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Tropane Alkaloid Isolated from Erythroxylum bezerrae Exhibits Neuropharmacological Potential in an Adult Zebrafish (Danio rerio) Model

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■■■Dear author, please mention the academic titles of the authors.■■■This study carried out to investigate the anti-inflammatory and antinociceptive effect of tropane alkaloid (EB7) isolated from *E. bezerrae*. It evaluated the toxicity and possible involvement of ion channels in the antinociceptive effect of EB7, as well as its anti-inflammatory effect in adult zebrafish (Zfa). Docking studies with EB7 and COX-1 and 2 were also performed. The tested doses of EB7 (4, 20 and 40 mg/kg) did not show any toxic effect on Zfa during the 96h of analysis (LD50 > 40 mg/kg). They did not produce any alteration in the locomotor behavior of the animals. Furthermore, EB7 showed promising pharmacological effects as it prevented the nociceptive behavior induced by hypertonic saline, capsaicin, formalin and acid saline. EB7 had its analgesic effect blocked by amiloride involving the neuromodulation of ASICs in Zfa. In evaluating the anti-inflammatory activity, the edema induced by κ-carrageenan 3.5 % was reduced by the dose of 40 mg/kg of EB7 observed after the fourth hour of analysis, indicating an effect similar to that of ibuprofen. Molecular docking results indicated that EB7 exhibited better affinity energy when compared to ibuprofen control against the two evaluated targets binding at different sites in the cocrystallized COX-1 and 2 inhibitors.

Introduction

Pain is one of the classic signs of inflammation, as inflammatory processes activate the nociceptive system, which generally helps homeostasis in the body and contributes to reducing the action of the agent causing damage, which is vital for its survival. To this end, specialized receptors are sensitized according to the stimulus generated and transmit alert information to the central nervous system (CNS), which plays an important role in the transmission between the body's external and internal environment, generating sensory experiences that trigger a cascade of reactions that can be expressed psychologically or behaviorally.[1]

Inflammation is a natural biological response of the organism, through which the host is protected against tissue damage and pathogen invasion, which can prevent the spread of these pathogens and even promote tissue repair.[2] Inflammation can be caused by various stimuli, including biological

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agents, chemical substances such as carrageenan and formaldehyde, and physical agents, as well as tissue malformation.[3] Flushing (redness), heat, swelling, and pain are classic signs of inflammation. The reaction is microscopically apparent in tissue at an affected site, such as the accumulation of neutrophils, monocytes, macrophages, and/or lymphocytes in a structure that may be disordered by edema, necrosis, fibrosis, lipidosis, malignancy, or infection.[4]

In this context, conventional anti-inflammatory therapy is based on drugs capable of inhibiting the production or action of mediators or preventing the recruitment and activation of neutrophils. Thus, the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and diclofenac, which act by inhibiting enzymes of the cyclooxygenase (COX) family, is common to limit the intensity of pain and improve the recovery process.[5]

The use of the zebrafish model in screening studies has made it possible to reduce the use of mammalian animals and, consequently, speed up research results, and Due to its similarity to other vertebrates in the development and organization of peripheral and central nociceptive processing systems. The zebrafish has a complex physiological system that recognizes and responds to painful stimuli, including various subtypes of nociceptors already identified with nociceptive circuit organization similar to that of mammals. It is important to note that the zebrafish expresses genes involved in pain responses and also exhibits specific protective pain behaviors when exposed to noxious stimuli, suggesting the existence of central mechanisms underlying pain responses. Regardless of the ways in which animal models are used for research, the investigator must adhere to the principles of the 3Rs: to minimize the number of animals used for scientific experiments without compromising the reliability of the results; to refine and improve the experimental conditions in which animals are involved; and, whenever possible, to replace the animal model with an alternative model. In this scenario, the zebrafish is gaining prominence for presenting specific experimental advantages over other animal models. Attributes such as genetic, anatomical, and physiological homology to mammals, external fertilization, high number of progeny, transparency of larvae, small size, and rapid development facilitate large-scale phenotypic approaches, while still maintaining the ability to adhere to the 3Rs. Thus, the use of the zebrafish model allows for the replacement, reduction, and refinement of the use of mammals in research, as well as the reduction of welfare-related issues for these animals.[4]

Indeed, new biologically active compounds are constantly being discovered and subsequently applied in sectors such as medicine and health. Among these bioactive molecules, alkaloids that have the tropane nucleus are among the oldest molecules used by humans. Most of them are esters of mono-, di-, tri-hydroxytropane, having a wide range of hydroxylation arrangements. It is noteworthy that alkaloids have some unique chemical characteristics that make them interesting candidates for use in medicine. They have basic properties, which make them soluble in water under acidic conditions and liposoluble under neutral and basic conditions. This is especially important for dissolution in the protonated form and membrane permeation in the deprotonated form. With these properties, alkaloids have been used in various applications in the treatment of plant and human diseases because they show strong biological effects in very small doses, such as anti-inflammatory, anticancer, analgesic, local anesthetic, neuropharmacological, antimicrobial, and antifungal activities.[5] In general, they have characteristics of neurotransmitters, demonstrating a role in the regulation, stimulation and induction of functions. These metabolites can interact with various targets, such as Na^+ channels, muscarinic receptors, acetylcholinesterase, and opioid receptors.[6]

In this context, plants of the genus *Erythroxylum* are responsible for biosynthesizing tropane alkaloids, which have shown biological activities such as anticholinergic, antiemetic, antidepressant, anesthetic and antitumor.[7] Thus, given recent progress in wellness science and the development of new drugs and analgesic techniques, the present study investigated the compound 3α-(3′,4′,5′-trimethoxycinnamoyloxy)-6β(3″,4″,5″-trimethoxybenzoyloxy)-7β-hydroxy-tropane (EB7) isolated from the bark of *Erythroxylum bezerrae*. This study evaluated the possible involvement of ion channels (TRPV1 – transient receptor potential vanilloid type 1, TRPA1 – transient receptor potential ankyrin 1 and ASIC – acid sensitive ion channels) in the antinociceptive behavioral action of EB7, as well as its anti-inflammatory effect.

Results and Discussion

There were no deaths or apparent anatomical changes in the animals during the analysis period of the acute toxicity study ($p > 0.05$). Thus, the tested doses of EB7 (4, 20 and 40 mg/kg) have pre-clinical safety as they did not show any toxic effect on Zfa during the 96 h of analysis (LD50 > 40 mg/kg).

The zebrafish has been used as a valuable model to evaluate drug candidates for toxicity and safety risks. This is related to the fact that this model shares physiological, morphological and histological similarities with mammals. Thus, Zfa was used as a model organism to evaluate the toxic effect of EB7. It should be noted that this is the first study that evaluated the acute toxicity (96 h) in Zfa of the species *Erythroxylum bezerrae*. As a result of acute toxicity, all tested doses of EB7 proved to be safe, as they were not toxic and there was no apparent anatomical change in the animals during the analysis period against ZFa.

The evaluation of the effect of EB7 on Zfa locomotor activity was investigated. Magalhães et al. (2017) adapted the open-field method performed in an aquarium for Zfa in Petri dishes to assess comprehensive locomotor and behavioral changes from the effects of analgesic drugs. Therefore, the same method was used to evaluate the action of EB7 on the Zfa locomotor system.

As shown in Figure 1, EB7 did not alter the locomotion of the adult Zfa in the open field test, as the treated animals showed locomotor activity significantly similar to that of the untreated group ($p > 0.05$ vs. Naive).

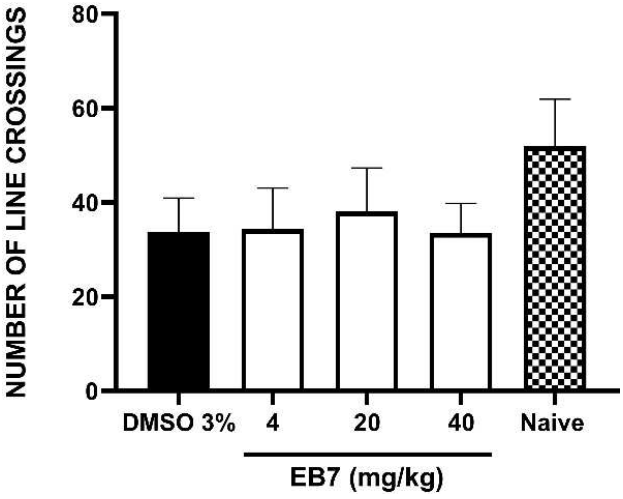

Figure 1. Effect of EB7 on Zfa locomotor behavior activity in the open field test. Each column represents the mean ± standard errors of the mean (n = 6/group) analyzed individually over 0–5 min. DMSO 3% (vehicle; 20 μL; i.p.). EB7 (4; 20 or 40 mg/kg; 20 μL; i.p.). Naive: untreated animals. One-way ANOVA followed by Tukey's test showed no difference between groups ($p > 0.05$ vs. naive).

Nociception is the physiological component of pain[1] and is associated with neural activation and sensory response. In this way, nociception is used in animal experimentation models of pain, in which the object of study is neural signaling and response to noxious stimuli.[32] Evidence indicates that pain-induced behavioral changes in fish are conserved across vertebrates. Zebrafish is rapidly emerging as a promising model organism to study nociceptive responses.[4,33,34] Homologs to human nociceptors, Transient Receptor Potential (TRP) ion channels (e.g., TRPA1 and TRPV1), acid sensing ion channels (ASICs) have already been characterized in zebrafish.[4,8,35–39] It investigated the effect of compound EB7 isolated from *E. bezerrae* on nociceptive behavior induced by formalin (TRPA1 channel agonist), capsaicin (TRPV1 channel agonist), hypertonic saline (TRPV1 channel agonist) and acid saline (ASIC channel agonist). TRPA1 are receptors that mediate sensitivity to harmful mechanical, thermal and chemical stimuli[40] such as cinnamaldehyde, allicin and formalin[41–43] and act in various isoforms, including humans, mice and zebrafish[44] investigated the locomotor behavior of Zfa larvae with the hypothesis that hyperlocomotion induced by activation of the zebrafish TRPA1 can serve as a phenotypic screen for discovering new anti-nociceptive drugs. This work used formalin as a chemical agent to induce nociception in Zfa. Nociception through activation of the TRPA1 receptor by formalin action has already been investigated in adult zebrafish through the intramuscular application (tail) of this noxious agent.[4,12,14]

One-way ANOVA statistical test followed by Tukey's test indicated that pre-treatment with EB7 prevented (**$p < 0.01$ vs 3% DMSO) the nociceptive behavior of hypertonic saline only at the lowest dose ■■■Dear author, please mention Figures 2, 3, 4, 5 to 6 before Figure 7.■■■(Figure 7A) while higher doses prevented (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ vs 3% DMSO) the harmful behavior of capsaicin and acid saline (Figure 2B and E), and the highest dose prevented (*$p < 0.05$; ***$p < 0.001$ vs 3% DMSO) the noxious behavior of formalin in the neurogenic and inflammatory phases (Figure 7C and D). Morphine pre-treatment prevented (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ vs 3% DMSO) noxious behavior in all nociceptive models analyzed (Figure 2).

The lowest and best effective doses of EB7 responsible for preventing the harmful behavior of acid saline, capsaicin, formalin and hypertonic saline were evaluated in the mechanism of action with antagonists (the 40 mg/kg doses of the formalin model in the neurogenic and inflammatory phases, as well as a dose of 4 mg/kg in the hypertonic saline model and doses of 20 mg/kg in the acid saline and capsaicin models).

The analgesic effect of EB7 via the TRPV1 channel was investigated using capsaicin and hypertonic saline models. Capsaicin is a hydrophobic molecule that directly activates the TRPV1 channel by binding to an intracellular site on the channel protein.[45] Because two amino acid residues (Ser-512 and Thr-550) required for capsaicin-mediated activation of TRPV1 in mammals are different at the analogous positions of TRPV1 in zebrafish (Thr-480 and Ile-518), zebrafish larvae do not show behavioral changes after administration of capsaicin by immersion in water.[36] However, pain behaviors can be observed after a single administration of capsaicin to the lips of adult zebrafish, and classic TRPV1 antagonists prevent these responses.[4,8,46,47] Furthermore, studies with adult Zebrafish indicate that capsaicin applied to the tail (i.m.) causes a noxious stimulus, reducing the animals' locomotion in the open field test in a petri dish.[12,14] EB7 blocked the noxious effect caused by capsaicin, demonstrating an analgesic effect at the highest doses analyzed (20 and 40 mg/kg) by recovering the animals' locomotor activity when compared to the control group (Figure 2B). However, this antinociceptive effect of EB7 was not blocked by pre-treatment with ruthenium red (non-specific TRPV1 channel antagonist), evidencing the non-neuromodulation of EB7 via TRPV1 neurotransmission in adult zebrafish.

According to [48], channels are cellular sensors involved in corneal formation and detecting harmful sensations. Corneal nociceptive fibers collectively respond to different chemical stimuli by activating TRPV1 channels. Studies confirm the induction of nociception by hypertonic saline solution applied to the corneal surface of zebrafish.[4,12,14,47,49] This test was performed to ascertain the effect of EB7 on corneal Zfa nociception. EB7 blocked the harmful effect caused by hypertonic saline on the cornea, demonstrating an analgesic effect at the lowest analyzed dose (4 mg/kg). On the other hand, this analgesic effect was not blocked by ruthenium red (non-selective antagonist of vanilloid receptors), indicating that EB7 did not present an antinociceptive effect by neuromodulating the TRPV1 channel under the described conditions.

Statistical analysis One-way ANOVA followed by Tukey's test indicated that pre-treatment with amiloride abolished (####$p < 0.0001$ vs. EB7) the antinociceptive effect of EB7 (Figure 3E), indicating that the EB7's antinociceptive effect occurs through the neuromodulation of ASIC channels. Pre-treatment with ruthenium red and camphor did not abolish the antinociceptive

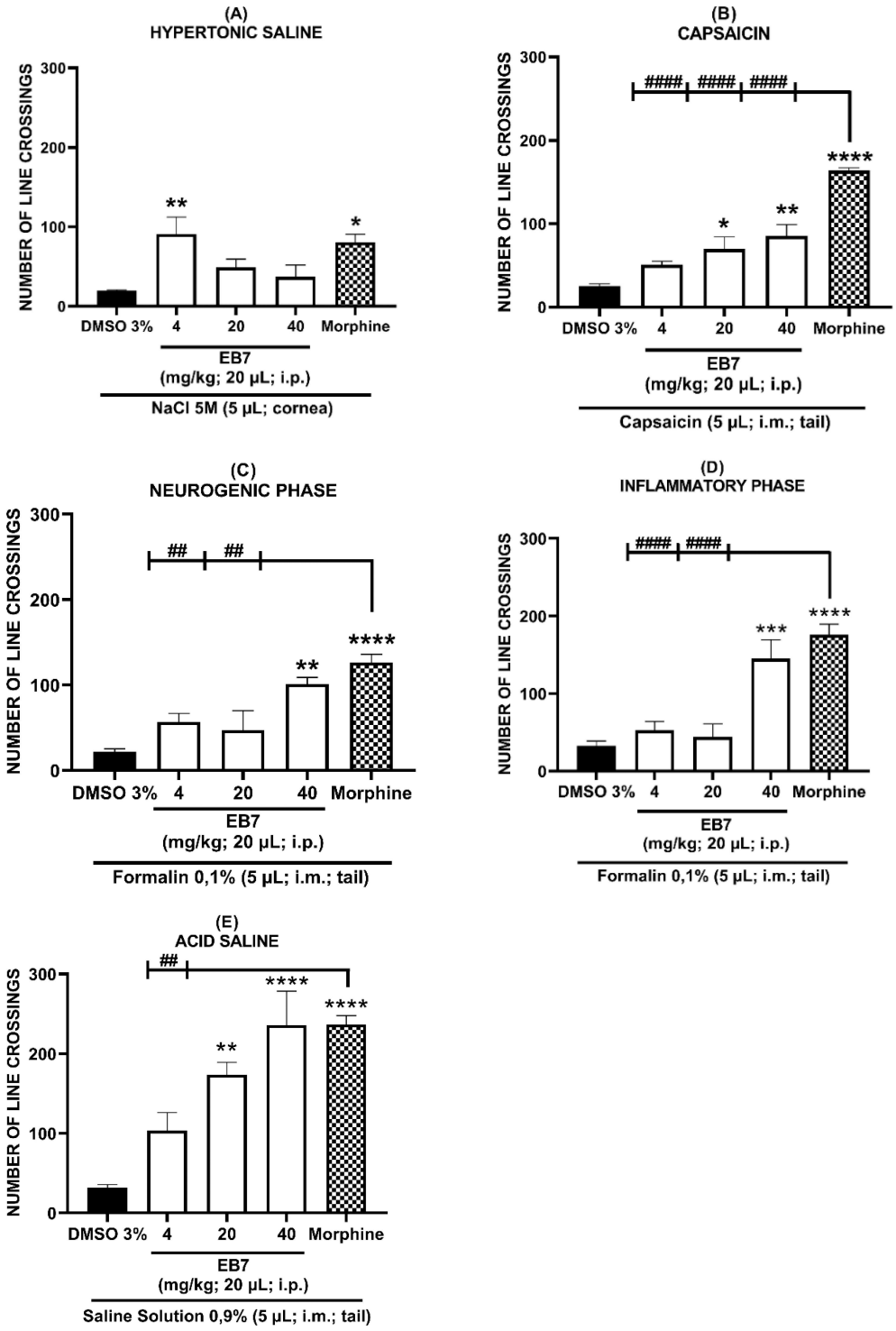

Figure 2. Effect of EB7 on the behavior of nociception induced in hypertonic saline (0–5 min) (A), capsaicin (10–20 min) (B), formalin neurogenic phase (0–5 min) (C), formalin inflammatory phase (15–30 min) (D) and acid saline (0–20 min) (E), in adult zebrafish. Each column represents the mean ± standard errors of the mean (n = 6/group). One-way ANOVA followed by Tukey's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs DMSO 3%. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs. Morphine).

effect of EB7 on nociception induced by hypertonic saline, capsaicin and formalin, respectively (Figure 3A, B, C and D).

ASICs are Na+ channels controlled by extracellular H+ and are widely expressed in mammals' central and peripheral nervous systems. Six ASICs have been characterized in zebrafish (zASICs; zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1 and zASIC4.2) that share up to 75% of amino acid sequences with those of rats and humans. Several substances have been described as potentially activating the ASIC channel through changes in pH.[39] For example, acetic acid induces pain in

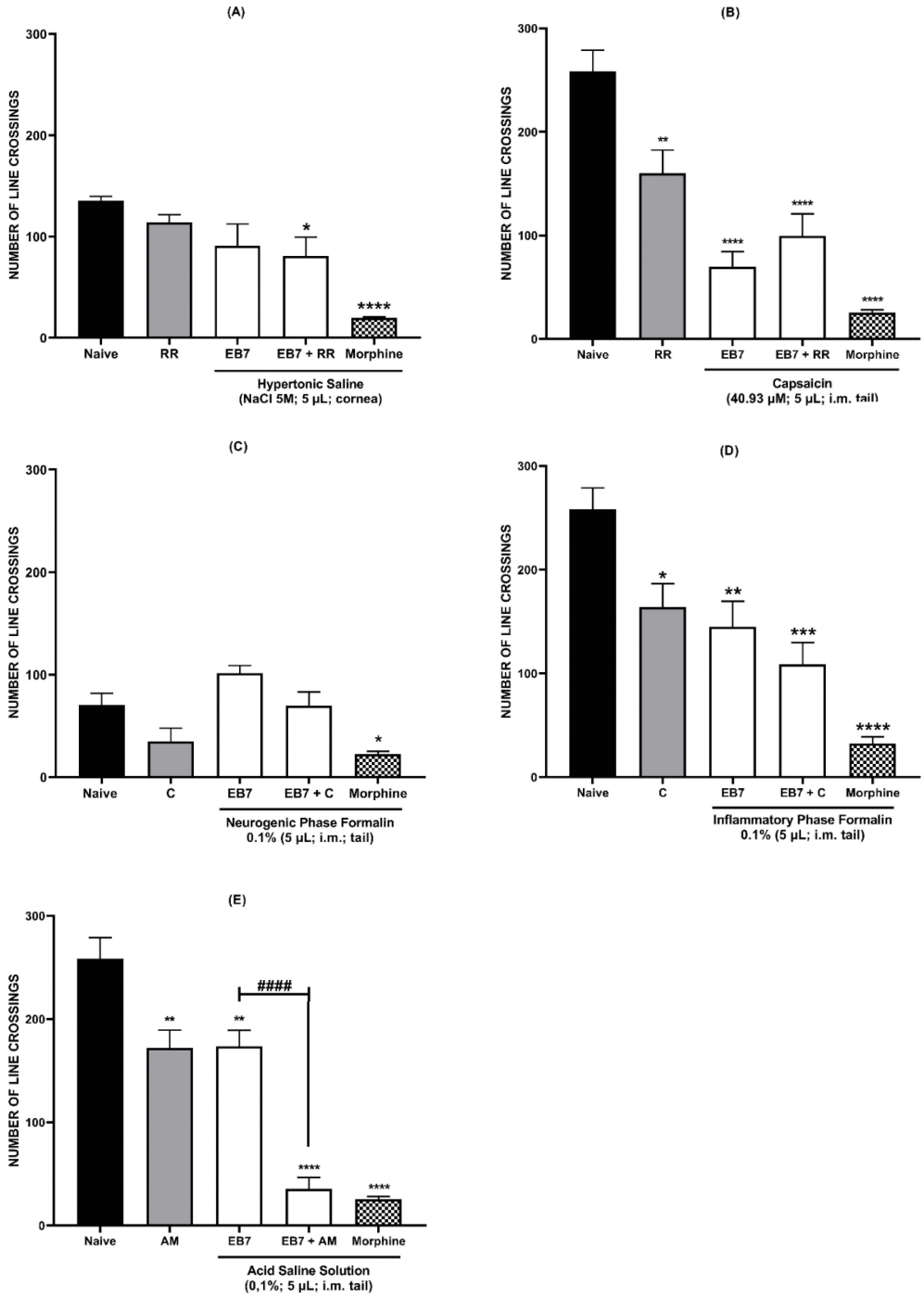

Figure 3. Effect of pre-treatment with ruthenium red (RR), camphor (C) and amiloride (AM) on the antinociceptive effect of EB7 in adult zebrafish, analyzed through nociception induced by hypertonic saline (0–5 min) (A), capsaicin (10–20 min) (B), formalin neurogenic phase (0–5 min) (C) and formalin inflammatory phase (15–30 min) (D) and acidic saline (0–20 min) (E) in zebrafish adult. Naive: untreated group. Each column represents a mean ± standard error of the mean (n = 6/fish). Two-way ANOVA followed by Tukey's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs Naive. $^{\#\#\#\#}p < 0.0001$ vs. EB7).

several fish species, including zebrafish.[12] For this, an acidic saline solution is used, which causes nociceptive behavior by reducing Zebrafish locomotion by activating ASIC channels when injected into the fish lip or tail.[4,8,12] Human acid-sensitive ion channels (ASIC) are ligand-gated ionotropic receptors expressed widely in peripheral tissues and sensory and central neurons. They are implicated in detecting inflammation, tissue injury, and hypoxia-induced acidosis. This makes ASIC channels promising targets for drug discovery in oncology, pain and ischemia, with several modulators having progressed through clinical trials.[4] The nociception induced by acidic saline was used to evaluate the antinociceptive effect of EB7, which, in the highest doses (20 and 40 mg/kg), prevented the animals from nociception. Furthermore, these effects were blocked by

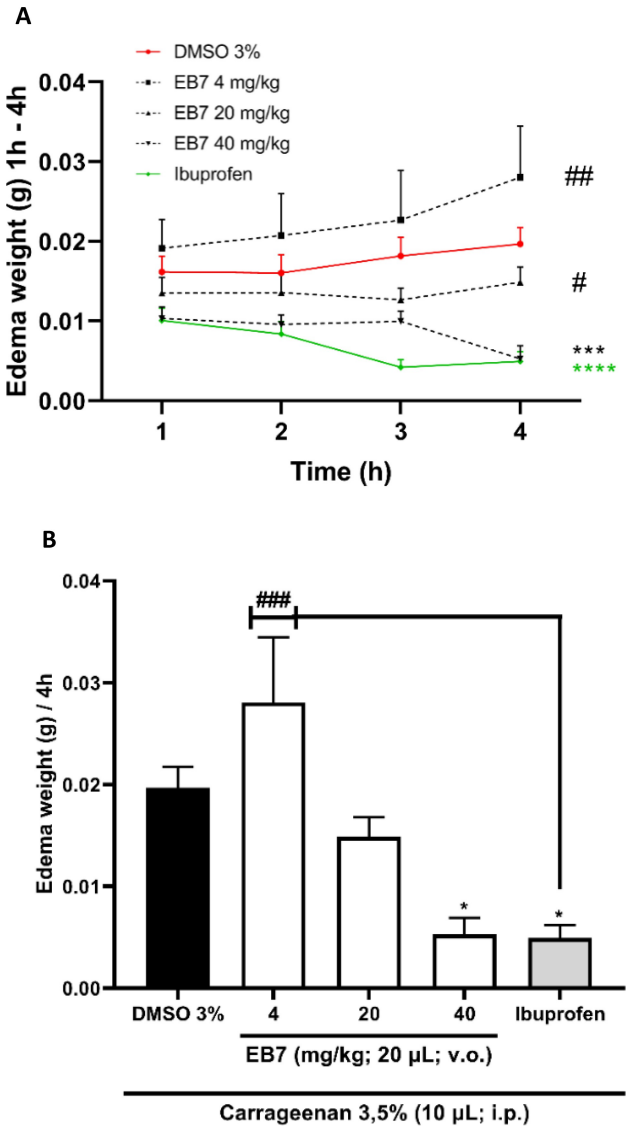

Figure 4. Effect of EB7 on 3.5% κ-CGN-induced abdominal edema in adult zebrafish. A: analysis during the period of 4 h consecutive. Each line represents a mean ± standard error of the mean (n = 6/fish). Two-way ANOVA followed by Tukey's test (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ vs DMSO 3%). ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs. Ibuprofen). B: result showing the fourth hour of analysis. Each column represents a mean ± standard error of the mean (n = 6/fish). Two-way ANOVA followed Tukey's test (*$p < 0.05$ vs. DMSO 3%; $^{\#\#\#}p < 0.001$ vs. Ibuprofen).

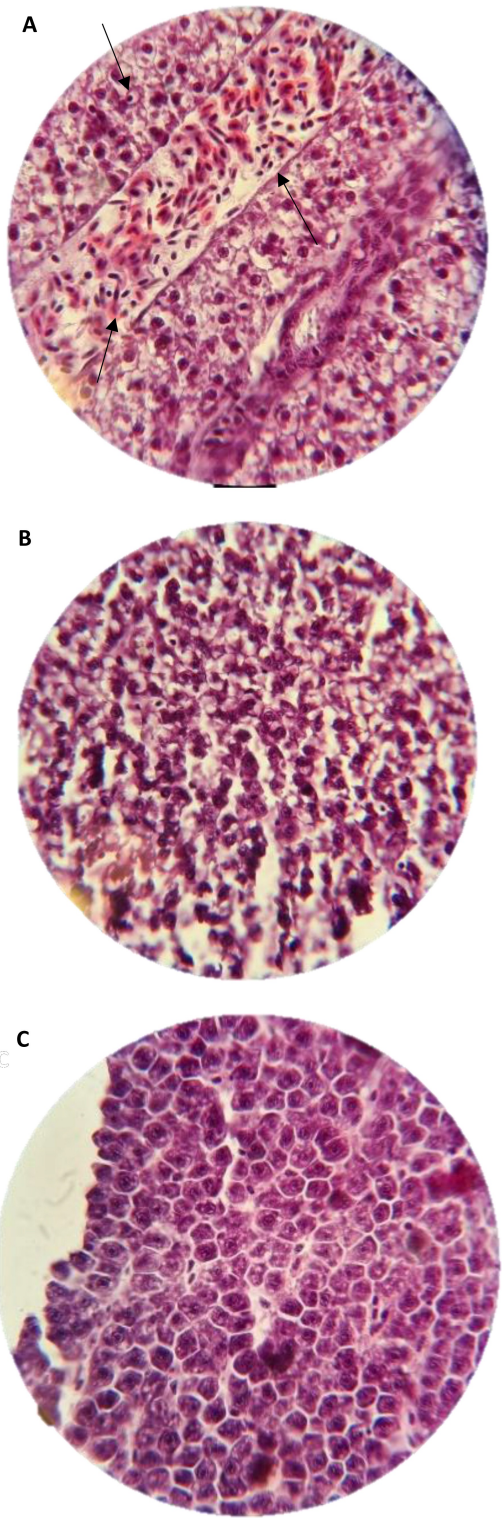

Figure 5. Histopathological analysis. A: Negative control (3% DMSO, 20 µL; p.o.). The presence of leukocyte infiltrates can be observed (black arrow) in the liver of Zfa after 4h of i.p. of κ-carrageenan 3.5%. B: treatment with ibuprofen (positive control – 100 mg/kg). C: treatment with EB7. Objective 100x. Coloring: H&E.

pretreatment with amiloride (ASIC channel antagonist),[50] indicating the efficiency of EB7 in acting as an antinociceptive neuromodulator of the ASIC channel in adult zebrafish.

Carrageenan applied intraperitoneally to adult zebrafish causes edema in the abdominal region as an inflammatory response, resulting from the upregulation of pro-inflammatory proteins and the accumulation of leukocytes in the edema-tous site.[51]

The κ-CGN-induced abdominal edema test was performed to ascertain the anti-inflammatory effect of EB7. The 40 mg/kg dose significantly reduced (***$p < 0.001$ vs. DMSO 3%) the inflammatory effect caused by κ-CGN, similar to the group treated with ibuprofen (****$p < 0.0001$ vs. DMSO 3%)

■■■Dear author, please mention Figure 8 before Figure 9.■■■(Figure 9A), reducing abdominal edema significantly

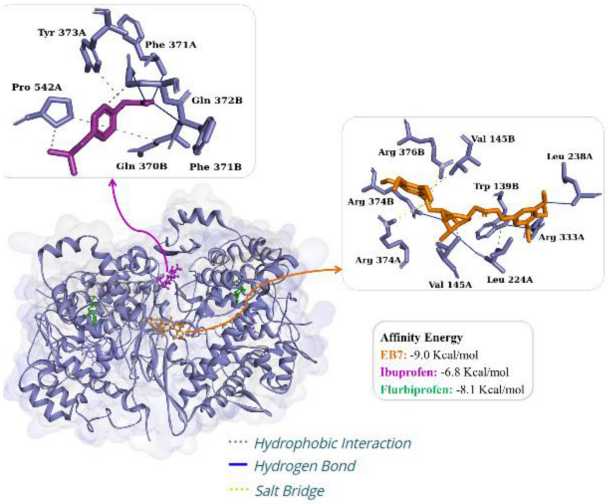

Figure 6. Interaction complex between EB7, Ibuprofen (control) and the co-crystallised inhibitor with COX-1.

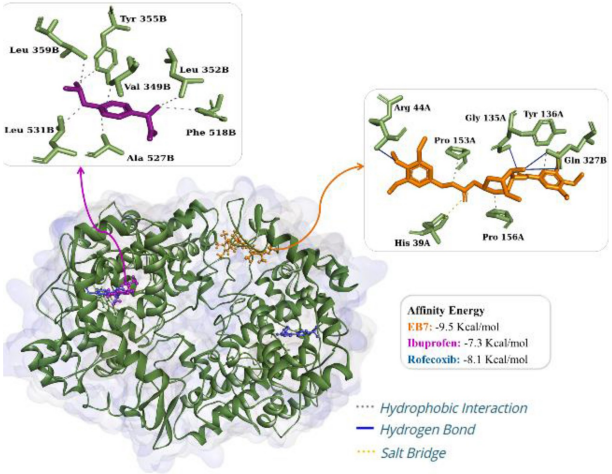

Figure 7. Interaction complex between EB7, Ibuprofen (control) and the co-crystallised inhibitor with COX-2.

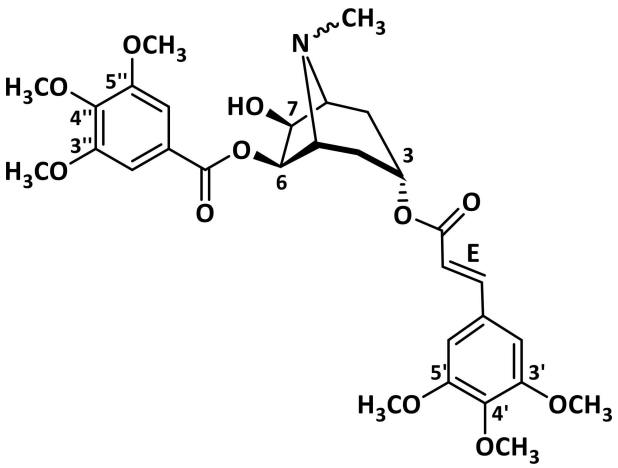

Figure 8. Structural representation of the compound 3α-(3′,4′,5′-trimethoxycinnamoyloxy)-6β(3″,4″,5″-trimethoxybenzoyloxy)-7β-hydroxy-tropane isolated from *E. Bezzerae.*

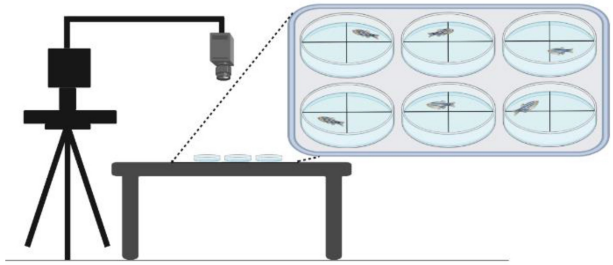

Figure 9. Analysis of locomotor activity by counting line crossings.

($^*p < 0.05$ vs. DMSO 3%) in the fourth hour of analysis (Figure 4).

Pre-treatment of Zfa prevented the animals from edema induced by κ-CGN, indicating a significant acute anti-inflammatory effect after the fourth hour of analysis, a result similar to the effect of ibuprofen, a selective inhibitor of cyclooxygenase (coxib), which in humans it has anti-inflammatory, antipyretic and analgesic activity, inhibiting the synthesis of prostaglandins mediated by one of the COX isoenzymes, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Zebrafish show homology with human COX-1 and COX-2, which are depressed by the action of NSAIDs such as ibuprofen, inhibiting the action of the COX enzyme on arachidonic acid and reducing the formation of prostaglandins, which are pro-inflammatory substances released of cells in tissue injury processes.[52] According to,[53,54] and,[55] the acute inflammatory response presents short-term events occurring in hours or days with a predominance of neutrophils, corroborating the findings in this study.

Histopathological analysis performed on whole Zfa indicates the presence of leukocyte infiltrates in the animals pretreated with 3% DMSO (negative control) after 4 h of analysis and the absence of leukocyte infiltrates in the slides of the groups pretreated with ibuprofen (positive control) and EB7 (40 mg/kg – a dose that showed similar results to ibuprofen in the acute inflammation test, $^*p < 0.05$ vs control), confirming the anti-inflammatory effect of EB7 in adult Zebrafish (Figure 5).

A leukocyte infiltrate resulting from an inflammatory process was observed in the negative control group (3% DMSO). In the positive control group and the group treated with EB7, there was an absence of these infiltrates, thus indicating that the treatment performed with EB7 (40 mg/kg) had a promising anti-inflammatory effect when analyzing the Zfa hepatocytes. A similar study,[4] demonstrates the presence of leukocyte infiltrates in Zfa during 4 hours after application of λ-carrageenan 3.5% also in the liver region, in addition to the formation of peritoneal edema due to the development of inflammatory processes, corroborating with the results of the histopathological slides of this study.

Through the results obtained, it was observed that the best pose presented values of RMSD (Root Mean Square Deviation) in the order of 0.504 Å (EB7) and 0.991 Å (Ibuprofen) against COX-1 ■■■Dear author, please mention Figure 10 before Figure 11.■■■(Figure 11) and the order of 0.278 Å (EB7), 1,590 Å (Ibuprofen) against COX-2 (Figure 6). Regarding the

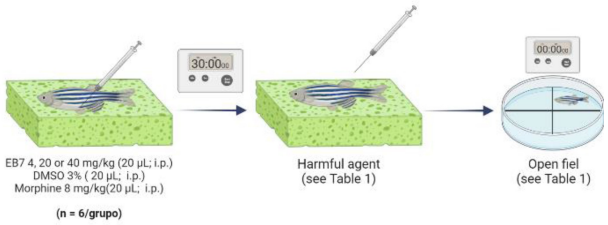

Figure 10. Evaluation of nociceptive behavioral activity through nociception induction.

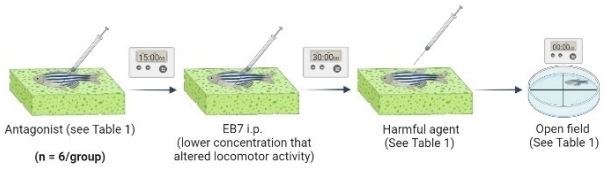

Figure 11. Scheme: mechanism of action of antinociceptive behavioral activity.

affinity energy (kcal/mol), it was ob served values in the order of −9.0 (EB7/COX-1), −6.8 (Ibuprofen/COX-1), −9.5 (EB7/COX-2) and −7.3 (Ibuprofen/COX-2).

Analyzing the interaction patterns, it was observed that the EB7/COX-1 complex had five hydrogen bonds involving the residues Trp 139B (2.87 Å), Leu 224A (2.89 Å), Leu 238A (2.50 Å), Arg 333A (3.04 Å), Arg 374B (2.40 Å), three hydrophobic interactions with Val 145A (3.55 Å), Val 145B (3.55 Å), Leu 224A (3.57 Å) and three Salt Bridges involving residues Arg 374A (5.30 Å), Arg 374B (3.77 Å), Arg 376B (4.67 Å).

The Ibuprofen/COX-1 complex showed five hydrophobic interactions involving residues Gln 370B (3.93 Å), Gln 372B (3.71 Å), Tyr 373A (3.72 Å), Pro 542A (3.69 and 3.79 Å) and three hydrogen bonds involving the residues Phe 371A (2.21 Å), Phe 371B (3.54 Å), Gln 372B (2.02 Å).

The EB7/COX-2 complex showed four hydrogen bonds, involving residues Arg 44A (2.67 Å), Gly 135A (2.60 Å), Gln 327B (2.42 and 3.32 Å), three hydrophobic interactions with Tyr 136A (3.65 Å), Pro 153A (3.56 Å), Pro 156A (3.24 Å) and a Salt Bridges with His 39A (5.08 Å). The Ibuprofen/COX-2 complex showed seven hydrophobic interactions, involving residues Val 349B (3.47 Å), Leu 352B (3.69 Å), Tyr 355B (3.39 Å), Leu 359B (3.73 Å), Phe 518B (3.77 Å), Ala 527B (3.46 Å), Leu 531B (3.35 Å).

The favorable values of affinity energy affirm the viability of forming receptor/ligand complexes. The redocking of the inhibitor flurbiprofen co-crystallized in COX-1 showed an RMSD value of 1,838 Å and an affinity energy of −8.1 kcal/mol. The flurbiprofen binding site is formed by residues Val 116, Arg120, Tyr 348, Val 349, Leu 352, Tyr 355, Leu 359, Leu 384, Tyr 385, Trp 387, Ile 523, Glu 524, Ala 527, Ser 530, Leu 531.[22] Interaction analysis showed that EB7 interacts in a region different from the binding site of the co-crystallized inhibitor and the control (Figure 6), indicating a possible synergistic effect with flurbiprofen and ibuprofen.

The redocking of the inhibitor rofecoxib co-crystallized in COX-2 showed an RMSD value of 1,410 Å and an affinity energy of −8.1 kcal/mol. The binding site for rofecoxib is formed by residues His 90, Arg 120, Val 344, Val 349, Ser 353, Tyr 355, Tyr 385, Trp 387, Arg 513, Phe 518, Val 523, Glu 524, Ser 530, Leu 531, Leu 352, Ala 527 (Orlando e Malkowski 2016). In the analysis of interactions, it was observed that EB7 interacts in another site of COX-2. Therefore, it does not interact with residues of the binding site of the co-crystallized inhibitor and the control, indicating a possible synergistic effect with rofecoxib and ibuprofen (Figure 7). It can also be observed that ibuprofen interacts with residues in the active site of COX-2, showing a similar effect to co-crystallized rofecoxib on the B chain of the enzyme.

Experimental Section

Sample

The stem bark of *Erythroxylum bezerrae* was collected in Serra das Almas (S 5°8′28.60″ and W 40°54′57.20″) in the Ibiapaba plateau, Ceará – Brazil, in April 2017 (rainy season). The plant was identified by Dra. M. I. B. Loiola, from the Laboratory of Plant Systematics and Ecology, Department of Biology, Federal University of Ceará (UFC), Fortaleza, Ceará – Brazil. A voucher specimen No. EAC 58211 (SIGEN: A50E3D9) was deposited at UFC's Prisco Bezerra Herbarium. The air-dried and powdered stem bark (1.8 Kg) and bark (3.0 Kg) of *E. bezerrae* was extracted by maceration with EtOH (2×8 L) and *n*-hexane (2×8 L) to yield 46.5 g and 9.2 g of crude extracts, respectively, after distillation of solvents under reduced pressure. The EtOH extract was suspended in a mixture of MeOH/H_2O 60:40 (200 mL), then partitioned with $CHCl_3$, AcOEt (100 mL×3 for each solvent) and n-BuOH (50 mL) to afford, after the solvents evaporation, 18.3, 2.6 and 2.4 g of soluble extracts, respectively. An aliquot of the $CHCl_3$ fraction (10.0 g) was chromatographed on a silica gel column (22.0 g) eluted with binary mixtures of CH_2Cl_2/MeOH (95:5, 90:10, 80:20, 50:50) and MeOH. Fraction CH_2Cl_2/MeOH 90:10 (4.0 g) was further re-chromatographed using the same eluents as before. The fraction CH_2Cl_2/MeOH 90:10 (1.9 g) was subjected to a silica gel flash column, eluted with CH_2Cl_2/i-PrOH, to yield two main fractions: FA (612.0 mg) and FB (314.0 mg). An aliquot of FA (200 mg) was subjected to HPLC analysis using a semi-preparative C-18 column, solvent system of H_2O (0.05% TEA)/ACN 50–100%, flow rate of 3.0 mL/min at 210–400 nm to afford the alkaloid 3α-(3′,4′,5′-trimethoxycinnamoyloxy)-6β-(3″,4″,5″-trimethoxybenzoyloxy)-tropane, (25.0 mg, t_R = 13.4 min, colorless crystals), designed erythrobezerrine C. The pure compound was identified as 3α-(3,4,5-trimethoxycinnamoyloxy)-6β(3,4,5-trimethoxybenzoyloxy)-7β-hydroxy-tropane (EB7) (Figure 8).

Drugs and Reagents

The following drugs and reagents were used in the study: formaldehyde, sodium chloride, dimethylsulfoxide purchased from Dinâmica, saline solution 0.9% purchased from Arboreto. Amiloride, capsaicin, camphor, ruthenium red, morphine and κ-carrageenan were purchased from Sigma–Aldrich (Brazil). Ibuprofen (Advil®) purchased from a commercial pharmacy. Acid saline: 0.1% acetic acid dissolved in saline solution 0.9%; pH: 3.28.

Obtaining and Acclimatizing Animals

The animals, Zebrafish (*D. rerio*) wild adults (Zfa), both sexes, aged between 60 and 90 days, sizes 3.5 ± 0.5 cm and weight 0.4 ± 0.1 g,

were acquired from Agroquímica: Comércio de Produtos Veterinários LTDA., local commercial supplier (Fortaleza, CE, Brazil). For acclimatization, the animals were transferred to a glass aquarium with a capacity of 52 L (28.5×32×57 cm, height (h)×width (L)×length (L)) located in the Natural Products Chemistry Laboratory and Synthetics and Bioassays with Zebrafish at the State University of Ceará. The density of 3 animals for each 1 liter of water was maintained. Aquarium water was treated with antichlorine (Protect-Plus®) and maintained at 25.0 ± 1.0 °C and pH 7.0 ± 0.2, temperature and pH parameters were measured regularly (once a day). Air pumps with a submerged filter were inserted in the aquarium to maintain the aeration of the water. The photoperiod used was a light/dark circadian rhythm of 12:12. Up to 24 h before the experiments, the animals were fed ad libitum with ration (Alcon Gold Spirulina Flakes®) or frozen Artemia salina twice daily. After the experiments, the animals were sacrificed by freezing by immersion in an ice bath (2–4 °C) until the loss of opercular movements. The experiments were carried out in accordance with the Ethical Principles of Animal Experimentation and approved by the Ethics Committee for the Use of Animals (CEUA) of the State University of Ceará (Approval n°. 04983945/2021).

General Protocol of Experiments and Treatments

On the day of the experiments, the Zfa (n = 6/group) were randomly distributed, transferred to a damp sponge, and specific treatments were performed for each experiment performed.[8] For intraperitoneal (*i.p.*), intramuscular (i.m; tail) and ocular treatments, insulin syringes (0.5 mL; UltraFine® BD) with a 30 G needle were used. For oral (p.o.) treatments, a 10–100 μL single-channel micropipette (BioPet® technologies) was used.

Acute Toxicity (96 h)

The acute toxicity study was carried out in Zfa according to the Organization for Economic Cooperation and Development Standard Method[9] to determine the lethal dose capable of killing 50% of the animals (LD50) in 96 h. The animals (n = 6/group) were treated with EB7 (4, 20 and 40 mg/kg; 20 mL; i.p.) and control (3% DMSO; 20 mL; i.p.). After the treatments, the animals were left to rest in a 1500 mL container, containing 1200 mL of aquarium water, for analysis of the mortality rate. Mortalities were recorded every 24 h until the 96 h of the experiment was completed. During the experiment period, the fish were fed normally, twice a day, with commercial flakes. The LD50 was determined using the Trimmed Spearman-Karber method with a 95% confidence interval.[10]

Assessment of Locomotor Activity (Open Field Test)

The open field test was performed to assess the presence or absence of changes in animal motor coordination,[11] whether due to sedation and/or muscle relaxation. Initially, fish (n = 6/group) were treated with EB7 (4, 20 and 40 mg/kg; 20 mL; i.p.), or 3% DMSO (vehicle; 20 mL; i.p.). For rest, each fish was kept in a 350 mL container containing 200 mL of aquarium water. After 30 min of treatments, the animals were placed individually in glass Petri dishes (90×15 mm; diameter (d)×height (h)), containing the same aquarium water, marked with four quadrants. Locomotor activity was evaluated by counting the number of line crossings made by the animals during five minutes of analysis,[12] as shown in Figure 9.

Induction of Nociceptive Behavior – Evaluation of the Nociceptive Response

Zfa (n = 6/group) were initially treated with EB7 (4, 20 and 40 mg/kg; 20 mL; *i.p.*) or vehicle (3% DMSO; 20 mL; *i.p.*). Subsequently, the Zfa were placed individually in glass beakers (250 mL) containing 150 mL of aquarium water and left to rest for 30 minutes. The Zfa were subsequently treated with an injection of noxious agents, administered *i.m.* or *i.p.*, for inducing nociception, as seen in Figure 10, and transferred to a glass Petri dish (90×15 mm; d×h), containing the same aquarium water, marked with four quadrants, and the locomotor activity was analyzed by counting the number of crossings of the line of quadrants marked on the plate.

Each noxious agent applied to the tail reduces the swimming activity of the fish and can be induced by acid saline (ASIC), hypertonic saline (TRPV1), capsaicin (TRPV1) and formalin in the neurogenic (TRPA1) and inflammatory (TRPA1) phases and the duration of analysis of nociceptive action varied according to the nociceptive model studied. The nociception model, its antagonist, nociception agent, analysis time (open field) and channel/receptor associated with the model are described in Table 1.

Mechanism of Action – Neuromodulation of ASICs

The doses of noxious agents and antagonists, as well as the time of analysis of the nociceptive action, were used according to the tests described by,[13,8] and.[14] In a simplified way, the mechanism for evaluating the antinociceptive activity can be seen in Figure 11.

Thus, the mechanism of action is carried out as follows: the antagonist was applied i.p. of the investigated model. After 15 minutes, the test sample was applied at the lowest effective dose (which had an antinociceptive effect). After 30 minutes, the noxious agent of the model under study was applied *i.p.* or *i.m.*, or on the cornea. After treat ments with nociceptive agents, the animals were added individually to a glass Petri dish (90×15 mm; d×h) divided into four quadrants and the antinociceptive response was quantified in terms of locomotor activity or line crossings performed during the specific time for each model described in Table 1. The antinociceptive activity was calculated individually during the analysis time of each nociception model.

Table 1. Assessment of antinociceptive behavioral activity.

Model	Antagonist	Harmful agent	Analysis time	Channel
Acid saline	Amiloride (200 mg/kg; i.p.)	Acid Saline 0,1% (5 μL; tail)	0–20 min.	ASIC
Hypertonic saline	Ruthenium Red (12 mg/kg; i.p.)	NaCl 5 M (5 μL; cornea)	0–5 min.	TRPV1
Capsaicin	Ruthenium Red (12 mg/kg; i.p.)	Capsaicin 40,93 mM (5 μL; tail)	10–20 min.	TRPV1
Formalin neurogenic phase	Camphor (30,4 mg/kg; i.p.)	Formalin 0,1% (5 μL; tail)	0–5 min.	TRPA1
Formalin inflammatory phase	Camphor (30,4 mg/kg; i.p.)	Formalin 0,1% (5 μL; tail)	15–30 min.	TRPA1

Abdominal Edema Induced by κ-Carrageenan 3.5 % (Inflammation Test)

Animals (n = 6/group) received EB7 (4; 20 or 40 mg/kg; 20 μL; p.o.) or vehicle (Negative Control, 3% DMSO; 20 μL; p.o.). The positive control was ibuprofen (100 mg/kg; 20 μL; p.o.). After 1 h, Zfa received i.p. of κ-carrageenan (κ-CGN) (3.5%; 10 μL). The animals' body weight (BW) was measured before treatment and 4 h after the induction of peritoneal edema. The animals were immediately sacrificed to stop the biological reactions at the end of the experiment.[4,14]

Histopathological Analysis

After the acute inflammation test, the animals were sacrificed by immersion in an ice bath for 10 minutes. Subsequently, whole animals exposed to κ-CGN (positive control, negative control and sample; n = 6/group) were fixed in a 10% formalin solution. Following the fixation, the whole fish was arranged, in right lateral decubitus, in plastic cassettes and subjected to a graded series of baths in ethanol, for dehydration, and xylene, for clearing, and, for inclusion, embedded in paraffin to obtain sagittal sections representative to allow the histopathological evaluation of the liver without losing the perspective of the whole animal (Figure 12). The fish were processed in an automatic tissue processor (Lupe®) and sectioned sagitally in a Leica® semiautomatic microtome, with 4 μm-thick sections and deparaffinized, following standard procedures and stained with hematoxylin and eosin.[15–17] The slides were analyzed using a LaboMed® Research Microscope Halogen Series Lx 400 optical microscope with a 100x objective. This procedure allows the visual evaluation and analysis of the entire structure of the fish, with emphasis on the liver.

Ligand and Receptor Preparation for Docking Simulations

The chemical structure of the 3α-(3,4,5-trimethoxycinnamoyloxy)-6β(3,4,5-trimethoxybenzoyloxy)-7βhydroxy-tropane (EB7) ligand obtained was drawn. The lowest energy conformer saved at physiological pH, using the MarvinSketch software,[18] and optimized using the Avogadro software,[19] configured to use a steepest descent algorithm with cycles of 50 interactions, applying the MMFF94 force field (Merck Molecular Force Field).[20,21] To investigate the mechanism of action of EB7 against cyclo-oxygenase I and II (COX-1 and COX-2), the structures of the targets were obtained from the Protein Data Bank repository (https://www.rcsb.org/), PDBs ID: 3N8Z[22] and 5KIR, respectively. In the receptor preparation stage, residues were removed, maintaining the prosthetic group Heme (COX-1) and protoporphyrin IX containing Co (COX-2), important residues for biological activity. Polar hydrogens and Kollman charges, and Gasteiger charges,[23] were also added using the Autodocktools™ software.[24]

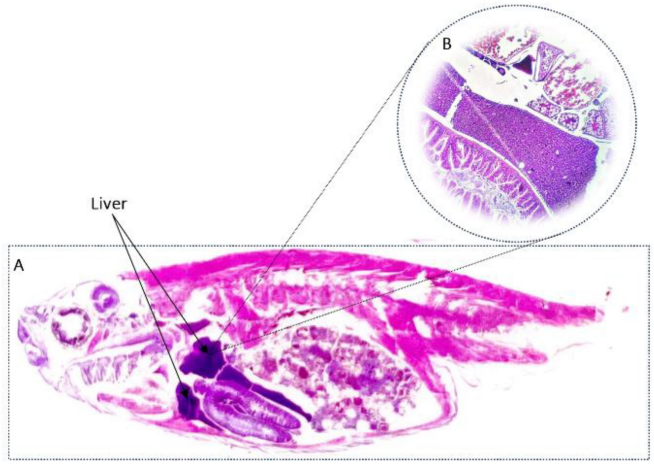

Figure 12. Whole-organism image of Zfa stained with H&E. (A) Representative sagittal cross-section of wild-type Zfa treated with EB7 (40 mg/kg) resulting 4h after i.p. injection of κ-CGN 3.5% i.p. (B) Highlighting of the animal's liver, using a LaboMed® Research Microscope Halogen Series Lx 400 optical microscope with a 40x objective.

Molecular Docking Simulation

Fifty independent molecular docking simulations were performed using AutodockVina, Lamarkian Genetic Algorithm (LGA) and Exhaustiveness 64.[25] The generated simulation grid was centered on involving the entire structure of the enzyme, using the axes (−35,044 x, 56,994 y, −11,088 z) and size (98 x, 92 y, 126 z) against COX-1; axes (31,116 x, 28,579 y, 23,619 z) and size (90 x, 104 y, 96 z) against COX-2. Simulations with Ibuprofen (control) were also performed to obtain comparative data. The redocking technique was performed with the co-crystallized inhibitors Flurbiprofen (COX-1) and Rofecoxib (COX-2) to validate the docking simulations. To select the best pose, the statistical parameter RMSD (Root Mean Square Deviation) was used as criteria, with values up to 2.0 Å,[26] and the affinity energy, considered ideal when it presents values equal to or less than −6.0 kcal/mol.[27]

Visualization of Binding Modes and Receptor-Ligand Interactions

Data analysis was performed using UCSF Chimera™,[28] Discovery studio visualizer™ viewer (Biovia 2016) and Pymol[29] software. Molecular interactions and hydrogen bonds were calculated using the Protein-Ligand Interaction Profiler (PLIP).[30,31]

Statistical Analysis

GraphPad Prism software (v. 8.0) was used to analyze the data statistically. Bar graphs represent means ± standard error of the mean for each group of 6 animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were subjected to variance one-way ANOVA analysis for the nociception model experiments and two-way ANOVA for the experiments evaluating the nociception mechanism and inflammation test, followed by the Tukey test. The level of statistical significance was set at 5% ($p < 0.05$).

Conclusions

The present study demonstrates the safety of low doses of EB7 and its pharmacological relevance as an inhibitor of nociceptive behavior through the neuromodulation of ASIC channels and anti-inflammatory effect preventing the infiltration of leukocytes in the hepatic tissue, binding to COX-1 and COX-2 in a region different from the active site, indicating synergism with ibuprofen or new binding sites responsible for the anti-inflammatory effect.

Author Contributions

Hortência Ribeiro Liberato and Ana Eloysa F. da Silva: Investigation, formal analysis, and writing – original draft. Jéssica Bezerra Maciel, Luana San De Oliveira Brito and Maria Izabel F Guedes: Supervision, writing e review and editing;. Hélcio Silva dos Santos, Jacilene Silva and Paulo Goberlânio De Barros Silva: Conceptualization, methodology, and determined the molecular structures. Antônio Wlisses Da Silva, Maria Kuerislene Amâncio Ferreira Francisco S. H. Da Silva and Arnaldo S Bezerra: Formal analysis, softwares, validation, and reviewed the manuscript.. Gabrielle S. Marinho and Otília Deusdênia Loiola Pessoa, Andreia Ferreira de Castro Gomes: Writing – original draft and aided in the analysis of the spectra. Andrelina N Coelho-de-souza, Jane Eire Silva Alencar De Menezes and Marcia Machado Marinho: Project administration and writing – review and editing.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Erythroxylum bezerrae* · Anti-inflammatory · Nociception · ASIC channels · Adult zebrafish

[1] J. Alves, *Acta Biomed Bras* **2017**, *8* (1), 56–68.
[2] M. Zhao, *Theranostics* **2021**, *11* (4), 1845–1863.
[3] M. G. Netea, *Nat. Immunol.* **2021**, *22* (2), 254–254.
[4] C.-S. I, *Front. Immunol.* **2022**, *29*, 1–21.
[5] R. L. Nahon, *Revista Brasileira de Medicina do Esporte* **2021**, *27* (6), 646–54.
[6] A. Sena, D. Silva, ■■First name is missing■■ Klc, M. Ra, *Scientia Naturalis* **2019**, *1 (1)*, 26–31.
[7] G. M. Pereira, *Phytochemistry* **2018**, *155*, 12–18.
[8] I. Soares, *Chem.-Biol. Interact.* **2019**, *299*, 37–43.
[9] Oecd (1992). URL www.oecd.org/chemicalsafety/risk-assessment/1948241.pdf.
[10] O. Arellano-Aguiar, S. Solis-Angeles, L. Serrano-García, E. Morales-Sierra, A. Mendez-Serrano, R. Montero-Montoya, *Journal of Fisheries Sciences* **2015**, *9*, 52–62.
[11] M. Ferreira, *Epilepsy Behav* **2021**, *117*, 107881–107881.
[12] A. Bezerra, *Biochem. Biophys. Res. Commun.* **2021**, *534*, 478–84.
[13] F. Batista, *Biomed. Pharmacother.* **2018**, *108*, 408–416.
[14] F. Silva, *Biochem. Biophys. Res. Commun.* **2020**, *533*, 362–67.
[15] D. A. Hirth, *Wound Repair Regen* **2012**, *20* (6), 918–945.
[16] G. C. Souza, *Pharmaceuticals* **2019**, *12* (4).■■■Dear author, please add the page number.■■■
[17] X. Wang, *Environ Sci Pollut Res* **2023**, *30*, 24212–24222.
[18] P. Csizmadia, MarvinSketch and MarvinView: Molecule Applets for the World Wide, Proceedings of the 3rd International Electronic Conference on Synthetic Organic Chemistry, **1999**, pp. 1775.
[19] M. D. Handwell, D. E. Curtis, D. Lonie, E. Vandermeersch, G. R. Zurek, ■■first name is missing■■ Hutchison, *J. Cheminform* **2012**, *4 (1)*, 17–17.
[20] T. A. Halgren, *J. Comput. Chem.* **1996**, *17* (5–6), 490–519.
[21] J. Neto, *Microb. Pathog.* **2021**, *155*, 104892–104892.
[22] R. S. Sidhu, *Biochemistry* **2010**, *49* (33), 7069–7079.
[23] G. Yan, *Int. J. Biol. Macromol.* **2014**, *64*, 213–236.
[24] R. Huey, G. M. Morris, S. Forli, ■■■Dear author, please add the *Journal title*, volume and page number.■■■**2012**.
[25] E. M. Marinho, *Microb. Pathog.* **2020**, *148*, 1–6.
[26] D. Yusuf, *J. Chem. Inf. Model.* **2008**, *48* (7), 1411–1422.
[27] J. Silva, *J Anal Pharm Res* **2021**, *10* (5), 177–194.
[28] E. F. Pettersen, *J. Comput. Chem.* **2004**, *25* (13), 1605–1612.
[29] W. L. Delano, ■■■Dear author, please add the *Journal title*, volume and page number.■■■**2020**.
[30] M. F. Adasme, *Nucleic Acids Res.* **2021**, *49* (1), 530–534.
[31] S. Salentin, *Nucleic Acids Res.* **2015**, *43* (1), 443–447.
[32] C. W. Woo, *Nat. Commun.* **2017**, 1–14.■■■Dear author, please add the volume number.■■■
[33] W. Bao, *Neuroscience* **2019**, *404*, 218–250.
[34] F. V. Costa, *Behav. Brain Res.* **2019**, *359*, 570–78.
[35] D. A. Prober, *The Journal of Neuroscience* **2008**, *28* (40), 10102–10112.
[36] P. Gau, *The Journal of Neuroscience* **2013**, *33* (12), 5249–60.
[37] E. Viña, *Histochem. Cell Biol.* **2015**, *143* (1), 59–68.
[38] M. J. Ko, *Sci. Rep.* **2019**, *9* (1), 2430–2430.
[39] N. Ohnesorge, *Frontiers in Neuroscience* **2021**, *15*, 632634–632634.
[40] J. F. Cordero-Morales, *Biophys. J.* **2012**, *102* (3), 23–23.
[41] D. M. Bautista, *Proceedings of the National Academy of Sciences* **2005**, *102* (34), 12248–52.
[42] C. R. Mcnamara, *Proc. Natl. Acad. Sci. USA* **2007**, *104* (33), 13525–13555.
[43] M. R. Due, *J. Neurochem.* **2014**, *128* (5), 776–86.
[44] D. Kokel, *Nat. Chem. Biol.* **2013**, *9* (4), 257–63.
[45] D. H. Kwon, *Nat. Struct. Mol. Biol.* **2021**, *28* (7), 554–63.
[46] A. Santos, *Arch. Biochem. Biophys.* **2019**, *664*, 149–56.
[47] O. A. Santos-Mayorga, *International Journal of Medicinal Plants and Natural Products* **2023**, *9* (2), 1–12.
[48] P. S. Launay, *Neurobiol. Dis.* **2016**, *88*, 16–28.
[49] A. Barros, *The FASEB Journal* **2019**, *33* (1).■■■Dear author, please add the page number.■■■
[50] T. D. Leng, *Neuroscience & Therapeutics* **2016**, *6*, 468–76.
[51] S. Y. Huang, *PLoS One* **2014**, *9* (8), 104414–104414.
[52] K. Mathews, *J. Small Anim. Pract.* **2014**, *55*, 10–68.
[53] C. Nucci, *J. Ethnopharmacol.* **2012**, *143* (1), 170–180.
[54] J. Ciemniecki, *Chemico Biological Interactions* **2016**, *246*, 63–68.
[55] T. S. Xiao, *Cell. Mol. Immunol.* **2017**, *14*, 1–3.

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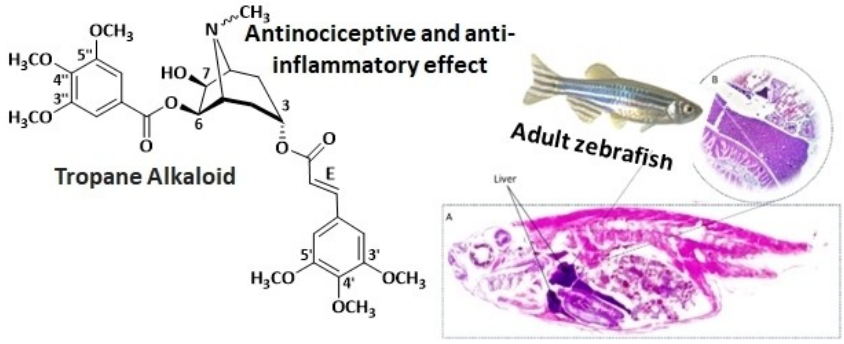

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1 – 13

Tropane Alkaloid Isolated from Erythroxylum bezerrae Exhibits Neuropharmacological Potential in an Adult Zebrafish (Danio rerio) Model

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