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Synthesis and Biological Activities of a Nitro-Shiff Base Compound as a Potential Anti-Inflammatory Agent

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Abstract

In order to discover a new compound having anti-inflammatory activity, a nitro-Schiff base was evaluated. The compound was synthesized and characterized by ^{1}H NMR and ^{13}C NMR. The cytotoxic activity was evaluated *in vitro* by hemolysis and MTT cell viability assay. To evaluate genotoxicity, the micronucleus assay was performed *in vivo*. The anti-inflammatory effects of the compound were examined using *in vivo* models of inflammation such as neutrophil migration assay, paw edema, and exudation assay. The production of NO was also estimated *in vivo* and *in vitro*. The data showed that the compound did not induce hemolysis at all the tested concentrations. Similarly, the compound did not induce cytotoxicity and genotoxicity to the cells. The neutrophil migration assay showed that the compound reduced the number of neutrophils recruited to the peritoneal cavity by approximately 60% at all the tested concentrations. In the exudation assay, the compound showed a reduction in extravasation by 24%. The paw edema model demonstrated a significant reduction in the paw volume at all the evaluated time points. The production of NO was decreased both *in vitro* and *in vivo*. These results suggest that the nitro-Schiff base compound efficiently inhibited inflammation and might be a good candidate for the treatment of inflammatory-associated conditions.

Keywords: Inflammation, Schiff bases, Nitro group.

1. Introduction

Inflammation is a mechanism of self-protection of the body against injurious agents that can damage the tissues (Medzhitov, 2010). It provides support to the body to eliminate harmful stimuli and helps in initiating the process of healing (Alessandri et al., 2013). Swelling, pain, leukocyte migration, and loss of function are the main signs of inflammation and are caused by the release of various inflammatory mediators (Serhan, 2017). Prolonged and severe inflammation contributes to the development of various diseases such as arthritis, chronic obstructive pulmonary disease, asthma, cancer, multiple sclerosis, and cardiovascular diseases (Freire and Van Dyke, 2013; Nathan and Ding, 2010).

Inflammatory response involves the recruitment of leukocytes, especially neutrophils (Mayadas et al., 2014) and macrophages (Nagatoshi and Kazuo, 2005). Neutrophils are the first cells to be recruited to the inflammatory sites and are responsible for the production of several inflammatory mediators (e.g. cytokines and chemokines) and reactive oxygen species (ROS) (Halade and Ma, 2016). These cells play a major role in the host defense through phagocytosis; however, neutrophils can play dual roles in inflammation. An increased neutrophil count contributes to the damage of tissues in inflammatory diseases, whereby they become inappropriately activated (Wright et al., 2010). In this context, numerous anti-inflammatory strategies are under investigation to control increased neutrophil migration in inflammatory diseases.

The main pharmacotherapy for inflammatory diseases comprises nonsteroidal anti-inflammatory drugs (NSAIDs). This class of drugs has antipyretic, anti-inflammatory, and analgesic properties, and is among the most commonly prescribed drugs in the world (Dwivedi et al., 2015). Despite good efficacy, their use, especially in high doses and for a prolonged period, is associated with several adverse effects such as gastrointestinal damage including gastric lesions, bleeding (Sostres et al., 2010; Tanaka et al., 2001), and cardiovascular disorders (Grosser et al., 2017).

Imines, also called azomethines or Schiff bases, are compounds containing the functional group –C=N–, which were first described by Hugo Schiff in the 19th century. Schiff bases form an important class of organic compounds having several applications in synthetic organic processes, organometallic chemistry, and also as pharmaceutical targets (Kajal et al., 2013; Qin et al., 2013). The functional group –C=N– brings a spectrum of biological activities to these compounds (Hameed et al., 2017). Schiff bases

have importance in the medical and pharmaceutical fields due to their reported biological activities including antitumor (Ceyhan et al., 2015; Hu et al., 2012), anti-inflammatory (Sondhi et al., 2006), analgesic (Pandey et al., 2012), nematicide (Al-Kahraman et al., 2010), and antimicrobial (Abirami and Nadaraj, 2015) activities. The present study evaluated the anti-inflammatory activities of a nitro-Schiff base derivate, (*E*)-2-(((4-nitrophenyl)imino)methyl) phenol (ENIMF).

2. Material and methods

2.1 Reagents

The following substances were used: salicylaldehyde (Sigma, China), 4-nitroaniline (Sigma, China), dimethyl sulfoxide (DMSO) (Vetec, Brazil), saline (JP Farma, Brazil), Triton X-100 (Vetec), thioglycolate (Himedia, India), Trypan blue (Gibco, USA), 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma, USA), isopropyl alcohol and hydrochloric acid (solubilizing solution) (Vetec), saline phosphate buffer (PBS) (Sigma, USA), ethylenediamine acid (EDTA) (Dinâmica, Brazil), colchicine (Geolab, Brazil), May-Grunwald stain (NewProv, Brazil), Giemsa stain (RenyLab, Brazil), dexamethasone (Aché, Brazil), carrageenan (Sigma, USA), ketamine (Syntec, Brazil), xylazine (Agener União, Brazil), rapid staining in hematology kit (Instant Prov, Brazil), Evans blue dye (Vetec), and lipopolysaccharide from *Escherichia coli* (LPS) (Sigma, USA).

2.2 Synthesis of ENIMF

The compound was synthesized with slight modifications of a reported procedure (Shahnaz et al., 2016). An ethanolic solution of salicylaldehyde (0.252 g, 2.06 mmol, 15 mL EtOH) was added drop-wise to an ethanolic solution of 4-nitroaniline (0.285 g, 2.06 mmol, 15 mL EtOH) and the mixture was stirred at 80 ^{o}C. After 3 h, the solvent was evaporated and the orange liquid was extracted with a chloroform/water mixture (1:1). The organic layer was then dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain an orange crystalline solid (E)-2-(((4-nitrophenyl)imino)methyl)phenol (Figure 1). ENIMF solubilization was performed using saline and DMSO (2%). The NMR conditions were as follows: ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 11.02 (s, 1H), 9.91 (s, 1H), 8.10−8.05 (m, 2H), 7.46−7.44 (t, *J* = 4.5 Hz, 1H), 7.40−7.35 (m, 1H), and 7.08−7.00 (m, 2H), 6.65−6.60 (m, 2H); and ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 196.83, 165.60, 137.22, 133.94, 133.21, 126.56, 125.44, 122.10, 120.06, 117.82, and 113.58.

2.3 Animals

Male and female Swiss mice weighing 20−22 g obtained from the Dom Bosco Catholic University (UCDB) (Campo Grande, MS, Brazil) were maintained at room temperature (23−25 °C) and controlled light/dark cycle, with free access to feed and water. The experimental protocol adhered to the Brazilian National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Committee for Ethics in Animal Experimentation (CEUA-UCDB, protocol no. 031/2017).

2.4 Hemolytic assay

The hemolytic assay was performed according to a previous report (Park et al., 2004) with minor modifications. The murine erythrocytes were washed with saline (0.9%) and centrifuged at 580 *g* at 4 °C for 2 min. The cells were distributed on a 96-well plate (KASVI) and incubated with different concentrations of ENIMF (100, 50, 25, 12.5, and 6.25 µg/mL). The positive control cells were treated with Triton X-100 (2%). The material was incubated at room temperature for 1 h and then centrifuged at 1400 *g* for 2 min at 10 °C. The reading was recorded at 415 nm (SpectraMax F3, Molecular Devices). All concentrations were evaluated in triplicate. The percentage of hemolysis was compared to the positive control and calculated according to the following formula:

$$\%\ \text{inibition} = \frac{\text{Abs treatment} \times 100}{\text{Abs positive control}}$$

2.5 Cell viability assay

The macrophage culture was performed according to a previous report (Zhang et al., 2008) with modifications. The macrophages were obtained from the peritoneal cavity of the Swiss mice (n = 10) 48 h after intraperitoneal (i.p) injection of thioglycolate (4%) (3 mL i.p.). After euthanasia (deepening anesthesia with ketamine 150 mg/kg and xylazine 7.5 mg/kg), the exudate was collected, washed (twice), and re-suspended in incomplete RPMI 1640 medium. Subsequently, the cells were counted in a Neubauer chamber. The cell viability was determined by the Trypan blue exclusion method (Strober, 1997). The samples with cell viability >90% were considered suitable for subsequent experiments. The cytotoxicity of ENIMF was determined by the salt dye MTT colorimetric assay (Sieuwerts et al., 1995; Takeuchi and Kobata, 1991). A suspension of peritoneal macrophages (1×10^5 cells/mL) was placed on 96-well plates and incubated with incomplete RPMI 1640 medium with different concentrations of ENIMF (200, 100, 50, 25, 12.5, 6.25, and 3.12 µg/mL) and kept in the presence of 5% CO_2 at 37 ºC. The cell viability was evaluated after 24 h and 48 h incubation with ENIMF. Following that, all medium was aspirated and the MTT solution (5 mg/mL

0.01 mL) was added and incubated for 4 h. After the incubation period, a solubilizing solution (0.01 mL) was added to solubilize the formazan crystals. The plates were shaken slightly at room temperature for 5−10 min and the reading was recorded at 540 nm (SpectraMax F3, Molecular Devices). Experiments in all the groups were performed in triplicate. The cell viability percentages were calculated relative to the untreated cell controls.

2.6 Micronucleus assay

The micronucleus assay was performed with the peripheral blood of the Swiss mice according to (Schmid, 1975) and (Heddle, 1973). The mice (n = 6) were treated with a concentration of 5 mg/kg of ENIMF (0.2 mL i.p.), vehicle (0.2 mL i.p.), or colchicine (5 mg/kg, 0.2 mL i.p.). After 24 h, the mice were anesthetized (ketamine 150 mg/kg and xylazine 7.5 mg/kg) and blood samples were obtained by caudal vein puncture for making blood smears. The slides were fixed and stained with May Grunwald and Giemsa stain (10%). The erythrocytes (2,000/slide) were counted, and the polychromatic cells were identified with or without micronuclei. The slides were analyzed under an optical microscope (Nikon® Eclipse 80i, oil immersion lens, 100/1.25). The data are expressed as the number of micronucleus/2000 cells.

2.7 Neutrophil migration

The mice (n = 6) were pretreated with subcutaneous *(*s.c.) vehicle (0.1 mL s.c.), dexamethasone (0.4 mg/kg, 0.1 mL s.c.), or ENIMF (5, 2.5, and 1.25 mg/kg s.c.) 15 min prior to the application of the inflammatory stimulus (carrageenan, 1.5% 0.1 mL i.p.). The negative control group received saline solution (0.9%, 0.1 mL i.p.). After 6 h, the animals were euthanized (deepening anesthesia with ketamine 150 mg/kg and xylazine 7.5 mg/kg) and the peritoneal cavity was washed with PBS/EDTA (0.3%, 3 mL). The total and differential cells present in the exudate were assessed. The total count was performed in a Neubauer chamber. The differential count was performed with slides of the exudate cell smear, which were stained and examined under an optical microscope (Nikon® Eclipse 80i, oil immersion lens, 100/1.25). The results are expressed as the number of cells × 10^5/mL (Moreno et al., 2006).

2.8 Exudation assay

The effects of ENIMF on the increase in carrageenan-induced permeability were performed according to a previous report (Graesser et al., 2002) with modifications. The mice (n = 5) were pretreated with vehicle (0.1 mL s.c.) or ENIMF (5 mg/kg s.c.) 15 min prior to the inflammatory stimulus (carrageenan, 1.5% 0.1 mL i.p.). After 45 min, Evans

blue dye (20 mg/kg) was intravenously (i.v.) administered (0.05 mL). After 4 h, the animals were euthanized (deepening anesthesia with ketamine 150 mg/kg and xylazine 7.5 mg/kg) and the peritoneal cavity was washed with PBS/EDTA and centrifuged at 220 *g* for 10 min at 10 °C. The absorbance of the supernatant was determined at 650 nm (SpectraMax F3, Molecular Devices). The results were compared to a nitrite standard curve (100–0.001 μM) and expressed as μM/mL.

2.9 Carrageenan-induced paw edema

The paw edema model was established according to a previous report (Winter et al., 1962). The mice (n = 5) were pretreated with ENIMF (5 mg/kg 0.1 mL s.c.), vehicle (0.1 mL s.c.), or dexamethasone (0.4 mg/kg, 0.1 mL s.c.). After 30 min, carrageenan (1.5%, 0.05 mL) (inflammatory stimulus) was injected sub-plantar (s.p.) into the left paw of all the mice, except the vehicle group that received the saline solution (0.9%, 0.05 mL s.p.). The paw volume was measured before and at 5, 10, 15, 30, 60, 120, and 240 min after carrageenan injection to assess the edema. The feet volumes were measured with a plethysmometer (Insight, Ribeirão Preto, Brazil). At the end of the experiment, the mice were euthanized (deepening anesthesia with ketamine 150 mg/kg and xylazine 7.5 mg/kg). The results are expressed in variation (Δ) of the increase in paw volume.

2.10 Nitric oxide (NO) production

The production of NO was quantified according to the Griess reaction method (Green et al., 1982; Stone et al., 2005) with some modifications. The peritoneal macrophages (2×10^5 cells/mL) were incubated with RPMI 1640 medium for 24 h for adhesion. Thereafter, the cells were incubated with different concentrations of ENIMF (200, 100, 50, 25, 12.5, 6.25, and 3.25 μg/mL), and after 12 h, LPS (200 ng/well) was added. After 24 h, the supernatant was collected and quantified with the Griess reaction method. To evaluate the production of NO *in vivo*, an aliquot of peritoneal exudate of ENIMF-treated mice was centrifuged at 400 *g* for 10 min at 10 °C and then incubated with Griess reagent as described above. The reading was obtained at 570 nm (SpectraMax F3, Molecular Devices). The results were compared to a nitrite curve (200 to 1 μM) and expressed as μM/mL.

2.11 Statistical analyses

All data analyses and graphical representations were performed by GraphPad Prism 6.0 program. The data are expressed as the means ± standard errors of the means (SEM). The means of the different treatment groups were compared by analysis of

variance (ANOVA). If significance was detected, individual comparisons were subsequently tested with Bonferroni's *t*-test for unpaired values. A *p*-value of < 0.05 was considered significant.

3 Results

3.1 Cytotoxic activity

The hemolysis assay was used to assess whether ENIMF could induce direct damage to the cell membrane. Table 1 demonstrates a weak hemolytic activity of ENIMF, even at the highest concentrations, as compared to that of the positive control (Triton X-100). The cellular toxicity of ENIMF was evaluated by MTT assay in murine macrophages (Table 1). ENIMF did not induce cytotoxicity after 24 h and 48 h of macrophage incubation. The cell viability was similar to that in the non-treated cells. However, the cells treated with DMSO 2% showed a reduction in viability at 24 h (50.6 ± 4.1%) and 48 h (54.1 ± 5.8%) as compared to that of the non-treated group. The data suggested the possibility of using ENIMF in *in vivo* experiments.

3.2 Genotoxic activity

To evaluate the genotoxic activity of ENIMF in murine erythrocytes, the micronucleus assay was performed. Figure 2 shows that ENIMF (5 mg/kg) did not induce an increase in micronucleus formation in the polychromatic erythrocytes as compared to that in the colchicine treated group. ENIMF-treated mice showed that the number of micronucleated cells (0.8 ± 0.4) was similar to that of the vehicle-treated group.

3.3 Anti-inflammatory activity

3.3.1 Cellular signs of inflammation

The effects of ENIMF on the cellular signs of inflammation were investigated. Figure 3 shows that ENIMF, in all the tested doses, could prevent carrageenan-induced leukocyte migration to the peritoneal cavity of mice (Figure 3A). The inhibition was 64%, 63%, and 61% at concentrations of 5, 2.5, and 1.25 mg/kg, respectively, as compared to the non-treated group (Figure 3A). Since neutrophils are the first-line immune cells recruited into the inflammatory site, the cell migration was evaluated. The data show that ENIMF reduced the recruitment of neutrophils to the mice peritoneal cavity as compared to the control group. ENIMF reduced (66%, 61%, and 65%) the number of neutrophils at concentrations of 5, 2.5, and 1.25 mg/kg, respectively (Figure 3B). The neutrophil migration observed in the ENIMF-treated mice was similar to that

in the dexamethasone-treated group (used as the anti-inflammatory standard drug). Based on this data, the ENIMF dose of 5 mg/kg was selected for subsequent experiments.

3.3.2 Vascular signs of inflammation

The effects of ENIMF on vascular signs of inflammation were investigated by Evans blue-labeled plasma protein extravasation assay. The results demonstrated that ENIMF (5 mg/kg) was able to reduce the carrageenan-induced increase in vascular permeability by 24% as compared to that in the control group. The ENIMF-treated mice showed exudation (1.3 ± 0.05) similar to that in the control group that received vehicle (1.1 ± 0.02) (Figure 4A). Because edema is a consequence of increased vascular permeability during inflammation, carrageenan-induced paw edema in the mice was evaluated. Figure 3 showed that ENIMF, at a dose of 5 mg/kg, inhibited edema at all-time points as compared to that in the untreated group. The anti-edematogenic effect of ENIMF after 4 h of treatment was 66% as compared to the control treatment (Figure 4B). This effect was similar to that of dexamethasone. DMSO (2%) used as the vehicle showed similar results as that in the saline-treated group (data shown in the supplementary material).

3.4 NO production

The anti-inflammatory activity of ENIMF was also evaluated based on NO production by macrophages stimulated with LPS. The results demonstrated that ENIMF caused a reduction in nitrite macrophage production at all doses in a dose-dependent manner (Figure 5A). To confirm that ENIMF could inhibit NO production, the nitrite concentration was assessed in the peritoneal exudate of the ENIMF-treated mice (Figure 5B). The data show that ENIMF, at concentrations of 5 and 2.5 mg/kg, reduced the concentration of nitrite (3.2 ± 0.9 and 2.5 ± 1.5, respectively) which was similar to that in the negative control group (0.7 ± 0.3). The data corroborate the results of *in vitro* NO production.

4 Discussion

The bioactivity of synthetic compounds is closely related to the profile of the substituents attached in their structure and may include a wide range of biological activities (Gonzalez et al., 2009; Sondhi et al., 2006). Among the compounds with potential pharmacological applications, nitro-derivatives are highlighted because they have an important pharmacophoric moiety in their molecules, which facilitate their use

in medicinal chemistry and pharmacology. ENIMF is a part of a class of compounds called imines or Schiff bases and has a nitro group (NO_2) in its structure. In nitroaromatic compounds, one or more nitro groups are directly attached to the aromatic ring system (Patterson and Wyllie, 2014). In the aromatic ring, it has an electron withdrawing group that can interact with proteins, amino acids, nucleic acids, and enzymes (Nepali et al., 2018; Olender et al., 2018; Strauss, 1979). Studies have shown that compounds containing a nitro group have a variety of biological activities including anti-*Trypanosoma cruzi* (Almeida et al., 2018), antifungal (Andrade et al., 2019), and anti-leishmania (Mendonça et al., 2019) activities.

The nitro group can induce toxicity (Nepali et al., 2018), and consequently, limits its use in drug designing. The toxicity of nitro-group correlates with ROS production by enzymatic reduction of the nitro groups, such as nitro radical anion, nitroso, and hydroxylamide. In the biological systems, these intermediates may react with the biomolecules and lead to adverse effects, such as mutagenicity and carcinogenesis (Olender et al., 2018; Plošnik et al., 2016). The possible toxic effect of the nitro group can be modified by modulating stereoelectronic properties and the appropriate position of the group and its substituents (Landge et al., 2016; Purohit and Basu, 2000).

Firstly, the toxicity of ENIMF to murine erythrocytes and macrophages was investigated. The data demonstrated that ENIMF did not induce erythrocyte membrane lysis and did not alter the viability of macrophages at 24 h and 48 h post exposure. Likewise, ENIMF also did not cause polychromatic micronucleus formation, suggesting that it does not trigger DNA damage during mitotic division. The absence of a genotoxic effect may be related to the capacity of ENIMF to inhibit NO synthesis. Once a high level of NO increases peroxynitrite production, it triggers undesirable effects on the target cells, such as cytotoxicity and mutagenicity (Kim, 2017). This preliminary data indicate that ENIMF can be used in *in vivo* studies.

Following this, the anti-inflammatory effects of ENIMF were evaluated. The onset of the inflammatory process is characterized by a cellular and vascular response in which a series of mediators trigger various processes, such as vessel dilation, increased capillary permeability, hyperemia, leukocyte recruitment, and nociceptor sensitization (Pober and Sessa, 2007). Though neutrophils are the first line of defense of the host organism, a high neutrophil count is detrimental in inflammation-related diseases (Headland and Norling, 2015). In this context, drugs capable of reducing the number of

neutrophils are crucial in controlling this condition. The data showed that ENIMF inhibited the recruitment of neutrophils to the mice peritoneal cavity, suggesting the anti-inflammatory effects of the compound on the cellular signs of inflammation.

The anti-inflammatory effects of ENIMF were also investigated on the vascular signs of inflammation. The results elucidated that ENIMF reduced vascular permeability and carrageenan-induced paw edema. Under normal physiological conditions, the endothelium allows selective transport of substances and is impermeable to macromolecules, such as albumin. In the presence of inflammation, this selective transport is affected, resulting in vascular leakage and, consequently, the development of edema (Radu and Chernoff, 2013; Yao et al., 2018). The results suggest that ENIMF can effectively inhibit the inflammatory response.

The data demonstrate that ENIMF inhibits leukocyte recruitment, exudation, and edema on carrageenan-induced models of inflammation. The mechanisms involved in the anti-inflammatory effects of ENIMF can be related to the inhibition of NO production. ENIMF inhibited NO production both in the peritoneal exudate and LPS-stimulated macrophage culture. The macrophages and neutrophils present in the peritoneal exudate are the possible sources of NO production during inflammation (Biswas et al., 2001; Wright et al., 1989).

NO is a mediator with several functions in the biological system. It exists in three different isoforms, of which two are constitutive forms (*e*NOS: responsible for the vascular tone and leukocyte aggregation and *n*NOS: acts as a neuromodulator) and one is an inducible form (*i*NOS: produced by macrophages and other cells and plays important roles in inflammatory processes). NO has a dual role in inflammation and may have pro- and anti-inflammatory characteristics. NO can activate and deactivate the gene expression of a diverse range of proteins, thereby modulating the activation profile of immune cells (Connelly et al., 2001). Inflammatory stimulus induces the production of high levels of NO, which regulate inflammatory signals such as vascular permeability, vasodilation, and leukocyte transmigration to the inflamed sites. NO production can be stimulated by pro-inflammatory cytokines, as well as by microbial products, such as LPS (Guzik et al., 2003; Korhonen et al., 2005; Pautz et al., 2010). NO synthesis inhibitors, especially selective *i*NOS inhibitors, are candidates for anti-inflammatory drugs (Coleman, 2001; Guzik et al., 2003; Korhonen et al., 2005).

Schiff base derivatives are reported as potential anti-inflammatory drugs (Arshad et al., 2014; Pandey et al., 2012; Sondhi et al., 2006) and the insertion of a nitro group

seems to augment the anti-inflammatory activities as compared to the other substituents (Ali et al., 2019; Gacche et al., 2006; Geronikaki et al., 2004; Rakesh et al., 2015). The antifungal, antimicrobial, and anti-leishmania activities of ENIMF have been reported (Abirami and Nadaraj, 2015; Al-Kahraman et al., 2010); however, there are no reports on its anti-inflammatory activities. The mechanism of action of anti-inflammatory compounds containing nitro groups is still unknown. Corroborating our data, studies with nitroaromatics have shown reduced NO production in LPS-activated macrophages and decreased expression of *i*NOS, cyclooxygenase-2 (COX-2), interleukin 1-β (IL1-β), and tumor necrosis factor-α (TNF-α) (Tumer et al., 2017). The nitro group also appears to be crucial for the inhibition of NO production (Cowart et al., 1998). Concomitantly, the activity of ENIMF may be related not only to the presence of the nitro group but also to azomethine binding of the Schiff base. It is also considered that the mechanism of action of ENIMF may involve other inflammatory pathways such as prostaglandin biosynthesis inhibition through COX inhibition, phospholipase A2 inhibition, or 5-lipoxygenase inhibition (Patil et al., 2019). In this regard, the present work introduces a new starting point for Schiff bases with nitro derivatives as potential anti-inflammatory agents.

5 **Conclusion**

A nitro-Schiff base was synthesized and evaluated for toxicity and anti-inflammatory activity. Collectively, the results support that ENIMF have an effective anti-inflammatory activity. The results encourage further studies that might help in a better understanding of the mechanism of action of this compound.

Competing interests

All authors declare that they have no conflict of interest.

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Credit Author Statement

Author	Credit Author Statement
Roriz, B	PhD student, responsible for conducting experiments in vivo (anti-inflammatory, analgesic and antimutagenic effect of ENIMF); data analysis, statistical analysis and writing - original draft.
Buccini, D.F	Post-doctoral student, assisted in the performance of in vitro tests of ENIMF (cytotoxicity and hemolytic activity), data analysis and writing - review & editing draft.
Santos, B. F	PhD student, performed the synthesis and analysis of the compound (*E*)-2-(((4-nitrophenyl)imino)methyl) phenol (ENIMF).
Silva, S. R	Master's student, helped to carry out studies to evaluate the genotoxicity of the ENIMF.
Domingues, N.L.C.	Senior researcher responsible for the design and supervision of the synthesis of the ENIMF, data analysis and writing - review draft.
Moreno, S.E	Supervisor of the PhD student Roriz, B., responsible for the conceptualization nd supervision of the project, data formal analysis; funding acquisition, writing – review draft.

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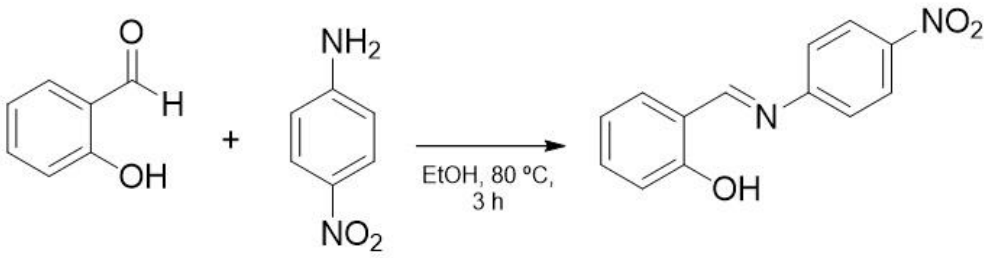

Figure 1. Synthesis of (*E*)-2-(((4-nitrophenyl)imino)methyl)phenol (ENIMF)

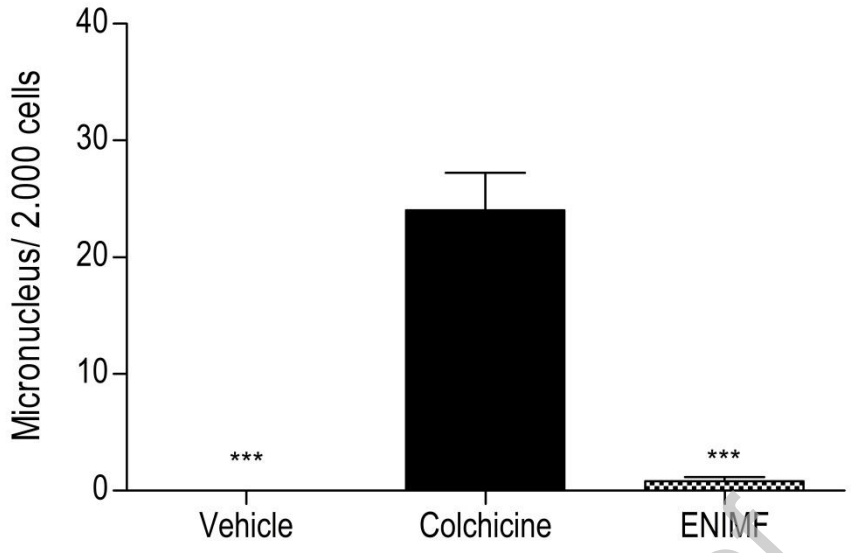

Figure 2. ENIMF did not induce micronucleus formation in the erythrocytes of mice peripheral blood. The mice received vehicle, colchicine (5 mg/kg), or ENIMF (5 mg/kg). After 24 h, the micronucleus frequency in the erythrocytes was evaluated. The results are expressed as the number of micronuclei in 2000 cells (mean ± SEM). One-way ANOVA followed by Bonferroni test was performed. $^{***}P < 0.001$ as compared to the control group treated with colchicine.

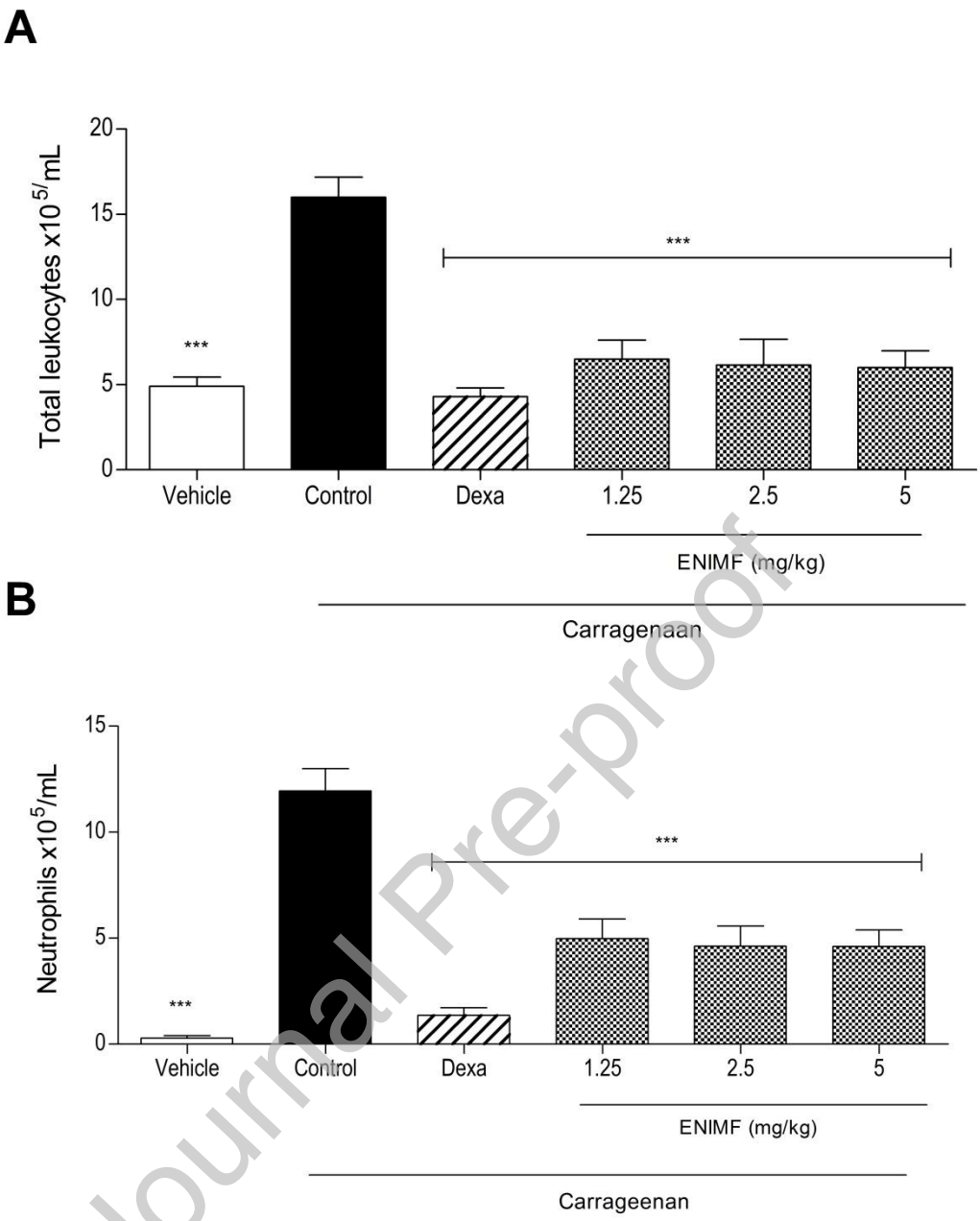

Figure 3. Inhibitory effects of ENIMF on the neutrophil migration to the inflammatory sites. The mice were pretreated with vehicle, ENIMF (1.25, 2.5, and 5 mg/kg) or dexamethasone (0.4 mg/kg) before injecting 1.5mg carrageenan (inflammatory stimulus). After 6 h, the total and differential cell counts were performed. **(A)** The number of total leukocytes and **(B)** the number of neutrophils immigrated into the mice peritoneal cavity are shown. The results are expressed as the cell number × 10^5/mL (mean ± SEM). One-way ANOVA followed by Bonferroni test was performed. $^{***}P<0.001$ as compared to the control group.

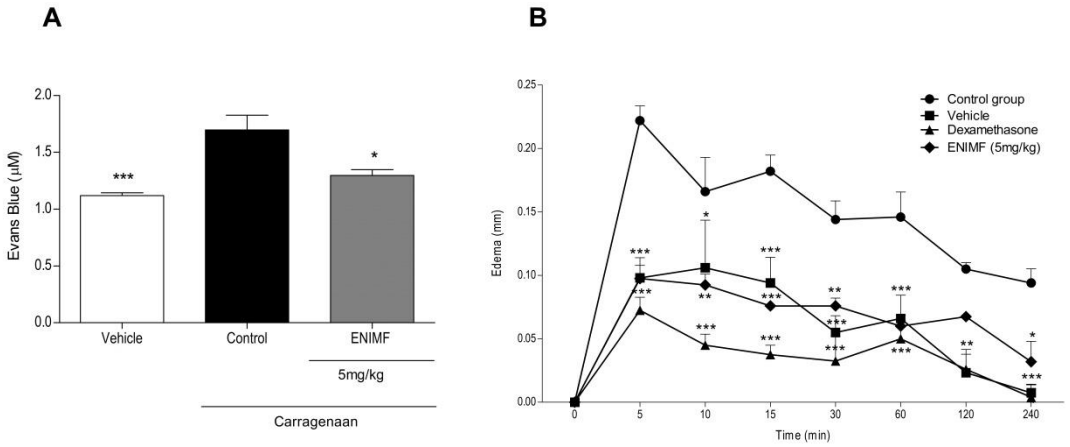

Figure 4. Inhibitory effects of ENIMF on exudation and edema. **(A)** Evaluation of the effects of ENIMF on the vascular permeability by Evans blue dye assay. The mice were pretreated with vehicle or ENIMF (5 mg/kg) before injecting 1.5% carrageenan (inflammatory stimulus). Evans blue dye was administered. After 4 h, quantification of the extracted Evans blue dye was performed. The results are expressed as Evans blue dye (µM/mL) (mean ± SEM). One-way ANOVA followed by Bonferroni test was performed. $^{***}P < 0.001$, and $^{*}P < 0.05$ as compared to the control group. **(B)** Evaluation of the effects of ENIMF on carrageenan-induced paw edema. The mice received vehicle, ENIMF (5 mg/kg), or dexamethasone (0.4 mg/kg). Carrageenan 1.5% (inflammatory stimulus) was injected into the left paw of all the mice, except the vehicle group that received the saline solution. The readings were recorded at 0, 5, 10, 15, 30, 60, 120, and 240 min after carrageenan injection. The results are expressed as the variation (Δ) in paw volume (mean ± SEM). Two-way ANOVA followed by Bonferroni test was performed. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared to the control group.

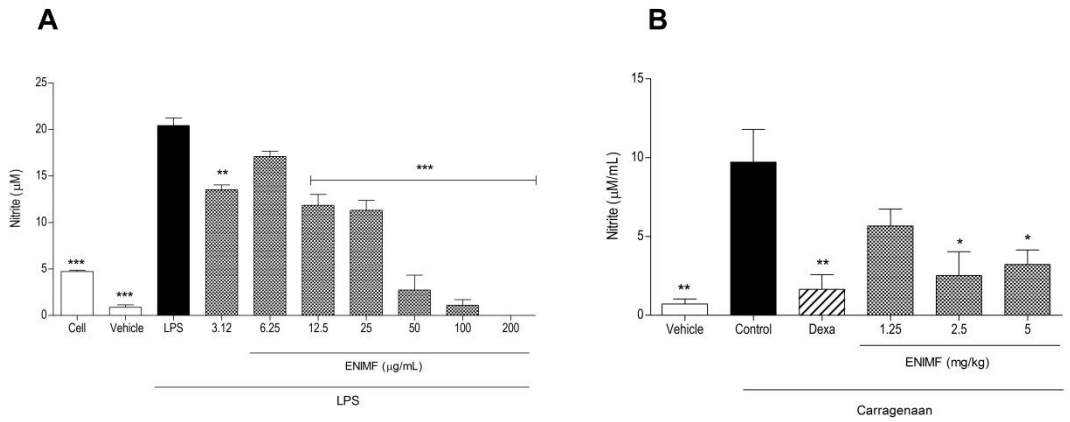

Figure 5. Effects of ENIMF on NO production. **(A)** NO production in the macrophages stimulated by LPS (200 ng) for 24 h. **(B)** NO production in the exudate of mice pretreated with vehicle, dexamethasone (0.4 mg/kg), or ENIMF (1.25, 2.5, and 5 mg/kg) and then subjected to an inflammatory stimulus (carrageenan 1.5 mg). Nitrite was measured by the Griess reaction method. The results are expressed as nitrite (µM/mL) (mean ± SEM). One-way ANOVA followed by Bonferroni test was performed. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ as compared to the control group.

Table 1. Evaluation of the *in vitro* cytotoxicity of ENIMF.

Treatment	**Hemolytic assay[a]**	**Cell viability at 24 h[b]**	**Cell viability at 48 h[c]**
Negative control	12.2 ± 0.9***	100 ± 8.6	100 ± 11.1
Vehicle	15.5 ± 0.6***	50.5 ± 4.1*	54.1 ± 5.8*
Positive control	100 ± 1.4	----------	----------
ENIMF (µg/mL)			
200	38.7 ± 8.1***	75.0 ± 4.8	79.6 ± 8.5
100	27.5 ± 5.8***	90.4 ± 4.4	68.7 ± 10.4
50	16.6 ± 0.5***	117.1 ± 2.5	104.9 ± 12.2
25	15.4 ± 4.0***	86.7 ± 1.9	80.5 ± 10.9
12.5	15.5 ± 1.1***	135.3 ± 0.0	120.4 ± 9.0
6.25	10.2 ± 1.2***	93.8 ± 4.6	106.0 ± 3.3
3.12	11.0 ± 0.6***	141.5 ± 14.6	134.6 ± 3.8

(a) Hemolytic activity in the murine erythrocytes. ***P <0.001 as compared to Triton X-100 (positive control). **(b)** and **(c)** Cell viability in ENIMF-treated murine macrophages at (b) 24 h and (c) 48 h. *P <0.05 as compared to the control (untreated). The results are expressed as percentages (mean ± SEM). One-way ANOVA followed by Bonferroni test was performed.

Graphical Abstract