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Withanicandrin Isolated from *Datura Ferox* Promotes Antinociception by Modulating the Asics and TRPS Channels and Anti-Inflammation in Adult Zebrafish

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This is the first study to analyze the anti-inflammatory and antinociceptive effect of withanicandrin, isolated from Datura Ferox leaves, and the possible mechanism of action involved in adult zebrafish (ZFa). To this end, the animals were treated intraperitoneally (i.p.) with withanicandrin (4; 20 and 40 mg/kg; 20 μL) and subjected to locomotor activity and acute toxicity. Nociception tests were also carried out with chemical agents, in addition to tests to evaluate inflammatory processes induced by κ-Carrageenan 1.5% and a Molecular Docking study. As a result, withanicandrin reduced nociceptive behavior by capsaicin at a dose of 40 mg/kg and by acid saline at doses of 4 and 40 mg/kg, through neuromodulation of TRPV1 channels and ASICs, identified through blocking the antinociceptive effect of withanicandrin by the antagonists capsazepine and naloxone. Furthermore, withanicandrin caused an anti-inflammatory effect through the reduction of abdominal edema, absence of leukocyte infiltrate in the liver tissue and reduction of ROS in thel liver tissue and presented better affinity energy compared to control morphine (TRPV1) and ibuprofen (COX-1 and COX-2).

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Introduction

Pain is the body's survival mechanism in the face of changes in homeostasis that can be stimulated through potential, real, or even non-existent tissue damage. Nociception comprises three mechanisms: transduction, which is caused by the activation of nociceptors through chemical, mechanical, or thermal stimuli; transmission, where afferent pathways carry the nerve impulse through the Central Nervous System – CNS; and the modulation that is responsible for blocking pain generated by the activation of nociceptive receptors.[1,2]

Many of these receptors are also activated in anti-inflammatory processes, demonstrating the direct association between pain and inflammation. When released, inflammatory mediators sensitize nociceptors, generating chemical changes that initiate an experience involving emotional, sensory, and behavioral components.[3,4]

Inflammation is a mechanism that aims to defend organisms from infections or injuries to repair damage to affected tissue, or that has lost function. This process presents five typical characteristics of its development that involve vascular and cellular changes: pain, heat (increase in local temperature), flushing (hyperemia), edema, and loss of function.[5]

The medications used for this type of condition are called anti-inflammatory drugs. They are divided into two classes: steroids and non-steroids. Steroids act similarly to the hormone cortisol and reduce the signals generated by the

inflammatory process. Still, they generate many adverse effects, and therefore, the choice often turns to non-steroids, which are responsible for inhibiting cyclooxygenase enzymes, preventing the effects of prostaglandins, and reducing the characteristic signs of inflammation. Still, even so, their indiscriminate use leads to reactions that can become serious for the body.[6]

Using plants with pharmacological action has demonstrated promising results in producing medicines that can help treat pain, such as analgesics, and inflammation, such as anti-inflammatories. To develop a drug with this potential, preclinical evaluations in animal models are necessary so that the compounds can proceed to the clinical stage. Among these compounds, withanolides are defined as vitasteroids, which are part of the secondary metabolites of several species, such as *Datura ferox*, known as ferocious prickly pear, and with reports of anti-inflammatory and analgesic action. However, research related to the pharmacological and biological activities of the withanicandrin compound isolated from *Datura ferox* is not in-depth, leaving room for the development of new studies.[7]

Studies report the relevance of ZFa as a model for studying natural products with anti-inflammatory and antinociceptive action.[8,9] ZFa have nociceptors homologous to those of humans, which demonstrates their effectiveness in preclinical nociception tests involving transient receptor potential (TRP) ion channels such as transient vanilloid potential (TRPV1) and acid detection ion channels (ASICs) .[10,11] Given the above, the antinociceptive and anti-inflammatory effect of withanicandrin in adult ZFa and the possible mechanisms involved were investigated.

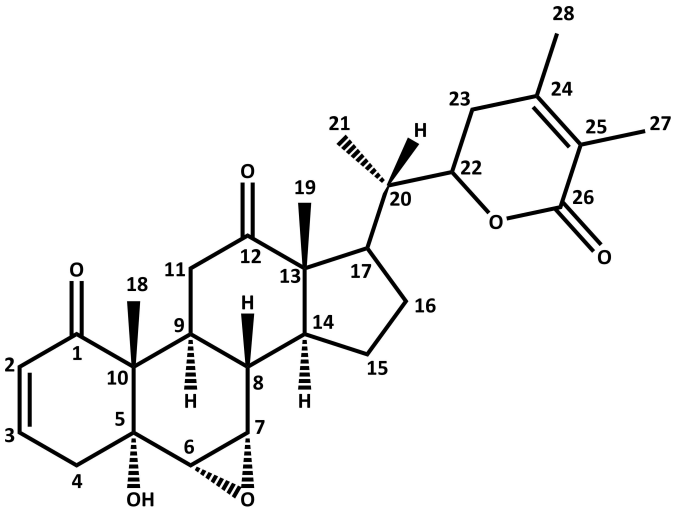

Figure 1. Chemical structure of withanicandrin withanolide.

Results and Discussion

Characterization

Withanicandrin (Vit) (Figure 1) was extracted from the dried and crushed leaves of *Datura ferox*. The ^{1}H-NMR spectrum of withanicandrin showed a distinct doublet at δ_H 6.59 (H-2) due to the coupling by the single proton at H-3. Another doublet observed at δ_H 3.07 (H-6) which is coupled to a single proton at C-7. The H-7 proton is coupled to 2 protons (H-6 and H-8) producing a doublet of doublets at δ_H 3.55. The doublet at δ_H 0.98 (H-21) was only one methyl proton that is coupled to H-20. The other 4 methyl protons (H-18, H-19, H-27 and H-28) were attached to quaternary carbons thus appearing as singlets. The ^{13}C-NMR spectrum of withanicandrin chemical shifts at δ_C 212.3 (C-12), 201.5 (C-1) confirmed the presence of two carbonyl carbons, the third carbonyl carbon can be identified as an ester carbonyl with a signal at δ_C 167.0 (C-26), four unsaturated carbons were identified at δ_C149.6 (C-24), 140.0 (C-3), 128.9 (C-2), 121.9 (C-25), four oxygenated carbons at δ_C 78.5 (C-22), 73.3 (C-5), 57.0 (C-7), 56.3 (C-6), three quaternary carbons at δ_C 57.8 (C-13), 51.6 (C-10), five methylene carbons at δ_C 36.8 (C-4), 38.5 (C-11), 27.2 (C-16), 23.7 (C-15), 30.1 (C-23), five methine carbons at δ_C 52.9 (C-14), 42.8 (C-17), 39.9 (C-20), 35.7 (C-8), 37.9 (C-9), and five methyl carbons at δ_C 20.6 (C-28), 14.8 (C-19), 13.7 (C-21), 12.6 (C-27), 11.5 (C-18). The NMR values of isolated withanicandrin were in good agreement with reported values.[12]

96 h Toxicity Test

Withanicandrin (4; 20 and 40 mg/kg; 20 μL; *i.p.*) was not toxic to ZFa, as it did not cause death during the 96 hours of follow-up after treatment with the sample in the three doses (LD_{50} > 40 mg/kg), as well as there was no change in the locomotor activity of the animals observed in the open field test.

Antinociceptive Activity

One-way ANOVA statistical analysis followed by Tukey's test indicated that the lowest and highest doses of withanicandrin (4 and 40 mg/kg; 20 μL; *i.p.*) significantly inhibited (**$p < 0.01$, **** $p < 0.0001$ vs. control) the nociception induced by acidic saline (Figure 2A), and the highest dose (40 mg/kg; 20 μL; *i.p.*) significantly inhibited it (**$p < 0.01$ vs. control) nociception induced by capsaicin (Figure 2B), similar to what occurred in the group treated with morphine (****$p < 0.0001$ vs. control) promoting analgesia in the animals.

Mechanism of Action of Withanicandrin Induced by Capsaicin and Acid Saline

The mechanism of action of withanicandrin analgesia indicated by the acid saline (ASIC channel agonist) and capsaicin

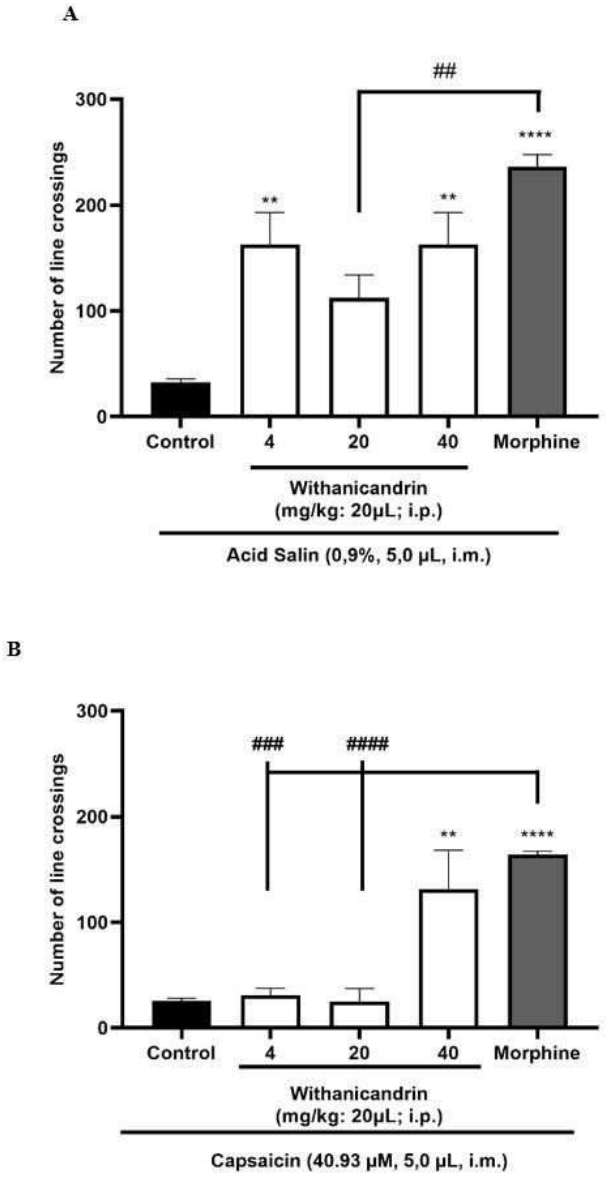

Figure 2. (A) Effect of withanicandrin on acidic saline-induced nociception in adult ZFa. (B) Effect of withanicandrin on capsaicin-induced nociception in adult ZFa. Each column represents the mean ± standard error of the mean (n = 6 groups). control: vehicle (DMSO 3.0%; 20 µL, i. p.). Morphine (8.0 mg/kg; 20 µL; *i. p.*). One-Way ANOVA followed by Tukey's test: (**$p < 0.01$; ****$p < 0.0001$ vs. control; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs. Morphine).

(TRPV1 receptor agonist) models were investigated using the antagonists naloxone (ASIC channel antagonist) and capsazepine (TRPV1 receptor antagonist) (Figure 3). The lowest effective dose of withanicandrin (4 mg/kg; 20 µL; i.p) was selected to investigate the mechanism of action with acid saline and that for capsaicin (40 mg/kg; 20 µL; *i.p*). As a result, the antinociceptive effect of withanicandrin (4 mg/kg; 20 µL; i.p) was blocked by naloxone ($^{\#\#\#\#}p < 0.0001$ naive vs Vit + nalox; Figure 4.) and by capsazepine ($^{\#\#}p < 0.01$ naive vs Vit + caps; Figure 4). The antagonists did not cause locomotor impairment and/or muscle relaxation in the animals.

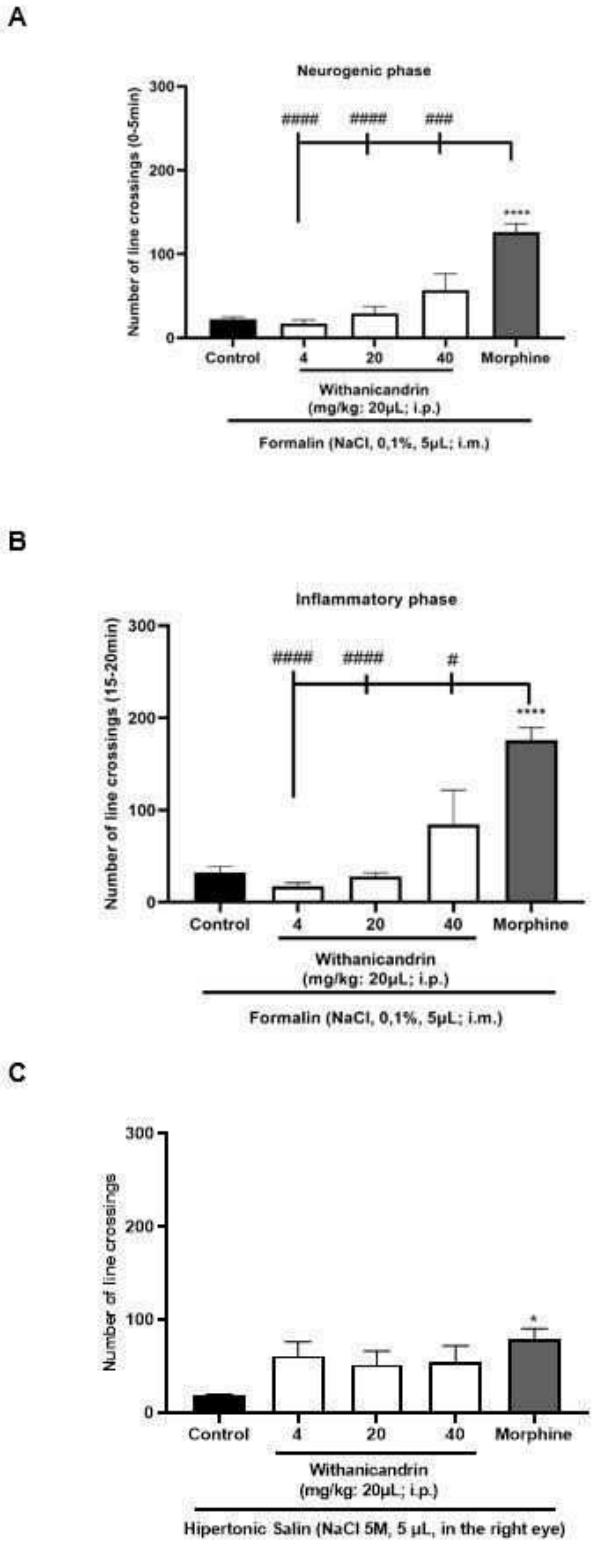

Figure 3. Effect of withanicandrin on formalin-induced nociception in the neurogenic (A) and inflammatory (B) phases in adult ZFa. (C) Effect of withanicandrin on hypertonic saline-induced nociception in adult ZFa. Each column represents the mean ± standard error of the mean (n = 6 groups). control (DMSO 3.0%; 20 µL, *i.p.*). Morphine (8 mg/kg; 20 µL; *i.p.*). One-way ANOVA with Tukey test. (*$p < 0.05$; ***$p < 0.001$, ****$p < 0.0001$ vs. Control, $^{\#} < 0.05$; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. Morphine).

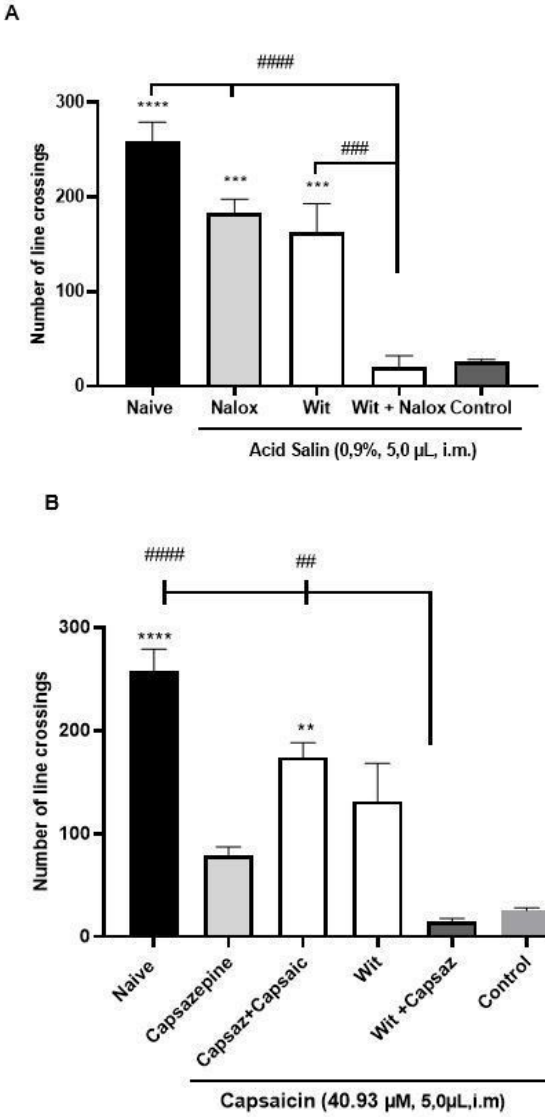

Figure 4. Effect of naloxone (A) and capsaicin (B) on the antinociception of withanicandrin (Wit) in adult ZFa. column represents the mean ± standard errors of the mean (n = 6/group). Nalox – Naloxone (0.2 mg/mL; 5 µL; *i.m.*); Naïve: untreated group. Caps. — Capsazepine (0.5 mg/mL; 5 µL; *i.m.*). Tukey's one-way ANOVA. (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs. control; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs. Withanicandrin).

Anti-Inflammatory Activity

The one-way ANOVA statistical analysis indicated that pre-treatment with withanicandrin significantly reduced (**$p <$ 0.01 vs. control) the abdominal edema induced by carrageenan, similar to what occurred with the positive control group ibuprofen and significantly different from the DMSO group 3% – negative control (Figure 5), indicating the anti-inflammatory effect of withanicandrin at all doses tested.

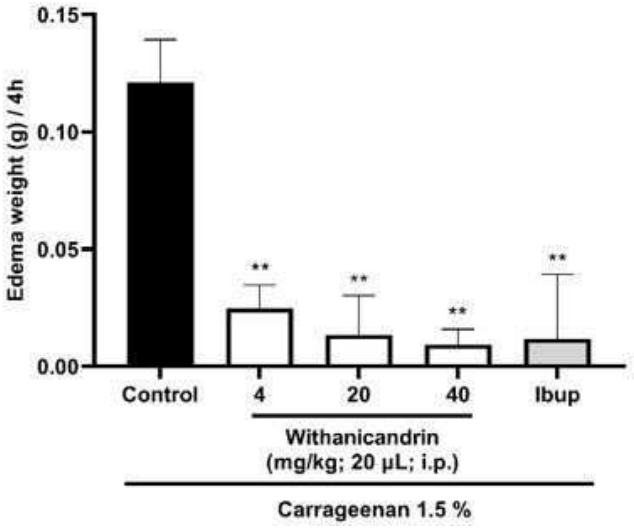

Figure 5. Effect of withanicandrin on abdominal edema induced by 1.5% κ-carrageenan in adult ZFa, analyzed throughout 4 h. Each column represents a mean ± standard error of the mean (n = 6/fish). One-way ANOVA with Tukey's post-hoc test (**$p < 0.01$ vs. control).

Histopathology in Whole Zebrafish Treated with 1.5% K-Carrageenan

Analysis on a histopathological slide performed on the entire ZFa after 4 hours of induction of abdominal edema with κ-carrageenan 1.5% indicates the presence of leukocyte infiltrates on the slide of the negative control group (DMSO 3%) (Figure 6A) and absence of leukocyte infiltrates in the abdominal edema slides of ZFa treated with ibuprofen (Figure 6B) and a 4 mg/kg dose of withanicandrin (Figure 6C).

Liver Tissue Teactive Oxygen Species Levels

Statistical analysis of withanicandrin (40 mg/kg) indicated a reduction in ROS levels in liver tissues when compared to the control group (*p > 0.05 vs. control), a result similar to that observed with the positive control ibuprofen (*$p > 0$ .05 vs. control) (Figure 7).

Molecular Docking

Through the results obtained, observed that the *best pose* presented RMSD (*Root Mean Square Deviation*) values in the order of 1,481 Å (Withanicandrin) and 1,674 Å (Morphine) compared to TRPV1 in the order of 1,872 Å (Withanicandrin) and 1,250 Å (Ibuprofen) compared to COX-1 and in the order of 1,592 Å (Withanicandrin), 1,379 Å (Ibuprofen) compared to COX-2. Regarding affinity energy (kcal/mol), observed values in the order of −11.8 (Withanicandrin /TRPV1), −6.7 (Morphin/TRPV1), −9.9 (Withanicandrin /COX-1), −6.5 (Ibuprofen/COX-1), −9.7 (Withanicandrin/COX-2) and −7.2 (Ibuprofen/COX-2).

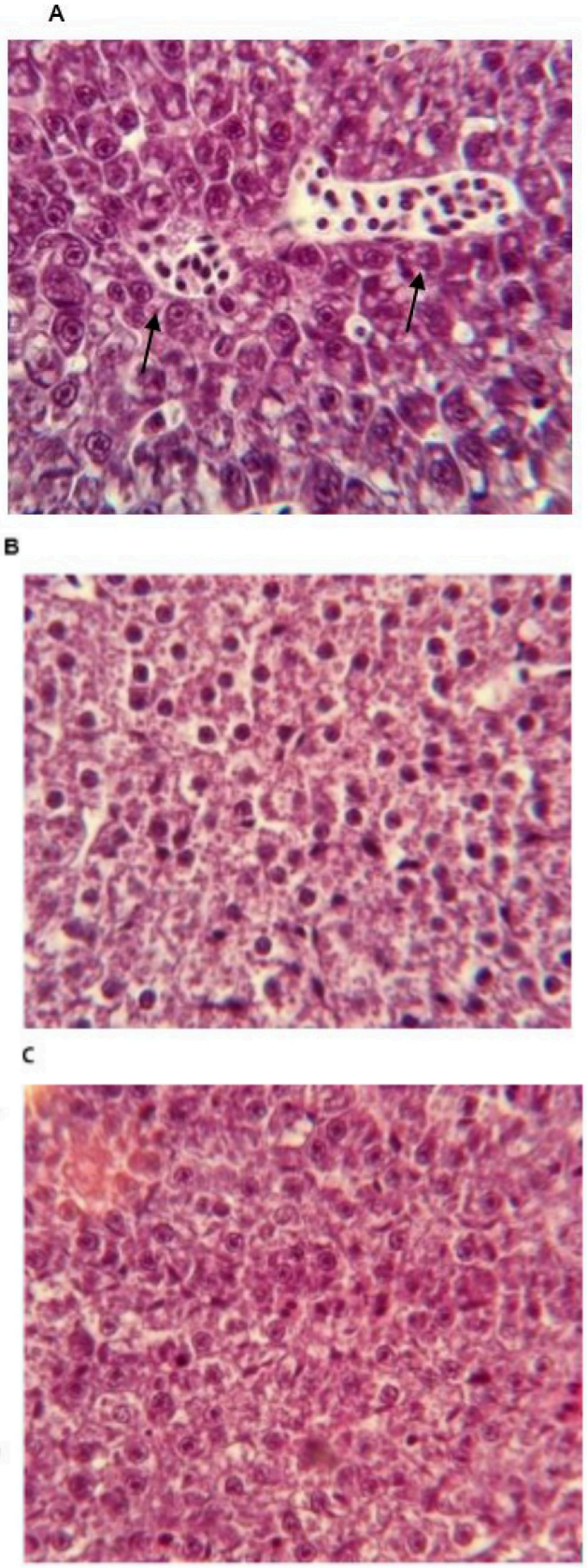

Figure 6. Histopathological slide analysis. (A) Negative control (DMSO 3%, 20 μL; i.p.) with leukocyte and red blood cell infiltrates (black arrow) in the Zfa liver 4 h after i.p. injection. of κ-carrageenan 1.5%. (B) positive control Ibuprofen (100 mg/kg) and (C) withanicandrin (4 mg/kg) with morphologically normal hepatocytes. 100x objective. Staining: H&E.

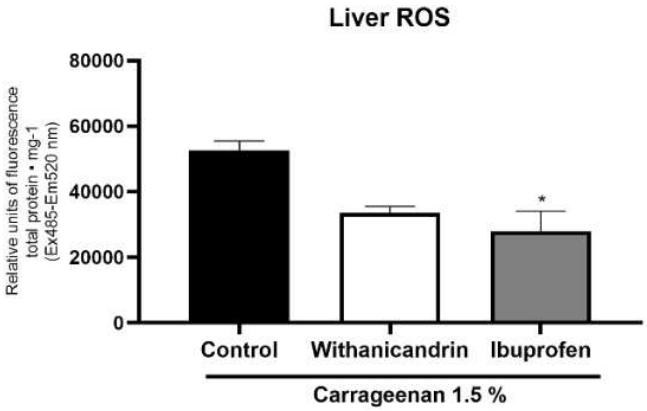

Figure 7. Effect of withanicandrin on hepatic oxidative stress induced by abdominal edema in adult ZFa. Values represent the mean ± standard error of the mean (SEM) for 6 fish/group (3 animals in duplicate). One-way ANOVA followed by Tukey (*$p > 0.05$ vs. control).

Analyzing the interaction patterns, observed that the withanicandrin/TRPV1 complex presented five hydrophobic interactions involving the residues Tyr-495D (3.66 Å), Glu-513D (3.18 Å), Lys-571D (3.93 Å), Leu-574D (3.28 Å), Gln-700D (3.41 Å), and three hydrogen bonds with residues Val-508D (2.52 Å), Tyr-511D (2.37 Å), Ser-512D (2.36 Å). The Morphine/TRPV1 complex showed five hydrophobic interactions involving residues Val-567 C (3.07 Å), Glu-570 C (3.49 Å), Lys-571 C (3.53 Å), Leu-574 C (3.59 Å), Ile-696 C (3.25 Å) and four hydrogen bonds with residues Tyr-511 C (2.78 Å), Ser-512 C (3.21 Å), Arg-557 C (2.08 and 3.29 Å) (Figure 8).

The withanicandrin/COX-1 complex showed five hydrophobic interactions involving residues Val-145 A (3.30 Å), Val-

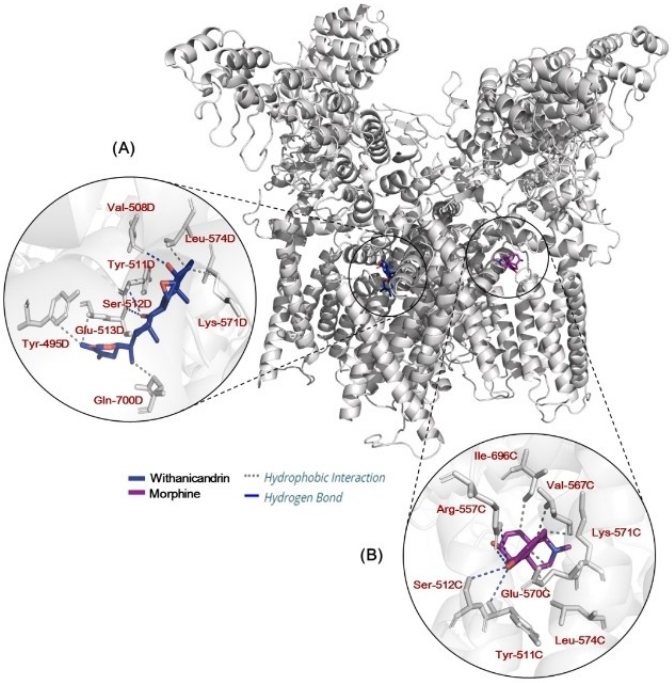

Figure 8. Interaction complex between Withanicandrin (A)

145B (3.64 and 3.78 Å), Leu-224 A (3.69 Å), Leu-224B (3.80 Å) and two bonds of hydrogen with residue Ser-143 A (2.21 and 3.71 Å). The Ibuprofen/COX-1 complex presented four hydrophobic interactions involving residues Gln-372B (3.89 Å), Tyr-373 A (3.84 Å), Pro-542 A (3.64 and 3.74 Å) and three hydrogen bonds involving residues Phe-371 A (2.57 Å), Phe-371B (3.35 Å), Gln-372B (2.13 Å) (Figure 9).

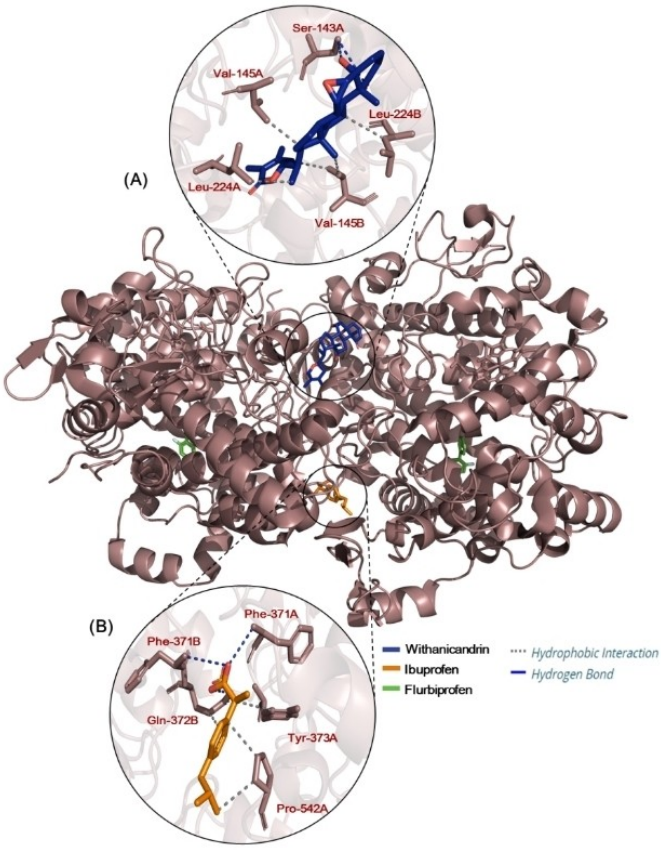

Figure 9. Interaction complex between Withanicandrin (A), Ibuprofen (B), and the co-crystallised inhibitor against COX-1.

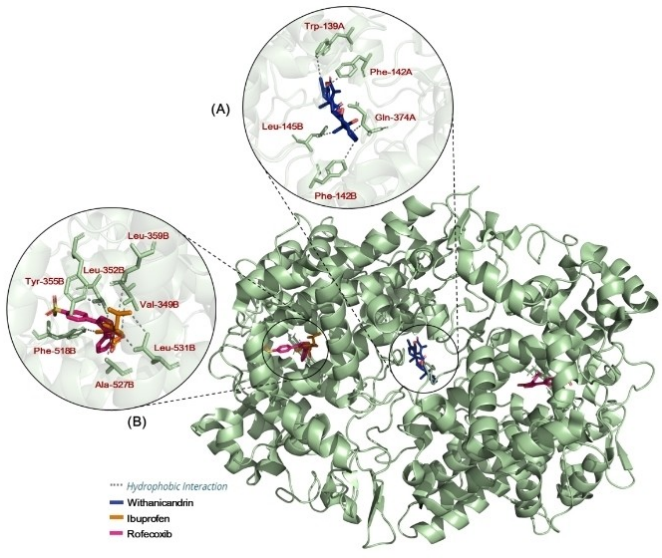

Figure 10. Interaction complex between Withanicandrin (A), Ibuprofen (B) and the co-crystallised inhibitor against COX-2.

The withanicandrin/COX-2 complex showed five hydrophobic interactions involving residues Trp-139 A (3.70 Å), Phe-142 A (3.33 Å), Phe-142B (3.60 Å), Leu-145B (3.94 Å) and Gln-374 A (3.68 Å). The Ibuprofen/COX-2 complex presented eight hydrophobic interactions involving the residues Val-349B (3.48 Å), Leu-352B (3.58 Å), Tyr-355B (3.30 Å), Leu-359B (3.76 Å), Phe-518B (3.63 Å), Ala-527B (3.51 Å), Leu-531B (3.30 and 3.48 Å) (Figure 10).

Although there are studies that analyze the pharmacological potential of other withanolide isolates, mainly related to cancer, it is believed that this is the first to analyze the analgesia and anti-inflammatory capacity of withanicandrin, a withanolide isolated from *Datura ferox* against nociceptive stimuli and inflammatory effects in an experimental ZFa model.

In this study, nociceptive tests were carried out through the induction of pain through chemical noxious stimuli, and the antinociceptive action of withanicandrin was investigated, as well as its pain neuromodulation mechanism in ZFa. Withanicandrin did not present toxicity in ZFa during the 96 h of analysis and did not cause interference in the animals' motor activities, supporting the results in the nociceptive tests of this study.[17]

The nociceptive system is part of a set of mechanisms that control the body's homeostasis, involving the transmission of painful signals through nerve fibers that can often be caused by chemical stimuli, activating nociceptors and causing the perception of pain and behavioral changes.[17] Acid Detection Ion Channels (ASICs) are part of a family of protic/NA^+ channels that are sensitized by changes in extracellular pH (5–7), causing a chemical modification that translates the protonation of some groups into an electrical signal, thus transmitting the nociceptive response.[38] In the experimental ZFa model, six ASICs have already been identified with properties and functions similar to those found in mammals and with high expression in the CNS.[39]

The effect of withanicandrin on the nociceptive behavior of adult ZFa induced by acidic saline (ASIC channel agonist) was investigated, and the highest doses of withanicandrin reduced the nociceptive behavior of the animals, similar to that observed with morphine. This antinociceptive effect of withanicandrin was blocked by naloxone, indicating that this effect occurs through the neuromodulation of ASIC channels.

In addition to ASICs, withanicandrin in its highest dose, it also interfered with the neuromodulation of TRPV1 receptors based on the nociceptive model induced by capsaicin, presenting an effect close to morphine. The TRPV1 receptor is part of the largest group of nociceptive ion channels, defined as TRPs. The activation of these channels occurs through the influx of Na^+ and Ca^+ through the plasma membrane, resulting in their depolarization, with capsaicin being the vanilloid with the most significant representation as a ligand activating nociception in these channels.[40] The analgesia caused by withanicandrin was blocked by capsazepine,

suggesting the participation of TRPV1 receptors in the antinociceptive effect of withanicandrin in adult ZFa.

Inflammation is a process directly linked to pain, as it can arise through a biological response to harmful triggers such as injuries or tissue stress.[2] Withanicandrin affected inflammation induced by κ-carrageenan in adult ZFa at all doses tested. This action can be associated with ASIC channels since, during the inflammatory process, the concentration of extracellular H^+ is elevated to significant levels, contributing to perceiving painful stimuli.[51] Through these channels, it was decisive for generating the nociceptive response. It may also be related to TRPV1 channels, given that these channels participate in the modulation and release of inflammatory mediators, in which one of the different means of activation is through temperature elevation, a characteristic that is predominant in inflammatory processes, as well as by through the perception of pain generated by κ-carrageenan in the development of the inflammation process.[42]

The anti-inflammatory effect of ibuprofen was also confirmed by reducing abdominal edema induced by κ-carrageenan in animals. Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that acts to inhibit prostaglandin synthesis, in which its precursor fatty acid (arachidonic acid) is mobilized by the cell membrane and converted into prostaglandin H through catalysis through prostaglandin H synthase (PGHS) known as cyclooxygenase (COX). Its two isoforms are COX 1 (ptgs1) and COX2 (ptgs2), and play an essential role in inflammation and nociception. Functional genes related to COX-1 and COX-2 have already been reported in ZFa due to their high expression in several cellular organelles, with prostaglandins E being more prevalent in ZFa.[43,44]

The histopathological study of the edematous animals was carried out. The region of the liver of Zfa treated with withanicandrin indicated the presence of morphologically normal hepatocytes and the absence of leukocyte infiltrates (Figure 7), unlike the histopathological slide of the negative control (DMSO 3%), which after 4 hours of injection of κ-carrageenan, still showed the presence of leukocytes, pointing to a possible continuity of the inflammatory process. A study on the normal anatomy and histology of ZFa highlighted that the majority of leukocytes have a structure and function similar to that of humans and that, in addition, the liver, responsible for metabolic homeostasis, also has equivalent functions, such as detoxification and protein synthesis.[45] When evaluating the histopathology of inflammatory processes in ZFa induced by 3.5% λ-carrageenan, showed the persistence of leukocytes such as neutrophils and lymphocytes infiltrated in the peritoneal cavity of the ZFa as well as in the liver and pancreas, corroborating the data obtained in this study.[46]

As for oxidative stress is considered a fundamental mechanism involved in the pathogenesis of abdominal edema after carrageenan application.[47] Previous studies indicate the overproduction of ROS after carrageenan administration, as it affects the integrity of the plasma membrane and increases its lipoperoxidation.[48,49] Withanicandrin protected liver tissues from oxidation caused by κ-carrageenan applied to the animals' peritoneum, reinforcing its potential anti-inflammatory effect in ZFa.

Regarding molecular docking, the favorable values of affinity energy confirm the feasibility of forming receptor/ligand complexes. Residues Tyr-511, Met-514, Leu-518, Leu-547, Thr-550, Arg-557, Glu-570, and Leu-670 have been reported to be critical for both agonists and antagonists. They are important residues involved in the pocket of TRPV1 binding.[50] The interaction profile showed that withanicandrin bound to TRPV1 in monomer D and morphine in monomer *C*. *Withanicandrin* showed a strong hydrogen bond with the critical residue Tyr-511D and morphine with the critical residues Tyr-511 C and Arg- 557 C. In addition, morphine interacts hydrophobically with the critical residue Glu-570 C (Figure 9).

The redocking of the inhibitor flurbiprofen co-crystallized in COX-1 showed an RMSD value of 1,813 Å and an affinity energy of −7.9 kcal/mol. The 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.[26] Interaction analysis showed that withanicandrin interacts in a different region of the binding site of the co-crystallized inhibitor and the control (Figure 10), indicating a possible synergistic effect with flurbiprofen and ibuprofen.

Redocking the inhibitor rofecoxib co-crystallized in COX-2 showed an RMSD value of 1,402 Å and an affinity energy of −8.0 kcal/mol. The binding site 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. In the analysis of interactions, observed that withanicandrin 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 10). It was also observed that ibuprofen interacts with residues in the active site of the enzyme, presenting a similar effect to rofecoxib co-crystallized in the B chain of COX-2.

Experimental Section

Obtaining Withanicandrin

The sample was isolated by Pinto et al. (2020) from the leaves of D. Ferox. The species was identified by Profa. Dr. Maria Iracema Bezerra Loiola, from the Laboratory of Systematics and Plant Ecology of the Department of Organic and Inorganic Chemistry at the Federal University of Ceará (UFC), Fortaleza, Ceará – Brazil.

The plant material was collected in Apuiarés-CE, cataloged under number 42384 in the Herbarium Prisco Bezerra (EAC) of the Federal University of Ceará – UFC, and registered in Sigen under number A86B918. The ^{1}H and ^{13}C NMR data are by with the literature.[12]

Drugs and Reagents

The drugs and reagents used in the nociception experiments were formalin (Formaldehyde; 0.1%), acidic saline, hypertonic saline, naloxone (Tocris Bioscience), capsaicin, camphor, capsazepine, ruthenium red and morphine obtained from Sigma- Aldrich (Brazil) and Dimethylsulfoxide (DMSO) obtained from Cequimíca. The drugs and reagents used in the inflammation experiments were κ-Carrageenan (Palazzo do Diet Light) and ibuprofen (Advilâ).

Zebrafish

Adult, wild zebrafish (Danio rerio) (ZFa) aged between 60 and 90 days (4.0 ± 0.1 g and between 3.5 ± 0.5 cm) of both sexes (n = 6/groups), obtained through the supplier Agroquímica: Comércio de Produtos Veterinários LTDA, from Fortaleza, Ceará. The handling and acclimatization of the animals were carried out in the Chemistry and Natural Products Laboratory of the State University of Ceará – LQPN/UECE. The animals were kept in a glass aquarium with a temperature of 25 °C and pH 7.0, with tap water treated with ProtecPlus® brand antichlorine and air pumps with submerged filters. After the experiments, the animals were sacrificed by immersion in cold water (2–4 °C) until loss of opercular movements.[13] (CONCEA, 2018). The Ethics Committee for the Use of Animals (CEUA) approved the experiment procedures under protocol n° 04983945/2021 of the State University of Ceará (UECE), Fortaleza, Ceará.

General Protocol

For the experiments, Zfa of both sexes were randomly selected, transferred to a moist sponge, treated with test samples or controls intraperitoneally (*i.p.*), and subsequently treated with h chemical agents armful agents intramuscularly (*i.m.*) in the tail or córnea.[14,15] Then, the animals were placed individually in a glass beaker (250 mL) containing 150 mL of aquarium water and kept at rest. For intraperitoneal (*i.p.*), intramuscular (*i.m.*) and topical corneal treatments, insulin syringes (0.5 mL; UltraFine® BD) with a 30G needle were used.

Assessment of Locomotor Activity (Open Field Test)

Fish (n = 6/group) were treated intraperitoneally (*i.p.*) with withanicandrin (4; 20 and 40 mg/kg 20 μL) or vehicle (control: DMSO 3%; 20 μL; *i.p.*), and after 30 minutes of treatments were taken to the open field test in a petri dish inserted individually in each dish (100×15 mm). The test was conducted to evaluate whether withanicandrin causes changes in the animals' motor coordination through sedation and/or muscle relaxation.[14]

Acute Toxicity 96 h

After the open field test, the animals were left at rest to analyze the mortality rate for a period of 96 h and, every 24 h, the number of dead fish in each group was recorded[16] and the lethal concentration capable of killing 50% of animals (LD_{50}). t was determined using the Trimmed Spearman-Karber mathematical method with a 95% confidence interval.

Treatments

In all nociceptive tests, animals (n = 6/group) were treated intraperitoneally (20 μL) with withanicandrin (4; 20 and 40 mg/kg), morphine (8 mg/kg – positive control) or vehicle (DMSO 3%). In inflammation tests, animals (n = 6/group) were treated intraperitoneally (20 μL) with withanicandrin (4; 20 and 40 mg/kg), ibuprofen (100 mg/kg) – positive control), κ- carrageenan (1.5%; 20.0 μL, *i.p.* – negative control) or vehicle (control, DMSO 3%, 20 μL).

Nociceptive Behavior Induced by Chemical Agents

The animals were pre-treated with withanicandrin (*i.p*) 30 minutes before receiving treatments with noxious stimulus *(i.m*): (1) Formalin (cation channel agonist, subfamily A, member 1 [TRPA 1] in the neuropathic and neurogenic phases; 0.1%; 5.0 μL); (2) Capsaicin (cation channel agonist with potential for transient receptor 1 of subfamily V [TRPV1]; 40.93 μM/ 5.0 μL); (3) Acid Saline (Agonist of Acid Detection Ion Channels (ASIC); 0.1% acetic acid dissolved in saline solution, pH 3.28/5.0 μL. Antinociceptive activity was evaluated individually for each type of treatment. The animals were placed individually in Petri dishes (100×15 mm), divided into quadrants, and the nociceptive response was quantified in terms of locomotor activity, that is, according to the number of line crossings performed during a period.[14] To verify the possible involvement of withanicandrin in the systems: TRPA1, TRPV1 and ASICs, tests were subsequently carried out with antagonists of these channels.[17] The animals (n = 6/group) were pretreated intraperitoneally (5.0 μL) with naloxone (8 mg/kg, ASICs channel antagonist), camphor (30.4 mg/kg, TRPA1 channel antagonist) capsazepine (20 mg/kg, TRPV1 channel antagonist) 15 minutes before pretreatment with the lowest effective dose of withanicandrin (4 mg/kg. *i.p.*). Antinociceptive activity was analyzed for each specific treatment. The animals were placed individually in a Petri dish (100×15 mm), divided into quadrants, and the nociceptive response was quantified in terms of locomotor activity (number of line crossings) performed during a specific period for each model described below in the results section.

Hypertonic Saline-Induced Corneal Nociception

The induction of nociception in the ZFa cornea was performed with hypertonic saline solution (TRPV1 agonist; 5 M NaCl; 5.0 μL),[15] applied to the right eye of the animals (n = 6/group) for 1 h after pretreatment with withanicandrin (4, 20 or 40 mg/kg; 20 μL; *i.p.*) or morphine (8 mg/kg; 20 μL; *i.p.*; positive control) or vehicle (DMSO 3%, 20 μL; *i.p.*). Antinociceptive activity was analyzed in the open field test in the Petri dish as previously described.

Induction of Edema by κ-Carrageenan 1.5%

The anti-inflammatory activity was investigated through the induction of abdominal edema induced by κ-carrageenan.[18] Animals (n = 6/group) received withanicandrin (4; 20 and 40 mg/kg; 20 μL; *i.p.*) or vehicle (control, DMSO 3%; 20 μL; *i.p.*). A group of animals were treated with the positive control - ibuprofen (100 mg/kg; 5.0 μL; *i.p.*). After 1 h of treatments, the fish were individually injected i.p. of κ-carrageenan (1.5%; 20.0 μL). The animals' body weight (BW) was measured before treatment and at 1-h intervals after induction of peritoneal edema over a 4-h period. The animals were immediately sacrificed to stop biological reactions at the end of the experiment.

Histopathology in Whole Zebrafish Treated with 1.5% κ-Carrageenan

Followed by the acute inflammation test by induction of edema by 1.5% κ-carrageenan, the animals treated with κ-CGN (positive control, negative control, and sample; n = 6/group) were sacrificed by immersion in an ice bath for 10 minutes and then fixed in 10% formaldehyde solution. After fixation, the whole fish was placed in the right lateral decubitus position in plastic cassettes and subjected to a graduated series of baths in ethanol for dehydration and xylene for clearing 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® semiautomatic microtome, with 4 μm thick sections, deparaffinized, following standard procedures and stained with hematoxylin and eosin (H&E) .[19] The slides were analyzed using a LaboMed® Research Microscope Halogen Serie Lx 400 optical microscope with a 100x objective, allowing visual evaluation and analysis of the entire structure of the fish, emphasizing the liver.

Liver Tissue Reactive Oxygen Species Levels

To verify the levels of reactive oxygen species (ROS) in the liver tissues of fish edematous with 1.5% κ-carrageenan, the lowest effective dose indicated in the acute inflammation test was used (40 mg/kg), and then the DCHF-DA.[20] After the carrageenan-induced abdominal edema test, the animals (n = 6/group) were euthanized on ice for liver extraction. Liver tissues from three animals (in duplicate) were macerated in Tris-HCl-EDTA, followed by centrifugation at 10,000×g for 10 min. Then, 200 μL of the supernatant was collected and mixed with 5 μL of DCHF-DA. The oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured to detect reactive oxygen species (ROS). The fluorescence intensity emission of dichlorofluorescein was recorded at 520 nm (with 480 nm excitation) 2 h after adding DCHF-DA to the sample. To interpolate the ROS results with the protein content of liver tissue samples, tissue protein quantification was performed using the Bradford method. Protein concentration was determined by UV-VIS light spectrophotometry at 280 nm using a standard BSA curve.

Molecular Docking

Ligand and Receptor Preparation for Docking Studies

The chemical structure of withanicandrin (CID12444955) was obtained from the PubChem repository (https://pubchem.ncbi.nlm.nih.gov/). The lowest energy conformer was saved at physiological pH using the MarvinSketch software[21] and optimized using Avogadro software,[22] configured to use the *steepest descent* algorithm with cycles of 50 interactions, applying the MMFF94 force field (*Merck Molecular Force Field*).[23,24]

To investigate the mechanism of action of withanicandrin against TRPV1, 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: 3 J5R,[25] 3 N8Z[26] and 5KIR,[27] respectively. In the receptor preparation stage, residues were removed, the prosthetic group Heme (COX-1) and protoporphyrin IX containing Co (COX-2), important residues for biological activity, were maintained, polar hydrogens and Kollman charges and Gasteiger charges were added[28] using Autodocktools™ software.[29]

Molecular Docking Simulation and Data Output

50 independent molecular docking simulations were performed using AutodockVina software,[30] *Lamarkian Genetic Algorithm* (LGA) and *Exhaustiveness* 64.[31] The generated simulation grid was centered on involving the entire enzyme structure using axes (0.514 x, 3.021 y, 7.304 z) and size (126 x, 126 y, 110 z) in front of TRPV1; 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) compared to COX-2. To obtain comparative data, simulations were carried out with Morphine (TRPV1 control) and Ibuprofen (COX-1 and COX-2 control). 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[32] and the affinity energy, considered ideal when it presents values equal to or lower than −6.0 kcal/mol, were used as criteria.[33]

Visualization of Binding Modes and Receptor–Ligand Interactions

Data analysis was performed using the software UCSF Chimera™,[34] *Discovery Studio Visualizer* ™ *viewer*[35] and *Pymol*.[36] Molecular interactions and hydrogen bonds were visualized using the Protein-Ligand Interaction Profiler (PLIP)[37] (Salentin et al. 2015).

Statistical Analysis

The results were expressed as mean values ± 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 analysis of variance (One-way ANOVA), followed by the Tukey test. All analyses were performed using GraphPad Prism v software. 6.01. The level of statistical significance adopted was 5% ($p < 0.05$).

Conclusions

Given the results, withanicandrin isolated from *Datura Ferox* was shown to have a promising pharmacological effect by inhibiting nociceptive behavior through neuromodulation of ASICs and TRPV1 channels, as well as an anti-inflammatory effect in ZFa, also generating hepatic protection from reactive oxygen species generated by carrageenan, without being toxic and without promoting changes in locomotion in the animal. The experimental data were corroborated by the molecular docking study, where withanicandrin showed better affinity energy compared to the controls morphine (TRPV1) and ibuprofen (COX-1 and COX-2), in addition, withanicandrin bound in a different site than the controls, indicating that it can be used alone or in combination with morphine and ibuprofen.

Author Contributions

Jéssica Bezerra Maciel, Maria Izabel F Guedes: Supervision, writing e review and editing; Hortência Ribeiro Liberato,

Andrelina Noronha Coelho-de-Souza and João Pedro Vieira da Silva: Investigation, formal analysis, and writing - original draft. Antônio Wlisses Da Silva and Maria Kuerislene Amâncio Ferreira: Formal analysis, software, validation, and reviewed the manuscript Hélcio Silva dos Santos, Francisco Sydney Henrique da Silva and Paulo Goberlânio De Barros Silva: Conceptualization, methodology, and determined the molecular structures. Francisco das Chagas L. Pinto, Emmanuel Silva 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.

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