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Potential anti-inflammatory effect of LQFM-021 in carrageenan-induced inflammation: The role of nitric oxide

Iziara F. Florentino [a, *], Daiany P.B. Silva [a], Dayane M. Silva [a], Carina Sofia Cardoso [a], André L.E. Moreira [b], Clayton L. Borges [b], Célia M. de A. Soares [b], Pablinny M. Galdino [c], Luciano M. Lião [d], Paulo C. Ghedini [a], Ricardo Menegatti [e], Elson A. Costa [a]

[a] Institute of Biological Sciences, Department of Pharmacology, Federal University of Goiás, Campus Samambaia, Goiânia, GO, Brazil
[b] Institute of Biological Sciences, Department of Biochemistry and Molecular Biology, Federal University of Goiás, Campus Samambaia, Goiânia, GO, Brazil
[c] Center of Biological Sciences and Health, Federal University of Western Bahia, Barreiras, BA, Brazil
[d] Chemistry Institute, Federal University of Goiás, Campus Samambaia, Goiânia, GO, Brazil
[e] Faculty of Pharmacy, Laboratory of Medicinal Pharmaceutical Chemistry, Federal University of Goiás, Goiânia, GO, Brazil

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ABSTRACT

The pyrazole compound LQFM-021 exhibits vasorelaxant, antinociceptive and anti-inflammatory activities. Furthermore, it has low toxicity, indicating that this compound may be considered to be a good prototype for the development of new analgesic/anti-inflammatory drugs. Therefore, the aim of this study was to investigate the potential anti-inflammatory activity of LQFM-021 using a model of carrageenan-induced inflammation as well as the mechanism of action and role of nitric oxide in this effect. Acute treatments with LQFM-021 (30 and 60 mg/kg p.o.) reduced paw edema formation dose-dependently 2 h after carrageenan injection. In the carrageenan-induced pleurisy test, LQFM-021 (30 mg/kg p.o.) reduced the leukocyte (polymorphonuclear) count in the pleural cavity, as well as decreased protein extravasation and myeloperoxidase activity. This dose of LQFM-021 increased the NO (nitrite/nitrate) and IL-4 levels and decreased the TNF-α and IL-1β levels in the pleural cavity. Moreover, pre-treatment with L-NAME reversed the effect of LQFM-021 on NO, leukocyte migration, and the TNF-α and IL-1β levels. Additionally, we observed that LQFM-021 showed weak inhibitory activity on cyclooxygenases, but reduced the PGE_2 levels in the pleural cavity. Immunoblot analyses showed that LQFM-021 promoted a decrease in COX-2 levels and increase in iNOS levels. In conclusion, we demonstrated that LQFM-021 has marked anti-inflammatory activity by reducing polymorphonuclear recruitment, which is associated with the inhibition of the production of inflammatory cytokines and eicosanoids. In addition, we found that the synthase/release of nitric oxide promoted by LQFM-021 is essential for the anti-inflammatory effect observed.

1. Introduction

Inflammation is a protective attempt by an organism to remove injurious stimuli and initiate the healing process. It is a complex response involving biochemical as well as immunological factors [1]. An important characteristic of inflammation is that it occurs in a sequence of steps marked by the migration of leukocytes from the blood to tissues [2]. Among the produced inflammatory mediators, tumor necrosis factor-a (TNF-a) and interleukin-1b (IL-1b) activate vascular endothelial cells and up-regulate adhesion molecules that mediate the rolling, cell adhesion, and extravasation of leukocytes to the site of tissue inflammation [3,4].

Another important factor involved in many cellular responses and the pathophysiology of inflammation is prostaglandin biosynthesis and release [5]. Classical non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used for the treatment of inflammatory conditions. The anti-inflammatory effects of NSAIDs are mainly due to their ability to inhibit cyclooxygenases (COX-1

* Corresponding author. Laboratório de Farmacologia de Produtos Naturais e Sintéticos – ICB-2, Sala 216, Universidade Federal de Goiás, CEP 74001-970, Goiânia, GO, Brazil.
E-mail address: iziara_bia@hotmail.com (I.F. Florentino).

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and COX-2), which are key enzymes involved in prostaglandin biosynthesis [6].

However, long-term ingestion of NSAIDs is associated with serious side effects, such as gastrointestinal ulcers, bleeding and, less frequently, kidney and liver damage, mainly by COX-1 inhibition [7]. To reduce the incidence of gastrointestinal toxicity, several highly selective COX-2 inhibitors have been developed since the 90s. Studies have demonstrated their association with significant cardiovascular side effects, leading to the reconsideration of their use [8]. Hence, drugs with moderate selectivity for COX-1/COX-2 were efficient and reduced the risk of both cardiac and gastrointestinal side-effects [9].

Therefore, there is a constant need for the discovery of novel and safer anti-inflammatory drugs. The pyrazole ring, bearing atoms of at least two different elements as a member of its ring, is considered to be a privileged structure and an attractive scaffold for drug discovery [10,11]. Pyrazole derivatives represent one of the most active classes of compounds and exhibit a broad spectrum of pharmacological activities, such as antimicrobial [12,13], analgesic [14,15], anti-inflammatory [16–18], antipyretic [15,19], cardiovascular [20] and anticancer [21,22] activities, among others effects.

Recently, some of the pharmacological activities of a new pyrazole compound, 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole, also named LQFM-021, have been tested and provided convincing evidence of its vasorelaxant, analgesic and anti-inflammatory activities; this compound also has low toxicity [23–25]. The mechanisms of action of the antinociceptive activity of LQFM-021 involve the activation of peripheral opioid receptors and the NO/cGMP/K_{ATP} pathway [24]. Furthermore, these effects occurred in the absence of apparent toxic effects, indicating that the pyrazole compound LQFM-021 may be a good prototype for the development of new analgesic/anti-inflammatory drugs.

Nitric oxide (NO) is a small signaling molecule that can diffuse into the cell and is synthesized through enzymatic and nonenzymatic pathways. This diffusible gas is produced by the conversion of *L*-arginine to *L*-citrulline by three distinct isoforms of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [26]. Several biological actions have been reflected in many publications on NO in the last few years. Despite this extensive research on inflammation, the role of NO in inflammation remains ambiguous because NO is reported to have both anti- and pro-inflammatory properties [27].

NO released during the inflammatory process can down-regulate the migration of neutrophils to inflammatory sites due to the decreased rolling and adhesion of neutrophils on the endothelium and also the induction of apoptosis in migrated neutrophils [2,28]. Some studies have observed that inhibitors of NOS increase neutrophil adhesion to endothelial cells [29,30], while NO donors decrease both adhesion and leukocyte transmigration to inflammatory sites [30,31]. In addition, some studies have shown that the molecular strategy of coupled NO donors in anti-inflammatory drugs increases the safety and therapeutic efficacy of these classical drugs [7,32–34]. The development of nitric oxide (NO)-releasing anti-inflammatory drugs has highlighted the important therapeutic benefits of modulating NO pathways.

2. Based on the studies mentioned above, the carrageenan-induced inflammation models are suitable for studying the relationships between migrating cells, exudates, NO, pro-inflammatory and cytokines [30], as well as the reported anti-inflammatory activity of LQFM-021. Therefore, the aim of this study was to evaluate the potential anti-inflammatory effects of LQFM-021 using an acute model of carrageenan-induced inflammation in mice. Additionally, we investigated the mechanism and role of nitric oxide on these effects.

2. Material and methods

2.1. Structure of LQFM-021

Compound 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-021) was synthesized by "Laboratório de Química Farmacêutica Medicinal" (LQFM), Faculty of Pharmacy, Federal University of Goias, according to the synthetic route described by Martins et al., [23]. The molecular weight of the compound is 230.07, and the chemical structure is shown in Fig. 1.

2.2. Drugs and chemicals

Ammonium format (Sigma-Aldrich St. Louis, MO, USA); Bradford reagent (Sigma-Aldrich St. Louis, MO, USA); Carrageenan (Sigma-Aldrich St. Louis, MO, USA); Dexamethasone (Decadron®, Ache, SP, Brazil); DMSO (Sigma-Aldrich St. Louis, MO, USA); Evan's blue (Merck, USA); Griess reagent (Acrôs organics, NJ, EUA); Heparin (Cristália, SP, Brazil); Hydrogen peroxide (Bioshop, GO, Brazil); Indomethacin (Indocid®, Merck Sharp & Dohme Farmacêutica-Ltda); *L*(+)- arginine (Acrôs organics, NJ, EUA); NG-nitro-*L*-arginine methyl ester (L-NAME) (Cayman Chemical Company, Ann Arbor, MI, USA); Nitrocellulose membranes (Ge Healthcare®, NJ, USA); *o*-dianisidine (Sigma-Aldrich St. Louis, MO, USA); Primary antibody by β-actin, COX-2 and iNOS (Sigma-Aldrich St. Louis, MO, USA and Cayman Chemical Company, Ann Arbor, MI, USA); Secondary antibody (Sigma-Aldrich St. Louis, MO, USA); Sodium azide (Sigma-Aldrich St. Louis, MO, USA); Türk liquid (Bioshop, GO, Brazil); Zinc sulfate (Sigma-Aldrich St. Louis, MO, USA); BCIP/NDT (Sigma-Aldrich St. Louis, MO, USA); LQFM-021 was dissolved in 10% (w/v) DMSO in saline, and all other drugs were dissolved in 0.9% (w/v) saline.

2.3. Animals

All experiments were performed with adult male *Swiss* albino mice (weighing 27–32 g) obtained from the Central Animal House

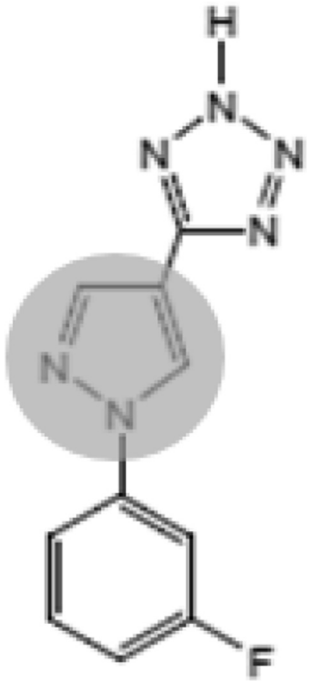

Fig. 1. Chemical structure of pyrazole compound LQFM-021: 5-(1-(3-Fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole. The molecular weight this compound is 230.07 and the pyrazole ring was marked.

of UFG. The animals were maintained at constant room temperature (22° ± 2 °C) under a 12-h light/dark cycle with free access to standard food and water. The animals were acclimatized for 7 days before the start of the experiments. Experimental groups of mice (n = 8) were use in this study. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal Experimentation and was approved by the Ethics Committee in Research of UFG (number 17/13).

2.4. Carrageenan-induced inflammation tests

2.4.1. Carrageenan-induced paw edema

The paw edema test was performed as described previously by Passos et al. [35]. The experimental groups of mice (n = 8) were treated with vehicle (10% (v/v) DMSO, 10 mL/kg p.o.), LQFM-021 (15, 30 and 60 mg/kg, p.o.), or indomethacin (10 mg/kg, p.o.) 1 h before injection of 50 μL of 1% (w/v) carrageenan in the right paw. The left paw, which received the same volume of a 0.9% (w/v) NaCl solution, was used as the control. Next, edema was measured by the difference in the volume between the paws (paw with carrageenan and saline). Edema was measured using a plethysmometer (Ugo Basile Co. - Italy) at different times (0, 1, 2, 3 and 4 h) after the injection of the phlogistic agent.

2.4.2. Carrageenan-induced pleurisy

The pleurisy test was performed as described previously by Lino et al. [36]. Each animal received 200 μL of Evan's blue (2.5 mg/mL) intravenously in 0.9% (w/v) saline 2 h after the experimental groups of mice (n = 8) were treated with vehicle (10% (v/v) DMSO, 10 mL/kg p.o.), LQFM-021 (30 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.). One hour after the treatments, animals received an injection of 100 μL of 1% carrageenan into the pleural cavity. The pleural exudate was collected with 1 mL of heparinized PBS 4 h after carrageenan administration. One aliquot was used to determine the total leukocyte content using Türk liquid in a Neubauer's chamber, and the other aliquot was used for the differential count of leukocyte mononuclear (MON) and polymorphonuclear (PMN) cells. The results of the total and differential counts were expressed as the absolute number of total cells and each type of cell, respectively, which was often more informative than their proportion. The final result was also adjusted according to the volume unit used in the performed evaluation and was expressed as the number of leukocytes × 10^6/mL. On the other hand, the other aliquot was used to determine the Evan's blue concentration using a spectrophotometer at 600 nm. The absorbance of the samples was compared with the standard curve of Evan's blue.

2.5. Analysis of the targets involved in the anti-inflammatory effect

2.5.1. Myeloperoxidase (MPO) assay

Measurement of the myeloperoxidase (MPO) activity was determined as described previously by Lino et al. [36]. Briefly, 20 μL of the pleural fluid samples was added to 360 μL of phosphate buffer pH 6.0 containing 0.167 mg/ml of *o*-dianisidine 2HCl and 0.0005% H_2O_2. The enzyme reaction was stopped after 15 min by adding 20 μL of 1% (w/v) sodium azide. The samples were centrifuged subsequently for 5 min at 300 g. The supernatant (100 μL) was transferred to a microplate well, and the absorbance was monitored at a wavelength of 450 nm.

2.5.2. Measurement of NO

Nitric oxide was measured as its breakdown products nitrite (NO_2^-) and nitrate (NO_3^-) using the *Griess* method. Briefly, pleural exudate samples of animals submitted to a pleurisy test were first deproteinated with 20% zinc sulfate and then centrifuged (3000 g, for 15 min). The supernatant was mixed with 1% ammonium format, sodium phosphate buffer, and a suspension of *Escherichia coli* (1:10). After 2 h of incubation, the samples were centrifuged (900 g, for 10 min). In duplicate, similar volumes of the supernatant and Griess reagent were added, and the absorbance at 490 nm was read after incubation for 10 min in a spectrophotometer. The absorbance of the samples was compared with that of the standard curve of sodium nitrite (0–20 μM), and the results are expressed in μM [36–38].

2.5.3. Measurement of cytokines

In another experiment, previously selected doses were used to determine the leukocyte count and to quantify the pro- and anti-inflammatory cytokines in the pleural exudate. Each group of mice (n = 8) was pre-treated with L-NAME (10 mg/kg, i.p. – NO synthase inhibitor) or saline (10 mL/kg i.p.); after 30 min, the animals were treated with vehicle (10 mL/kg p.o.), LQFM-021 (30 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.). The concentration of TNF-α, IL-1β and IL-4 in the pleural exudate samples was assessed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Ebioscience®). Samples were collected 4 h after the induction of pleurisy and centrifuged at 1200 g for 10 min at 4 °C, and the supernatant was separated and stored (−70 °C) until the assay [39].

2.5.4. Measurement of prostaglandin E_2 (PGE_2)

Each group of mice (n = 8) was pre-treated with *L*-name (10 mg/kg, i.p. – NO synthase inhibitor) or saline (10 mL/kg i.p.); after 30 min, the animals were treated with vehicle (10 mL/kg p.o.), LQFM-021 (30 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.). The concentration of PGE_2 in pleural exudate samples was determined by ELISA using a commercial kit (Cayman Chemical). Samples were collected 4 h after the induction of pleurisy, with PBS and 10 μM indomethacin, and then were centrifuged at 1200 g for 10 min at 4 °C, followed by separation and storage (−70 °C) of the supernatant until the assay. Then, the basal PGE_2 values were removed from all of the samples [40].

2.5.5. In vitro cyclooxygenase inhibition assay

The ability of LQFM-021 and indomethacin (positive control) to inhibit COX-1/COX-2 was determined using a colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical, Catalogue Nº. 760111) according to the protocol recommended by the supplier [41].

2.5.6. Immunoblot analysis

Western blot analysis was performed to evaluate the COX-2 and iNOS protein levels in pleural exudates of animals submitted to carrageenan-induced pleurisy, as described previously by McCartney-Francis et al. [39] and Hatanaka et al. [42], with slight modifications. We used the same groups as those in item 4.5.3. The protein concentration was determined by the Bradford protein assay using a standard curve of BCA and was subjected to western blot analysis. Each protein extract was separated on 12% SDS-PAGE gels and was stained with Coomassie brilliant blue or was transferred to nitrocellulose membranes (Ge Healthcare®). β-Actin (cytosolic protein) was used as the housekeeping endogenous control. The membranes were incubated in 0.05% (v/v) Tween-20 plus Tris-buffered saline (TBS) containing 1% (w/v) skim milk and then were incubated with primary antibodies to β-actin (1:5000 dilution), iNOS (1:200 dilution - Sigma Aldrich, Co., St. Louis, MO) and COX-2 (mouse) Polyclonal (1:200 dilution - Cayman Chemical Company, USA). The blots were washed (3 times) with 0.1% Tween 20/PBS-buffered saline. The antibody anti-mouse IgG coupled to

alkaline phosphatase was used as the secondary antibody (1:20000 dilution - Sigma Aldrich, Co., St. Louis, MO). Thereafter, the membranes were washed, and the reactions were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT). The protein levels were evaluated through densitometry, were quantified using Image-*J* Software and were expressed as arbitrary units of the ratio to β-actin.

2.6. Statistical analysis

All the results are expressed as the mean ± S.E.M. The data were analyzed statistically by one-way ANOVA followed by *Tukey's* test as post-hoc or by two-way ANOVA followed by *Bonferroni's* test as post-hoc. All statistical analyses were carried out using Graph Pad Prism version 5.00. Values of $P \leq 0.05$ were considered significant. Others information about statistical analyses are present in supplementary material.

3. Results

3.1. Carrageenan-induced paw edema

Treatments with LQFM-021 reduced paw edema formation from the 2nd hour in a dose-dependent manner. Over this time, compared with the control group (92.2 ± 4.3 μL), treatments with LQFM-021 (30 or 60 mg/kg p.o.) reduced edema by 25.3% ($P \leq 0.01$) and 33.7% ($P \leq 0.001$), respectively. After the 3rd hour, compared with the control group (82.2 ± 7.6 μL), the same doses reduced edema by 25.5% and 33.0% ($P \leq 0.01$), respectively. At the 4th hour, the control group 73.2 ± 6.9 μL reduced edema by 30.4% and 29.0% ($P \leq 0.05$), respectively. LQFM-021, at a dose of 15 mg/kg, did not reduce edema formation at any time. The group treated with indomethacin (10 mg/kg, p.o.), an anti-inflammatory positive control, decreased paw edema at all times after carrageenan injection (Fig. 2).

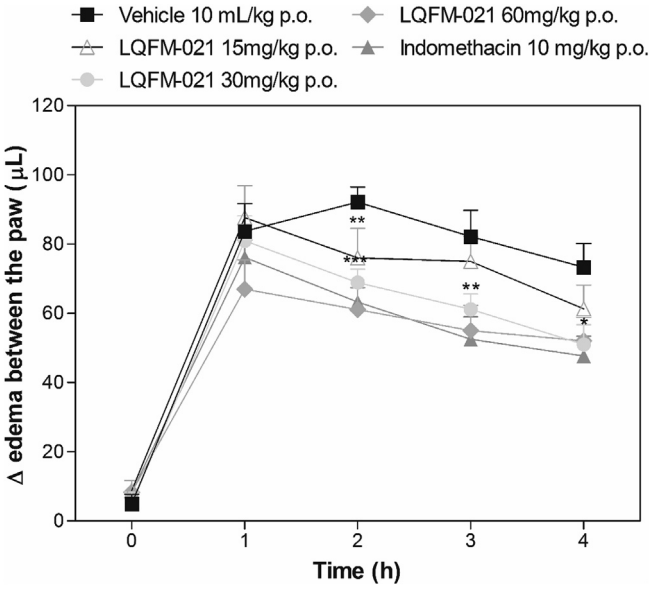

Fig. 2. Anti-edematogenic effect of LQFM-021 (15, 30, and 60 mg/kg, p.o.) and indomethacin (10 mg/kg, p. o. — positive control) on the carrageenan-induced edema test, in mice (n = 8). Treatment with LQFM-021 (30, and 60 mg/kg) began to reduce the edema starting from the 2nd hour until 4th hour. Treatments with indomethacin reduced the edema at all hours after the treatment and LQFM-021. The values were expressed as mean ± SEM of the difference between the paws, in μL. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, compared with control group, according to two-way ANOVA followed by *Bonferroni's* post-hoc test.

3.2. Carrageenan-induced pleurisy

The injection of carrageenan into the pleural cavity of mice induced an acute inflammatory response characterized by the accumulation of fluid containing a large number of leukocytes. Compared with the control group ($5.03 \pm 0.47 \times 10^6$/mL), treatment with LQFM-021 30 mg/kg and dexamethasone 2.0 mg/kg in the positive control group reduced the leukocyte count in the pleural cavity by 38.2% ($P \leq 0.01$) and 60.0% ($P \leq 0.001$), respectively. These reductions were due to a decrease in polymorphonuclear cells of 39.8% (Fig. 3A). We also observed that LQFM-021 reduced the Evan's blue concentration in the pleural exudates by 30.9% ($P \leq 0.01$) compared with that in the control group (4.56 ± 0.32 μg/mL), Fig. 3B. In addition, Fig. 3C shows that treatment with LQFM-021 decreased the myeloperoxidase activity by 43.0% ($P \leq 0.05$) compared with that in the control group (192.5 ± 23.5 mU/mL). Similar results were observed with the positive control dexamethasone.

3.3. NO pathway in inflammation

The involvement of the NO pathway in inflammation was analyzed with the pre-treatment of mice with L-NAME or NO synthase (NOS) inhibitor (10 mg/kg, given 15 min before the treatments). When L-NAME was administered per se, at the dose tested, it was did not product notably different results from the control group. This dose significantly reversed the reduced leukocyte count exhibited by LQFM-021 (30 mg/kg p.o.) in the pleural cavity.

Treatment with *L*-arginine, a nitric oxide precursor (100 mg/kg i.p.), reduced the leukocyte count in the pleural cavity similar to LQFM-021; this effect was reversed by pre-treatment with L-NAME (Fig. 4A). NO in the pleural exudate was analyzed using the Griess reaction. Treatments with LQFM-021 or *L*-arginine increased the nitrite/nitrate levels by 102% ($P \leq 0.01$) and 177% ($P \leq 0.001$), respectively, compared with those of the control group (5.68 ± 1.35 μM). However, pre-treatment with L-NAME also reversed the effect of both LQFM-021 and *L*-arginine on the NO levels (Fig. 4B).

3.4. Measurement of cytokines

In addition to the LQFM-021 effect on the leukocyte count, we observed that this compound also caused significant changes in the production/release of some anti- and pro-inflammatory cytokines. In our experiments, LQFM-021 30 mg/kg showed significant inhibition of TNF-α (% inhibition of 55.60, $P \leq 0.01$) and IL-1β (% inhibition of 26.38, $P \leq 0.05$). When L-NAME was administered per se, at the dose tested, it did not produce notably different results from the control group. This dose could significantly reverse the inhibitory effect of LQFM-021 on these cytokine levels (Fig. 5A–C). In this experiment, we observed that LQFM-021 increased the production of the levels of the anti-inflammatory cytokine IL-4 compared with the inflamed group (% increase in 84.68) ($P \leq 0.01$). However, pre-treatment with L-NAME increased this effect on IL-4 levels (Fig. 5D). As expected, dexamethasone showed the expected profile through inhibition of pro-inflammatory cytokines (% inhibition of TNF-α by 70.25 and IL-1β by 87.86, $P \leq 0.001$) and increased the levels of the anti-inflammatory cytokine IL-4 (% increase of 261.42, $P \leq 0.001$) (Fig. 5).

3.5. Measurement of prostaglandin E_2 (PGE_2)

PGE_2, a major product of the enzymatic reaction catalyzed by

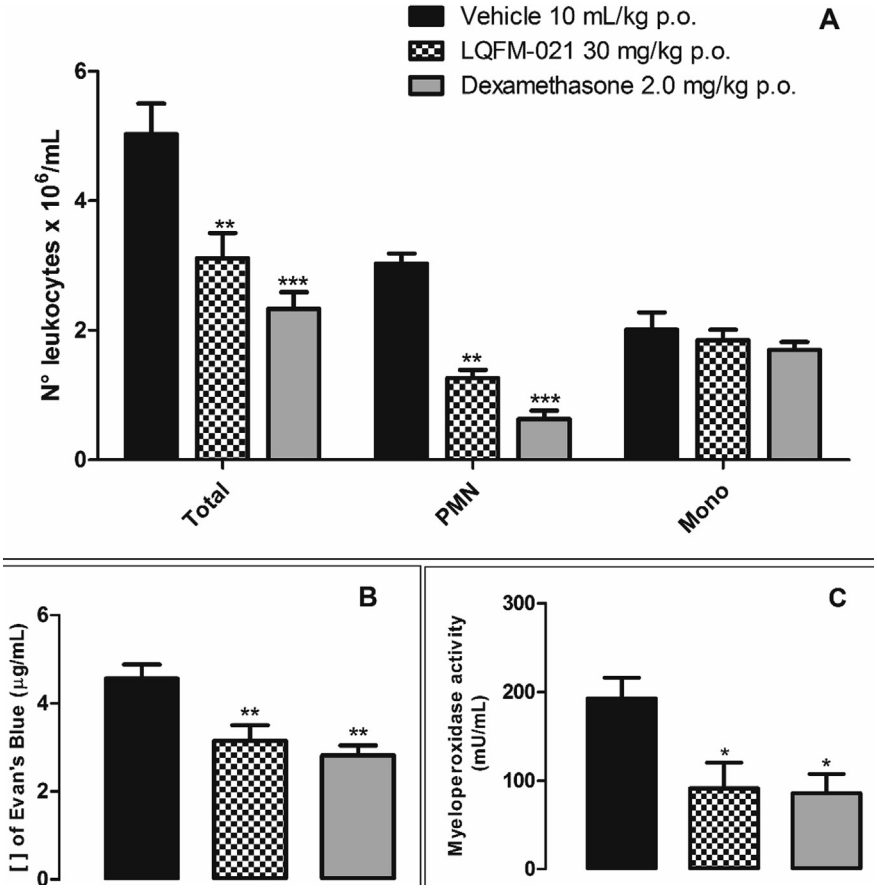

Fig. 3. Anti-inflammatory effect of LQFM-021 (30 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o. - positive control) on the carrageenan-induced pleurisy test, in mice. (A) Represent leukocytes count into pleural cavity (total leukocytes; PMN, polymorphonuclear; MON, mononuclear). (B) Represent Evan's blue concentration (µg/mL). (C) Myeloperoxidade enzyme activity (mU/mL). Values are expressed as mean ± SEM of 8 mice. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001, compared with control group, according to ANOVA followed by *Tukey's* test.

COX-2, was also analyzed. Compared with the control group (5.04 ± 0.50), treatment with LQFM-021 30 mg/kg showed significant inhibition of the PGE_2 levels (% inhibition of 57.7, P ≤ 0.001). Treatment with L-NAME did not produce notably different results from the control group, but significantly reversed the inhibitory effect of LQFM-021. Dexamethasone showed the expected profile and inhibition of PGE_2 levels (% inhibition 70.6, P ≤ 0.001). The basal value (180 pg/mL) was removed from all of the samples (Fig. 6).

3.6. In vitro cyclooxygenase inhibition assay

LQFM-021 inhibited both COX-1 and COX-2, with an IC50 of 3160 µM for COX-1 and an IC50 of 1520 µM for COX-2; the selectivity index (SI; COX-1 IC50/COX-2 IC50) was 2.08. However, compared with the classical inhibitor of COX, Indomethacin showed an IC50 of 14 µM for COX-1 and IC50 of 86.8 µM for COX-2 (SI 0.16), and we observed that LQFM-021 showed weak inhibitory activity on these enzymes (Table 1).

3.7. Immunoblot analysis

Furthermore, to continue the study of the possible anti-inflammatory targets of LQFM-21, cyclooxygenase (COX-2) and iNOS were quantified by western blotting. Interestingly, COX-2 protein expression was up-regulated by the injection of carrageenan compared to the control group (2.28 ± 0.15), and treatments with LQFM-021 and dexamethasone promoted down-regulation of this enzyme (decrease by 35.1% and 54.4%, respectively, P ≤ 0.05). Previous pre-treatment with L-NAME did not change this parameter, as shown in Fig. 7A. However, iNOS, which is primary responsible for the production of NO in inflammatory processes, was up-regulated by the injection of carrageenan (1.55 ± 0.03), and LQFM-021 increased this up-regulation (increase by 20%, P ≤ 0.05). L-NAME and dexamethasone significantly decreased these enzyme levels (decreases of 25.8 and 65.8%, respectively, P ≤ 0.05). These results indicated that the LQFM-021 increase of NO production is associated with the up-regulation of iNOS expression (Fig. 7B).

4. Discussion

Nitric oxide is a signaling molecule that is responsible for diverse physiological and pathophysiological processes. In this work, we present a prevailing hypothesis regarding the role of NO on the anti-inflammatory effect of LQFM-021. This hypothesis is based on the previous observation that the maintenance of the production of nitric oxide is very important for the antinociceptive effect of LQFM-021 [24]. Another important fact about this pyrazole compound is that sub-chronic administration induced anti-nociceptive and anti-inflammatory activities in CFA-induced arthritis, but did not induce alterations in the toxicology and maintenance of the gastric integrity [25]. In this study, we investigated the acute anti-inflammatory activity of LQFM-021, as well as the possible targets and role of nitric oxide in this activity, by

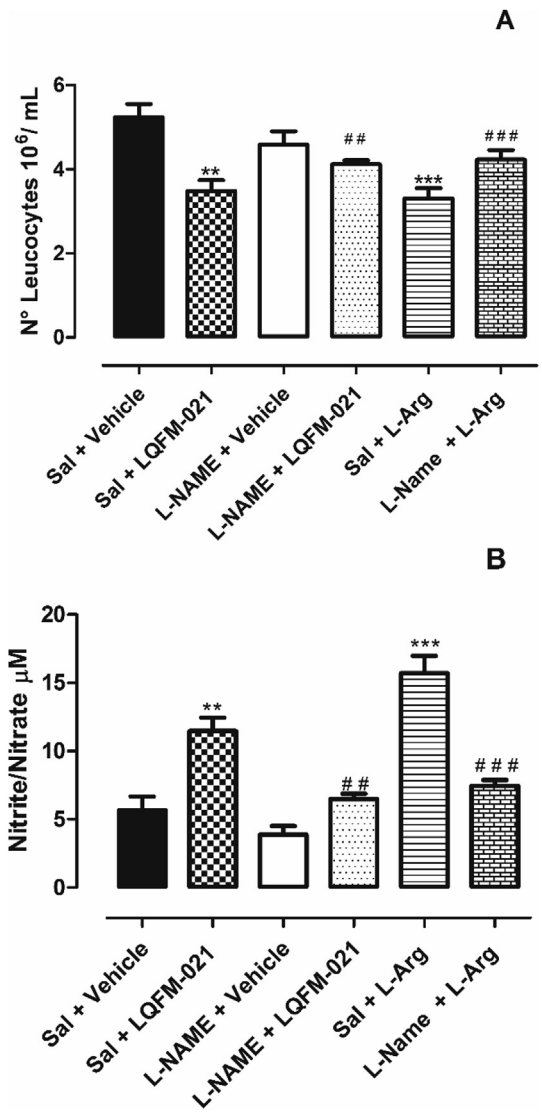

Fig. 4. Effect of pre-treatment with saline (10 mL/kg i.p.) or L-NAME (10 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg, p.o.) and L-arginine (100 mg/kg, p.o. - positive control) on leukocytes count and NO concentration in the pleural cavity. (A) Represent leukocytes count to pleural cavity. (B) Represent Nitrite/Nitrate µM measured by *Griess* method. Vertical bars represent mean ± SEM of 8 mice. **P = 0.01 and ***P = 0.001 (compared control group) and # #P = 0.01 and # # #P = 0.001 (compared with respectively treated group) according to ANOVA followed by post-hoc *Tukey's* test. Abbreviations: Sal: saline and L-Arg: L-arginine.

carrageenan-induced paw edema and pleurisy tests.

Injection of carrageenan into the plantar surface of the paw promotes a biphasic acute inflammatory response [43]. The first phase is mediated by the release of histamine and serotonin, followed by the subsequent release of bradykinin. The late edema phase is dependent on cytokine and prostaglandin production by resident cells and neutrophil infiltration [3]. Our results clearly demonstrated that LQFM-021 has an anti-inflammatory effect in acute models of inflammation when administered acutely. Administration of LQFM-021 (15, 30 and 60 mg/kg) induced inhibition of paw edema in a dose-dependent manner after the second hour. This protocol was initially chosen considering the treatment schedule with LQFM-021 used in our previous study [24].

Carrageenan-induced pleurisy is another model that is widely used to evaluate leukocyte recruitment as well as the production of mediators that play important roles in acute inflammation. In this test, the highest neutrophil accumulation was observed four hours after the injection of carrageenan, as demonstrated in the present study and supported by other studies [2,36,39]. The polymorphonuclear content induced by carrageenan was markedly inhibited by treatment with LQFM-021 at a dose of 30 mg/kg, the effective dose in paw edema. A similar result was observed with the same dose as that in a previous study on the CFA-induced arthritis model [25].

Myeloperoxidase (MPO) activity is commonly considered to be an indicator of neutrophil infiltration [36]. In the current assay, our results demonstrated that LQFM-021 inhibited the leukocyte content, especially polymorphonuclear cells, which was also evident from the reduced MPO activity, as measured in pleural cavity. This result suggests that the anti-inflammatory effect of LQFM-021 was related to inflammatory events involving neutrophil migration. Another signal of inflammation was the exudate concentration, which was also significantly reduced by treatment with LQFM-021. This result was in accordance with the effect observed in carrageenan-induced paw edema in another study [25]. In addition, LQFM-021 also showed anti-inflammatory activity in the croton oil-induced ear edema and CFA-induced paw edema, in mice (Supplementary material).

In this regard, several pyrazole-based agents have also been developed and demonstrated excellent anti-inflammatory activity [11]. The synthesized pyrazole derivatives increased inhibition of paw edema formation at 6 h to a higher degree than inhibition of COX-2 [44]. Another study conducted by our group showed that the pyrazole derivative FPPT reduced edema formation as well as the leukocyte content in carrageenan-induced paw edema and pleurisy with similar doses used in this study in rats [45].

During the inflammatory process, production and release of cytokines, chemokines, prostaglandins and nitric oxide occur, resulting in the down-modulation of leukocyte recruitment to the inflammatory site [3,28]. Therefore, in this study, we verified the influence of the NO pathway on the leukocyte migration induced by carrageenan. Our results demonstrated that treatment with LQFM-021 and *L*-arginine (nitric oxide precursor) decreased the leukocyte count and L-NAME at the dose used, but did not the leukocyte counts. However, this dose of L-NAME was sufficient to reverse the effects of both LQFM-021 and the *L*-arginine leukocyte counts in the pleural cavity. Regarding the evaluation of the NO (nitrite/nitrate) levels in the pleural exudate, we observed a similar effect with LQFM-021, and *L*-arginine increased the NO levels. Pre-treatment with L-NAME also reversed the effects of both. The results shown here suggest that NO increased by LQFM-021 modulated this anti-inflammatory effect.

The role of nitric oxide in inflammation is complex because it is thought to have both positive and negative effects. Some studies in the literature have shown that NO is a mediator that contributes to the inflammatory response, stimulating the production of inflammatory cytokines and peroxynitrite [46,47]. Although the beneficial effects of NO during inflammatory insult have also been reported [2,3,28], Iwata et al. [30] showed, in a carrageenan-induced pleurisy model, that administration of an NO donor or NO substrate (*L*-arginine) reduced the migration of inflammatory cells and edema formation, lowered oxidative stress, and normalized antioxidant enzyme activities. Other studies have suggested the importance of more research concerning the role of NO in osteoarthritis, as well as the use of NO-donating drugs to provide a new therapeutic option for the treatment of inflammatory diseases [48,49].

In addition, we evaluated LQFM-021's effects on the production of some cytokines and mediators that play important roles in neutrophil recruitment. We demonstrated that carrageenan administration increased the leukocyte count and the TNF-α and IL-1β levels in the pleural exudate. These cytokines were markedly

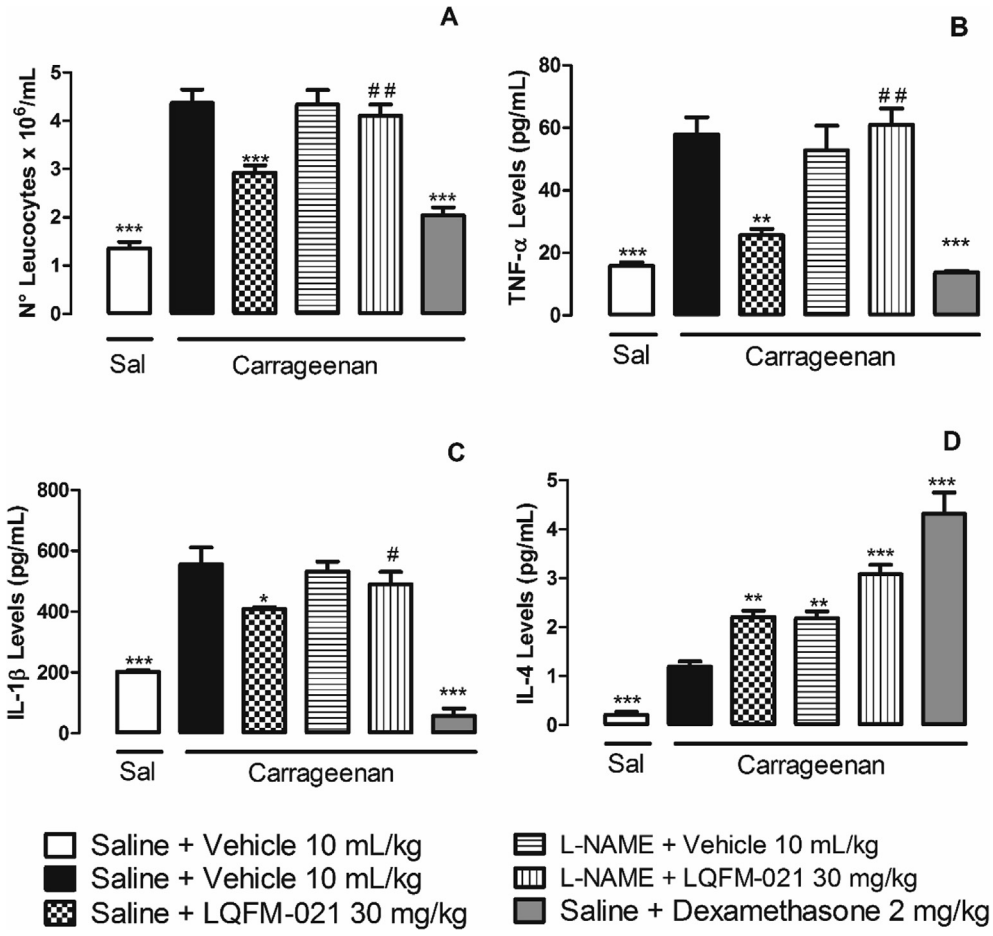

Fig. 5. Effect of pre-treatment with saline (10 mL/kg i.p.) or L-NAME (10 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o. - positive control) on leukocyte migration and cytokines levels. (A) Represent leukocyte migration to pleural cavity. (B) Represent tumor necrosis factor-alpha (TNF-a) levels. (C) Interleukin 1β (IL-1β) levels. (D) Interleukin 4 (IL-4) levels. The results were expressed in pg/mL. Vertical bars represent mean ± SEM of 8 mice. *P = 0.05, **P = 0.01 and ***P = 0.001 (compared control group - Saline + vehicle and received carrageenan) and #P = 0.05 e ##P = 0.01 (compared with respectively treated group) according to ANOVA followed by post-hoc *Tukey's* test.

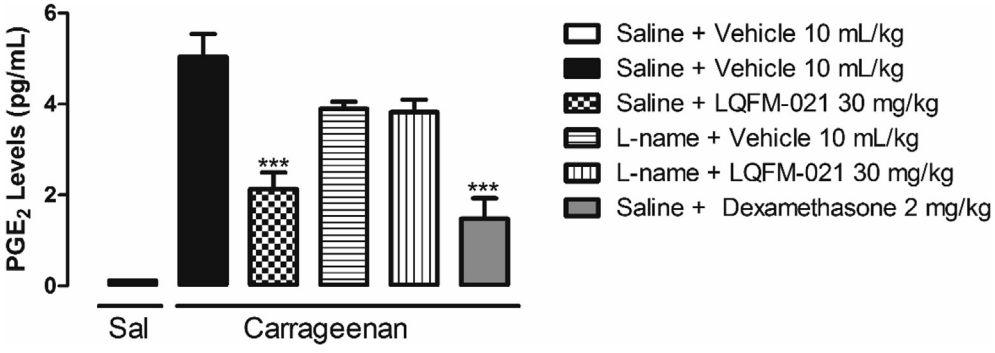

Fig. 6. Effect of pre-treatment with saline (10 mL/kg i.p.) or L-NAME (10 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o. - positive control) on PGE_2 levels. The results were expressed in pg/mL. Vertical bars represent mean ± SEM of 8 mice. **P = 0.01 and ***P = 0.001 (compared control group - Saline + vehicle and received carrageenan) and ##P = 0.01 (compared with respectively treated group) according to ANOVA followed by post-hoc *Tukey's* test.

reduced by treatment with LQFM-021, as well as the leukocyte count. It has been shown that the TNFα and IL-1β cytokines play important roles in leukocyte recruitment in the models of inflammation [50,51]. These cytokines enhanced the expression of several prostanoids that was blocked by COX-2-selective inhibitors [52]. We also observed that L-NAME per se, at the dose used, did not cause significant changes in the cytokine levels or leukocyte count; however, pretreatment with L-NAME reversed the LQFM-021 effects in these parameters.

In contrast to these results, the data obtained showed that both LQFM-021 and L-NAME produced an increase in the IL-4 Levels, but the association between LQFM-021 and L-NAME had a synergic effect, suggesting that this action may be independent of the release of nitric oxide at the inflammatory site. It is important to

Table 1
The in vitro test compound concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC50, μM) is the mean of two determinations acquired using an ovine COX-1/COX-2 assay Kit (Cayman Chemicals Inc., Ann Arbor, MI, USA). In vitro selectivity index (COX-1 IC50/COX-2 IC50).

Compound	IC50 COX-1 (μM)	IC50 COX-2 (μM)	COX-2 Selectivity Index (SI)
LQFM-021	3160	1520	2.08
Indomethacin	14	87	0.16

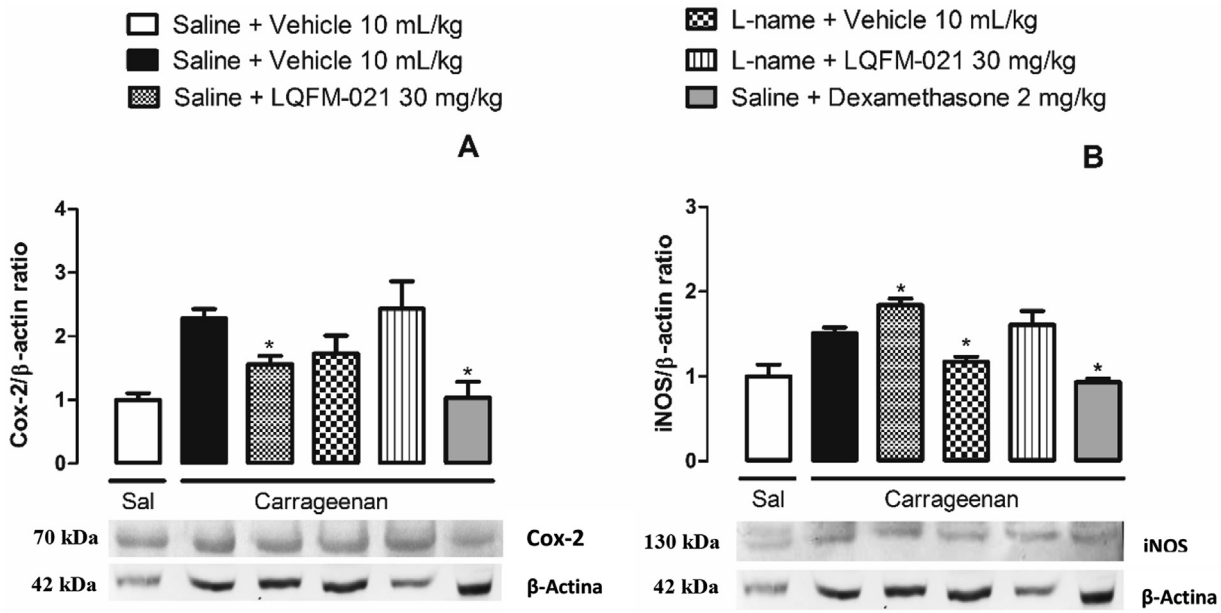

Fig. 7. Representative images of immunoblot analysis for cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) in the pleurisy test. The graphical shown the effect of pre-treatment with saline (10 mL/kg i.p.) or L-NAME (10 mg/kg i.p.) given 15 min prior to treatment with vehicle (10 mL/kg p.o.), LQFM-021 (30 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o. - positive control). Figure (A) Represent COX-2, (B) Represent iNOS. Protein levels were expressed in arbitrary units, as the ratio of signal intensity for the target protein relative to β-actin, which represented the relative values among all samples. Data are presented as mean ± SEM of groups of mice. *P ≤ 0.05 versus the control group (Saline + vehicle and received carrageenan), according to ANOVA followed by post-hoc *Tukey's* test.

consider that IL-4 promotes a decrease in the synthesis of inflammatory cytokines, such as IL-1β, TNFα, and IL-6, in the cell cultures of chondrocytes [53], as well as an anti-inflammatory effect in the course of osteoarthritis [51,54].

Other mediators that are clearly involved in the inflammatory reaction induced by carrageenan in our model are prostaglandins [55]. NSAIDs present anti-inflammatory effects that are likely to inhibit prostaglandin production by the blockade of the cyclooxygenase isoforms [5]. Therefore, it is possible that LQFM-021 inhibits COX-2 activity, contributing to the anti-inflammatory effect. Thus, the clinical efficacy of structurally distinct NSAIDs, all of which share this capacity to inhibit prostanoid production, points to the importance of these mediators in the promotion of inflammatory diseases [55].

In this study, we found that LQFM-021 reduced the PGE_2 levels in the pleural exudate. We also observed that this pyrazole compound promoted inhibition of the activities of both isoforms, COX-1 and COX-2, with a good selectivity index (COX-1/COX-2) of 2.08. Moreover, we concluded that LQFM-021 showed a weak ability to inhibit these enzymes due to the IC50 of this compound compared with the IC50 of indomethacin, a classical NSAID, or other pyrazole compounds that have an IC50 similar to that of indomethacin and other classical NSAIDs [11,34,56].

Additionally, immunoblot analyses showed that the COX-2 levels were increased after carrageenan administration and treatment with an anti-inflammatory dose of LQFM-021 promoted a decrease in the COX-2 levels. This fact may contribute to the effect found with the PGE_2 levels in the pleural exudate and their marked anti-inflammatory activity observed in this study, as well as the anti-inflammatory and antinociceptive effects in previous works [24,25].

The results found in immunoblot analyses with iNOS showed that LQFM-021 up-regulated these enzyme levels, justifying the effect on the NO levels. The search for mechanisms underlying LQFM-021-induced anti-inflammatory activity demonstrated in this study revealed that the inhibition of prostaglandin synthesis in inflamed tissue is not the only pathway for this response. This suggests a plausible link between this work and previous studies, where we showed that the antinociceptive mechanisms for LQFM-021 may also involve the nitric oxide (NO)/cGMP/K_{ATP} signaling pathway [24], as well as justified the effect of LQFM-021 on gastric mucosa protection in sub-chronic administration in rats [25]. Thus, we infer that nitric oxide plays an important role in the anti-inflammatory effect of LQFM-021 similar to other pharmacological effects observed with this pyrazole compound.

5. Conclusion

In summary, LQFM-021 showed marked anti-inflammatory activity in the acute models of paw edema and pleurisy induced by carrageenan in mice. This activity was characterized by the reduction of edema formation associated with the reduction of polymorphonuclear recruitment, besides inhibiting biochemical pathways with a crucial role in the development of the inflammatory response and modulating cytokines and prostaglandin synthesis/release. In addition, we observed that the release of nitric oxide promoted by LQFM-021 is essential for the anti-inflammatory effect.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.niox.2017.04.006.

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