 \pm 0.01^{a,c}$	79.1 ± 4.3	$0.07 \pm 0.02^{a,c}$	77.8 ± 5.9	$0.02 \pm 0.02^{a,c}$	92.5 ± 3.8

Values of the delta of the paw edema are expressed in mean ± SEM ($n = 6$). Different letters in the same column are significantly different. $^{a}p < 0.001$ significantly different from saline. $^{b}p < 0.001$ significantly different from diclofenac. $^{c}p < 0{,}05$ significantly different from free-quercetin (QU-SUSP)

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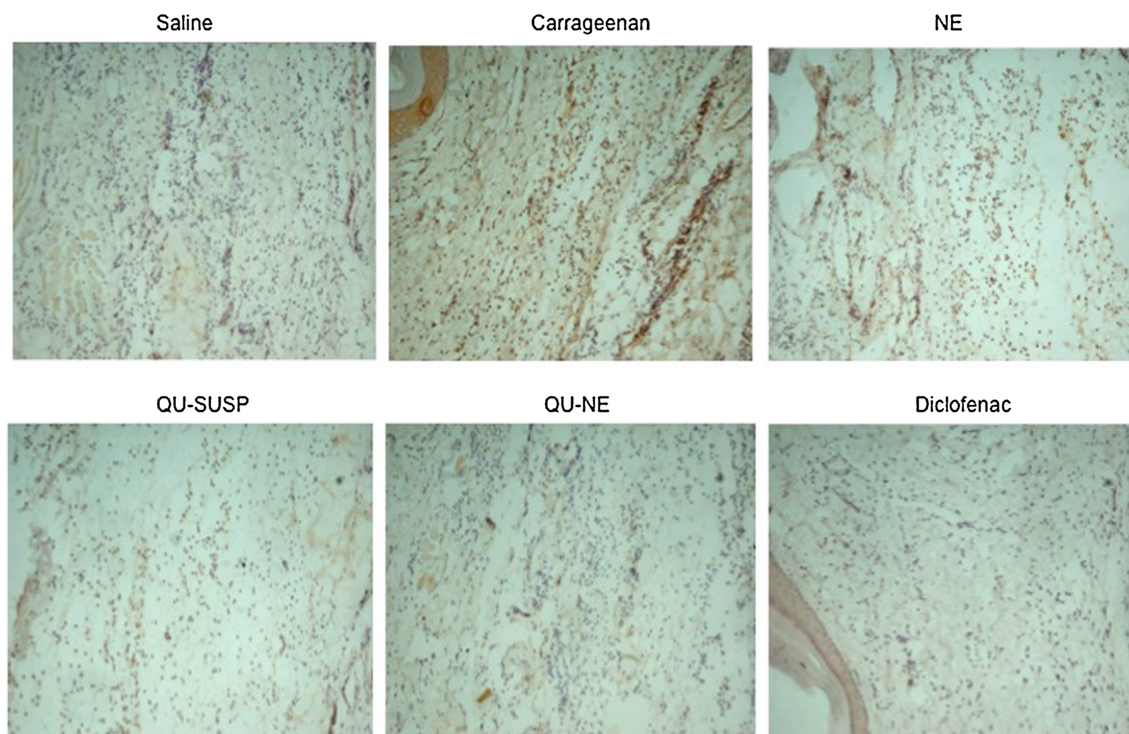

Fig. 3 Quercetin nanosized emulsion reduced the NF-κB expression in the paw stimulated with carrageenan. Rats were treated with saline or carrageenan (1% per paw) and then the paws were isolated at the 3 h. Rats were treated orally with unloaded nanosized emulsion (NE), free quercetin (QU-SUSP), quercetin nanosized emulsions (QU-NE) (20 mg kg^{-1}, p.o.) and diclofenac (10 mg kg^{-1}, i.p.), 90 min before carrageenan injection and then immunohistochemistry was performed for NF-κB. Representative images of phospo-p65 NF-κB imunohistochemistry staining of saline (**a**), carrageenan (**b**), NE (**c**) QU-SUSP (**d**), QU-NE (**e**) and Diclofenac (**f**) were stained with periodic acid-Schiff (original magnifications, ¥200).

but not after the treatment with free quercetin. This result suggests that the delivery system developed improves the oral bioavailability of quercetin. The oral bioavailability of several flavonoids such as berberine (42), puerarin (43) and curcumin (44) also showed significant improvement when compared to non-emulsion based oral formulations.

When emulsions are used as oral delivery systems, the pharmacokinetic behaviors, body distribution, gastric empty time, and many other physiological parameters may be modified to improve the oral bioavailability of target bioactives. There are three primary mechanisms by which lipids and lipophilic excipients affect drug absorption, bioavailability and disposition after oral administration. These are: (i) change of composition and character of the intestinal milieu, (ii) the recruitment of intestinal lymphatic drug transport, and (iii) the interaction with enterocyte-based transport processes (16). In this context, we tested the nanocarriers *in vitro* intestinal permeability in the Caco-2 cell model. As a result, free quercetin permeated better and faster when compared to the QU encapsulated into lipid nanocarrier in this assay. Here we note a limitation of this methodology since it was tested for 90 min. QU-NE release studies indicate an *in vitro* drug release of 24hs.

It is well documented that the NF-κB pathway can be activated in the inflamed tissue. Yoon et al. (2011) (45) investigated the effect of QU in primary cultured orbital fibroblasts from Graves' Orbitopathy and the results showed that this flavonoid significantly attenuated the IL-1β or TNF-α concentration *via* reduction of activation of NF-κB pathway. Wan

Fig. 4 Effect of diclofenac, free quercetin (QU-SUSP), unloaded nanosized emulsion (NE), and quercetin nanosized emulsion (QU-NE) on the lipid peroxidation. Each point represents the mean ± SEM of six rats. **Statistically significant compared with control group at $p < 0.01$.

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Table III Biochemical and Hematological Analysis

	AST (U/dl)	ALT (U/dl)	UREA (md/dL)	Creatinine (md/dL)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)
Saline	136.8 ± 21.7	47.2 ± 6.4	50.3 ± 1.8	0.37 ± 0.01	18.6 ± 1.9	78.4 ± 1.6	3.05 ± 0,5
Diclofenac	133 ± 42.6	122.3 ± 16.7	151.3 ± 35.6	0.63 ± 0.11	40.3 ± 1.9	58 ± 2.1	1.6 ± 0.7
QU-SUSP	128 ± 19.1	49,5 ± 3.1	53.1 ± 1.0	0.37 ± 0.02	25.9 ± 4.1	71.5 ± 3.9	2.5 ± 0.43
NE	115.2 ± 21.5	45.5 ± 3.4	52.3 ± 1.8	0.40 ± 0.03	20.1 ± 2.1	78 ± 2.1	1.8 ± 0.5
QU-NE	122.3 ± 13.8	38.3 ± 4.7	44.5 ± 3.6	0.40 ± 0.03	17.3 ± 2	80,3 ± 1,9	2.3 ± 0.5

Values are expressed as the mean ± SEM

et al. (2014) (46) reported that liposomal quercetin can effectively inhibit acute hepatitis and hepatic fibrosis induced by Con A in rats and also showed that the inhibitory effect of quercetin was associated with its ability to modulate the NF-κB signal. Rogerio *et al.*, 2010 (20) demonstrated in an airways allergic inflammation models in mice induced by ovalbumin that reduction of IL-4, IL-5 concentrations, P-selectin expression and the eosinophil recruitment in the airways in animals treated with QU-NE, but not QU-SUSP, it was consequence of reduction of NF-κB activation. Our study corroborates this result since we have demonstrated an inhibition of NF-κB only when QU-NE was administrated in our model.

Flavonoids may inhibit NF-κB by acting as antioxidants, since NF-κB is a redox-sensitive transcription factor (8,47). The antioxidant and anti-inflammatory activity of flavonoids were closely related since reactive oxygen species were produced during the inflammation process by phagocytic cells. Reactive oxygen species may be involved in the cycloxygenase- and lipoxygenase-mediated conversion of arachidonic acid into pro-inflammatory intermediates (48). Quercetin blocked both the COX-2 and lipooxygenase pathways at relatively high concentrations, while at lower concentrations it blocked mainly the lipoxygenase pathway (49). In order to evaluate the antioxidant activity of QU-NE it was done the peroxidation lipid test. QU-NE, but not free QU, demonstrated antioxidant effects in our model. These findings corroborate a study of Ghosh *et al.* (2009) (50). As drinking water contaminated by arsenic is a well-known cause of chronic poisoning through causing oxidative stress, quercetin nanocapsule, was successfully demonstrated to improve efficacy against arsenic-induced oxidative damage in liver and brain tissues than unformulated free quercetin. These results showed that the change in pharmacokinetics, modulated by the drug encapsulation in nanocarriers, could lead to a change in pharmacodynamics, but not to a change in a pharmacological property.

The improvement in antioxidant and anti-inflammatory property was also demonstrated for other flavonoids encapsulated in nanoformulations. The effect of nanoemulsion system on the anti-inflammatory activity of curcumin was evaluated using the mouse ear inflammation model. While curcumin suspension showed no inhibition effect, orally dosed curcumin emulsion (618.6 nm) or nanoemulsion (79.5 nm) exhibited significant reduction in 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse ear edema by 43 and 85%, respectively (51). The anti-oxidation ability of a-tocopherol nanocarrier was increased in all liver, kidney and brain (52). Oral administration of resveratrol and puerarin nanoformulattions was proven to effectively reduce inflammation, colon inflammation and colon cancer proliferation in rodents (53–55).

NSAIDs are widely used to help relieve musculoskeletal pain and inflammation, but can cause serious upper gastrointestinal side effects including dyspepsia, peptic ulceration, and hemorrhage (56). Thus, further research is need to develop new drugs or dosage forms with less adverse effects. In addition to anti-inflammatory activity, QU may also have gastric ulcer protective effect through the reduction of the lipid peroxidation and an increase of the activity of antioxidant enzymes (57). The possibility to use quercetin as an anti-inflammatory agent with protective effect against ulcer makes this compound even more interesting when compared to NSAIDs.

QU-NE has not demonstrated toxic effects (no alteration on hematological or biochemical parameters and genetic damages) in our analyses. Earlier studies have suggested that QU at higher doses (3 g/kg) does not exhibit toxic and harmful effect in mice (58,59). As for humans, doses of 1.2 g per day are safe and well tolerated (60). Thus, QU-NE seems to be within of the tolerable limits.

CONCLUSION

The results presented herein showed that QU-NE reduced significantly the paw edema. This is probably due to the reduction of activation NF-κB pathway. Quercetin may inhibit NF-κB acting as antioxidant by reducing the lipid peroxidation. Furthermore QU-NE did not show toxic effects. This indicates that QU-NE has a potential application in the treatment of inflammatory diseases.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflicting interests.

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