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Anti-inflammatory and antinociceptive activity profile of a new lead compound – LQFM219

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ABSTRACT

LQFM219 is a molecule designed from celecoxibe (COX-2 inhibitor) and darbufelone (inhibitor of COX-2 and 5-LOX) lead compounds through a molecular hybridisation strategy. Therefore, this work aimed to investigate the antinociceptive and anti-inflammatory activities of this new hybrid compound. The acute oral systemic toxicity of LQFM219 was evaluated via the neutral red uptake assay. Acetic acid-induced abdominal writhing and CFA-induced mechanical hyperalgesia were performed to evaluate the antinociceptive activity, and the anti-oedematogenic activity was studied by CFA-induced paw oedema and croton oil-induced ear oedema. Moreover, the acute anti-inflammatory activity was determined by carrageenan-induced pleurisy. In addition, cell migration, myeloperoxidase enzyme activity, and TNF-α and IL-1β levels were determined in pleural exudate. Moreover, a redox assay was conducted using electroanalytical and DPPH methods. The results demonstrated that LQFM219 was classified as GHS category 4, and it showed better free radical scavenger activity compared to BHT. Besides, LQFM219 decreased the number of writhings induced by acetic acid and the response to the mechanical stimulus in the CFA-induced mechanical hyperalgesia test. Furthermore, LQFM219 reduced oedema formation, cell migration, and IL-1β and TNF-α levels in the pleural cavity and inhibited myeloperoxidase enzyme activity. Thus, our study provides that the new pyrazole derivative, LQFM219, demonstrated low toxicity, antinociceptive and anti-inflammatory potential *in vitro* and in vivo.

1. Introduction

Inflammation is a normal and essential response, ranging from a localized response to a generalized response, and is generally classified as acute or chronic depending on the cellular and molecular events involved [1,2]. The acute inflammatory response can be triggered by tissue damage and/or infection that is recognised by resident macrophages via receptors of the innate immune system, such as family of the Toll-like receptors (TLRs) [2]. This process is characterised by the production and release of pro-inflammatory mediators, such as bradykinin, prostaglandins (PGs), leukotrienes, and cytokines [3], and it occurs through the activation of a cascade of arachidonic acid via the action of cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes [4].

The beginning of the inflammatory process occurs through the delivery of serotonin and histamine, which is followed by the recruitment of polymorphonuclear (PMN) cells, among which neutrophils are the most abundant. These cells are important for the elimination of pathogens by releasing reactive oxygen species (ROS) and reactive nitrogen species present in their granules, as well as increasing the

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expression of myeloperoxidase (MPO), which is responsible for the oxidation of pathogens [5,6]. The elimination of the pathogen is followed by the phase of resolution and repair tissue by anti-inflammatory mediators produced by resident macrophages. If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires a chronic characteristic, requiring pharmacological intervention in many cases [7,8].

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most used drugs in clinics worldwide due to their anti-inflammatory, analgesic, and antipyretic properties. NSAIDs have a diversity of compounds, with varied chemical structures that share similar therapeutic and adverse effects. The main mechanism of action related to NSAIDs is the inhibition of the cyclooxygenase enzymes (COX-1 and COX-2), thus reducing the synthesis of PGs and thromboxane (TX). The prolonged use of this class of drugs can cause damage to the gastrointestinal, renal, and cardiovascular systems [9].

Based on this, this work aimed to design a new drug with anti-inflammatory and antioxidant properties, as well as improved safety and tolerability. We describe the synthesis and biological evaluation of LQFM219 (**3**), which was designed from celecoxibe (**1**) and darbufelone lead compounds through a molecular hybridisation strategy. Celecoxibe (**1**) is a COX-2 inhibitor, while darbufelone (**2**) is a dual inhibitor of COX-2 and 5-LOX. To design LQFM219 (**3**), A and B scaffolds from celecoxibe (**1**) and the C scaffold from darbufelone (**2**) were maintained (Fig. 1). Our results demonstrated that LQF219 showed anti-inflammatory and antinociceptive activity through the reduction of pro-inflammatory cytokines (IL-1 and IL-6) as well as the activity of the MPO enzyme.

2. Experimental

2.1. Chemicals

Synthesis of 2,6-di-*tert*-butyl-4-((1-phenyl-1H-pyrazol-4-yl)methyl) phenol (3) (LQFM219) [10]: To a heated heterogeneous mixture of formic acid (3 mL), 1-phenyl-1*H*-pyrazol-4-yl)methanol (**7**) (172 mg, 1 mmol), 2,6-di-*tert*-butylphenol (**8**) (206 mg, 1.00 mmol), and 2 mL of $CHCl_3$ were added, and the mixture was heated at 65 °C for 8 h. At the end of the reaction, the residue was split between water and CH_2Cl_2. The spelling formed was precipitate was filtered off under vacuum and dried. The phases were separated, the aqueous layer was extracted

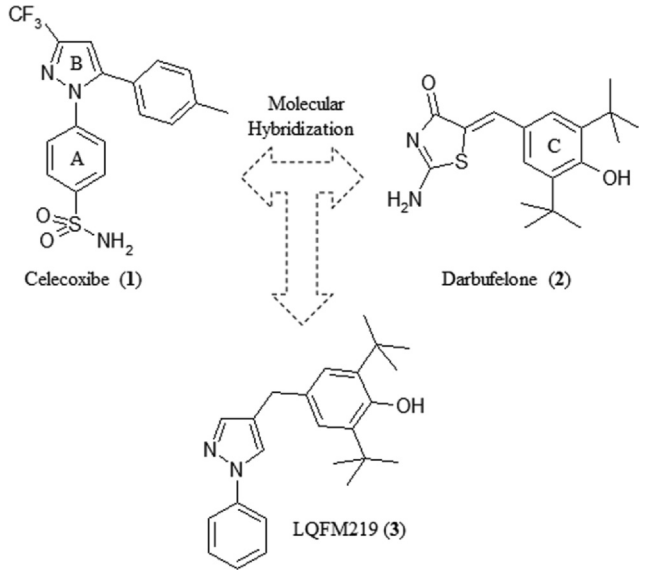

Fig. 1. Structural design of 2,6-di-*tert*-butyl-4-((1-phenyl-1H-pyrazol-4-yl)methyl)phenol (LQFM219) (**3**) from celecoxibe **(1)** and darbufelone **(2)** lead compounds.

3 × 15 mL with CH_2Cl_2, and the combined organic layers were dried with sodium sulphate and concentrated *in vacuo*. The crude product was crystallised from (MeOH/H_2O = 1:9) to 2,6-di-*tert*-butyl-4-((1-phenyl-1*H*-pyrazol-4-yl)methyl)phenol (**3**) (181 mg, 50%) as a beige solid (m.p. = 122–124 °C, Rf = 0.60 [hexene/AcOEt = 7:3]). IR_{max} (KBr) cm^{-1}: 3630 (ν O–H); 3093 (ν C–H); 2916 (ν C–H); 1599 (ν C = C); and 752 (ν Ar-1,4) (**Fig. S1**, Supplementary material); ^1H NMR (500.13 MHz) $CDCl_3$ (δ): 7.57 (brs, H3); 7.68 (brd, *J* = 0.7 Hz, H5); 3.81 (s, H6); 7.65 (dd, *J* = 8.5; 1.2 Hz, H2′); 7.42 (ddd, *J* = 8.5; 7.5; 1.0 Hz, H3′); 7.24 (tt, *J* = 7.5; 1.2 Hz, H4′); 7.42 (ddd, *J* = 8.5; 7.5; 1.0 Hz, H5′); 7.65 (dd, *J* = 8.5; 1.2 Hz, H6′); 7.05 (s, H3″); 7.05 (s, H5″); 1.43 (s, H8″); 5.08 (s, OH) (**Fig. S2** and **Table 2**, Supplementary material); 2D NMR (HSQC/HMBC-125.76 MHz) $CDCl_3$/TMS (δ): 141.3 (C-3); 123.8 (C-4); 125.4 (C-5); 30.4 (C-6); 140.4 (C-1′); 118.7 (C-2′); 129.4 (C-3′); 126.0 (C-4′); 129.4 (C-5′); 118.7 (C-6′); 152.3 (C-1″); 136.1 (C-2″); 125.0 (C-3″); 131.1 (C-4″); 125.0 (C-5″); 136.1 (C-6″); 34.6 (C-7″); 30.4 (C-8″) (**Figs. S3, S4**; **Table 2**; Supplementary material); $[M + H]^+ m/z$ of 363.2385 (**Fig. S5**, Supplementary material).

2.2. X-ray crystallography

Single crystals of LQFM219 (**3**) were obtained in acetone and selected for x-ray diffraction data collection. A clear light-yellow, plate-like specimen was mounted in a Bruker-AXS Kappa Apex II Duo diffractometer operating with an IμS microsource of Cu-Kα radiation. The raw data were processed using the APEX 2 suite of programs and corrected for absorption effects using the multi-scan method with SADABS [11]. The structure of the solution was obtained by direct methods using SHELXS [12], and the final refinement was performed with full-matrix least-squares on F^2 using SHELXL [12]. The programs ORTEP-3 and SHELXS/SHELXL [12] were used in the WinGX [13] software package.

2.3. Electroanalytical assay

Voltammetric experiments were carried out with a potentiostat/galvanostat (μAutolab III®) controlled with NOVA 2.1® software (Metrohm). The measurements were obtained with a three-electrode system consisting of a glassy carbon electrode, a Pt wire, and the Ag/AgCl/KCl (3 M), representing the working electrode, the auxiliary electrode, and the reference electrode, respectively. Assays were performed in the potential range from 0 V to 1 V. The experimental conditions for DPV analysis were as follows: pulse amplitude, 50 mV; pulse width, 0.5 s; and scan rate, 10 mV s^{-1}. The square wave voltammetry (SWV) parameters were as follows: pulse of 50 mV, frequency of 50 Hz, and potential increment of 2 mV, resulting in an effective scan rate of 100 mV s^{-1}. The voltammograms were plotted with Origin8® software. All experiments were conducted in triplicate (n = 3) in a 2.0 mL one-compartment electrochemical cell at room temperature (21 ± 1 °C), and the main electrolyte used was 0.1 M phosphate buffer (PBS) (pH 7.0).

2.3.1. Electrochemical index

The electrochemical index (EI) considers parameters such as the anodic peak potential (Epa), which is related to the electron donation capacity, and the anodic peak current, which correlates with the analyte concentration (Ipa). Thus, since Epa is inversely proportional to the ease of electron donation and Ipa is directly proportional to the electroactive species concentration, EI is calculated using the following equation:

$$EI = \sum_{i=1}^{n} \frac{Ipan}{Epan}$$

where, in a voltammogram with n peaks, Ipan and Epan are the current and potential, respectively, of the n^{th} peak.

2.3.2. DPPH radical scavenging assay

The radical scavenging assay was performed with DPPH reagent. A mixture of 2.7 mL of DPPH solution with 0.3 mL methanol was prepared, and the absorbance read at 517 nm was c.a. 0.7, which was used as the control. In the assays, the 300 μL methanolic portion was substituted with the stock solution. The antioxidant capacity was expressed as the IC_{50} value, representing the concentration (mM) of the sample that produces 50% DPPH discoloration relative to the control solution. All samples were read in a 1 cm glass recipient at room temperature 5 min after the stock solution transference to the recipient with DPPH.

2.3.3. ABTS radical scavenging assay

The radical scavenging assay was performed with ABTS reagent. An aqueous solution of 140 mM potassium persulfide was prepared and an aqueous solution of 7 mM ABTS. Five millilitres of the ABTS solution was mixed for each 84 μL of potassium persulfide solution, and the mixture was left protected from light for 16 h. After this period, the resulting solution was read, representing a mixture of 2.7 mL of ABTS solution with 0.3 mL of methanol. Next, the absorbance read at 734 nm was c.a. 0.7, which was used as a control. In the assays, the 300 μL methanolic portion was substituted with the stock solution. The antioxidant capacity was expressed as the IC_{50} value, representing the concentration (mM) of the sample that produced 50% ABTS discoloration relative to the control solution. All samples were read in a 1 cm glass recipient at room temperature 5 min after stock solution transference to the recipient with ABTS.

2.4. Toxicology

2.4.1. Cell culture

Balb/c 3 T3-A31 fibroblasts were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). 3 T3 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The cells were passaged three times a week and used in the exponential growth phase. Cell viability was analysed using a TC20TM automated cell counter (Hercules, CA, USA) according to the manufacturer's instructions. A value of $>$ 90% was considered satisfactory for conducting the assays.

2.4.2. In vitro prediction of acute oral toxicity

Acute oral toxicity estimation *in vitro* was performed by evaluation of the cytotoxicity of LQFM219 (**3**) using Balb/c 3T3 cells according to ICCVAM [14]. Briefly, the Balb/c 3 T3-A31 cells (3 × 104 cells/well) were seeded in 96-well plates overnight. The cells were then treated with 8 different concentrations of LQFM219 (**3**) (200–12.5 μg/mL) for 48 h in an incubator (Thermo Scientific, USA) with a humidified atmosphere at 37 °C in 5% CO_2. Next, the solutions were removed from the plates, and 100 μL of DMEM containing 5% (v/v) FBS and neutral red (NR, 0.25 mg/mL) was added to each well. After 3 h of incubation, the NR medium was discarded, and the cells were washed with PBS. The PBS was decanted from the plate, and 100 μL of NR desorb solution (50 EtOH: 1 acetic acid: 49 water) was added to all wells. The plates were shaken, and the absorbance was measured at 550 nm using a spectrophotometer (Thermo Scientific Multiskans Spectrum, Boston, MA, USA). Each concentration was tested in six replicates in three independent experiments. Cell viability was expressed as a percentage of the control, and the IC_{50} value was obtained and used to estimate the LD_{50} (median lethal dose) based on the following equation created with the in-house model previously established by Vieira (2011) [15]: Log (LD_{50}) = 0.545 × log (IC_{50}) + 0.757. The estimated LD_{50} value was then used to classify the probable acute oral systemic toxicity of LQFM219 (**3**), according to the Globally Harmonized Classification and Labelling of Chemicals (GSH).

2.5. Pharmacology

2.5.1. Animals

All experiments were performed using adult male Swiss albino mice (27–32 g) obtained from the Central Animal House 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 acclimatised for 7 days before the start of the experiments. Experimental groups of mice (n = 8) were used 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 approved by the Ethics Committee in Research of UFG (number 17/13).

2.5.2. Drugs and chemicals

The chemicals used in this study were acetic acid (Merck AG, Darmstadt, Germany), DMSO (Sigma-Aldrich St. Louis, MO, USA), carrageenan (Sigma-Aldrich, St. Louis, MO, USA), croton oil (Sigma Chemical Co., St. Louis, MO, USA), dexamethasone (Decadron®, Ache, Brazil), heparin (Cristália, SP, Brazil), hydrogen peroxide (Bioshop, GO, Brazil), *o*-dianisidine (Sigma-Aldrich, St. Louis, MO, USA), sodium azide (Sigma-Aldrich, St. Louis, MO, USA), Türk liquid (Bioshop, GO, Brazil), and LQFM219 (**3**), which were dissolved in 10% DMSO in distilled water. All other drugs were dissolved in distilled water. The doses of the LQFM219 (**3**) were based on the doses of LQFM182 [16] used in previous studies.

2.5.3. Anti-nociceptive activity

2.5.3.1. Acetic acid-induced abdominal writhing test. The acetic acid-induced nociception was performed as previously described by Koster et al. [17]. Groups of mice (n = 8) were treated by gavage (p.o.) with vehicle (10% DMSO, 10 mL/kg) or LQFM219 (**3**) at doses of 25, 50, or 100 mg/kg or dexamethasone (1 mg/kg, positive control) 60 min before the application of acetic acid solution (1.2% v/v; 10 mL/kg, i.p.). The number of abdominal constrictions (writhing) was counted for each animal over a period of 30 min after acetic acid injection; the results are expressed as the mean ± SD of the number of writhings.

2.5.3.2. CFA-induced mechanical hyperalgesia test. Experimental groups of male mice (n = 8) were treated with vehicle (control, 10% DMSO, 10 mL/kg p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone (1 mg/kg, positive control) 1 h before the injection of 20 μL of CFA (1 mg/mL) in the right paw. The left paw, which received the same volume of 0.9% (w/v) NaCl solution, was used as the control. The nociceptive threshold in response to the mechanical stimulus was measured from exposure of the inflamed and the non-inflamed hind paw to an increasing force until the appearance of a nociceptive reaction (vocalisation or paw withdrawal). The stimulation was stopped when the mouse struggled to withdraw the corresponding paw, and the threshold value was recorded. Measurements were alternately performed for each paw. The cut-off for each animal was 450 g. The nociceptive threshold was evaluated by the differences between the two paws (Δ) at 0, 2, 4, 8, and 24 h after CFA injection using a Raldall Selito analgesimeter (Insight EFF-440, Brazil). The baseline was established before the treatments (time 0) for each animal. The results are expressed as the means ± SD in grams [18,19].

2.5.4. Anti-inflammatory activity

2.5.4.1. CFA-induced paw oedema test. The paw oedema test was performed as previously described by Passos et al. (2007) and Nascimento et al. (2016) [20,21]. Experimental groups of male mice (n = 8) were treated with vehicle (control, 10% DMSO, 10 mL/kg, p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone (1 mg/kg, positive control) 1 h before injection of 20 μL of CFA (1 mg/mL) in the right paw. The left paw, which received the same volume of 0.9% (w/v) NaCl solution, was used as the control. Oedema was then measured by

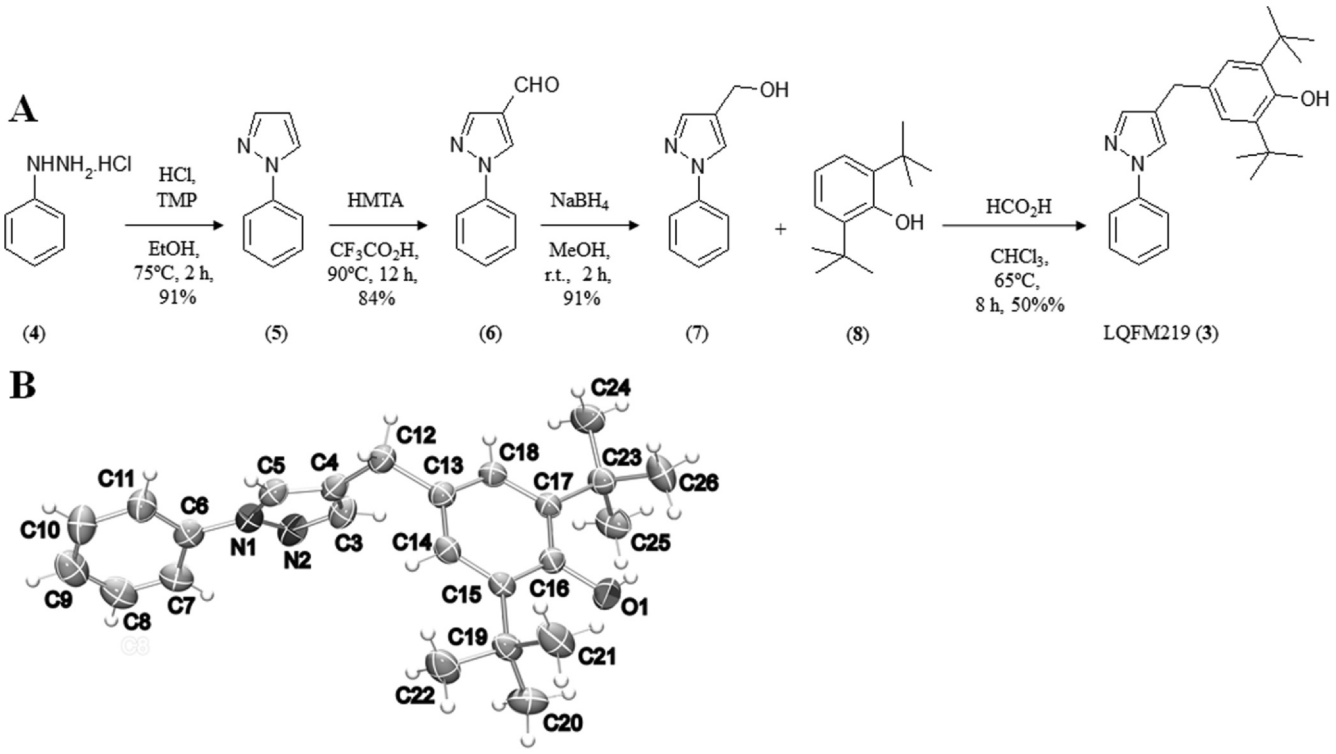

Fig. 2. (A) Synthetic route used for the preparation of LQFM219 (3). (B) ORTEP view of the compound.

the difference in volume between the paws (paw with carrageenan and saline) at different times (0, 2, 4, 8, and 24 h) after injection of CFA using a plethysmometer (Ugo Basile Co., Italy).

2.5.4.2. Croton oil-induced ear oedema test. Experimental groups of male mice (n = 8) were treated with vehicle (control, 10% DMSO, 10 mL/kg, p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone (1 mg/kg, positive control), and 60 min later cutaneous inflammation was induced by applying 25 μL of a solution of croton oil in 2.5% acetone (v/v) to the inner surface of the right ears of the mice. The same volume of acetone was applied to the left ear. Four hours after croton oil-induced inflammation, the mice were euthanised, and a 6-mm-diameter plug was obtained from both treated and untreated ears with a punch. The inflammatory response (oedema) was monitored by measuring the difference in weight (mg) between the two plugs [22].

2.5.4.3. Carrageenan-induced pleurisy test. The pleurisy test was performed as previously described by Saleh et al. (1999) [23]. Experimental groups of mice (n = 8) were treated with vehicle (10% [v/v] DMSO, 10 mL/kg, p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone (1 mg/kg, positive control) 1 h before injection of 100 μL of 1% carrageenan into the pleural cavity. The pleural exudate was collected with 1 mL of heparinised PBS 4 h after carrageenan administration. One aliquot was used to determine the total leukocyte count using Türk liquid in a Neubauer's chamber, and the other aliquot was used to measure the MPO activity and cytokine levels.

2.5.4.4. Myeloperoxidase (MPO) assay. MPO activity was measured as previously described by Lino et al. (2013) [24]. Briefly, 40 μL of the pleural exudate samples from the animals treated with vehicle (10% [v/v] DMSO, 10 mL/kg, p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone was added to 360 μL of phosphate buffer (pH 6.0) containing 0.167 mg/mL of *o*-dianisidine, 2 HCl, and 0.0005% H_2O_2. The enzymatic reaction was stopped after 15 min by the addition of 20 μL of 1% (w/v) sodium azide. The samples were subsequently centrifuged 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. The results are expressed as the means ± SD of enzymatic activity in mU/mL.

2.5.4.5. Measurement of cytokines. The pleural exudates of mice treated with vehicle (10% [v/v] DMSO, 10 mL/kg, p.o.), LQFM219 (**3**) (100 mg/kg, p.o.), or dexamethasone (1 mg/kg, positive control) were also used to determine the concentrations of TNF-α and IL-1β using an immunosorbent assay kit (ELISA) (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. The results are expressed as the means ± SD in pg/mL [25,26].

2.6. Statistical analysis

All the results are expressed as the mean ± SD The data were analysed statistically by one-way ANOVA followed by *Tukey's* post-hoc test or by two-way ANOVA followed by *Bonferroni's* post-hoc test. Values of $p \leq 0.05$ were considered significant. All statistical analyses were carried out using Graph Pad Prism® version 5.00.

The assays were carried out in three independent experiments. The cytotoxicity results, expressed as the mean ± SD of six replicates, were transformed into percentages of the control, and IC_{50} values were obtained by non-linear regression.

3. Results

3.1. Synthesis of 2,6-di-tert-butyl-4-((1-phenyl-1H-pyrazol-4-yl)methyl) phenol - LQFM219 (3)

As illustrated in Fig. 2**A**, the synthetic route began with a condensation reaction between 1-(phenyl)hydrazine hydrochloride (**4**) and 1,1,3,3-tetramethoxy propane, as described by Finar and Hurlock [27], to provide 1-(phenyl)-1-*H*-pyrazole (**5**) with a yield of 91%. The chemoselective and regiospecific formylation of 1-(phenyl)-1-*H*-pyrazole

Table 1
Electrochemical index, DPPH, and ABTS IC_{50} of LQFM219 (**3**) and BHT.

	EI (μA/V)	DPPH IC_{50} (mM)	ABTS IC_{50} (mM)
LQFM219 (**3**)	0.9211	0.27 ± 0.012*	0.32 ± 0.003*
BHT	0.7098	0.25 ± 0.012*	0.38 ± 0.004*

* Values given as the average ± SD (n = 3).

(**5**) to 1-(phenyl)-1-*H*-pyrazole-4-carbaldehyde (**6**) was carried out under Duff's conditions, with 84% yield [28]. In turn, the 1-(phenyl)-1-*H*-pyrazole-4-carbaldehyde (**6**) was reduced to 1-phenyl-1*H*-pyrazol-4-yl)methanol (**7**) through the use of $NaBH_4$ in methanol, with a 91% yield [29]. Finally, 1-phenyl-1*H*-pyrazol-4-yl)metanol (**7**) was reacted with 2,6-di-*tert*-butylphenol (**8**) through a Friedel-Crafts alkylation reaction using HCO_2H as a catalyser to obtain 2,6-di-*tert*-butyl-4-((1-phenyl-1*H*-pyrazol-4-yl)methyl)phenol (LQFM219) (**3**), with a yield of 50% [10]. LQFM219 (**3**) was obtained after four synthetic steps, with a 35% global yield.

3.2. X-ray crystallography

The ORTEP view of LQFM219 (**3**) is illustrated in Fig. 2**B**, as well as crystal data and structure refinement for LQFM219 (**3**) (Table 1, Supplementary material).

3.3. Electroanalytical assay

In LQFM219 (**3**) voltammograms, it was possible to observe two anodic peaks: Epa1 c.a. 0.05 V and Epa2 c.a. 0.5 V (Fig. 3**A**). In the cyclic voltammetry (Fig. 3**B**), only the major second anodic peak, Epa2 c.a. 0.5 V, was observed, which demonstrated no signs of reversibility. Slight reversibility was observed for both oxidative processes (Fig. 3**C**); however, due to their amplitude, it is safe to assume that these processes are quasi-irreversible. Individual analysis of the EI and the DPPH/ABTS IC50 calculated results showed that LQFM219 (**3**) had better antioxidant capacity than 2,6-di-*tert*-butyl-4-methylphenol (BHT) since higher EI values and lower IC_{50} infer higher antioxidant capacity (Table 1).

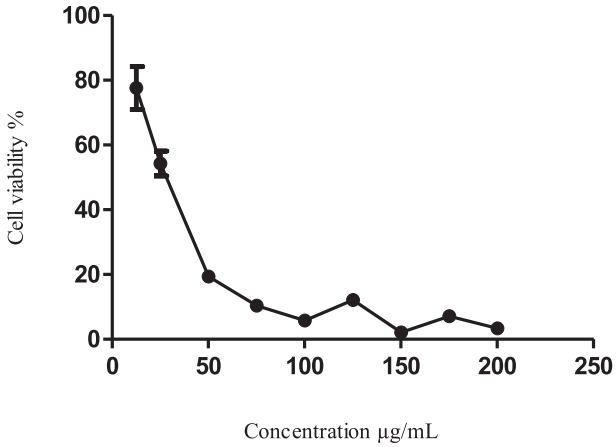

Fig. 4. Cytotoxic activity of LQFM219 in murine fibroblast (BALB/3T3) cells after 48 h of treatment with different concentrations of compound (200–12.5 μg/mL). Cytotoxic effects were determined by the neutral red uptake assay. The results represent the mean ± SD of three independent experiments in six replicates.

3.4. In vitro assay to estimate the LD_{50} value

The cytotoxic effects of the eight concentrations of LQFM219 (**3**) (200–12.5 μg/mL) on Balb/c 3T3-A31 cells determined by the NRU assay are shown in Fig. 4. LQFM219 (**3**) inhibited cell proliferation in a concentration-dependent manner. Using these findings as a basis, the

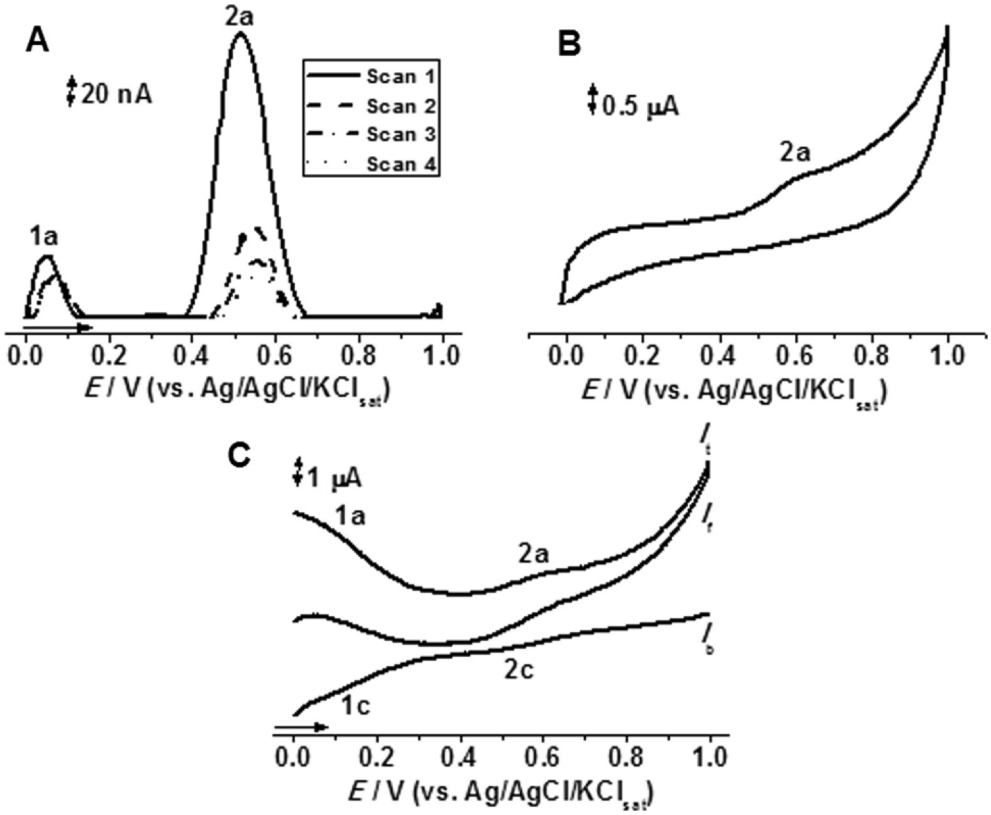

Fig. 3. Differential pulse (A), cyclic (B), and square wave (C) voltammograms of a 1 mM LQFM219 stock solution in PBS (pH 7.0) with glassy carbon.

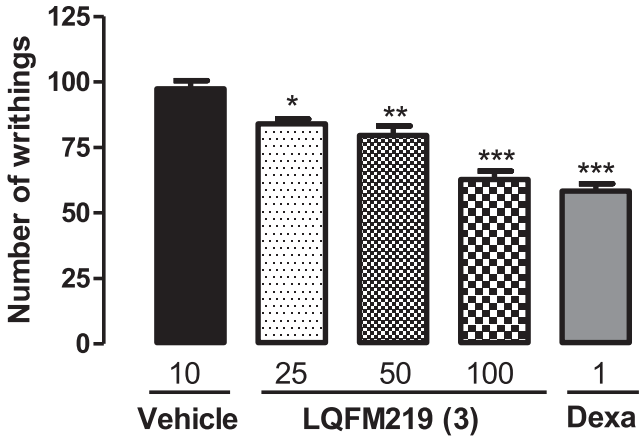

Fig. 5. Effect of LQFM219 (**3**) (25, 50, and 100 mg/kg, p.o.) on the number of acetic acid-induced writhings in mice (n = 8). Vertical bars represent the mean ± SD of the number of writhings in 30 min for each experimental group. Dexamethasone (Dexa, 1 mg/kg) was used as a positive control. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 (compared with the control group), according to ANOVA followed by *Tukey's* test.

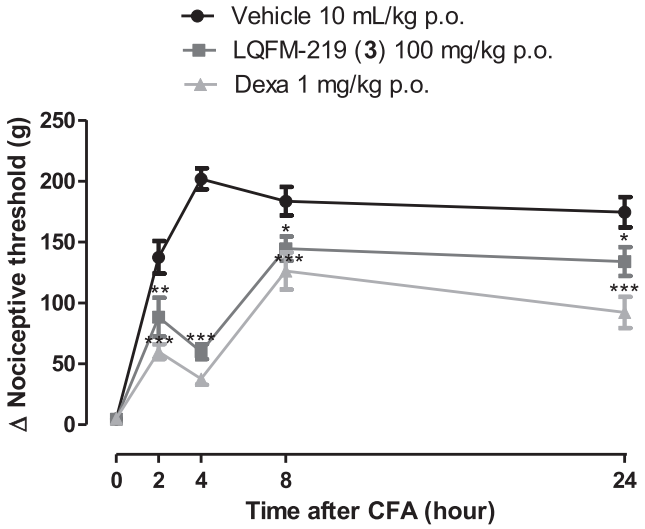

Fig. 6. Antihyperalgesic effect of LQFM219 (3) (100 mg/kg, p.o.) and dexamethasone (Dexa, 1 mg/kg; p.o., positive control) on the CFA-induced mechanical hyperalgesia test in mice (n = 8). Treatment with LQFM219 (3) and dexamethasone began to reduce mechanical hyperalgesia starting from hour 2 until hour 24 after CFA injection. The values are expressed as the mean ± SD of the difference between non-inflamed and inflamed paws, in grams (g). *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001, compared with the control group, according to two-way ANOVA followed by *Bonferroni's* post-hoc test.

LD_{50} was estimated to be 369.1 mg/kg. Therefore, LQFM219 (**3**) was classified as GHS category 4 (300 mg/kg > LD_{50} < 2000 mg/kg).

3.5. Anti-nociceptive activity

3.5.1. Acetic acid-induced abdominal writhing test

To evaluate the possible antinociceptive activity of LQFM219 (**3**), the acetic acid-induced abdominal writhing test was performed. This test is a visceral pain model that is widely employed and has been frequently used to evaluate peripheral antinociceptive activity [30]. The oral treatments with LQFM219 (**3**) at doses of 25, 50, or 100 mg/kg decreased the number of writhings induced by acetic acid in a dose-dependent manner, compared with the control group (vehicle, 10% DMSO, 10 mL/kg), from 97.4 ± 8.7 (group treated with vehicle) to 84.0 ± 5.0 (13.7%, P ≤ 0.05), 79.6 ± 10.1 (18.2%, P ≤ 0.01), and 62.7 ± 8.9 (35.6%, P ≤ 0.001), respectively. The positive control for this test, dexamethasone (1 mg/kg), reduced the number of writhings by 58.4 ± 7.9 (40.1%, P ≤ 0.001) (Fig. 5).

3.5.2. CFA-induced mechanical hyperalgesia test

The treatment with LQFM219 (**3**) (100 mg/kg) reduced the difference in the nociceptive threshold between the non-inflamed and inflamed paw of animals in response to a mechanical stimulus consistently in the paw pressure test. At hour 2 of the test, compared with the control group (137.5 ± 32.7 g), treatments with LQFM219 (**3**) (100 mg/kg) and with dexamethasone (1 mg/kg) reduced mechanical hyperalgesia by 35.8% and 55.7% (P ≤ 0.001), respectively. At hour 4 of the test, compared with the control group (201.9 ± 22.9 g), treatments with LQFM219 (**3**) (100 mg/kg) and with dexamethasone (1 mg/kg) reduced mechanical hyperalgesia by 70.3% and 81.4% (P ≤ 0.001), respectively. At hour 8 of the test, compared with the control group (183.6 ± 31.1 g), treatments with LQFM219 (**3**) (100 mg/kg) and with dexamethasone (1 mg/kg) reduced mechanical hyperalgesia by 21.2% (P ≤ 0.05) and 31.2% (P ≤ 0.001), respectively. At hour 24 of the test, compared with the control group (174.6 ± 33.0 g), treatments with LQFM219 (**3**) (100 mg/kg) and with dexamethasone (1 mg/kg) reduced mechanical hyperalgesia by 23.2% (P ≤ 0.05) and 47.2% (P ≤ 0.001), respectively (Fig. 6).

3.6. Anti-inflammatory activity

3.6.1. CFA-induced paw oedema test

Treatments with LQFM219 (**3**) (100 mg/kg) consistently reduced paw oedema formation. At hour 2 of the test, compared with the control group (62.5 ± 7.1 μL), treatments with LQFM219 (**3**) and with dexamethasone (1 mg/kg) reduced oedema by 46.0% (P ≤ 0.01) and 62.0% (P ≤ 0.001), respectively. At hour 4, compared with the control group (93.7 ± 15.1 μL), oedema was reduced by 45.3% and 65.3% (P ≤ 0.001), respectively. At hour 8, compared with the control group (125 ± 20.7 μL), oedema was reduced by 27.0% (P ≤ 0.05) and 68% (P ≤ 0.001), respectively. At hour 24, compared with the control group (177.5 ± 15.8 μL), oedema was reduced by 33.1% (P ≤ 0.05) and 71.1% (P ≤ 0.001), respectively (Fig. 7).

3.6.2. Croton oil-induced ear oedema test

Croton oil contains 12-O-tetradecanoylphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. In this test, oral treatments with LQFM219 (**3**) (100 mg/kg) reduced ear oedema formation by 57.6% (P ≤ 0.001) compared with the control group (13.4 ± 3.3 mg). The positive control, dexamethasone (1 mg/kg), reduced ear oedema by 78.3% (P ≤ 0.001), as shown in (Fig. 8**)**.

3.6.3. Carrageenan-induced pleurisy test

Injection of carrageenan into the pleural cavity of mice induced an acute inflammatory response characterised by the accumulation of leukocytes. Oral treatments with LQFM219 (**3**) (100 mg/kg) and with dexamethasone (1 mg/kg) reduced the total number of leukocytes to 3.42 ± 0.9 leukocytes × 10^6/mL (31.3% reduction, P ≤ 0.001) and 1.7 ± 0.5 leukocytes × 10^6/mL (65.1% reduction, P ≤ 0.001), respectively, when compared to the control group (5.0 ± 0.6 leukocytes × 10^6/mL), as shown in (Fig. 9**A)**.

3.6.4. Myeloperoxidase (MPO) assay

In the carrageenan-induced pleurisy test, LQFM219 (**3**) (100 mg/kg) also decreased myeloperoxidase activity to 75.4 ± 7.7 mU/mL (69.0% reduction, P ≤ 0.001) compared with the control group (242.9 ± 65.6 mU/mL). A similar result was observed for the positive control, dexamethasone (1 mg/kg, p.o.), which resulted in a decrease to 117.7 ± 28.4 mU/mL (51.5% reduction, P ≤ 0.001), as shown in (Fig. 9**B)**.

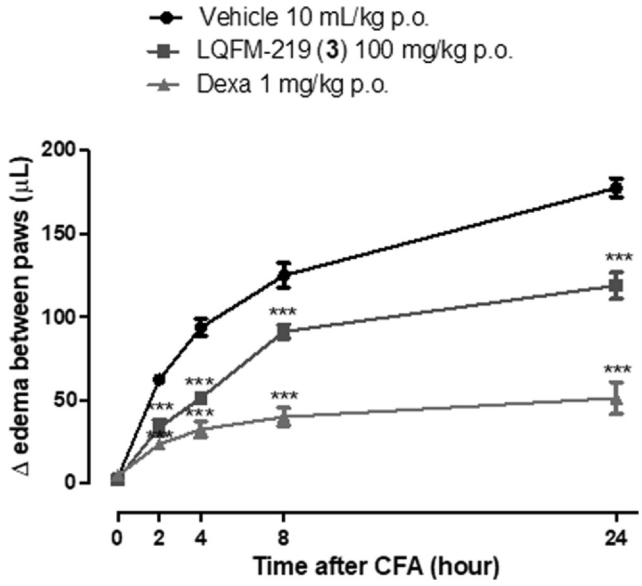

Fig. 7. Anti-oedematogenic effect of LQFM219 (**3**) (100 mg/kg, p.o.) and dexamethasone (Dexa, 1 mg/kg; p.o., positive control) on the CFA-induced oedema test in mice (n = 8). Treatment with LQFM219 (**3**) and dexamethasone began to reduce oedema starting from hour 2 until hour 24 after CFA injection. The values are expressed as the mean ± SD of the difference between paws, in μL. ***p ≤ 0.001, compared with the control group, according to two-way ANOVA followed by *Bonferroni's* post-hoc test.

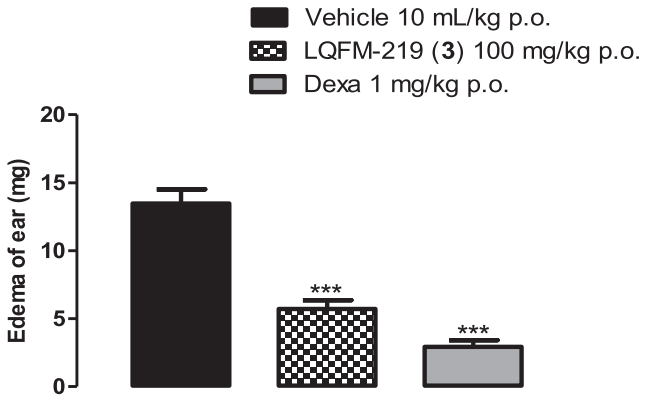

Fig. 8. Anti-oedematogenic effect of LQFM219 (**3**) (100 mg/kg, p.o.) and dexamethasone (Dexa, 1 mg/kg; p.o., positive control) on croton oil-induced ear oedema in mice. Values are the mean ± SD (n = 8) of differences in weight between right and left ear plugs. ***p ≤ 0.001, compared with the control group, according to ANOVA followed by *Tukey's* test.

3.6.5. Measurement of cytokines

In addition to the effect of LQFM219 (**3**) on leukocyte counts, we observed that this compound also caused significant changes in the production/release of some pro-inflammatory cytokines. In our experiments, LQFM219 (**3**) (100 mg/kg) caused a significant reduction in TNF-α levels (percent inhibition of 54.0%, P ≤ 0.001) compared with the control group (18.2 ± 5.7 pg/mL) (Fig. 10**A**). The same dose also reduced IL-1β levels (percent inhibition of 48.4%, P ≤ 0.01) compared with the control group (122.1 ± 50.4 pg/mL) (Fig. 10**B**). As expected, dexamethasone (1 mg/kg) showed the expected profile through inhibition of pro-inflammatory cytokines (percent inhibition on TNF-α of 88.2% and IL-1β of 95.5%, P ≤ 0.001) (Fig. 10**A, B**).

4. Discussion

The present study reported the synthesis and pharmacological profile of a new compound, LQFM219 (**3**), which was designed from celecoxibe (**1**) and darbufelone (**2**) using a molecular hybridisation strategy. The new compound, LQF219, showed an anti-inflammatory and antinociceptive effect that involves a reduction in the oedema induced by different phlogistic agents, a decrease in the levels of pro-inflammatory cytokines, and inhibition of the migration of polymorphonuclear cells in carrageenan-induced pleurisy.

Initially, the toxicity of the new compound, LQFM219 (3), was determined *in vitro*. LQFM219 (3) was classified as GHS category 4 (300 < LD50 < 2000 mg/kg), whereas darbufelone (1) showed a greater potential to promote acute oral toxicity since it was classified as GHS category 3 [4]. According to OECD, chemicals classified under GHS category 4 have low/moderate acute toxicity potential [31]. The results obtained for the cytotoxicity assay allowed us to estimate the DL50 for compound LQFM219 (3) as 369.1 mg/kg, demonstrating that this compound may be useful for the development of new drugs.

The acetic acid model was used to assess the antinociceptive activity of LQFM219 since it induces pain indirectly via the release of some endogenous mediators, such as prostaglandins and cytokines that reduce the threshold of nociception and stimulate nociceptive fibres [32]. In this test, oral treatments with LQFM219 (3) at doses of 25, 50 or 100 mg/kg decreased the number of writhings induced by acetic acid in a dose-dependent manner. Moreover, LQFM219 (3) at a dose of 100 mg/kg significantly inhibited the mechanical hyperalgesia associated with CFA. CFA induced inflammatory pain by regulating NF-κB activation and increases in PGE2, TNF-α, IL-1β, and IL-6, which are capable of sensitising nociceptive neurons, favouring hyperalgesia [33,34]. We used dexamethasone as a positive control in the experiment since several studies have reported its potential to reduce inflammatory pain [35–37]. As expected, dexamethasone treatment reduced the CFA-induced pain at all times measured. Therefore, these data suggest that part of the antinociceptive effect might be associated with a possible anti-inflammatory activity of LQFM219 (3). Thus, we investigated the anti-inflammatory activity of LQFM219 (**3**) by CFA-induced paw oedema and croton oil-induced ear oedema.

CFA and croton oil produce acute inflammation by a local release of mediators, such as cytokines and prostaglandins, which are involved in the formation of oedema, leukocyte migration, vasodilation, and hyperalgesia [38–41]. LQFM219 (**3**) was able to inhibit CFC oedema formation 24 h after the stimulus. A similar effect was observed for CFA-induced hyperalgesia. In addition, at a dose of 100 mg/kg, LQFM219 (**3**) inhibited ear oedema formation induced by croton oil. As expected, treatment with dexamethasone (1 mg/kg), the positive control, inhibited the oedema induced by CFA and croton oil. Together, these data demonstrate the antiedematogenic effect of the new compound LQFM219 (3). The results corroborate some previous studies, which indicates antinociceptive and anti-inflammatory activities of pyrazole derivatives [24,42–44]. Furthermore, Lino and collaborators (2017) demonstrated anti-inflammatory and antinociceptive activities for the compound LQFM091, derived from darbufelone [45].

To investigate the action of LQFM219 (**3**) on different parameters of the inflammatory process, the carrageenan-induced pleurisy test was performed. Carrageenan-induced pleurisy is a model extensively used to assess leukocyte recruitment, especially neutrophils. Different studies have shown an accumulation of neutrophils 4 h after injection with carrageenan in the pleural region, as well as the production and release of mediators that play important to inflammatory response [25,46–48]. Our data demonstrated that treatment with LQFM219 (3) (100 mg/kg) reduced leukocyte migration. It is known that MPO is more abundant in immune cells, such as neutrophils, and the level of activity is directly proportional to the neutrophil concentration in inflamed biological liquids or tissues [49,50]. Consequently, we performed the MPO activity and observed that LQFM219 (3) reduced MPO activity, suggesting that the action of the new compound could be due to a preferential reduction in neutrophils or a direct inhibition of MPO enzyme activity.

The pro-inflammatory cytokines are involved in cellular

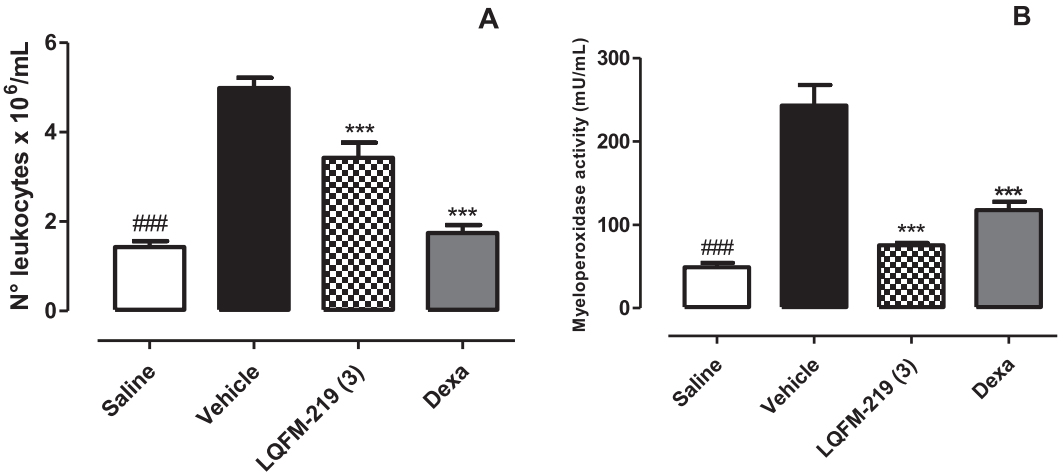

Fig. 9. Anti-inflammatory effect of LQFM219 (**3**) (100 mg/kg, p.o.) and dexamethasone (Dexa, 1 mg/kg; p.o., positive control) on the carrageenan-induced pleurisy test in mice. (A) Leukocyte counts in the pleural cavity. (B) Myeloperoxidase enzyme activity (mU/mL). Values are expressed as the mean ± SD of 8 mice. ***p ≤ 0.001, compared with the carrageenan group, ###p ≤ 0.001 (Saline × Carrageenan), according to ANOVA followed by *Tukey's* test.

recruitment, acute phase protein release, increases in vascular permeability, and hyperalgesia, in addition to inducing the production of other pro-inflammatory mediators, contributing to the progression of inflammation [51–54]. Thus, we analysed the levels of IL-1β and TNF-α in the pleural exudate after the challenge with carrageenan. The treatment with LQFM219 (**3**) markedly reduced the concentrations of IL-1β and TNF-α in the pleural exudate, suggesting that the reduction of cell migration and antinociceptive effect observed was due, in part, to the reduction of these pro-inflammatory cytokines.

Oxidative stress plays a critical role in acute inflammatory responses, such as lung injury [55] and several preclinical studies have shown that compounds with antioxidant effects also have considerable anti-inflammatory activity [56,57]. Therefore, we investigated the possible antioxidant effect of LQFM219 (3). As we demonstrated in cyclic voltammetry assays, LQFM219 (3) showed better antioxidant capacity than BHT, a gold standard antioxidant that is commonly used in the foods, pharmaceuticals, petroleum products, rubber, and oil industries. These findings indicate that the presence of BHT in the chemical structure of LQFM219 (3) may have contributed positively to the anti-inflammatory effect demonstrated by this compound.

5. Conclusion

In conclusion, the present findings show, for the first time, that LQFM219 (**3**), a molecule designed from celecoxibe and darbufelone, has antinociceptive, anti-inflammatory, and antioxidant activities. The anti-inflammatory activity was characterised by a reduction of leukocyte migration in the pleural exudate, associated with inhibition of MPO activity, reduced levels of TNF-α and IL-1β, and free radical scavenger activity, demonstrating that this compound may be useful for the development of new analgesic and anti-inflammatory drugs.

CRediT authorship contribution statement

Gustavo M. Galvão: Methodology, Investigation, Data curation, Writing - original draft. **Iziara F. Florentino:** Methodology, Investigation, Data curation. **Germán Sanz:** Methodology, Investigation. **Boniek G. Vaz:** Formal analysis, Supervision. **Luciano M. Lião:** Formal analysis, Supervision. **José R. Sabino:** Formal analysis, Supervision. **Carina S. Cardoso:** Methodology, Investigation. **Daiany P.B. da Silva:** Methodology, Investigation, Data curation. **Elson A. Costa:** Formal analysis. **Andreia L.P. Silva:** Methodology, Investigation. **Artur C.G. da Silva:** Methodology, Investigation, Data curation, Writing - original draft. **Marize C. Valadares:** Formal analysis, Supervision. **Jacqueline A. Leite:** Supervision. **Eric de S. Gil:** Formal analysis, Supervision. **Ricardo Menegatti:** Conceptualization, Project administration, Supervision.

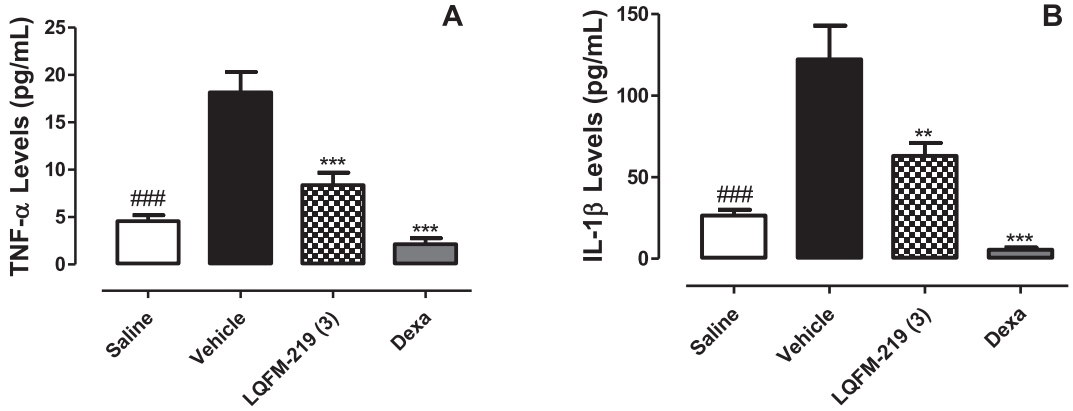

Fig. 10. Effect of LQFM219 (**3**) (100 mg/kg, p.o.) and dexamethasone (Dexa, 1 mg/kg; p.o., positive control) on cytokine levels. (A) Tumor necrosis factor-alpha (TNF-α) levels. (B) Interleukin 1β (IL-1β) levels. Vertical bars represent the mean ± SD of 8 mice. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001, compared with the control group, ###p ≤ 0.001 (saline × carrageenan), according to ANOVA followed by *Tukey's* test.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106893.

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