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Anti-Inflammatory and Antinociceptive Potential of Hydroethanolic Extract of *Bauhinia guianensis* Aubl. in Zebrafish (*Danio rerio*)

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Abstract: The leaves and stems of *Bauhinia guianensis* Aubl. are used in traditional Amazonian phytotherapy for the treatment of pain and inflammation. This study investigates the anti-inflammatory and antinociceptive effects of hydroethanolic extracts from *B. guianensis* Aubl. leaves and stems (HELBg and HESBg, respectively) in *in vivo* models of inflammation and hyperalgesia. *Danio rerio* experimental animals were submitted to the acute inflammation test, induced by intraperitoneal (ip.) administration of carrageenan 20 µg / animal (abdominal edema), the groups were previously treated orally with saline solution 2 µl / animal (SS), Dimethyl sulfoxide 2 µl / animal (DMSO), Indomethacin 10 mg/kg, HELBg 100 mg/kg and HESBg 100 mg/kg, n = 12 per experimental group, to evaluate inhibition of edema and alterations histopathology of the liver, intestine and kidney of these animals. The antinociceptive effect was observed from the body curvature index and the behavioral responses of *Danio rerio*, after an experimental protocol for the induction of hyperalgesia, by ip. administration of 10 µl / animal of 2.5% acetic acid, the animals were previously orally treated with saline solution 2 µl/animal (SS), dimethyl sulfoxide 2 µl/animal (DMSO), morphine 2.5 mg/kg, HELBg 100 mg/kg and HESBg 100 mg/kg, n = 5 per experimental group. In carrageenan-induced edema, the group treated with HESBg inhibited edema formation over the 3 hours of the experiment. Maximum edema was inhibited by 54% ($p < 0.05$) when compared to the control group. Both HELBg and HESBg prevented body curvature index changes ($t_{(df=3\,8)}$ =, 6.96 and $t_{(df=3,\,8)} = 6.61$, respectively, both $p < 0.0001$). In the behavioral parameters sensitive to antinociceptive pharmacological modulation, due to the abdominal constriction induced by acetic acid, the administration of HELBg and HESBg resulted in an improvement in swimming activity, by increasing the distance covered ($F_{(df=3,\,16)} = 6.50$ and $F_{(df=3,\,16)} = 7.72$, respectively, both $p < 0.0001$), decrease in freezing time ($F_{(df=3,\,16)} = 2.04$ and $F_{(df=3,\,16)} = 1.28$, respectively, both $p < 0.0059$), increase in the number of ascents to the upper area of the tank ($F_{(df=3,\,16)} = 33.02$ and $F_{(df=3,\,16)} = 35.62$, respectively, both $p < 0.0009$) and decreased time spent in that area ($F_{(df=3,\,16)} = 101.19$ and $F_{(df=3,\,16)} = 103.59$, respectively, both $p < 0.0038$). It is reasonable to suppose that both extracts modulated the variations induced by carrageenan and acetic acid through the inhibition of prostaglandin biosynthesis, decreasing the release of inflammatory mediators and the sensitization of peripheral nociceptors and, consequently, the perception of pain. These results suggest that HELBg and HESBg have anti-inflammatory and antinociceptive activities, probably of peripheral origin and linked to the inhibition of prostaglandin biosynthesis.

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1. Introduction

The centuries-old use of native plant species reflects the valuable resources available to traditional peoples and communities in the Amazon, especially those who live in remote areas and dense forests, who resort to native medicinal flora due to the essential components disseminated through ethnomedicinal practice [1–3]. A growing body of evidence describes the promising role of natural compounds in reducing pain and inflammation [4,5]. A bioprospecting approach that reinforces these popular perception results should be strongly encouraged in pharmacological research [6].

Although there have been great efforts to develop drugs of synthetic origin in recent years, with anti-inflammatory and antinociceptive action, the importance of ethnopharmacology as a valuable source of therapeutic compounds is known due to the vast biosynthetic capacity derived from constituents phytochemicals [5,7]. In the Amazonian socioeconomic scenario, the availability of pharmaceutical products is not always accessible, corroborating for communities to depend solely on knowledge of traditional medicine for everyday needs [8,9].

In this context, the species *Bauhinia guianensis* Aubl. (*B. guianensis* Aubl.), from the Fabaceae family. It is a spontaneous plant, usually known as the "tortoise ladder," due to the growth of the stem lianas in a flattened, twisted shape with alternating curves that resemble a ladder. This liana species is native to the Amazon Biome and is found in Bolivia, Brazil, Colombia, Guyana, French Guiana, Peru, Suriname, and Venezuela. In Brazil, it occurs mainly in the northern region. However, it has also been identified in the State of Maranhão, in remnants of the Atlantic Forest Biome in the State of São Paulo [6].

Historically, the stems and leaves of *B. guianensis* Aubl. they are widely used in Amazonian ethnomedicinal practice to relieve abdominal pain and treat inflammatory processes [10]. Despite the many reports of the popular medicinal use of *B. guianensis* Aubl. in the Amazon, few scientific studies have described its toxicity potential, phytochemical characterization [6,11], and pharmacological potential of this species [10–12].

Among the studies that evaluate the bioprospecting potential of the species, the studies by Carvalho et al. [11], who evaluated the methanolic extract of the stem bark of *B. guianensis* Aubl. (100 mg/kg) in Swiss mice with induction of paw edema by carrageenan, in which they observed a significant inhibition of edema formation in animals treated with the extract, when compared to the control group. The study also tested the dichloromethane extract of *B. guianensis* Aubl. in Swiss mice. The authors observed significant inhibition of the algogenic process, produced by inducing abdominal contortions with acetic acid.

Other studies have described tests with hydroalcoholic extract of *B. guianensis* Aubl., against the anticonvulsant activity in Swiss mice, with seizures induced by pentylenetetrazol and by auricular electroshock. After using the extract, there was a significant latency of the seizure threshold [12]. However, further studies are needed to establish the pharmacological profile of *B. guianensis* Aubl. compatible with its therapeutic potential as an anti-inflammatory and antinociceptive agent [6].

In this context, inflammation is a necessary and beneficial process for the body, restructuring homeostasis and restoring tissue normality [13]. It acts as a defense and repair strategy. However, this mechanism can be compelled in the intense and uncomfortable presence of acute clinical manifestations (heat, redness, edema, and pain), subacute, extensive systemic or chronic repercussions, with manifestations of disabling symptomatic conditions and cumulative tissue damage, e.g., deformities and functional losses [14–16].

The inflammatory process is extensively studied due to its pathophysiological relevance. Faced with the complex series of restorative and protective responses to tissue injury, there are anti-inflammatory agents: steroidal and non-steroidal (NSAIDs) [17]. Currently used anti-inflammatory

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agents, such as NSAIDs, inhibit cyclooxygenase and therefore prostaglandin synthesis. These also have antinociceptive activity, since prostaglandins are implicated in mediating nociception [18]. However, NSAIDs can cause undesirable side effects [19]. Although widely available on the market, they are not necessarily accessible to Amazonian populations, who seek native plant sources to relieve inflammation. Research on substances or medications that offer fewer adverse effects and are capable of preventing or mitigating the successive events of inflammation are strongly encouraged [20,21].

In terms of application sciences, there is a lot of biomedical research about inflammation in experimental model *Danio rerio* [22,23]. This animal model has been widely used in tests for drug discovery, due to its characteristics, such as easy genetic manipulation, high prolificity, external fertilization, rapid development, transparent embryo [24,25]. In addition to the advantages of providing quickly translatable data in a spectrum of tissues, organs and systems. It has high genetic homology with mammals, robust phenotypes and high-throughput genetic and chemical screening, making it a powerful tool to evaluate *in vivo* natural and synthetic substances [26].

In addition, the small size, associated with the low body weight of the adult Danio rerio, allows the use of reduced amounts of the compound tested, due to the calculation of the dose, being considered an advantageous and relevant model for the screening of new anti-inflammatory substances [25,27]. This is because it is possible to assess behavioral and tissue alterations, edema formation, accumulation of inflammatory cells in the exudate, mediators, signaling pathways, gene expression, production of specific proteins [4].

Therefore, the present study aims to investigate the anti-inflammatory and antinociceptive effects of hydroethanolic extracts from *B. guianensis* Aubl. leaves and stems in models of inflammation and hyperalgesia *in vivo*, reinforcing the traditional Amazonian use, particularly important in the decision of the tested activities.

2. Results

2.1. Anti-inflammatory potential of HESBg and HELBg in the inhibition of edema by carrageenan in Danio rerio

The first group studied received saline only orally and PBS ip. (SS/PBS), therefore, there was no induction of inflammatory edema. In the other groups, the administration of carrageenan in the peritoneum of *Danio rerio* produced visible edema, reaching a maximum peak after the third hour of the stimulus, mainly in the group that received only the DMSO extract diluent. The group treated with HESBg considerably inhibited swelling over the 3 monitored hours. The percentage of inhibition was around 15% in the HELBg group, and the group treated with HESBg had 54% of the edema volume reduced, compared to 51% in the control group (indomethacin) ($p < 0.05$), Figure 1.

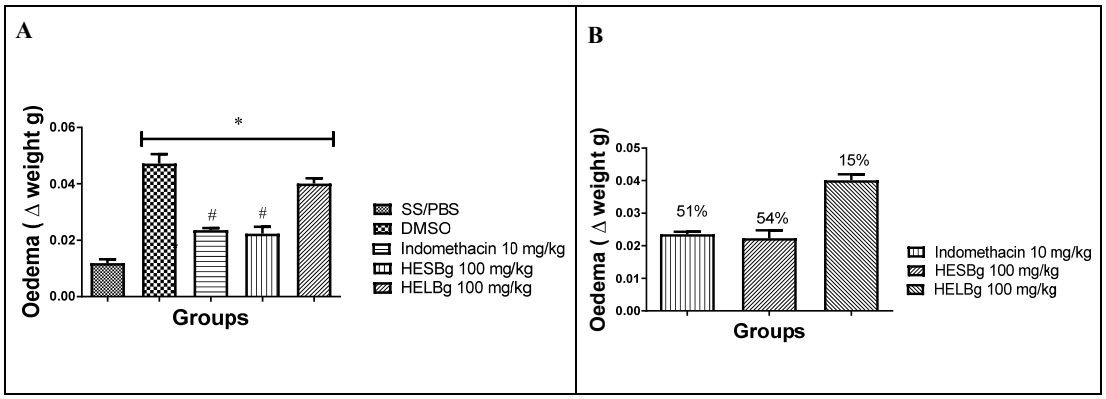

Figure 1. Effect of oral administration of saline and PBS (SS/PBS, 2 µl), DMSO 2 µl, Indomethacin 10 mg/kg, HESBg 100 mg/kg and HELBg 100 mg/kg on edema induced by carrageenan (20 µg/animal). * $p < 0.05$ ANOVA followed by Tukey's test, n = 12 (A). Inhibition of the percentage obtained with the oral treatment of doses of indomethacin, HESBg and HELBg in carrageenan edema (B).

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2.2. Histopathological evaluation of the inflammation test in Danio rerio

2.2.1. Histopathological evaluation of the liver

In the histopathological analysis of the animals' livers (Figure 2 A), the Histopathological Change Index (IHA) of the SS and HESBg groups was 0.5 ± 0.288, Indomethacin; 0.75 ± 0.250 (Figure 2 A), indicating a normal state of the organ after the application of PBS, and carrageenan in the indomethacin and HESBg groups. Microscopic reading of the slides showed only grade I changes, such as cytoplasmic vacuolation (Figure 3 A).

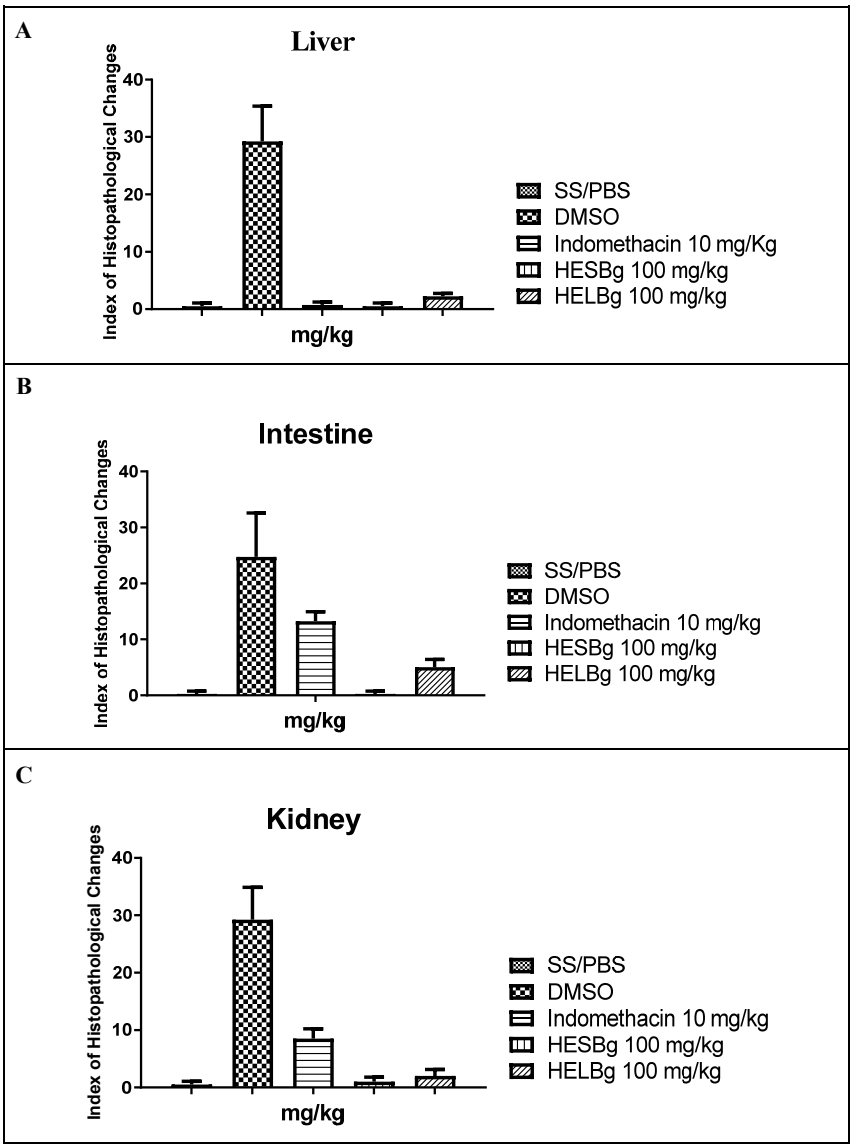

Figure 2. Effect of oral administration of saline and PBS (SS/PBS, 2 µl), DMSO 2 µl, Indomethacin 10 mg/kg, HESBg 100 mg/kg and HELBg 100 mg/kg on the Index of Histopathological Changes in the Liver (A), intestine (B) and kidney (C) of Danio rerio with the application of carrageenan (20 µg/animal). Histopathological Change Index (HAI): HESBg and HELBg were not organ toxic. * $p < 0.05$ ANOVA followed by Tukey's test, n = 12.

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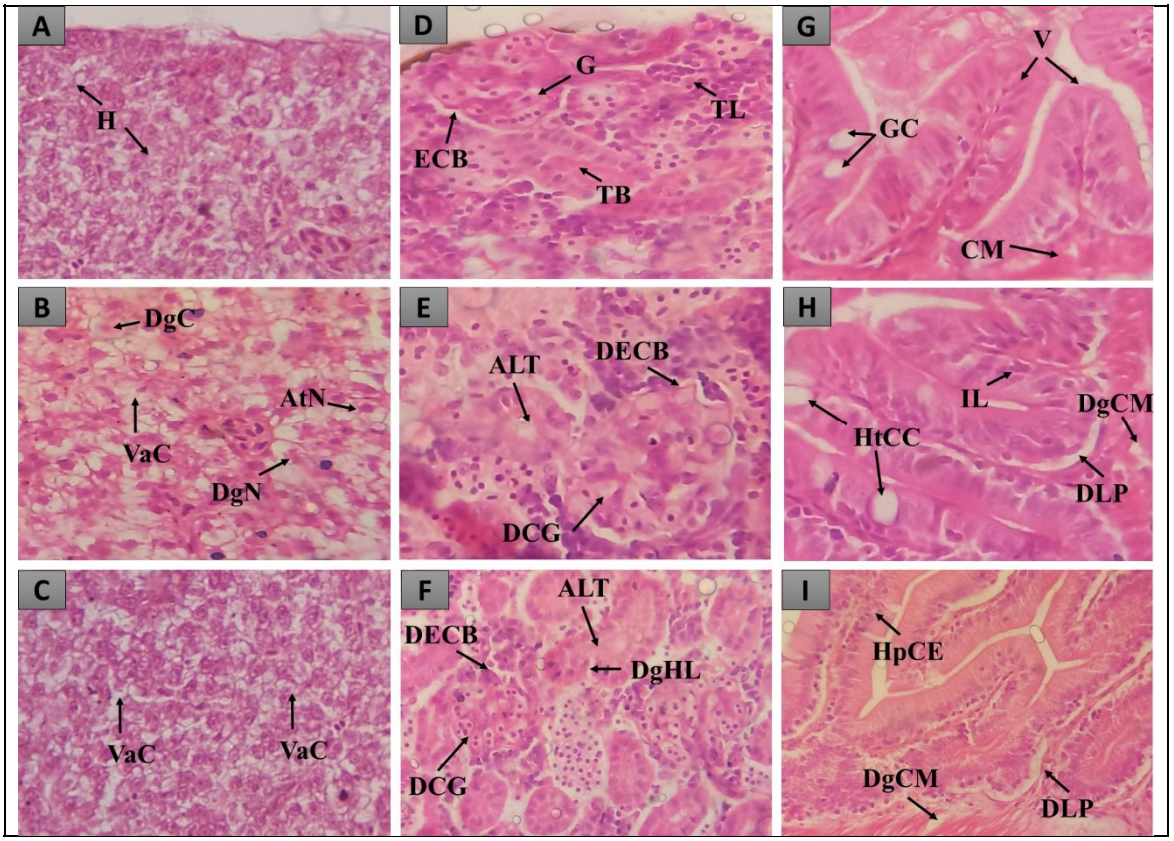

Figure 3. Histopathological alterations observed in the liver, intestine and kidneys of zebrafish in the different treatments. In A, B and C, liver tissue with normal hepatocytes (H), cytoplasmic vacuolation (VaC), nuclear atypia (AtN), nuclear degeneration (DgN) and cellular degeneration (DgC) is observed; In D, E and F, there is renal tissue with normal glomerulus (G), Bowman's capsule space (ECB), tubules (TB), lymphoid tissue (TL), increased tubular lumen (ALT), dilated capillaries of glomerulus (DCG), Bowman's capsule space narrowing (BDEC), mild tubular hyaline degeneration (DgHL); In G, H and I, intestinal tissue with normal goblet cells (GC), lymphocytic infiltration (IL), villi (V), goblet cell hypertrophy (HtCC), muscle layer degeneration (DgCM) is observed, epithelial cell hyperplasia (HpCE). Staining (H&E).

The DMSO group had an IHA of 29.25 ± 3.065 (Figure 2 A), with significant alterations classified as moderate to severe (levels I, II and III) Table 1, including nuclear atypia (I), nuclear degeneration (II) and cellular degeneration (III) (Figure 3 B and C).

Table 1. Histopathological evaluation of *Danio rerio* inflammation model.

Treatment groups	Histopathology [1]		
	Liver	Intestine	Kidneys
SS	Normal		
DMSO	Moderate to severe		
Indomethacin	Normal		Mild to moderate
HESBg	Normal		
HELBg	Normal		

[1] Effects of PBS or carrageenan administration on the analyzed organs, according to Carvalho et al. [28]; Poleksic and Mitrovic-Tutundzic [29]; Rigolin-Sá [30].

The animals in the HELBg group showed liver tissue with an IHA 2.25 ± 0.250, which classifies this organ as functionally normal (Figure 2 A). The histopathological changes observed were cytoplasmic vacuolation (I) and nuclear atypia (I) (Figure 3 A and B).

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2.2.2. Histopathological evaluation of the intestine

The intestinal tissue of the SS and HESBg groups obtained an IHA of 0.25 ± 0.250, and of the HELBg 5.00 ± 0.707, classifying the organs as normal (Figure 2 B), that is, with the presence of grade I alterations, such as lymphocytic infiltration and goblet cell hypertrophy (Figure 3 G and H).

The DMSO group had an IHA of 24.75 ± 3.924 and classification of changes from moderate to severe (Figure 2B), namely, lymphocytic infiltration, hypertrophy of goblet cells (I) and degeneration of the muscle layer (III) (Figure 3G, H and I).

The Indomethacin control group had an IHA of 13.25 ± 0.853, that is, classifying the organs with mild to moderate alterations (Figure 2 B), including epithelial cell hyperplasia (I) (Figure 3 G and H).

2.2.3. Histopathological evaluation of the Kidneys

The changes registered in the renal tissue of the groups treated with SS, HESBg, HELBg and Indomethacin were classified with IHA of 0.5 ± 0.288; 1.0 ± 0.408; 2.0 ± 0.577 and 8.5 ± 0.866, respectively, demonstrating a functionally normal organ (Figure 2 C). In the histopathological analysis, these kidneys showed grade I alterations, such as an increase in the tubular lumen, dilation of the glomerulus capillaries (Figure 3 D, E and F).

In the kidneys of the DMSO group, histopathological damage was observed, IHA equal to 29.25 ± 2.810 (Figure 2 C), with a decrease in Bowman's capsule space (I), mild tubular hyaline degeneration (I), being classified as moderate to severe (Figure 3 E and F).

2.3. Antinociceptive potential of HESBg and HELBg in the modulation of behavioral phenotypes after intraperitoneal injection of acetic acid in Danio rerio

The first group studied received saline only orally and PBS ip. (SS/PBS), therefore, PBS alone did not change the body curvature index ($t_{(df=3)} = 3.50$, $p < 0.0001$). Administration of acetic acid (2.5%, ip.) affected *Danio rerio* body curvature index ($t_{(df=3)} = 13.83$, $p < 0.0001$) compared to its baseline conditions ($t_{(df=3)} = 3.29$, $p < 0.0001$) and/or compared to the control (PBS), DMSO does not influence the measured abdominal constriction behavior, Figure 4.

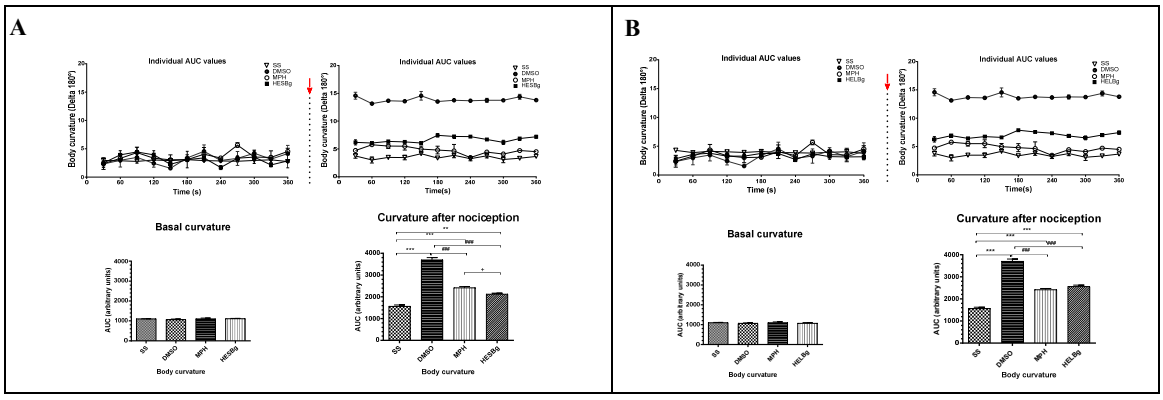

Figure 4. Behavioral phenotypes (body curvature index) at baseline and after intraperitoneal injection of 2.5% acetic acid in *Danio rerio* with different treatments (SS 2 µl, DMSO 2 µl, Morphine 2.5 mg/Kg, HESBg 100 mg/kg, B HELBg 100 mg/kg). Results expressed as mean ± S.P.M analyzed using one-way Analysis of Variance (one-way ANOVA), followed by Tukey's post test of multiple comparisons, n = 5 *$p < 0.05$.

Oral treatments such as Morphine, HESBg and HELBg standard prevented changes in body curvature index after ip injection. of acetic acid ($t_{(df=3, 8)} = 4.83; 6.61; 6.96$; $p < 0.0001$) Figure 4.

In Figure 5, animals that received PBS ip. demonstrated baseline behaviors, such as locomotion ($F_{(3.16)} = 8.12$, $p < 0.0001$), duration of the animal's freezing behavior ($F_{(3.16)} = 0.66$, $p < 0.0059$), transitions to the upper area of the tank ($F_{(3.16)} = 38.68$, $p < 0.0009$) and time spent in this area ($F_{(3.16)} = 98.64$, $p < 0.0038$).

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The other animals, such as the DMSO group, when receiving acetic acid ip., it was seen how this influenced the behavior, as it reduced locomotion ($F_{(3,16)} = 4.47$, $p < 0.0001$), transitions to the upper area ($F_{(3.16)} = 10.52$, $p < 0.0009$). In addition, it increased the duration of the animal's freezing behavior ($F_{(3.16)} = 3.54$, $p < 0.0059$) and the time spent in the upper area of the tank ($F_{(3.16)} = 250.71$, $p < 0.0038$).

While fish treated with Morphine, HESBg, and HELBg had an improvement in swimming activity by increasing the distance covered ($F(3.16) = 7.14; 7.72; 6.50$, $p < 0.0001$); decrease in freezing time ($F_{(3.16)} = 1.82; 1.28; 2.04$, $p < 0.0059$); increase in the number of ascents to the upper area of the tank ($F_{(3.16)} = 31.63; 35.62; 33.02$, $p < 0.0009$); decrease in time spent in this area ($F_{(3.16)} = 112.61; 103.59; 101.19$, $p < 0.0038$).

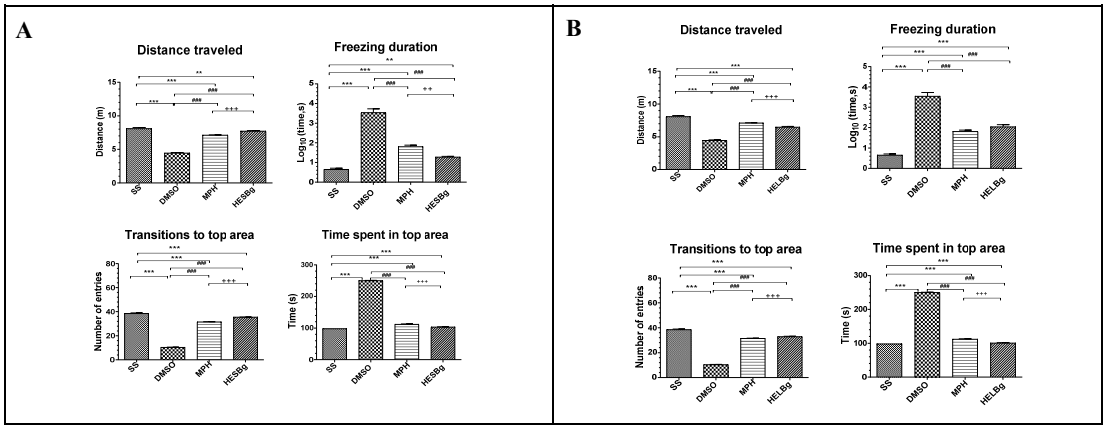

Figure 5. Behavioral phenotypes (locomotor and exploratory endpoints were assessed by distance walked, freezing duration, number of entries and time spent in the upper area) after intraperitoneal injection of 2.5% acetic acid in *Danio rerio* with different treatments (SS 2 µl, DMSO 2 µl, Morphine 2.5 mg/Kg, A HESBg 100 mg/kg, B HELBg 100 mg/kg). Results expressed as mean ± S.P.M analyzed using one-way Analysis of Variance (one-way ANOVA), followed by Tukey's post-test of multiple comparisons, n = 5 *$p < 0.05$.

3. Discussion

In the investigation of the anti-inflammatory effect of *B. guianensis* Aubl. Saline solution (SS), Dimethyl sulfoxide (DMSO), Indomethacin, HELBg, and HESBg were used as a treatment protocol, administered orally 60 minutes before the acute inflammatory induction by carrageenan [4]. Consequently, abdominal edema was formed, characterized by inflammatory swelling due to the local accumulation of low molecular weight catabolic products and extra fluid due to increased tissue osmotic pressure [31].

In the absence of treatment, and extreme cases, inflammation can lead to serious complications and death [16]. The induction of edema ip. by the usual method of administration of the bioproduct carrageenan, derived from seaweed, consists of immunological sensitization with cell migration and consequent inflammatory events [32]. Based on this methodology, evaluating substances with anti-inflammatory potential is considered an excellent alternative for screening promising compounds [16].

The acute anti-inflammatory activity of *B. guianensis* Aubl. was previously described by Carvalho et al. [11], in the evaluation of paw edema induced by carrageenan in albino Wistar rats. The study demonstrated that at a dose of 100 mg/kg of body weight, the methanolic extract of the species significantly inhibited the *in vivo* edema model. The bioactive compounds of this plant species were examined only in rodent models, being tested in the present study in a *Danio rerio* model, which reduces the difficulty of the procedure itself, improving the experimental design as a whole [15].

The SS control group did not receive the dose of carrageenan, being compared for receiving only the saline solution orally and via ip. PBS, carrageenan solubilizing extender. The DMSO negative control group was treated with this organic solvent to rule out any anti-inflammatory effects associated with the extracts [21,22].

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The animals that received the standard drug, indomethacin, had a decrease in edema. This non-steroidal anti-inflammatory drug is widely used as a positive control in screening new anti-inflammatory molecules [33], as evidenced in other studies that obtained similar inhibitory inflammation responses in *Danio rerio* [22,23]. It is noted that the HESBg achieved a higher percentage of inhibition of abdominal edema than the anti-inflammatory used, and the HELBg, even with a lower percentage of inhibition than the control, also showed an inhibitory effect against edema. When evaluating substances with anti-inflammatory potential, similar mechanisms should be addressed. However, the compounds differ in their specific mechanisms of action. The effects are concentrated in the different inflammatory mediators. For example, NSAIDs inhibit cyclooxygenase and prostaglandin synthesis [14].

It is believed that the results of this analysis suggest an anti-inflammatory activity in the extracts obtained from the species *B. guianensis* Aubl., due to the presence of procyanidins (PCs), formed by the condensation of the flavan-3-ol subunits (catechin and epicatechin), important secondary metabolites of the flavonoid class [34], with numerous pharmacological properties, including anti-inflammatory, antiallergic [11,35] and antioxidant, for stabilizing cell membranes [36].

Several studies report the therapeutic potential of these compounds. Sun et al. [37] evaluated the cytoprotection of PCs against H2O2-induced oxidative cellular toxicity in tendon-derived stem cells. Kim et al. [38] demonstrated that PCs present in ethanolic grape seed extracts improved collagen-induced arthritis (CIA) in mice, negatively regulating the expression of Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), phosphorylated synovial protein IκBα, as well as inhibition of nuclear translocation of nuclear factor-κB (NF-κB) subunits (p65 and p50) in an *in vivo* model of experimental arthritis. Ma et al. [39] investigated the anti-inflammatory effects of PCs and the mechanism underlying these effects in bovine mammary epithelial cells (MAC-T) stimulated by lipopolysaccharides (LPS), demonstrating a decrease in inflammatory cytokines (IL-6, IL-1β and TNF-α) and oxidative stress indicators (cyclooxygenase 2 - COX-2 and iNOS) after LPS induction in MAC-T cells. However, little information is available on PCs' immunomodulatory role in the carrageenan inflammation model in *Danio rerio*.

The potential of flavonoids to inhibit enzymes that metabolize arachidonic acid (AA) has been described since 1980 [40]. Since then, regulation of the AA pathway has been recognized as one of the main mechanisms by which flavonoids, such as procyanidins, exert their anti-inflammatory effects [41].

During inflammation, COX-2 is essential, as its modulation regulates the inflammatory response. At the transcriptional level, COX-2 can be regulated by pro-inflammatory stimuli, including LPS, pro-inflammatory cytokines, and growth factors, stimulating the MAPK and NF-κB pathways with consequent transcription of the COX gene -two [18]. In this context, procyanidins inhibit COX-2 by gene transcription, protein expression, or enzymatic activity. Thus, the literature demonstrates that extracts rich in procyanidin are dose-dependent inhibitors of COX-2 protein expression [42,43].

Consequently, if COX-2 is inhibited, there will be low production of prostaglandin (PG) [34]. Therefore, the strong evidence of the inhibitory potential of PCs on COX-2 activity, gene and protein expression, consistent with the reduction of PG secretion, as reported by Martinez-Micaelo et al. [44] and Terra et al. [45], when evaluating PCs in primary human macrophages.

The histopathology results of the present study showed the induction of acute inflammation by ip. caused alterations in important organs, such as the liver, intestine, and kidneys. *Danio rerio* is a well-established model for addressing multiple systems. Testing liver responses to many toxins enables high-throughput toxicity detection, screening, and drug discovery [46]. Furthermore, several intestinal functions and immune genes are conserved between *Danio rerio* and mammals, making this teleost an exciting organism to investigate fundamental processes underlying intestinal inflammation and injury [47]. This model organism has been instrumental in the analysis of organogenesis and kidney disease, such as kidney-related ciliopathies and acute kidney injury, as well as in the search for new therapies due to the structural and functional simplicity of the kidney [48].

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Animals in the SS group had similar observations to the findings by Borges et al. [4]. The second group received the DMSO extender. Thus, the inertia of this solvent in preventing tissue damage led to tissue changes arising from the inflammatory process. The histopathological alterations observed in the liver, intestine, and kidneys of this negative control group can be attributed to different factors, such as oxidative stress, accumulation of toxic substances or lack of adequate supply of nutrients; direct damage to the muscles; accumulation of proteins and other materials inside the tubules, respectively [4,22].

The third group received a standard drug, Indomethacin, with histopathological alterations that did not influence *Danio rerio* liver and intestinal function which could jeopardize some functions of that body. Indomethacin is a non-selective COX-1/COX-2 inhibitor. When used in a study with *Danio rerio* in the larval phase, Westhoff et al. [49] demonstrated serious dose-dependent renal side effects when analyzing histological sections of larvae treated with indomethacin, with alterations in renal development during nephrogenesis.

The groups of plant extracts studied did not develop histopathological alterations capable of influencing the functioning of the organs. Suggesting that the components present in these tested bioproducts could explain the specific mechanisms of action of secondary metabolites derived from the stem and leaf of *B. guianensis* Aubl. In terms of modulation of the inflammatory response, the mechanisms underlying the anti-inflammatory effects found may be related to the presence of procyanidins, which, among other activities, are responsible for modulating several fundamental pathways for regulating inflammation. cellular homeostasis, such as the AA pathway (through regulation of eicosanoid-generating enzymes), production and secretion of inflammatory mediators, such as cytokines or nitric oxide, and modulation of MAPKs and NF-κB pathways [50].

In inflammation, pro-inflammatory mediators can be produced by tissue infiltration and immune cells. These pro-inflammatory mediators can induce pain [51]. Thus, the present study also investigated the antinociceptive effect of *B. guianensis* Aubl., using SS/PBS as a treatment protocol; DMSO, Morphine, HELBg, and HESBg, administered orally 30 minutes before induction of hyperalgesia by ip acetic acid. Consequently, the injection of acetic acid resulted in modifications in the body curvature index and the behavioral phenotypes of *Danio rerio*. This response was characterized by a notable abnormal abdominal constriction, which justifies the observed locomotor alterations [52].

Pain is present in several human disorders. It occurs when nociceptors in primary afferent nerve fibers transmit painful signals in response to harmful mechanical, thermal, or chemical stimuli, which activate the central nervous system (CNS) for pain perception and behavioral responses to pain [53]. While acute pain is temporary, has a protective purpose, and is beneficial, when it becomes chronic, it lacks the protective purpose and severely degrades patients' quality of life [54]. It is considered a public health problem due to its prevalence impact on quality of life and health systems [51].

Among experimental models, evolutionarily conserved, underlying nociception, *Danio rerio* emerges as a promising organism to study nociceptive-type responses. Among homologs with humans, *Danio rerio* presents Transient Receptor Potential (TRP) ion channels, such as TRPV1 and TRPA1; acid-sensing ion channels (ASICs) and toll-like receptors (TLRs) [55].

Induction of hyperalgesia by noxious stimuli such as acetic acid ip. in *Danio rerio*, consists of increased sensitivity to pain, with changes in curvature and behavior, suggesting a local nociceptive effect. The evaluation of substances with antinociceptive potential, based on this methodology, is particularly viable for the study of new compounds with antinociceptive properties since *Danio rerio* has physiological and neuroanatomical structures for nociceptive responses like mammals [56,57].

The species *B. guianensis* Aubl. it is widely used in experimental models about underlying processes of nociception and pain. It was reported for its pain-modulating effect in a model of abdominal writhing induced by acetic acid in mice treated with the methanolic extract of the species [11]. Cechinel Filho et al. [58] demonstrated a considerable analgesic effect in hydroalcoholic and ethyl acetate extracts (10 mg/kg) of the leaves, stems, bark, and roots of the species in the model of pain caused by 0.6% acetic acid in mice, the extracts exhibited greater efficacy compared to controls (aspirin and paracetamol). Willain Filho et al. [59] observed inhibition of abdominal constriction

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caused by both acetic acid and formalin in rodents treated with hydroalcoholic extract of *B. guianensis* Aubl. The authors proposed that the extract's mechanism of action would be related to the modulation of opioid receptors.

A similar species of the genus *Bauhinia* (*Bauhinia microstachya*) has also been described in the literature for its antinociceptive and antihyperalgesic activity. When hydroalcoholic extracts of aerial parts of *B. microstachya* were tested in models of pain induced by intraplantar injection of capsaicin and acetic acid ip. in mice, in addition to hyperalgesia induced by intraplantar injection of various phlogistic agents: carrageenan, bradykinin, capsaicin, substance P and adrenaline, there was a significant antinociceptive effect of extracts obtained from *B. microstachya* on chemical nociception induced by capsaicin and by acetic acid in mice. As well as, the extract of this species reduced the hyperalgesia induced by phlogistic agents in rats, substances involved in the transmission of pain [60].

The SS control group did not receive the dose of acetic acid, that is, it represented a comparison by receiving only the saline solution orally and via ip. PBS, diluent of acetic acid, demonstrating the behavior and body curvature common to the baseline [57].

The DMSO negative control group was thus treated to avoid bias of association of the organic solvent of the extracts with any effects [61]. Acetic acid provided a consistent reduction in locomotion, reflecting less exploratory activity, to the point of hypolocomotion of the animal for a prolonged period. It also decreased the behavior of climbing to the top of the tank, and when it was at the top, it stayed longer, which may be inferred from the stress factor caused by the pain reflex [62–64].

Notably, locomotor deficits are usually associated with animal welfare. The positive control group, treated with morphine, exhibited antinociceptive activity on ip. acetic acid-induced nociception. It is possible that the activation of opioid receptors in *Danio rerio* has negatively modulated variations in the body curvature index and mitigated other behaviors [52].

The present study demonstrated that the administration of HESBg and HELBg presented anti-nociceptive activity in the nociception induced by a harmful agent in an adult *Danio rerio* model, the HESBg with greater significance, negatively modulating the nociception, with better effects in the index behavior and body curvature, when compared to controls. Given the effectiveness of the extracts in preventing nociceptive response, it is the relationship of this endpoint with nociception and its good predictive validity.

This result may be associated with the procyanidins present in the extracts of *B. guianensis* Aubl., due to the suppression of the development of peripheral and central sensitization in the animals. As described by Cady et al. [65], when investigating the effects of extracts rich in procyanidins obtained from grape seeds on neurons and glial cells in the trigeminal ganglia and in the caudal trigeminal nucleus in response to persistent inflammation of the temporomandibular joint induced in Sprague Dawley rats, thus, the extracts suppressed the inflammatory responses caused by prolonged stimulation of the trigeminal nerves, as well as significantly increased the basal expression of MKP-1 in neurons and glial cells within the trigeminal ganglia, as well as in neurons and glia in the nucleus trigeminal caudal.

Grape extracts rich in procyanidins were also examined in a model of pain and structural alterations of osteoarthritis produced by monosodium iodoacetate (MIA) in the knee joint of rats, which it was demonstrated that the treatment with the extracts attenuates the pain induced by MIA and the histological changes in the knee joint. For the authors, the antinociceptive and antiarthritic effects of the extracts were mediated by the inhibition of cartilage damage, synovitis, and fracture of the subchondral bone, reduction in the production of nitrotyrosine and matrix metalloproteinase-13 and suppression of osteoclastogenesis [66].

The effectiveness of the administration of HESBg and HELBg, in a model of inflammation and pain (*Danio rerio*) in the activities observed in the present study revealed that such extracts offer an excellent opportunity for the study of relevant biomarkers of the mechanisms involved, considering that more tests Sensitive tests may be necessary to evaluate the cellular response, involving mechanisms underlying the anti-inflammatory effects found, for example, evaluation of cytokines or nitric oxide and the modulation of MAPKs and NF-κB pathways. Also, the results in the pain model

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can be further evaluated through relevant pain-related biomarkers to follow the responses of molecular and physiological changes underlying the behavioral and curvature responses observed in the *Danio rerio* model.

Possibly an anti-inflammatory and antinociceptive mechanism triggered by procyanidins present in *B. guianensis* Aubl.

4. Materials and Methods

4.1. Drugs and reagents

Freeze-dried stem and leaf extracts of *B. guianensis* Aubl. (HESBg and HELBg) dissolved in Dimethyl sulfoxide (DMSO), with dose selected from the toxicity assay and based on previous studies [11], concentration of 0.02% m/v; 0.9% saline solution (SS); 300 µg kappa carrageenan, solubilized in phosphate buffered saline (PBS, pH 7.2, 0.128 mg/mL); indomethacin 10 mg; 2.5% acetic acid [52]; morphine 2.5 mg; ethylenediaminetetraacetic acid (EDTA); alcohol 70, 80, 90 and 100%; 100% xylol; paraffin; hematoxylin and eosin dyes [4,11,27]. The reagents used were purchased from Sigma Chemical Company (St. Louis, Millstone, USA).

4.2. Experimental animals

Females and males of the *Danio rerio* species (zebrafish), wild lineage AB, adults approximately six months old, 3.7 to 4 cm long, weighing around 550 mg, supplied by Power Fish Piscicultura, Itaguaí, were used. -Rio de Janeiro, Brazil. Kept on the Zebrafish Platform of the Drug Research Laboratory, Department of Biological and Health Sciences (Federal University of Amapá-UNIFAP-Brazil). The animals remained in an adaptation period (40 days), circadian cycle of 12 hours (light from 7:00 a.m. to 7:00 p.m.), temperature 23 ± 2 °C, and feeding twice a day with commercial flake feed (Alcon Colors, Santa Catarina, Brazil). The aquariums were monitored under ideal conditions (pH 6.0–8.0; conductivity 8.2 ± 0.2; daily cleaning of the recirculation water system) [27,28]. The experiments followed the rules established for the care of animals, approved by the Ethics Committee on Animal Use—CEUA—UNIFAP with protocol number 007/2020.

4.2.1. Treatment groups and routes of administration

The animals were fasted for 24 hours before the experiments, and the groups were treated with different substances, Table 2, according to each test:

Table 2. List of drugs used in experiments, concentrations and potential effects in adults *Danio rerio.*

Substance	Concentration/Dose	Potential Effect
Acetic Acid	2.5%	Noxious stimulus
Carrageenan	300 µg	Noxious stimulus
DMSO	0,1%	-
HELBg	100 mg/kg	Anti-inflammatory
HESBg	100 mg/kg	Anti-inflammatory
Indomethacin	10 mg/kg	Anti-inflammatory
Morphine	2,5 mg/kg	Anti-inflammatory
PBS	0,128 mg/mL	-
Saline solution	0,9%	-

[1] Concentrations were taken from Borges et al. [4] and Costa et al. [52].

Inflammation: control group (SS+PBS, SS 2 µl/animal p.o. + PBS 20 µL ip., carrageenan extender), negative control group (DMSO+CAR, DMSO 2 µl/animal p.o. + carrageenan 20 µL ip.), positive control group (Indomethacin 10 mg/kg animal p.o. + carrageenan 20 µL ip.), groups treated with HELBg and HESBg (100 mg/kg animal p.o. + carrageenan 20 µL ip.), triplicates with n = 12 animals

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per experimental group. The treatments by the gavage method occurred 60 minutes before carrageenan administration, according to the methodology described by Borges et al. [4].

Hyperalgesia: control group (SS+PBS, SS 2 µl/animal p.o. + PBS 10 µL ip., acetic acid diluent), negative control group (DMSO+AAC, DMSO 2 µl/animal p.o. + AAC 10 µL ip.), control group positive (Morphine 2.5 mg/kg animal p.o. + AAC 10 µL ip.), groups treated with HELBg and HESBg (100 mg/kg animal p.o. + AAC 10 µL ip.), n = 5 per experimental group, adhering to the 3R principles of ethical animal experimentation. Oral treatments occurred 30 minutes before the administration of acetic acid, according to the methodology described by Costa et al. [52].

For the treatments by the gavage method, a volumetric pipette (HTL Lab Solutions Co., São Paulo, Brazil) was used. While the method of injecting substances into the peritoneum was with the aid of a BD Ultra-fine™ 30U syringe (needle size 6 mm x 0.25 mm).

4.3. Inflammation induction protocol with carrageenan and measurement of inflammatory edema Danio rerio

The process followed the protocol by Borges et al. [4], in which individually weighed animals (beginning and end of the trial, initial weight-Wi and final weight-Wf) on an analytical scale (FA2104N, Bioprecisa Co., São Paulo, Brazil), received a thermal shock as anesthesia, in water at 8–10 °C (the marked opening of the caps and the reduced swimming rhythm indicated the anesthetized state) — this method was preferred over other known anesthetic methods in order to avoid any additional drug being factored in concomitantly with the effects of the examined compounds.

Subsequently, they were placed on a damp sponge with a hollow center to hold the animal, positioning it with the abdomen facing upwards to receive the injection. The needle was inserted at a 45° angle in the midline of the abdomen, between the pelvic and pectoral fins. IP Administration. it took an average of 10 seconds, then the animals were placed in separate tanks under the same environmental conditions as the system (25 ± 2 °C) for recovery from anesthesia and observation of edema formation. It is important to emphasize that this protocol allowed a quick evaluation of the animal's resistance, through the return of the swimming activity, otherwise it could be replaced by another animal.

Inflammation Inhibition (II) was evaluated based on the difference in body weight (≠ BW = Wf — Wi) of each animal, subsequently calculating the percentage of inflammation inhibition (% II) [22], according to (1):

$$\% \text{ II} = \frac{100\% — \text{Mean (treatment HESBg or HELBg) } 100\%}{\text{Mean negative control}} \quad (1)$$

At the end of the experiment (5h00 after the carrageenan injection), the animals were photographed with a digital camera (Canon T7 rebel, EEFS 55-250 mm lens, macro 1.1m/3.6ft, Nagasaki, Japan) and, after euthanasia in water cold (< -2°C), were immediately stored in Bouin's solution for tissue fixation.

4.3.1. Histopathological analysis of the inflammation test in Danio rerio

For embedding, the animals were fixed in Bouin's solution for 24 hours and decalcified in EDTA solution for 24 hours. Subsequently, they received a treatment for tissue dehydration, in a progressive series of alcohol concentrations (70, 80, 90 and 100%) for 1h00 each, followed by diaphanization with impregnation in xylol and inclusion in paraffin. The blocks were sectioned into 5 µm cuts using a microtome (Brand Rotary Microtome Cut 6062, Slee Medical, Germany) and placed on glass slides for tissue staining with hematoxylin and eosin [27].

Histopathological analyzes of the liver, intestine and kidneys were observed on an Olympus BX41-Micronal Microscope and photographed with a digital camera (MDCE-5C USB 2.0.h, Qingdao, China).

4.3.2. Evaluation of histopathological alterations of the inflammation test in Danio rerio

The index of histopathological alterations (HAI) was defined by the extension of the tissue alterations observed in the liver, intestine, and kidneys, classified in stages I, II and III. That is, IHA

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= 0 to 10 (healthy organ), 11 to 20 (mild to moderate changes), 21 to 50 (moderate to severe changes) or > 100 (irreversible changes) [28,29]. The index were calculated according to the following equation:

$$I = \frac{\sum_{i-1}^{na} ai + 10 \sum_{i-1}^{nb} bi + 10^2 \sum_{i-1}^{nc} ci}{N} \quad (2)$$

(2) a: first stage alterations; b: second stage alterations; c: third-stage alterations; na: number of alterations considered as a first stage; nb: number of alterations considered as second stage; nc: number of alterations considered as the third stage; N: number of fish analyzed per treatment.

4.4. Hyperalgesia induction protocol with acetic acid and behavioral analysis

The animals were randomly selected and placed individually in the observation tanks (15 × 13 × 10 cm, length × height × width), 10 cm of water depth, in environmental conditions similar to those on the platform. The basal behavioral activity of the fish was recorded for 6 minutes with a digital camera (Canon T7 rebel, EEFS 55-250 mm lens, macro 1.1m/3.6ft, Nagasaki, Japan). Then, the oral treatments were administered, according to the group (SS, DMSO, Morphine, HELBg, and HESBg), after 30 minutes of the ip injection was performed. of PBS in the control group (SS) and the other acetic acid. The treatment with morphine defined in this study was chosen because it is a classic opioid analgesic, clinically validated and potent, recognized in experimental pain models, with sensitivity previously described in *Danio rerio* [52].

The animals were gently handled in a damp sponge with a hollow center, behaving so that the abdomen was facing upwards to receive the AAC injection. The needle was inserted at a 45° angle in the midline of the abdomen, between the pelvic and pectoral fins. After the protocol, the fish returned to the water, observing their swimming activity, with a view to a more accurate analysis of the acute pain responses. if the behavioral recordings [52].

It is recorded for 6 min using a digital camera. This study performed all behavioral tests between 9:00 a.m. and 4:00 p.m. After the experimental procedures, the animals were euthanized in ice water (< -2°C).

4.4.1. Behavioral parameters

Possible swimming behaviors related to nociception in *Danio rerio* were recorded 6 minutes before (baseline values) and after ip. injection. of AAC, as [52]. All recorded behaviors were analyzed in automated video tracking software (ToxTrac version 2.61, Umeå University, San Diego, CA) at 30 frames/sec to quantify distance traveled (m), freeze duration (sec), transitions for the upper area (number of entries), and time spent in the upper area of the tank (s). Said program is optimized using an algorithm for tracking animals, such as fish, insects, and rodents [67].

Freezing was defined as complete immobility of the animal (≥ 2 s), except for eyes and brachial arches. The body curvature index observed Hyperalgesia, like abdominal constriction. Frontal fingerprints were taken every 30 seconds (totaling 24 photos per fish). Body curvature was measured using ImageJ 1.45 software for Windows (NIH, Bethesda, MD, USA) [68].

To establish the curvature of the body, three points were used on the animal: frontal (front of the head), central (middle of the body – between the anal and dorsal fins), and posterior point (caudal fin). To calculate the value of the body curvature index, the average curvature angle of the 180° group was subtracted. Double-blind trained observers observed temporal variations in the body curvature index (inter-rater reliability > 0.90) and expressed as the area under the curve (AUC). Both observers and data analysts were unaware of treatment groups. Only the person who treated the animals knew about these conditions but did not analyze the data.

4.5. Statistical analysis

The normality of the data and the homogeneity of the variances of the induction of hyperalgesia and inflammation were analyzed using the Kolmogorov-Smirnov and Bartlett tests. The significance of the results obtained in inflammatory activity was determined using the one-way ANOVA test (Analysis of variance), followed by Tukey's multiple comparisons test, and the IHA was established

by one-way ANOVA (Kruskal-Wallis) and by the Student-Newman-Keuls test, comparing the means between the control and treatment groups, considering significant values of $p < 0.05$ [4] and highly significant values of $p < 0.01$. Data were expressed as mean ± standard error of the mean (S.E.M.).

Changes in body curvature index and behavioral activity were analyzed by paired Student's t-test, one-way analysis of variance (one-way ANOVA). Tukey's multiple comparison test further assessed differences between groups for significant ANOVA data. Results were expressed as mean ± standard error of the mean (S.E.M.), and non-parametric data (freezing duration) were previously transformed into log. The significance level was set at $p \leq 0.05$ in all analyses. Graph Pad Prism ® 5.03 software (GraphPad Software Inc., San Diego, CA, USA) was used.

5. Conclusions

The treatment with extract of stems and leaves of *Bauhinia guianensis* Aubl., in a model of inflammation induced by carrageenan, can significantly reduce edema formation. The treatment does not cause tissue alterations that compromise the function of important organs (liver, intestine, and kidneys) of *Danio rerio*. Both extracts from *B. guianensis* Aubl. negatively modulated nociception, with better effects on behavioral and body curvature index. The observed antinociceptive and anti-inflammatory activities are probably of peripheral origin and linked to the inhibition of prostaglandin biosynthesis.

Author Contributions: R.C.R.K., study design and wrote the manuscript on the results obtained R.C.R.K, A.M.F., G.C.S., implemented the inflammation and pain methodologies; A.A.S., P.F.S., performed the statistical tests, J.C.T.C., coordinated and supervised the research project.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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