Anxiolytic-like effect of natural product 2-hydroxy-3,4,6-trimethoxyacetophenone isolated from Croton anisodontus in adult zebrafish via serotonergic neuromodulation involvement of the 5-HT system

Antonio Wlisses da Silva¹ · Maria Kueirislene A. Ferreira² · Emanuela L. Rebouças¹ · Francisco Rogenio S. Mendes³ · Atilano Lucas dos S. Moura² · Jane Eire S. A. de Menezes² · Márcia Machado Marinho⁴ · Emmanuel Silva Marinho⁴ · Hélcio S. Santos¹,²,³,⁵ · Alexandre M. R. Teixeira¹,³

Received: 25 March 2021 / Accepted: 22 June 2021 © The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract
Benzodiazepines are highly effective in combating anxiety; however, they have considerable adverse effects, so it is important to discover new safe anxiolytic agents. This study was designed to investigate the effect of the natural product 2-hydroxy-3,4,6-trimethoxyacetophenone (HTMCX) on anxiety and seizure behavior in adult zebrafish and its possible mechanisms of action. The acute toxicity of 96 h of HTMCX was analyzed, and the open and light/dark field tests (n = 6 animals/group) were used to assess the anxiety behavior of animals treated with HTMCX. In addition, the mechanisms of action were investigated with antagonists of the GABA_A, 5-HT receptors, and molecular anchorage study. Pentylenetrazole (PTZ) was used to induce seizure by immersion. As a result, acetophenone HTMCX (1, 3 and 10 mg/kg; v.o.) was non-toxic and affected locomotor activity. The higher doses (3 and 10 mg/kg; v.o.) produced signs of anxiolytic action in the light/dark test, and this effect was reversed by the pizotifen (antagonist 5HTR_1 and 5HTR_2A/C), having the potential to form a complex with 5HTR_1B. However, the anxiolytic effect of HTMCX has not been abolished by flumazenil (antagonist GABA_A), cyproheptadine (antagonist 5HTR_2A), and granisetron (antagonist 5HTR_3A/3B). Therefore, HTMCX demonstrated an anxiolytic effect, suggesting that the 5HTR_1 and 5HTR_2C receptors may be involved in the pharmacological performance of this acetophenone in the central nervous system.

Keywords Croton · Anxiety · Zebrafish · 5HTR_1/2C

Introduction
Neurological diseases affect millions of people worldwide (Goni et al. 2021). According to the World Health Organization (WHO), approximately 264 million people suffer from some type of anxiety disorder, and Brazil contains one of the highest rates of this pathology (WHO 2017). Psychotherapeutic anxiety treatments such as “gold standard” therapy (in other words, cognitive-behavioral therapy; CBT) are commonly used; however, the results of the effects obtained are of little impact, especially in disorders such as post-traumatic stress disorder (PTSD), social anxiety disorder (SAD), and panic disorder (Carpenter et al. 2018). Studies report that CBT is effective when used in combination with benzodiazepine pharmacotherapy...
Material and methods

Drugs, reagents, and natural product

Granisetron hydrochloride (Corepharma/Middlesex, NJ, EUA), Pizotifene maleate (Central Manipulation Pharmacy/São Paulo, SP, Brazil), Fluoxetine (Eli Lilly/Indiana, IN, EUA), Cyproheptadine (Evidence Soluções Farmacêuticas/Fortaleza, CE, Brazil), Diazepam and Pentilenetetrazol (Sigma-Aldrich/Missouri, EUA) Flumazenil (Roche Pharmaceutical/Welwyn Garden City, United Kingdom). In this work, the 2-hydroxy-3,4,6-trimethoxyacetophenone natural product was extracted, isolated, purified, and characterized from the stem bark of *C. anisodontus* (Fig. 1) (Santiago et al. 2018).

Zebrafish

The fish (from 90 to 120 days; 0.4 ± 0.1 g, 3.5 ± 0.5 cm), wild, male and female, were purchased at a local store (Fortaleza, CE) and kept for a week before the experiments in a glass aquarium (30×15×20 cm) of 10 L (n = 3/L), at a temperature of 25 ± 2 °C, in light–dark cycles for 24 h and with chlorinated water (ProtecPlus®) and air pump with submerged filters, at 25°C and pH 7.0, under a 14:10 h circadian cycle (light/dark). The animals received ration for fish (Alcon Gold Spirulina Flakes®) 24 h before the experiments. All procedures were approved by the Animal Use Ethics Committee of the State University of Ceará (CEUA-UECE; # 3,344,801/2017).

General treatment protocol

Adult fish were randomly selected from males and females (n = 6/group), anesthetized in cold water (2–4 °C) and transferred to a wet sponge; then they were treated orally (v.o.) using a 20-μL automatic pipette. The samples applied orally in the groups of animals were as follows: HTMCX (dose of 1; 3 or 10 mg/kg), diazepam (10 mg/kg; 20 μL; v.o.) (positive control), and 3% DMSO (Vehicle: diluent the drugs tested). In tests to evaluate the mechanism of action, groups of animals were treated orally with flumazenil (4 mg/kg; 20 μL; v.o.), cyproheptadine (32 mg/kg; 20 μL; v.o.), pizotifen (32 mg/kg; 20 μL; v.o.), and granisetron (20 mg/kg; 20 μL; v.o.). Fluoxetine (positive control) was applied to the animals intraperitoneally (0.05 mg/kg; i.p.) using insulin syringe (0.5 mL; Ultrafine® BD) (Gonçalves et al. 2020). After treatment, the fish were transferred (individually) to beakers (250 mL) containing 150 mL of water and left to recover before testing. After the tests, the fish were sacrificed by immersion in cold (BZDs) and when used as long-term monotherapy (Bandelow et al. 2012; Watanabe et al. 2007).

Although BZDs have a proven high effectiveness in combating anxiety disorders such as PTSD, SAD, and panic disorder, they are generally recommended in practical clinical guidelines as short-term auxiliary options, preferably with regular dosing, either to help relieve patients with anxiety and acute agitation or await a response to antidepressants (Bandelow et al. 2012; Katzman et al. 2014); however, prolonged use can cause serious adverse health effects (Chen et al. 2020), such as cognitive change, impaired memory (Smink et al. 2010), increased risk of dementia, and Alzheimer’s disease (de Gage et al. 2014; Wu et al. 2009), in addition to the risk of falling and traffic accidents, as well as tolerance and dependence on BZDs (Voyer et al. 2009; Yu et al. 2017). Therefore, selective serotonin reuptake inhibitors (SSRIs) are currently the first-line drugs of choice for the treatment of these anxiety disorders, due to the combination of efficacy and safety; however, their effects may take time to occur (Bandelow et al. 2012; Katzman et al. 2014).

Although benzodiazepines have high effectiveness in combating anxiety, they have considerable adverse effects, which is why selective serotonin reuptake inhibitors (SSRIs) are currently the first-line drugs of choice for the treatment of anxiety disorders due to a combination of efficacy and safety. However, its effects may take time to occur. The pathological state of anxiety is associated with changes in different neurotransmission pathways, such as serotonergic, noradrenergic, GABAergic, dopaminergic, and nitrergic pathways (Martin et al. 2009; Spolidorio et al. 2007). Animal models are generally used to investigate the anxiolytic action of new compounds that act on these neuromodulation pathways.

The zebrafish (*Danio rerio*) has received attention as an animal model for pharmacological studies of anxiety because it has conserved neurotransmitters, and in addition, its genome has more than 80% of ortholog genes related to human diseases (Khan et al. 2017). Serotonergic neurotransmission in zebrafish has been shown to be similar to a mammal in terms of its physiology and pharmacology (Connors et al. 2014; Maximino et al. 2010; Panula et al. 2010), and studies have shown that the activation of serotonergic receptors through the use of selective agonists alters anxiety-like behaviors in zebrafish (Nowicki et al. 2014).

Modern pharmacological research indicates that natural products derived from plants have an anxiolytic effect through neuromodulation GABAergic or serotonergic (Lei et al. 2015). Based on the reported data, this study aimed to evaluate the anxiolytic and anticonvulsant effect of 2-hydroxy-3,4,6-trimethoxyacetophenone isolated from *C. anisodontus* and the possible mechanisms of action in adult zebrafish.
water (2–4 °C) for 10 min until the end of the opercular movement (Matthews and Varga 2012).

**Toxicity to adult Zebrafish (ZFa)**

Acute toxicity of HTMCX (dose of 1, 3 or 10 mg/kg; v.o.) was performed against the ZFa for lethal dose (LD$_{50}$) for 96 h in accordance with the guidelines of the Organization for Economic Cooperation and Development (OECD) (Test Guideline No. 203; Fish, Acute Toxicity Testing 2019). After the OFT (see the section below), the fish (n = 6/group) were transferred to tanks, separated by treatment groups, and observed for 96 h. Dead fish in each group were counted, and the LD$_{50}$ was determined.

**Open field test (OFT)**

The OFT was performed to identify changes in the locomotion of the zebrafish, whether due to anxiolytic effect, sedation, and/or muscle relaxation (Ahmad and Richardson 2013). The animals (n = 6/group) received 20 μL of HTMCX orally at a dose of 1 mg/kg, 3 mg/kg, or 10 mg/kg. The negative control group in each experiment was treated with the Vehicle (3% DMSO; 20 μL), and the positive control group received diazepam (10 mg/kg; 20 μL; v.o.). An untreated group (Naive) was also included. After 1 h, the fish were transferred to Petri dishes (10 cm × 15 cm; with quadrants at the bottom of the plate) containing water from the tank. Locomotor activity was assessed based on the number of times that each individual crossed the lines drawn in the Petri dishes in 5 min (Gonçalves et al. 2020).

**Anxiolytic evaluation (LDT)**

An animal’s anxiety behavior can be observed through the light/dark test (LDT). Similar to rodents, the zebrafish naturally avoids bright areas (Gonçalves et al. 2020). The experiment was carried out in a glass aquarium (30 cm × 15 cm × 20 cm) divided into a light area and a dark area. The water tank was filled to 3 cm with tap water without chlorine and without drugs, which simulated a new shallow environment different from the conventional housing aquarium and capable of inducing anxiety behavior. For zebrafish (n = 6/group), 20 μL of HTMCX were administered orally at doses of 1 mg/kg, 3 mg/kg, or 10 mg/kg. The negative and positive control groups consisted of 3% DMSO and 10 mg/kg diazepam solution, respectively. An untreated group (Naive) was also included. After 1 h, zebrafish were placed individually in the clear zone, and the anxiolytic effect was measured based on the time spent in the clear zone within 5 min of observation (Gebauer et al. 2011).

**Evaluation of the mechanism of action**

The receptor(s) involved in the HTMCX anxiolytic type effect was identified by pretreatment (treatment with antagonists before HTMCX application and positive controls) with flumazenil (GABAA antagonist) and cyproheptadine serotonergic antagonists (5-HTR$_{2A}$ antagonist), pizotifen (antagonist of 5-HTR$_1$ and 5-HTR$_{2A/C}$), and granisetron (5-HTR$_{3A/B}$ antagonist) before LDT (Benneh et al. 2017). Zebrafish (n = 6/group) were pretreated with flumazenil (4 mg/kg; 20 μL; v.o.), cyproheptadine (32 mg/kg; 20 μL; v.o.), pizotifen (32 mg/kg; 20 μL; v.o.), or granisetron.
(20 mg/kg; 20 μL; v.o.). After 15 min, the highest effective dose of HTMCX (10 mg/kg; 20 μL; v.o.) found in the pilot test was administered (see “Anxiolytic evaluation (LDT)” section). The 3% DMSO (Vehicle; 20 μL; v.o.) was used as a negative control. Diazepam (Dzp; 10 mg/kg, 20 μL; v.o.) and fluoxetine (Flx; 0.05 mg/kg; i.p.) were used as GABA_A and 5-HT agonists, respectively. After 1 h of the treatments, the animals were submitted to the light/dark test described in the previous section.

Pentylenetetrazole-induced seizure (PTZ)

PTZ-induced seizure reversal was investigated (Siebel et al. 2015). The animals (n=6/group) were treated with HTMCX (10 mg/kg; 20 μL; v.o.), diazepam (10 mg/kg; 20 μL; v.o.), and Vehicle (3% DMSO; 20 μL; v.o.). An untreated group (n=6/group) was included (Naive). After 1 h, the animals were individually exposed by immersion in 7.5 mM PTZ, promptly dissolved in water in a 250-mL beaker, and the behavior similar to seizure in three stages was evaluated: stage I, dramatically increased swimming activity; stage II, swirling swimming behavior; and stage III, clonus-like seizures, followed by loss of posture when the animal falls to one side and remains immobile for 1–3 s. At the end of the evaluation of the three stages of the test, the animals were euthanized on the ice.

Docking of the anxiolytic effect

The structure of the human 5-HT_{1B} receptor (PDB 4IAQ) was obtained from the Protein Data Bank (https://www.rcsb.org/), identified as “Crystal structure of the chimeric protein of 5-HT1B-BRIL in complex with dihydroergotamine (PSI Community Target),” deposited with a resolution of 2.80 Å, being determined by X-ray diffraction, classified as signaling protein and electron transport, Homo sapiens organisms, Escherichia coli, and Spodoptera frugiperda expression system (Wang et al. 2013). Molecular docking simulations were performed using the AutoDock Vina code (version 1.1.2) (Trott and Olson 2010). The grid box was defined by centralizing the entire protein, with parameters of 70 Å x 100 Å x 76 Å and dimensions (x, y, z) = (-21,133, -0.855, 17,314), with 50 independent simulations with 20 poses each. To study the possible interaction mechanism of HTMCX on the GABAergic system, molecular docking simulations were performed. The protein target structure was obtained from the Protein Data Bank repository (https://www.rcsb.org/); it is identified as “Crystal structure of a human gamma-aminobutyric acid receptor, the GABA (A) R-beta3 homopentamer” (PDB 4COF), deposited with a resolution of 2.97 Å, determined by X-ray diffraction and classified as transport protein in Homo sapiens organism (Miller and Aricescu 2014). To perform docking simulations, the AutoDock Vina code (version 1.1.2) was used, using the Lamarckian Genetic Algorithm (Trott and Olson 2010). The grid box was centralized throughout the protein and defined with the parameters of 98 Å x 98 Å x 126 Å and dimensions (x, y, z) = (-1.357, -1.094, 139.084). Fifty independent simulations were carried out, obtaining 20 poses in each one. The Biovia Discovery Studio visualizer from Dassault Systèmes Biovia Corporation (BIOVIA Discovery Studio 2016) and UCSF Chimera (Pettersen et al. 2004) codes were used to analyzing the results.

Statistical analysis

The results were expressed as mean ± standard deviation from the mean for in vivo tests (n=6/group). After confirming the normality and homogeneity distribution of the data, differences between the groups were subjected to analysis of variance (one-way ANOVA), followed by the Tukey test, using the GraphPad Prism v software. 7.0. The level of statistical significance was considered to be 5% (p<0.05).

Results

Toxicity to adult Zebrafish (ZFa)

The natural product HTMCX was non-toxic to adult zebrafish up to 96 h of analysis (LD_{50}> 10 mg/kg), as there was no death and did not cause any apparent anatomical changes in the animals during this period.

Open field test (OFT)

HTMCX [***p < 0.01, ****p < 0.001 (1; 3 or 10 mg/kg)] and diazepam [***p < 0.001 (10 mg/kg)] significantly decreased the locomotor activity of the adult zebrafish compared to control groups (Naive and Vehicle) (Fig. 2A).

Anxiolytic evaluation (LDT)

HTMCX (3 or 10 mg/kg) increased (****p < 0.0001 vs. Naive or Vehicle) the time of the animals in the clear region of the aquarium in the LDT (Fig. 2B). This effect was significantly similar to the effect of diazepam (Dzp; 10 mg/kg; v.o.), positive control. Only animals treated with the lowest dose of HTMCX (1 mg/kg) did not show anxiolytic behavior, as they had the same anxiety behavior as the negative control groups (Naive and Vehicle).

Involvement of the GABAergic system (GABA)

The ZFa treated with HTMCX + flumazenil did not have the anxiolytic behavior reversed, unlike animals treated...
with Dzp + flumazenil that remained most of the time in the dark region of the LDT test. However, flumazenil reversed (##p < 0.01 vs. Dzp) the anxiolytic effect of diazepam (Dzp; 10 mg/kg; v.o.) but not HTMCX (10 mg/kg; v.o.) (Fig. 2C).

**Involvement of the serotonergic system (5-HT)**

**System involvement 5-HTR$_{2A}$**

Cyproheptadine did not reverse the anxiolytic effect of HTMCX (10 mg/kg, v.o.); however, it reduced (####p < 0.0001 vs. Flx) the anxiolytic effect of fluoxetine (Flx; 0.05 mg/kg; i.p.) (Fig. 2D).

**Involvement of 5-HTR$_1$ and 5-HTR$_{2A/2C}$ systems**

Pizotifen reduced (#####p < 0.0001 vs. HTMCX or Flx) the anxiolytic effect of HTMCX (10 mg/kg, v.o.) and fluoxetine (Flx; 0.05 mg/kg; i.p.), indicating that HTMCX has an anxiolytic effect through neuromodulation of the 5-HTR$_1$ and 5-HTR$_{2C}$ channels (Fig. 2E).

**Involvement of 5-HTR$_{3A/3B}$ systems**

Granisetron did not reverse the anxiolytic effect of (10 mg/kg, v.o.); however, it reduced (#####p < 0.0001 vs. Flx) the anxiolytic effect of fluoxetine (Flx; 0.05 mg/kg; i.p.) (Fig. 2F).

**Pentylenetetrazole-induced seizures (PTZ)**

HTMCX (10 mg/kg; v.o.) did not reverse the convulsive behavior induced by PTZ, unlike [(*p < 0.01, stage I; **p < 0.001, stage II; ***p < 0.01, stage III)] of Dzp (10 mg/kg; v.o.) that delayed the onset of the three stages of the seizure in the aZF compared to the control groups (Naive and Vehicle) [(*p < 0.01, stage I and stage II; **p < 0.01, stage III)] (Fig. 3).

---

**Fig. 2** Effect under the locomotor behavior of zebrafish (Danio rerio) adult in the OFT (A) and anxiolytic-like effect in the LDT (B) of the HTMCX (20 μL; v.o.). Anxiolytic-like effect of HTMCX after pretreatment with flumazenil (C), cyproheptadine (D), pizotifen (E), and granisetron (F) in adult zebrafish in the LDT (0–5 min). Naive-untreated animals. Dzp, diazepam (10 mg/kg; 20 μL; v.o.). Vehicle (3% DMSO) (20 μL; v.o.). The results are expressed as mean values ± S E M. (n = 6/group). ANOVA followed by the Tukey test (**p < 0.01, ***p < 0.001, ****p < 0.0001 compared with negative control groups—Naive or Vehicle; ####p < 0.01, #####p < 0.0001 compared to the HTMCX or Flx or Dzp groups). Dzp diazepam, Cipro cyproheptadine, Gstn granisetron, Piz pizotifen; Flx fluoxetine.
Docking of the anxiolytic effect

The best conformation simulation between HTMCX and the receptor coupled to the human G 5-HT1B protein presented a RMSD (root mean square deviation) value in the order of 1.45 Å and an affinity energy value in the order of -6.0 kcal mol\(^{-1}\). The trimethoxyacetophenone-5-HT1B complex formed showed four interactions, two hydrophobic with residues ILE130A and VAL201A and two hydrogen bonds of strong intensity with residues THR134A and ASP129A (Table 1). Regarding the interaction with the GAB\(_{\alpha}\) receptor, it was possible to identify the interactions with the GLN64C, TYR535D, ALA639D, THR640D, THR640D, TYR62C, and PHE638D residues, coupling in a different region of the diazepam site that interacts with the TRP241B, LEU735C, ALA738C, PHE739C, TRP241B, TRP241B, and TRP241B residues (Table S1, supplementary material).

Table 1 Interactions between the 5-HT1B receptor and the HTMCX ligand

| Ligand | Receptor | Interaction | Distance (Å) |
|--------|----------|-------------|--------------|
| HTMCX  | ILE130A* | Hydrophobic | 3.89 Å       |
|        | VAL201A* | Hydrophobic | 3.86 Å       |
|        | ASP129A  | H-bond      | 2.77 Å       |
|        | THR134A* | H-bond      | 2.51 Å       |

*2GM orthostatic site residue

can also indicate more specific behaviors, such as anxiety. Adult zebrafish introduced individually in OFT swim initially near the walls of the Petri dish (Gupta et al. 2014), a thigmotaxis response to detect predators (Peitsaro et al. 2003); this fear and anxiety behavior makes animals move freely throughout the space of the Petri dish during the 5 min of analysis in the open field, as observed in the Naive and Vehicle groups (Fig. 2A). The groups of animals that were treated with the HTMCX natural compound and Diazepam (positive control) presented locomotor impairment (Fig. 2A), a result similar to those obtained with anxiolytic drugs that decreased locomotor activity of adult zebrafish in the open field (Benneh et al. 2017; Gebauer et al. 2011) confirm that anxiolytic compounds decrease locomotor activity in adult zebrafish, this has been observed in research with anxiolytic compounds that act via serotonergic neuromodulation (Ferreira et al. 2020; Gonçalves et al. 2020). In addition, HTMCX was non-toxic during the 96 h of analysis.

Zebrafish are anxious under natural conditions, preferring dark environments. Moreover, new environments are potentially risky and trigger anxiety in them. The preference of these animals for the clear region of the aquarium is characterized by the action of anxiolytic substances in the central nervous system (CNS) (Maximino et al. 2011). LDT was performed to confirm the possible effect of the HTMCX anxiolytic type observed in OFT. The higher doses of acetophenone HTMCX and diazepam significantly increased the time of the animals in the clear region of the aquarium (Fig. 2B), allowing the assumption that this natural product has an acute anxiolytic effect, confirming the results observed in the OFT.

LDT and OFT showed that HTMCX and diazepam have similar effects on zebrafish. Benzodiazepines have a depressant/sedative effect on the CNS of zebrafish and mammals through positive allosteric neuromodulation of the GAB\(_{\alpha}\) receptor, which causes hypnotic effects, locomotor...
Serotonergic neuromodulation is also involved in the mechanisms of anxiety mediation, and the role of serotonin (5-HT) in this disorder is widely investigated (Maximino et al. 2014). Anxiety behavior in LDT is positively associated with extracellular levels of 5-HT in the zebrafish brain (Gonçalves et al. 2020). Thus, high levels of 5-HT can cause effects similar to those of anxiety, while low levels generally cause anxiolytic behaviors (Nowicki et al. 2014).

Considering the hypothesis that serotonergic neurotransmission mediates anxiolytic effects, the participation of this system in the anxiolytic action of HTMCX after pretreatment with cyproheptadine antagonists (a 5-HTR2A antagonist), pizotifen (a 5-HTR1A and 5-HTR2A2C antagonist), and granisetron (a 5-HTR3 antagonist) was evaluated. Fluoxetine was used as a positive control. Unlike granisetron (5-HTR3A/3B; Fig. 2E) and cyproheptadine (5-HTR2A; Fig. 2D), pretreatment with pizotifen significantly inhibited the acute anxiolytic effect of HTMCX, suggesting that the mechanism of action of this acetophenone involves the 5-HTR1A and/or 5HT2c serotonergic receptors (Fig. 2D). However, it does not involve the 5HT2A receptor because cyproheptadine did not reverse the anxiolytic effect of HTMCX on LDT. In addition, it was observed that all the mentioned antagonists reversed the effects of fluoxetine (Fig. 2C, D, and F).

5HT1A receptor agonist substances exhibit antidepressant and/or anxiolytic effects. 5-HT1A is an inhibitory receptor coupled to protein G, and its activation reduces the rate of firing of serotonergic neurons and promotes the synthesis, renewal, and release of 5-HT in various areas of the CNS (Gonçalves et al. 2020). Studies have shown that zebrafish treated with 5HT1A receptor agonists showed anxiolytic behaviors (Bennet et al. 2017; Gonçalves et al. 2020; Maximino et al. 2014). Therefore, HTMCX is possibly a 5-HT1A agonist, as it prevented the animals’ anxiolytic effect (Fig. 2E).

The serotonin 2C receptor (5-HTR2c) is a molecular target of drugs developed for the treatment of behavioral conditions, such as eating and mood disorders, anxiety, and motor behavior (Lee et al. 2010). The genetic similarities of mammalian 5-HTR2c with that of zebrafish indicate the use of this model to study the role of the 5-HT2c receptor in the behavior, development, and discovery of drugs that act in this channel. Reports indicate that 5-HTR2c activation induces panic and/or anxiety, while blocking it causes anxiolytic effect (Chagraoui et al. 2016). Thus, HTMCX may have competed with pizotifen and was replaced by it in 5-HT2c, being indicative of the action of this acetophenone in the anxiolytic effect under this route.

For the study of the anxiolytic effect in silico, via serotonergic neuromodulation of HTMCX, the structure of the receptor coupled to the human G 5-HT1B protein was selected due to its orthostatic site composed of residues conserved in practically the entire family of the receptor 5-HT (Morgan et al. 2013). The dihydroergotamine agonist (2GM) is complexed in the protein at the orthostatic site, composed of residues ILE130, CYS133, THR134, VAL201, ALA216, PHE331, ASP352, and THR355. After the molecular docking simulations, it was possible to observe that the HTMCX coupled in the same region of the orthostatic site (Fig. 4), showing four interactions that vary between 2.51 Å and 3.89 Å in distance, with three interactions with the residues of the dihydroergotamine sites ILE130A, VAL201A, and THR134A (two hydrophobic interactions and a hydrogen bond of strong intensity) and a hydrogen bond with the ASP129A residue (Table 1).

**Conclusion**

The results of this study show that HTMCX acetophenone was non-toxic and demonstrated an anxiolytic effect in adult zebrafish. The HTMCX acetophenone exhibited an anxiolytic effect through the 5-HT1A and 5-HT2c systems, being able to form a complex in the same region of the orthosteric site. The results confirm the pharmacological relevance of HTMCX as an anxiety inhibitor, enabling new therapeutic approaches to be investigated.
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00210-021-02116-z.

Author contribution AWS performed the experiments, analyzed the data, prepared figures and tables, and authored and wrote the manuscript. MKAF and JESAM conceived and designed the experiments. ELR, FRSM, and ALSM analyzed the data and reviewed drafts of the manuscript. MMM and ESM performed the docking molecular study and reviewed the paper. HSS and AMRT contributed reagents/materials/analysis tools, supervised the research, and reviewed the manuscript. All authors read and approved the final version of the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Funding This research was funded by Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – FUNCAP (grant number BP4-0172–00075.01.00/20 for Hélcio Silva dos Santos), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (finance code 001 for Antonio Wlisses da Silva), and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (grant number 305719/2018–1 for Alexandre Magno Rodrigues Teixeira).

Data availability All data generated or analyzed during this study are included in the supplementary material.

Code availability The authors thank Centro Nacional de Processamento de Alto Desempenho (CENAPAD) of the Federal University of Ceará (UFC) for the use of the AutoDock Vina software and to the Centro Nordestino de Aplicação e Uso da Ressonância Magnética Nuclear (CENAUREM) for the NMR measurements. The Discovery Studio Visualizer and UCSF Chimera free software were used to analyzing the results of the research.

Declarations

Ethics approval All procedures performed in studies involving zebrafish-based animal were approved by the Animal Use Ethics Committee of the State University of Ceará (CEUA-UECE; #3344801/2017). All methods were performed in accordance with the relevant guidelines and regulations.

Consent to participate All authors gave their consent to participate in this study.

Consent for publication All authors gave their consent for the publication of this manuscript.

Conflict of interest The authors declare no competing interests.

References

Ahmad F, Richardson MK (2013) Exploratory behaviour in the open field test adapted for larval zebrafish: impact of environmental complexity. Behav Processes 92:88–98. https://doi.org/10.1016/j.beproc.2012.10.014
Bandelow B, Sher L, Bunevicius R, Hollander E, Kasper S, Zohar J, Möller H-J (2012) Guidelines for the pharmacological treatment of anxiety disorders, obsessive–compulsive disorder and post-traumatic stress disorder in primary care. Int J Psychiatry Clin Pract 16:77–84. https://doi.org/10.3109/13651501.2012.66714
Benneh CK, Biney RP, Mante PK, Tandoh A, Adongo DW, Woode E (2017) Maerua angolensis stem bark extract reverses anxiety and related behaviours in zebrafish—Involvement of GABAergic and 5-HT systems. J Ethnopharmacol 207:129–145. https://doi.org/10.1016/j.jep.2017.06.012
BIOVIA Discovery Studio (2016) Dassault Systèmes Biovia Corporation, San Diego, USA
Carpenter JK, Andrews LA, Witcraft SM, Powers MB, Smits IAJ, Hofmann SG (2018) Cognitive behavioral therapy for anxiety and related disorders: a meta-analysis of randomized placebo-controlled trials. Depress Anxiety 35:502–514. https://doi.org/10.1002/da.22728
Chagraoui A, Thibaut F, Skiba M, Thuillez C, Bourin M (2016) 5-HT2C receptors in psychiatric disorders: a review. Prog

Fig. 4 5-HT1B receptor interaction complex with acetophenone (A) and the co-crystallized inhibitor dihydroergotamine (B)
Neuro-Psychopharmacol Biol Psychiatry 66:120–135. https://doi.org/10.1016/j.nppbp.2015.12.006
Chen Y-T, Liu C-Y, Chang C-M, Lai Y-M, Wang B-H, Yang T-Y, Hsu S-C (2020) Perceptions, clinical characteristics, and other factors associated with prolonged and high daily dose of benzodiazepine use among patients with anxiety or depressive disorders. J Affect Disord 271:215–223. https://doi.org/10.1016/j.jad.2020.03.077
Connors KA, Valenti TW, Lawless K, Sackerman J, Onaivi ES, Brooks BW, Gould GG (2014) Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabimoid CB receptors on zebrafish behavior in novel environments. Aquat Toxicol 151:105–113. https://doi.org/10.1016/j.aquatox.2013.12.005
de Gage SB et al. (2014) Benzodiazepine use and risk of Alzheimer’s disease: case-control study. BMJ-Brit Med J 349:g5205. https://doi.org/10.1136/bmj.g5205
WHO (2017) Depression and other common mental disorders: global health estimates. Geneva: World Health Organization 1–24. Licence: CC BY-NC-SA 3.0IGO
Ferreira MKA et al. (2020) Anxiolytic-like effect of chalcone N-[4’-[2E]-3-(3-nitrophenyl)-1-(phenyl)prop-2-en-1-one] acetamide on adult zebrafish (Danio rerio): involvement of the 5-HT system. Biochem Biophys Res Commun 526:505–511. https://doi.org/10.1016/j.bbrc.2014.09.022
Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, Sallinen V (2010) The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis 34:463–473. https://doi.org/10.1016/j.nbd.2009.01.018
Penninga EI, Graudal N, Ladekarl MB, Jürgens G (2016) Adverse events associated with flumazenil treatment for the management of suspected benzodiazepine intoxication – a systematic review with meta-analyses of randomised trials. Basic Clin Pharmacol Toxicol 118:40–48. https://doi.org/10.1111/bcpt.12434
Pettersen EF, Goddard TD, Huang CC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1955–1972. https://doi.org/10.1002/jcc.20084
Santiago RNS et al. (2018) Crystal structure, vibrational spectra and quantum chemical parameters of 2-hydroxy-3,4,6-trimethoxyacetophenone isolated from the Croton anisodontus Müll. Arg. (Euphorbiaceae). J Mol Struct 1171:815–826. https://doi.org/10.1016/j.molstruc.2018.06.064
Shaikh MF, Sancheti J, Satheye S (2013) Effect of Eclipta alba on acute seizure models: a GABA(A)-mediated effect. Indian J Pharm Sci 75:380–384. https://doi.org/10.4103/0250-474x.117432
Siebel AM et al. (2015) Role of adenosine signaling on pentyleneetetra- zole-induced seizures in zebrafish. Zebrafish 12:127–136. https://doi.org/10.1016/j.zeb.2014.1004
Smink BE, Egberts AGC, Lusthof KJ, Uges DRA, de Gier JJ (2010) The relationship between benzodiazepine use and traffic accidents. CNS Drugs 24:639–653. https://doi.org/10.1007/s12640-010-0035-1
Spolidorio PCM, Echeverry MB, Iyomasa M, Guimarães FS, Del Bel EA (2007) Anxiolytic effects induced by inhibition of the nitric oxide-cGMP pathway in the rat dorsal hippocampus. Psychopharmacology 195:183–192. https://doi.org/10.1007/s00213-007-0890-0
OECD (2019) Test No. 203: fish, acute toxicity test, OECD guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264099661-en
Trott O, Olson AJ (2010) Software news and update AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, Efficient Optimization, and Multithreading. J Comput Chem 31:455–461. https://doi.org/10.1002/jcc.21334

Voyer P, Préville M, Roussel M-E, Berbiche D, Béland S-G (2009) Factors associated with benzodiazepine dependence among community-dwelling seniors. J Community Health Nurs 26:101–113. https://doi.org/10.1080/07370010903034375

Wang C et al (2013) Structural basis for molecular recognition at serotonin receptors. Science 340:610. https://doi.org/10.1126/science.1232807

Watanabe N, Churchill R, Furukawa TA (2007) Combination of psychotherapy and benzodiazepines versus either therapy alone for panic disorder: a systematic review. BMC Psychiatry 7:18. https://doi.org/10.1186/1471-244X-7-18

Wu C-S, Wang S-C, Chang IS, Lin K-M (2009) The association between dementia and long-term use of benzodiazepine in the elderly: nested case–control study using claims data. Am J Geriatr Psychiatry 17:614–620. https://doi.org/10.1097/JGP.0b013e3181a65210

Yu N-W et al (2017) Association of benzodiazepine and Z-drug use with the risk of hospitalisation for fall-related injuries among older people: a nationwide nested case–control study in Taiwan. BMC Geriatr 17:140. https://doi.org/10.1186/s12877-017-0530-4

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.