

# Bullfrog Oil Reduces the Carrageenan-induced Edema in Wistar Rats by *in vitro* Reduction of Inflammatory Mediators

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**Abstract:** Bullfrog oil (BFO) is a natural product from the adipose tissue of the amphibian *Rana catesbeiana* Shaw, a bio-product rich in polyunsaturated fatty acids, which claims anti-inflammatory activity. The objective of this work was to evaluate the cytotoxicity and the anti-inflammatory activity of BFO using *in vivo* and *in vitro* assays. Thus, the *in vitro* cytotoxicity was assessed by the MTT assay. Additionally, the *in vivo* anti-inflammatory activity was performed by the carrageenan-induced paw edema model in Wistar rats, followed by histological analysis. Moreover, the BFO effect on inflammatory pathways was investigated by *in vitro* evaluation of the nitric oxide (NO) synthesis, and type-6 interleukin (IL-6) and tumor-necrosis-factor (TNF) levels. *In vivo* experiments showed that BFO administered by intragastric route produced a significant anti-inflammatory effect, which was as substantial as indomethacin, the positive control. Histopathological analysis confirmed these results, showing the absence of the edema and minimal signs of inflammation in the paws of rats treated with BFO. The MTT results showed that BFO at all tested concentrations had no toxic effect against a macrophage cell line, not affecting the cell viability. In addition, after 48 hours of treatment, the BFO itself and its blend with Cetiol®-V (1:1<sub>v/v</sub>) at 200 µg.mL<sup>-1</sup> were able to reduce the NO synthesis, and the IL-6 and TNF levels up to 35 ± 2%, 40 ± 6%, and 12 ± 3%, respectively. Therefore, these results provide unprecedented scientific evidence of the anti-inflammatory effect of BFO, suggesting its potential as a new candidate for the development of pharmaceutical products with anti-inflammatory activity.

**Key words:** histological analysis, inflammation, natural products, *Rana catesbeiana* oil, rat paw edema

## 1 Introduction

Zootherapy, a therapeutic strategy widely used by several cultures, aims the treatment of medical conditions based on the use of therapeutic substances, as natural oils, obtained from animals<sup>1,2</sup>. These oils may present a renewable character, low toxicity, high biodegradability and chemical composition based on different saturated and unsaturated fatty acids<sup>3,4</sup>. Additionally, the presence of substances with pharmacological activity makes these products potential candidates for therapeutic purposes<sup>5,6</sup>.

In this scenario, bullfrog oil (BFO) is a noteworthy natural oil extracted from the adipose tissue of the amphibian *Rana catesbeiana* Shaw, popularly known as bullfrog. The species, originated from North America, was introduced in Brazil at around the 1930s. This amphibian has an important contribution in the economy, since its farming and marketing increased over the years, mainly because of its meat's nutritional value. Nonetheless, during the meat processing, the bullfrog adipose tissue has been discarded. In the last years, this tissue has been used to obtain the BFO

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Accepted December 9, 2019 (received for review August 20, 2019)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

by a biotechnological reuse process<sup>7)</sup>.

In this regard, the *in natura* BFO has been used by the folk medicine as an anti-inflammatory, anti-allergic and healing agent. These properties have been attributed to the presence of unsaturated and saturated fatty acids on its chemical composition<sup>7, 8)</sup>. Previous studies from our research group elucidated the BFO chemical composition, highlighting the presence of myristic (1.4%), arachidonic (0.7 – 8.4%), palmitic (10.3%), oleic (30%), stearic (2.5%), docosahenaxoic - DHA (0.8%) and eicosapentaenoic - EPA (17.6%) fatty acids, in addition to cholesterol (9.5%) and ethyl-iso allocholate (3.5%)<sup>6, 9, 10)</sup>. However, despite its broad popular use, especially against inflammatory conditions, there is a lack of scientific evidence supporting such therapeutic property. To date, a limited number of studies have shown scientific evidence of the BFO potential for the development of pharmaceutical products, as showed by previous reports from our research group in which promising antimicrobial and antibiofilm activities were investigated<sup>7, 11)</sup>. In addition, the cytotoxic activity of BFO (not only *in natura*, but also carried out by nanostructured systems) against melanoma cells was also elucidated<sup>6, 9)</sup>. Nevertheless, no report was found in the literature to support its anti-inflammatory activity, specially the evaluation of the inflammatory pathways involved in this process.

Therefore, the present study was carried out aiming to evaluate the anti-inflammatory activity of BFO using *in vitro* and *in vivo* classical models of inflammation.

## 2 Experimental Procedures

### 2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Cultilab (São Paulo, SP, Brazil). Cetiol® V (decyl oleate) was purchased from Emb-racaps (Porto Alegre, RS, Brazil). The MTT reagent ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)), lipopolysaccharides (LPS), L-NAME (N-nitro-L-arginine methyl ester hydrochloride), Griess Reagent, Sodium thiopental, Indomethacin,  $\kappa$ -carrageenan, Formalin solution, and Hematoxylin and Eosin were from Sigma-Aldrich Inc (St. Louis, MO, USA). Saline solution came from Arboreto (Minas Gerais, MG, Brazil).

### 2.2 Bullfrog oil

*In natura* BFO, a gift from the Asmarana Industry of Natural Products (Natal, RN, Brazil), was stored at room temperature and protected from light exposure. For the *in vivo* experiments, BFO was diluted with decyl oleate (Cetiol® V) to reach different tested concentrations. Cetiol® V was chosen as a BFO vehicle for the *in vivo* studies due to its biocompatibility and chemical composition, display-

ing a single compound also presented on BFO (therefore, not affecting the chemicals in the solution). Furthermore, BFO displayed adequate miscibility with this compound, excluding the need of further co-solvents<sup>12, 13)</sup>.

### 2.3 Biological

Adult male Wistar albino rats (200-250 g) were used in the *in vivo* study. The animals were carried out according to the Guide for the Care and Use of Laboratory Animals<sup>14)</sup> and to our institutional guidelines of animal care. Indeed, the experimental work with animals was conducted under the duration period of the protocol approved by the Ethics Committee on Animal Use of the Federal University of Rio Grande do Norte, Protocol Number 001, August/2007. Animals 6 to 7 weeks-old were used for the experiments. They were housed at  $25 \pm 2^\circ\text{C}$  inside of clean polypropylene cages in groups of five ( $n = 5$ ). They had free access to water and standard laboratory chow. Two weeks before experiments, animals were manipulated once a day in the experimental room for acclimation and prevention of stress on the day of experiment, when they were placed in the experimental room at least one hour prior to the tests.

The Raw 267.4 cell lineage from American Type Culture Collection (ATCC), used on the *in vitro* study, was donated by the Laboratory of Biotechnology of Natural Polymers (BioPol), from the Federal University of Rio Grande do Norte (Natal, RN, Brazil).

### 2.4 *In vitro* cytotoxic evaluation – MTT assay

In order to evaluate the cytotoxicity of the BFO, *in natura*, and the blend of BFO and Cetiol® V (BFO-CV) (1:1  $v/v$ ), the colorimetric MTT assay was performed in triplicate against murine macrophage cells (Raw 264.7), which were incubated into 96 well-plates ( $5 \times 10^3$  cell/well) for 24 hours with DMEM supplemented with 10% FBS. After placement and cells adherence, the medium was replaced by FBS-free DMEM for 24 hours. Then, the cells were treated for 24 and 48 hours with BFO and BFO-CV at concentrations of 50, 100 and 200  $\mu\text{g.mL}^{-1}$ . Finally, 100  $\mu\text{L}$  of MTT reagent (1  $\text{mg.mL}^{-1}$ ) was added to each well and allowed to react for 4 hours, with subsequent dissolution of the formazan crystals in 100  $\mu\text{L}$  of analytical grade ethanol and absorbance reading by an Elisa Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA, USA) set at 570 nm. The results were expressed as the relative percentage to the untreated cells (negative control = 100% viability). To reach the BFO concentrations mentioned above, samples were diluted in DMSO at 1% aqueous solution. Thus, both the DMSO at 1% aqueous solution and the Cetiol® V, used as diluents, were also assayed to evaluate their effect on Raw 264.7 cells.

### 2.5 Carrageenan-induced paw edema

The anti-inflammatory activity of the BFO was evaluated

using the carrageenan-induced paw edema model in rats<sup>15</sup>. Dose selection and time of pre-treatment for the test were based on preliminary studies carried out in our laboratory. Before the treatments, the rats were fasted (with water *ad libitum*) for 2h. Then, the animals, 6 -7 weeks old, were separated in 5 different groups (n=5 animals/group). Each group was treated by intragastric administration with one of the following samples: 0.9% saline solution (10 mL/kg) (negative control), indomethacin (10 mg/kg) (positive control), or BFO (50, 100 and 200 mg/kg) in Cetiol® V solution. The inflammation induction was performed after 1 hour of treatment in which 100 µL of a freshly prepared 1% κ-carrageenan suspension (1000 µg per paw) was injected in the sub-plantar region of the right paw of all animals. At the same time, 100 µL of 0.9% saline solution was injected in the contralateral paw as a control. Subsequently, the increase in paw thickness was measured with a digital caliper over 4 h. Edema was expressed according to the Equation (1):

$$\text{Edema (\%)} = \frac{\text{Right paw} - \text{Left paw}}{\text{Right paw of Negative control}} \times 100 \quad (\text{Eq. 1})$$

Wherein, *Right paw* was the thickness of the paw injected with 1% κ-carrageenan suspension and *Left paw* was the thickness of the paw injected with 0.9% saline solution, both from rats treated with BFO or indomethacin. *Right paw of Negative control* means the thickness of the paw injected with 1% κ-carrageenan suspension in rats treated with 0.9% saline solution.

## 2.6 Histological analysis

Samples of paws were collected 4 h after carrageenan injection for histological examination. Tissue slices were fixed in 10% buffered formalin, embedded in paraffin, and posteriorly sectioned. Sections of 5 µm were stained with hematoxylin and eosin, and further examined under an optical light microscope for the assessment of inflammation indicatives.

## 2.7 *In vitro* inflammatory pathway evaluation: nitric oxide synthesis, and IL-6 and TNF levels

### 2.7.1 Nitric Oxide (NO) synthesis

NO production was evaluated by measuring NO released from Raw 264.7 macrophages stimulated with LPS 2 µg.mL<sup>-1</sup> for 1 hour, according to the Griess reaction. The cells were seeded in 24-well plates (3 × 10<sup>5</sup> cells/well) and maintained in DMEM supplemented with 10% FBS at 37 ± 2°C and 5% CO<sub>2</sub> atmosphere. After the LPS stimulation, the cells were treated with BFO and BFO-CV at 50, 100 and 200 µg. mL<sup>-1</sup> for 24 and 48 hours. Then, the supernatant (100 µL) was removed and incubated with 100 µL of Griess reagent for 10 minutes. Moreover, the absorbance of the supernatant was evaluated by an Elisa Multiskan Ascent Microplate

Reader (Thermo Labsystems, Franklin, MA, USA) set at 540 nm. Untreated cells were used as negative control and L-NAME, at 70 µg.mL<sup>-1</sup>, was used as NO inhibitor.

### 2.7.2 IL-6 and TNF levels

The evaluation of the IL-6 and the TNF levels was performed according to the kit Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine (BD) (BD Biosciences, San Diego, CA, USA). The cells were cultivated in the same conditions as in the NO production assay and, after treatment using BFO and BFO-CV at 50, 100 and 200 µg. mL<sup>-1</sup> for 48 hours, the cell supernatant (50 µL) and the reagents provided by the kit (50 µL of capture beads and 50 µL of the phytoerythrin antibody) were incubated for 2 hours at dark conditions. Subsequently, the wash buffer presented in the kit (1 mL) was added and this mixture was centrifuged at 200 g for 10 minutes. After centrifugation the supernatant was removed, and the pellet was resuspended in 200 µL of wash buffer. The IL-6 and TNF levels were measured using a flow cytometer (FACSCANTO II, BD Bioscience, Franklin Lakes, NJ, USA) apparatus. Untreated cells were used as negative control and L-NAME at 70 µg.mL<sup>-1</sup> was used as anti-inflammatory control.

## 2.8 Statistical analysis

All the results were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA), followed by the Bonferroni's test, or by the one-way ANOVA followed by the Tukey's test, when appropriate. *P* values lower than 0.05 were considered statistically significant. All statistical analyses were performed using the GraphPad Prism version 5.00 for Windows (San Diego, California, USA).

## 3 Results

### 3.1 *In vitro* cytotoxic evaluation

In order to evaluate the BFO cytotoxicity in a macrophage cell lineage (Raw 264.7), the MTT assay was performed. This *in vitro* assay is based on the activity of mitochondrial enzymes<sup>16</sup>. Based on its result, the cytotoxicity of pharmaceutical samples may be inferred. Thus, the BFO and BFO-CV at 50, 100 and 200 µg.mL<sup>-1</sup> were tested. These concentration were chosen according to previous work of our research group, wherein BFO concentrations up to 0.5 mg.mL<sup>-1</sup> were not able to promote cytotoxic effect<sup>11</sup>.

Accordingly, the results are demonstrated in **Figs. 1A** (24 hours of treatment) and **1B** (48 hours of treatment). **Figure 1A** shows that after 24 hours of cell exposure no significant decrease (*p* > 0.05) in cell toxicity was found for all tested concentrations, hence, the samples did not interfere on the cell viability by this assay. In addition, after 48 hours of treatment (**Fig. 1B**), the cell viability ranged between 90

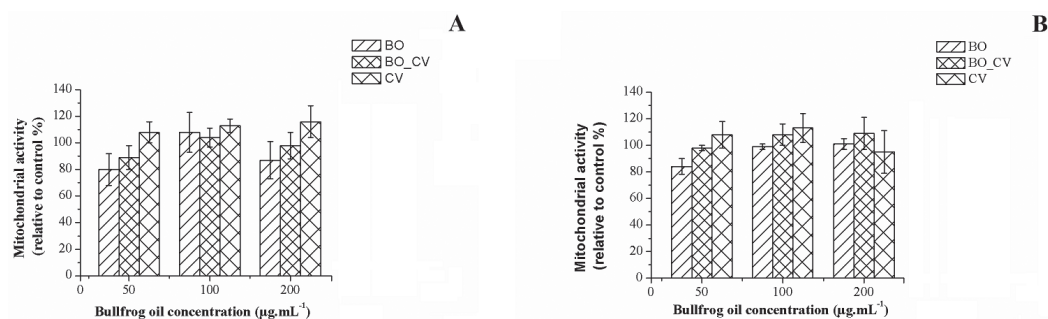


Fig. 1 Evaluation of the *in vitro* cytotoxicity of bullfrog oil. A: mitochondrial activity percent by MTT assay after 24 hours of treatment; B: mitochondrial activity percent by MTT assay after 48 hours of treatment. BFO: Bullfrog oil; BFO-CV: blend of bullfrog oil and Cetiol® V; CV: Cetiol® V.

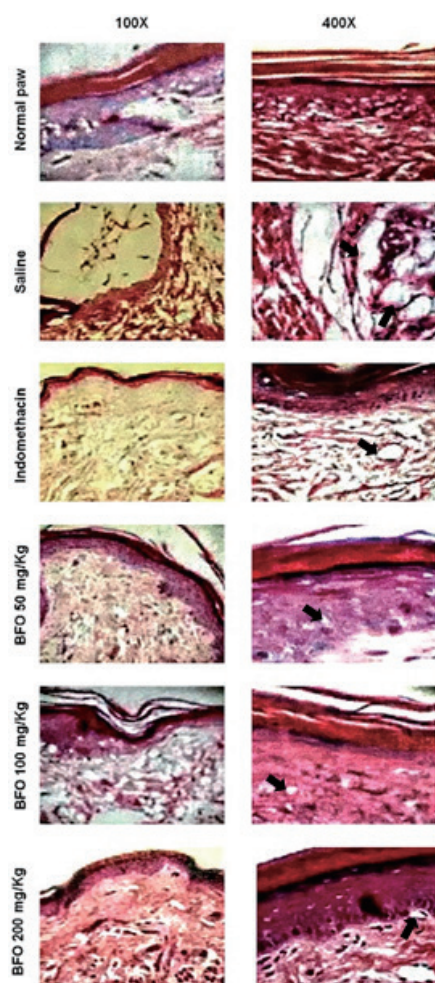


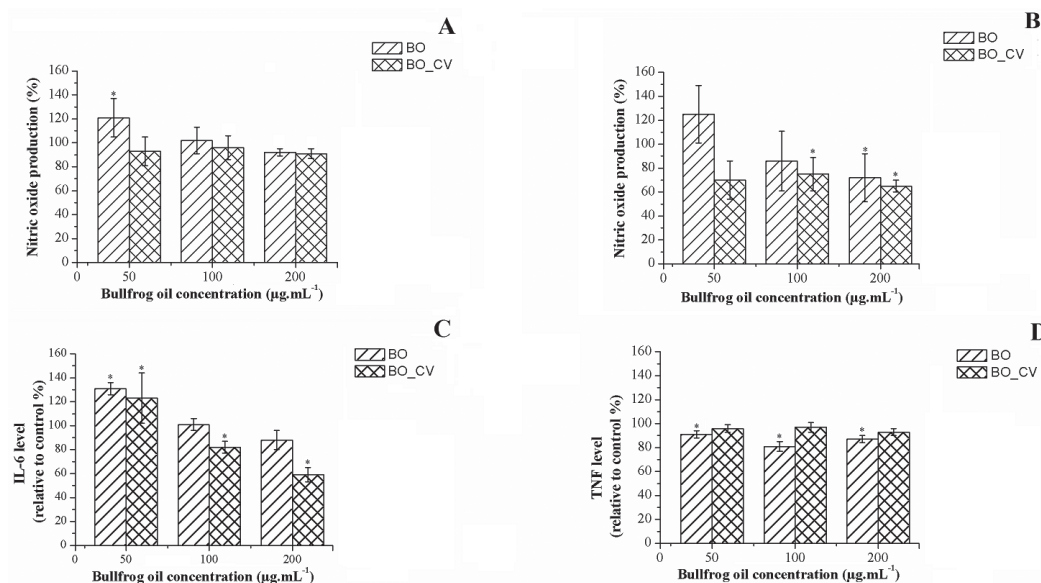
Fig. 2 Histological analysis of the right paws of rats 4 h after the carrageenan injection (10 mg/kg) observed under an increase of 100x (left micrographs) and 400x (right micrographs). "Normal paw" group represents the histological aspect of the left paws treated only with 0.9% saline (absence of inflammogen). "Saline" group represents the histological aspect of the left paws treated only with 0.9% saline after the inflammation induction. "Indomethacin" group represents the histological aspect of the left paws treated with the positive control (indomethacin drug) after the inflammation induction. "BFO 50, 100, and 200 mg/Kg" groups represent the histological aspect of the left paws treated with bullfrog oil (BFO) at different concentrations after the inflammation induction. Black arrows indicate the extracellular spaces.



**Table 1** Inhibition (%) of paw edema in rats after oral pre-treatment with indomethacin and bullfrog oil at 50, 100 and 200 mg/kg from 1 to 4 hours after carrageenan-induced inflammation.

Treatment	Dose (mg/kg)	Inhibition (%)			
		1 h	2 h	3 h	4 h
Indomethacin	10	73.3 ± 10.0***	71.8 ± 8.1***	60.7 ± 7.9***	54.3 ± 15.1**
Bullfrog oil	50	66.8 ± 15.0**	54.9 ± 14.1***	44.8 ± 15.2**	66.3 ± 9.1***
Bullfrog oil	100	48.8 ± 7.8*	53.6 ± 9.7***	63.6 ± 7.0***	49.9 ± 7.2*
Bullfrog oil	200	100.0 ± 0.0***	79.0 ± 6.4***	49.4 ± 5.2***	56.2 ± 11.6**

Data show the mean ± SEM (n=5/group). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , when compared to the control group using the two-way ANOVA followed by the Bonferroni's test.



**Fig. 3** Evaluation of the *in vitro* anti-inflammatory pathway of bullfrog oil. A: Nitric oxide dosage after 24 hours of treatment; B: Nitric oxide dosage after 48 hours of treatment; C: IL-6 dosage after 48 hours of treatment; D: TNF dosage after 48 hours of treatment. BFO: Bullfrog oil; BFO-CV: a blend of bullfrog oil and Cetiol® V. \* Significant statistical difference ( $p < 0.05$ ) when compared to the control group (cells treated only with culture media).

and 100% against all tested samples, highlighting the absence of BFO cytotoxicity against this macrophage cell line. Similar results were observed for both BFO-CV and Cetiol® V, suggesting that both BFO and Cetiol® V are non-cytotoxic bioactive oil and vehicle, respectively.

### 3.2 *In vivo* assay

The BFO anti-inflammatory effect was initially evaluated by the *in vivo* induced-carrageenan edema in Wistar rats. The sub-plantar injection of carrageenan induced a gradual increase in the paw thickness of the control group. The effect was evident from the first hour after the inflammation induction, peaked around the third hour, and persisted over 4 h (Fig. 2). In addition, the treatment with indomethacin (control anti-inflammatory drug) showed a significant inhibitory effect on the paw edema (inhibition: 54.3 ± 15.1%,  $p < 0.001$ ) (Table 1).

Additionally, as it can be seen in Table 1, the BFO at all

tested concentrations produced significant anti-inflammatory effect after the first hour following the inflammogen stimulus, with inhibition ranging up to 100% (200 mg/kg, first hour,  $p < 0.001$ ). Histological examinations of the right paws demonstrated that the animals injected with carrageenan and treated with saline exhibited intense leukocyte infiltration, vasodilatation and edema, characteristic of the inflammatory reaction (Fig. 2). After treatment with indomethacin or BFO, the inflammatory parameters were reduced, especially a marked reduction in the cell recruitment at the inflammation site (Fig. 2).

### 3.3 Investigation of the *in vitro* bullfrog oil anti-inflammatory pathway

The BFO did not present cytotoxicity against macrophage cells (Raw 264.7) according to the MTT assay and showed a remarkable *in vivo* anti-inflammatory effect, demonstrated by the edema reduction in rats. Based on

this, its modulation on inflammatory pathways was investigated by the *in vitro* evaluation of nitric oxide (NO) production, and IL-6 and TNF levels.

**Figure 3A** shows the NO levels of BFO and BFO-CV after 24 hours of treatment. It is possible to notice that the BFO at  $50 \mu\text{g.mL}^{-1}$  promoted a significant increase ( $p < 0.05$ ) of 20% in the NO level, while this sample at 100 and  $200 \mu\text{g.mL}^{-1}$  and all tested concentrations of BFO-CV were not able to interfere in the NO levels. On the other hand, after 48 hours of treatment (**Fig. 3B**) the BFO at  $50 \mu\text{g.mL}^{-1}$  did not change the NO levels compared to its effect at 24 hours of treatment, while all other samples promoted a decrease in the NO levels, as it can be seen for BFO at  $100 \mu\text{g.mL}^{-1}$  ( $14 \pm 8\%$ ) and for BFO at  $200 \mu\text{g.mL}^{-1}$  ( $28 \pm 12\%$ ). Similarly, BFO-CV at 100 and  $200 \mu\text{g.mL}^{-1}$  displayed a significant decrease, in the NO levels, ( $p < 0.05$ ) of  $25 \pm 11\%$  and  $35 \pm 1\%$ , respectively, suggesting an interference on the inflammatory process.

Additionally, further investigations were performed in order to evaluate the BFO effect on possible inflammatory pathways by the dosage of IL-6 and TNF, which are mediators responsible for the cellular interaction regulation in the inflammatory process<sup>17</sup>. **Figure 3C** shows an significant increase ( $p < 0.05$ ) on the IL-6 production for BFO and BFO-CV at  $50 \mu\text{g.mL}^{-1}$ , corroborating the data from the NO dosage, in which, at this concentration, an immunostimulatory effect was observed due to the increase on the NO production. On the other hand, BFO at high concentrations decreased the IL-6 levels up to 12% while BFO-CV potentiated the BFO activity, showing an IL-6 dose-dependent inhibitory effect, promoting a significant decrease ( $p < 0.05$ ) in the IL-6 levels in 20% and 40% at  $100 \mu\text{g.mL}^{-1}$  and  $200 \mu\text{g.mL}^{-1}$ , respectively.

Accordingly, the TNF levels were also assessed and the results show that only the BFO samples were able to promote a significant decrease ( $p < 0.05$ ) in the TNF levels (up to 12%) at all tested concentrations, while BFO-CV decreased the TNF levels at approximately 5% (**Fig. 3D**). Hence, these data allow us to suggest that the BFO is able to reduce the NO synthesis by the inhibition of the TNF production, promoting the attenuation of the inflammatory process<sup>18, 19</sup>.

## 4 Discussion

### 4.1 *In vitro* cytotoxicity evaluation

The BFO is an animal oil that presents a promising anti-inflammatory activity based on its chemical composition. Previous to the investigation of its pharmacological activity, the BFO cytotoxicity was evaluated by the *in vitro* MTT assay. The results showed that both BFO and BFO-CV were not able to decrease the cell viability at all tested times and concentrations, suggesting an absence of toxicity

of these products against macrophages (Raw 264.7). These data are in accordance to previous literature reports regarding the cytotoxicity evaluation of BFO against an epithelial cell lineage (3T3) and a macrophage cell lineage (Raw 264.7)<sup>6, 29</sup>. These reports revealed that both BFO and its emulsified system, at concentrations up to  $100 \mu\text{g.mL}^{-1}$ , did not show cytotoxicity up to 72 hours of treatment against these cell lineages. Therefore, the obtained data in this work allow us to highlight the BFO as a non-toxic bioactive oil as well as its potential as an important raw material for pharmaceutical product development.

### 4.2 *In vivo* assay

The rat paw edema induced by the injection of different inflammogens is one of the most used models for anti-inflammatory activity evaluation of new drugs and natural products. Among these inflammogens, the carrageenan is an inflammatory inductor made of a complex group of galactose-related polysaccharides<sup>20, 21</sup>.

Carrageenan acts in a biphasic behavior: (i) the vascular phase (from 1 to 2 h), in which the induction of histamine, serotonin and bradykinin release is observed, producing a rapid increase in the vascular permeability with consequent edema formation, and (ii) the cellular phase (after three hours of administration), wherein the prostaglandins are produced, allowing the maintenance of the edema due to their potent vasoactive action and the recruitment of inflammatory cells<sup>15, 20, 22</sup>. Therefore, as it happened in this study, all signs of inflammation (edema, hyperalgesia, and erythema) were developed immediately after sub-plantar injection, as a result of the action of several pro-inflammatory agents<sup>22, 23</sup>.

Furthermore, indomethacin was used in this study as the anti-inflammatory control drug due to its ability to decrease the prostaglandins production and attenuate the signs of inflammation and edema, as confirmed by the  $53.3 \pm 15.3\%$  of edema reduction (**Table 1**). Additionally, the use of saline as negative control revealed that the untreated rat paw presented an edema formation characterized by the vasodilatation process, as consequence of the first phase of the inflammation process, as previously discussed. The histopathological aspect of the edema is represented by the clear spaces between cells in the tissue slide and confirmed by the migration of the leukocytes, mainly neutrophils, as corroborated by Sadeghi *et al.*<sup>24</sup> and Makni<sup>25</sup>.

Furthermore, the BFO anti-inflammatory activity was assessed by the above-mentioned model and evaluated by both the macroscopic analysis of the edema progression and the histopathological analysis. In **Fig. 2** (BFO at all concentrations) it is possible to observe a reduction in the extracellular space (indicated by the black arrows in the images with 400x magnification) caused by the edema after BFO treatment, which was reinforced by the paw edema reduction promoted by the BFO, as showed in the **Table 1**.

This result can be related to the BFO chemical composition. In fact, as already reported by previous chemical characterization studies, the BFO is rich in fatty acids, especially oleic, linolenic, stearic, palmitic and myristic acids in addition to EPA and DHA<sup>7, 8)</sup>. Such compounds may be used as anti-inflammatory agents, especially the *n*-3 fatty acids<sup>26)</sup>. All these substances act not only in the reduction of the pro inflammatory compounds such as NO, IL-6 and TNF, but also in the reduction of the expression of adhesion molecules and major histocompatibility complexes (MHC)<sup>27)</sup>.

These results are in agreement with the work from Arruda *et al.*<sup>28)</sup>. This study performed an investigation regarding the anti-inflammatory activity of fish oil by *in vivo* injection of carrageenan in rats using a single dose of oil as treatment. As the BFO, the fish oil has a substantial amount of *n*-3 polyunsaturated fatty acids (PUFAs), such as DHA and EPA. In accordance to our results, Arruda *et al.*<sup>28)</sup> also attributed the *in vivo* anti-inflammatory activity of the oil to the effect of the *n*-3 fatty acids on the production and/or release of inflammatory mediators and edema decrease<sup>28)</sup>.

#### 4.3 Investigation of the *in vitro* bullfrog oil anti-inflammatory pathway

Based on the *in vivo* results, it is possible to suggest the use of BFO as an important raw material able to decrease the inflammation signs after intragastric administration in rats. To this regard, its inflammatory pathway was investigated by the *in vitro* evaluation of inflammatory mediators such as NO, IL-6 and TNF levels.

The nitric oxide (NO) level released by the Raw 264.7 cells was measured in order to evaluate the BFO potential in modulating the inflammatory process and to clarify the pathways involved on the BFO anti-inflammatory activity evidenced by the *in vivo* assay. Indeed, the NO is defined as an endogenous free radical produced by the macrophages during the inflammatory response due to the membrane receptors activation after contact with bacterial components (poly and lipopolisaccharides). These bacterial components interact with macrophages receptors promoting an intracellular signaling cascade that activates the NO synthase enzyme (NOS) and, thus, enables the production of NO from the L-arginine<sup>30, 31)</sup>. To this regard, the NO has an important role on the inflammation and infectious processes, once it induces lipid peroxidation, DNA damage and protein oxidation because of its reaction with other free radicals (reactive oxygen species – ROS and/or hydrogen peroxide – H<sub>2</sub>O<sub>2</sub>) during the host defense<sup>32)</sup>. In addition, the excessive NO production in the inflammatory process is responsible for promoting pathogen cell damage. Nonetheless, high levels of NO may also promote deleterious effects in the immune system cells as in the proteins of the cellular matrix, leading to tissue damage and a pro-inflammatory effect<sup>32, 33)</sup>.

flammatory effect<sup>32, 33)</sup>.

The data obtained after 24 hours of treatment suggest that the BFO at 50 µg.mL<sup>-1</sup> induces the NO production, acting as a pro inflammatory agent. However, because the BFO-CV and the Cetiol® V itself did not promote changes in the NO levels, it is possible to infer that the pro-inflammatory response of BFO at 50 µg.mL<sup>-1</sup> can be related to the ratio of the chemical compounds from the BFO, as well as their concentration in the cellular medium. This result suggests that, at the aforementioned concentration of BFO, no anti-inflammatory fatty acids exists in the medium to prevent the NO production, favoring the inflammatory response<sup>33, 34)</sup>.

On the other hand, after 48 hours of treatment, a significant decrease ( $p < 0.05$ ) in the NO levels was observed in all samples, except for the BFO at 50 µg.mL<sup>-1</sup>, suggesting that this oil was able to promote an anti-inflammatory effect by the NO level reduction. In addition, it was also possible to observe that BFO-CV at 50 µg.mL<sup>-1</sup> had an anti-inflammatory activity statistically different ( $p < 0.05$ ) from the BFO itself. This can be attributed to the Cetiol® V chemical composition, since this oily vehicle is made of an ester derived from the oleic acid, a fatty acid able to promote anti-inflammatory synergic effect<sup>35)</sup> and thus, enhances the BFO anti-inflammatory activity. Additionally, studies that performed *in vitro* anti-inflammatory evaluation of natural oils obtained from animal sources reported that chemical compounds such as palmitic, oleic, stearic and arachidonic fatty acids (also identified in the BFO chemical composition) are directly involved in the inflammatory process<sup>36)</sup>. Therefore, it is possible to suggest that the BFO modulates the inflammatory pathway related to the NO production.

Moreover, two pro-inflammatory cytokines (IL-6 and TNF) were measured in order to improve the evaluation of the BFO effect on the inflammatory pathway. The IL-6 levels obtained after cell treatments at the lower concentrations of BFO and BFO-CV demonstrated that both products stimulated the IL-6 production. These data were in accordance to the NO level results and can be attributed to the pro-inflammatory compounds (arachidonic and docosahexaenoic fatty acids) from the BFO in the cell medium. Indeed, these compounds can modulate the inflammatory response, resulting on eicosanoids and cytokine synthesis<sup>37)</sup>. On the other hand, the highest tested BFO concentrations were able to reduce the IL-6 levels, showing that both samples have an anti-inflammatory response. IL-6 cytokine is a pro-inflammatory compound of the inflammation acute phase, responsible for activating the T-helper cells, for inhibiting the T regulatory cells, and for assisting the modulation of the adaptive immunological response<sup>38)</sup>. It is also found at high levels in chronic inflammatory processes such as rheumatoid arthritis, osteoporosis, Crohn's disease and psoriasis<sup>17, 38)</sup>. In fact, the decrease on the IL-6 levels promoted by the BFO and the BFO-CV at the highest

tested concentrations allow us to suggest a dose-dependent bullfrog oil anti-inflammatory effect.

Finally, the TNF dosage results revealed that all samples were able to decrease more than 10% of the TNF levels released by the macrophage cells at all tested concentrations. Indeed, TNF is a pro inflammatory mediator produced by immune cells, such as macrophages, T-lymphocytes and NK-cells, during the inflammatory process. Therefore, TNF is related to the NO synthesis, which is responsible for modulating the inflammatory response<sup>18)</sup>.

The TNF decrease observed after BFO treatment may also be attributed to the saturated and unsaturated fatty acids, such as the oleic, linoleic and docosahexaenoic acids (existing on the BFO chemical composition), which can inhibit the effect of the pro-inflammatory compounds<sup>37, 39)</sup>. Indeed, studies that performed the anti-inflammatory evaluation of isolated *n*-3 polyunsaturated fatty acids showed that these compounds were able to reduce the gene expression of IL-6, TNF and IL-1 $\beta$ <sup>39)</sup>.

Hence, it is possible to suggest that the BFO anti-inflammatory effect can be associated to the decrease in the NO, IL-6 and TNF levels, promoted by the *n*-3 PUFAs presented in BFO chemical composition. Moreover, the *in vitro* results corroborate the obtained data from the *in vivo* assay, since the reduction of NO, IL-6 and TNF levels were directly related to the decrease of the edema and neutrophils infiltration.

## 5 Conclusion

This study provided unprecedented scientific evidence of the inflammatory pathways involved on the effect of BFO in reducing the inflammation in a rat model. The obtained data from the *in vivo* and *in vitro* studies corroborate themselves, allowing us to suggest that the BFO anti-inflammatory activity has a direct correlation to the decrease of the inflammation mediators such as NO, IL-6 and TNF. These results could rationalize the traditional medicine use of this product in the treatment of inflammation and related diseases. This study also highlights the promising potential of BFO as it becomes an oil of interest for the development of pharmaceutical products with anti-inflammatory activities. In addition, since BFO is extracted from the adipose tissue of the *Rana catesbeiana* Shaw, a bio-product of the food industry, it can be inferred that this product could be exploited as a potential source of natural pharmacological agents, thereby, promoting the added-value to this oil.

## Acknowledgements

We gratefully acknowledge the assistance and coopera-

tion of the Pharmacy and Medicinal Surgery departments. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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