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Heterocyclic Hydroxychalcone Promotes Anxiolytic and Anticonvulsant Effects through Gabaergic Neuromodulation in Wild Adult Zebrafish

Milena Lira Furtado[1], Antonio Wlisses da Silva[2], Naubert Bezerra De Melo[1], Roberto Lima de Albuquerque[1], Dráulio Sales da Silva[3], Jane Eire Silva Alencar de Menezes[1], Elton Patrick Barbano[3], Emmanuel Silva Marinho[1], Márcia Machado Marinho[3], Andreia Ferreira de Castro Gomes[4], Sônia Maria Costa Siqueira[1] and Hélcio Silva dos Santos[1,2,3,*]

[1]*State University of Ceará, Graduate Program in Natural Sciences, Natural Products Chemistry Laboratory, Fortaleza, Ceará, Brazil*

[2]*State University of Ceará, Northeaste Biotechnology Network, Fortaleza, Ceará, Brazil*

[3]*Center for Exact Sciences and Technology, State University of Ceará, Fortaleza, Ceará, Brazil*

[4]*Center for Molecular and Environmental Biology, University of Minho, School of Sciences, Department of Biology, Braga, Portugal*

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Abstract:

Benzodiazepines are drugs used to treat anxiety disorders and epilepsy due to their hypnotic, anxiolytic and anticonvulsant functions. But due to existing adverse effects such as decreased psychomotor activity and impaired memory, new and safer drugs are sought. Therefore, this study seeks to evaluate the anxiolytic and anticonvulsant action of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one against wildadult zebrafish, and the mechanisms of action involved in such effects.The acute toxicity of chalcone was analyzed during 96 hours, and the anxiolytic behavior of fish treated with chalcone was evaluated in light/dark tests and open field tests (n=6 animals/group). In the test to evaluate the anticonvulsant effect of the sample, Pentylenetetrazole (PTZ) was used to induce seizures by immersion. Next, we investigated the mechanism of action with the γ-aminobutyric acid (GABA) antagonist and performed a molecular coupling study. The results showed that chalcone at doses of 4, 20, and 40 mg/kg was non-toxic and altered fish locomotion (****p<0.0001 vs. control). All analyzed doses exhibited possible anxiolytic effects (* p < 0.05, *** p < 0.001 vs. control) in the light/dark test. This effect was blocked by the antagonist flumazenil (FMZ) at a dose of 40 mg/kg (# p < 0.05 vs. Fmz+Chalcone). In addition to exhibiting anxiolytic effects, chalcone demonstrated anticonvulsant effects via GABA, as confirmed by a binding/activity study (### p < 0.01, #### p < 0.0001 vs. Fmz+Chalcone).

*Corresponding Author
E-mail: helciodossantos@gmail.com

1. INTRODUCTION

Anxiety disorder is one of the leading causes of disability in the world. It is a normal response to circumstances that can generate fear, apprehension, doubt or expectation, causing symptoms such as chest pain, fatigue, palpitations and sleep disturbance [1,2]. In addition to presenting more prevalent psychiatric conditions, it can trigger other psychiatric disorders, such as depression and epilepsy [3].

Epilepsy is a neurological disorder characterized by spontaneous and unpredictable seizures due to excessive abnormal neuronal activity in the brain, affecting an estimated 70 million people worldwide. There are effective anticonvulsant drugs to treat seizures. However, these drugs often cause adverse effects [4-6].

Benzodiazepines are among the most used drugs in the world in the primary treatment of anxiety-related disorders due to their depressant action on the central nervous system (CNS). They are also used for sleep disorders and anticonvulsants [7]. Some effects are caused by the benzodiazepine medication, such as drowsiness and decreased reflexes. Inappropriate use leads to decreased cognition, sedation, tolerance and dependence [8,9].

Different neurotransmitter systems have been linked to anxiety and seizure disorders, such as GABA and the monoamines serotonin (5-hydroxytryptamine or 5-HT). Being GABA, the main inhibitory neurotransmitter of the CNS, acts practically in every region of the brain, acting in different physiological processes, such as in response to stressors, motor processes, pain control, fear and anxiety [10]. GABAergic neurotransmission occurs through the interaction of GABA with its receptors, which are mainly classified into two main types: ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptor [11]. $GABA_A$ acts by activating the rapid hyperpolarizing negative ion channel (Cl^-) and diffuses through a concentration gradient to hyperpolarize mature postsynaptic neurons [12]. Compounds with high affinity for the $GABA_A$ receptor can enhance the activity of the GABAergic system and promote anxiolytic and anticonvulsant effects.

Chalcones are an important chemical class in medicinal chemistry because they have many interesting biological activities, including antinociceptive, anxiolytic, anti-inflammatory and anticonvulsant [13-15]. Its structure is formed by aromatic ketones of natural or synthetic origin, consisting of an α, β -unsaturated carbonyl system joining two aryl rings, probably the main responsible for its biological activities, and with a carbon skeleton with several replaceable hydrogens. This structure can undergo several changes that result in different effects on biological metabolism [16,17]. Biological activities of chalcones may be related to their small structure and Michael acceptor characteristics (unsaturated molecule conjugated with an electronegative group that attracts electrons), which make them easily bind to other biological molecules [18]. Their ability to interact with other molecules and enzymes allows chalcones to intervene and react with several important cellular mechanisms. Chalcones can be considered Michael acceptors due to the presence of an α,β-unsaturated carbonyl group in their structure[19].Studies have revealed important neuropharmacological effects of chalcones, such as anxiolytic, anticonvulsant [13,17], neuroprotective and anti-Alzheimer's effects [19].

Research has used zebrafish (*Danio rerio*) as a model for investigating new chalcones with anxiolytic and anticonvulsant effects [13,17]. Zebrafish have been chosen as a model for studies of new compounds with neuropharmacological effects because they are low cost and have a high degree of morphological, physiological, and genetic homology with humans [20]. Their central nervous system has a general organization and neural circuits very similar to those of mammals, with all the classic neurotransmitters found in vertebrates, and their motor, sensory, and endocrine systems are well developed [21]. These characteristics make zebrafish a key model for obtaining low-cost preclinical results.

Due to the side effects caused by benzodiazepines, the field of neuropharmacology has been extensively studied. Several studies are being conducted to search for compounds that have promising results against anxiety and seizures. Chalcones have been evaluated and have shown a promising effect against both [13,17]. This study aims to evaluate the anxiolytic and anticonvulsant potential of chalcone (E)-3-(furan-2-yl)-1-(2-hydroxyphenyl) prop-2-en-1-one, together with the interaction study ligand/protein.

2. MATERIALS AND METHODS

2.1. Drugs and Reagents

Flumazenil (FMZ) (Roche Pharmaceutical/Welwyn Garden City, United Kingdom), Diazepam (DZP) and

Pentylenetetrazole(PTZ) (Sigma-Aldrich, Missouri, EUA).

2.2. Synthesis and Chemical Characterization of Chalcone

The chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl) prop-2-en-1-one was synthesized by a Claisen-Schmidt condensation reaction in a basic medium (Figure **1**). An ethanolic solution of 2-hydroxyacetophenone (2 mmol) was added to a solution of furfural (2 mmol), followed by the addition of 10 drops of 50% m/v aqueous NaOH with stirring for 48 h at room temperature. After 48 h, the reaction mixture was neutralized with dilute HCl (10%) and added ice water. The product was obtained as a yellow solid. The formed solid was filtered under reduced pressure and washed with cold water. The chemical structure of chalcone was determined and confirmed by 1H, 13C and infrared NMR, which were obtained using a Bruker DPX-300 platform operating at frequencies of 300 MHz for hydrogen and 75 MHz for carbon. The infrared absorption spectrum was obtained using a Bruker vacuum spectrometer (VERTEX 70V).

2.3. Zebrafish

Wild, male and female fish (*Danio rerio*) (aged 90 to 120 days; 0.4 ± 0.1 g, 3.5 ± 0.5 cm) were obtained from Agroquímica: Comércio de ProdutosVeterinários LTDA, a supplier located in Fortaleza-Ceará. The animals were kept in a 10 L glass aquarium (30 × 15 × 20 cm) at a temperature of 25 ± 2 °C, in cycles of 10 to 14 h (light/dark), with chlorinated water pH 7.0 (ProtecPlus®) using an air pump with submerged filters. The animals received fish food (Spirulina®) 24 h before the experiments. In each experiment, the animals were randomly selected from the aquarium, and the doses of chalcone tested were chosen based on studies in the literature that used doses of 4 mg/kg, 20 mg/kg, and 40 mg/kg [13,17]. Before each treatment, the animals were anesthetized in ice water, and after the experiments, they were euthanized by immersion in ice water (2–4 °C) for 10 min until loss of opercular movements. All procedures were approved by the Ethics Committee on Animal Use of the State University of Ceará (CEUA-UECE; nº 04983945/2021) in accordance with ethical principles involving experiments with animals.

2.4. Acute Toxicity

An acute chalcone toxicity study was performed on adult zebrafish (*Danio rerio*) in accordance with Organization for Economic Co-operation and Development (OECD) guidelines [22]. The fish (n = 6/group) were treated orally (*v.o*) with chalcone (4, 20 and 40 mg/kg; 20 µL), and 20 µL of 3% dimethylsulfoxide (DMSO) was used as a negative control. After the treatments, the mortality rates of the animals were observed. Every 24 h up to 96 h, the number of dead fish in each group and the lethal concentration capable of killing 50% of the animals (LD_{50}) were recorded, being determined by the mathematical method Spearman-Karber Trimming with a 95% confidence interval [23].

2.5. Locomotor Activity

The open field test was performed to analyze the motor coordination of the fish. The animals (n=6/group) were orally treated with the test drug (4, 20 and 40 mg/kg; 20 µL). Two groups of animals (n=6/group) treated with the control (3% DMSO, 20 µL) and diazepam (4 mg/kg; 20 µL) were included. After 1h of treatments, the animals were added to Petri dishes (10 x 15 cm) containing the same aquarium water, marked with four quadrants, and locomotor activity was analyzed by counting the number of lines crossing (CL) during 5 minutes [24].

2.6. Anxiolytic Effect

The anxiolytic effect was investigated through the light and dark test. Zebrafish have an aversion to lighted areas, similar to rodents when they are anxious. The animals (n = 6 / group) were orally treated with the sample (4, 20 and 40 mg/kg; 20 µL), control (3% DMSO, 20 µL) and diazepam (4 mg/kg; 20 µL). After 1 hour of treatment, the animals were individually added to the light zone of the aquarium (30 x 15 x 20 cm), which was divided into light and dark zones, and the behavior of the zebrafish was observed for 5 minutes. The anxiolytic effect was measured based on the time the fish spent in the light zone of the aquarium; the

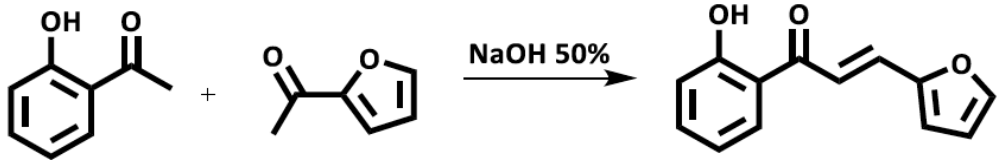

Figure 1: Scheme of the synthesis of chalcone,

longer the time spent in the light area, the greater the anxiolytic behavior and the less anxiety the zebrafish had [25].

2.7. Involvement of the GAB Aergic System

The involvement of the GABAergic system was also assessed by the previously described light and dark test after pretreatment with flumazenil, a $GABA_A$ antagonist [26]. Zebrafish (n=6/group) were pretreated with FMZ (4 mg/kg; 20 µL; v.o). After 15 min, chalcone (4, 20 and 40 mg/kg; 20 µL; p.o.) was administered. 3% DMSO (20 µL; v.o) was used as a control, and Diazepam (4 mg/kg, 20 µL; v.o) was used as a $GABA_A$ agonist. After 1 h of treatments, the animals were submitted to the light/dark test described in the previous section.

2.8. Anticonvulsant Effect

PTZ-induced seizure reversal was investigated by Siebel *et al*. [27]. The animals (n = 6/group) were treated with chalcone (4, 20 and 40 mg/kg; 20 µL; v.o.), Diazepam (4 mg/kg; 20 µL;v.o.) and control (DMSO 3%; 20 µL; v.o.). After 1 h, the animals were individually exposed to 7.5 mM PTZ, dissolved in water in a 250 mL beaker, and convulsive behavior in three stages was assessed: stage I, a drastic increase in swimming activity; stage II, whirlpool behavior; and stage III, convulsions similar to clonus, followed by loss of posture when the animal fell to the side and remained immobile for 1-3 s. At the end of the evaluation of the three test stages, the animals were euthanized on ice.

2.9. Assessment of GABAergic Neuromodulation in Seizures

The fish (n = 6/group) were pre-treated with FMZ (4 mg/kg; 20 µL; ***V.O***.) [13]. After 15 min, they were treated with chalcone (40 mg/kg; 20 µL;v.o.), Diazepam (4 mg/kg; 20 µL;v.o.) and control (3% DMSO; 20 µL;v.o.). After 1 h of treatment, the animals were individually exposed to PTZ (7.5 mM) and the three seizure stages were evaluated as described above.

2.10. Statistical Analysis

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 data, differences between groups were submitted to analysis of variance (one-way ANOVA) and two-way ANOVA in experiments with antagonists, followed by Tukey's test. All analyzes were performed using GraphPad Prism v.8.0 software. The adopted statistical significance level was 5% ($P < 0.05$).

2.11. Molecular Docking

To investigate the anxiolytic and anticonvulsant effect of chalcone against the GABA receptor, molecular docking simulations were performed, with the receptor structure obtained from the Protein Data Bank repository (https://www.rcsb.org/), PDB ID: 6HUP [28], identified as "CryoEM structure of human full-length alpha1beta3gamma2L GABA(A)R in complex with DZP (Valium), GABA and megabody Mb38" [29]. Target preparation was performed using the methodology proposed by Mendes *et al*. [30], where residues were removed, polar hydrogens and Gasteiger charges were added [31] using the AutodockTools™ software [32]. 50 independent simulations were performed and 20 poses each, using the Auto Dock Vina software [33], configured to run the Lamarkian Genetic Algorithm (LGA), *Exhaustiveness* 64 [34] and a simulation grid centralized in the target to involve the entire protein structure using the axes: 125.281 (x), 139.534 (y), 136.018 (z) and size: 126Å (x), 100Å (y), 126Å (z) (MENDES *et al*., 2023).To validate the docking simulations, the redocking technique was performed with the drug DZP co-crystallized in the GABA receptor [17]. The statistical parameter RMSD (*Root Mean* Square Deviation) with values up to 2.0 Å [35] and affinity energy, with values below -6.0 kcal/mol [36,37]. Data were analyzed using UCSF Chimera™ [38], *Pymol* [39] and *Discovery studio visualizer™ viewer* [40]. The Protein-Ligand Interaction Profiler (PLIP) server [41] was used to visualize molecular interactions and hydrogen bonds.

3. RESULTSAND DISCUSSION

3.1. Chemical Characterization

In the ^{1}H NMR spectrum (Figure **2**), the signals at 7.56 (*J*= 14.0) and 8.00 (J= 15.6) are attributed to two doublets referring to α, β unsaturated hydrogens, whose coupling constant (J) confirms the E-stereochemistry of the double bond. The signals at 8.00 (d, *J* = 11.2 Hz, H6'), 7.10 (d, *J* = 8.4 Hz, H3'), 7.54-7.79 (m, H4') and 7.01 (t, *J* = 7.5 Hz, H5') refer to the aromatic hydrogens of the phenolic ring. While the signals at 7.54-7.79 (m, H5), 6.85 (d, *J* = 3.4 Hz, H3) and 6.62 (dd, *J* = 8.3; 2.6 Hz, H4) refer to the aromatic hydrogens of the furan ring.

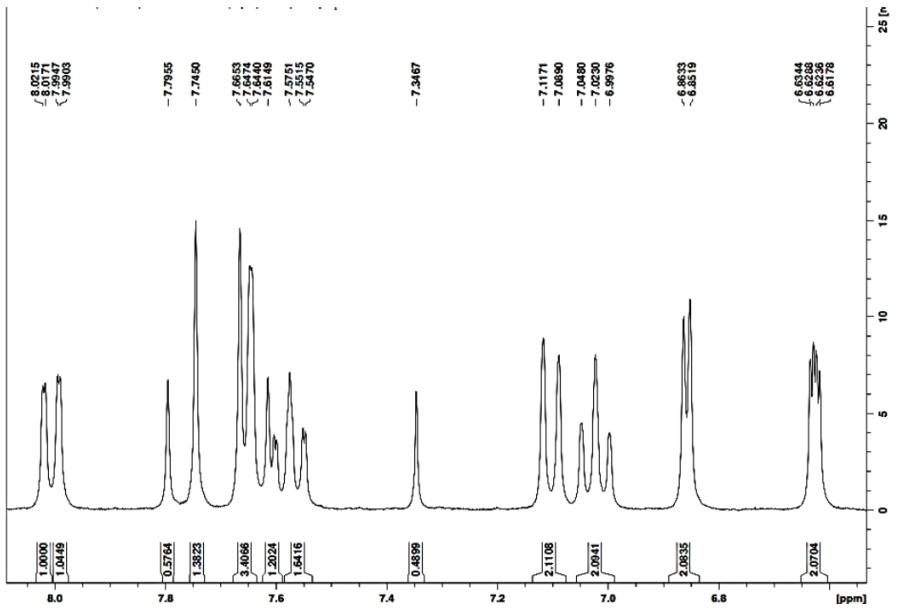

Figure 2: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one.

In the ^{13}C NMR spectrum (Figure **4**), a signal refers to α, β unsaturated carbonyl at 193.4. The ketone absorbs at 203.8. However, the presence of α, β unsaturation causes a shift to a high field, and the likely cause is charge shifting by the benzene ring or the double bond, which makes the carbonyl carbon less electron deficient. The α and βolefinic carbons are 136.1 and 118.5, respectively. At 163.6 (C-2'), 131.2 (C-4'), 129.6 (C-6'), 120.1 (C-1'), 118.8 (C-5') and 117.6 (C-3') are the signs referring to the carbons present in the phenolic ring. While the signals at 151.6 (C-1), 145.4 (C-5), 117.1 (C-4) and 112.9 (C-3) refer to the furan ring carbons (Table **1**). In the infrared absorption spectrum (Figure **6**), the most characteristic absorptions of chalcone are the conjugated ketone (C=O) at 1638, the double bond (C=C) at 1581, the C-O bond at 1258 and 1014 cm^{-1} confirming the presence of the furfuryl ring and the phenol (OH) stretch at 3129 cm^{-1} [42]. The structure of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one was determined by interpreting ^{1}H NMR spectra (Figure **2**), ^{13}C (Figure **4**) and infrared (Figure **6**), data obtained from ^{1}H and ^{13}C NMR of chalcone are shown in (Table **1**).

3.2. Acute Toxicity and Locomotor Activity

During the 96 h of analysis, one animal died per tested dose, indicating that the sample was nontoxic to adult zebrafish (LD50 > 40 mg/kg), and the agent did not cause apparent anatomical changes in the animals during this period (P >0.05). The animals' locomotion was altered by chalcone at all concentrations [****p<0.0001 (4, 20 and 40 mg/kg)] and Diazepam (4 mg/kg) (****p<0, 0001), as the animals had significantly reduced locomotor activity when compared to the control group ($F_{4, 25}$ = 1.73; Figure **7**).

The open field test evaluates anxiety-related behavior since several behavioral parameters are analyzed, such as distance covered and freezing. Decreased locomotion may indicate effects related to central nervous system depression [26]. The locomotor behavior of adult zebrafish with the addition of chalcone was evaluated. With this, it was possible to observe that the fish had a reduction in locomotion compared to the control (DMSO 3%) and behavior similar to diazepam (Figure **7**). Studies report that anxiolytic drugs such as benzodiazepines and chalcones reduce the locomotion of adult zebrafish in the open field test [13,17,30].

3.3. Anxiolytic Effect

One-way ANOVA statistical analysis followed by Tukey indicated that all doses of chalcone significantly increased the time the animals spent in the light region of the aquarium (* p <0.05, *** p <0.001 vs. Control) ($F_{4, 25}$ = 3.32; Figure **8**), causing anxiolytic behavior similar to the positive control DZP (4 mg/kg) (** p <0.01 vs Control).

The anxiety behavior of zebrafish is similar to that of rodents, preferring dark environments in addition to new environments that induce anxiety in animals [43]. The light/dark test was performed to evaluate the

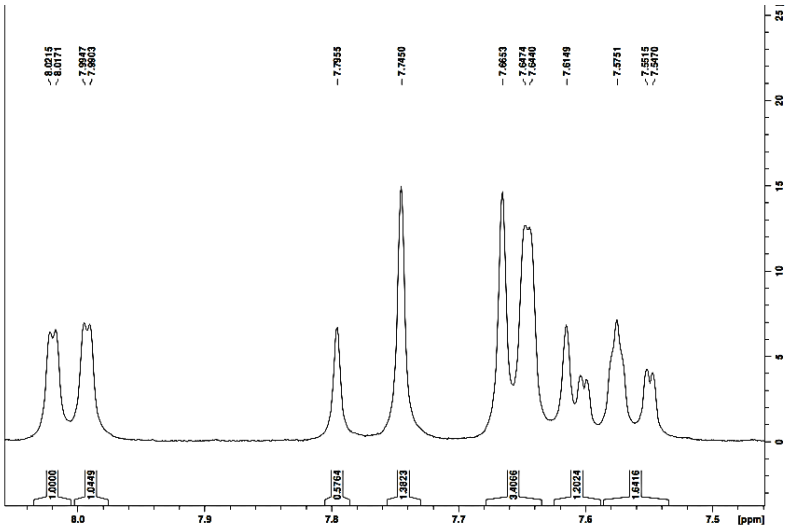

Figure 3: Expansion of the ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one.

Table 1: ^{1}H and ^{13}C NMR Data of Chalcone (E)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one in $CDCl_3$

C	$\delta_{C(ppm)}$	$\delta_{H\ (ppm)}$
1´	120.1	
2´	163.6	
3´	117.6	7.10 (d, *J* = 8,4 Hz)
4´	131.2	7.54-7,79 (m)
5´	118.8	7.02 (t, *J* = 7,5 Hz)
6´	129.6	8.00 (d, *J* = 11,2 Hz)
C=O	193.4	
1	151.6	
2		
3	112.9	6.85 (d, *J* = 3.4 Hz)
4	117.1	6.62(dd, *J* = 8.3; 2.6 Hz)
5	145.4	7.54-7.79 (m)
C_α	118.5	7.56 (d, *J* = 14.0 Hz)
C_β	136.1	8.00 (d, *J* = 15.6 Hz)

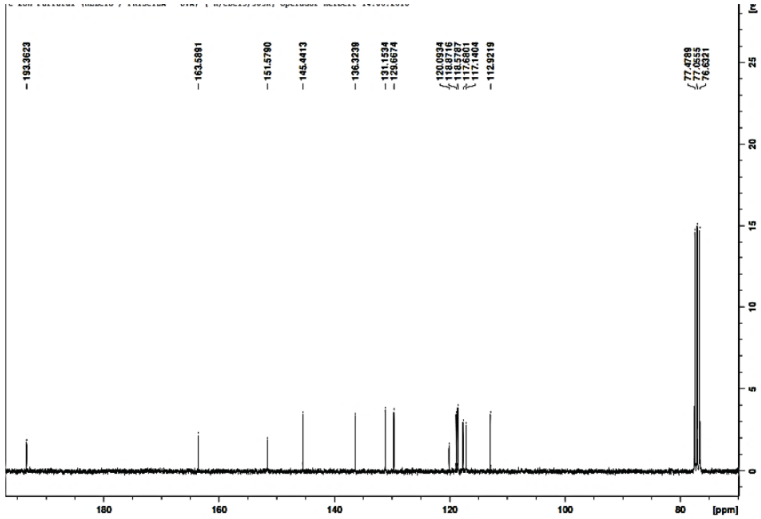

Figure 4: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one.

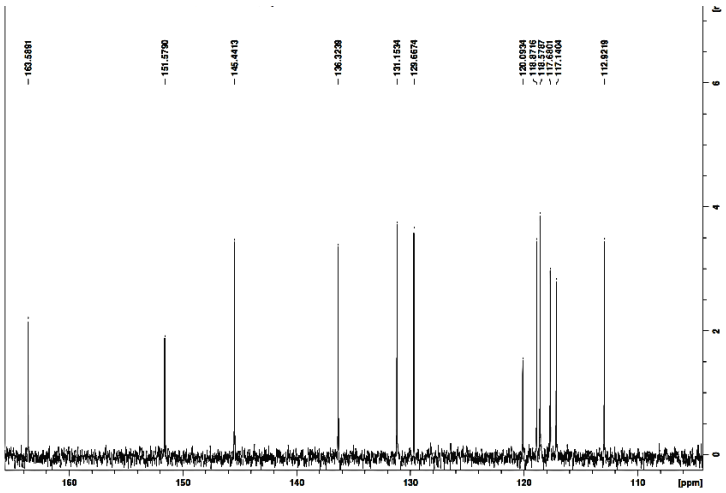

Figure 5: ^{13}C NMR spectrum expansion ($CDCl_3$, 125 MHz) of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one.

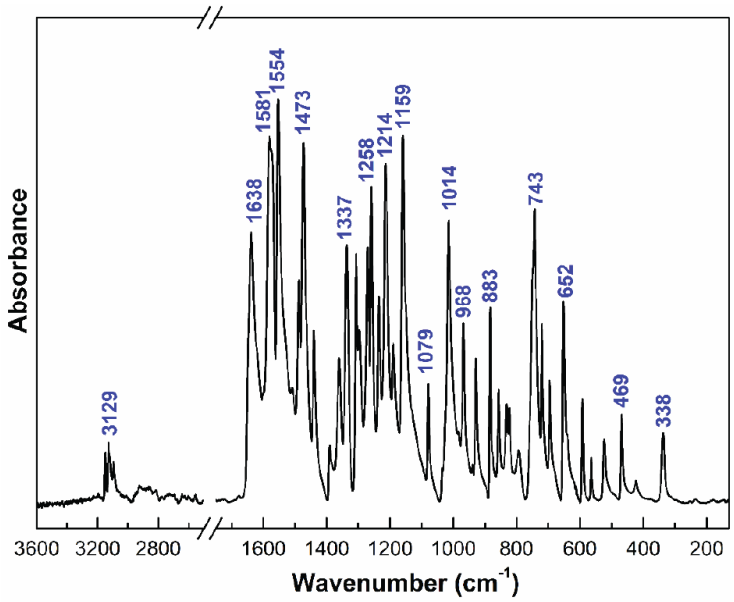

Figure 6: Infrared spectrum of chalcone (*E*)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one in the spectral range from 3600 to 2400 cm^{-1} and 1750 cm^{-1} to 40 cm^{-1}. mg/kg).

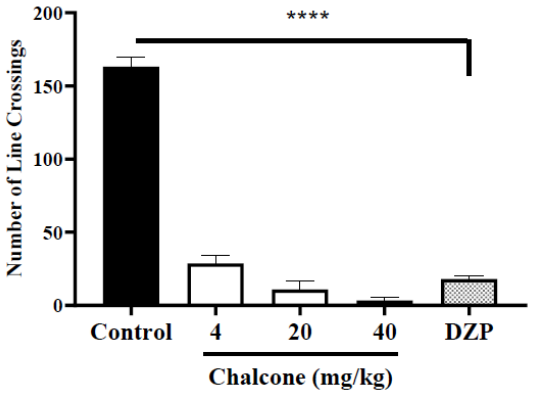

Figure 7: Effect of chalcone on locomotor behavior of adult zebrafish in the Open Field Test (0–5 min). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Tukey's test (****p<0.0001 vs. Control).

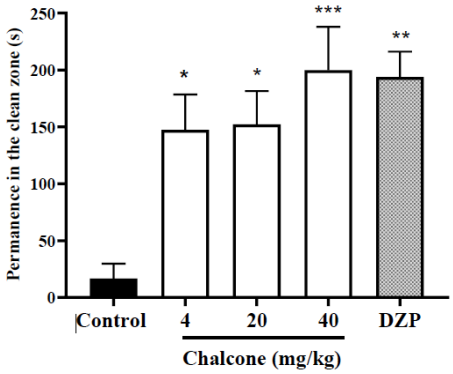

Figure 8: Effect of chalcone on zebrafish anxiety in the light/dark test (0–5 min). Values represent the mean ± standard error of the mean for 6 animals/group; One-way ANOVA followed by Tukey's test for the light/dark test (*p<0.05, **P<0.01, ***p<0.001 vs. Control).

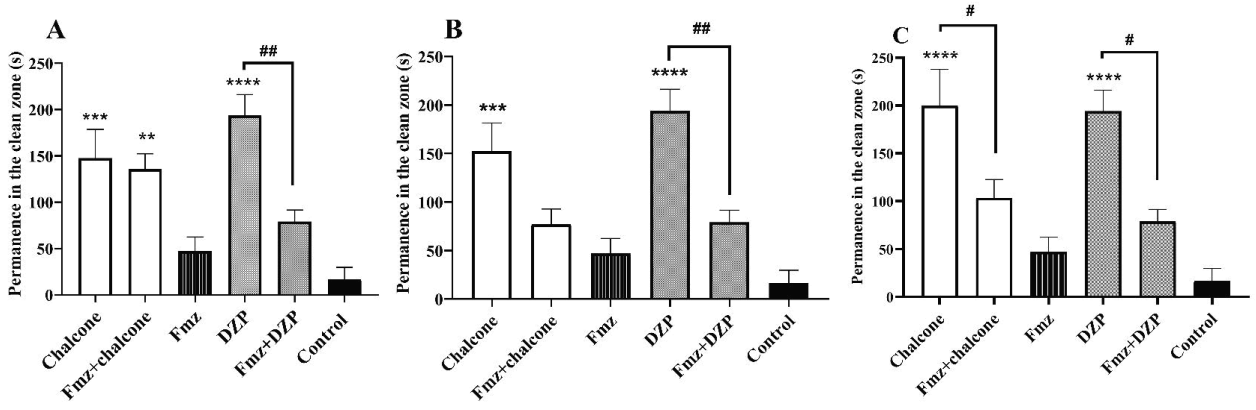

Figure 9: Anxiety mechanism via GABA of chalcone at doses of 4 mg/kg (**A**), 20 mg/kg (**B**) and 40 mg/kg (**C**) in the light/dark test (0–5 min). Values represent the mean ± standard error of the mean for 6 animals/group; (One-way ANOVA and two-way ANOVA in experiments with antagonists) followed by Tukey's test (**p<0.01, *** p <0.001, **** p<0.0001 vs Control; # p <0.05, ## p<0.01 vs. Fmz+DZP; # p<0.05 vs. Fmz+Chalcone).

anxiolytic effect of the sample on zebrafish, where the animals increased their time in the light region of the aquarium compared to the control (Figure **8**), as they were less anxious, and the result was superior to the positive control - DZP, with 66.6% remaining in the light by the group treated with chalcone at the highest dose (40 mg/kg) and 64% remaining in the light by the group treated with DZP, demonstrating a superior anxiolytic effect.

3.4. Involvement of the GABAergic System

The investigation of the mechanism of action via GABAergic neurotransmission was carried out with pretreatment with flumazenil in the animals. Flumazenil blocked the effect of chalcone at the highest dose (40 mg/kg) and DZP, as the pretreated animals spent most of their time in the dark region of the aquarium (anxiety behavior), a result significantly different from that observed in groups treated only with the highest dose of chalcone or DZP (# P < 0.05 vs. Chalcone or DZP; $F_{5, 25} = 12.14$). The 20 mg/kg dose of chalcone also had its effect partially blocked by pretreatment with flumazenil (p > 0.05), unlike the lower dose (4 mg/kg) of chalcone, which after pretreatment continued to cause anxiolytic behavior in the animals (Figure **9**). These results indicate the involvement of GAB Aergic neurotransmission in the anxiolytic effect of chalcone.

The mechanism of action related to the anxiolytic effect of the sample was investigated using flumazenil, a GABAA receptor modulator known to antagonize the anxiolytic, sedative and hypnotic effects of benzodiazepines [44,45]. These effects occur because benzodiazepines increase the GABA response by potentiating the opening of GABA-activated Cl- channels, hyperpolarizing the intracellular environment, and reducing neuronal excitability. Dysfunction of these channels results in anxiety disorders and epilepsy [46]. The anxiolytic effect of chalcone at a dose of 40 mg/kg was blocked by flumazenil, similar to what occurs with DZP, which had its anxiolytic action blocked by the antagonist (Figure **9C**), reducing the time spent by zebrafish in the clear zone of the aquarium, as they were more anxious. That is, the anxiolytic effect of chalcone may occur through interactions involving the GABAergic system.

3.5. Involvement of the Serotonergic System (5-HT)

Serotonergic neuromodulation was investigated by pretreatment with the antagonists pizotifen ($5\text{-}HT_1$ and $5\text{-}HT_{2A/2C}$) and granisetron ($5\text{-}HT_{3A/3B}$). Both antagonists blocked the anxiolytic effect of chalcone at the highest dose (40 mg/kg) and of the positive control Fluoxetine [# p<0.05 vs. Chalcone; #### p<0.0001 vs. Flx (40 and 0.05 mg/kg respectively)] (Figure **10**; $F_{5, 25} = 22.9$ – A; $F_{5, 25} = 34.25$ – B).

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter responsible for shaping mood, emotion, and defensive, social, and anxiety behaviors [47]. Anxiety behavior in the light-dark test is positively associated with extracellular levels of 5-HT in the zebrafish brain [24]. The results reveal that in addition to chalcone being capable of causing anxiolytic behavior by modulating the GABAergic pathway, as shown in previous results, it also modulates the serotonergic pathway, potentially acting on serotonin receptors 1, 2, and 3. These results demonstrate the

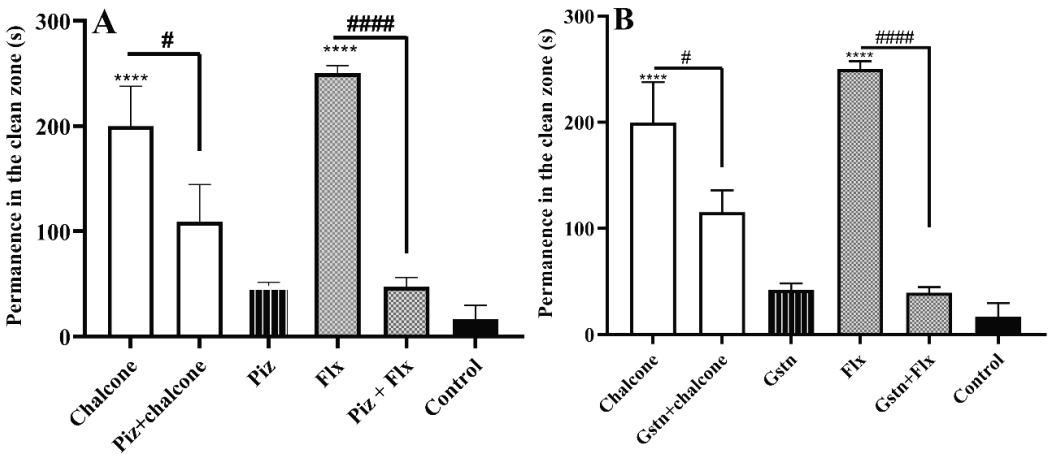

Figure 10: Effect of pizotifen (**A**) and granisetron (**B**) on the anxiolytic effect of chalcone in the light/dark test (0–5 min). The values represent the mean ± standard error of the mean for 6 animals/group; (one-way ANOVA and two-way ANOVA in experiments with antagonists) followed by Tukey's test (**** p<0.0001 vs Control; # p<0.05, #### p<0.0001 vs Chalcone or Flx).

relevance of this chalcone for new biochemical and molecular studies that better clarify the mechanism of action involved in this effect.

3.5. Anticonvulsant Effect

One-way ANOVA indicated that the three doses of chalcone significantly delayed PTZ-induced convulsive behavior in all three stages (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p<0.0001 vs Control) when compared to the control (3% DMSO), an effect significantly lower than DZP (4 mg/kg) (# p < 0.05, # # # p <0.001, # # # # p<0.0001 vs DZP), which delayed the onset of seizures longer in the three stages (**** p<0.0001 vs Control) ($F_{4, 25}$ = 0.68 – Stage I; $F_{4, 25}$ = 1.12 – Stage II; $F_{4, 25}$ = 1.43 – Stage III; Figure **10**).

These results were similar to those obtained in other studies conducted with chalcones in adult zebrafish [13,17]; however, hydroxychalcone did not delay PTZ-induced seizures like Diazepam did. Mendes *et al*. [17] reported that anticonvulsant compounds delay the latency time to enter each stage of PTZ-induced seizures. Therefore, heterocyclic hydroxychalcone may be anticonvulsant, but higher doses may be necessary to cause an effect similar to Diazepam. Hydroxy-chalcone was responsible for delaying the tonic/clonic stage of seizures (stage 3) by up to 49.9%, while DZP delayed up to ~71% of PTZ-induced seizures.

3.6. Assessment of GABAergic Neuromodulation in Seizures

The mechanism of action of the anticonvulsant effect of chalcone at a dose of 40 mg/kg was investigated with flumazenil. Pre-treatment with flumazenil blocked the anticonvulsant behavior of the animals caused by the higher dose of chalcone and DZP in all three stages (#### p < 0.0001, ## p < 0.01 vs. chalcone or DZP). These results indicate that the anticonvulsant effect of chalcone occurs through GABAergic neurotransmission similar to DZP ($F_{5, 25}$ = 61.21 – Stage I; $F_{5, 25}$ = 77.69 – Stage II; $F_{5, 25}$ = 33.77 – Stage III; Figure **11**).

Pentylenetetrazole is a convulsant that acts allosterically on the $GABA_A$ receptor [48]. All doses of chalcone analyzed (4, 20 and 40 mg/kg) delayed the convulsive behavior induced by PTZ in the three stages in zebrafish (Figure **11**), a result considered partial when compared to Diazepam (4 mg/kg), which also increased the latency time for the onset of seizures in the three stages when compared to the control. Other studies show that chalcones have a possible anticonvulsant effect [13,17]. These results corroborate the data obtained from the mechanism of action of the $GABA_A$ receptor with flumazenil (Figure **12**), demonstrating that there is an anticonvulsant action of chalcone (40 mg/kg) on the GABAergic system since the sample, as well as Diazepam, did not delay the onset of convulsive crises in the three stages in animals when pre-treated with flumazenil.

Although the results obtained provide relevant information, this study has some limitations that should be considered when interpreting the data. One of the main limitations is the absence of dose-response curves for all tests performed. These limitations do not invalidate the findings of this study, but indicate the need for more comprehensive future studies, with optimized experimental protocols and complementary approaches that allow a more detailed assessment of

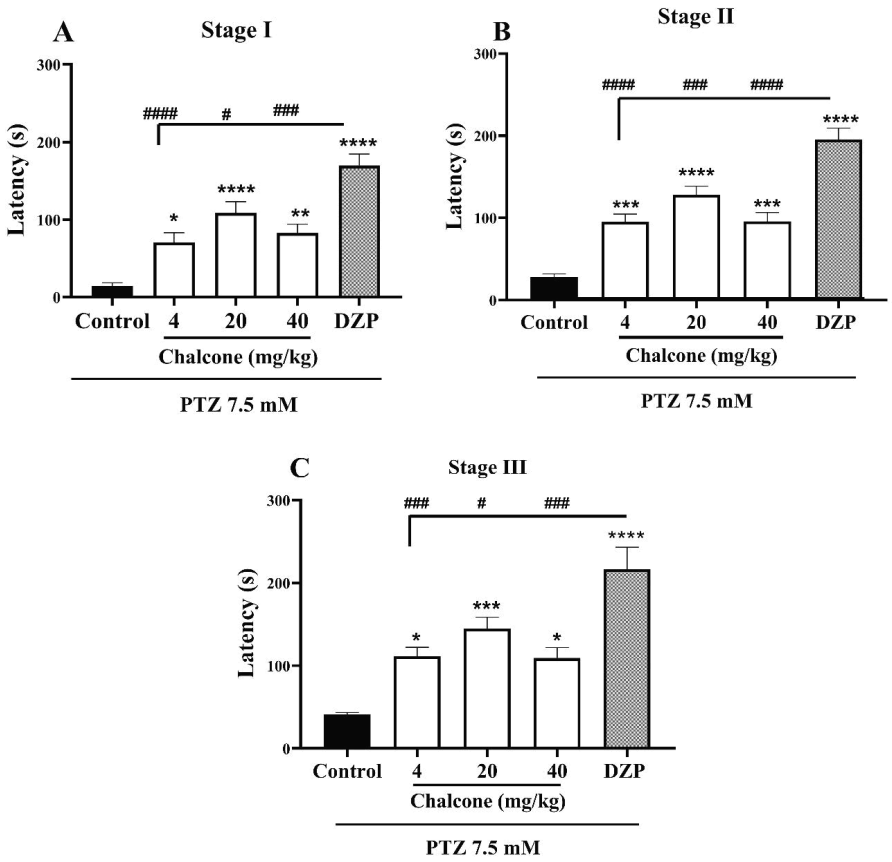

Figure 11: Effect of chalcone on pentylenetetrazole-induced seizure (3 stages) in adult zebrafish. Stage I (**A**), Stage II (**B**), Stage III (**C**). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P< 0.0001 vs Control; #P < 0.05, ## #P < 0.001, ####P< 0.0001 vs DZP).

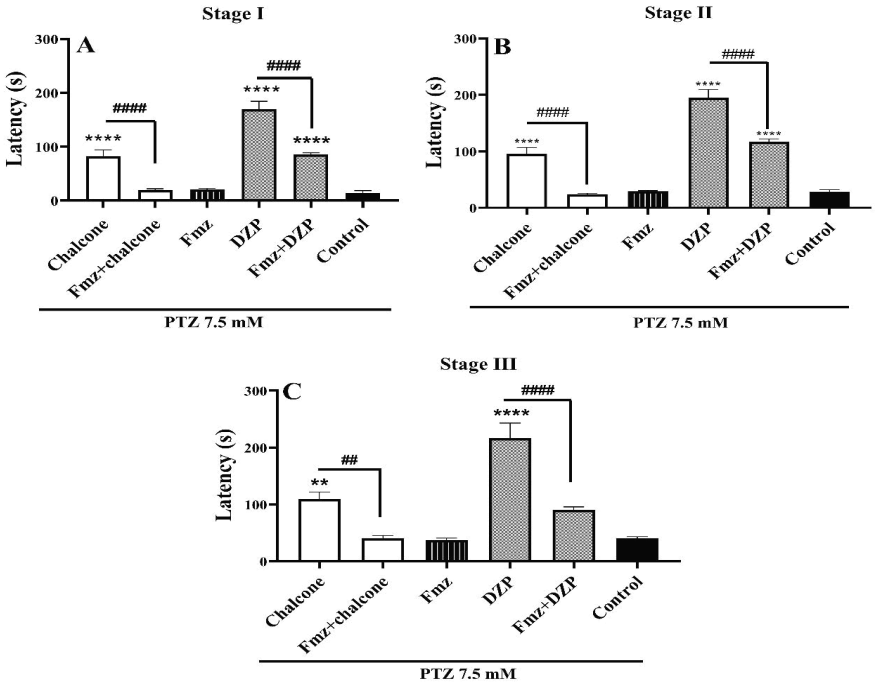

Figure 12: Anticonvulsant effect of chalcone (40 mg/kg) after pretreatment with flumazenil in the pentylenetetrazol-induced seizure test in adult zebrafish. Stage I (A), Stage II (B), Stage III (C). Values represent mean ± standard error of mean for 6 animals/group; (ANOVA one-way) and (ANOVA two-way in experiments with antagonists) followed by Tukey's test (**P < 0.01, ****P < 0.01 vs Control; ## p< 0.01, #### P < 0.0001 vs Fmz+chalcone; #### P < 0.0001 vs Fmz+DZP).

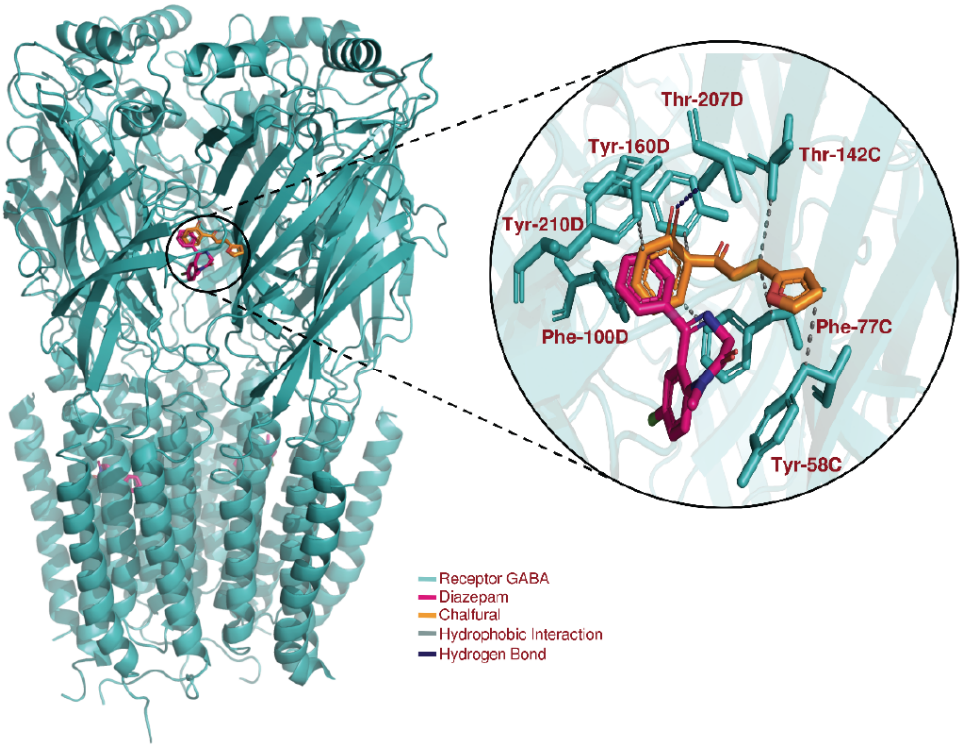

Figure 13: Complex interaction of the receptor GABA with Chalcone and Diazepam.

the effects of hydroxy chalcone and the mechanisms involved.

Molecular Docking

Through the study of molecular docking, we observed that the best poses of Chalcone presented an RMSD value lower than the reference value (2.0 Å), in the order of 1,049 Å, and the DZP presented a value in the order of 0.016 Å, remaining within ideality statistic. Concerning affinity energy, the Chalcone/GABA and DZP/GABA complexes showed values around -8.4 kcal/mol and -7.8 kcal/mol, respectively, indicating that chalcone had a higher affinity when compared to DZP.To evaluate the possible interaction through the GABA receptor, a molecular docking study was performed to determine the protein-ligand and protein-protein interactions, thus being able to explain the anxiolytic and anticonvulsant effects of chalcone (KHAN *et al*., 2016).

Analyzing the interaction patterns, it was observed that the Chalcone/GABA complex is formed by seven hydrophobic interactions involving the residues Tyr 58C (3.62 Å), Phe 77C (3.49 and 3.65 Å), Phe 100D (3.67 Å), Thr 142C (3.94 Å), Tyr 160D (3.88 Å), Tyr 210D (3.66 Å) and a strong H-bond with the residue Thr 207D (2.90 Å).The binding site for Diazepam co-crystallized between the C and D chains of the GABA receptor is formed by the residues Tyr 58C, Asn 60C, Phe 77C, Phe 100D, His 102D, Ser 159D, Tyr 160D, Glu 189C, Val 203D, Gln 204D, Ser 205D, Ser 206D and Tyr 210D [49,50]. It was observed that Chalcone bound in the same region as the binding site of Diazepam, having in common interactions with residues Tyr 58C, Phe 77C, Phe 100D and Tyr 210D, being an indication of action like DZP (Figure **13**).

Molecular docking results showed that chalcone had lower affinity energy against the GABA receptor than DZP, in addition to binding in the same region of the Diazepam binding site, indicating a similar effect to the reference drug. Therefore, the anxiolytic and anticonvulsant effects of chalcone may be modulated by GABA.

5. CONCLUSION

This study provides evidence that the chalcone evaluated is non-toxic and has anxiolytic and anticonvulsant effects in adult zebrafish. The GABAergic and serotonergic systems may be involved in the effects of hydroxychalcone on anxiety. The interaction of chalcone with the GABA receptor may form a complex in the same binding region as diazepam. The results obtained in this study have relevant translational potential, as they provide preliminary data that may support further investigations

of the effect of heterocyclic hydroxychalcone on the central nervous system in more complex biological models. These findings corroborate the feasibility of additional preclinical studies in mammals, contributing to the understanding of the physiological effects of the substance in organisms more similar to humans.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The work was approved by the Ethics Committee on the Use of Animals of the State University of Ceará (CEUA-UECE; nº 04983945/2021) following the Ethical Principles of Animal Experimentation.

CONSENT FOR PUBLICATION

The author(s) Consent with the publication of this work.

AVAILABILITY OF DATA AND MATERIAL

Data is available on request.

COMPETING INTERESTS

All authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this work.

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AUTHORS' CONTRIBUTIONS

Milena Lira Furtado: Investigation; Formal analysis; Writing - original draft. Hélcio Silva dos Santos: Co-supervision; Writing - review and editing. Antonio Wlisses da Silva: Project administration; Conceptualization. Jane Eire Silva Alencar de Menezes: Funding; Formal analysis; Writing - review and editing. Sônia Maria Costa Siqueira: Methodology; Formal analysis. Emmanuel Silva Marinho: Software; Formal analysis; Writing - review and editing. Walber Henrique Ferreira Ribeiro: Methodology; Formal analysis. Márcia Machado Marinho: Software, Visualization. Naubert Bezerra De Melo: Methodology; Formal analysis.

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REFERENCES

[1] Bandelow B, Michaelis, S.; Wedekind D. Treatment of anxiety disorders. Dialogues Clin Neurosci 2017; 19: 93-107. https://doi.org/10.31887/DCNS.2017.19.2/bbandelow

[2] Karino CA, Laros JA. Ansiedade em situações de prova: evidências de validade de duas escalas. Psico-USF 2014; 19: 23-36. https://doi.org/10.1590/S1413-82712014000100004

[3] Kysil EV, Meshalkina DA, Frick EE, *et al*. Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. Zebrafish 2017; 14: 197-208. https://doi.org/10.1089/zeb.2016.1415

[4] Barbalho PG, Carvalho B de S, Lopes-Cendes I, *et al*. Cyclooxygenase-1 as a potential therapeutic target for seizure suppression: Evidences from zebrafish pentylenetetrazole-seizure model. Front Neurol 2016; 7. https://doi.org/10.3389/fneur.2016.00200

[5] Buchanan-Peart K-AR, Oribhabor GI, Khokale R V, *et al*. Cannabis, More Than the Euphoria: Its Therapeutic Use in Drug-Resistant Epilepsy. Cureus 2020; 12. https://doi.org/10.7759/cureus.9299

[6] Sun S, Zhu C, Ma M, *et al*. A pentylenetetrazole-induced kindling zebrafish larval model for evoked recurrent seizures. bioRxiv 2019; 1-24. https://doi.org/10.1101/787580

[7] Robert Peter Biney, Charles Kwaku Benneh, James Oppong Kyekyeku, *et al*. Attenuation of Anxiety Behaviours by Xylopic Acid in Mice and Zebrafish Models of Anxiety Disorder. Pharm Biosci J 2018; 6: 07-16. https://doi.org/10.20510/ukjpb/6/i3/173546

[8] Senra ED, Queiroz GS, Brito Y de F, *et al*. Efeitos colaterais do uso crônico e indiscriminado de benzodiazepínicos: Uma revisão narrativa / Side effects of chronic and indiscriminate use of benzodiazepines: A narrative review. Brazilian J Dev 2021; 7: 102013-102027. https://doi.org/10.34117/bjdv7n11-023

[9] Souza ARL de, Opaleye ES, Noto AR. Contextos e padrões do uso indevido de benzodiazepínicos entre mulheres Contexts and patterns of undue use benzodiazepine among women. Cien Saude Colet 2012; 18: 1131-1140. https://doi.org/10.1590/S1413-81232013000400026

[10] Mishra A, Goel RK. Modulatory Effect of Serotonergic System in Pentylenetetrazole-Induced Seizures and Associated Memory Deficit: Role of 5-HT_{1A} and 5-$HT_{2A/2C}$. J Epilepsy Res 2020; 9: 119-125. https://doi.org/10.14581/jer.19012

[11] Phootha N, Yongparnichkul N, Fang Z, *et al*. Plants and phytochemicals potentials in tackling anxiety: A systematic review. Phytomedicine Plus [Internet] 2022; 2: 100375. https://doi.org/10.1016/j.phyplu.2022.100375

[12] Karim N, Khan I, Abdelhalim A, *et al*. Stigmasterol can be new steroidal drug for neurological disorders: Evidence of the GABAergic mechanism via receptor modulation. Phytomedicine [Internet] 2021; 90: 153646. https://doi.org/10.1016/j.phymed.2021.153646

[13] Ferreira MKA, da Silva AW, dos Santos Moura AL, *et al*. Chalcones reverse the anxiety and convulsive behavior of adult zebrafish. Epilepsy Behav 2021; 117. https://doi.org/10.1016/j.yebeh.2021.107881

[14] Mohamad AS, Akhtar MN, Zakaria ZA, *et al*. Antinociceptive activity of a synthetic chalcone, flavokawin B on chemical and thermal models of nociception in mice. Eur J Pharmacol 2010; 647: 103-109. https://doi.org/10.1016/j.ejphar.2010.08.030

[15] Ferreira MKA, Freitas WPO, Barbosa IM, *et al*. Heterocyclic chalcone (E)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-3-(thiophen-2-yl) prop-2-en-1-one derived from a natural product with antinociceptive, anti-inflammatory, and hypoglycemic effect in adult zebrafish. 3 Biotech [Internet] 2023; 13: 1-21. https://doi.org/10.1007/s13205-023-03696-8

[16] Goodarzi M, Saeys W, De Araujo MCU, *et al*. Binary classification of chalcone derivatives with LDA or KNN based on their antileishmanial activity and molecular descriptors selected using the Successive Projections Algorithm feature-selection technique. Eur J Pharm Sci [Internet] 2014; 51: 189-195. https://doi.org/10.1016/j.ejps.2013.09.019

[17] Silva Mendes FR, Wlisses da Silva A, Amâncio Ferreira MK, *et al*. GABAA receptor participation in anxiolytic and anticonvulsant effects of (E)-3-(furan-2-yl)-1-(2hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one in adult zebrafish. Neurochem Int 2022; 155. https://doi.org/10.1016/j.neuint.2022.105303

[18] Gargantilla M, López-Fernández J, Camarasa MJ, *et al*. Inhibition of xpo-1 mediated nuclear export through the michael-acceptor character of chalcones. Pharmaceuticals 2021; 14. https://doi.org/10.3390/ph14111131

[19] Villa SM, Heckman J, Bandyopadhyay D. Medicinally Privileged Natural Chalcones: Abundance, Mechanisms of Action, and Clinical Trials. Int J Mol Sci 2024; 25. https://doi.org/10.3390/ijms25179623

[20] Khan KM, Collier AD, Meshalkina DA, *et al*. Zebrafish models in neuropsychopharmacology and CNS drug discovery. Br. J. Pharmacol 2017. https://doi.org/10.1111/bph.13754

[21] Siebel AM, Menezes FP, Capiotti KM, *et al*. Role of adenosine signaling on pentylenetetrazole-induced seizures in zebrafish. Zebrafish 2015. https://doi.org/10.1089/zeb.2014.1004

[22] OECD. Test No 203: Fish, Acute Toxicity Testing, Section 2: Effects on Biotic Systems. Guidel Test Chem [Internet] 2019; 10.

[23] Arellano-Aguilar O, Solis-Angeles S, Serrano-García L, *et al*. Use of the Zebrafish Embryo Toxicity Test for Risk Assessment Purpose: Case Study. J Fish 2015. p. 051-062.

[24] Gonçalves NGG, De Araújo JIF, Magalhães FEA, *et al*. Protein fraction from Artocarpus altilis pulp exhibits antioxidant properties and reverses anxiety behavior in adult zebrafish via the serotoninergic system. J Func Foods 2020; 66: 103772. https://doi.org/10.1016/j.jff.2019.103772

[25] Gebauer DL, Pagnussat N, Piato ÂL, *et al*. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. Pharmacol Biochem Behav 2011; 99: 480-486. https://doi.org/10.1016/j.pbb.2011.04.021

[26] Benneh CK, Biney RP, Mante PK, *et al*. Maerua angolensis stem bark extract reverses anxiety and related behaviours in zebrafish—Involvement of GABAergic and 5-HT systems. J Ethnopharmacol 2017; 207: 129-145. https://doi.org/10.1016/j.jep.2017.06.012

[27] Siebel AM, Menezes FP, Schaefer IC, *et al*. Rapamycin suppresses PTZ-induced seizures at different developmental stages of zebrafish. Pharmacol Biochem Behav 2015; 139: 163-168. https://doi.org/10.1016/j.pbb.2015.05.022

[28] Masiulis S, Desai R, Uchański T, *et al*. GABAA receptor signalling mechanisms revealed by structural pharmacology. Nature 2019; 565: 454-459. https://doi.org/10.1038/s41586-018-0832-5

[29] Xavier J da C, Ferreira MKA, da Silva AW, *et al*. Anxiolytic-like and anticonvulsant effect in adult zebrafish (Danio rerio) through gabaergic system and molecular docking study of chalcone derived from natural products. Biointerface Res Appl Chem 2021; 11. https://doi.org/10.33263/BRIAC116.1402114031

[30] Mendes FRS, da Silva AW, Ferreira MKA, *et al*. GABAA and serotonergic receptors participation in anxiolytic effect of chalcones in adult zebrafish. J Biomol Struct Dyn [Internet] 2023; 41: 12426-12444. https://doi.org/10.1080/07391102.2023.2167116

[31] Yan J, Zhang G, Pan J, *et al*. α-Glucosidase inhibition by luteolin: Kinetics, interaction and molecular docking. Int J Biol Macromol [Internet] 2014; 64: 213-223. https://doi.org/10.1016/j.ijbiomac.2013.12.007

[32] Huey R, Morris GM, Forli S. Using autodock 4 and autodock vina with autodocktools : a tutorial 2012;

[33] Trott O, Olson AJ. Autodock vina. J Comput Chem 2010;

[34] Marinho EM, Batista de Andrade Neto J, Silva J, *et al*. Virtual screening based on molecular docking of possible inhibitors of Covid-19 main protease. Microb Pathog 2020; 148: 1-6. https://doi.org/10.1016/j.micpath.2020.104365

[35] Yusuf D, Davis AM, Kleywegt GJ, *et al*. An alternative method for the evaluation of docking performance: RSR vs RMSD. J Chem Inf Model 2008; https://doi.org/10.1021/ci800084x

[36] Silva J, Rocha MN da, Marinho EM, *et al*. Evaluation of the ADME, toxicological analysis and molecular docking studies of the anacardic acid derivatives with potential antibacterial effects against Staphylococcus aureus. J Anal Pharm Res 2021; 10: 177-194.

[37] Shityakov S, Förster C. In silico predictive model to determine vector-mediated transport properties for the blood-brain barrier choline transporter. Adv Appl Bioinforma Chem AABC 2014; 7: 23-36. https://doi.org/10.2147/AABC.S63749

[38] Pettersen EF, Goddard TD, Huang CC, *et al*. UCSF Chimera - A visualization system for exploratory research and analysis. J Comput Chem 2004; 25: 1605-1612. https://doi.org/10.1002/jcc.20084

[39] DeLano WL. The PyMOL Molecular Graphics System, Version 2.3. Schrödinger LLC 2020.

[40] BIOVIA DS. Discovery Studio Modeling Environment, Release 2017, San Diego. Dassault Systèmes 2016.

[41] Adasme MF, Linnemann KL, Bolz SN, *et al*. PLIP 2021: Expanding the scope of the protein-ligand interaction profiler to DNA and RNA. Nucleic Acids Res 2021; 49: W530-W534. https://doi.org/10.1093/nar/gkab294

[42] Xavier J da C, de Almeida-Neto FWQ, Rocha JE, *et al*. Spectroscopic analysis by NMR, FT-Raman, ATR-FTIR, and UV-Vis, evaluation of antimicrobial activity, and in silico studies of chalcones derived from 2-hydroxyacetophenone. J Mol Struct [Internet] 2021; 1241: 130647. https://doi.org/10.1016/j.molstruc.2021.130647

[43] Facciol A, Gerlai R. Zebrafish Shoaling, Its Behavioral and Neurobiological Mechanisms, and Its Alteration by Embryonic Alcohol Exposure: A Review. Front Behav Neurosci 2020; 14: 1-12. https://doi.org/10.3389/fnbeh.2020.572175

[44] Cao Y, Yan H, Yu G, *et al*. Flumazenil-insensitive benzodiazepine binding sites in GABAA receptors contribute to benzodiazepine-induced immobility in zebrafish larvae. Life Sci [Internet] 2019; 239: 117033. https://doi.org/10.1016/j.lfs.2019.117033

[45] Griffin CE, Kaye AM, Rivera Bueno F, *et al*. Benzodiazepine pharmacology and central nervous system-mediated effects. Ochsner J 2013; 13: 214-223.

[46] Calcaterra NE, Barrow JC. Classics in chemical neuroscience: Diazepam (valium). ACS Chem Neurosci 2014; 5: 253-260. https://doi.org/10.1021/cn5000056

[47] Herculano AM, Maximino C. Serotonergic modulation of zebrafish behavior: Towards a paradox. Prog Neuro-Psychopharmacology Biol Psychiatry [Internet] 2014; 55: 50-66. https://doi.org/10.1016/j.pnpbp.2014.03.008

[48] Shaikh MF, Sancheti J, Sathaye S. Effect of Eclipta alba on acute seizure models: A GABAA-mediated effect. Indian J Pharm Sci 2013;

[49] Rose AS, Bradley AR, Valasatava Y, *et al*. NGL viewer: Web-based molecular graphics for large complexes. Bioinformatics 2018; 34: 3755-3758. https://doi.org/10.1093/bioinformatics/bty419

[50] Masiulis S, Desai R, Uchański T, *et al*. GABAA receptor signalling mechanisms revealed by structural pharmacology. Nature 2019; https://doi.org/10.1038/s41586-018-0832-5