Inflammopharmacology
https://doi.org/10.1007/s10787-017-0438-9

ORIGINAL ARTICLE

Anti-inflammatory activity of nanoemulsions of essential oil from *Rosmarinus officinalis* L.: in vitro and in zebrafish studies

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Received: 5 November 2017 / Accepted: 26 December 2017

Abstract
The essential oil from *Rosmarinus officinalis* L. (OERO) has bioactive compounds with anti-inflammatory activity. The objective of this study was to evaluate the anti-inflammatory potency of nanoemulsions containing essential oil of *Rosmarinus officinalis* L. (NOERO, NECHA, NECULT, and NECOM) in vitro and in vivo. This study was accomplished in a quantitative format through tests with diphenyl picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), cellular antioxidant activity (CCA), determination of nitric oxide production, cellular viability and anti-inflammatory activity in zebrafish. OERO's were submitted to the analysis-coupled gas chromatography–mass spectrometry (GC–MS), which highlighted 1,8-cineol and camphor as major compounds. NOEROs were obtained by a low-energy method and presenting the medium size smaller than 200 nm. The efficiency of encapsulation by spectrometry and gas chromatographic analysis was 67.61 and 75.38%, respectively. In the CCA assay, all of the samples presented percentage values of inhibition similar to the quercetin pattern, indicating antioxidant activity. In the test for determination of NO·, all of the samples inhibited the production of NO· when compared to LPS, and NOEROS were more effective than OEROS to 5 µg/mL. In the cell viability assay, the cells remained viable after contact with the samples, demonstrating an absence of cytotoxicity. This study showed that all nanoemulsions (NECHA, NECULT, and NECOM) showed no toxicity to macrophages, besides demonstrating antioxidant activity and potentiation of the essential oil effect in the proliferation of viable fibroblasts. Nanoemulsions has also shown the ability to potentiate the anti-inflammatory action of essential oils by exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide. The results obtained with NECHA in zebrafish confirm the hypothesis that prominent terpenic compounds, alpha-pinene, 1,8-cineole, and camphor, became more available at the target sites, inhibiting the inflammatory process in this animal species.

Keywords *Rosmarinus officinalis* · Essential oil · Nanoemulsions · Anti-inflammatory · In vitro · In vivo · Zebrafish

Introduction

The Rosemary, scientific name *Rosmarinus officinalis* L., is a medicinal plant of the Lamiaceae family, that presents erect subshrub feature with little branch and height of up 1.5 m (Lorenzi and Matos 2002). Their leaves, quite aromatic, and stem are used popularly for medicinal purposes, ingested as tea (Marchiori 2004). It has been widely used in traditional medicine and as a flavouring in food (Ohno et al. 2003).

The essential oil of *Rosmarinus officinalis* L. (EORO), from the leaves, has been related to the presence of chemical compounds with specific properties (Celiktas et al. 2007). The chemical profile of EORO may vary according to environmental conditions; however, the most known chemotypes in the literature are cineoliferous (higher concentration of 1,8-cineole), camphorliferous (high concentration of camphor), and verbenoniferous (predominance of verbenona) (Napoli et al. 2015). Studies report bactericidal and fungicidal properties (Mekonnen et al. 2016),

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Published online: 05 February 2018

larvicide (Duarte et al. 2015), insecticide (Badreddine et al. 2015), acaricide (Martinez-Velazquez et al. 2011), antioxidant (Rašković et al. 2014), antinociceptive (Faria et al. 2011), anti-caries (Freires et al. 2015), and anti-inflammatory (Melo et al. 2011).

Among the bioactive compounds of EORO related to anti-inflammatory activity, the most reported is 1,8-cineole. The compound is a relatively abundant monoterpene and can reach a concentration of up to 40% by volume in the cineoliferous chemotype (Napoli et al. 2015).

Essential oils are lipophilic compounds that exhibit low interaction capacity with water. This issue has been considered a technological challenge. Pharmaceutical industries have used colloidal transport systems to encapsulate lipophilic compounds, enabling their dispersion in aqueous media (McClements and Rao 2011). Nanoemulsions are promising systems for drug delivery with little solubility in water and have already been proposed to be associated with the essential oil of *Rosmarinus officinalis* L. (Duarte et al. 2015). They consist of systems with thermodynamic stability, where oil/drugs, with lipophilic characteristics, is dispersed in an aqueous environment. They present a wide superficial area with low tension, thermodynamic stability, and solubilization capacity (Ostertag et al. 2012).

In studies for the screening of natural products, the zebrafish has stood out as an experimental model due to the increasing number of publications in recent years using the animal in biological assays for the isolation of bioactive molecules from plant extracts (Santos et al. 2016). For the evaluation of the anti-inflammatory activity of grape seed extract, Kao et al. (2010) infected with *S. aureus* adult zebrafish pretreated with grape seed extract and observed a lower frequency of death and a reduced inflammatory response in the animals. In a study by Wang et al. (2013), the anti-inflammatory effects of *Gentiana dahurica* (Gentianaceae) ethanolic extract on zebrafish and RAW 264.7 cells were evaluated, with orotic acid and liriodendron exhibiting inflammatory inhibitory activity and none of the compounds being cytotoxic.

Yang et al. (2014) used lipopolysaccharide (LPS) to induce an inflammatory response in zebrafish larvae. The quantitative reverse transcription polymerase chain reaction (RT-PCR) showed elevated levels of IL-1β, IL-6 and TNF-α. Cytokines were involved in the inflammatory process, caused by increased macrophages and neutrophils at the site where the LPS was injected, suggesting LPS as a possible new model of inflammation induction in zebrafish.

On the other hand, Huang et al. (2014) demonstrated the efficacy of adult zebrafish as an animal model for the carrageenan-induced abdominal oedema test. The substance administered intraperitoneally presents a direct induction of inflammatory oedema, making possible the evaluation of small volumes of drugs or compounds.

Due to the presence of very sensitive organs, zebrafish can be useful to evaluate the toxic effects of substances through the histopathology of damaged organs (Carvalho et al. 2017). Studies have been carried out with zebrafish as an experimental model for evaluating the anti-inflammatory activity of several compounds (Santos et al. 2016). With a relatively small size and body weight, the adult zebrafish requires a considerably small amount of the compounds to be tested in the carrageenan inflammation assay, which represents a significant advantage in its use in laboratories (Huang et al. 2014).

As nanoemulsions have the potential to enhance the penetration of lipophilic substances through cell membranes, these systems may be an appropriate vehicle for the transport of EORO, increasing its bioavailability and improving its anti-inflammatory effect. The effect of nanoemulsion containing essential oil of *Rosmarinus officinalis* L. (NEORO) on the inhibition of inflammation was evaluated by in vitro and in vivo (in zebrafish) assays.

Materials and methods

Chemical products

All the reagents used in the in vitro tests were obtained from Sigma Chemical Company (ST. Louis, Millstone, USA). The purified water was obtained from the Millipore Direct® Q3 equipment (Millipore Corp., MA, USA). Dulbecco´s Modified Eagle´s Medium (DMEM) low glucose and the fetal bovine serum (FBS) were obtained from the manufacturer GIBCO®, and trypan blue solution from Sigma-Aldrich.

Vegetal materials

Three samples of essential oil of *Rosmarinus officinalis* L. (EORO), denominated OECULT, OECHA, and OECOM, were used. The first two were extracted in the Laboratory of Phytopharmaceutical Nanobiotechnology of UNIFAP, and the other was acquired from a specialised company. The sample OECULT was obtained from the leaves of the rosemary cultivated in the field of cultivation of medicinal plants of the Goiana Agency of Technical Support, Rural Extension and Agricultural Research (EMATER) from Goiânia, GO, with exsiccate stocked in the Herbarium of the Federal University of Goiás, under nr. 49581, characteristics according to those described in British Pharmacopeia (2009).

The denominated sample OECHA was obtained from the dry leaves of rosemary sold for the preparation of tea by the company Native Natural Products Ltda, in a sealed package from Lot 209. The essential oil, OECOM, was acquired from the company, Florien, containing the decision of a

botanical analysis, and showing organoleptic, physiochemical, and microbiological characteristics besides the following descriptions: essential oil of rosemary obtained from the leaves; lot 056757.

Extraction of the essential oil

The extractions of OERO's were accomplished according to Fernandes et al. (2013). The essential oil was obtained by hydrodistillation using the apparel Clevenger. The samples were triturated in a turbolizer and transferred to a 5 L volumetric balloon containing distilled water. The balloon was placed on a heating platform, and the vaporised water carried the vegetable oil through the coobation tube, promoting the separation of phases in the sifter tube (De Groot and Schmidt 2016). The collected sample was stored and cooled to 4 °C for subsequent analyses.

Gas chromatography–mass spectrometry

Coupled gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Shimadzu system/GC 2010 coupled to a self-gun Shimadzu/AOC-5000 and mass detector (Shimadzu MS2010 Plus) with an electron impact of 70 eV, and equipped with a fused silica column of DB-5MS (Agilent Advanced J & W 30 m × 0.25 mm × 0.25 µm). The parameters were as follows: split ratio, 1:20; helium as carrier gas (65 kPa); injection volume, 1.0 µl; injector temperature, 250 °C; detector temperature, 250 °C; initial column temperature, 50 °C for 1 min; and heating rate, 5 °C min^{-1} to 250 °C. The total analysis time was 35 min, and the identification of compounds was performed using the NIST 5.0 equipment library.

Nanoemulsions preparation

The nanoemulsions were prepared through the low energy load methodology described by Fernandes et al. (2013). For a final mass of 50 g, 90% of water was used, 5% of OERO, and 5% of Tween 20. Initially, an organic phase was prepared, adding OERO and the tensioactive into a beaker. The mixture was agitated using a magnetic agitator (750 rpm) for 30 min. Next, the aqueous phase was added, with a flow of ≈ 0,5 mL/min with continuous agitation for 60 min. The stability of all of the emulsions was evaluated 1, 30, and 60 days after the preparation through macroscopic analysis (colour, visual aspect, phase separation, creaming, and sedimentation) (Falcão et al. 2007). During this period, all of the emulsions were maintained at room temperature (25 ± 2 °C) in glass test tubes (Borges et al. 2017).

Droplet size analysis

The droplet size and polydispersity were determined by photon correlation spectroscopy using a Zetasizer 5000 (Malvern Instruments, Malvern, UK). Each emulsion was diluted using ultra-pure Milli-Q water (1:25). Measures were performed in triplicate. An average droplet size was expressed as the mean diameter (Orafidiya and Oladimeji 2002).

Determination of encapsulation efficiency

The encapsulation efficiency (EE %) of NECHA nanoemulsion was determined according to the techniques described by Natrajan et al. (2015), Rivera et al. (2015), Hosseini et al. (2013) and Ixtaina et al. (2015), based on the free (unencapsulated) oil content.

Hexane (2 mL) was first added to the nanoemulsion (2 mL) and the mixture vortexed. After that, centrifugation was performed at 4000 rpm for 30 min to separate the unencapsulated oil. The supernatant was collected and quantified in a UV–vis spectrometer (Model 1240, Shimadzu, Kyoto, Japan) at 306 nm, and also by mass spectrometry (GC–MS) coupled to gas chromatography (Shimadzu MS2010 Plus), following the technique previously described. The amount of free oil was evaluated in triplicate from a standard curve obtained with different concentrations of the essential oil of *R. officinalis* (OECHA), used to prepare the nanoemulsion, diluted in hexane (Natrajan et al. 2015; Rivera et al. 2015). For the curve, the peak area value of camphor, which is one of the major compounds of OECHA, was used.

The percentage of encapsulation efficiency of the nanoemulsion was calculated by the following equation:

$$\%EE = \frac{(\text{Total oil} - \text{Free oil})}{(\text{Total oil})},$$

where total oil corresponds to the total amount of oil present in the nanoemulsion, and free oil corresponds to the amount of unencapsulated oil.

Cultivation and maintenance of cells

Macrophages J774A1 were provided from the ascites of mice (*Mus musculus*) of the strain BALB/cN. The fibroblasts of the lineage MRC5 were obtained from healthy human lung (Homo sapiens). They were obtained from Fiocruz Manaus. The cells were maintained frozen in liquid nitrogen and stored in the Laboratory of Biological Activity (BIOPHAR) in the Federal University of Amazonas with DMSO + RPMI without serum (macrophages) or bovine fetal serum (fibroblasts).

The macrophages were cultivated according to the conditions adapted to the Laboratory of Biological Activity II of the Federal University of Amazon (UFAM), with cultivation mean from

Dulbecco´s modified eagle's medium (DMEM)—GIBCO, low glucose, supplied with 10% bovine fetal serum (BFS)—GIBCO, penicillin (100 U/mL), and streptomycin (100 U/mL), and incubated with 5% CO_2 to 37 °C. After the formation of cellular confluence (monolayer), the trypsin was applied, and the mean DMEM, containing 10% BFS, was used for the resuspension. The cells were distributed among cell culture plates (96 wells) in density (nr of cells/mL) according to the necessity of each assay. All the procedures involving the cellular cultivation were accomplished in a vertical laminar flow chapel (VECO).

DPPH chemical assays

The chemical test for the evaluation of the reducing potential of the stable free radical diphenyl picrylhydrazyl (DPPH) was accomplished in agreement with the method described by Molyneux (2004) in a plate of 96 wells. The solution of DPPH (0.9 mmol/L) was prepared in Ethanol. The final volume of each well of the plate (300 µL) was obtained with 30 µL of the first dilution (sample of 10 mg + 1 mL of Dimethyl sulfoxide—DMSO) or 30 µL of the second dilution (100 µL of the first dilution + 900 µL of DMSO), this dilution resulted in the amount of 389 µg of nanoencapsulated oil (25 times less than oil), and 270 µL of DPPH was added in each well. The gallic acid was used as pattern and DMSO as a negative control. After the addition of the solution of DPPH, the plate was kept at rest in the dark at room temperature. The absorption was measured in microplates reader DTX800 (Beckman Coulter) to 517 nm. The results were obtained using the following formula:

$$\% \text{ inhibition} = 100 \times \left[1 - (\text{Abs dilution } 2 - \text{Abs dilution } 1/\text{Abs control})\right].$$

ABTS chemical assays

The assay for evaluation of the reducing potential with the radical preformed monocation of 2,29-azinobis-(3-ethylbenzothiazoline-6 sulfonic)—ABTS + was accomplished as the method described by Re et al. (1998), using a plate of 96 wells. An oxidized solution was prepared ABTS (ABTS+) with 10 mg of ABTS + 5 mL of deionised water MiliQ + 5 mL of potassium persulfate to 5 mM. The solution was stored in an amber flask and kept in a magnetic mixer (potency 1) for 16 h. The final volume of each well of the plate (300 µL) was obtained with 30 µL of the first dilution (10 mg of sample + 1 mL of DMSO) or 30 µL of the second dilution (100 µL of the first dilution + 900 µL of DMSO) added 270 µL of ABTS in each well. The gallic acid was used as a pattern and DMSO as the negative control. This dilution resulted in the amount of 389 µg of nanoencapsulated oil (25 times less than oil). After the addition of the solution of ABTS, the plate was kept at rest, in the dark, at room temperature for 1 h. The absorption was measured in a microplates reader DTX800 (Beckman Coulter) at 714 nm. The results were obtained using the following formula: $\%$ inhibition $= 100$ x $\left[1 - \left(\text{Abs dillution2} - \text{Abs dillution1}/\text{Abs control}\right)\right]$.

Cellular antioxidant activity

Antioxidant activity in cells was evaluated in the MRC5 fibroblasts lineage obtained from a human lung (*Homo sapiens*), according to the method described by Wolfe and Liu (2007). 6×10^4 cells were cultivated by a well in a plate of 96 wells. After 24 h of incubation in a CO_2 chamber at 37 °C, the medium was removed, and the wells were washed with PBS. The cells were treated in triplicates with 100 µL of dichlorofluorescein diacetate (DCFH-DA) to 25 µM, followed by incubation for 1 h in a CO_2 chamber at 37 °C. After the incubation period, the solution was discarded, and the cells were washed with PBS. Afterwards, 50 µL of solution containing different concentrations of the samples were added (50, 5, 0.5 and 0.05 µg/mL, corresponding to the concentrations of 0.01621 µg, 0.1621 µg, 1.621 µg, and 16.21 µg of the nanoencapsulated oil), together with 50 µL 2,2′-azobis (amidinopropane) dihydrochloride (ABAP) to 60025 µM. The fluorescence readings were accomplished in the microplate reader (DTX 800, Beckman) with excitement 485 nm and emission 535 nm for 60 min. As a pattern for the test, quercetin was used in the concentrations of 50, 5, 0.5, and 0.05 µg/mL. The median inhibitory concentration was calculated for the time of 60 min according to the formula:

$$\% \text{ inhibition} = 100 - (F \text{ sample} / F \text{ control}) \times 100$$

where F = fluorescence to the 60 min − fluorescence to the 0 min.

Cell viability assay (Alamar Blue)

Cytotoxicity was evaluated by the Alamar blue method according to Nakayama et al. (1997). Cultivated macrophages were used in the concentration of 5×10^3 cells by well in plates of 96 wells. After 24 h of incubation and adherence of the cells, those were treated with the samples in the concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56 µg/mL, corresponding to the concentrations of 0.5065, 1.013, 2.026, 4.052, 8.105 and 16.21 µg of the nanoencapsulated oil, respectively, and triplicate for 72 h. For the positive control of cell death, doxorubicin (5 µg/mL) was used (Sigma Aldrich 20, Germany) and as a negative control, DMSO, to evaluate the influence of the diluents in the cells. After the treatment period (72 h), 10 µL de Alamar Blue was added (resazurin 0.4%) (diluted 1:10 = 950 µL of DMEM + 50 µL of Alamar). After the metabolization of the resazurin, 3 h for

J774A1, the reading of the fluorescence was accomplished in the apparel DTX800 (Beckman Coulter); 540 nm of excitement and 585 emission nm were applied. The viability was calculated according to the formula:

$$\% \text{ Viability} = \text{Ft} \times 100/\Delta\text{Fb Fb}$$

where Ft = (fluorescence of the cell + half + substance + resazurin) and ΔFb = (fluorescence of the cell + half + resazurin).

Viability of fibroblasts test (Trypan blue)

The fibroblasts MRC5 (1×10^3 cells/mL) were seeded onto a plate. Then, the cells were treated with complete medium only (control cells), NECHA, OECHA, NECONTROL, or Ascorbic Acid (positive control) at 12.5 µg/mL (0.10 µg of nanoencapsulated oil) for 24, 48, and 72 h. After that, the cells were removed from the plate, and an aliquot of cell suspension was diluted with trypan blue solution (1:10) (Sigma-Aldrich, St. Louis, MO, USA). The cells were then observed under light microscopy (Nikon T1-SM, Eclipse, Konan, Tokyo, Japan) and the viability of the cells was estimated using a Neubauer Chamber.

NO· production assay

The assay was accomplished measuring the accumulation of nitric oxide (NO·) in a medium of culture, using the Griess reaction (Green et al. 1982). The macrophages were plated at a density of 1×10^6 cells/mL in plates of 96 wells, following the adhesion per 24 h at 37 °C, in an atmosphere containing 5% CO_2. After adherence, the mean was removed and added to the cultivation mean of DMEM, supplied with 10% of BFS with a volume of 100 µL/well. The cells were stimulated by the addition of lipopolysaccharide (LPS) of gram-negative bacteria *Escherichia coli* (final concentration 1 µg/mL) and treated together with the samples in the concentration of 5, 10, 25, 50, and 100 µg/mL, corresponding to 8.11, 16.21, 40.52, 81.05, and 162.1 µg of the nanoencapsulated oil. For the experiment control, the cells were cultivated with and without LPS in the cultivation mean of DMEM containing 10% of BFS. After this, the cells were incubated for more than 24 h at 37 °C, 5% CO_2, and the cell supernatant was collected. To determine the production of NO·, 100 µL were removed from the cell supernatant, and 100 µL of the Griess reagent was added. Stock solutions of naftali chloride-1-ethylenediamine—Merck ($C_{12}H_{16}Cl_2Na_2$) dissolved at 0.1% in H_3PO_4 (5%) and of sulfanilamide—sigma ($C_6H_8N2O_2S$) dissolved at 1% in H_3PO_4 (5%) were used for the preparation of this reagent. Before the usage, the solutions were added in a proportion of 1:1, forming the Griess Reagent as known. After the 15-min incubation period, the samples were analysed in the microplates reader (DTX 800, Beckman) at 560 nm. The calculation of the concentrations of nitrite was accomplished based on pattern curves using different concentrations of $NaNO_3$ (15 µM up to 1000 µM).

Study in zebrafish with NECHA

Animals

Zebrafish (*Danio rerio*) males weighing 400–1000 mg were used, which were purchased from the company, Acqua New Aquarium and Fish LTDA, ME., located in Itagassu-PE-Brazil and kept quarantined on the Zebrafish Platform of the Laboratory of Research in Pharmaceuticals of the University Federal University of Amapá—UNIFAP, Macapá, Amapá, Brazil.

The animals were kept in tanks, in which the water conditions were controlled for 2 months before the trials, as described by Carvalho et al. (2017). The experiments were carried out according to the norms established for the care of animals, and the project was approved by the Ethics Committee on Animal Use—CEUA—UNIFAP, the Federal University of Amapá with protocol number 0021/2015.

Routes of administration and treatment groups

The treatments were performed in a double-blind manner, and the different test substances were coded. Diclofenac, dexamethasone, saline, NECONT, and NECHA were administered by gavage, as described by Collymore et al. (2013) 1 h before the application of carrageenan.

The animals were divided into six groups ($n = 5$/group): group A—control PBS, a substance used to solubilize carrageenan, was applied intraperitoneally, along with saline via gavage; group B—with the application of carrageenan via intraperitoneal and saline via gavage; group C—Diclofenac (0.5 mg/kg, Sigma Co., São Paulo, Brazil) with the application of carrageenan intraperitoneally; group D—Dexamethasone (0.5 mg/kg, Sigma Co., São Paulo, Brazil) with the application of carrageenan intraperitoneally; group E—NECONT group (control nanoemulsion, Tween 20, and water) with application of carrageenan intraperitoneally; and group F—NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg, described by Borges et al. (2017)) with the application of carrageenan intraperitoneally.

Induction of inflammation with carrageenan and measurement of oedema

The induction of inflammation in the adult zebrafish was performed according to the method described by Huang et al. (2014), and carrageenan (Carrageenan kappa, Sigma Co., Lot 16HO616) was injected intraperitoneally into a volume of 20 µL (300 µg) in PBS. The animals were individually

anaesthetized in water (8–10 °C) for approximately 3 min before injection.

All animals were weighed individually at the beginning and at the end of the experiment (5 h after carrageenan injection) on an analytical balance (FA2104N, Bioprecisa Co., São Paulo, Brazil). They were photographed at the end of the experiment using a camera (Samsung WB150F, Brazil) and, after euthanasia, were immediately stored in Bouin's solution for histopathological study.

Behavioral analysis

The behaviour of the animals was evaluated over 1–5 h after the injection of carrageenan. The behavioural parameters observed were those described by Souza et al. (2016). The behaviour of zebrafish was classified into three stages: I with increased swimming activity and presence of tail tremors, II with circulatory movement in swimming and loss of balance, and III with loss of motility, rest at the bottom, and death.

Histopathological study

After the experiment, the animals were stored in identified cassettes and immersed for 24 h in Bouin's solution. Afterwards, they were descaled with EDTA (Ethylenediaminetetracetic acid, Sigma Co., São Paulo, Brazil) for 24 h. After decalcification, alcohol dehydration was performed at 70, 80, 90, and 100%, for 1 h each. Then diaphanization with xylol and inclusion with paraffin was performed. The 5 µm sections were obtained on a microtome (Rotary Microtome Cut 6062, Slee Medical, Germany), transferred to glass slides and stained with Hematoxylin and Eosin (Souza et al. 2016). Histological analysis was performed under an optical microscope. Histological changes in gills, liver, kidneys, and gut were evaluated according to parameters presented by Meletti and Rocha (2003), Souza et al. (2016) and Carvalho et al. (2017).

To define the degree of tissue change, the Histopathological Change Index (HCI) for the gills, liver, kidneys and intestine was used, which classified the organs as normal when the HCI values ranged from 0 to 10, moderately altered when the values are from 11 to 20, changed in moderate to severe degree when the HCI is between 21 and 50, and severe with irreversible changes to values greater than 100 (Poleksic and Mitrovic-Tutundzic 1994; Rigolin-Sá 1998; Takashima and Hibiya 1995).

Statistical analysis

In the results obtained with assays, the significance was determined through the ANOVA test to compare the means between the control and treated groups, considering significant values of $p < 0.05$ (Sokal and Rohlf, 1995), and highly significant values of $p < 0.01$. In the zebrafish study, one-way ANOVA (Analysis of variance) was used, followed by Dunnett's multiple comparison test, and to histopathological change index, Kruskal–Wallis one-way ANOVA and Student–Newman–Keuls test were used. Values of $p < 0.05$ were considered statistically significant. Data were expressed as a mean ± error standard mean. The graphs were recorded by the program GraphPad Prism 6.0.

Results

The essential oils of *Rosmarinus officinalis* L. (OECULT and OECHA) presented an average yield of 2.5 and 1.0%, respectively. In the characterization of these oils and the OECOM commercial essential oil, the major components identified were: 1,8-cineole (16.84, 50.82 e 33.70%), camphor (30.93, 19.16 and 27.68%) and alfa-pinene (14.24, 10.12 and 8.13%), respectively (Table 1 and Fig. 1). It should be noted that OECULT essential oil also presented 10.20% beta-myrcene.

In the measurement of droplet size and polydispersity index (PDI), all nanoemulsions had a mean droplet size less than 100 nm and PDI less than 1. NECOM showed mean droplet of 89.87 ± 0.083727 nm and PDI of 0.193 ± 0.008 nm, while in NECULT, the mean droplet value was 98.01 ± 0.302900 nm and polydispersity of 0.182 ± 0.001 nm, and NECHA showed mean droplet of 77.32 ± 1.192000 nm and PDI of 0.239 ± 0.006 nm (Fig. 2).

The efficiency of encapsulation by spectrometry determined the free oil content of 32.39 ± 0.069%, with 67.61 ± 0.069% of the encapsulated oil. In the gas chromatographic analysis (GC–MS) the free oil content was 24.62 ± 0.090% and 75.38 ± 0.090% encapsulated.

In the chemical assays, the nanoemulsions (NECHA, NECULT, and NECOM) demonstrated free radical reducing action in ABTS and DPPH. In the ABTS assay, it was observed that, in the concentration of 100 µg/mL, all the samples had values of reductive activity lower than 20%, indicating a low profile of this activity to the gallic acid standard (90.38 ± 0.13%). At the concentration of 1000 µg/mL, the oils had a low free radical reducing activity profile compared to gallic acid (91.99 ± 0.21), and the nanoemulsions showed an absence of this activity when compared to their respective essential oils, NECHA (20.09 ± 0.84) and OECHA (54.67 ± 1.98), NECULT (17.27 ± 0.75) and OECULT (46.64 ± 5.17), NECOM (10.67 ± 0.83) and OECOM (24.50 ± 3.95) (Fig. 3).

In the DPPH radical assay, at the concentrations tested, all nanoemulsions presented inexpressive values of reducing activity (< 10%, Fig. 3), while in the cellular antioxidant

Table 1 Chemical constituents of *Rosmarinus officinalis* L. essential oil (EORO), of different origins, determined by GC–MS analysis

Peak	RT (min)	Compound	(%) GC–MS OECOM	(%) GC–MS OECULT	(%) GC–MS OECHA	IR exp.	IR lit.*
1.	4.872	α-thujene	0.11	–	–	928	926
2.	5.054	α-pinene	8.13	14.24	10.12	935	939
3.	5.546	β-Thujene	–	0.35	–	955	971
4.	5.424	Camphene	1.68	4.48	3.26	950	954
5.	6.045	Sabine	0.21	–	–	955	976
6.	6.152	β-pinene	0.58	1.27	1.10	979	979
7.	6.347	3- octanone	–	0.24	–	987	983
8.	6.482	β-myrcene	0.90	10.20	0.65	993	990
9.	6.911	α-phellandrene	0.77	0.37	0.13	1007	1002
10.	7.282	α-Terpinene	0.45	0.87	0.33	1018	1017
11.	7.532	o-cymene	1.65	1.67	1.92	1026	1026
12.	7.674	Limonene	21.99	4.49	2.14	1030	1031
13.	7.773	1,8-cineole	33.70	16.84	50.82	1033	1033
14.	8.666	γ -Terpinene	0.39	1.18	0.16	1059	1059
15.	9.724	Terpinolene	0.20	0.93	0.22	1091	1088
16.	10.128	β-linalool	0.16	1.67	1.01	1102	1098
17.	10.350	***	0.44	0.36	–	1108	***
18.	10.662		–	–	0.10	1216	
19.	11.897	Camphor	27.68	30.93	19.16	1147	1146
20.	12.620	Isopinocamphone	–	0.20	–	1165	1160
21.	12.736	Borneol	0.32	1.61	4.32	1168	1169
22.	13.194	Isopinocamphone	–	1.06	0.83	1179	1173
23.	13.739	α-terpineol	0.12	2.23	2.98	1193	1188
24.	13.899	α-campholenal	0.20	–	–	1197	1125
25.	14.005	Myrtenol	–	0.19	–	1199	1194
26.	14.532	Verbenone	0.18	3.71	–	1213	1205
27.	17.723	Bornyl acetate	–	0.38	0.26	1288	1288
28.	23.220	β-caryophyllene	0.12	0.54	0.29	1421	1427
29.	29.850		–	–	0.19	1960	

(IRexp: IR calculado, ** IRlit: IR table for compound, * not identified)

activity assay, the nanoemulsions presented antioxidant activity like that of the standard quercetin. There was no significant difference between the effects caused by the essential oils and their respective nanoemulsions, nor was there a dose–response effect, and the inhibitory responses to OECULT, NECULT, OECOM, OECHA, NECHA, and quercetin were 92, 82, 93, 91, 90, and 96%, respectively (Fig. 4).

In the cytotoxicity assay in J774 cells (cell viability), none of the samples tested showed a significant reduction in the viability of the macrophages compared to the diluent, DMSO, after 24 h of treatment (Fig. 5). The solubilization of DMSO samples presented 100% cell viability. When compared to DMSO, all essential oil samples showed an increase in the amount of cells at all concentrations tested (1.5, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL, respectively). Among the nanoemulsions, only in the concentration of 1.5 µg/mL, NECULT (56.3 ± 4.17) and NECOM (71.3 ± 1.0) presented a reduction in the number of cells to DMSO (100 ± 10.0). However, they presented a relevant quantity of viable cells in this concentration. In comparison to their respective oils, the NECHA nanoemulsion showed an increase in the number of cells at all concentrations, tested with values of cell viability similar to those demonstrated by OECHA. NECULT and NECOM presented lower numbers of cells when compared to OECULT and OECOM, but maintained cellular viability (Fig. 5).

In the test of the viability of fibroblasts (Trypan blue), nanoemulsion (NECHA), after 72 h of treatment, showed higher values of 16, 24, and 48% of fibroblast proliferation compared to NECONTROL, OECHA, and the untreated cells, respectively (Fig. 6).

The action of the different essential oil samples of *Rosmarinus officinalis* at concentrations of 5, 25, and 50 µg/

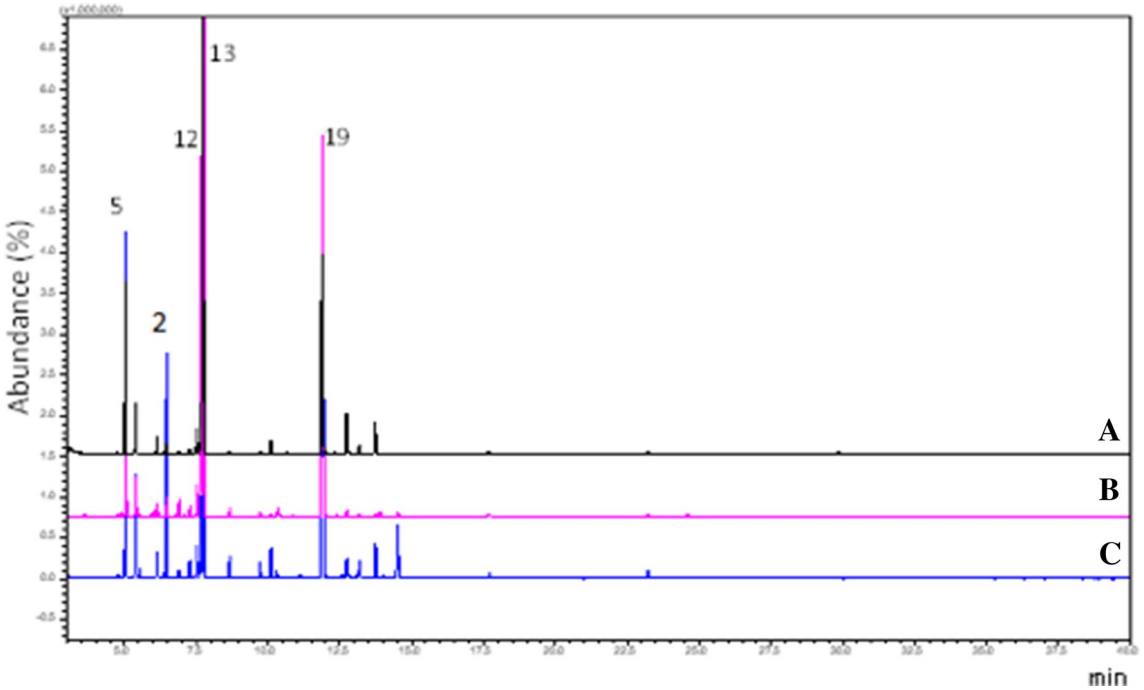

Fig. 1 Chromatogram obtained by analysis of essential oils by coupled gas chromatography–mass spectrometry (GC–MS) where: **A** = OECOM corresponding to **12**—Limonene (21,99%), **13**—1,8-cienole (33,70%), **19**—Camphor (27,58%); **B** = OECULT: corresponding to **2**—α-pinene (14,24%), **13**—1,8-cienole (16,84%), **19**—Camphor (30,93%) and **C** = OECHA: corresponding to **2**—α-pinene (10,12%), **13**—1,8-cienole (50,82%), **19**—Camphor (19,16%)

mL and their nanoemulsions corresponding to 1.621, 3.242, 8.105, 16.21, and 32.42 µg/mL of the nanoencapsulated oil on the production of NO· in µmol/L are shown in Fig. 7. The essential oils (OECHA, OECULT, and OECOM) inhibited the dose-dependent production of nitric oxide in the macrophages, with OECHA IC_{50} = 10.05 µg/mL, OECULT = 4.45 µg/mL, and OECOM = 11.81 µg/mL. With nanoemulsions, this inhibition occurred non-dose-dependent (Fig. 7).

In the NO· test, it is possible to observe that, in the lower concentrations, the nanoemulsions reduced or maintained the NO· production in the presence of LPS when compared to the essential oils. The NECHA nanoemulsion demonstrated a higher inhibitory effect of NO· than the OECHA essential oil at the concentrations of 25 µg/mL (3.0 ± 0.1, 5.6 ± 2.8) and 5 µg/mL (4.0 ± 2.4, 6.7 ± 3.5). The NECOM (4.5 ± 0.2) nanoemulsion also showed higher inhibition of NO· than OECOM (8.1 ± 6.5) and 5 µg/mL oil. At this same concentration, NECULT (5.9 ± 0.9) maintained an inhibitory activity similar to that of OECULT (5.5 ± 3.8) (Fig. 7).

In the zebrafish study, 40% of the animals in group C and 20% in groups B, D, and E showed an increase in swimming activity in the first hour after administration of carrageenan, and 20% in groups B, C, and E rested on the bottom of the beaker. In the second hour after the injection of carrageenan, 20% of the animals in group C and 40% of group D presented rest in the background. After 3 h, 20% of groups B and E and 60% of group C rested at the bottom, 20% of animals in groups A and B increased swimming activity, and 20% in groups D, E, and F showed loss of balance. Four hours after the application of carrageenan, 20% of the animals of groups A, B, D, E and 60% in group C rested in the background. In the last hour, 5 h after carrageenan, 60% in group C and 20% in groups A, B, D, E, and F rested on the bottom of the beaker. The animals that presented loss of balance corresponded to 40% in groups D and F. They presented loss of balance 20 and 40% of the animals in groups A and D, respectively. No death occurred in any of the groups (Table 2).

Group A (control, treated with saline solution by gavage) presented behavioural changes of level I only 3 h after the application of PBS, and level II from the fourth hour and, there was no oedema formation (Fig. 8). While group B that received carrageenan, and was treated with saline presented the three levels of behavioural changes and increased in 272 mg (Fig. 8) when compared to the initial body weight, demonstrating the formation of inflammatory oedema.

The groups previously treated with diclofenac showed more behavioural changes than those treated with dexamethasone, in addition to losing 160 mg of initial body weight,

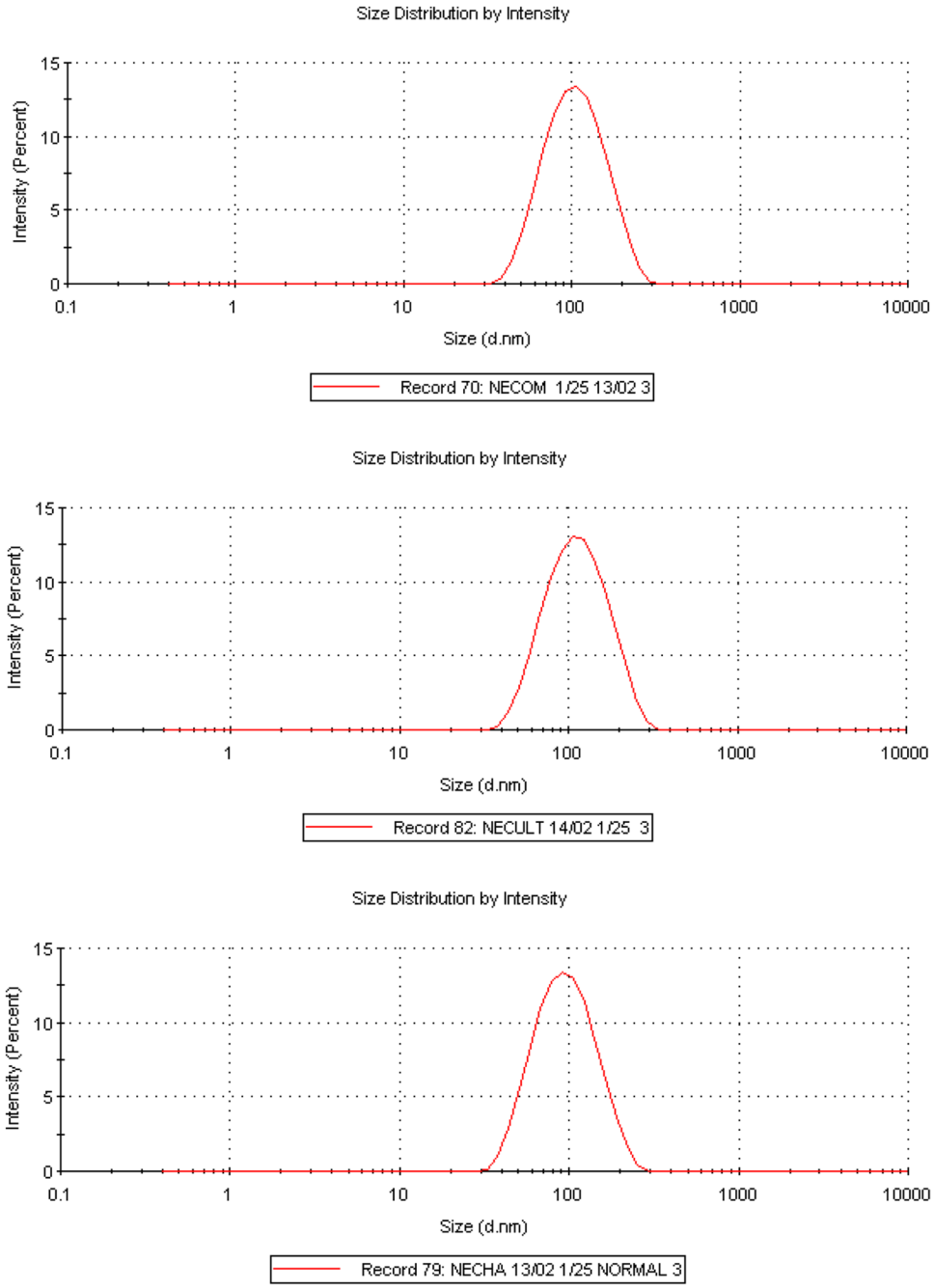

Fig. 2 Particle size distribution of NECOM (mean droplet—89.87 ± 0.083727 nm; polydispersity 0,193 ± 0.008 nm), NECULT (mean droplet—98.01 ± 0.302900 nm; polydispersity 0,182 ± 0.001 nm) and NECHA (mean droplet—77.32 ± 1.192000 nm; polydispersity 0.239 ± 0.006 nm)

while the dexamethasone group lost 32 mg of their initial body weight (Fig. 8).

Group E (NECONT) presented the three levels of behavioral changes with body weight gain of 304 mg (Fig. 8), and group F (NECHA) did not present behavioral changes in the first two hours after the injection of carrageenan, showing changes in levels II and III only after the third hour, with a body weight loss of 241 mg and inhibition of 77.99% of abdominal edema (Fig. 8 and Table 2).

In the histopathological study of the gills, group A (intraperitoneal PBS + saline via gavage) presented no histopathological alterations (Fig. 9a). The HCI value was zero and, consequently, the percentage of total tissue changes was zero (Table 3). Group B (intraperitoneal carrageenan + saline via gavage) showed the fusion of the secondary lamellae, removal of the primary lamella, and displacements of epithelial cells, such as level I alterations, epithelial rupture (level II), and aneurysm (level III) (Fig. 9b). The HCI value was 23, which indicated moderate to severe changes in the organ, with 71.4% of total alterations (Table 3).

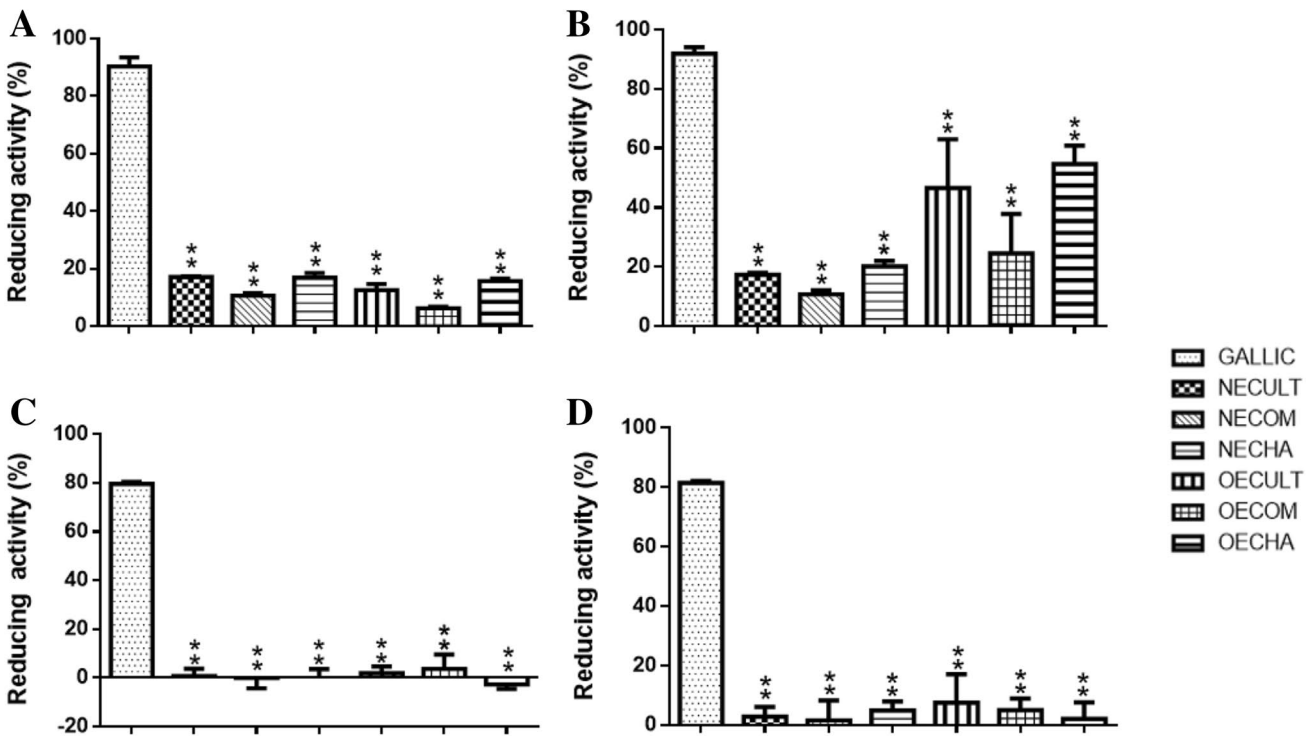

Fig. 3 Reducing activity (%) of NECULT, NECOM, NECHA, OECULT, OECOM and OECHA in ABTS and DPPH assay. **a** Reducing activity at 100 µg/mL in ABTS; Mean ± SD, triplicate. **b** Reducing activity at 1000 µg/mL in ABTS; Mean ± SD, triplicate. **c** Reducing activity at 100 µg/mL in DPPH; Mean ± SD, triplicate. **d** Reducing activity at 1000 µg/mL in DPPH; Mean ± SD, triplicate. Significance was determined using ANOVA Test (*$p < 0.05$; **$p < 0.01$ compared to gallic acid)

Group C (diclofenac) showed only level I changes in gills, the fusion of secondary lamellae, the detachment of primary lamellae, and hyperplasia of epithelial cells (Fig. 9c). Its HCI (0) indicated that the organ remained normal and the total changes present in the tissue was 42.8% of changes in the gills (Table 4). The group treated with dexamethasone had an HCI of 22, indicating the presence of moderate to severe changes in the gills. The percentage of total changes was 57.1% (Table 3). In the histopathological analysis, the fusion of secondary lamellae and displacement of epithelial cells (Level I), epithelial rupture (Level II), and aneurysm (Level III) (Fig. 9d).

The group E (NECONT) presented the fusion of secondary lamellae, displacement of epithelial cells (Level I), HEC hyperplasia of epithelial cells (I), epithelial rupture (Level II), and aneurysm (III), with HCI of 22 indicating moderate alterations to severe (Fig. 9e, Table 4). The group F (NECHA) presented level I changes, such as fusion of secondary lamellae, the detachment of primary lamella and detachment of secondary lamella, and only epithelial rupture as level II alteration (Fig. 9f). The HCI was 3, demonstrating that the organ was functionally normal and the alterations presented in the tissue corresponded to 57.1% (Tables 3 and 4).

In the hepatic tissue, group A (intraperitoneal PBS and saline solution by gavage) presented only level I changes, such as increased cell volume and loss of cell contour (Fig. 10a). The HCI was zero, indicating that the organ remained normal after the application of PBS. Histopathological changes were 15.4% (Table 3). The group B (treated with saline solution and intraperitoneal injection of carrageenan) presented histopathological alterations of level I and II: increased cell volume (I), loss of cell contour (I), nuclear degeneration (II), vessel rupture, hyperemia (II), and nuclear vacuolization (II) (Fig. 10b). He presented 53.8% of hepatic alterations with HCI of 10, with mild to moderate alterations (Table 4).

The animals of group C (Diclofenac) presented a normal liver (HCI of 4) with 30.7% of hepatic tissue alterations. The histopathological changes observed were loss of cell contour (I), loss of nucleus contour (I), cell disruption (II), and vessel rupture (II) (Fig. 10c). In the same way, group D, treated with dexamethasone, also presented organs with normal functionality (HCI of 3) and 38.4% of tissue alterations (Tables 3, 4). Only cell rupture was identified as level II alteration, and all others were level I: loss of cell contour, increased nuclear volume, decreased glycogen, and the decreased relative frequency of nucleus occurrence (Fig. 10d).

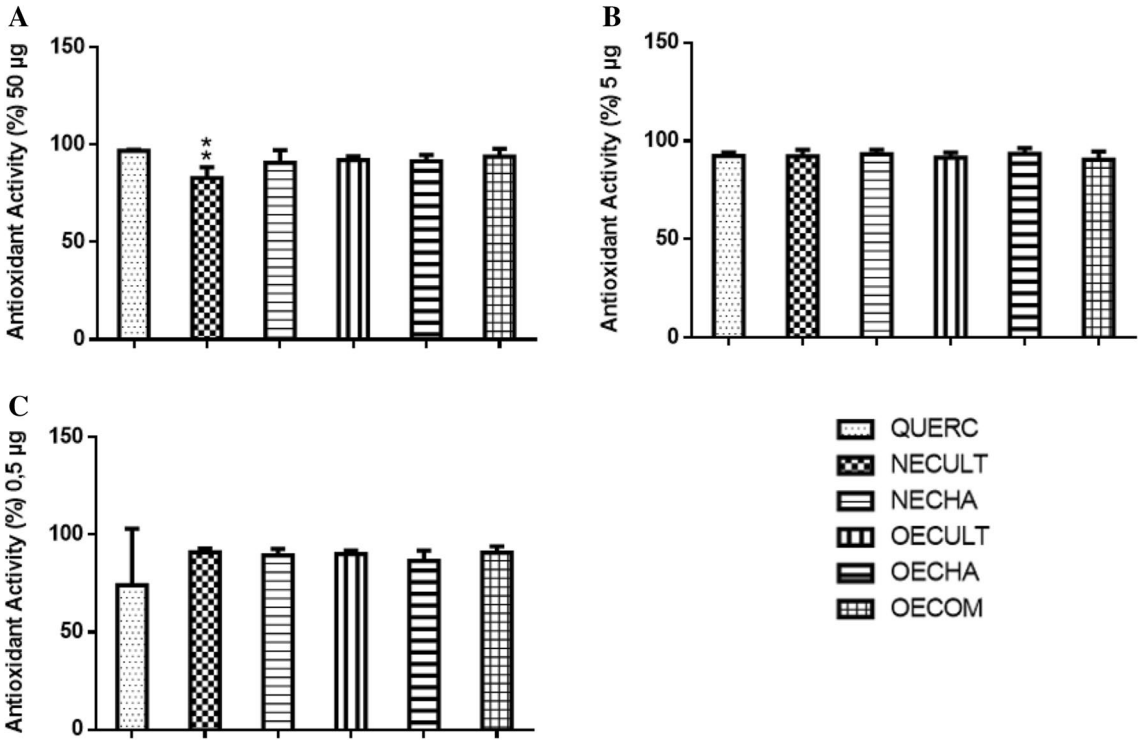

Fig. 4 Antioxidant activity (%) of NECULT, NECHA, OECULT, OECHA and OECOM in Cellular Antioxidant Activity (CAA) Assay. **a** Antioxidant activity (%) at 50 µg/mL in CAA; Mean ± SD, triplicate. **b** Antioxidant activity (%) at 5 µg/mL in CAA; Mean ± SD, triplicate. **c** Antioxidant activity (%) at 0.5 µg/mL in CAA; Mean ± SD, triplicate. Significance was determined using ANOVA Test (*$p < 0.05$; **$p < 0.01$ compared to Quercetin)

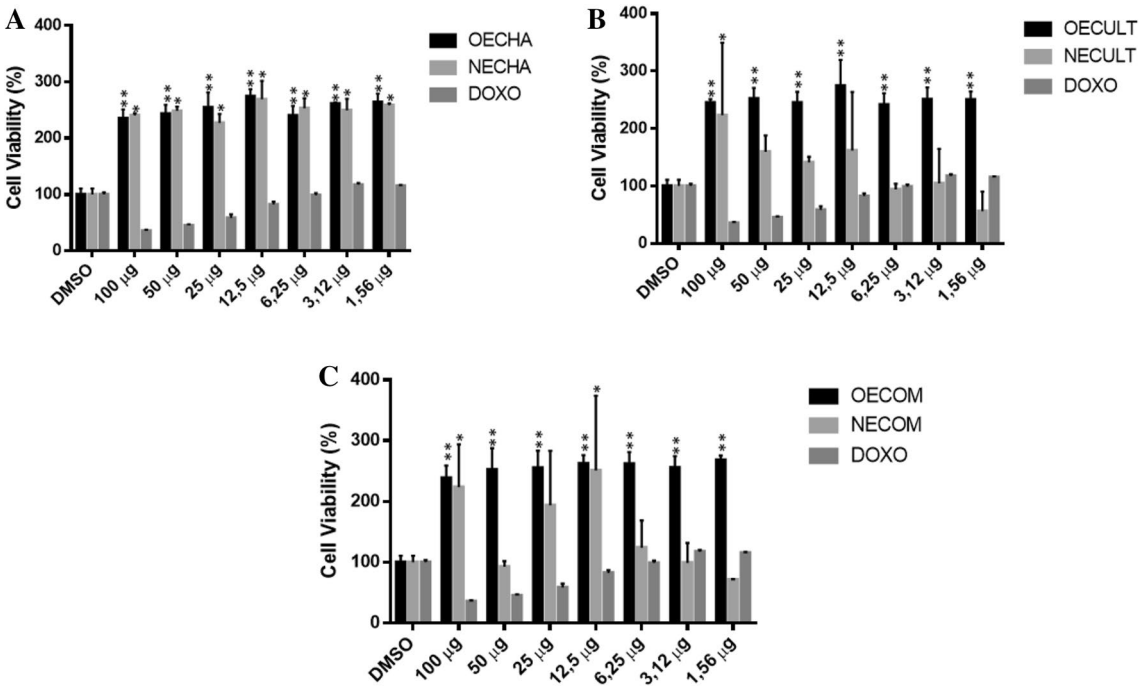

Fig. 5 Viability (%) of J774 cells with OECHA and NECHA (**a**), OECULT and NECULT (**b**), OECOM and NECOM (**c**) at 1.56–100 µg/mL compared to doxorubicin (DOXO) curve and DMSO dilu-ent. The values are the mean ± SD from three independent experiments. Significance was determined using ANOVA Test (*$p < 0.05$; **$p < 0.01$ compared to DMSO)

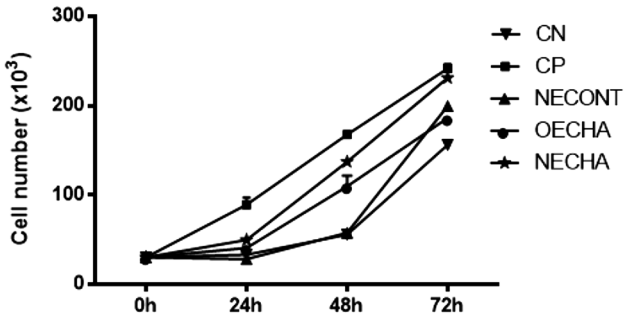

Fig. 6 Effect of OECHA, NECHA and NECONTROL on viability of fibroblasts in the trypan blue test at 12.5 µg/mL. CN: cells without any treatment. CP: ascorbic acid. Data are the mean ± standard error (SE) of three independent experiments

The animals of group E (NECONT) presented several level II hepatic alterations, such as nuclear degeneration, cell disruption, vessel rupture, hyperemia, nuclear vacuolization, and only decreased glycogen as level I alteration (Fig. 10e). Its HCI was 10, indicating mild to moderate changes and 46.1% of histopathological changes (Table 3 and 4). The F group (NECHA) presented functionally normal liver (HCI = 0), with increased nuclear volume (I), increased vessel volume (I), cytoplasmic degeneration (II), and cell disruption (II) (Fig. 10f) and 30.7% liver changes (Table 3).

As for the kidneys, group A did not present any histopathological alteration, with HCI equal to zero. Group B presented histopathological changes of the three levels: hypertrophy of tubular cells (I), dilation of glomerular capillaries (I), increase in tubular lumen (II), tubular degeneration (II), cytoplasmic degeneration of tubular cells (II), and necrosis (III), HCI of 28, with moderate to severe alterations and with 53.8% renal tissue changes.

Groups C and D (diclofenac and dexamethasone) presented similar histopathological changes, such as vessel dilatation (I) and tubular degeneration (II). Group C also showed hyperemia, increased space of Bowman's capsule, and glomerular degeneration, and animals of group D tubular disorganisation (Fig. 11c, d). Both maintained the functionally normal organs with HCIs of 6 and 2, respectively (Table 4). Of the total histopathological changes in

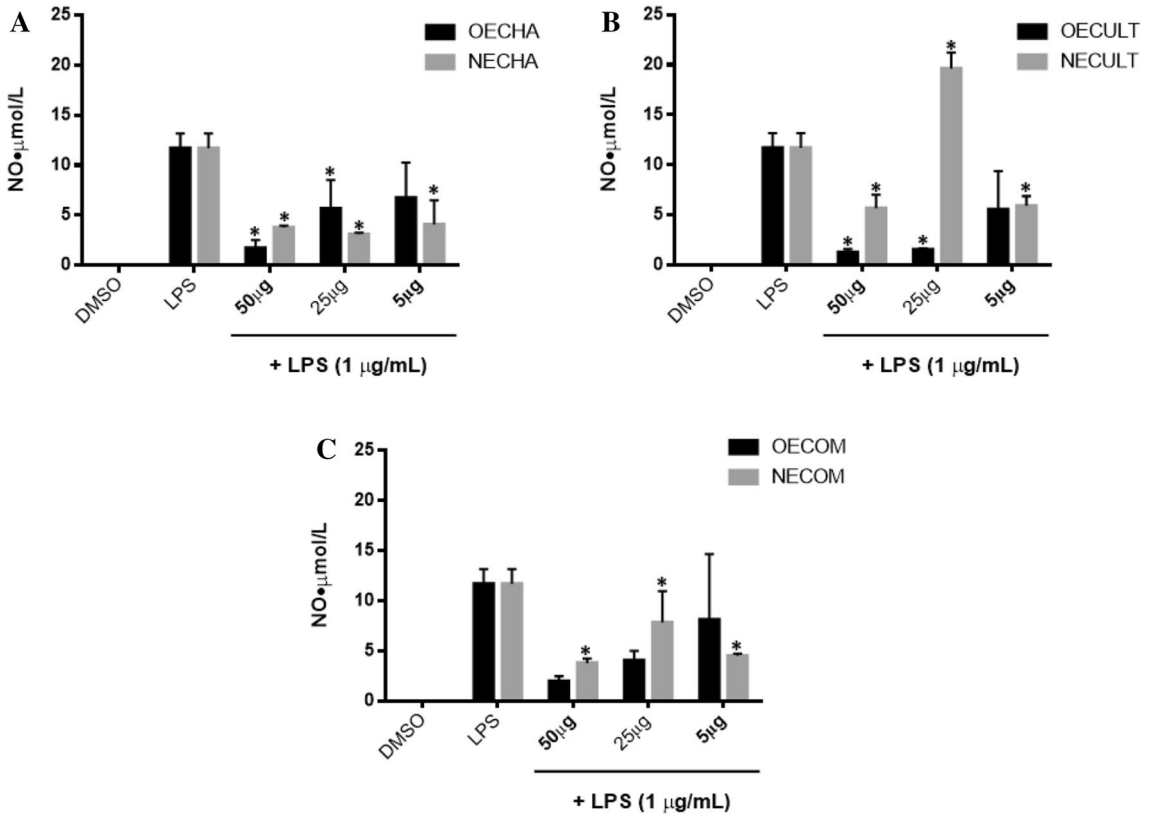

Fig. 7 Effect of the OECHA and NECHA (**a**), OECOM and NECOM (**b**), OECULT and NECULT (**c**) on NO· production in LPS-stimulated J774 cells. Production of NO· was assayed in culture supernatants of macrophages stimulated with LPS (1 µg/mL) for 24 h in the presence of the compounds (5, 25, 50 µg/mL). At 0 the control whit the DMSO diluent. The essential oils presented the following IC_{50} values: OECHA = 10.05 µg/mL, OECULT = 4.45 µg/mL and OECOM = 11.81 µg/mL. The NO· values are the mean ± SD from three independent experiments. Significance was determined using ANOVA Test (*$p < 0.05$; **$p < 0.01$ compared to LPS)

Table 2 Behavioral alterations after treatment with **A** control PBS, **B** application of carrageenan via intraperitoneal and saline via gavage, **C** diclofenac (0.5 mg/kg) with the application of carrageenan intraperitoneally, **D** Dexamethasone (0.5 mg/kg) with the application of carrageenan intraperitoneally, **E** NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and **F** NECHA (OECHA essential oil nanoemulsion, 498 µg/kg) on *D. rerio* at different observation time

Behavioral parameters	Zebrafish with behavioral parameter alteration per group (%)					
	A	B	C	D	E	F
1 h						
Stage I		20%	40%	20%	20%	
Stage II						
Stage III		20%	20%		20%	
2 h						
Stage I						
Stage II						
Stage III			20%	40%		
3 h						
Stage I	20%	20%				
Stage II				20%	20%	20%
Stage III		20%	60%		20%	
4 h						
Stage I						
Stage II				40%		40%
Stage III	20%	20%	60%	20%	20%	
5 h						
Stage I						
Stage II	20%			40%		
Stage III	20%		60%	20%	20%	20%

Stage I: (1) increase swimming activity, (2) tail tremors; Stage II: (1) Circular swimming movement, (2) loss of posture; Stage III: (1) Loss of motility; (2) Animal deposition in the base of the beaker, (3) Death

the kidneys, diclofenac had 38.4% and dexamethasone 23% (Table 4).

The animals that were treated with NECONT (Group E) presented the three levels of histopathological changes: vessel dilatation (I), tubular disorganization (I), tubular degeneration (I), cytoplasmic degeneration of tubular cells (II), hyperemia (II), and necrosis (III) (Fig. 11e), with an HCI of 27, with moderate to severe alterations, and 53.8% of tissue alterations (Tables 3, 4). In animals treated with NECHA (Group F), histopathological changes were observed, such as vessel dilatation (I), tubular disorganization (I), increased space of Bowman's capsule (I), tubular degeneration (II), and cytoplasmic degeneration of tubular cells (II), with an HCI of 7 and 38.4% of tissue changes (Fig. 11f, Tables 3, 4).

The intestinal tissue group A (PBS and treated with saline solution) presented cell degeneration (I), degeneration of muscle layer (I) and necrosis (III) (Fig. 12a), with 20% of total histopathological changes (Table 3) and HCI of 20, indicating mild to moderate changes (Table 4). Group B (injection of carrageenan and treated with saline) presented several histopathological changes: increased leukocyte infiltration (I), detachment of epithelial lining (I), hypertrophy of epithelial cells (I), hypertrophy of globet cells (I), cell degeneration (I), detachment of lamina propria (II), fusion of villous (II), villous degeneration (II), hemorrhage in the lamina propria (II), and necrosis (III) (Fig. 12b), with HCI of 29, alterations moderate to severe, and 66.6% total alterations (Tables 3, 4).

The animals treated with diclofenac and dexamethasone (Groups C and D) presented several changes in intestinal tissue, such as detachment of epithelial lining (I), increased leukocyte infiltration (I), villous atrophy (I), vacuolization of enterocytes (I), cell degeneration (I), villous degeneration (II), detachment of lamina propria (II), fusion of villous (II), and necrosis (III) (Fig. 12c, d). These changes were classified as moderate to severe, with HCI of 24.8 and 25, respectively (Table 4). Tissue changes were 46.6 and 53.3% (Table 3).

Group E (treated with NECONTROL) presented several changes. Among them were detachment of lamina propria (II), villous degeneration (II), leukocyte infiltration (I), vacuolization of enterocytes (I), hemorrhage in the lamina propria (II), partial or (II), necrosis (III) (Fig. 12e), with HCI of 30 (Table 4), indicating moderate to severe changes, and 60% changes in intestinal tissue (Tables 2, 3) The group, F (NECHA), presented leukocyte infiltration (I), vacuolization of enterocytes (I), hyperplasia of goblet cells (I), cell degeneration (I), degeneration of muscle layer (I), hypertrophy of epithelial cell hemorrhage in the lamina propria (II), and necrosis (III) (Fig. 12f). These changes were classified as moderate to severe with an HCI of 23 and with 53.3% tissue changes (Tables 3 and 4).

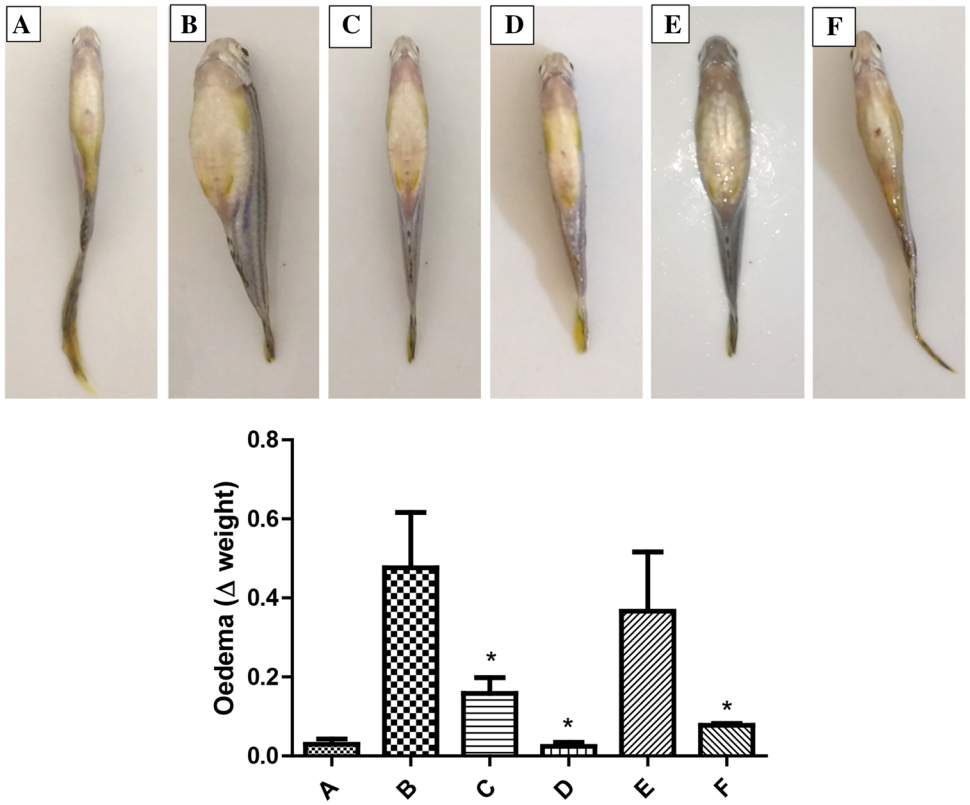

Fig. 8 Effect of the oral treatment with **a** PBS, **b** saline, **c** Diclofenac (0.5 mg/kg), **d** Dexamethasone (0.5 mg/kg), **e** NECONT (control nanoemulsion) and **f** NECHA (OECHA essential oil nanoemulsion, 498 µg/kg) on oedema induced by carrageenan (300 µg, intraperitoneal) in *D. rerio*. *$p < 0.05$, ANOVA, Dunnett's Multiple Comparison Test

Discussion

The chemical composition found in the OERO samples is as described by Takayama et al. (2016) where 1,8-cineole and camphor were found as major compounds of *Rosmarinus officinalis* essential oil collected in the Brazilian Midwest region. The same major compounds were found in the essential oil obtained from samples of *Rosmarinus officinalis* L. collected from three different geographical origins: Beja, Sidi Bouzid, and Gabes, Tunisia (Hcini et al. 2013). In samples of *Rosmarinus officinalis* L. collected in Turkey, 1,8-cineole and camphor were among the major components of essential oil (Türkmen et al. 2014). In a study by Salido et al. (2003), the chemical composition of twelve essential oil samples of *Rosmarinus officinalis* L., harvested at four different sites in Southern Spain at different periods, was analyzed, and it was observed that 1,8-cineole remained constant among the compounds regardless of the collection period while the other components varied according to the life cycle of the plant.

The nanoemulsions containing the essential oil of *Rosmarinus officinalis* L. (NEORO) presented a translucent and bluish-like appearance, as described by Forgiarini et al. (2000), with droplet sizes smaller than 200 nm and polydispersity indexes below 1 (Table 3). Similar results were obtained by Solans et al. (2005) and Solè et al. (2012).

NECHA maintained a higher amount of the encapsulated OERO than the essential oil oregano nanoparticles with EE of 21–47% (Hosseini et al. 2013), and than the essential oil of *Lippia sidoides* with EE of 55% (Oliveira et al. 2014).

The results obtained in this study can be compared to the study carried out with the essential oils of *Mentha piperita*, *Eucalyptus globulus* encapsulated in chitosan nanoparticles and analyzed by UV vis spectrometry, with EE values higher than 70% (Pijpers 2017) and oil of saffron showed 71.1% (Natrajan et al. 2015). According to Pijpers (2017) encapsulation efficiency values higher than 70%, it suggests that the encapsulation technique was successful. Although the encapsulation techniques of essential oils are different among the studies cited here, the results of encapsulation efficiency show values similar to those found in the present study (67–75%), suggesting that NECHA was effective in keeping most of OERO effectively encapsulated.

The low reductant activity of the nanoemulsions observed in the DPPH assay (Fig. 3) may be related to the fact that the antioxidant compounds present in the essential oil were encapsulated in the nanoemulsions, which possibly hindered their release and the consequent interaction with the chemical agent employed. As the tests with ABTS and DPPH show the reduction activity and not the antioxidant activity, it can be stated that the nanoemulsions did not present reductive

Table 3 Percentage of alteration presented by each tissue after treatment with **A** control PBS, **B** application of carrageenan via intraperitoneal and saline via gavage, **C** diclofenac (0.5 mg/kg) with the application of carrageenan intraperitoneally, **D** dexamethasone (0.5 mg/kg) with the application of carrageenan intraperitoneally, **E** NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and **F** NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg) on *D. rerio*

Group/tissue	Stage I	Stage II	Stage III	Total	%
Gills					
A	0/5	0/1	0/1	0/7	0
B	3/5	1/1	1/1	5/7	71.4
C	3/5	0/1	0/1	3/7	42.8
D	2/5	1/1	1/1	4/7	57.1
E	3/5	1/1	1/1	5/7	71.4
F	3/5	1/1	0/1	4/7	57.1
Liver					
A	2/7	0/6	0/0	2/13	15.4
B	2/7	5/6	0/0	7/13	53.8
C	2/7	2/6	0/0	4/13	30.7
D	4/7	1/6	0/0	5/13	38.4
E	1/7	5/6	0/0	6/13	46.1
F	2/7	2/6	0/0	4/13	30.7
Kidney					
A	0/5	0/7	0/1	0/13	0
B	2/5	4/7	1/1	7/13	53.8
C	2/5	3/7	0/1	5/13	38.4
D	2/5	1/7	0/1	3/13	23.0
E	3/5	3/7	1/1	7/13	53.8
F	3/5	2/7	0/1	5/13	38.4
Intestine					
A	2/8	0/6	1/1	2/15	20
B	5/8	4/6	1/1	10/15	66.6
C	4/8	2/6	1/1	7/15	46.6
D	5/8	2/6	1/1	8/15	53.3
E	3/8	5/6	1/1	9/15	60
F	6/8	1/6	1/1	8/15	53.3

It is shown in every tissue the percentage of alteration observed on to alteration total in each stage. The percentage were determined about fish number per group ($n = 5$). According to Poleksic and Mitrovic-Tutundzic (1994), Rigolin-Sá (1998) and Takashima and Hibiya (1995)

activity, but this does not exclude the possibility of their presenting antioxidant activity.

These results can be compared to those described by Ha et al. (2015), which tested lycopene-containing nanoemulsions with different particle sizes in DPPH and ABTS assays. This study demonstrated that the incubation period of the sample with the reagent might have influenced the release of the encapsulated compounds in some nanoemulsions. The authors stated that it would take a longer time for reactions with free radicals to occur in the nanoemulsions that did not exhibit reductive activity because the release of the molecules may have occurred slowly as they were protected by the structures of the droplets. Thus, it is understood that the relatively short incubation period used in the present study may have influenced the non-release of the active principles present in the nanoemulsions, implied by the expression values for the reductive activity.

The samples that presented the most relevant results in the ABTS test (NECHA, NECULT, OECHA, OECULT, and OECOM) were used in the cellular antioxidant assay (CAA). In this assay, at the three concentrations tested (0.05, 0.5, 5, and 50 µg/mL), they presented high values of antioxidant activity, with no significant difference to the quercetin standard (Fig. 4). It should be noted that the concentrations of the nanoencapsulated oils in the nanoemulsions corresponded three times less than those of the pure essential oils (0.01621, 0.1621, 1.621, and 16.21 µg), and these had the same antioxidant effect as pure oils. Possibly, this result can be explained by the fact that the lipophilicity of the cell membranes allowed the penetration of the micelles, present in the nanoemulsions, in the intracellular medium, promoting the decapsulation of the active antioxidant principles and, consequently, their ability to act.

A study by Sessa et al. (2013) involved polyphenols extracted from grape marc, which were encapsulated in the form of nanoemulsions and evaluated for the protective role of these formulations in preventing degradation and, in improving the distribution through biological membranes, preserved a high antioxidant activity. Two chemical tests were carried out to measure the radical-reducing activity, and, in one of them, one of the nanoemulsions presented low values of this activity. In the antioxidant assay with the cells, the nanoemulsions presented values relevant to the antioxidant activity, and these results were significantly higher for the encapsulated grape marc polyphenols than for the non-encapsulated polyphenols, suggesting the fundamental role of the nanoemulsions in the release through biological membranes.

In the J774 cell viability assay, it can be seen that none of the samples tested showed cytotoxicity. All the results presented different profiles of cellular viability to the antineoplastic doxorubicin curve, indicating that the cell viability profiles of the oils and nanoemulsions were not dose-dependent (Fig. 5).

The absence of cellular toxicity presented by nanoemulsions was similar to the study by Teixeira et al. (2017), who tested various nanoemulsions containing D-α-tocopherol and tween 80 surfactants at different concentrations, and observed that none showed toxicity to the cells.

To evaluate the action of nanoemulsification on cell proliferation, the viability of a fibroblast assay was performed in the presence of trypan blue, which is a diazo dye used to

Table 4 Histopathological Change Index (HCI) presented by groups of animals after treatment with **A** control PBS, **B** application of carrageenan via intraperitoneal and saline via gavage, **C** diclofenac (0.5 mg/kg) with the application of carrageenan intraperitoneally, **D** dexamethasone (0.5 mg/kg) with the application of carrageenan intraperitoneally, **E** NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and **F** NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg) on *D. rerio*

Organs	A	B	C	D	E	F
Gills	0.2 ± 0.03	22.6 ± 0.33	0.6 ± 0.00*	22.4 ± 0.20	22.4 ± 0.43	2.6 ± 0.07*[a]
Liver	0.4 ± 0.03	10.4 ± 0.40	4.4 ± 0.06*	3.0 ± 0.09*	10.2 ± 0.32	4.0 ± 0.16*[a]
Kidney	0.0 ± 0.00	28.4 ± 0.46	6.4 ± 0.07*	2.4 ± 0.16*	26.6 ± 0.25	4.6 ± 0.05*[a]
Intestine	20.4 ± 0.45	29.0 ± 0.51	24.8 ± 0.32*	25.0 ± 0.23*	30.6 ± 0.72	23.2 ± 0.36*[a]

Values are expressed as the mean ± SEM ($n = 5$/group). Kruskal–Wallis one-way Analysis of Variance (ANOVA) $p < 0.001$. *$p < 0.05$ versus B group and [a]$p < 0.05$ versus E group (Student–Newman–Keuls test)

measure the viability of the cells through the penetration of the dye into the intracellular medium.

In the mechanism of this assay, one has to consider that the cell membrane is formed by a highly selective lipid bilayer; therefore, in cells considered viable, the dye does not penetrate. Cells with damaged membranes show distinct blue staining in their cytoplasm. In this way, this dye allows the discrimination between viable cells and cells with damaged membranes, considered as dead cells (Tran et al. 2011). MRC5 fibroblasts were used for this assay. Fibroblasts are common connective tissue cells with high protein synthesis capacity to maintain the fibres and fundamental substance of the extracellular matrix in the tissues. They also produce growth factors that control cell proliferation and differentiation, acting on healing processes (Junqueira and Carneiro 2013).

The NECHA nanoemulsion, the essential oil of *Rosmarinus officinalis* (OECHA), and the control nanoemulsion (NECONTROL) were evaluated in the cell viability test, and it was possible to observe that NECONTROL did not significantly interfere, when compared to untreated cells (negative control), on cell proliferation, thus evidencing that the surfactant Tween 20, used in the preparation of nanoemulsions, did not influence this process. After 72 h of treatment, NECHA presented 48%, and ascorbic acid 54% increased cell proliferation.

In this essay, it should be noted that the essential oil of *Rosmarinus officinalis* (OECHA) delivered in the form of nanoemulsion (NECHA) was used at a concentration 125 times lower than the OECHA. Thus, it is possible to suggest that this nanoencapsulated oil potentiated the effect on fibroblast proliferation (Fig. 6). Anuchapreeda et al. (2012) evaluated the cytotoxicity of a nanoemulsion containing curcumin in several cell lines and stated that control nanoemulsion (containing Tween 80) did not influence on cell viability. Rampersad (2012) reported that in metabolism assays, there is the drawback of no differentiation between cells that are actively dividing and those that are quiescent, which may result in overestimation of cell numbers. However, he claims that an increase in cell proliferation can be considered indicative of cell viability.

In the inhibition test of nitric oxide production (NO·) in vitro, LPS was used as a positive control of this production. NO· plays an important role in various inflammatory conditions, being produced in the form of nitric oxide synthase (iNOS) from the amino acid L-arginine (Abdelwahab et al. 2012). Several stimuli, such as LPS, can significantly increase the production of NO·by macrophages, which are cells derived from monocytes, capable of acting as antigen presenting to lymphocytes, in addition to, in some cases, phagocytosis. They play an important role in acute and chronic inflammatory reactions (Verma et al. 2009). In aerobiosis, NO· reacts with oxygen to produce stable nitrate (NO_3^-) and nitrite ($NO_2\cdot$) whose quantities can be determined using Griess's reagent (Islam et al. 2016).

In the evaluation of NO· production in macrophages, in general, all samples of *Rosmarinus officinalis* oils and nanoemulsions inhibited production in macrophages when compared to cells receiving only LPS. Among them, the NECHA and NECOM nanoemulsions were characterised by a reduction in this production when compared to the non-encapsulated oils at the lowest concentration tested. This shows that while being distributed at only 5 µg/mL, nanoemulsions were able to potentiate the effects of essential oil in reducing the production of the pro-inflammatory mediator, nitric oxide (Fig. 7). Justo et al. (2015) observed that rosemary extract obtained by supercritical CO_2 extraction inhibited the release of NO· by peritoneal macrophages and J774 cells; maintaining reduced cytotoxicity when dispersed in DMSO at a concentration lower than 2.79 mg/mL.

In the study with zebrafish, it should be considered that when in contact with foreign substances, zebrafish may present altered behaviour (Mathur et al. 2011), including derivatives of plant species, as described by Ribeiro (2013) for the ethanolic extract composition of *Spilanthes acmella*. In evaluating the toxicity of nanoemulsion with perillyl alcohol, Souza et al. (2016) observed that behavioural changes in zebrafish begin with increased excitability, leading to loss of balance, rest in the background, and probably death.

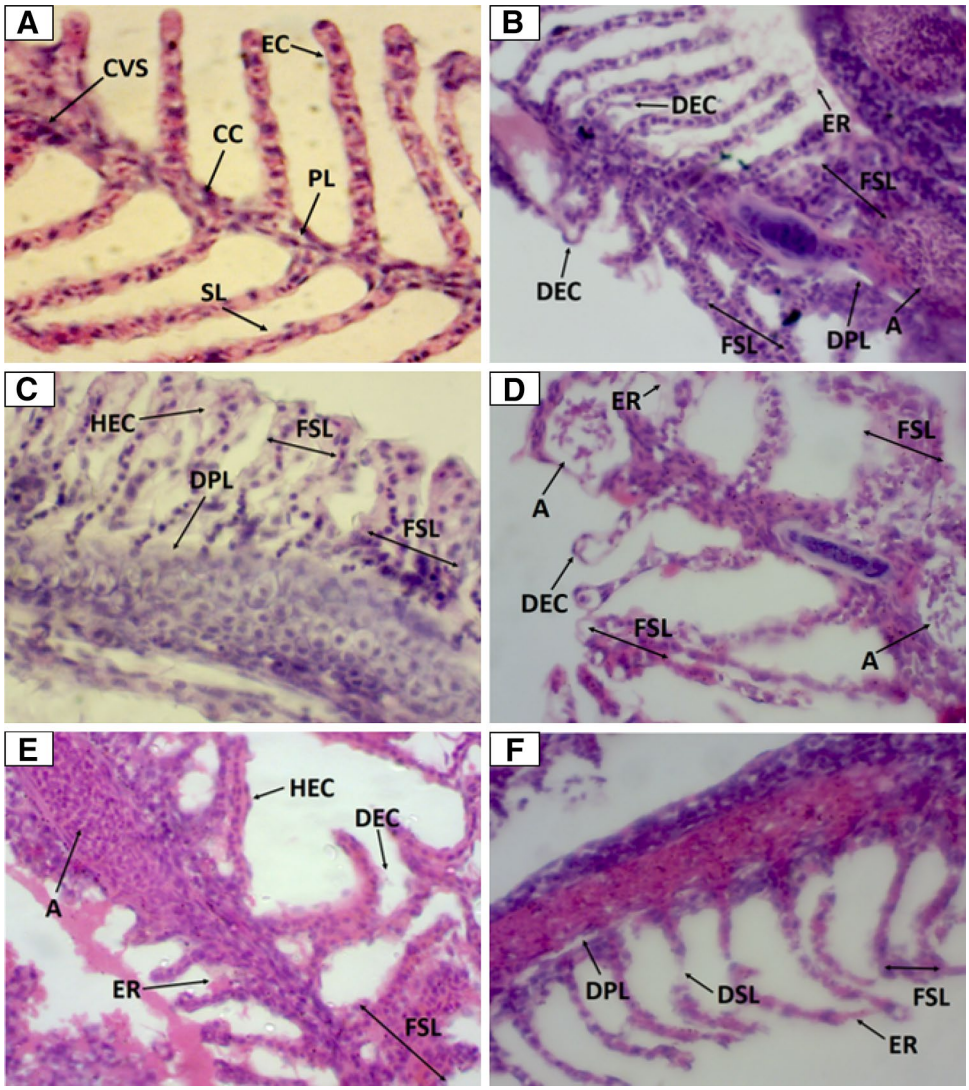

Fig. 9 Longitudinal cross section of *Danio rerio* gills, all figures were magnified by 9400, H & E. **a** Negative control (PBS intraperitoneal + saline by gavage) showing *EC* epithelial cells, *CC* chloride cells, *PL* primary lamellae, *CVS* central venous sinusoids, *SL* secondary lamellae; **b** Positive control (carrageenan intraperitoneal + saline by gavage) showing *FSL* fusion of secondary lamellae, *DPL* detachment of primary lamella, *DEC* displacement of epithelial cells, *FSL* fusion of secondary lamellae, *ER* epithelial rupture, *A* aneurism; **c** carrageenan intraperitoneal + diclofenac by gavage showing *DPL* detachment of primary lamella, *HEC* hyperplasia of epithelial cells, *FSL* fusion of secondary lamellae; **d** carrageenan intraperitoneal + dexamethasone by gavage showing epithelial rupture, *A* aneurism, *DEC* displacement of epithelial cells, *FSL* fusion of secondary lamellae; **e** nanoemulsion control (carrageenan intraperitoneal + NECONT) by gavage showing *DEC* displacement of epithelial cells, *HEC* hyperplasia of epithelial cells, *A* aneurism, *FSL* fusion of secondary lamellae, *ER* epithelial rupture, *DEC* displacement of epithelial cells; **f** carrageenan intraperitoneal + NECHA by gavage showing *DPL* detachment of primary lamella, *DSL* detachment of secondary lamella, *ER* epithelial rupture, *FSL* fusion of secondary lamellae

The application of carrageenan (300 µg) in the abdomen of the animals produced oedema that was very visible in the control groups (B and E), with maximum peak at the 5th hour after the application (Fig. 8). The reactions observed in this study were described by Huang et al. (2014) as typical symptoms of zebrafish inflammation, with the presence of oedema, leukocyte marker (MPO), proinflammatory proteins such as TNF-α and iNOS.

The different previous treatments of the animals with diclofenac (Group C), dexamethasone (Group D) and NECHA (Group F) inhibited the formation of oedema in 49.5, 10.35, and 77.99%, respectively (Fig. 8). Borges et al. (2017) demonstrated the anti-inflammatory action of NECHA in rats at the dose of 498 µg/kg orally and demonstrated the involvement of the camphor compound in this action. In the in vitro assays, this nanoemulsion was

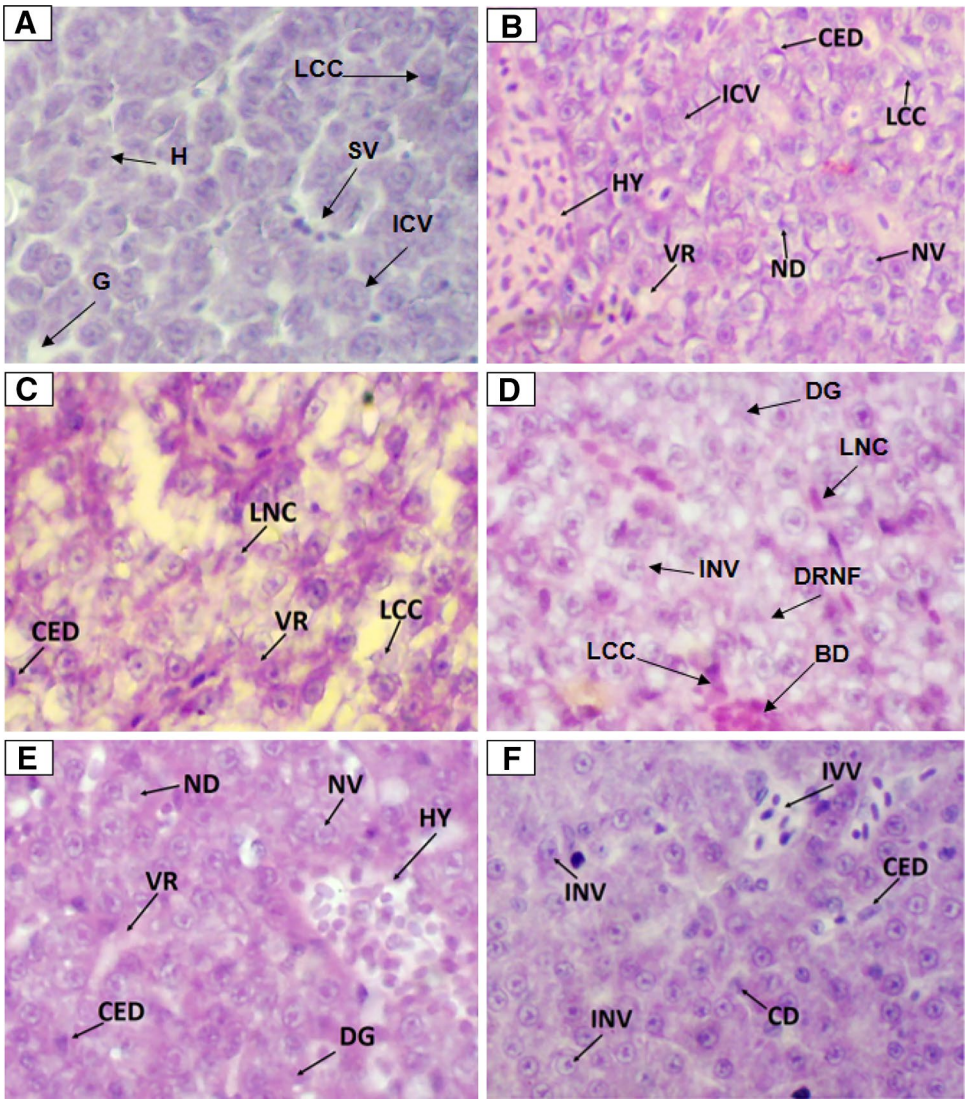

Fig. 10 Longitudinal cross section of *Danio rerio* liver, all figures were magnified by 9400, H & E. **a** negative control (PBS intraperitoneal + saline by gavage) showing *H* hepatocytes, *SV* sinusoids vessels, *G* glycogen, *ICV* increased cell volume, *LCC* loss of cell contour; **b** positive control (carrageenan intraperitoneal + saline by gavage) showing *VR* vessels rupture, *HY* hyperemia, *CED* cell disruption, *NV* vacuolization nuclear, *ND* nuclear degeneration, *LCC* loss of cell contour, *ICV* increased cell volume; **c** carrageenan intraperitoneal + diclofenac by gavage showing *LCC* loss of cell contour, *LNC* loss of nucleus contour, *CED* cell disruption, *VR* vessels rupture; **d** carrageenan intraperitoneal + dexamethasone by gavage showing *BD* bile ducts, *LCC* loss of cell contour, *LNC* loss of nucleus contour, *INV* increased nuclear volume, *DG* decreased glycogen, *DRNF* decreased relative frequency of nucleus occurrence; **e** nanoemulsion control (carrageenan intraperitoneal + NECONT) by gavage showing *DG* decreased glycogen, *CED* cell disruption, *ND* nuclear degeneration, *VR* vessels rupture, *NV* vacuolization nuclear and *HY* hyperemia; **f** carrageenan intraperitoneal + NECHA by gavage showing *INV* increased nuclear volume, *IVV* increased vessels volume, *CED* cell disruption, *CD* cytoplasmic degeneration

highlighted concerning activity. It should be emphasised that in the composition of this essential oil were found alpha-pinene, 1,8-cineole, and camphor compounds that are strictly related to the anti-inflammatory and anti-allergic actions of essential oils (Borges et al. 2017). These results in zebrafish confirm the hypothesis described by Huang et al. (2014) that compounds with anti-inflammatory properties can modulate the responses induced by carrageenan in adult zebrafish.

According to Petrillo (2012), dexamethasone can be up to thirty times more potent than cortisol, its natural analogue, whose mechanism of action occurs from its accumulation in the cellular cytoplasm. Dexamethasone was used as a positive control for the inhibition of the inflammatory process

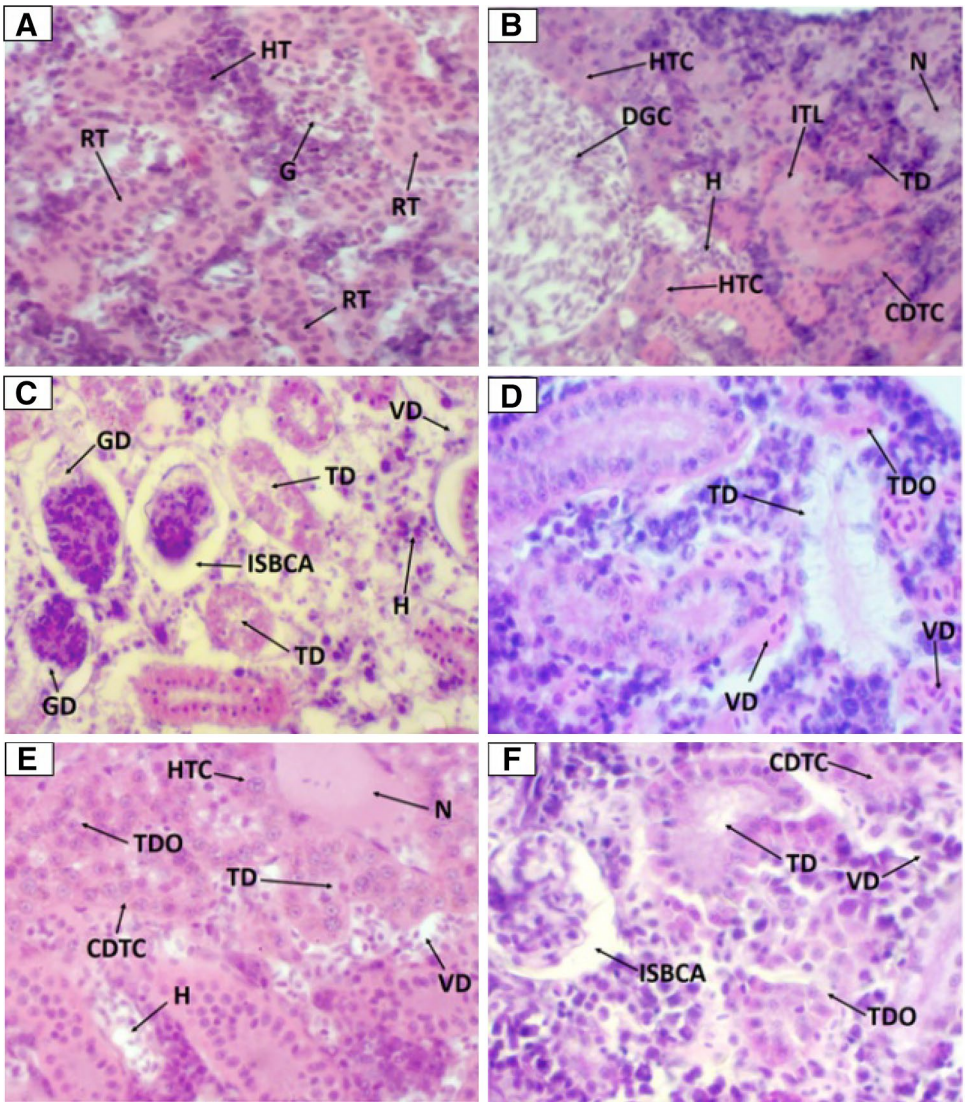

Fig. 11 Longitudinal cross section of *Danio rerio* kidney, all figures were magnified by 9400, H & E. **a** negative control (PBS intraperitoneal + saline by gavage) showing *RT* renal tubules, *HT* hematopoietic tissue, *G* glomerulus; **b** positive control (carrageenan intraperitoneal + saline by gavage) showing *HTC* hypertrophy of tubular cells, *DGC* dilation of Glomerular capillaries, *ITL* increase in tubular lumen, *TD* tubular degeneration, *CDTC* cytoplasmic degeneration of tubular cells, *N* necrosis, *H* hyperemia; **c** carrageenan intraperitoneal + diclofenac by gavage showing *VD* vessels dilatation, *H* hyperemia, *TD* tubular degeneration, *ISBCA* increased space of Bowman's capsule, *GD* glomerular degeneration; **d** carrageenan intraperitoneal + dexamethasone by gavage showing *VD* vessels dilatation, *TD* tubular degeneration, *TDO* tubular disorganization; **e** nanoemulsion control (carrageenan intraperitoneal + NECONT by gavage) showing *VD* vessels dilatation, *TD* tubular degeneration, *TDO* tubular disorganization, *HTC* hypertrophy of tubular cells, *CDTC* cytoplasmic degeneration of tubular cells, *H* hyperemia, *N* necrosis; **f** carrageenan intraperitoneal + NECHA by gavage showing *VD* vessels dilatation, *TD* tubular degeneration, *TDO* tubular disorganization, *ISBCA* increased space of Bowman's capsule, *CDTC* cytoplasmic degeneration of tubular cells

in zebrafish since it inhibits the recruitment of macrophages and neutrophils to the site of inflammation and decreases fish mortality (Yang et al. 2014). In this study NECHA was more effective when compared to diclofenac and dexamethasone. This fact demonstrates that the nanoencapsulation of the essential oil, OECHA, whose composition directs it to the anti-inflammatory activity, was potentiated because at the dose of 498 µg/kg per oral administration, was highly effective (Fig. 8).

Carvalho et al. (2017) have shown that because zebrafish is a small animal, injection of an inflammatory agent in the abdominal region, such as carrageenan, can provoke reactions in other vital organs such as gills, liver, intestine, and kidneys. This fact was detected in groups B and E (negative controls) that presented histopathological changes in all these organs

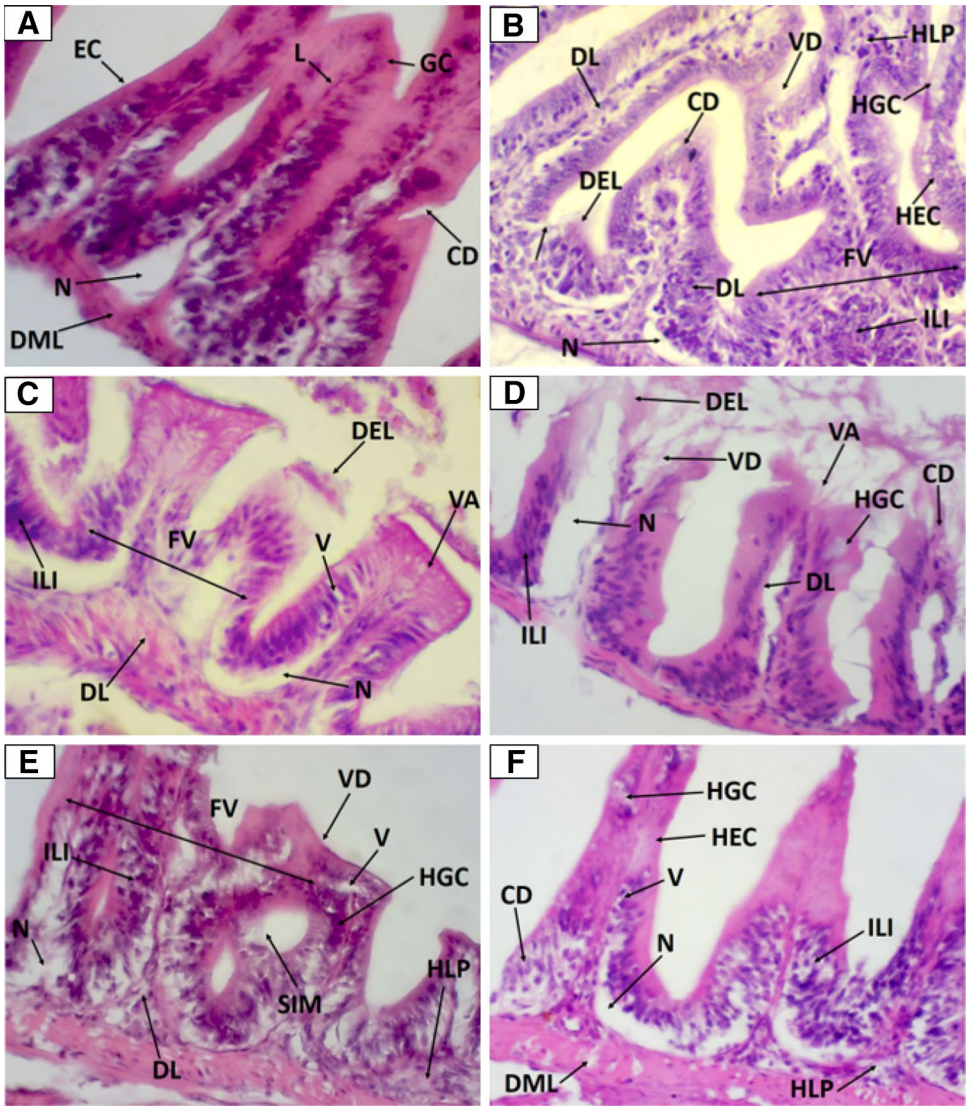

Fig. 12 Longitudinal cross section of *Danio rerio* intestine, all figures were magnified by 9400, H & E. **a** Negative control (PBS intraperitoneal + saline by gavage) showing *L* lamina propria, *DML* degeneration of muscular layer, *GC* goblet cell, *EC* enterocyte cell, *CD* cell degeneration, *N* necrosis; **b** positive control (carrageenan intraperitoneal + saline by gavage) showing *ILI* leukocyte infiltration, *DEL* detachment of the epithelial lining of the apex of the intestinal villous, *CD* cell degeneration, *DL* detachment of the lamina propria, *FV* partial or complete fusion of villous, *VD* villous degeneration, *HLP* hemorrhage in the lamina propria, *HEC* hypertrophy of epithelial cells of the lamina propria, *HGC* hyperplasia of globet cells, *N* necrosis; **c** carrageenan intraperitoneal + diclofenac by gavage showing *V* vacuolization of enterocytes, *ILI* leukocyte infiltration, *DL* detachment of the lamina propria, *FV* partial or complete fusion of villous, *DEL* detachment of the epithelial lining of the apex of the intestinal villous, *VA* villous atrophy, *N* necrosis; **d** carrageenan intraperitoneal + dexamethasone by gavage showing *ILI* leukocyte infiltration, *HGC* hyperplasia of globet cells, *N* necrosis, *DL* detachment of the lamina propria, *VD* villous degeneration, *DEL* detachment of the epithelial lining of the apex of the intestinal villous, *VA* villous atrophy; **e** nanoemulsion control (carrageenan intraperitoneal + NECONT by gavage) showing *N* necrosis, *ILI* leukocyte infiltration, *V* vacuolization of enterocytes, *DL* detachment of the lamina propria, *SIM* sloughing of the intestinal mucosa, *HGC* hyperplasia of globet cells, *VD* villous degeneration, *FV* partial or complete fusion of villous, *HLP* hemorrhage in the lamina propria; **f** carrageenan intraperitoneal + NECHA by gavage showing *CD* cell degeneration, *DML* degeneration of muscular layer, *N* necrosis, *V* vacuolization of enterocytes, *HGC* hyperplasia of globet cells, *HEC* hypertrophy of epithelial cells of the lamina propria, *ILI* leukocyte infiltration, *HLP* hemorrhage in the lamina propria

(Table 3). Gills are structures that allow fish to efficiently extract oxygen from water for use in metabolic reactions, regulating the acid–base balance and allowing the excretion of toxic waste (Holden et al. 2012; Houlihan et al. 1982).

According to Mazon et al. (2002), epithelial cell distension promotes haemorrhages and aneurysms in the gill tissue, as observed in groups B, D, and E. Other histological alterations observed, such as lamellar epithelial cells

(present in C and E) and epithelial cells identified in B, D, and E), indicate the attempt to adapt the tissue to the new pathophysiological conditions (Carvalho et al. 2017). One of the ways of tissue adaptation is to reduce the passage of water and blood into the secondary lamellae (Souza et al. 2016).

In this zebrafish study, diclofenac treatment was more effective than dexamethasone in preventing histopathological changes caused by carrageenan in the gills, liver, intestine, and kidneys. This fact can be explained by the time it was evaluated, 5 h after the application of carrageenan, whereas in the study of Huang et al. (2014), with methylprednisolone intraperitoneally, it was 24 h after application.

The liver is the vital organ for the detoxification process of substances, and any dysfunction in its tissue can be harmful to the animal and even cause death (Carvalho et al. 2017). Hepatocytes are the most common cell type in zebrafish. Its cytoplasm contains abundant glycogen and stores lipids and iron, produces proteins and amino acids, and aids in the detoxification of several compounds (Holden et al. 2012). The groups of diclofenac, dexamethasone, and NECHA presented livers with normal functionality (Table 3).

The changes observed in this study are common in cases of inflammation. Changes in tissue may lead to hepatic dysfunction (Carvalho et al. 2017). None of the zebrafish groups presented serious alterations, such as biliary stagnation, excretion of bile pigments, and metabolic insufficiency. The hepatocyte vacuolization observed in groups B and E (Negative controls), which received carrageenan with saline via gavage and carrageenan with NECONT via gavage, respectively, may be related to the reduction of glycogen accumulation in the cells (Rodrigues 1994). When intense, it can change the functioning of the liver. According to Carvalho et al. (2017), the fact that zebrafish are very agile and have accelerated metabolism may justify the presence of vacuolization in the hepatocytes, indicating dysfunction in their metabolism. Groups B and E also presented hyperemia. This condition can be considered a defence mechanism, which aims to increase the number of blood cells in the tissue and, consequently, increases oxygenation and the arrival of nutrients (Takashima and Hibiya 1984).

The adult zebrafish's kidney contains the nephrons responsible for the filtration of blood residues and the uptake of salt and water. It presents regions with lymphoid, hematopoietic, steroidogenic, and endocrine cells. Therefore, the main role of the kidneys in Teleostei freshwater fish is to eliminate the large volume of water entering the fish through the mouth, not having to store it (Holden et al. 2012). All treatments maintained functionally normal organs; however, the NECHA-treated group presented a response equal to that of the animals treated with diclofenac (38.4%), suggesting that the nanoemulsion may have a similar action as that of the nonsteroidal anti-inflammatory for the inhibition of the effects of carrageenan-induced inflammation in the kidneys (Table 3).

Zebrafish do not have stomachs, so the intestinal bulb, which precedes the oesophagus has an absorption function and acts as a food reservoir. The mucosal layer is formed by goblet cells, dispersed inflammatory cells, and by enterocytes, which probably absorb lipids. Through these cells, the intestinal epithelium performs the absorption of nutrients and acts on the immune response (Holden et al. 2012). The results show that anti-inflammatories were ineffective in reducing the histopathological changes observed in intestinal tissue; however, animals treated with NECHA showed moderate to severe changes.

All groups studied had many histopathological changes in the intestine. They may be related to the invasive technique of intraperitoneal injection, which can damage the intestine. Invasive procedures in fish can generate inflammation in the intestinal lamina propria and cause leukocyte infiltration in the epithelial tissue of the intestine (Carvalho et al. 2017). In addition to causing increased defence cells (Roberts and Ellis 2012). Exposure of zebrafish to toxic substances can cause damage to the intestinal mucosa, impairing organ physiology (Carvalho et al. 2017). Epithelial cell hypertrophy, observed in groups B and E (negative controls), can be considered a defence mechanism, serving as a barrier to reduce the entry of these substances into the intestinal epithelium (Takashima and Hibiya 1984). This process can lead to necrosis, change observed in all groups, and to vacuolation, present in groups C, E, and F. These changes may compromise the body's ability to absorb nutrients (Carvalho et al. 2017).

In a study carried out by Juerges et al. (2003), 1,8-cineol was effective in reducing airway inflammation caused by acute asthma in humans. The anti-inflammatory property of 1,8-cineol has also been demonstrated by Santos and Rao (2000), through the significant reduction of carrageenan-induced rat paw inflammation oedema, a decrease in cotton fibre-induced granuloma formation, and the reduction of Evan Blue dye induced by intraperitoneal acetic acid. Juerges (2014) demonstrated that 1,8-cineol was able to dose-dependently reduce arachidonic acid metabolism, inhibiting the formation of Leukotriene-B_4 (LTB_4), Prostaglandin-E_2 (PGE_2), and Interleukin-1β (IL-1β) and, consequently, the production of Tumor Necrosis Factor α (TNF-α), interrupting the continuity of inflammatory activity.

By comparing the in vitro results with the zebrafish results with the NECHA nanoemulsion, it is possible to state that, in the form of the OECHA essential oil, it has potentiated the involvement of the terpenoid compounds on the inflammatory pathways involved in the carrageenan-triggered pathophysiology of this animal species.

Conclusion

This study showed that all nanoemulsions (NECHA, NECULT, and NECOM) showed no toxicity to macrophages, cells with a relevant role in the inflammatory response, besides demonstrating antioxidant activity and potentiation of the essential oil effect in the proliferation of viable fibroblasts, which are connective tissue cells acting in the healing processes. These results demonstrate that the essential oil, in the form of nanoemulsions, increased the bioavailability of the active principles since they require a lower concentration of the essential oil to present antioxidant effect, similar to that of the unencapsulated oil. Nanoemulsions have also demonstrated the ability to potentiate the anti-inflammatory action of essential oils by exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide. The results obtained with NECHA in zebrafish confirm the hypothesis that prominent terpenic compounds, alpha-pinene, 1,8-cineole, and camphor, became more available at the target sites, inhibiting the inflammatory process in this animal species.

Acknowledgements We thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES) for the grant bestowed, Assistance from Capes—Senior internship (Proc. 88881.120909/2016-01, and CNPq Process number 407768/2013-0 (Rede Amazônica de Pesquisa em Biofármacos – RAPBioFar). J.C. Tavares Carvalho acknowledges to DGAPA-UNAM—Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México—Programa de Estancias de Investigación en la UNAM (PREI-2017).

Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants, but with animals (Zebrafish - Danio rerio) that was approved by the Ethics Committee of the Amapá Federal University.

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