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Neuromodulation of acid-sensitive ion channels (ASICs) and anti-inflammatory potential by lichenxanthone in adult zebrafish (*Danio rerio*): Experimental and docking studies

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The xanthone lichenxanthone did not show toxic effects (LC_{50} > 1.0 mg/mL). lichenxanthone prevented nociceptive behavior induced by acidic saline, and its analgesic effect was blocked by amiloride, highlighting the involvement of neuromodulation of acid-sensitive ion channels (ASICs). In the analysis of anti-inflammatory activity, concentrations of 0.1 and 0.5 mg/mL of lichenxanthone reduced the edema induced by k-carrageenan 3.5 %, observed from the fourth hour of analysis. This effect was similar to that observed with ibuprofen (positive control). No leukocyte infiltrates were observed in lichenxanthone, suggesting that the compound acts in the acute inflammatory response. The results of the molecular docking study revealed that lichenxanthone exhibited better affinity energy when compared to the ibuprofen control against the two targets evaluated.

Introduction

Inflammation is a natural biological response of the body to pathological injuries. Its main function is to protect the body against tissue damage and invasion of pathogens, preventing the spread of these pathogens and even promoting tissue repair and restoring the body's homeostasis.[1,2] The inflammatory response promotes the development of signaling cascades, increased levels of oxidants and pro-inflammatory molecules, activation of transcription factors and gene expression of cytokines.[3]

Mainly characterized by the activation of immune and non-immune cells, inflammation can be caused by various stimuli, including biological agents, chemical substances, such as carrageenan, and physical agents.[4]

Acute inflammation is activated by pathogen-associated molecular patterns or damage-associated molecular patterns and terminated by the body's homeostatic regulatory mechanisms.[5] If inflammation regulatory mechanisms are impaired, or the causes are not eliminated, inflammation can become chronic and affect multiple organs in the body.[2]

The reaction is microscopically apparent in the tissue of 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.[6]

The release of inflammatory mediators during the inflammatory process, such as cytokines, chemokines, prostaglandins, leukotrins, histamine, adenosine triphosphate, reactive oxygen species and protons, has the potential to activate both immune and neuronal cells.[7] Under the influence of these inflammatory mediators, changes occur in the expression and functioning of nociceptive ion channels present in sensory neurons. These changes increase the general excitability of peripheral nociceptive fibers, thus triggering the sensation of inflammatory pain.[8]

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Tissue acidosis, standing out as a potent marker in inflammatory processes, tumor growth or ischemia, plays a crucial role in the origin of unwanted pain and hyperalgesia. The primary recognition of acidosis is attributed to acid-sensitive ion channels (ASICs).[9] Activation of ASICs mainly triggers $Na+$ influx. As such, ASICs are part of the degenerin/epithelial $Na+$ channel (DEG/ENaC) superfamily, whose characteristic is the high permeability to $Na+$ that can be blocked by amiloride.[10,11]

ASIC channels are implicated in neurological diseases and pain sensation, but no potent and selective small molecule inhibitors of ASICs are available.[12] In particular, ibuprofen is an effective allosteric inhibitor of $H+$-evoked ASIC1a currents, and mutations in the pulse and first transmembrane domain reduce the apparent affinity for ibuprofen. This evidence suggests that ASICs are targets of nonsteroidal anti-inflammatory drugs (NSAIDs).[13]

The main medications used for acute pain include selective cyclooxygenase 2 (COX-2) inhibitors and non-selective cyclooxygenase-1 (COX-1) inhibitors. Selective COX-2 inhibitors have risks associated with kidney and heart disease, while non-selective COX-1 inhibitors can lead to gastrointestinal ulcers and kidney problems.[14] Despite these findings, these drugs continue to be fundamental and widely recommended in the main clinical protocols for pharmacological pain treatment.[15]

For this reason, the search for natural compounds, especially those derived from plants, that have fewer side effects and better efficacy is constant.[16] Given this scenario, plants of the *genus Erythroxylum* have gained prominence, as they are responsible for biosynthesizing compounds, which have biological activities, such as anticholinergic, antiemetic, antidepressant, anesthetic and antitumor.[17]

Thus, this study investigated the compound 1-hydroxy-3,6-dimethoxy-8-methylxanthen-9-one (lichenxanthone), isolated from the bark of *Erythroxylum bezerrae*. The study also evaluated the possible involvement of ASICs in the antinociceptive behavioral action of lichenxanthone and its anti-inflammatory effect using the adult zebrafish (*Danio rerio*) animal model, not yet described in the literature. Histopathological analysis was performed to verify the presence of leukocyte infiltrates in the animals' liver. A molecular docking study was conducted to predict the possible and best binding affinity of the isolated compounds against cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).

Results and Discussion

There were no deaths or apparent anatomical changes in the animals during the analysis period of the acute toxicity study ($p > 0.05$). Therefore, the tested concentrations of *Lichenxanthone* (0.1, 0.5 and 1.0 mg/mL) have preclinical safety as they did not present any toxic effect on Zfa during the 96 h of analysis ($LC_{50} > 1.0$ mg /mL).

According to Hussain et al.,[18] a behavioral response is widely recognized as a more robust and sensitive outcome than mortality in toxicological studies. As can be seen in Figure 1, lichenxanthone did not alter Zfa locomotion 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). In other words, lichenxanthone did not cause sedation and/or muscle relaxation in the animals.

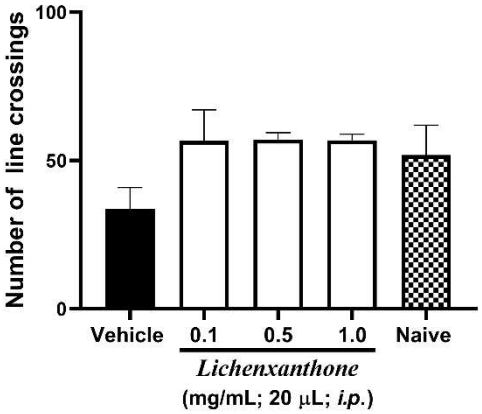

Figure 1. Effect of lichenxanthone on Zfa locomotor behavior activity in the open field test. Each column represents the standard errors of the mean (n = 6/group) analyzed individually over 0–5 min. Vehicle (DMSO 3%; 20 μL; *i.p.*). Naive: group of animals without treatment. One-way ANOVA followed by Tukey's test.

A study conducted by Lima et al.[19] investigated the nociceptive response induced by chemical stimuli, including acidic saline, in adult zebrafish. Morphine was used as a positive control in this study. The results revealed that morphine reversed the nociception caused by chemical stimuli, resulting in an increase in the locomotor activity of the fish compared to groups treated with vehicle alone. These findings suggest that fish subjected only to the injection of harmful agents exhibit a reduction in locomotor activity. At the same time, those pre-treated with substances that have analgesic effects similar to morphine present increased locomotor activity.

From the one-way ANOVA statistical test followed by the Tukey test, it was observed that pre-treatment with lichenxanthone (1.0 mg/mL) prevented the nociceptive behavior induced by acidic saline solution (****$p < 0.0001$ vs. Control) (Figure 2). Pre-treatment with morphine prevented harmful behavior in all nociceptive models studied.

Therefore, the mechanism of action was performed with the ASIC channel antagonist (amiloride) with the lowest effective concentration of lichenxanthone (1.0 mg/mL).

It was indicated by the Two-way ANOVA statistical test followed by the Tukey test that pre-treatment with the antagonist amiloride inhibited the antinociceptive effect of *Lichenxanthone*, (&&&&$p < 0.0001$ vs. lichenxanthone) (Figure 3), thus indicating that the antinociceptive effect of lichenxanthone occurs through the neuromodulation of ASIC channels.

An increase in the expression of the ASIC subunit was observed in the dorsal root ganglion during inflammatory episodes, and inflammatory mediators can modify this increase. Additionally, ASIC inhibitors, such as amiloride, have been shown to attenuate hyperalgesia in mice and rats and alleviate

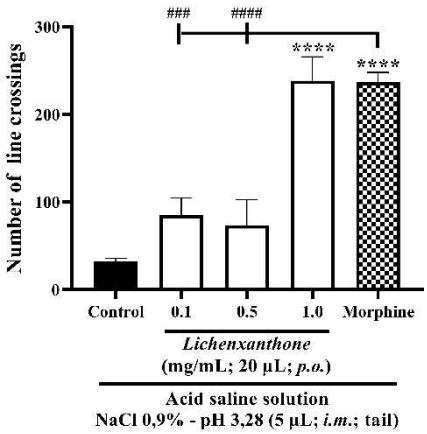

Figure 2. Effect of lichenxanthone on nociception behavior induced by acidic saline solution (0–20 minutes) in adult zebrafish. Control: DMSO 3%. Each column represents the standard errors of the mean (n = 6/group). One-way ANOVA followed by Tukey's test (****p < 0.0001 vs. Control. ###p < 0.001; ####p < 0.0001 vs. Morphine).

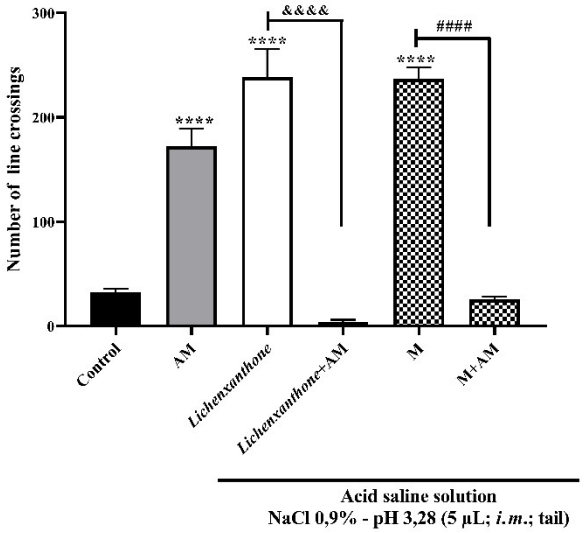

Figure 3. Effect of pre-treatment with amiloride, an ASICs antagonist, on the antinociceptive effect of lichenxanthone in Zfa, analyzed through nociception induced by acidic saline solution (0–20 min). Control: DMSO 3%. M: Morphine. AM: Amiloride. Each column represents a mean standard error of the mean (n = 6/fish). Two-way ANOVA followed by Tukey's test (****p < 0.0001 vs. Control. &&&&p < 0.0001 vs. *Lichenxanthone.* ####p < 0.0001 vs. Morphine).

acid-induced pain in humans, suggesting an additional contribution of ASICs to pain perception.[20]

Animal models are widely used to understand the pathophysiological mechanisms of the inflammatory process.[21] Orso et al.[22] highlight that k-carrageenan can induce inflammatory events and metabolic disorders in different experimental models, including zebrafish.

The abdominal edema test induced by κ-CGN 3.5% was performed to investigate the anti-inflammatory effect of lichenxanthone. The lowest concentrations of lichenxanthone (0.1 and 0.5 mg/mL) were able to significantly reduce abdominal edema caused by κ-CGN 3.5% in the fourth hour of analysis (**p < 0.01; ***p < 0.001 vs. DMSO 3%) (Figure 4).

Many reports highlight the anti-inflammatory activity of compounds, such as alkaloids[23] and diterpenoids,[24] from species of the genus *Erythroxylum*. However, the availability of data on other secondary metabolites isolated from this genus, especially when investigating the species *E. bezerrae*, remains limited, making research even more challenging.

As a detoxifying organ, the liver is often exposed to cytotoxic substances.[25] Charlie-Silva et al.[26] revealed that neutrophils and lymphocytes infiltrating the peritoneal cavity of adult zebrafish exhibited short-term persistence but triggered a robust pattern of inflammation with systemic impact, sufficient to induce the formation of edema.

Histopathological analysis carried out on the entire Zfa shows the presence of leukocyte infiltrates in animals pre-treated with 3% DMSO (negative control) after 4 hours of analysis and the absence of leukocyte infiltrates in the slides of groups pre-treated with ibuprofen (positive control) and lichenxanthone (0.1 mg/mL – concentration that presented a similar result to ibuprofen in the acute inflammation test, **p < 0.01 vs. Control), demonstrating the anti-inflammatory effect of lichenxanthone in adult zebrafish (Figure 5).

The inflammatory process promotes the synthesis of prostaglandins (PGs) PGI2 and PGE2, while prostaglandin synthesis is inhibited by COX inhibitors. Therefore, the COX-1 and COX-2 blocking effect of lichenxanthone was investigated in the molecular docking study.

Through the molecular docking study, it was observed that the best pose presented an RMSD value in the order of 1.704 Å (lichenxanthone) and 0.991 Å (Ibuprofen) against COX-1 and in the order of 1.640 Å (lichenxanthone), 1.590 Å (Ibuprofen) against to COX-2. Regarding the affinity energy (kcal/mol), it was observed values in the order of −8.3 (lichenxanthone/COX-

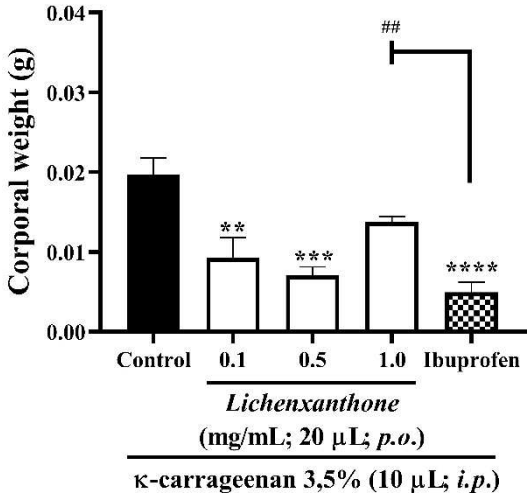

Figure 4. Effect of lichenxanthone on abdominal edema induced by 3.5% κ-CGN showing the fourth hour of analysis of the acute inflammation test in adult zebrafish. Each column represents a mean standard error of the mean (n = 6/fish). Control: 3% DMSO (20 µL; *p.o.*). Ibuprofen: positive control (20 µL; *p.o.*). Two-way ANOVA followed by Tukey's test (**p < 0.01; ***p < 0.001 ****p < 0.0001 vs. Control; ##p < 0.01 vs. Ibuprofen).

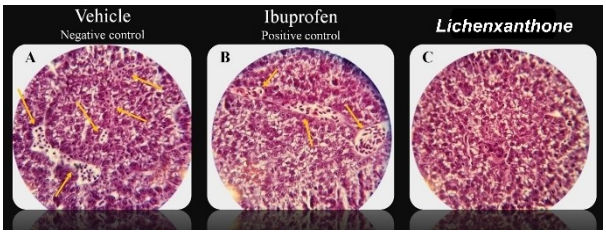

Figure 5. Histopathological analysis of lichenxanthone. A: Negative control (3% DMSO, 20 μL; *p.o.*). B: treatment with ibuprofen (positive control – 1.0 mg/mL). C: treatment with lichenxanthone (0.1 mg/mL; 20 μL; *p.o.*). Leukocyte infiltrates can be observed in the Zfa liver 4 hours after injecting 10 μL *i.p.* of 3.5% κ-carrageenan (yellow arrows). 100x objective. Staining: H&E.

1), −6.8 (Ibuprofen/COX-1), −9.1 (lichenxanthone/COX-2) and −7.3 (Ibuprofen/COX-2).

Analyzing the interaction patterns, it was observed that the lichenxanthone/COX-1 complex presented four strong H-bonds involving the residues Gln 44B (2.10 Å), Cys 47B (2.78 Å), Gln 461B (2.34 Å), Glu 465B (1.81 Å) and two hydrophobic interactions involving residues Tyr 39B (3.99 Å), Pro 153B (3.35 Å).

The Ibuprofen/COX-1 complex presented five hydrophobic interactions involving residues Gln 370B (3.93 Å), Gln 372B (3.71 Å), Tyr 373 A (3.72 Å), Pro 542 A (3.69 and 3.79 Å) and three hydrogen bonds involving the residues Phe 371 A (2.21 Å), Phe 371B (3.54 Å), Gln 372B (2.02 Å). The lichenxanthone/COX-2 complex presented four strong H-bonds involving the residues Cys 36B (2.47 Å), His 39B (1.94 Å), Arg 44B (2.45 Å), Gln 461B (2.64 Å) and a hydrophobic interaction with Leu 152B (3.57 Å).

The Ibuprofen/COX-2 complex presented seven hydrophobic interactions involving the 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) and Leu 531B (3.35 Å).

Redocking of the co-crystallized inhibitor flurbiprofen against COX-1 showed an RMSD value of 1,838 Å and an affinity energy of −8.1 kcal/mol. Lichenxanthone presented a better affinity energy value than the co-crystallized inhibitor and the control. The flurbiprofen binding site is formed by residues Val 116, Arg120Gln, 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.[27]

Interaction analysis showed that lichenxanthone interacts in a different region of the binding site of the co-crystallized inhibitor and the control (Figure 6), indicating a possible synergistic effect with flurbiprofen and ibuprofen.

Redocking the inhibitor rofecoxib co-crystallized against COX-2 showed an RMSD value of 1,410 Å and an affinity energy of −8.1 kcal/mol. Lichenxanthone presented a better affinity energy value than rofecoxib and the control ibuprofen. The rofecoxib binding site is formed by residues His 90, Arg 120, Val 344, Val 349, Leu 352, Ser 353, Tyr 355, Tyr 385, Trp 387, Arg 513, Phe 518, Val 523, Glu 524, Ala 527, Ser 530, Leu 531.[28] The interaction analysis showed that lichenxanthone interacts in another site of COX-2. Thus, it does not interact with residues of the binding site of the co-crystallized inhibitor and the control,

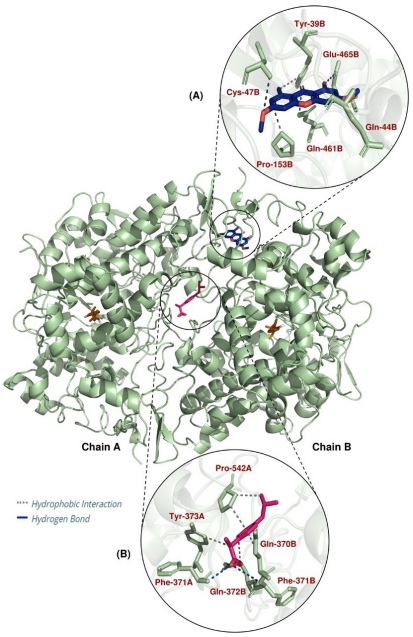

Figure 6. Binding sites against COX-1. (A) lichenxanthone (blue). (B) Ibuprofen (pink) and co-crystallised inhibitor (orange).

indicating a possible synergistic effect with rofecoxib and ibuprofen (Figure 7). The analysis also showed that ibuprofen interacts with residues in the binding site of the co-crystallized inhibitor, having in common interactions with residues Val 349B, Leu 352B, Tyr 355B, Phe 518B and Leu 531B, indicating that ibuprofen has a similar effect to co-crystallized rofecoxib on the B chain of COX-2.

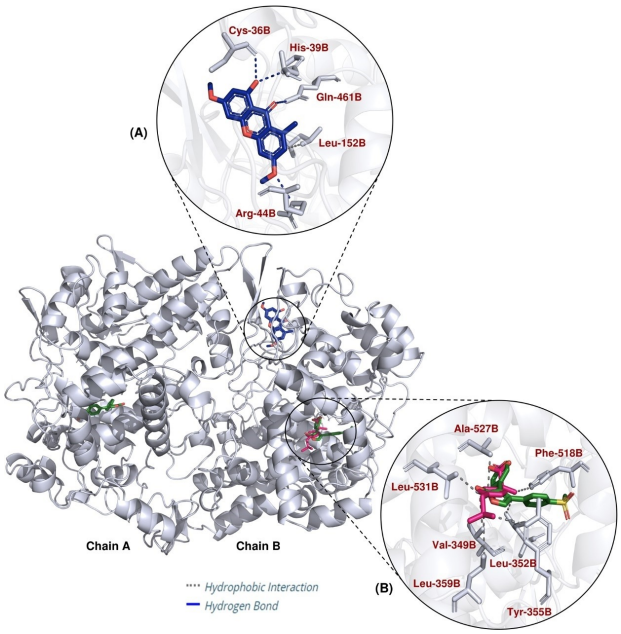

Figure 7. Binding sites against COX-2. (A) lichenxanthone (blue). (B) Ibuprofen (pink) and co-crystallised inhibitor (green).

Experimental Section

Sample

The pure compound 1-hydroxy-3,6-dimethoxy-8-methylxanthen-9-one (*lichenxanthone*) (Figure 8) was extracted from the stem bark of the species *Erythroxylum bezerrae* (supporting information). The plant material was collected in April 2017 (rainy season) in Serra das Almas, Crateús, Ceará – Brazil (S 5°8′28.60″ e W 40°54′57.20″). The plant was identified by Dr. M. I. B. Loiola from the Laboratory of Systematics and Plant Ecology, Department of Biology at the Federal University of Ceará (UFC), Fortaleza, Ceará – Brazil. An exsiccate No. EAC 58211 (SIGEN: A50E3D9) was deposited at the UFC Prisco Bezerra Herbarium. The characterizations were performed at the Laboratory of Phytochemical Analysis of Medicinal Plants II (LAFIPLAM II). Dr. O. D. L. Pessoa provided the compound.

Drugs and reagents

The following drugs and reagents were used: dimethyl sulfoxide and acetic acid purchased from Dinâmica, 0.9% saline solution purchased from Arboreto. Amiloride, morphine and κ-carrageenan were purchased from Sigma-Aldrich (Brazil). Ibuprofen (Advil) was purchased from a commercial pharmacy. Acidic saline solution: 0.1% acetic acid dissolved in saline solution; pH: 3.28.

Obtaining and acclimatizing animals

The animals (*Danio rerio*), both sexes, aged between 60 and 90 days, size 3.5 ± 0.5 cm and weight 0.4 ± 0.1 g, were purchased 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 x 32 x 57 cm, height (h) x width (W) x length (L)) located in the Chemical Bioassay Laboratory -Pharmacological and Environmental Studies at the State University of Ceará. The density of 3 animals for every 1 liter of water was maintained. The aquarium water was treated with antichlorine (ProtectPlus) and maintained at 25.0 ± 1.0 °C and pH 7.0 ± 0.2. These parameters were measured regularly (1x a day). Air pumps with submerged filters were inserted into the aquarium to maintain water aeration. The photoperiod used was a circadian light/dark rhythm of 12:12. Up to 24 h before the experiments, the animals were fed ad libitum with food (Alcon Gold Spirulina Flakes) twice daily. After the experiments, the animals were sacrificed by immersion in an ice bath (2–4 °C) until loss of opercular movements. The experiments were carried out in accordance with the Ethical Principles of Animal Experimentation and approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Ceará (Approval no. 04983945/2021).

General protocol of experiments and treatments

The animals (n = 6/group) were randomly distributed and transferred to a damp sponge on the day of the experiments. Specific treatments were carried out for each experiment.[29] For intraperitoneal (*i.p.*) and intramuscular (*i.m*; tail) treatments, insulin syringes (0.5 mL; UltraFine BD) with a 30G needle were used. For oral (*p.o.*) treatments, a 10–100 μL single-channel micropipette (BioPet technologies) was used.

Acute toxicity (96 h)

The study of acute toxicity in adult zebrafish (Zfa) was carried out according to the methodology proposed by the Organization for Economic Cooperation and Development Standard Method[30] to determine the lethal concentration capable of killing 50% of the animals (LC_{50}) in 96 h.

The animals (n = 6/group) were exposed to the substance under study *Lichenxanthone* (0.1 or 0.5 or 1.0 mg/mL; 20 mL; *p.o.*) and control (DMSO 3%; 20 mL; *p.o.*). After treatments, the groups were left to rest in a 2500 mL container containing 2000 mL of aquarium water to analyze the mortality rate.

Mortality was recorded at 24, 48, 72 and 96 hours. The number of adult zebrafish killed in each group was recorded, and the lethal concentration capable of killing 50% of the animals (LC_{50}) was determined.[31] During the experiment, the fish were usually fed twice daily.

Assessment of locomotor activity (open field test)

The open field test is performed to evaluate whether or not there were changes in the animals' motor coordination, whether as a result of sedation and/or muscle relaxation.[32] The assessment of locomotor activity can be explored through open field testing in a simple aquarium and in Petri dishes.[33]

The open field test in Petri dishes, proposed by Ahmad and Richardson,[34] was adapted to evaluate the locomotor activity of adult zebrafish under the action of analgesic drugs.[35] Therefore, the animals are positioned individually in glass Petri dishes (90×15 mm; diameter (d) x height (h)) containing the same water as the aquarium, marked with four quadrants. A video recording is made for a specified period of time for each analysis. Afterwards, line crossings are counted individually (Figure 9). The values are taken for statistical treatment, and the locomotor activity is determined through analysis of variance.

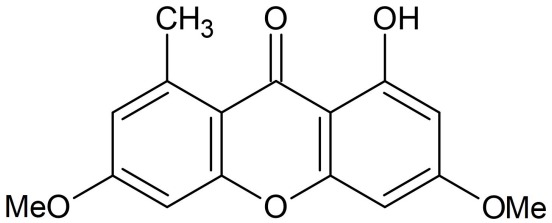

Figure 8. Structural representation of the compound 1-hydroxy-3,6-dimethoxy-8-methylxanthen-9-one.

Figure 9. Illustration of the open field test to determine locomotor activity by counting line crossings.

Animals (n = 6/group) were treated with *lichenxanthone* (0.1, 0.5 and 1.0 mg/mL; 20 mL; *p.o.*) or 3% DMSO (vehicle; 20 mL; *p.o.*). A group without treatment (Naïve; n = 6) was included. For rest, each fish was kept in a 500 mL container containing 350 mL of aquarium water. After 60 minutes of treatments, the animals were taken to the open field, as illustrated in Figure 2. The number of line crossings was recorded during 0–5 minutes.[36]

Induction of nociceptive behavior – assessment of the nociceptive response

Zfa (n = 6/group) were pretreated with *lichenxanthone* (0.1 or 0.5 or 1.0 mg/mL; 20 mL; *p.o.*) or vehicle (3% DMSO; 20 mL; *p.o.*). Subsequently, the animals were placed individually in glass beakers (250 mL) containing 150 mL of aquarium water and left to rest for 30 minutes.

Subsequently, to induce nociception, the groups of animals were treated with an injection of 20 μL of acidic saline solution, administered *i.m.* After treatment, the animals were analyzed directly in an open field. Locomotor activity was analyzed by counting the number of line crossings performed by the animals during 0–20 minutes of analysis.[37]

To verify the possible involvement of *lichenxanthone* in ASICs, a subsequent test of the mechanism of action was carried out with antagonists of this channel.

Mechanism of action – neuromodulation of ASICs

20 μL of amiloride, an ASICs antagonist, was administered intraperitoneally. After 15 minutes, the lowest effective concentration of *lichenxanthone* was applied. Nociception was induced by an acidic saline solution (5 μL) applied to the animals' tails 30 minutes after pretreatment with *lichenxanthone*. Subsequently, the animals were taken to the open field, and the blockade of the antinociceptive response was quantified by counting line crossings performed for 0–20 minutes.[38]

Abdominal edema induced by κ-carrageenan 3.5% (inflammation test)

The anti-inflammatory activity was carried out under abdominal edema induced by carrageenan, as described by Silva et al..[39] The animals (n = 6/group) received pre-treatment with *lichenxanthone* (0.1, 0.5 or 1.0 mg/mL; 20 μL; *p.o.*) or vehicle (control, 3% DMSO solution; 20 μL; grandma.). Ibuprofen (2.5 mg/mL; 20 μL; *p.o.*) was used as a positive control. After 1 h, groups of animals were injected with κ-carrageenan (κ-CGN) (3.5%; 10 μL; *i.p.*). The animals' body weight (BW) was measured before treatment and 4 hours after induction of peritoneal edema. The animals were sacrificed immediately to stop biological reactions at the end of the experiment.[40]

Histopathological Analysis

After the acute inflammation test, the animals were sacrificed by immersion in an ice bath for 10 minutes. Then, groups of whole animals exposed to κ-CGN were fixed in a 10% formaldehyde solution. Following fixation, whole fish were placed in the right lateral decubitus position in plastic cassettes and subjected to a graduated series of baths in ethanol and xylene and, for inclusion, embedded in paraffin to obtain representative sagittal sections, to allow histopathological evaluation of the liver without losing perspective of the entire animal. The fish were processed in an automatic tissue processor (Lupe®) and sagittally sectioned in a Leica® semi-automatic microtome, with 4 μm thick sections and deparaffinized, following standard procedures and stained with hematoxylin and eosin (H&E).[2] The slides were analyzed using a LaboMed® Research Microscope Halogen Serie Lx 400 optical microscope with a 100x objective.

Molecular docking against COX-1and COX-2

The chemical structure of the ligand 1-hydroxy-3,6-dimethoxy-8-methylxanthen-9-one (*lichenxanthone*) was designed. The lowest energy conformer was saved at physiological pH using MarvinSketch software,[41] the conformer was optimized using Avogadro software,[42] configured to use steepest descent algorithm with cycles of 50 interactions, applying the MMFF94 force field (Merck Molecular Force Field).[43,44]

To investigate the mechanism of action of *lichenxanthone* against cyclooxygenase I and II (COX-1 and COX-2), the structures of the targets were obtained from the Protein Data Bank repository , PDBs ID: 3 N8Z[27] and 5KIR[28] respectively. In the stage of preparing the receptors, the residues were removed, the prosthetic group Heme (COX-1) and protoporphyrin IX containing Co (COX-2), important residues for biological activity, were added, the polar hydrogens, the charges Kollman and Gasteiger loads[45] using AutodocktoolsTM software.[46]

Molecular docking study

50 independent molecular docking simulations were performed using AutodockVina software,[46] Lamarkian Genetic Algorithm (LGA) and Exhaustiveness 64.[47] The simulation grid generated was centralized to involve 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) in front of COX-1; axes (31,116 x, 28,579 y, 23,619 z) and size (90 x, 104 y, 96 z) against COX-2. Simulations were carried out with Ibuprofen (control) to obtain comparative data. To validate the docking simulations, the redocking technique was performed with the co-crystallized inhibitors Flurbiprofen (COX-1) and Rofecoxib (COX-2). To select the *best pose*, the statistical parameter RMSD (Root Mean Square Deviation) with values up to 2.0 Å[48] and the affinity energy with values equal to or lower than −6.0 kcal/mol were used as criteria.[49]

Visualization of binding modes and receptor-ligand interactions

Data analysis was performed using the software UCSF ChimeraTM,[50] Discovery studio visualizerTM viewer[51] and Pymol.[52] Molecular interactions and hydrogen bonds were visualized using the Protein-Ligand Interaction Profiler (PLIP) server.[53]

Statistical analysis

The GraphPad Prism software (v. 8.0.1) 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 normality of distribution and homogeneity of the data, the differences between the groups were submitted to one-way ANOVA analysis of variance for the open field experiments and two-way ANOVA for the other experiments, followed by Tukey's test. The level of statistical significance was set at 5% ($p < 0.05$).

Conclusions

The present study highlights the safety of low doses of *lichenxanthone*, highlighting its pharmacological importance as an inhibitor of nociceptive behavior. The properties of this compound were verified through neuromodulation of ASIC channels and the acute anti-inflammatory effect demonstrated by *lichenxanthone.* Histopathological analysis revealed the absence of leukocyte infiltrates in the groups of animals that received treatment with *Lichenxanthone*, suggesting a promising potential anti-inflammatory effect when examining the liver tissue of adult zebrafish. Through the molecular docking study, we inferred that *Lichenxanthone* exhibited better affinity energy compared to the ibuprofen control against the two targets evaluated. However, *lichenxanthone* did not present significant interactions against COX-1 and COX-2 compared to co-crystallized inhibitors and ibuprofen control. Therefore, the present study suggests using *lichenxanthone* as a model to generate new molecules that can present significant interactions with cyclooxygenase I and II and, thus, act in their inhibition. Therefore, the results indicate that 1-hydroxy-3,6-dimethoxy-8-methylxanthen-9-one represents a promising target in investigating new therapeutic options for treating pain and inflammation. Furthermore, this study aims to contribute to the advancement of knowledge related to the species *E. bezerrae*, a plant native to northeastern Brazil, and to the discovery of its bioactive properties in scientific literature.

Author's contributions

Hortência Ribeiro Liberato and Maria Eduarda Uchoa Bezerra: 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 and Maria Kuerislene Amâncio Ferreira: Formal analysis, softwares, validation, and reviewed the manuscript. Gabrielle S Marinho and Otília Deusdênia Loiola Pessoa: Writing - original draft and aided in the analysis of the spectra. Andreia Ferreira de Castro Gomes, 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: Xanthone · *Erythroxylum bezerrae* · Acute toxicity · Locomotor behavior · Neuromodulation of ASICs · Anti-inflammatory · Molecular docking

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